# UNIVERSITA'DEGLI STUDI DI MESSINA

## Dipartimento di Scienze Veterinarie


DOTTORATO DI RICERCA IN SANITÀ PUBBLICA ANIMALE E SICUREZZA ALIMENTARE XXXII CICLO

Settore Scientifico Disciplinare VET/06

# Vectors and hosts other than dog in the epidemiology of *Leishmania infantum*

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**Preface**

The following thesis is written in a 'thesis by publication' format and includes five published articles in peer-reviewed international journals, and one manuscript submitted for publication.

This thesis entitled "Vectors and hosts other than dogs in the epidemiology of *Leishmania infantum*" is divided in three main chapters and further sections:

**Chapter 1**. includes Section 1.1. and Section 1.2, containing two research articles published in the international journal 'Parasites & Vectors' and focusing on main domestic animal reservoirs and the importance of the adoption of effective preventative measures to reduce the risk of infection and disease spreading control;

**Chapter 2**. consists of three Sections: Section 2.1 presents a paper published in the journal 'BioMed Research International' journal; Section 2.2 comprises an article published in the journal 'Acta Tropica' and Section 2.3 includes a paper submitted for publication in 'PLoS One'. The chapter focuses on the vectors of *Leishmania*. In particular, innovative capture strategies, new method for quicker and reliable species identification, and original data on *Leishmania* infection rate and blood sources are presented in three sections, respectively;

**Chapter 3.** consists of an article accepted for publication in 'Comparative Immunology, Microbiology & Infectious Diseases'. Survey included in this chapter provides first evidence of the circulation of *L. infantum* in sylvatic animals suggesting their participation in the epidemiology of *L. infantum* in well-known hyper-endemic area of the Mediterranean basin.

The investigations presented in form of research articles herein included were mainly carried out at the Department of Veterinary Sciences of the University of Messina during the candidate's PhD course.

A collaboration with the Institute of Tropical Medicine of The New University of Lisbon (Portugal) has been agreed for the execution of molecular procedures included in the research article in Section 2.3. Also, a collaboration with the Department of Veterinary Medicine of the University of Bari has been established for the execution of molecular analysis performed in the surveys reported in Sections 1.2, 2.1 and Section 3.

The study in Section 1.1 was partially funded by Merial, now part of Boehringer-Ingelheim Animal Health, 29 Avenue Tony Garnier, 69007 Lyon (cost of consumables and publication fees). The study in Section 2.3 was partially funded by 'Research and Mobility' program (molecular analysis) and by Bayer Animal Health GmbH, 40789 Monheim, Germany (cost of consumables and publication fees).

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**General introduction**

Leishmaniasis is a complex of vector-borne diseases, caused by more than 20 protozoan species of the genus *Leishmania* (order Kinetoplasta; family Trypanosomatidae). Worldwide, leishmaniasis is classified as one of the most emergent 'neglected zoonotic diseases', representing the 2nd and the 4th most common cause of death and illness, respectively among all tropical diseases, surpassed only by malaria and lymphatic filariasis (WHO 2018; Alvar et al., 2012; Bern et al., 2008; Mathers et al., 2007). In Europe, *Leishmania infantum* is the causative agent of zoonotic visceral (VL) and cutaneous leishmaniasis (CL) in humans, and of a generalized disease in dogs known as canine leishmaniosis (CanL). Visceral leishmaniosis is the most severe clinical form of the disease, it is fatal if left untreated in more than 95% of cases within 2 years after the onset of clinical signs, but also even treated, VL may result in fatal outcome in 10–20% of cases (Alvar et al., 2012). Approximately 875 new human cases are reported in Europe each year, although the underestimation of subclinical infection has not been excluded (Alvar et al., 2012; Quinnell and Courtenay, 2009; Dujardin et al., 2008).
*Leishmania* is a digenetic protozoan parasite that resides in the midgut of the vector in the flagellated promastigote form, and within the reticulo-endothelial cells of a vertebrate host in the amastigote form. The natural transmission cycle involves phlebotomine sand flies (Diptera: Psychodidae: Phlebotominae), as unique hematophagous insects able in the transmission of the pathogen (Maroli et al., 2013). The amastigote parasites are ingested by vector during the blood meal and potential transmission depends on the injection of promastigotes forms when sand fly feeds a new host. Dog is regarded as the main reservoir host of the parasite, and based on serological surveys, canine leishmaniosis (CanL) affects approximately 2.5 million dogs in Mediterranean and peri-Mediterranean areas, with different prevalence rates in reason of geographical climate conditions that influence presence and abundancy of sand fly vectors (Moreno and Alvar, 2002). In addition to dogs, several other domestic and wild mammals have been suspected to have an epidemiological role in *Leishmania* transmission (Tomassone et al., 2018; Millán et al., 2014; Gramiccia et al., 2011). Specifically, the role of cat in the epidemiology of the infection has recently gain attention more than ever before, and prevalence rate of *L. infantum* infection has increased in some endemic foci, reaching 60% in certain cat population (Pennisi and Persichetti, 2018; Pennisi et al., 2015). Recent epidemiological studies suggest that the occurrence of feline leishmaniosis (FeL) might be higher than that currently thought, since *Leishmania* is not usually considered by practitioners as causative agent of disease in this animal species (Brianti et al., 2019; Otranto et al., 2017; Iatta et al., 2019). Cats infected with *L. infantum* are able to transmit protozoa to sand fly vectors, and thus may be considered as reservoirs of infection with an epidemiological role (Maia and Campino, 2011; Da Silva et al., 2010; Maroli et al., 2007). *Leishmania infantum* infection has also been increasingly reported in wild mammals that are present in urban areas, including rodents, as well as in free ranging wildlife including canids, felids, mustelids and lagomorphs (Tomassone et al., 2018; Millán et al., 2014). Nevertheless, the potential of wild species for transmitting VL is still unclear. Most of the wild animals exposed to *Leishmania* are naturally resistant and capable of eliminating the pathogen (Millán et al., 2014, McCall et al., 2013, Sobrino et al., 2008); while certain species are susceptible asymptomatic hosts able to infect sand fly vectors potentially contributing for transmission of VL (Jiménez et al., 2014; Molina et al., 2012). Xenodiagnosis is a tool that allows to evaluate the capability of a given infected vertebrate host to transmit the pathogen to the biological vector and, among sylvatic mammals, this ability has only been established in hares (*Lepus granatensis*), rabbits (*Oryctolagus cuniculus*) and black rats (*Rattus rattus*) (Jiménez et al., 2014; Zanet et al., 2014; Molina et al., 2012). Interestingly, the potential of naturally infected wild hares in transmitting leishmaniosis to humans has been strongly suggested in the southwest of Madrid (Spain), where a large and unusual outbreak of leishmaniosis with 446 human infections cases (n=286 CL; n=160 VL) was recorded between 2009 and 2012 (Moreno et al., 2014; Arce et al., 2013; Carrillo et al., 2013; Molina et al., 2012). Infection rate recorded among dogs was only 1.6-2%, while *L. infantum* infection was detected in a 30% of

asymptomatic screened hares (Arce et al., 2013; Carrillo et al., 2013). Xenodiagnosis confirmed the capability of hares to infect sand flies and, the identification of blood meals confirmed hares as preferred sand flies' food source (Gonzalez et al., 2017; Martín-Martín et al., 2014; Jimenez et al., 2013; Molina et al., 2012). Therefore, unconventional vertebrate hosts may play a chief role as reservoir of human infection in particular scenarios, and this finding highlights the importance to better clarify the role of species other than dogs in the epidemiology of *L. infantum* (Gonzalez et al., 2017; Moreno et al., 2014; Jiménez et al., 2013; Molina et al., 2012).

The transmission of *Leishmania* parasites involves complex ecological interactions between parasite-vector and hosts and the epidemiology of vector-borne diseases is constantly changing. Changes in the risk of pathogen transmission are mainly associated with variations in sand fly vector distribution and with uncontrolled movements of infected animals from endemic foci to free territories (Alten et al., 2016; Maia and Cardoso, 2015; Fischer et al., 2011). Additionally, the urbanization increases the contact with wildlife leading to exchange of pathogens between man and sylvatic animals, including *Leishmania*. The severity and the zoonotic nature of leishmaniasis make its prevention mandatory and nowadays, prophylactic measures are mainly focused on dog, being the use of repellents products the most effective tool for prevention of *L. infantum* infection in this animal species (Brianti et al., 2016; Brianti et al., 2014; Otranto et al. 2013). Also, the treatment of infected/diseased dogs is a key factor to reduce their infectiousness to vectors, diminishing the epidemiological risk for human and other healthy mammals (Otranto et al., 2013; Mirò at al., 2011). Nevertheless, increased in *Leishmania* reports in some endemic foci as a failure of control strategies, has been repeatedly rationalized with the underestimated role of vertebrate hosts other than dogs in *Leishmania* transmission (Millán et al., 2014).

Surveys included in this thesis were carried out in Sicily (southern Italy) a well-known hyper-endemic area for CanL where a 54.6% incidence rate of infection has been reported in unprotected shelter dogs (Brianti et al., 2014), and where almost 50% of the human population live in areas at risk for *Leishmania* infection (Cardo et al., 2006). Considering the endemicity and the zoonotic potential of leishmaniasis, this doctoral thesis aims to provide original data on the epidemiology and transmission dynamics of this complex vector-borne disease in order to improve the current knowledge and the ongoing control strategies. Studies included in the thesis focused on three main research lines, namely domestic animal reservoirs, vectors and wild animal reservoirs. In detail, Chapter 1 highlights the need to employ comprehensive preventive strategies in dog, that is still regarded as the main domestic reservoir hosts of *Leishmania infantum* and source of human infection. Chapter 2 aims to provide information on phlebotomine sand flies as unique insects able to transmit *Leishmania*, including innovative capture strategies, new method for quicker and reliable species identification, and original data on *Leishmania* infection rate and blood sources, to assess the risk for transmitting of *Leishmania* species and to gain indirect data on alternative hosts for *Leishmania*. Finally, Chapter 3 provides first evidence of the circulation of *L. infantum* in sylvatic animals suggesting their participation in the epidemiology of *L. infantum* in well-known hyper-endemic area of the Mediterranean basin.

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**Abstract**

This doctoral thesis aims to provide original data on the epidemiology and transmission dynamics of this complex vector-borne disease in order to improve the current knowledge and the ongoing control strategies. The thesis is presented in form of "thesis by publication" and is composed by three chapters.

**Chapter 1** is divided into 2 sections. Survey included in Section 1.1 provide evidence-base data on the efficacy and safety of the concomitant use of two antiparasitic drugs, namely Frontline Tri-Act® and NexGard Spectra® in dogs for six months. This combination is now proposed as a comprehensive prevention strategy in dogs where the risk of leishmaniosis and other vector-borne zoonotic diseases overlaps. Additionally, in Section 1.2, a case of feline leishmaniosis with a long-term follow-up is described, with the aim to provide data on clinical manifestations, pathological abnormalities, diagnosis and treatment of this poorly documented disease of cat, and to emphasize the susceptibility of this animal species to *Leishmania* infection, advocating the adoption of effective preventive measures in cats that live in endemic areas.

**Chapter 2** is divided into 3 sections focused on phlebotomine sand flies as unique proven vectors able to transmit *Leishmania* infection. In Section 2.1, the attractiveness of light traps equipped with different colored LED to phlebotomine sand fly endemic in the Mediterranean basin is described. Light trap equipped with UV LED showed a higher attractiveness to *P. perniciosus*, representing an effective alternative technology in sand flies monitoring to be employed in entomological surveys. In Section 2.2, the employment of MALDI-TOF mass spectrometry as a novel method for rapid, simple and reliable phlebotomine sand fly species identification, compared to the conventional morphological method is described. MALDI-TOF produced distinct, consistent and reproducible specie-specific protein spectra of the sand fly species analyzed, with no differences between males and females. Gained results strongly support its advantageous use in entomological surveys as reliable and quicker approach to sand fly species identification, improving the knowledge on the spread of these vectors of medical importance. In Section 2.3, natural *Leishmania* infection and blood-feeding preferences were molecularly investigated in wild caught phlebotomine sand flies in Sicily along two transmission seasons in order to assess the risk of transmission of *Leishmania* spp., and to recognize potential reservoir hosts for leishmaniosis. The detection of *L. infantum* DNA in *P. perniciosus* confirms the role of this species in the maintenance and spread of leishmaniosis in Sicily. The blood-feeding preference of *P. perniciosus* to rabbit incites to better clarify the hypothesis on the involvement of this wild host in the epidemiology of leishmaniosis as sylvatic reservoir. Finally, the presence of *L. infantum, L. tarentolae* and *Trypanosoma* sp. DNA in *S. minuta*, together with the anthropophilic feeding-behaviour observed, spurs to clarify the ability of this species in the transmission of pathogens to humans and other warm-blooded animals.

In **Chapter 3**, the circulation of *Leishmania infantum* in sylvatic mammals was molecularly and serologically investigated, providing first evidence of infection in Sicily. A low prevalence of infection was recorded, suggesting a minimal involvement of these animal populations in the epidemiology of leishmaniosis in Sicily. Nevertheless, the intrinsic constrains in diagnose *Leishmania* infection in wild animals do not definitively allow to exclude their involvement as alternative sylvatic hosts and future studies are needed to better define their role in the epidemiology of leishmaniosis in Sicily.

**Chapter 1**

Section 1.1

# Six-month field efficacy and safety of the combined treatment of dogs with Frontline Tri-Act® and NexGard Spectra®

Adapted from:

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**Parasites & Vectors 2018 Jul 16;11(1): 425.**

**Abstract**

**Background:** Safety and efficacy of the combined monthly use of spot-on fipronil 6.76% w/v / permethrin 50.48% w/v (Frontline Tri-Act®) and chewable tablets of afoxolaner 1.9% w/w / milbemycin oxime 0.4% w/w (NexGard Spectra®) in dogs was evaluated in a field study over a period of 6 months.

**Methods:** Forty-one healthy dogs living in highly endemic area for canine leishmaniosis and other canine vector borne diseases (VBD) were included in the study at the beginning of the *Leishmania* transmission season. Sixteen dogs were pet dogs living each in a single household; twenty-five dogs were hunting dogs living in three kennels. At inclusion, the dogs were ELISA (rapid test) negative for antibodies to *Anaplasma*, *Borrelia*, *Ehrlichia*, and for antigens of *Dirofilaria*. The dogs were also negative for blood microfilariae at the Knott's test, and no clinical or hematological abnormalities were observed. Of the included dogs, six hunting, apparently healthy, dogs were ELISA (rapid test) positive to *Leishmania*, and some were naturally infected by gastrointestinal nematodes (58.5%) and/or infested by fleas (58.5%) and ticks (9.8%). All the included dogs were treated at Days 0, 28, 56, 84, 112 and 140, and followed-up for efficacy until the study end (Day 168).

**Results:** No adverse events related to the two products, nor skin reactions, general signs, or changes in the hematological profile, were observed during the study. At Day 14, anthelminthic efficacy was 100% for *Toxocara canis*, *Toxascaris leonina* and *Capillaria aerophila*, while few hunting dogs were still shedding eggs of *Trichuris vulpis* (1/25 hunting dog) and Ancylostomatidae (9/25 hunting dogs). All pet dogs were nematode free at the end of the study. Hunting dogs were free of roundworms and whipworms. Twenty-four hours after the first treatment, 95.8% of the ectoparasite infested dogs were free from fleas and ticks. Ectoparasites were significantly controlled during the 6-month study period, with 100% efficacy on both fleas and ticks from Day 56 to Day 168. Blood and serum samples collected on Day 168 were tested for vector-borne pathogens using same methods of the inclusion and no new seroconversions or circulating blood microfilariae were observed.

**Conclusions:** Concomitant use of Frontline Tri-Act® and NexGard Spectra® in dogs for six months was well tolerated. The combination was effective in controlling fleas, ticks, gastro-intestinal nematodes, and neither new seroconversion to the tested vector-borne pathogens nor blood microfilariae were detected in treated dogs at the end of the study.

**KEYWORDS:**

Afoxolaner/milbemycin oxime; Canine vector-borne diseases; Ectoparasites; Efficacy; Fipronil/permethrin; Nematodes; Safety

## 1. Background

Dogs are continuously exposed to parasitic infections and vector-borne diseases (VBD), with some of them being of zoonotic concern [1, 2]. The risk is higher in animals living in communities (e.g. kennels or shelters) and/or with access to the outdoors [3]. Despite better attention to canine health and the use of highly effective anti-parasitic products, recent studies conducted throughout Europe have shown that intestinal nematode infections remain a common occurrence in dogs [4-6]. A recent survey conducted on 1390 owned dogs reported that more than a third of them were shedding eggs/cysts of endoparasites, i.e. nematodes, cestodes and/or protozoans [7]. The ratio of endoparasitic infections is higher if kennel dogs are included in the epidemiological surveys [8].

Ectoparasite infections, mainly caused by fleas and ticks, constantly challenge dogs [9]. Their role as vectors of many pathogens highlights the need for an efficacious year-round strategy of control [10, 11]. Canine leishmaniosis (CanL), caused by *Leishmania infantum*, and canine dirofilariosis, due to *Dirofilaria immitis* or *Dirofilaria repens*, are among the most diffused and common VBD of dogs throughout Europe [12-15]. Several drivers are currently influencing the epidemiology of these diseases. Changes in vector presence, abundance and activity, as well as increased movements of animals from endemic areas into free territories and *vice versa*, seem to play key roles in this change in epidemiology [16]. As a result, the current distribution of CanL and canine heartworm disease (CHD) due to *D. immitis* is overlapping in several European countries, including Albania [17], Greece [18], France [2], Italy [3], Portugal [13, 14] and Spain [19]. This means that dogs living in these areas are at risk for both diseases during at least 4–6 months per year on average, which corresponds to the period of activity of the respective vectors (i.e. sand flies and mosquitoes).

Advances in research for the control of endo- and ectoparasites have resulted in the development of several highly effective veterinary products for the prevention of canine VBD. However, a single product that guarantees prevention against endoparasites, ectoparasites and canine VBD is not yet available. Therefore, the combined use of different drugs is likely to represent the only preventative solution to protect dogs under particular epidemiological settings [20]. Recently, two new antiparasitic products, namely Frontline Tri-Act® (Merial, now part of Boehringer Ingelheim), a topical solution combination of fipronil 6.76% w/v and permethrin 50.48% w/v, and NexGard Spectra® (Merial, now part of Boehringer Ingelheim), a chewable tablet containing afoxolaner 1.9% w/w and milbemycin oxime 0.4% w/w, have been launched onto the veterinary market. The former is a spot-on formulation, effective for treatment and prevention of flea infestations, including *Ctenocephalides felis* and *Ctenocephalides canis* [21, 22] and ticks, including *Dermacentor reticulatus*, *Rhipicephalus sanguineus* (*sensu lato*) and *Ixodes ricinus* [23, 24]. The product has shown a repellent and insecticidal activity against mosquitoes and stable flies [25, 26], and against phlebotomine sand flies, the vectors of CanL, under laboratory conditions [27]. Moreover, the efficacy in preventing CanL in a cohort of naturally exposed dogs has been recently demonstrated in a hyperendemic area of Greece [18]. NexGard Spectra® is a systemic insecticidal and acaricidal product in a chewable formulation licensed for treatment and prevention of ectoparasite infestations (fleas and ticks), and treatment of gastrointestinal nematodes [7]. This product, due to the presence of milbemycin oxime, is also effective for the prevention of CHD [28].

The concomitant use of these two products could represent a reliable solution for a comprehensive protection against both endo- and ectoparasites, including the prevention of CanL and CHD. Currently, no information on their concomitant administration is available in literature. Therefore, the aim of the present study was to assess the safety and the efficacy of the monthly concurrent treatments with Frontline Tri-Act® and NexGard Spectra® in dogs for a period of six months.

## 2. Methods

### 2.1 Study design

The study was a non-controlled, un-blinded, field trial conducted on privately owned dogs. Animals were included in the study after collection of the owner written informed consent. The study was conducted in Sicily and Calabria regions (southern Italy) from May 2016 to January 2017.

Seventy-four dogs with a minimum body weight of 2 kg and older than 8 weeks were screened at the pre-inclusion visit on study day -7 (Day -7). Each animal was physically examined, and individual faecal and blood samples were collected for analyses (see Sample analyses section). Only dogs with satisfactory general health condition, not infected by agents of chronic debilitating diseases or VBD (i.e. anaplasmosis, borreliosis, ehrlichiosis and leishmaniosis), and without circulating microfilariae, were enrolled.

On Day 0, 41 dogs were included in the study and sampled again for faeces, checked for the presence of ectoparasites, and simultaneously treated with the two testing products at therapeutic doses and in compliance with manufacturer's instructions. Frontline Tri-Act® pipette was applied directly on animal's skin and divided in two approximately equal spots (i.e. at the base of the skull and between the blades of shoulders). NexGard Spectra® was administered to dogs initially by offering the chew for spontaneous ingestion or by putting it directly into the mouth, if the dog had not eaten the drug spontaneously. After treatment, each dog was observed for 2 h in order to detect any side effects (e.g. ptyalism, vomiting or skin reactions). All enrolled animals remained with their owners and kept as per normal routine for the entire study. The owners were instructed to assess the animal's health status daily and to notify any adverse event or behaviour abnormalities immediately.

After initial Day 0 treatment, the dogs were followed-up on Days 1, 14, 28, 56, 84, 112, 140 and 168, and were retreated with the two testing products every 28 days (± 2) after clinical check and sampling (**Table 1**). At each visit the dogs were clinically examined by evaluation of body temperature, body weight, nutritional status, respiratory, cardiovascular and gastrointestinal systems, and checked for tick and flea presence by thumbing and combing the fur for ~3 min. Individual faecal samples were collected and screened for gastrointestinal parasites using copromicroscopical analyses at each visit. Blood samples were collected from each dog on Days -7, 28, 84 and 168.

**2.2 Sample analyses**

Faecal samples were processed with Baermann-Wetzel technique [29] for the detection of broncho-pulmonary larvae and by a modified McMaster technique with a cut-off of 15 eggs per gram (epg) [30-33].

Blood was collected from a peripheral vein (jugular or cephalic) using a standard technique and stored in 2.5 ml anticoagulant ($K_3$ EDTA) tube and in 2.5 ml cloth activator tube. A complete cells blood count (CBC), including red blood cells (RBC), haemoglobin (HGB), haematocrit (HCT), white blood cells (WBC) and platelets (PLT), was performed on all blood samples using an automated haematology analyser (Benesphera **H32 VET, Avantor Performance Materials Inc., Center Valley, PA, USA).

The presence of circulating microfilariae was assessed using a modified Knott's test [34] on Days -7 and 168. Observed microfilariae were identified to species level using morphometric criteria [34].

Sera samples collected on Days -7 and 168 were tested by rapid ELISA assays for *L. infantum* using the Canine *Leishmania* Antibody Test Kit produced by IDEXX® (IDEXX laboratories, Westbrook, ME, USA), and for *Anaplasma* spp., *Borrelia* spp., *Ehrlichia* spp., and *D. immitis* using SNAP® 4Dx Plus (IDEXX laboratories, Westbrook, ME, USA) following the producer's recommendations.

**2.3 Safety assessments**

Data on clinical parameters (body temperature, body weight, nutritional status, respiratory, cardiovascular and gastrointestinal systems) and CBC collected during clinical examinations and from analyses were compared to the data collected at the pre-inclusion visit, which were considered as baseline values. Pre-treatment haematological blood parameters (Day -7) were compared with values obtained at Days 28, 84 and 168 by means of one-way analysis of variance (ANOVA) for repeated measures.

The same statistical analysis was performed in order to evaluate the effect of time on dog's body weight values. When significant differences were found, Bonferroni's post hoc comparison was applied. A $P$-value < 0.05 was considered statistically significant. The statistical analysis was performed using the software GraphPad Prism version 5.1 (GraphPad Software, San Diego, California, USA).

Data on local tolerance of the spot-on treatment were also collected and the application sites were particularly examined for changes of the hair coat and skin.

### 2.4 Efficacy assessments

Efficacy against endo- and ectoparasites was evaluated in the animals that scored positive at the inclusion. The effect (curative or preventive) against ectoparasites was assessed 24 h after the initial treatment, at Day 14, and then monthly until Day 168. The efficacy is expressed as a percentage of flea or tick free dogs as no ectoparasite counts were performed. Due to the poor correlation between the egg numbers and parasite infestation for nematodes in dogs, the number of infected dogs at each sampling time-point assessed the efficacy against gastrointestinal and respiratory nematodes. The percentage of nematode free dogs was calculated for each parasite species.

## 3. Results

### 3.1 Enrolment

Among the 74 dogs screened, 41 dogs, with a mean age of 3.6 years and a weight ranging from 3.4 to 32 kg, fulfilled the inclusion criteria and were included in the study at Day 0 (**Table 2**). The 33 excluded dogs had abnormal haematological parameters or tested positive to CanL or other vector borne diseases (**Table 3**). Six of the included hunting dogs were, however, seropositive to *Leishmania* but did not show clinical signs or pathological alterations and, therefore, were kept included because co-housed in the same pen with other enrolled dogs.

Of the 41 enrolled dogs, 16 were pet dogs living each in a single household, and 25 were hunting dogs living in three kennels (6 in kennel 1, 7 in kennel 2, and 12 in kennel 3). One dog was lost by the owner after Day 84, one hunting dog deceased as outcome of hierarchy aggression by another dog (after Day 112), two other dogs did not come for veterinary visit and sampling on Days 84, 112, and 140 because of owner's unavailability, but came back on Day 168. For statistical analysis, only complete dog cases at Day 168 ($n$ = 37) were used.

### 3.2 Safety

Neither adverse events related to the two products, nor skin reactions or general signs were observed during the study. No change of clinical examination parameters (i.e. body temperature, nutritional status, heart rate and respiratory rate) was recorded. The mean body weight showed a non-significant slight increase from the baseline, i.e. from 16.1 to 17.3 kg ($\Delta$ = 1.22, $P$ = 0.949).

Mean values of haematological parameters were within physiological limits of the species throughout the study period. However, a statistically significant increase of HCT ($F_{(3, 160)}$ = 36.21, $P$ < 0.0001), HGB ($F_{(3, 160)}$ = 44.35, $P$ < 0.0001) and PLT ($F_{(3, 160)}$ = 3.08, $P$ = 0.029) compared to the baseline values was observed on Days 84 and 168 (Table 4).

### 3.3 Efficacy assessments

At Day 0, 24 dogs (58.5%) were found infected with gastrointestinal nematodes, i.e. 18.8% (3/16) pet dogs and 84% (21/25) hunting dogs ($\chi^2$ = 17.1137, $df$ = 1, $P$ < 0.0001). At inclusion, six parasite species/taxa were identified, with *Trichuris vulpis* (20/41, 48.8%) and Ancylostomatidae (15/41, 36.6%) as the most frequent species, followed by *Capillaria aerophila* (8/41, 19.5 %), *Toxascaris leonina* (7/41, 17.1%), *Toxocara canis* (6/41, 14.6%) and *Dipylidium caninum* (3/41, 7.3%).

At Day 14 visit, no eggs of *C. aerophila*, *T. canis* and *T. leonina* were found at coproscopy, while some hunting dogs were still shedding eggs of *T. vulpis* (1/25) and Ancylostomatidae (9/25). All pet dogs were found nematode free from Day 14 to Day 168. Except for the Ancylostomatidae, all the hunting dogs were nematode free from Day 112 assessment up to the end of the study (Table 5). None of the enrolled dogs tested positive at the Baermann-Wetzel technique neither at the pre-inclusion nor throughout the study period.

On Day 0 visits the percentage of dogs infested by fleas was 58.5% (3/16 pet dogs and 21/25 hunting dogs; $\chi^2$ = 17.1137, $df$ = 1, $P$ < 0.0001). On Day 1, 40/41 (97.6%) of the dogs were flea-free and none of them was found infested by fleas on the following scheduled controls, except on Day 28 when seven hunting dogs (28%) scored flea positive (Table 6).

On Day 0, tick infestation was observed in 1/16 (6.2%) pet dogs and 3/25 (12%) of the hunting kennel dogs; 24 h post-treatment the pet dog was still infested by ticks, while none of the animals scored positive for ticks in the other follow-ups (Table 6).

At inclusion and at Day 168, none of the enrolled dogs scored positive for *Anaplasma* spp., *Borrelia* spp., *Ehrlichia* spp. and *D. immitis* using the SNAP® 4Dx Plus test. No microfilariae were detected in blood samples, and all dogs scored ELISA (rapid test) negative to *L. infantum* antibodies with the exception of the six that were already positive at the inclusion.

## 4. Discussion

This study demonstrates how the concurrent use of Frontline Tri-Act® and Nexgard Spectra® in dogs for six months is effective and well tolerated. These findings are largely in line with what has been previously observed with the single use of both products under laboratory and field conditions [7, 18, 22, 24, 35].

Haematological parameters remained within the reference ranges [36], with a significant increase of HCT, HGB and PLT observed on Days 84 and 168. This could be attributable to a general improvement of health conditions in treated dogs as they were dewormed and freed from ectoparasites. Also, it could be the result of increased physical activities [37], as the majority of these animals were hunting dogs and the last part of the study overlapped with the hunting season.

The species of gastrointestinal nematodes identified in the present field trial match with those reported in other surveys conducted throughout Europe [7]. The overall frequency of intestinal parasite infections reported here (from 14.6% to 48.8% according to species/taxa) was considerably high, especially in hunting dogs living in kennels with 84% of mixed infestation by endo- and ectoparasites. This underlines, once again how owned dogs may be significantly infected by intestinal parasites even if adult and apparently healthy pets [38]. All dogs included, whipworms and hookworms were the species with the highest frequencies (48.8 and 36.6%, respectively). The presence of *C. aerophila* in 8/41 (19.5%) of the dogs, seven hunting dogs and one pet dog, is of particular interest. This trichuroid nematode infects the lungs of domestic (i.e. dogs and cats) and wild carnivores (e.g. foxes) and may represent a zoonotic hazard [39, 40]. Capillariosis in dogs and cats is increasingly diagnosed throughout Europe and the frequency of infection reported here is the highest

ever reported in dogs so far [5, 41, 42], confirming how certain animal categories (e.g. hunting dogs) may be at great risk of infection.

The efficacy against intestinal parasites reported in the present study largely matches that observed previously in an extensive European multicentre field study [7]. At Day 112 and after, all dogs were free of whipworms, roundworms, and *Capillaria*. Conversely, the efficacy against hookworms was lower to what has previously been observed under both natural and experimental conditions [7, 43]. It is important, however, to point out that all the dogs, which were continuously shedding Ancylostomatidae eggs, were hunting kennel dogs living with other untreated dogs in highly contaminated environment. Egg shedding would be related to re-infections, but also to regular coprophagy and passive migration of eggs from infected faeces. It is likely that re-infections could not be prevented due to the epidemiological situation in the three kennels. In kennel 1, the only hookworm infected dog became negative, in kennel 2, 4/7 infected dogs remained infected, and in kennel 3, from 8/12 at the beginning, 6/10 dogs were infected at the end of the study. Indeed, the pre-patent period of *Ancylostoma* can be as short as 14 days, and their control in kennels may require regular anthelmintic treatments administered every 2 weeks until control is obtained, and then monthly administrations [38, 44].

In the present trial fleas and ticks were efficiently controlled in treated animals starting from 24 hours post-treatment when 97.6% of flea and tick pre-infested animals were cured. Tick infestations were totally prevented as no ticks were found on dogs treated from Day 14 up to Day 168. On Day 28, a few hunting dogs (7/25) were found infested by fleas, but, interestingly, the majority of fleas collected during this visit were dead or moribund (data not shown). Thereafter, all dogs were flea-free. As already mentioned, the majority of dogs included in this trial were hunting dogs housed in kennels where other not included infested dogs were present, thus indicating a high regular exposure. Although acting with a different mode of action, both products here tested claim curative and preventative efficacy against ectoparasites of dogs; therefore, a synergistic action between them could not be ruled out and it may provide clues for the high efficacy profile here observed. However, it is important to note that the combined use of these products was primarily intended to provide a simultaneous protection against some VBD, especially CanL and CHD [7, 10, 24].

Despite this study was primarily designed to assess the safety and efficacy of the combined used of the two tested products, it also allowed the collection of supplementary data on the incidence of some vector-borne pathogens (VBP) in treated dogs. The study was indeed conducted in a hyper-endemic area for CanL, where a 54.6% incidence rate of infection has been reported in unprotected shelter dogs [45]. Most of the dogs screened at Day -7 were not enrolled in the study because they were already infected or diseased by *L. infantum* (see Table 3). The selected study area is also endemic for VBP transmitted by ticks, i.e. *Anaplasma phagocytophilum*, *Babesia canis*, *Ehrlichia canis* and *Rickettsia conorii* [46] and canine dirofilarioses, which have been reported in the same animal population [47, 48]. In the present trial dogs were enrolled in May and completed the study in December after they had been exposed to one entire CanL transmission season. Despite the high risk of transmission, no negative treated dogs scored seropositive to *L. infantum* at study end; analogously, no seroconversion against other VBD causing pathogens (i.e. *Anaplasma* spp., *Borrelia* spp., *Ehrlichia* spp. and *D. immitis*) or blood microfilariae were detected in dogs at the end of the study. Tough the limitations in terms of sensibility of the sole use of serological tests for the diagnosis of some VBP such as *L. infantum* (e.g. exposed/infected dogs that may have not seroconvert yet), results of the current trial further corroborate what has been observed in a previous study specifically designed to assess the efficacy of the association permethrin/fipronil in reducing the risk of transmission in naturally exposed dogs living in a CanL hyper-endemic area [18].

**5. Conclusions**

The present study showed that the simultaneously administration of Frontline Tri-Act® and Nexgard Spectra® in dogs during a 6-month period is safe and provides a high efficacy in controlling ecto- and endoparasite infestations. In addition, neither new seroconversion to *Anaplasma* spp., *Borrelia* spp., *Ehrlichia* spp., *L. infantum* and *D. immitis* nor blood microfilariae were detected in treated dogs at the end of the study. By virtue of the repellent effect of the association fipronil/permethrin (Frontline Tri-Act®) against sand flies, and thanks to the efficacy of afoxolaner/milbemycin oxime (Nexgard Spectra®) in preventing canine heartworm disease, the concomitant use of these products offers a broad spectrum of protection which may be regarded as a safe and effective prevention strategy for dogs, particularly those living in or travelling to geographical areas where the risk of CanL and CHD is overlapping.

**Abbreviations**

CanL: Canine leishmaniosis; CBC: Cells blood count; CHD: Canine heartworm disease; ELISA: Enzyme-linked immunosorbent assay; HCT: Haematocrit; HGB: Haemoglobin; PLT: Platelets; RBC: Red blood cells; VBD: Vector-borne diseases; VBP: Vector-borne pathogens; WBC: White blood cells.

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**Availability of data and materials**

All data generated or analysed during this study are included in this published article.

**Authors' contributions**

EB, EN, FB and LH conceived and designed the study. JMA, EB, EN and GG carried out the field activities. JMA, EN, FA and GG carried out the laboratory work and statistical analyses. JMA and EN drafted the first version of the manuscript. EB, FA, GG, SG, FB and LH critical reviewed the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The protocol and the study design were approved by the Ethical Committee of the Department of Veterinary Sciences of the University of Messina (no. 003/16). Animals were included in the study only after the signature of an informed consent by the owner.

**Consent for publication**

Not applicable.

**Competing interests**

FB and LH are employees of Merial, now part of Boehringer-Ingelheim Animal Health, 29 Avenue Tony Garnier, 69007 Lyon. The other authors declare that they have no competing interests.

**Publisher's Note**

**Table 1** Dates and scheduled operations

| Activity | Pre-inclusion (Day -7) | Inclusion (Day 0) | Day 1 visit | Day 14 visit | Day 28 visit | Day 56 visit | Day 84 visit | Day 112 visit | Day 140 visit | Day 168 visit |
|---|---|---|---|---|---|---|---|---|---|---|
| **Clinical examination** | + | + | + | + | + | + | + | + | + | + |
| **CBC** | + | | | | + | | + | | | + |
| **Copromicroscopy (McMaster + Baermann)** | + | + | | + | + | + | + | + | + | + |
| **Blood microfilariae** | + | | | | | | | | | + |
| **Ectoparasites** | + | + | + | + | + | + | + | + | + | + |
| ***Anaplasma* spp. serology** | + | | | | | | | | | + |
| ***Ehrlichia canis* serology** | + | | | | | | | | | + |
| ***Leishmania infantum* serology** | + | | | | | | | | | + |
| **Treatment (Frontline Tri-Act+NexGard Spectra)** | | + | | | + | + | + | + | + | |

**Table 2** Characteristics of the 41 dogs included in the study

| **Dogs' variables** | | | |
|---|---|---|---|
| **Age** | **≤12 months** | **12-60 months** | **>60 months** |
| | 14 | 15 | 12 |
| **Size** | **Small (≤7 kg)** | **Medium (7-18kg)** | **Large (>18kg)** |
| | 4 | 24 | 13 |
| **Hair coat** | **Long** | **Medium** | **Short** |
| | 6 | 7 | 28 |
| **Life style** | **Pet** | **Hunting** | |
| | 16 | 25 | |
| **Gender** | **Female** | **Male** | |
| | 19 | 22 | |
| **Breed** | **Cross-breed** | **Pure breed** | |
| | 21 | 20 | |

**Table 3** Clinical assessment and evaluation of the 74 dogs screened at the pre-inclusion visit

| | |
|---|---|
| *Leishmania infantum* seropositive dogs | 25/74 (33.8%) |
| *Ehrlichia canis* seropositive dogs | 2/74 (2.7%) |
| *Anaplasma* spp. seropositive dogs | 1/74 (1.4%) |
| Gastrointestinal parasite positive dogs | 29/74 (39.2%) |
| Fleas positive dogs | 26/74 (35.1%) |
| Ticks positive dogs | 4/74 (5.4%) |
| Alterations of the haematological parameters | 19/74 (25.7%) |
| Owners not complying with the study protocol | 2/74 (2.7%) |

**Table 4** Mean values ± standard deviation (M±SD) of haematological parameters recorded in dogs during the study. Dogs were treated with Frontline Tri-Act and Nexgard Spectra on study Day 0 and thereafter every 28 (± 2 days) up to Day 140

| | | **Study days** | | | | |
|---|---|---|---|---|---|---|
| | | **Day -7** | **Day 28** | **Day 84** | **Day 168** | |
| ***Parameters*** | ***RR*[a]** | ***M±SD*** | ***M±SD*** | ***M±SD*** | ***M±SD*** | ***P value*** |
| **RBC** | ***5.5-8.5x10$^6$/µL*** | 6.8±0.9 | 7.0±0.8 | 7.2±0.7 | 7.1±0.4 | 0.12 |
| **WBC** | ***6-17x10$^3$/µL*** | 12.9±2.9 | 12.0±2.5 | 12.1±2.3 | 11.9±12.6 | 0.07 |
| **HCT** | ***39-56 %*** | 42.2±6.2 | 43.3±4.8 | 46.2±4.4* | 52.4±4.2* | <0.001 |
| **HGB** | ***11-19 g/dL*** | 14.3±2.2 | 14.42±1.5 | 14.9±1.4 | 18.3±2.2* | <0.001 |
| **PLT** | ***117-460x10$^3$/µL*** | 231.6±84.1 | 233.6±75.3 | 244.1±96.7 | 284.8±108.2* | <0.05 |

*Abbreviations:* Red blood cells (RBC), white blood cells (WBC), haematocrit (HCT), haemoglobin (HGB) and platelets count (PLT).

** Statistically significant different vs Day-7.*

[a] Reference range [36]

**Table 5** Number and percentage of infested dogs examined on pre- and post-treatment days

| | Number of positive dogs per endoparasite species (%) | | | | | |
|---|---|---|---|---|---|---|
| **Study days** | *Trichuris vulpis* | Ancylostomatidae | *Capillaria aerophila* | *Toxocara canis* | *Toxascaris leonina* | *Dipylidium caninum** |
| **Day 0** | 20/41 (48.8%) | 15/41 (36.6%) | 8/41 (19.5%) | 6/41 (14.6%) | 7/41 (17.1%) | 3/41 (7.3%) |
| **Day 14** | 1/41 (2.4%) | 9/41 (22.0%) | 0/41 (0%) | 0/41 (0%) | 0/41 (0%) | 0/41 (0%) |
| **Day 28** | 2/41 (4.9%) | 15/41 (36.6%) | 4/41 (9.8%) | 0/41 (0%) | 0/41 (0%) | 1/41 (2.4%) |
| **Day 56** | 1/41 (2.4%) | 15/41 (36.6%) | 0/41 (0%) | 0/41 (0%) | 0/41 (0%) | 0/41 (0%) |
| **Day 84** | 1/39 (2.6%) | 10/39 (25.6%) | 0/39 (0%) | 1/39 (2.6%) | 0/39 (0%) | 0/39 (0%) |
| **Day 112** | 0/37 (0%) | 12/37 (32.4%) | 0/37 (0%) | 1/37 (2.7%) | 0/37 (0%) | 0/37 (0%) |
| **Day 140** | 0/37 (0%) | 13/37 (35.1%) | 0/37 (0%) | 0/37 (0%) | 0/37 (0%) | 0/37 (0%) |
| **Day 168** | 0/37 (0%) | 10/37 (27%) | 0/37 (0%) | 0/37 (0%) | 0/37 (0%) | 0/37 (0%) |

* Dogs found infected with *Dipylidium caninum* were treated with praziquantel, since this tapeworm species is not targeted by the two products tested in the study

**Table 6** Number of infested dogs and percentages of ectoparasite free dogs in the scheduled follow-ups**.** Dogs were treated with Frontline Tri-Act and Nexgard Spectra on study Day 0 and thereafter every 28 (± 2 days) up to Day 140

| | **Study days** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Day -7** | **Day 0** | **Day 1** | **Day 14** | **Day 28** | **Day 56** | **Day 84** | **Day 112** | **Day 140** | **Day 168** |
| **Fleas n/total (% free dogs)** | 20/41 (51.2%) | 24/41 (41.5%) | 1/41 (97.6%) | 0/41 (100%) | 7/41 (83.0%) | 0/41 (100%) | 0/39 (100%) | 0/37 (100%) | 0/37 (100%) | 0/37 (100%) |
| **Ticks n/total (% free dogs)** | 1/41 (97.6%) | 4/41 (90.2%) | 1/41 (97.6%) | 0/41 (100%) | 0/41 (100%) | 0/41 (100%) | 0/39 (100%) | 0/37 (100%) | 0/37 (100%) | 0/37 (100%) |

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**Chapter 1**

Section 1.2

**Treatment and long-term follow-up of a cat with leishmaniosis**

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**Abstract**

**Background:** *Leishmania* infection in cats is being increasingly reported in endemic areas. Nevertheless, only a few clinical cases have been described in cats, and even fewer have provided information on the response to treatment and a proper follow-up. Here we report a case of feline leishmaniosis not associated with any other disease or co-infection and document its response to allopurinol treatment and long-term follow-up data.

**Results:** A 6-year-old domestic shorthair female cat was referred for nodular blepharitis, mucocutaneous ulcerative lesions of the mouth and lymph node enlargement. The cat was moderately anemic, hyperglobulinemic and tested negative for feline leukemia virus and feline immunodeficiency virus. Fine needle aspirates of nodules and mucocutaneous lesions showed the presence of numerous amastigote forms of *Leishmania. Leishmania* infection was further confirmed by serology (IFAT test, 1:640) and real-time PCR (RT-PCR) on blood and conjunctival swabs. The cat was treated with allopurinol (20 mg/kg SID), which was clinically effective, although the cat remained *Leishmania*-positive in serology and RT-PCR on blood and conjunctival swabs. Allopurinol treatment was interrupted after seven months because of the healing of all lesions and lack of compliance by the owner. After two years, the cat relapsed displaying almost the same clinical signs and clinicopathological alterations. On this occasion, the parasite was isolated by culture and identified as belonging to *L. infantum*. Allopurinol treatment was started again but was interrupted several times because of the itching side effect observed. The cat worsened progressively and died two months after the relapse without any chance to shift the treatment to another molecule (e.g. meglumine antimoniate or miltefosine).

**Conclusions:** Out of all documented cases of feline leishmaniosis, the present case has the longest follow-up period and it is one of the few in which the parasite was isolated and identified. It further confirms the potential progression of *Leishmania* infection to disease in cats even in the absence of comorbidities. Veterinarians practicing in endemic areas should be aware of this susceptibility, properly include feline leishmaniosis in the differential diagnosis and propose preventative measures to those cats at risk.

**KEYWORDS:**

Allopurinol; Cat; Feline leishmaniosis; HSP70; ITS1; *Leishmania infantum*; PCR-RFLP; Sequencing analysis; Treatment

**1. Background**

Leishmaniosis, caused by *Leishmania infantum*, is one of the most important vector-borne zoonotic diseases worldwide [1]. Dogs are regarded as the main reservoir hosts of *Leishmania infantum* in endemic areas but the role of other domestic and sylvatic animals in the epidemiology of the infection has recently gained prominence [2-4].

Infection by *L. infantum* in cats has been increasingly reported in the same areas where canine leishmaniosis is endemic [4, 5]. Although the proportion of infected cats is always lower than that recorded in dogs living in an endemic area, recent epidemiological studies have suggested that the occurrence of feline leishmaniosis (FeL) might be higher than that currently thought [6]. Despite the increased interest on FeL, little information is available on clinical features, management and treatment of infected cats.

Cats are naturally infected by the same *Leishmania* species affecting dogs and humans worldwide, but progression to active disease is rare and information on adaptive immune response and mechanisms responsible for susceptibility or resistance of feline patients is lacking [7].

Of the few clinical cases reported in the literature, about the half are associated with concurrent immunosuppressive conditions, e.g. feline leukaemia virus (FeLV), feline immunodeficiency virus (FIV), diabetes or neoplasia, thus suggesting that these conditions may act as promoting factors [7]. The most recurrent clinical features in *Leishmania* infected cats are cutaneous lesions including ulcerative, crusty, nodular or scaly dermatitis [5, 7, 8]. These lesions are mainly found on the head and neck and less often on the trunk and legs. The histopathological findings of skin lesions display a diffuse granulomatous dermatitis with macrophages containing many amastigotes forms, or a granulomatous perifolliculitis and lichenoid tissue reaction/interface dermatitis, with a lower parasite load [9]. The most frequent non-cutaneous clinical signs, which have been found alone or in combination, are lymph node enlargement, ocular lesions, gingivostomatitis and decreased appetite [5]. Clinicopathological changes include hyperproteinemia with hypergammaglobulinemia and hypoalbuminemia associated with a reduced albumin/globulin ratio and biochemical abnormalities (e.g. increase of azotemia and hepatic enzymes) [10-12].

Cats affected by FeL are treated with drugs and protocols/dosages prescribed to dogs with the long-term oral administration of allopurinol being the most frequently used treatment [5]. This drug provides clinical improvement and it is generally well tolerated [5]. However, data on clinical signs, pathological alterations, diagnosis, treatment and long-term follow-up are lacking on cats with FeL [13-16].

This study reports clinical, diagnostic and therapeutical findings observed in a domestic shorthair cat with leishmaniosis along with long-term follow-up data, thus providing more evidence-based information on this scantly documented disease of cats**.**

**2.Methods**

Complete cells blood count, including red blood cells (RBC), haemoglobin (HGB), haematocrit (HCT), white blood cells (WBC) and platelets (PLT), was performed on a $K_3$EDTA blood sample using an automated haematology analyser (HeCo Vet C, SEAC, Florence, Italy). Values of serum proteins (i.e. albumin, globulins), creatinine and alanine amino-transferase (ALT) were assessed using commercially available kits by means of an automated UV spectrophotometer (Slim, SEAC). Serum protein fractions were assessed using an automated system (Sel Vet 24, SELEO Engineering, Naples, Italy) according to the manufacturer's instructions. Infection by FeLV and/or FIV was first tested using an ELISA rapid assay (SNAP Combo FeLV antigen/FIV antibody, IDEXX Laboratories, Westbrook, ME, USA) and further assessed by PCR (FeLV) and nested PCR (FIV) [6]. Smears of the material collected by fine-needle-aspiration of cutaneous lesions were stained using May-Grünwald-Giemsa quick stain (Bio-Optica, Milan, Italy) and microscopically

observed at low (200×) and high magnification (1000×). An immunofluorescence antibody test (IFAT) for antibodies against *L. infantum* and real-time PCR (RT-PCR) for parasite kinetoplast DNA from blood and conjunctival swabs were performed as described elsewhere [17, 18].

The parasite was isolated in EMTM and Sloppy Evans medium cultures, and the strain was identified using PCR-restriction fragment length polymorphism and sequencing analysis of ITS1 spacer and the HSP70 gene [19].

**3. Results**

In October 2014, a 6-year-old domestic shorthair female cat living in the urban area of the city of Messina, southern Italy (38°11′39″48N, 15°33′1″80E) was referred to a private veterinary clinic with dermal, oral and ocular alterations. Physical examination revealed areas of nodular dermatitis on the eyelids and left carpal region, blepharitis, conjunctivitis, mucocutaneous ulcerative lesions on the mouth and lymph node enlargement (Fig. 1 a, b). The cat was moderately anemic and hyperglobulinemic at the complete blood count and serum protein electrophoresis (Table 1), respectively, and tested negative for FeLV and FIV. The initial differential diagnosis included eosinophilic granuloma complex, feline gingivostomatitis complex and neoplasia (e.g. squamous carcinoma or lymphoma) and *Poxvirus* or *Mycobacterium* infections. The cat therefore underwent palliative treatment consisting of antibiotic and corticosteroid (enrofloxacin 5 mg/kg PO SID and prednisone 2.5 mg/kg SID). All the above conditions were excluded according to cytology results, while the presence of numerous macrophages containing intracytoplasmic forms consistent with amastigotes of *Leishmania* (Fig. 2) was documented on fine-needle-aspirates of dermal and mucocutaneous lesions. The cat scored positive to IFAT with a 1:640 IgG titre and positive to RT-PCR on both blood and conjunctival swabs. After the diagnosis of leishmaniosis, palliative treatments were suspended and the cat was treated with days and allopurinol (20 mg/kg PO SID). Three months after commencing allopurinol therapy, nodular dermatitis and conjunctivitis resolved, while ulcerative lesions on the mouth improved significantly (Fig. 1c). Despite the clinical improvement, the cat was persistently positive to *L. infantum* at both serology (1:320) and RT-PCR on blood and conjunctival swabs. Therefore, the treatment was continued with allopurinol at the same dose regime. After seven months, allopurinol was suspended because of a lack of compliance by the owner and due to potential side effect (i.e. intense itch and scratch on the trunk) observed in the last weeks of treatment. Indeed, while potential causes of the itch were excluded (e.g. flea infestation) this sign ceased a few days after the suspension of allopurinol without any other therapy. On this follow-up the cat was apparently healthy (Fig. 1d) but further serological and RT-PCR studies were declined by the owner.

After two years, in September 2017, the cat relapsed showing almost the same clinical signs upon clinical examination (Fig. 3) and hematological and biochemical abnormalities were observed as well (Table 1). In particular, the cat showed hypochromic and microcytic anemia, while the protein profile analysis highlighted hyperproteinemia and alteration of electrophoresis (Fig. 4) with hypergammaglobulinemia, hypoalbuminemia and, consequently, a reduced albumin/globulin ratio (0.2) [20, 21].

On this occasion, the parasite was isolated in culture starting from the material collected by fine-needle-aspirate of the nodular lesion on the eyelid, and identified as belonging to *L. infantum*. Unfortunately, the cat owner allowed only domiciliary consultations, and was not compliant in collecting samples (e.g. urine) nor in allowing medical interventions such as sedation or cystocentesis. This impaired the accurate evaluation of alterations and the proper staging of the disease. In addition, despite the suspected adverse reaction observed during the first course of treatment, allopurinol was prescribed again at the same dose regime due to the reluctance of the owner in using other drugs with a complicated route of administration (e.g. subcutaneous injection) or those which were expensive. The treatment with allopurinol was,

however, interrupted several times because of the occurrence of the intense itching observed soon after starting this therapy. The cat worsened progressively due to the irregular treatment administration and died two months after the relapse without any chance to shift the treatment to another molecule (e.g. meglumine antimoniate or miltefosine).

**4. Discussion**

Here, we report the clinical signs, pathological findings, allopurinol treatment and 38-month follow-up period of a cat affected by FeL with no other concomitant infections or diseases. Since FeL infection is not usually regarded by practitioners, even in *Leishmania*-endemic areas, its diagnosis is usually not included in the panel of the diagnostic agents for this animal species. The present report, however, confirms the susceptibility of cats to *L. infantum* infection and the progression to disease even in the absence of concurrent immunosuppressive conditions [8]. Significant associations have been found between retroviral infection (i.e. FIV) and FeL, and it has been estimated that about half of the FeL cases reported in literature were associated with impaired immune-competence caused by co-infections or comorbidities [7]. In the present case, FIV and FeLV infections were excluded and cytological examination performed on mucocutaneous ulcerous and nodular dermatitis documented only the presence of numerous *Leishmania* amastigotes and granulomatous reaction. Although the cat was not tested for other vector-borne diseases (i.e. ehrlichiosis, anaplasmosis, bartonellosis), clinical presentation, laboratory abnormalities and an especially good response to specific therapy for leishmaniosis made these co-infections unlikely.

Nodular dermatitis, mucocutaneous lesions and ocular disorders are the most frequent signs of clinical FeL usually associated with clinicopathological alterations such as anaemia, leukocytosis, hyperglobulinemia and hypoalbuminemia, as reported for canine leishmaniosis [10-12]. All the above signs and alterations, alone or in combinations, should always alert clinicians to include leishmaniosis in the differential diagnosis process of diseased cats that reside in or have travelled to *Leishmania* endemic areas. The long-term administration of allopurinol was clinically effective resulting in the apparent resolution of the lesions in about seven months. However, the treatment was not effective in curing the infection/eliminating the parasite as demonstrated by molecular and cytological tests in the subsequent follow-ups. It is, however, difficult to assess whether the relapse observed after two years was induced by a reactivation of the previous infection (as suggested by the reappearance of same lesions) or by further re-infections. Indeed, during these two years the cat was not protected with any preventative measure against sand flies and therefore it cannot be excluded that it was subjected to further infective bites. Notably, a matrix collar impregnated with imidacloprid and flumethrin, licensed for the use in cats, has recently proved to be effective in reducing *L. infantum* infection in a cohort of naturally exposed cats [22]. As in dogs, preventative measures by means of repellent products should be adopted for the prevention against sand fly bites and for reducing the risk of *L. infantum* infection [23]. As demonstrated through xenodiagnosis [24], cats with leishmaniosis are infective to sand flies and thus may participate in sustaining the parasite cycle and spreading the disease. Despite the fact that cats are not regarded as a primary reservoir host, the parasitic load in this animal species may be high [25] as observed in this case where a high number of amastigotes was observed in microscopic fields from skin lesions aspirates and successfully used to isolate the parasite in culture. Although the isolation and characterization of *Leishmania* parasites from infected cats are rarely reported [5, 26, 27], the strain herein identified was *L. infantum*, the most common species circulating among dogs, humans and other animal species in the Mediterranean area [2].

Long-term administration of allopurinol is regarded as the most effective treatment for FeL [7]. Although the molecule is generally well tolerated, information on pharmacokinetic and pharmacodynamics as well as safety are lacking for cats. In the present study, itching and scratching were observed and considered as related side effects in the first and especially in the second course of treatment; these side effects were observed soon after starting the therapy. So far, the sole side

effects of allopurinol treatment are the elevation of hepatic enzymes and toxicity to kidneys in cats [12, 28], although very recently dermatological signs compatible with a cutaneous adverse drug reaction were described in a *L. infantum*-positive cat treated with allopurinol [29]. Therefore, even though allopurinol currently has the most extensive clinical experience available for FeL, its administration to cats needs to be strictly monitored and fine-tuned according to clinical response, owner compliance and safety.

The case herein reported describes the course of a FeL case that survived 38 months after the first diagnosis with a well-maintained quality of life, except in the last months after the relapse in which it worsened rapidly despite treatment attempts. According to a recent retrospective evaluation of 14 cases of FeL, the median survival time is three months after the first diagnosis and it seems that there are no significant differences among treated cats (median time five months), not treated (median time one month) and FIV co-infected (median two and a half months). Therefore, the expectancy of life of FeL patients is not significantly influenced by therapy or retroviral coinfection [12]. However, treatment of FeL should be always attempted since it may provide a better quality of life to diseased animals, a longer survival time and a significant reduction of the parasite load which, in turn, may result in a lower infectivity to sand flies.

## 5. Conclusion

This study further confirms the potential progression of *Leishmania* infection to disease in a cat patient even in the absence of comorbidities. Although the long-term allopurinol treatment provided an improvement of the clinical manifestation, it was unsuccessful in controlling the disease. In addition, despite the drug being well tolerated, side effects may appear and the patients should be systematically monitored during the treatment course. Veterinarians practicing in endemic areas should be aware of the susceptibility of cats to *Leishmania* infection, properly include FeL in the differential diagnosis and propose preventative measures to those cats at risk.

## Abbreviations

FeL: feline leishmaniosis; FeLV: feline leukemia virus; FIV: feline immunodeficiency virus; PCR: polymerase chain reaction; IFAT: immunofluorescence antibody test; EMTM: Evans' modified Tobie's medium.

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## Availability of data and materials

All data generated or analyzed during this study are included in this published article. The parasite strain is stored at the Istituto Superiore di Sanità, Rome, Italy, under the accession code MFEL/IT/2017/ISS3203-Gatto BBB106-Messina001.

## Authors' contributions

EB conceived and designed the study. NC, EN, JMA, GG and FA carried out the veterinary examination and laboratory work. EB drafted the first version of the manuscript. RI, SG, MG and DO critically reviewed the manuscript. All the authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The cat was managed in line with the GCP principles. The cat was not included in any experimental study and all the procedures to which the animal underwent were approved by the owner and performed for diagnostic, treatment and/or follow-up purposes.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Fig. 1 a** Clinical signs observed in the leishmaniotic cat at the first veterinary examination (October 2014). Note the nodular dermatitis of eyelids on the left carpal region and the nodular conjunctivitis in the right eye. **b** Clinical signs on the face of the cat at the beginning of allopurinol treatment (October 2014). Note the vast ulcerous area of the mucocutaneous junction of the lips. **c** Three-month follow-up. **d** Seven-month follow-up

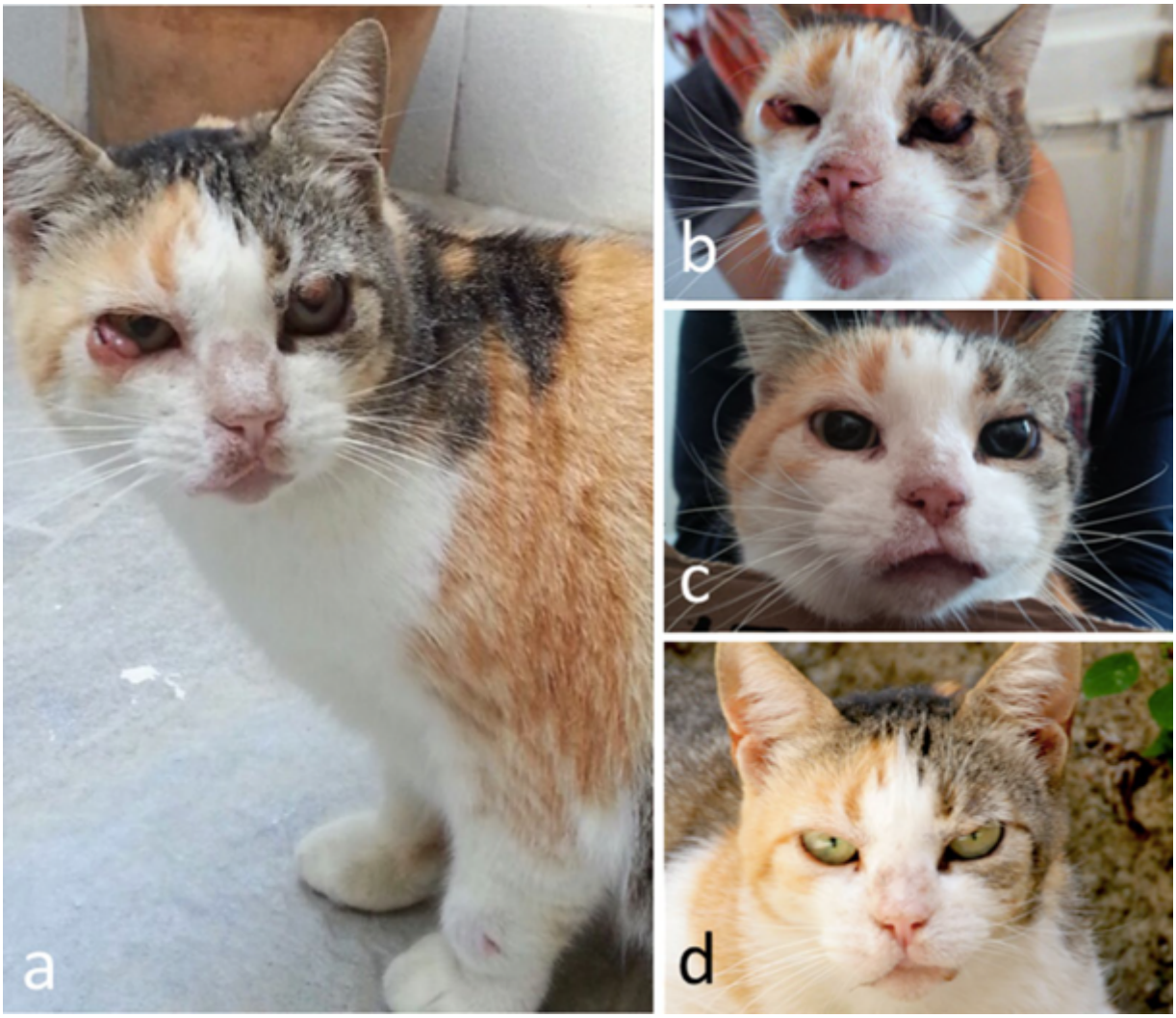


**Table 1** Haematological and biochemical parameters determined in the leishmaniotic cat at the first veterinary examination before treatment (October 2014), and at the relapse (September 2017)

| | **October (2014)** | **September (2017)** | **Reference range** |
|---|---|---|---|
| Haematology[a] | | | |
| WBC ($\times 10^3/\mu l$) | 23.4 | 13.7 | 6.0–17.0 |
| RBC ($\times 10^6/\mu l$) | 6.2 | 4.4 | 5.50–8.5 |
| HCT (%) | 27.5 | 27.1 | 37–55 |
| HGB (g/dl) | 8.9 | 7.8 | 12–18 |
| MCV (fl) | 44.1 | 61 | 60–77 |
| MCH (g/dl) | 14.3 | 17.5 | 20–25 |
| MCHC (%) | 32.4 | 28.6 | 32–36 |
| RDW (%) | 26.8 | 16.6 | 15–27 |
| PLT ($\times 10^3/\mu l$) | 288 | 183 | 200–500 |
| Blood chemistry[b] | | | |
| Total protein (g/dl) | 8.1 | 9.0 | 5.4–7.8 |
| Albumin (g/dl) | 2.9 | 2.1 | 2.1–3.3 |
| Total globulins (g/dl) | 5.2 | 7.9 | 2.8–5.1 |
| A/G | 0.6 | 0.2 | 0.6–1.2 |
| Creatinine (mg/dl) | 1.1 | 0.9 | 0.8–1.8 |
| ALT (U/l) | 24 | 23 | 6–83 |

[a] **Reference range** [18]

[b] **Reference range** [19]

**Fig. 2** Cytology of the fine needle aspirate of the nodular skin lesion on the eyelid. Note the high load of Leishmania infantum amastigotes. May-Grünwald-Giemsa quick stain, 400Å~. Scale-bar: 30 µm

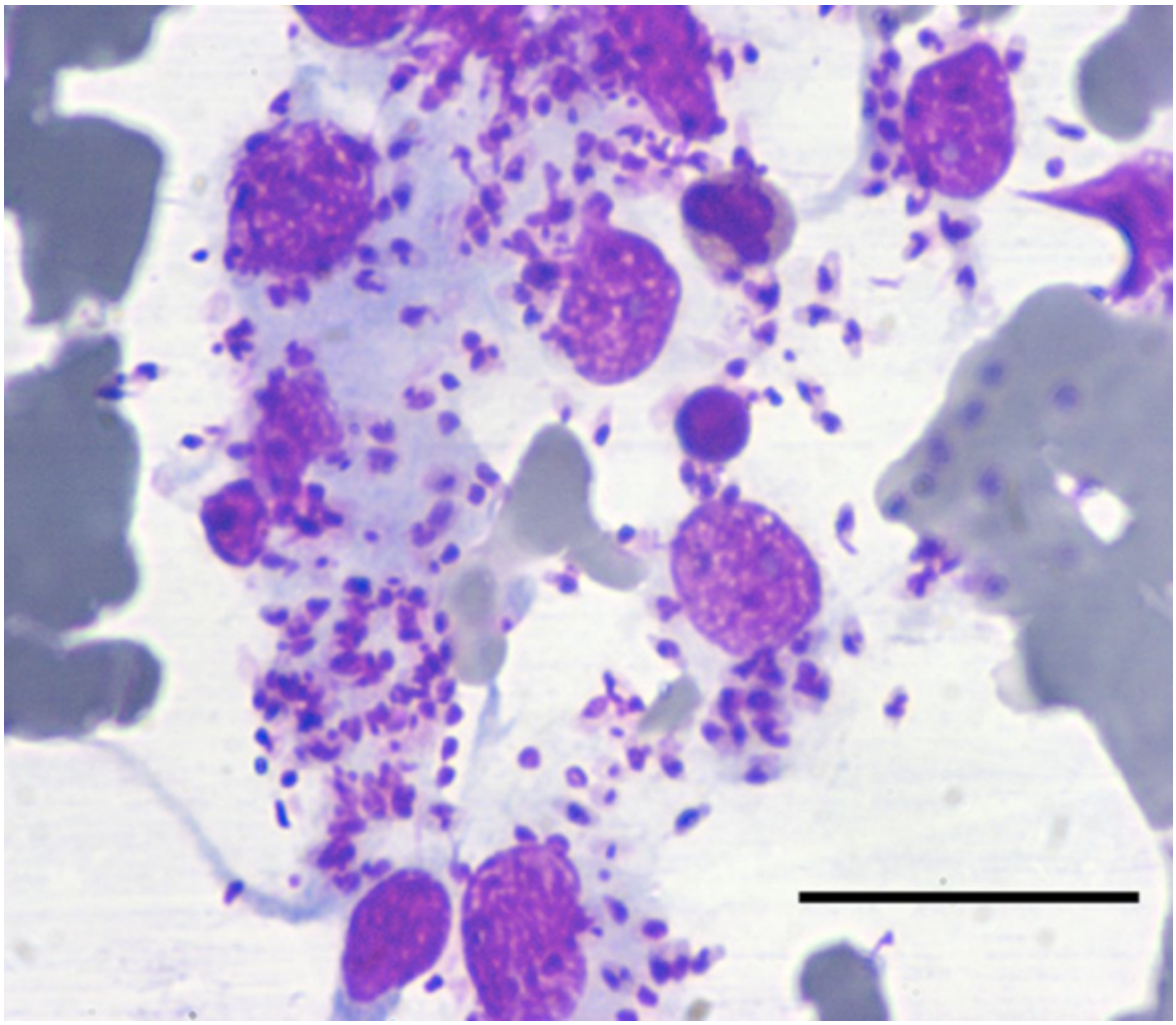
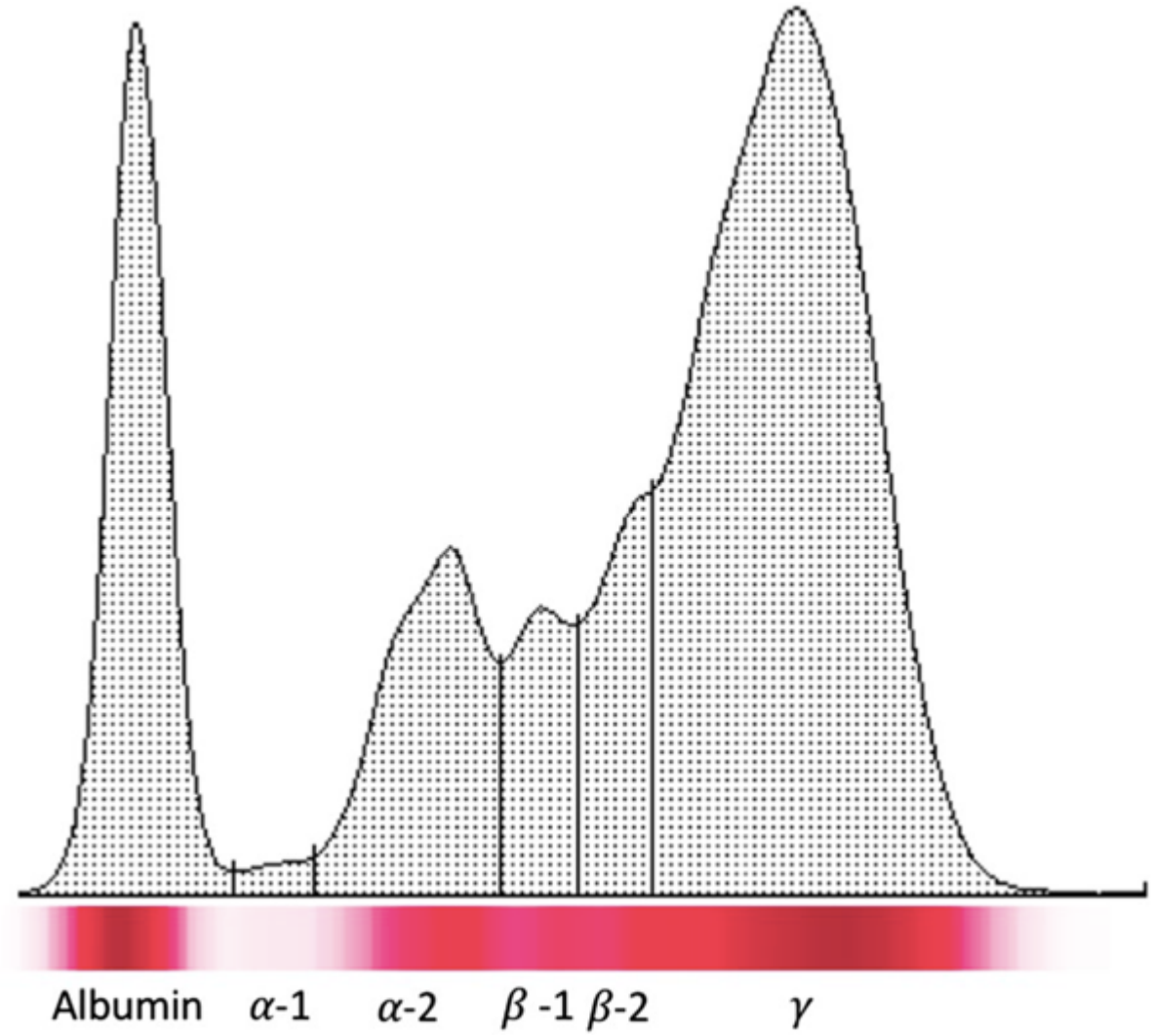


**Fig. 3** Clinical signs observed in the leishmaniotic cat at the relapse in September 2017

**Fig. 4** Cellulose acetate electrophoretograms of serum proteins of the leishmaniotic cat at the relapse in September 2017



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**Chapter 2**

Section 2.1

## Do Different LED Colours Influence Sand Fly Collection by Light Trap in the Mediterranean?

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**Abstract**

Light traps represent the most used attractive system to collect and monitor phlebotomine sand flies. Recent studies have suggested that light traps can be easily upgraded by the use of light-emitting diode (LED) with positive effects on trap design, weight, and battery life. However, scant data on the effect of different LED colours on the attractiveness to phlebotomine sand fly species are available in literature. In this study, the capture performances of light traps equipped with different LED colours on phlebotomine sand fly species indigenous in the Mediterranean area were evaluated. Phlebotomine sand fly collections were performed using a classical light trap (CLT), equipped with a traditional incandescent lamp, and five Laika 4.0 light traps supplied, each with LED of different colours and wavelengths: (i) white; (ii) red; (iii) green; (iv) blue; (v) UV. Light traps were set for three consecutive nights fortnightly from May to October 2017 and climate data recorded using a meteorological station. A total of 411 phlebotomine sand flies (191 males and 220 females), belonging to three different species, namely, *Phlebotomus perniciosus* (n= 298, 141 males and 157 females), *Sergentomyia minuta* (n=110, 48 males and 62 females), and *Phlebotomus neglectus* (n=3, 2 males and 1 females) were collected. Abundance of capture was influenced by colours of LED and time. The highest number of phlebotomine sand flies was captured on June ($P<0.01$) and by UV LED ($P<0.01$). As regard to species, *P. perniciosus* was mainly captured by UV LED on June ($P<0.01$). No effect of time ($P>0.05$) or LED colour ($P>0.05$) was recorded for *S. minuta* and *P. neglectus*. According to the results of the present study light trap equipped with UV LED can represent an effective tool for the capture of sand fly species in the Mediterranean area.

## 1.Introduction

Phlebotomine sand flies (Diptera: Psychodidae) are small nocturnal insects that act as vectors of various infectious and parasitic agents including canine and human *Leishmaniosis*. These insects play a crucial role in the epidemiology of relevant diseases being some of great veterinary and medical importance; their monitor and control are, therefore, of pivotal importance.

The system for trapping phlebotomine sand flies can be categorized into attractive (e.g., light traps and $CO_2$) or passive (e.g., sticky traps) traps, and the different methods may influence the capture outcomes including specific attraction to sand fly species in a given area [1]. Although phlebotomine sand flies are nocturnal/crepuscular insects and their flight activity increases in relation to the decrease of the daily light intensity [2], they are contemporarily attracted by artificial light; thus, light traps are largely employed for the collection of these insects. Interestingly, insects are attracted by light of different colours and intensity relating to their retina structure and to the presence of photoreceptors. Honeybees, for instance, have three photoreceptors (i.e., ultraviolet, blue, and green); in butterfly the retinas have six or more photoreceptor classes [3]. It has been demonstrated that the eyes of the adult sand fly *Lutzomyia longipalpis* reacted maximally to light in the ultraviolet region (at 340 nm) with a secondary peak in the blue-green-yellow region at 520-546 nm [4].

Light trap methods have recently been improved by the use of light-emitting diode (LED) [5-8]. LED light traps have some advantages compared to traditional light trap models including the low electric consumption resulting in a longer battery life and in a longest lifetime of the lamp. The LED technology allows easily customizing the colour and the intensity of the light. Some authors showed that LED of different colours could have different power of attraction demonstrating, for instance, that *Phlebotomus papatasi* is more attracted by red LED [9], while *Nyssomyia whitmani* and *Lutzomyia longipalpis* seem to be more attracted by blue and green LED, respectively [10]. However, no data on the effect of different LED colours on other *Phlebotomus* species present in Mediterranean area are available in the literature. Therefore, the aim of the present study is to evaluate the capture performances of light traps equipped with different LED colours to phlebotomine sand fly species endemic in the Mediterranean area.

## 2. Materials and Methods

*2.1. Study Area and Collection Procedures.* The study was conducted from May to October 2017, which corresponds to the sand fly season in the study area [11], in the municipality of Messina, an area highly endemic for canine leishmaniosis where the presence of competent sand fly species has been previously reported [11, 12]. The traps were placed in a suburban area nearby the horse stables of the Department of Veterinary Sciences of the University of Messina (38°13′59″N; 15°32′48.99″E; 263 m a.s.l.).

Sand fly collection was performed using both a classical light trap (named CLT), equipped with a traditional incandescent lamp (12V, 8W) and five Laika 4.0 light traps supplied, each with LED of different colours and wavelengths: (i) white/455 nm; (ii) red/620 nm; (iii) green/530 nm; (iv) blue/470 nm; (v) UV/395 nm. The six traps were set opposite to a stonewall at 50 cm above the ground [11] and at about 3 meters apart from each other (Figures 1(a) and 1(b)). Both CLT and LED traps were placed for three consecutive days twice a month (six days per month), from May to October 2017. Traps were switched-on before sunset (18:00) and left working for 13 hours (up to 7:00 a.m.). Net-bags of traps were collected and replaced after each day of collection. Temperature (°C), relative humidity (RH%), and wind intensity (WI) were recorded using a meteorological station placed in the same area of traps.

*2.2. Sand Fly Identification.* Phlebotomine sand flies collected were initially separated from other insects, differentiated by sex and stored in vials containing 70% ethanol. For species identification, the external genitalia of males and the head and posterior last tergites of females were dissected, cleared, and slide-mounted as described elsewhere [8]. Identification was performed using morphological keys [13].

*2.3. Data Analysis.* Due to the limited number of phlebotomine sand flies collected in each sampling session, the data were merged according to the month of capture. Two-way analysis of variance (ANOVA) was applied in order to evaluate the effect of time (i.e., month) and traps (i.e., CLT or LED traps) on abundance and sand fly species. When significant differences were found, Bonferroni's post hoc comparison was applied.

For the best represented species, Pearson's chi-square analysis was applied to evaluate statistically significant difference in the number of male and female specimens.

Statistically significant values were set for $P$ values $< 0.05$. The statistical analyses were performed using the STATISTICA software package (STATISTICA 7 for Windows, Stat Software Inc., Tulsa, Oklahoma).

**3. Results**

A total of thirty-six sampling days were carried out throughout the study, but no phlebotomine sand flies were captured in the first four (May) and in the last six capture days (October). The number of captured phlebotomine sand flies along with environmental parameters recorded during the sampling days are summarized in Table 1. Overall, 411 specimens, belonging to three different species, namely, *Phlebotomus perniciosus* (n=298, 141 males and 157 females), *Sergentomyia minuta* (n=110, 48 males and 62 females), and *Phlebotomus neglectus* (n=3, 2 males and 1 females) were collected (Table 2). A statistically significant effect of time (month) and trap model found that the highest number of specimens was collected in the month of June and by blue LED, UV LED, and CLT (Figure 2). Similarly, in regard to species, *P. perniciosus* was mainly captured in June ($P<0.01$) and by UV LED (Figure 3); no differences in the number of male and female specimens of *P. perniciosus* captured by the three more efficient traps (i.e., blue LED, UV LED, and CLT traps) were observed (Figure 4). Neither effect of sampling period nor of trap model was found on the number of *S. minuta* and *P. neglectus* captured during the study.

**4. Discussions**

The advent of LED technology has substantially improved light trap performances. Despite the fact that previous surveys have demonstrated how light trap using LED technologies can be valid alternative to classical models, the attractiveness of different colours LED has not been explored in deep, so far. The present study investigated the capture performances of light trap equipped with LED of different colour and wavelength on sand fly species endemic in the Mediterranean area. According to the herein results, it may be speculated that light trap equipped with UV LED of 395 nm has a higher attractiveness compared to other coloured LED traps. This is particularly evident for the species *P. perniciosus* which is one of the main vectors of leishmaniosis by *L. infantum* to both humans and dogs [14].

The species captured in the present survey are the same reported in other previous investigations performed in the same area [11, 12, 15], where *P. perniciosus* and *S. minuta* were the most abundant species. Though the well-known role of *P. perniciosus* is being competent vector of *L. infantum,* that of *S. minuta* is still unclear and needs further investigations. Recently, [16] reported the first molecular detection of *L. infantum* in *S. minuta* in southern Portugal and the first isolation of human blood as meal source in an engorged female of *S. minuta.* These findings opened a debate on the potential role

of this species in the transmission of leishmaniosis and its involvement in the epidemiology deserves further researches including, but not limited to, protozoan isolation from engorged specimens as well as experimental transmission.

The limited presence of *P. neglectus* here observed is consistent with previous reports in southern Italy [11, 17] and justified by the higher abundance of this species in northern Italian regions which are featured by cooler climate [18, 19].

The attractiveness of different light technologies and colours could bias the estimation of sand fly population in a specific area. In fact, as demonstrated by a study that compared the attractiveness of different LED colours, *Phlebotomus papatasi* was attracted four times more by red LED compared to blue and green and twice with respect to incandescent lamp [9]. A study performed in Brazil demonstrated that Hoover Pugedo light traps equipped with green or blue LED or incandescent lamps showed the same attractiveness power for *Nyssomyia whitmani* and *Lutzomyia longipalpis* [10]. In another study, the CDC and Disney traps were, respectively, more efficient in the capture of *Lutzomyia ovallesi* and *Lutzomyia olmeca* compared to LED light trap [20]. In a previous study conducted by our group [8] the attractiveness of a Laika trap equipped with both white and UV LED was compared to CLT, no significant differences in the trapping performance of the two traps were recorded in that study. Here, the Laika model equipped with UV LED showed the best capture performances in general and to *P. perniciosus* in particular. These findings suggest that the eyes of *P. perniciosus* react maximally to light in the ultraviolet region as already observed for *Lutzomyia longipalpis* [4]. However, spectral sensitivities using electroretinograms need to be determined in order to clarify whether the greater attractiveness of the UV LED for this species is linked to a higher sensitivity of the eyes to wavelength of the ultraviolet region (395 nm) or to the low brightness emitted by this LED colour.

In addition to trapping methods, sand fly abundance and richness may be influenced considerably by other variables including animals' presence and environmental and climate variables [21]. In particular, the environmental factors have been used to predict and elucidate the distribution of diseases transmitted by vectors [22]. The climatic factors including temperature, humidity, wind, and rainfall could influence the distribution of vectors including sand fly species [22, 23]. A previous study conducted in the same area recorded two peaks of sand fly collection on August and September [11], in the present study instead only one peak was observed in the month of June. Moreover, phlebotomine sand flies were captured earlier in the here survey compared to the aforementioned study in which the first positive trapping session was observed in late June. In regard to this difference, it is interesting to notice that the summer season in which the present study was conducted was featured by very hot temperature especially in July (28.6 ± 3.2°C) and August (28.8 ± 5.0) and high humidity in September (69.3 ± 13.1%) and October (72.5 ± 15.6%) caused by intense rains. These particular climatic conditions may explain the decline in the number of sand flies during the hottest months and their complete absence in October.

In conclusion, this study showed how light trap equipped with UV LED of 395 nm has superior performances to other LED traps equipped with blue or green or white or red lamps for the capture of sand flies including important *Leishmania* vector species such as *P. perniciosus*. By virtue of their performances and technology, UV LED trap may represent a highly efficient alternative to CLT for the capture and monitoring of the sand fly species endemic in the Mediterranean.

**Data Availability**

Data supporting the findings of this study is included within the article.

**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

**Authors' Contributions**

Ettore Napoli and Francesca Arfuso contributed equally to this paper.

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FIGURE 1: Classical light trap (CLT) and five Laika traps equipped with red, green, white, UV, and blue LEDs placed in the study area. (a) Diurnal vision; (b) nocturnal vision.

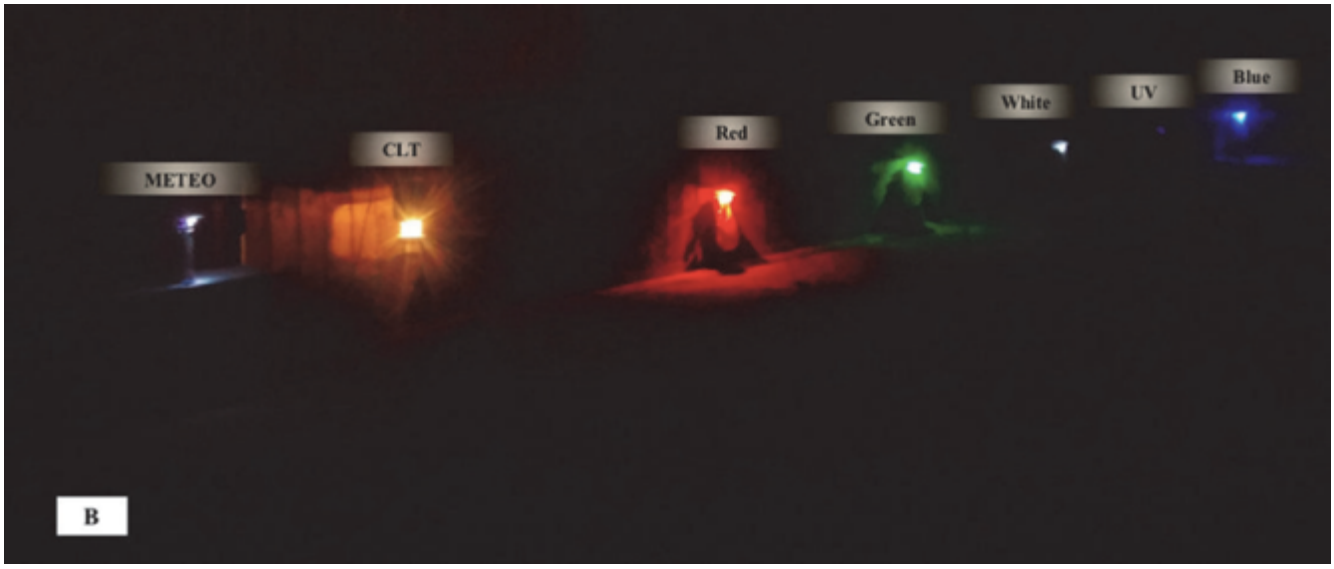



TABLE 1: Number of phlebotomine sand flies captured in the study along with environmental parameters recorded during the trapping days. Environmental parameters are provided as means of values recorded during each trapping day (i.e. from 6 p.m. to 7 a.m.).

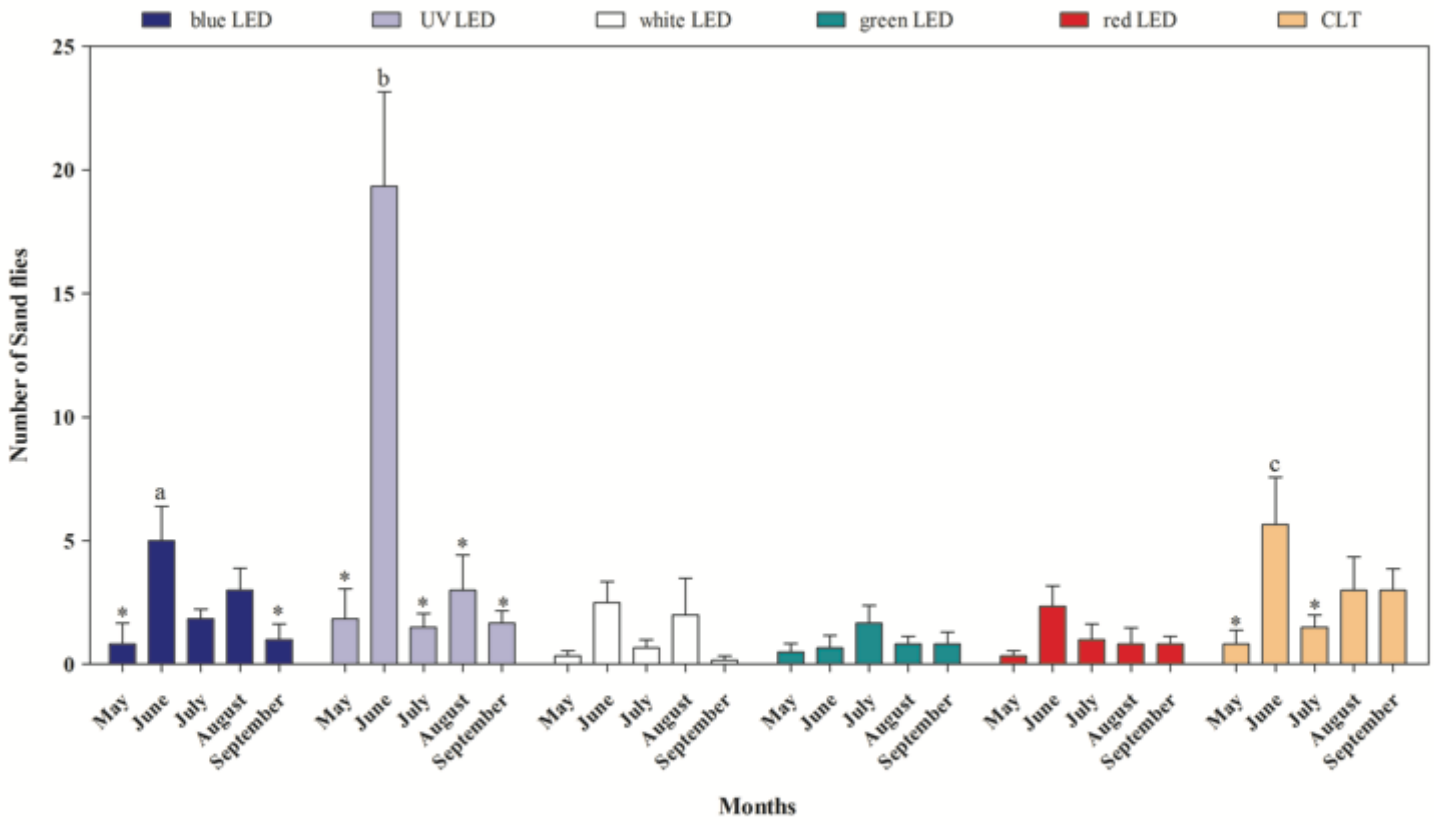
| Month | Sampling day | Sand fly number | Temperature (°C) | Relative Humidity (%) | Wind Intensity (km/h) |
|---|---|---|---|---|---|
| **May** | **15** | 0 | 23 | 46 | 23 |
| | **16** | 0 | 24 | 64 | 18 |
| | **17** | 0 | 22 | 58 | 20 |
| | **24** | 0 | 23 | 63 | 24 |
| | **25** | 15 | 21 | 57 | 17 |
| | **26** | 13 | 25 | 49 | 12 |
| **June** | **13** | 41 | 24 | 57 | 21 |
| | **14** | 29 | 25 | 49 | 28 |
| | **15** | 46 | 24 | 61 | 26 |
| | **27** | 61 | 28 | 55 | 10 |
| | **28** | 12 | 29 | 50 | 13 |
| | **30** | 24 | 28 | 63 | 14 |
| **July** | **11** | 10 | 31 | 45 | 17 |
| | **12** | 10 | 33 | 36 | 19 |
| | **13** | 15 | 30 | 60 | 26 |
| | **25** | 3 | 25 | 73 | 25 |
| | **26** | 7 | 25 | 67 | 19 |
| | **27** | 4 | 27 | 54 | 22 |
| **August** | **1** | 7 | 33 | 30 | 19 |
| | **2** | 6 | 33 | 28 | 21 |
| | **3** | 3 | 34 | 27 | 27 |
| | **29** | 9 | 25 | 87 | 17 |
| | **30** | 39 | 23 | 84 | 11 |
| | **31** | 12 | 24 | 74 | 10 |
| **September** | **11** | 14 | 24 | 73 | 19 |
| | **12** | 8 | 22 | 84 | 20 |
| | **13** | 2 | 27 | 50 | 18 |
| | **25** | 8 | 22 | 62 | 15 |
| | **26** | 10 | 22 | 64 | 9 |
| | **27** | 3 | 21 | 82 | 9 |
| **October** | **9** | 0 | 17 | 88 | 8 |
| | **10** | 0 | 19 | 96 | 10 |
| | **11** | 0 | 26 | 68 | 13 |
| | **16** | 0 | 20 | 57 | 9 |
| | **17** | 0 | 20 | 65 | 9 |
| | **18** | 0 | 21 | 61 | 18 |

TABLE 2: Number and percentages of phlebotomine sand fly species captured by each trap model in the study.

| Traps | Species | Study period | | | | |
|---|---|---|---|---|---|---|
| | | **May** n/total (%) | **June** n/total (%) | **July** n/total (%) | **August** n/total (%) | **September** n/total (%) |
| **blue LED** | *Phlebotomus perniciosus* | 1/5 (20) | 20/30 (66.7) | 3/11 (27.3) | 9/18 (50) | 4/6 (66.7) |
| | *Sergentomyia minuta* | 4/5 (80) | 10/30 (33.3) | 8/11 (72.7) | 9/18 (50) | 2/6 (33.3) |
| | *Phlebotomus neglectus* | 0/5 (0) | 0/30 (0) | 0/11 (0) | 0/18 (0) | 0/6 (0) |
| **UV LED** | *Phlebotomus perniciosus* | 10/11 (90.9) | 104/116 (89.7) | 8/9 (97.1) | 14/18 (77.8) | 6/10 (60) |
| | *Sergentomyia minuta* | 1/11 (9.1) | 12/116 (10.3) | 1/9 (2.9) | 4/18 (22.2) | 4/10 (40) |
| | *Phlebotomus neglectus* | 0/11 (0) | 0/116 (0) | 0/9 (0) | 0/18 (0) | 0/10 (0) |
| **white LED** | *Phlebotomus perniciosus* | 2/2 (100) | 9/15 (60) | 0/4 (0) | 9/12 (75) | 1/1 (100) |
| | *Sergentomyia minuta* | 0/2 (0) | 5/15 (33.3) | 4/4 (100) | 3/12 (25) | 0/1 (0) |
| | *Phlebotomus neglectus* | 0/2 (0) | 1/15 (6.7) | 0/4 (0) | 0/12 (0) | 0/1 (0) |
| **green LED** | *Phlebotomus perniciosus* | 1/3 (33.3) | 2/4 (50) | 3/10 (30) | 0/5 (0) | 4/5 (80) |
| | *Sergentomyia minuta* | 2/3 (66.7) | 2/4 (50) | 7/10 (70) | 5/5 (100) | 0/5 (0) |
| | *Phlebotomus neglectus* | 0/3 (0) | 0/4 (0) | 0/10 (0) | 0/5 (0) | 1/5 (20) |
| **red LED** | *Phlebotomus perniciosus* | 1/2 (50) | 10/14 (71.4) | 0/6 (0) | 0/5 (0) | 2/5 (40) |
| | *Sergentomyia minuta* | 1/2 (50) | 4/14 (28.6) | 6/6 (100) | 5/5 (100) | 2/5 (40) |
| | *Phlebotomus neglectus* | 0/2 (0) | 0/14 (0) | 0/6 (0) | 0/5 (0) | 1/5 (20) |
| **CLT** | *Phlebotomus perniciosus* | 5/5 (100) | 33/34 (97.1) | 5/9 (55.6) | 15/18 (83.3) | 17/18 (97.1) |
| | *Sergentomyia minuta* | 0/5 (0) | 1/34 (2.9) | 4/9 (44.4) | 3/18 (16.7) | 1/18 (2.9) |
| | *Phlebotomus neglectus* | 0/5 (0) | 0/34 (0) | 0/9 (0) | 0/18 (0) | 0/18 (0) |

Legend: blue LED, UV LED, white LED, green LED, red LED= Laika traps equipped with blue, UV, white, green and red LEDs respectively; CLT = Classical light trap equipped with incandescent lamp.

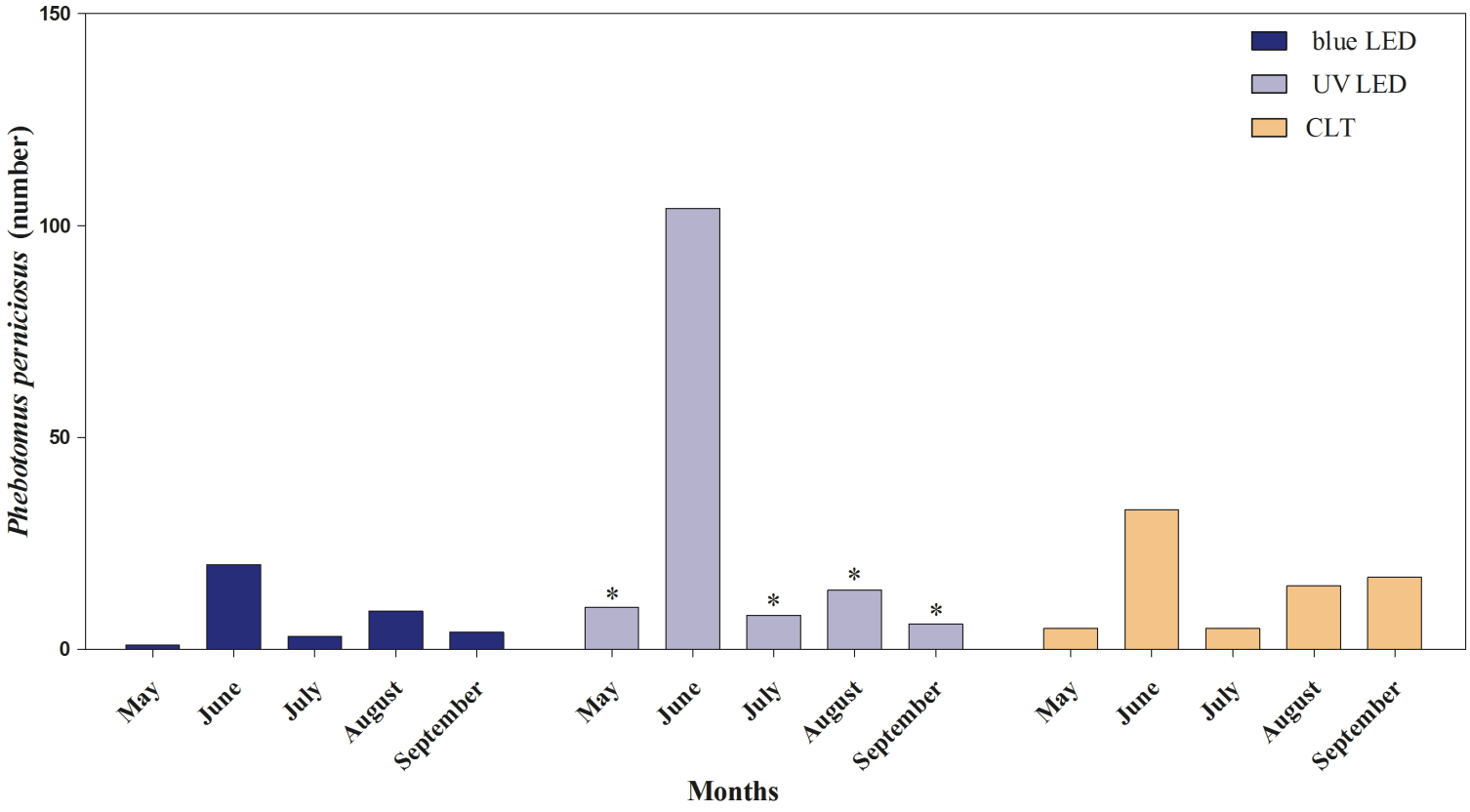
FIGURE 2: Total number of phlebotomine sand flies captured by classical light trap (CLT) and five Laika traps equipped with red, green, white UV, blue LEDs.




***Statistically significant effect of LED colours:*** *[a]vs UV LED and green LED (P<0.01); [b]vs white LED, green LED and red LED (P<0.001); [c]vs UV LED, white LED, green LED and red LED (P<0.05)*

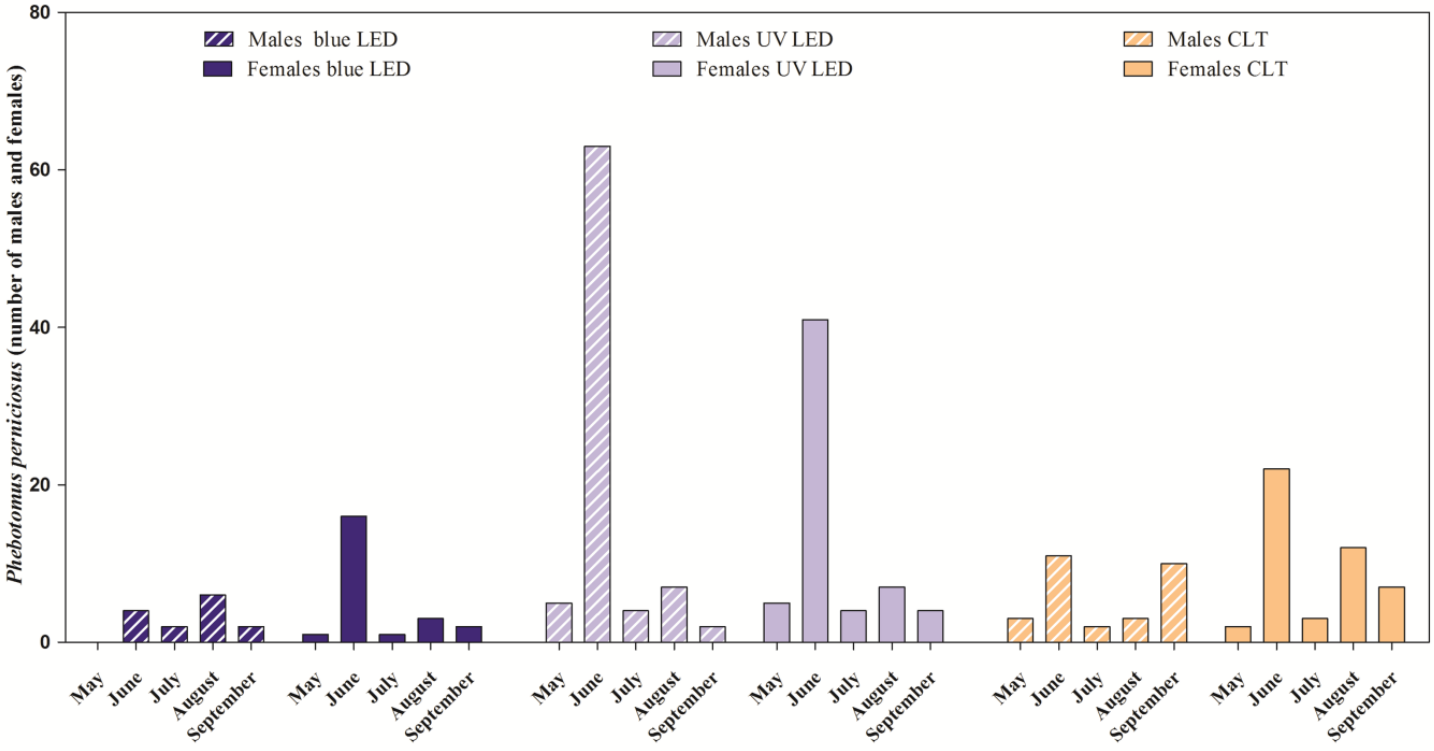
***Statistically significant effect of months:*** **vs June (P<0.01)*

FIGURE 3: Total number of *Phlebotomus perniciosus* captured by classical light trap (CLT) and blue and UV Laika traps.




***Statistically significant effect of months:*** **vs June (P<0.01)*

FIGURE 4: Number of males and females of *Phlebotomus perniciosus* captured by classical light trap (CLT) and blue and UV Laika trap along with results of Pearson's chi-square test.




| Months | Traps | Pearson's chi-square results | |
|---|---|---|---|
| | | chi-square value | *P* value |
| May | blue LED | 0.83 | 0.36 |
| | UV LED | 0.92 | 0.34 |
| | CLT | 0.09 | 0.76 |
| June | blue LED | 0.48 | 0.05 |
| | UV LED | 0.50 | 0.48 |
| | CLT | 0.49 | 0.48 |
| July | blue LED | 0.24 | 0.62 |
| | UV LED | 0.90 | 0.34 |
| | CLT | 0.09 | 0.76 |
| August | blue LED | 3.60 | 0.06 |
| | UV LED | 0.79 | 0.37 |
| | CLT | 0.26 | 0.61 |
| September | blue LED | 0.00 | 1.00 |
| | UV LED | 0.28 | 0.60 |
| | CLT | 0.63 | 0.41 |

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**Chapter 2**

Section 2.2

# Identification of phlebotomine sand flies through MALDI-TOF mass spectrometry and in-house reference database

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**Abstract**

Phlebotomine sand flies are vectors for many pathogens responsible for human and animal diseases worldwide. Their identification at species level is of importance in epidemiological studies and control programmes. MALDI- TOF MS has been increasingly investigated as an alternative approach to the conventional identification of arthropods species. To establish an in-house protein spectra database for a quick and reliable species identification of phlebotomine sand flies, 166 field-caught sand fly specimens, morphologically identified as *Phlebotomus perniciosus* (no = 56; 26 males and 30 females), *Phlebotomus neglectus* (no = 4 males), *Phlebotomus sergenti* (no = 6; 4 males and 2 females) and *Sergentomyia minuta* (no = 100; 45 males and 55 females), were subjected to MALDI-TOF MS analyses. Out of 166, 149 specimens (89.8%) produced consistent species-specific protein spectra. Good quality database for *P. perniciosus* and *S. minuta* were generated; no databases have yet constructed for *P. neglectus* and *P. sergenti* due to the low number of specimens examined. The identification of 80 sand flies (no = 20 *P. perniciosus*; no = 60 *S. minuta*) were confirmed using the new generated SuperSpectra as validation test. The results reported support the use of MALDI-TOF MS for rapid, simple and reliable phlebotomine sand fly species identification suggesting its usefulness in accurate survey studies, ultimately improving biological and epidemiological knowledge on these important vectors of pathogens.

**KEYWORDS:**

Species identification; Protein spectra; *Phlebotomus* spp.; *Sergentomyia minuta*; MALDI-TOF MS.

## 1.Introduction

Phlebotomine sand flies (Diptera, Psychodidae) are hematophagous insects of high significance in human and veterinary medicine due to their involvement in the transmission of viruses (*Bunyaviridae*, *Reoviridae* and *Rhabdoviridae*), bacteria (*Bartonella*), and protozoa of the genus *Leishmania* (Maroli et al., 2013; Gaglio et al., 2014, 2018; Halada et al., 2018a). Indeed, in terms of global disease burden, leishmaniasis is the third most important vector-borne disease, after malaria and lymphatic filariasis (WHO, 2002, 2018). Sand flies of the genus *Phlebotomus* and *Lutzomyia* are the only proven vectors of the species *Leishmania infantum*, the causative agent of human and canine leishmaniosis. The transmission of *Leishmania* parasites involves complex ecological interactions between parasites–vectors and hosts. Changes in vector distribution are associated with changes in the risk of pathogen transmission and therefore monitoring activities of arthropod vectors becomes of importance in surveillance programs. Under the above circumstances the accurate species identification of sand flies is of paramount importance to prevent the emergence of leishmaniasis and to establish proper vector control programmes (Lafri et al., 2016). The conventional identification of sand fly species based on morphological features is laborious, time-consuming and needs a certain degree of expertise (Depaquit et al., 2013). Furthermore, identification is hampered by the lack of comprehensive morphological keys, minute species-distinctive characters and by the existence of phenotypic plasticity among populations (Prudhomme et al., 2012; Ready, 2013). Molecular-based approaches have been established for several phlebotomine species (Depaquit, 2014; Dvorak et al., 2014; Latrofa et al., 2011, 2012); however, this method is expensive and its validity may be impaired by the genetic variability at the target locus (Depaquit et al., 2002; Dvorak et al., 2011). In the last few years, the matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), a routine method to identify and classify mainly bacteria for clinical diagnostics (Sauer and Kliem, 2010) has been investigated as an alternative approach to the conventional identification of arthropod species (Kaufmann et al., 2011; Karger et al., 2012; Kaufmann et al., 2012; Yssouf et al., 2013a, 2013b, 2014a, 2014b; Dvorak et al., 2014). Advances in technology/computer science and the development of well validated comprehensive databases of protein mass spectra enabled automation of MALDI-TOF MS and associated data analysis. Noteworthy, this approach showed important application in the species identification of a wide range of common as well as esoteric bacteria and fungi (Patel, 2015). Efforts are underway to enhance and/or build databases *ex novo* for identification of esoteric organisms, and with some exceptions, MALDI-TOF MS is proving useful for their identification. MALDI-TOF MS has been first applied on *Culicoides* species identification (Kaufmann et al., 2011, 2012) and on several mosquito species (Muller et al., 2013; Yssouf et al., 2013b; Schaffner et al., 2014). Mediterranean phlebotomine sand fly species reared in laboratory colonies (Dvorak et al., 2014; Mathis et al., 2015; Halada et al., 2018a) and/or wild captured have also been evaluated by MALDI-TOF MS (Mathis et al., 2015; Lafri et al., 2016; Halada et al., 2018b) displaying their potentialities for many applications in vector biology and epidemiology of vector-borne disease (Yssouf et al., 2016).

However, MALDI-TOF MS has never been employed as a tool for monitoring phlebotomine sand fly fauna from Italy. The current study aimed to assess the suitability of MALDI-TOF MS to distinguish field-caught phlebotomine sand fly species and to evaluate whether the obtained protein mass spectrum represents a unique protein pattern allowing unambiguous species identification. This survey could represent the first step towards the establishment of an in-house protein spectra database that would enable quick and reliable species identification.

## 2. Material and methods

*2.1. Captures and morphological identification of phlebotomine sand flies*

Phlebotomine sand flies used in the study were captured from May to August 2018 by light traps placed inside 3 dog shelters located in the province of Syracuse (2) and Catania (1), Sicily, Southern Italy. Sand flies were stored in 70% ethanol and identified to species level using morphological keys (Dantas-Torres et al., 2014). Briefly, each specimen was dissected with entomological needles, and the external genitalia of males or the head and posterior last tergites of females were cut off, cleared and slide-mounted as described elsewhere (Gaglio et al., 2017).

A total of 262 unfed and/or not-gravid sand fly specimens, identified as *Phlebotomus* perniciosus (no = 80; 41 males and 39 females), *P. neglectus* (no = 4 males), *P. sergenti* (no = 6; 4 males and 2 females) and *Sergentomyia minuta* (no = 172; 67 males and 105 females) were analysed for SuperSpectra generation and validation.

*2.2. MALDI-TOF MS analysis and SuperSpectra generation*

Sample preparation and MALDI-TOF MS analysis followed a previously described protocol optimized for sand flies (Dvorak et al., 2014; Lafri et al., 2016). Briefly, dissected thoraxes of specimens were homogenized in 10 µL of 25% formic acid (Sigma-Aldrich, Buchs, Switzerland) in 1.5 mL microtubes with sterile disposable pestles. After short centrifugation (10,000 rpm for 60 s), 1 µL of supernatant of each sample, corresponding to protein extract, was deposited in duplicate onto a steel target plate (VITEK®MS, bioMérieux, France). Thereafter, 1 µL of a MALDI matrix was directly overlaid on each spot sample on the target plate. The MALDI matrix was prepared daily as a solution of sinapinic acid (30 mg/mL, Sigma-Aldrich, Buchs, Switzerland) in 60% acetonitrile, 40% $dH_2O$ with 0.3% trifluoroacetic acid (TFA) (Sigma-Aldrich, Buchs, Switzerland).

The target plate with samples and matrix was allowed to air-dry at room temperature and placed for analysis in a MALDI-TOF Mass Spectrometry Axima™ Confidence machine (Shimadzu-Biotech Corp., Kyoto, Japan) with detection in the linear, positive mode at a laser frequency of 50 Hz and within a mass range from 2 to 30 kDa. For each target plate the calibration was conducted using spectra of the reference strain *Escherichia coli* (ATCC®8739™). This bacterial test standard was introduced in all target plates for each analysis in order to control loading on mass spectrum, matrix quality and MALDI-TOF apparatus performance as well.

Thorax homogenate supernatant from 166 sand fly specimens was analyzed by MALDI-TOF MS in order to create an in-house database and to assess intra-species reproducibility of protein mass spectrum. The processed spectra were exported as peak lists with *m/z* values for each peak and signal intensity in the ASCII format. The mass protein fingerprints analysis and dendogram generation, based on whole spectra including all signals passing the peak detection criteria, were performed by using the SARAMIS™ Premium software package (spectral archive and microbial identification system, AnagnosTec, Potsdam-Golm, Germany) as described elsewhere (Kaufmann et al., 2011).

Peak lists were trimmed to a mass range of 2–30 kDa and peaks with a relative intensity below 1% were removed. Biomarker mass patterns, referred as "superspectra", were calculated using the SARAMIS™ SuperSpectra™ tool by using 10 specimens per species with 4 technical replicates each (quadruplicates). Specificity of these potential biomarker masses was determined by comparison against the whole SARAMIS™ spectral archive.

For the validation of the generated SuperSpectra™, a total of 96 specimens (no = 24, *P. perniciosus*, no = 15 males and no = 9 females; no = 72, *S. minuta*, no = 22 males and no = 50 females) were analysed in duplicate, and the obtained mass fingerprints were imported into SARAMIS™ software. The threshold for identification was set at 75% biomarker matches based on the reference data set, according to the SARAMIS™ user guideline. Spectra containing less than 30 data counts and peak relative intensity <1% were considered low quality.

Kappa analysis was performed with the aid of the statistical software Prism v. 4.00 (Graphpad Software Ldt., USA, 2003) in order to evaluate the degree of agreement between MALDI-TOF MS and morphological identification.

*2.3. Molecular identification of sand flies*

Portions of sand fly specimens which showed a low-quality spectrum by MALDI-TOF MS analysis and/or which were not correctly identified by the in-house protein spectra database, were subjected to molecular investigation. Briefly, the genomic DNA was extracted from abdomen of 33 sand fly specimens using DNeasy Blood & Tissue Kit (Qiagen, GmbH, Hilden, Germany) after homogenization by thermal shock.

The molecular identification was performed by amplification and sequencing of mitochondrial DNA (mtDNA) fragment encompassing cytochrome b (cytb) and NADH dehydrogenase subunit 1 (nd1) regions (cytb-nd1∼500 bp) using primers (PhleF/PhleR) and PCR run protocol elsewhere described (Latrofa et al., 2011). Amplified products were examined on 2% agarose gels stained with GelRed (VWR International PBI, Milan, Italy) and visualized on a GelLogic 100 gel documentation system (Kodak, New York, USA). Amplicons were purified and sequenced, in both directions using the same primers as for PCR, employing the Big Dye Terminator v.3.1 chemistry in an automated sequencer (3130 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA).

Sequences were aligned using the ClustalW program (Larkin et al., 2007) and compared with those available in GenBank using the BLASTn tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**3. Results**

Out of 166 sand flies identified morphologically (no = 56 *P. perniciosus,* 26 males and 30 females; no = 4 *P. neglectus,* males; no = 6 *P. sergenti*, 4 males and 2 females and no = 100 *S. minuta*, 45 males and 55 females), 149 (89.8%) showed high quality spectra with more than 30 data counts at MALDI-TOF MS analysis. Low quality spectra were obtained from 16 specimens (i.e., no = 6 *P. perniciosus*, no = 1 *P. neglectus,* no = 2 *P. sergenti* and no = 7 *S. minuta*), whereas no spectra were obtained for one specimen of *P. sergenti*.

Representative spectra obtained from male and female specimens belonging to the species *S. minuta, P. perniciosus*, *P. neglectus* and *P. sergenti* are reported in Fig. 1, Fig. 2, Fig. 3, Fig. 4.

Dendrograms of MALDI-TOF MS reporting the percentage of similarity generated from representative species is given in Fig. 5. The protein spectra showed several species-unique peaks within the mass range of 2–7 kDa, which allowed reliable species identification of the analysed sand flies. The majority of protein peaks of spectrum patterns overlapped in both gender of the same species.

SuperSpectra™ were generated for the species of *P. pernicious* and *S. minuta*, whereas it was not possible to generate the biomarker masses for *P. neglectus* and *P. sergenti,* due to the low number of specimens available.

Following validation test, 80 out of 96 insects (83.3%) (no = 20 *P. perniciosus*, no = 12 males and no = 8 females; no = 60 *S. minuta*, no = 15 males and no = 45 females) were correctly identified by the new generated SuperSpectra™. The correct identification was achieved starting from 75% biomarker matches based on the reference data set, according to the SARAMIS™ user guideline. No misidentification occurred when comparison against the whole SARAMIS™ spectral archive was performed. Overall, out of 16 specimens that produced poor quality spectra, 14 (no = 4 *P. perniciosus* and no = 10 *S. minuta*) yielded no result upon automated identification with SuperSpectra™. Finally, two

specimens, morphologically identified as *S. minuta,* despite the good quality spectra obtained were not identified by the new generated SuperSpectra™.

Kappa analysis showed moderate agreement between MALDI-TOF and morphological identification (Table 1).

BLAST analysis of the mtDNA sequence of phlebotomine sand fly specimens, corroborated morphological identification for 26 out of 33 specimens analyzed showing high nucleotide identities with sequences available in GenBank database (i.e., up to 100% for *P. pernicious* accession number: JF766958; 98% for *P. neglectus* accession number: JF766962; 99% for *S. minuta* accession number: JF766980). The 7 specimens not identified by molecular analysis were belonging to the species *P. sergenti* (no = 3) and *S. minuta* (no = 4) according to morphology.

**4. Discussion**

In the present study the usefulness of MALDI-TOF MS analysis for the identification of phlebotomine sand fly population of the Mediterranean region has been assessed, and protein mass spectra profiles have been provided for species examined.

The applications of this method on multicellular organisms including medically important insects have been recently investigated (Kaufmann et al., 2011, 2012) and reference database of biomarker mass sets was established for many species of biting midges to make possible their automated database-based identification. Sand flies are ideal candidates for MALDI-TOF MS towards taxonomical identification both for their minute species-specific morphological features, which make the conventional identification arduous, and for their high medical significance (Dvorak et al., 2014; Mathis et al., 2015; Lafri et al., 2016; Halada et al., 2018a, b).

Indeed, according to previous studies, the protein mass spectra obtained by MALDI-TOF MS from phlebotomine sand fly samples were intra-species reproducible and inter-species specific (Lafri et al., 2016; Mathis et al., 2015; Halada et al., 2018a, b; Dvorak et al., 2014). Specimens analyzed in the present study produced distinct, consistent and reproducible species-specific protein spectra, and despite minor gender-specific peaks, no substantial differences between males and females of the respective species were detected. In line with previous surveys where both sexes of the sand fly species were analysed (Dvorak et al., 2014; Mathis et al., 2015; Lafri et al., 2016), in the current study thorax was used for mass protein spectra analysis, therefore, the exclusion of the terminal body sections of specimens in the acidic extraction prior MALDI-TOF analysis, probably removed some of the sex-specific proteins. The present study also reports the mass protein spectrum of *P. neglectus* which has never been previously presented in scientific literature. Distinct and reproducible mass protein peaks were recorded for *P. neglectus* despite the low number of specimens. The generation of an in-house database for this sand fly species remains one of the tasks for future studies.

The protein spectra herein obtained from *P. perniciosus*, *P. sergenti* and *S. minuta* displayed profiles with a lower mass range in comparison with the spectra previously proposed (Dvorak et al., 2014; Mathis et al., 2015; Lafri et al., 2016). This difference in mass protein range might be due to the storage method (i.e., 70% ethanol at room temperature) of sand fly specimens herein adopted. Indeed, though the storage of samples in ethanol is a common method of preservation under field conditions, storing samples in alcohol for long period of time induces protein precipitation, reducing their solubility and, consequently, leading to a decrease in qualitative and quantitative spectra profiles, notably for masses above 9 kDa (Mathis et al., 2015; Lafri et al., 2016). However, it is also showed that identification to species level is unaffected by this constrain as references masses are in the lower range (Mathis et al., 2015). The results obtained in the present study showed that the protein mass fingerprints of phlebotomine sand flies obtained by MALDI-TOF MS approach allow a reliable species identification by using the respective database and software (SARAMIS™). In particular,

the in-house reference spectra database established for the most representative species *P. perniciosus* and *S. minuta* was evaluated with 96 specimens identified morphologically.

The species identification by MALDI-TOF MS revealed consistent results with the morphological identification for more that 80% of tested samples highlighting the moderate specificity of MALDI-TOF MS analysis. Ten specimens belonging to the species *S. minuta* and 4 specimens belonging to the species *P. perniciosus* showed spectra with low data counts and relative intensity (<30 and <0.9%, respectively) and they were not identified probably due to a low spectra quality. According to the findings of a previous survey (Mathis et al., 2015) a threshold of 30 data counts together with a peak of relative intensity <1% seems to be useful criterion to exclude spectra from future analysis.

Noteworthy, among the specimens showing low quality protein spectra, those with threshold >20 data count and with relative intensity >0.6% were correctly identified by MALDI-TOF MS corroborating morphological identification and furtherly confirmed by the molecular analysis. Though molecular analysis corroborated morphological identification, no amplification of *cytb*-nd1 region occurred in 7 specimens (no = 3, *P. sergenti*; no = 4, *S. minuta*) out of the 33 sand flies characterized by a low-quality spectrum and/or not correctly identified by the in-house protein spectra database. Noteworthy, despite the good quality spectra, 2 specimens recognized morphologically as *S. minuta* were not identified by MALDI-TOF MS and by molecular analysis due failing the *cytb*-nd1 region amplification. This inconsistence could be related to faults in extraction and/or operator procedures both in spectrometry and molecular analysis, or to inexact morphological identification because of cryptic species. This last hypothesis is worthy of interest but it should be confirmed by more detailed molecular investigations.

## 5. Conclusions

In this study an in-house database for the sand flies *P. perniciosus* and *S. minuta* was created allowing a SuperSpectrum generation; whereas the databases for the species *P. neglectus* and *P. sergenti* are still developing due to the low number of specimens available. Overall, the findings obtained in the current study support the potential of MALDI-TOF MS for rapid, simple and reliable phlebotomine sand fly species identification. MALDI-TOF-based identification of these important insect vectors would facilitate survey studies and improve biological and epidemiological knowledge on these medically important vectors. The power of MALDI-TOF MS in the species identification is strongly connected with the quality of the available mass protein spectra database, therefore the improvement and update of phlebotomine sand fly species database by collaborative work of expert taxonomists and experienced users of mass spectrometers will be imperative to provide quality species identification into the foreseeable future.

## Abbreviations

MALDI-TOF MS: matrix-assisted laser desorption/ionization time of flight mass spectrometry; dH2O: distilled water; TFA: trifluoroacetic acid; mtDNA: Mitochondrial DNA; cytb: cytochrome b; nd1: NADH dehydrogenase subunit 1; PCR: polymerase chain reaction.

## Conflict of interests

All authors have declared that there is no conflict of interest.

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Not applicable.

**Author contributions**

Arfuso F. and Brianti E. conceived and designed the study. Arfuso F., Lupia A. and Giarratana F. carried out the MALDITOF MS experiments and analyzed the data. Gaglio G. and Caracappa G. carried out captures and morphological identification of sand flies; Latrofa M.S. and Otranto D. performed molecular identification analyses; Arfuso F. and Brianti E. drafted the first version of the manuscript. All the authors critically revised the manuscript and approved the submitted version.

**Fig. 1.** Representative matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra of male (♂) and female (♀) specimens of *Sergentomyia minuta*.

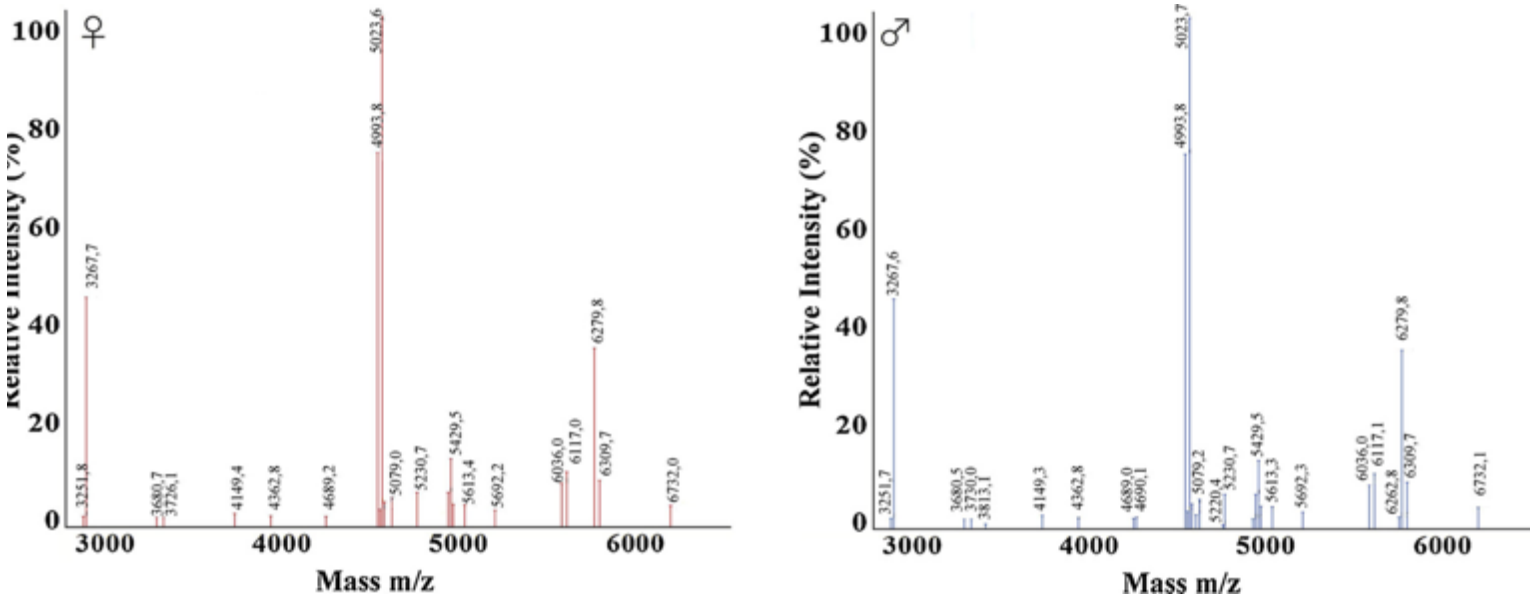

**Fig. 2.** Representative matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra of male (♂) and female (♀) specimens of *Phlebotomus perniciosus*.

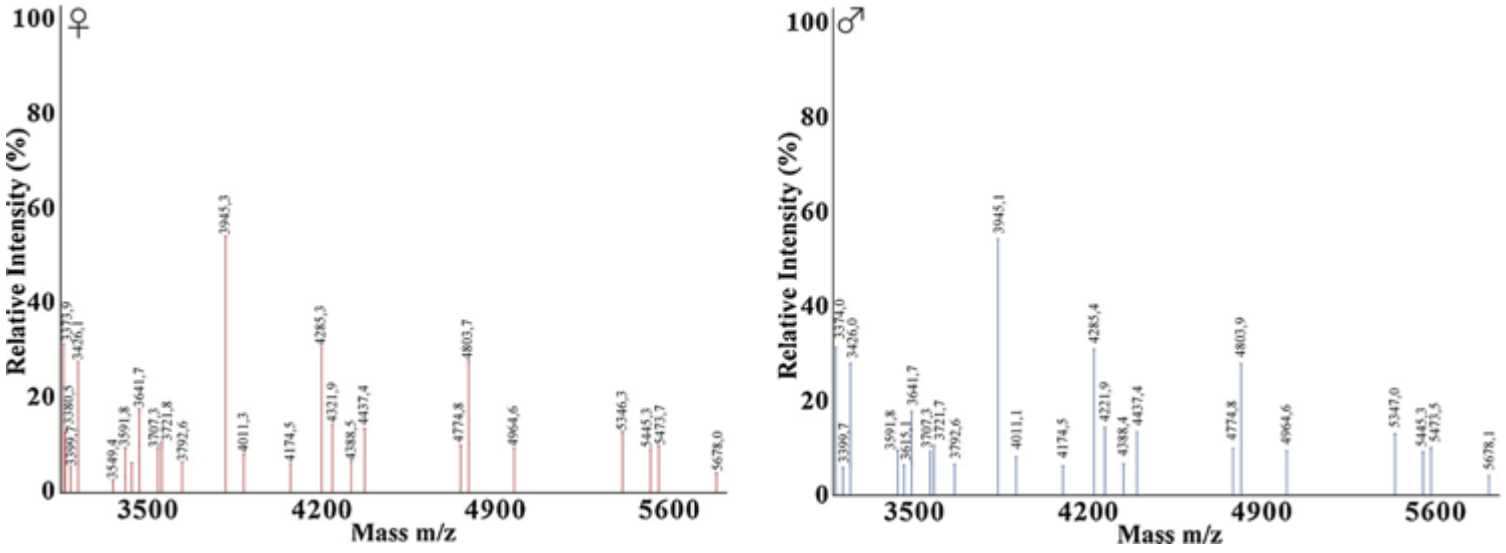

**Fig. 3.** Representative matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra of male (♂) specimens of *Phlebotomus neglectus*.

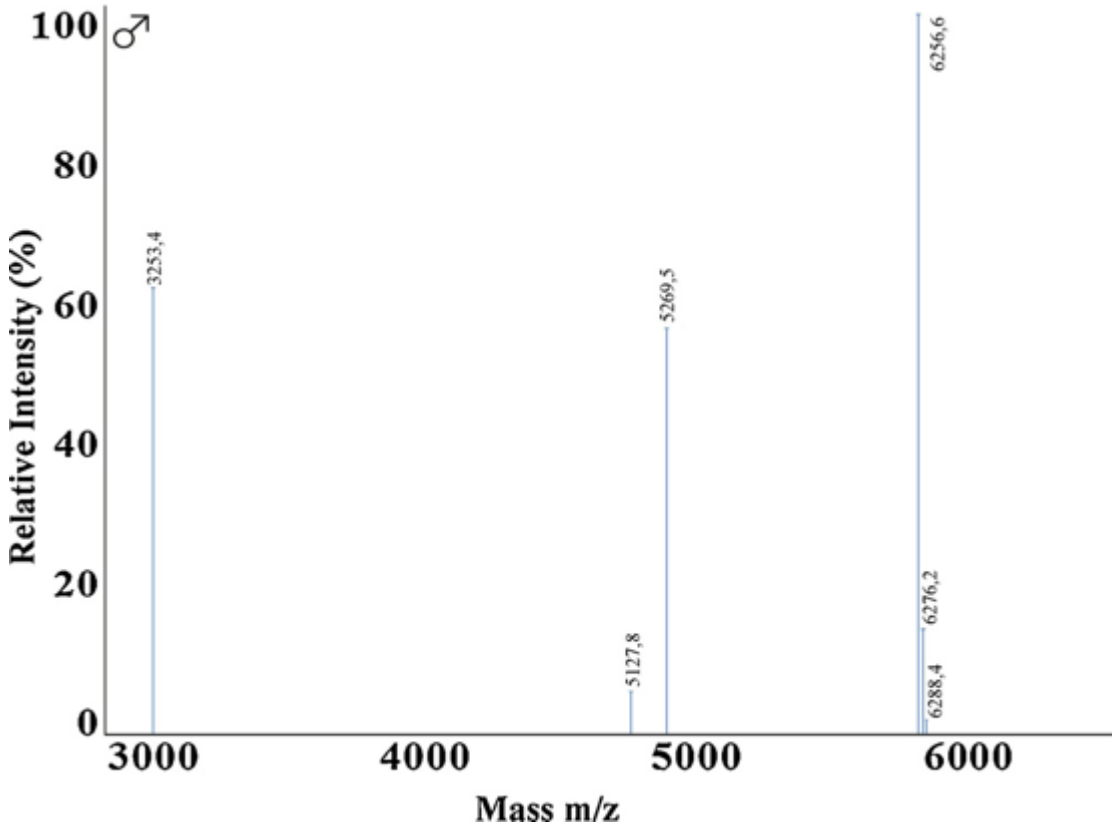

**Fig. 4.** Representative matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra of male (♂) and female (♀) specimens of *Phlebotomus sergenti*.

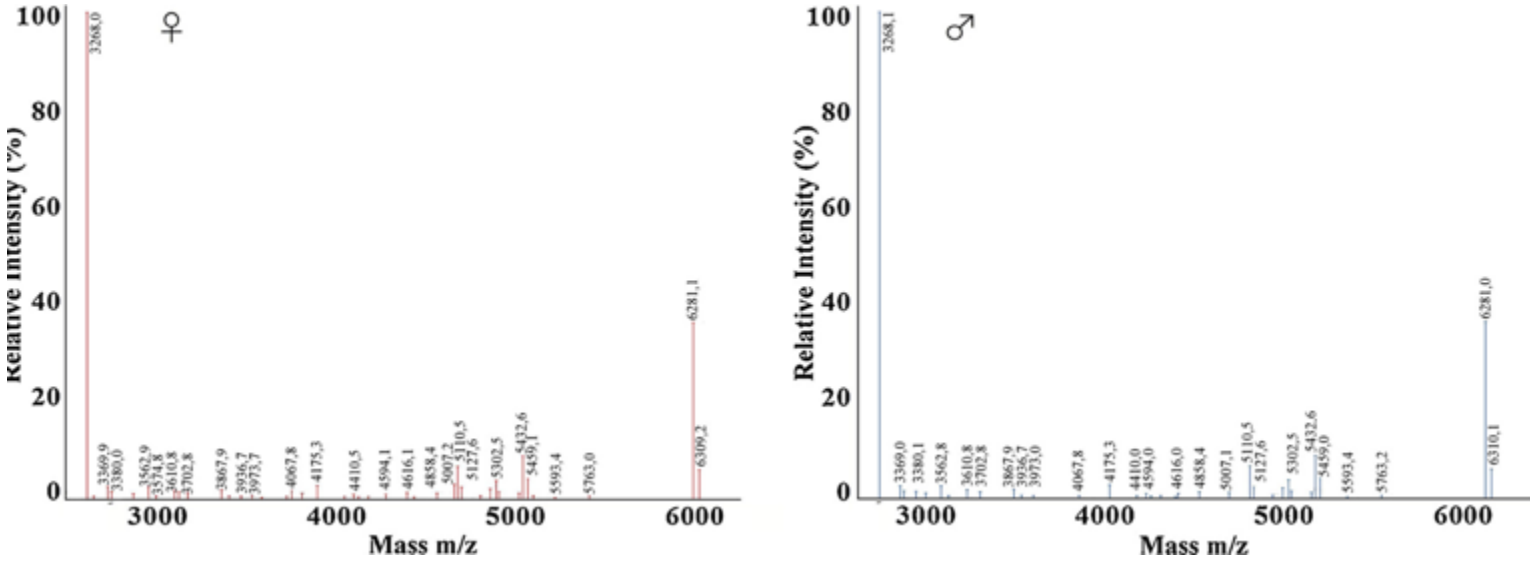

**Fig. 5.** Dendrogram of matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra generated from representative specimens of each species (*Sergentomyia minuta*, *Phlebotomus perniciosus*, *P. neglectus*, *P. sergenti*).

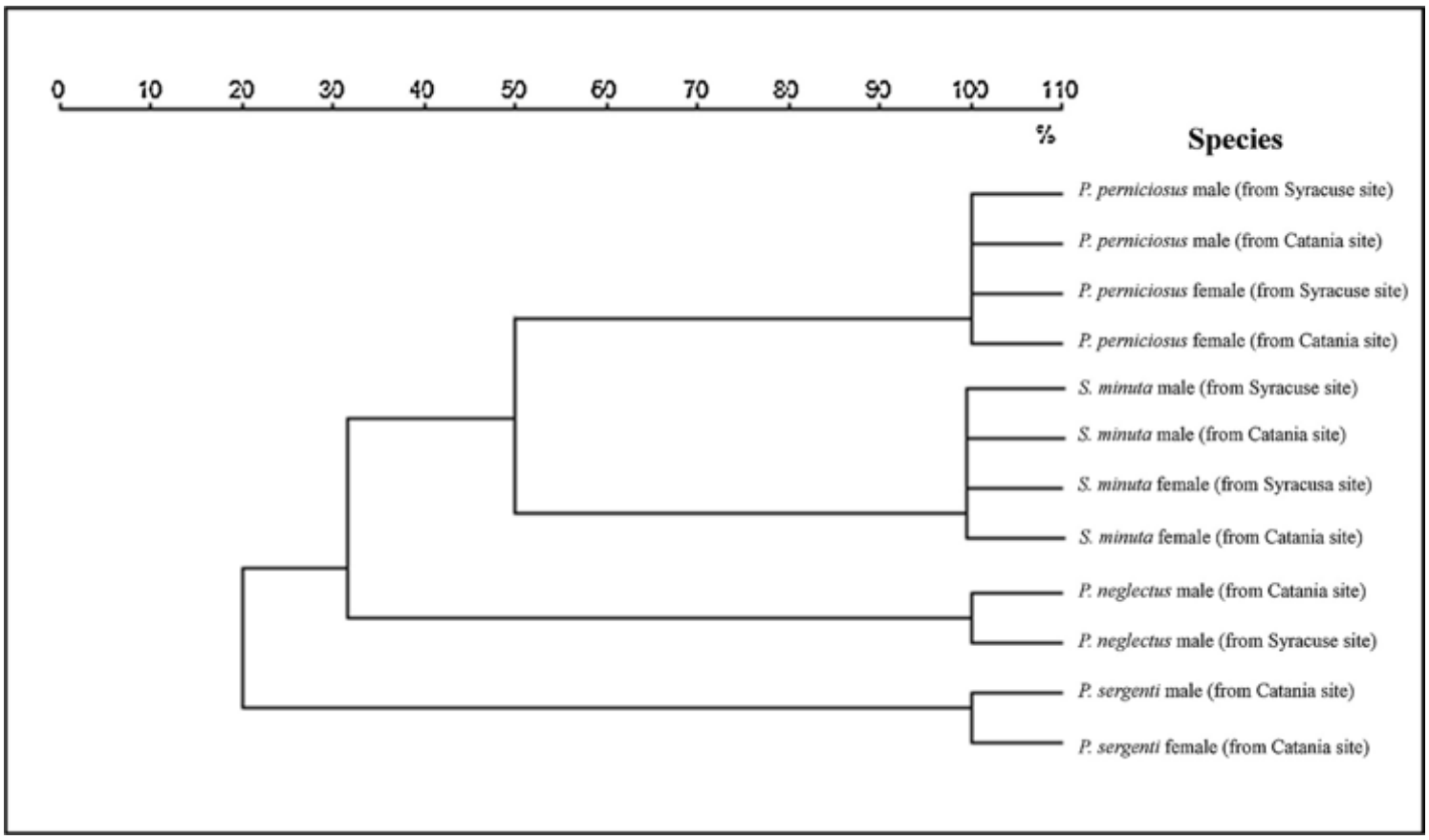

**Table 1** Degree of agreement between MALDI-TOF MS and morphological identification of sand fly species.

| | **Morphology** | **MALDI-TOF MS** |
|---|---|---|
| **Number of identified sand flies** | 96 | 80 |
| ***Kappa analysis*** | | |
| *Observed Agreement* | 83.3 % | |
| *Expected by chance Agreement* | 58.3 % | |
| *95 % Confidence Interval* | 0.43 – 0.77 | |
| ***Kappa value*** | 0.6 | |

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**Chapter 2**

Section 2.3

# *Leishmania* infection and blood feeding preferences of phlebotomine sand fly species common in the Mediterranean area

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**Abstract**

In this study, the presence of *Leishmania* DNA and blood feeding sources in phlebotomine sand fly species typical of the Mediterranean area were investigated.

A total of 1,866 female sand flies including 176 blood fed specimens were sampled over two seasons in five selected sites in Sicily (southern Italy). *Sergentomyia minuta* (no=1,264) and *Phlebotomus perniciousus* (no=594) were the most abundant species in all the sites, while other three species from the genus *Phlebotomus (i.e. P. sergenti* no=4*, P. perfiliewi* no=3 and *P. neglectus* no=1) were sporadically captured.

Twenty-eight (1.5%) out of 1,866 tested sand flies scored positive to *Leishmania* spp.. *Leishmania tarentolae* DNA was identified in 26 specimens of *S. minuta*, while the DNA of *Leishmania donovani* complex was detected in *S. minuta* (no=1) and *P. perniciosus* (no=1). Interestingly, seven *S. minuta* specimens (0.4%) tested positive to *Trypanosoma* sp. DNA.

Blood sources of 108 out of 176 blood fed females were successfully identified. Wild rabbits (27/82) represented the most preferred mammal species for *P. perniciosus*, while *S. minuta* mainly fed on humans (16/25). In both species, several other vertebrate hosts including horse, goat, pig, dog, chicken, cow, cat, donkey and rat were recognized as feeding sources.

Results of this study confirm the chief vectorial role of *P. perniciosus* in the maintenance and spread of leishmaniosis in the Mediterranean area; also, vectors' feeding preference herein described puts forward the hypothesis on the involvement of lagomorphs as sylvatic reservoirs of *Leishmania*. The detection of *L. donovani* complex and *Trypanosoma* sp. DNA in *S. minuta*, together with the anthropophilic feeding-behaviour herein observed, incites to clarify the capacity of this species in the transmission of pathogens to humans and other warm-blooded animals.

**KEYWORDS:**

*Leishmania donovani* complex, *Leishmania tarentolae*, *Trypanosoma* sp., sand flies, blood sources.

## 1. Background

Phlebotomine sand flies (Diptera: Phlebotominae) are insects of great interest in human and veterinary medicine. They are vectors of a wide plethora of viral and bacterial pathogens and recognised as the sole hematophagous arthropods proven to transmit protozoa of the genus *Leishmania* such as *Leishmania infantum*, the causative agent of canine leishmaniosis (CanL) in dogs and visceral (VL) or cutaneous (CL) leishmaniosis in humans in the Mediterranean area [1].

Sand flies are distributed throughout many regions of the world and their biodiversity and phenology have been investigated particularly in *Leishmania* endemic areas. Only sand flies of the genus *Phlebotomus* are competent vectors for *Leishmania* transmission and *P. perniciosus* is the most widespread species in western Europe [2].

Surveillance on phlebotomine sand fly vectors is pivotal to assess the risk for transmission of endemic *Leishmania* species, but it also crucial to monitor the risk for introduction of new *Leishmania* species in free territories [2-3].

Several studies have investigated the presence of *Leishmania* DNA in phlebotomine sand flies, and the infection rate in *P. perniciosus* varies from 0.13 % to 50% according to the epidemiological context [4-5]. Although *L. infantum* is the only species widespread in Europe that causes illness, human cases due to non-indigenous *Leishmania* species are increasingly reported [6].

In Italy, imported leishmaniosis cases are mainly associated with international travellers and/or refugees coming from endemic zones, and consist of chronic forms diagnosed many months after entering the country [7-8]. Noteworthy, anthroponotic species such as *Leishmania tropica* and *Leishmania donovani* are included among the causative agents of imported human cases, and the risk of introduction in Europe of these species is higher since suitable sand fly vectors are present [3,6].

Sicily, one of the two major Italian islands, is located in the centre of the Mediterranean Sea; the island is characterized by a typical temperate climate, with mild and wet winters and hot dry summers. This island is a well-known hyper-endemic region for CanL with a prevalence of infection up to 40% in dogs which are regarded as the main domestic reservoir hosts and source for human infection [9-10]. According to entomological surveys on *L. infantum* vectors carried out in Sicily, *Phlebotomus perniciosus* has been recognised as the most abundant species, followed by *P. neglectus* and *P. perfiliewi* much less frequently detected [11-13]. The presence of *P. sergenti* has been also sporadically reported along the east side of the Island [11-13] implying the risk for *L. tropica* transmission. Besides Sicily, *P. sergenti* has also been caught in other European regions, including the Iberian Peninsula, the south of France, the Canary Islands, the Balearic Islands, the island of Corsica and Greece [1]. Although no autochthonous cutaneous leishmaniosis cases (ACL) caused by *L. tropica* have been detected in south-western Europe so far, Sicily is an area potentially susceptible to the introduction of this protozoa species due to its proximity to northern Morocco, an emerging ACL area [14], and to the large migratory flow of people between the two territories. As matter of fact, the flow of immigrants from endemic regions of North Africa and Middle East, together with the rising number of reports of *L. tropica* human infections in Italy [6,7,15-16], could constitute the first step in its potential spread in Sicily and subsequently across other areas of southern Europe where the competent vector has been described [7]. *Sergentomyia minuta*, another sand fly species, has been largely reported in the Mediterranean area, including Sicily. This sand fly species is not annotated among proven vectors of *L. infantum*; however, the recent detection of *L. infantum* DNA in captured specimens of the species could suggest its potential participation in the epidemiology of leishmaniosis [4,17-18].

Female sand flies are hematophagous insects and take blood meals on many vertebrate hosts including reptiles, birds and mammals [1]. Although several studies demonstrated that sand flies display an opportunistic feeding behaviour and obtain

blood from a variety of domestic and wild animals and humans as well [4,19-20], surveys conducted in the context of a large outbreak of human leishmaniosis in Madrid showed that *Phlebotomus perniciosus* preferably feeds on lagomorphs [21-23]. Interestingly, the role of both hares and wild rabbits as reservoir hosts of leishmaniosis has been elucidated by xenodiagnoses, and these lagomorph species have been imputed as the main source of human leishmaniosis in Madrid [23-26]. Therefore, the identification of blood meal sources in wild-caught sand flies provides information on host-feeding patterns under natural conditions, which, in turn, results in data on potential reservoir hosts and essential knowledge for the establishment of efficient control strategies.

Given all the above considerations, the aim of the present study was to investigate *Leishmania* infection rate and blood sources of phlebotomine sand fly species typical of the Mediterranean area that were caught in Sicily along two transmission seasons. These data would improve the current knowledge on sand flies feeding behaviour as well as their vectorial role and would assist the refinement of control strategies and monitoring plans.

## 2. Materials and Methods

### *2.1. Sand fly collection and identification*

Sand flies used in this study were sampled during two different entomologic surveys carried out in Sicily in 2017 and 2018 (**Fig 1**). In 2017, sand flies were captured in a suburban area (Site A) nearby the didactic farm of the Department of Veterinary Science of the University of Messina using a classic light trap (CLT), equipped with a traditional incandescent lamp (12V, 8W) and five Laika 4.0 light traps with LED of different colours. Traps were placed before sunset until sunrise for three consecutive nights each month from May to October [27]. In 2018, sand fly captures were performed in four different sites, i.e. in three shelters located in the suburban area of Syracuse (sites B and C) and Catania (site D) and in one farm (site E) situated in a rural area of the municipality of Messina. In each of the above sites, light traps (i.e. CLT) were placed from May to October and left working for two consecutive nights twice a month.

In laboratory, sand flies were differentiated by sex with the aid of a stereomicroscope and thereafter processed for identification. Briefly, the head and posterior last tergites of females were dissected, cleared and slide-mounted for microscopic observation as described elsewhere [11], and identified to species level using morphological keys [28]. Females were further ranked as unfed (no visible blood in the abdomen), blood fed (presence of blood in the abdomen) and gravid (presence of eggs). Finally, the thorax and the abdomen of each specimen were individually transferred into 2mL vials containing 70% ethanol and stored for DNA analysis.

**Fig 1. Geographical characteristics of sand fly sampling sites and availability of vertebrate hosts.**

### *2.2. DNA extraction and Leishmania spp. detection*

Genomic DNA was extracted from portions (i.e., thorax and abdomen) of sand flies stored in 70% ethanol using the Citogene® Cell and Tissue kit (Citomed, Portugal) according to the manufacturer's instructions. The DNA extracted was suspended in 30 µl of sterile water and stored at + 4°C until future analysis.

### *2.3. Trypanossomatidae DNA amplification*

The presence of Trypanossomatidae DNA in unfed, blood fed and gravid females was firstly screened using a one-step PCR protocol with a set of primers targeting sections of the ribosomal internal transcribed spacer (ITS-rDNA) [29]. For further molecular characterisation of ITS-rDNA positive samples, a two-step PCR protocol using a set of primers targeting

conserved regions of the mitochondrial cytochrome b gene (cytB) was performed [30]. An additional one-step PCR with specific primers for small subunit ribosomal DNA gene (SSU-rDNA) partial amplification [31] were carried out, to characterise the samples where the presence of Trypanosoma spp. was suggested by BLAST analysis of cytB sequences (S1 Table).

**S1 Table. PCR protocols performed for detection and characterization of Trypanosomatidae DNA and blood source identification.**

*2.4. Blood source identification*

Identification of blood sources was conducted by the amplification of a 350 bp segment of the host mitochondrial *cytB*, using the modified vertebrate-universal specific primers (cytB1-F and cytB-2-R) on blood fed sand fly specimens [32]. The *cytB* PCR was carried out with 5 µL of extracted DNA in a final volume of 25 µL, using 12.5 µL of NZYTaq 2× Green Master Mix (Nyztech, Portugal) and 1.5 µL of each primer (10 pmol/µL). Amplification was performed as follows: one cycle at 94°C for 5 min, followed by 40 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 min, followed by final elongation at 72°C for 7 min [4] (S1 Table). Electrophoresis of PCR products was carried out in 1.5% agarose gel stained with 2.5 µL Greensafe premium® (Nzytech, Portugal), using a 100 bp DNA ladder as a molecular weight marker and final amplicons were visualized under UV light.

*2.5. Sequence analysis*

PCR products were purified and sequenced by Sanger's method (StabVida, Portugal), using the same primers used for the PCR reactions. Nucleotide sequences obtained were examined using 4Peaks v1.8 (Nucleobytes, Netherlands) and analysed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find positional homologs. Obtained sequences were deposited at the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp/).

*2.6. Phylogenetic analysis*

Multiple sequence alignments of nucleotide datasets were performed using the iterative G-INS-I method as implemented in MAFFT v7 [33]. The obtained alignments were optimized via Gblocks [34], followed by their manual correction considering the encoding reading frame. Phylogenetic trees were constructed using the Maximum Likelihood method under the best-fitting evolutionary model (GTR + G +I; GTR - general time reversal, G - gamma distribution, I - proportion of invariant sites), selected based on the corrected Akaike information criterion, as suggested by Mega v6 [35]. The stability of the obtained tree topologies was assessed by bootstrapping with 1000 resamplings of the original sequence data. The generated trees were edited for display using FigTree v1.4.3 (available at http://tree.bio.ed.ac.uk/software/figtree/).

*2.7. Statistical analysis*

Statistical analysis was performed only with data from sites where a large number of sand flies was captured. Variables were tested for normality of distribution using the Shapiro-Wilk test. As assumption of normality was not valid ($P < 0.05$), nonparametric analysis was carried out. Chi-square analysis was performed to evaluate the difference in host preference within and between sand fly species. A $P$ value < 0.05 was considered as statistically significant. Data were analyzed using statistical software Prism v4.00 (GraphPad Software Ltd., USA).

## 3. Results

### *3.1. Sand fly identification and Leishmania/ Trypanosoma infection*

During the two entomological surveys, a total of 3,090 sand flies (no=1,866 females and no=1,224 males) were captured. Species and details on phlebotomine sand flies collected in each site are summarized in Table 1. Among female sand flies, *S. minuta* was the most abundant species (no=1,264; 67.7%); followed by four species from the genus *Phlebotomus*: *P. perniciosus* (no=594; 31.8%); *P. sergenti* (no=4; 0.2%); *P. perfiliewi* (no=3; 0.2%) and *P. neglectus* (no=1; 0.1%). One hundred seventy-six females were blood fed (9.4%) and 113 were gravid (6.1%) (Table 2).

*Leishmania* DNA was detected in twenty-eight female sand flies (1.5%) out of 1,866. In detail, 26 out of 1,264 (2.1%) *S. minuta* specimens (no=21 unfed; no=4 blood-fed and no=1 gravid) scored positive to *Leishmania tarentolae* DNA; whereas *Leishmania donovani* complex DNA was identified in a *S. minuta* unfed female (1/1264; 0.1%) and in a *P. perniciosus* blood-fed female (1/594; 0.2%). Seventeen out of the 26 *L. tarentolae* positive females were captured in rural biotype (Site E), whereas 9 were collected in periurban environments (Site A-D). Both *L. donovani* complex positive females were captured in rural environment (Site E).

The 26 *cytB* sequences obtained from *S. minuta* revealed >99% sequence identity and 100% sequence coverage with reference sequences of *L. tarentolae* (eg. accession number: LC092878). The *cytB* sequences obtained from *S. minuta* and *P. perniciosus* specimens, revealed >99% identity and 100% sequence coverage with *Leishmania donovani* complex sequences (accession numbers: CP022652; KX061917). All *ctyB*-rDNA obtained sequences were submitted to DDBJ (DDBJ Accession numbers: LC464942 to LC464968; LC471400). Based on phylogenetic analysis, 24 out of 26 obtained *L. tarentolae* sequences amplified from *S. minuta*, segregate together with *L. tarentolae* reference sequences in a monophyletic cluster, supported by a high bootstrap value (i.e. 99). The two *L. donovani* complex sequences herein amplified from *S. minuta* and *P. perniciosus* extracts, segregate in the *Leishmania donovani* complex cluster (Fig 2).

In addition to *Leishmania*, *Trypanosoma* DNA was detected in seven specimens of *S. minuta*, captured in rural Site E (no=1) and periurban biotopes (Site A, no=3; Site B, no=1; Site C, no=2). The 7 *cytB* obtained sequences revealed >95% sequence identity and a sequence coverage >80% with a reference sequence of *Trypanosoma lewisi* of Chinese origin (accession number: KR072974) [36]. The sequences were submitted to DDBJ (DDBJ Accession numbers: LC471393 to LC471399). The *SSU*-rDNA nucleotide sequences showed both 100% identity and coverage with the sequence of *Trypanosoma* sp. isolated from Gecko (accession number: AJ620548) [37]. The *SSU*-rDNA obtained sequences were submitted to DDBJ (DDBJ Accession numbers: LC471401 to LC471407). The phylogenetic tree shows that the obtained sequences share the same common ancestry of *Trypanosoma varani* (Accession number: AJ005279) forming together with *Trypanosoma* sp. isolated from Gecko a monophyletic cluster supported by a high bootstrap value (i.e. 92); whereas 2 sequences are different. These two sequences also sharing the same common ancestry of *T. varani* but segregate independently (Fig 3).

**Tab 1. Total sand flies collected during the two entomological surveys.**

**Tab 2. Female sand flies sampled and molecularly analysed.**

**Fig 2.** Maximum likelihood phylogenetic tree based on unambiguous multiple-*Leishmania* cytB sequences alignment, using the GTR+G+I model of evolution. At specific branch nodes bootstrap values (from 1000 random replicates of the original dataset) ≥75% are shown. The size bar indicates the number of nucleotide substitutions per site. The tree was rooted using as an outgroup a reference sequence of *Trypanosoma brucei brucei* (M94286). The sequences obtained in this study are identified with "*" and their respective accession numbers underscored.

**Fig 3.** Maximum likelihood phylogenetic tree based on unambiguous multiple-*Trypanosoma* SSU-rDNA sequences alignment, using the GTR+G+I model of evolution. At specific branch nodes bootstrap values (from 1000 random replicates of the original dataset) ≥75% are shown. The size bar indicates the number of nucleotide substitutions per site. The tree was rooted using as an outgroup a reference sequence of *Trypanosoma vivax* (U22316). The sequences obtained in this study are identified with "*" and their respective accession numbers underscored.

*3.2 Identification of sand flies' blood meals*

The majority of blood-fed females were captured in site E (149/176; no=128 *P. perniciosus* and no=21 *S. minuta*) and underwent statistical analysis, whereas the remainder 27 specimens were caught in the other sites (A, no=4 *P. perniciosus*, no=1 *S. minuta*; B, no=6 *P. perniciosus*; C, no=11 *S. minuta*; D, no=4 *S. minuta*, no=1 *P. sergenti*) and were not statistically analysed (Table 2). Host mitochondrial *cytB* was successfully amplified from 169 out of 176 engorged females, and the vertebrate hosts of 108 blood meals were correctly identified (Table 3**)**.

Wild rabbit (*Oryctolagus cuniculus*) (no=28) and human (*Homo sapiens*) (no=24) were the most frequent blood sources. Other vertebrate hosts were also found, i.e., goat (no=16), horse (no=13) pig (no=9), dog (no=9), rooster (no=3), cow (no=3), cat (no=1), donkey (no=1) and rat (no=1). Blood sources for each sand fly species and site of capture are shown in Fig 4.

Identification of blood source was successfully achieved in two out of four engorged *S. minuta* specimens that scored positive to *L. tarentolae* DNA, being human (*Homo sapiens*) and donkey (*Equus asinus*); whereas host mitochondrial *cytB* partial gene was not amplified from the blood-fed *P. perniciosus* positive to *L. donovani* complex DNA.

Wild rabbits (27/82; 32.9%) represented the most preferred mammal species for *P. perniciosus*, while *S. minuta* mainly fed on humans (16/25; 64%). Results of statistical analysis on frequency of blood sources for *P. perniciosus* and *S. minuta* from site E are summarized in Table 4. A statistically significant higher frequency of blood meal on rabbit by *P. perniciosus* compared to *S. minuta* ($\chi^2$ = 183.1; $P$ = 0.01), and on human by *S. minuta* compared to *P. perniciosus* ($\chi^2$ = 27.7; $P$ <0.001), was detected.

**Tab 3. *CytB* sequences analysis for each sample, percentages of BLAST identity and sequences accession numbers.**

**Fig 4**. **Vertebrate hosts identified per each sand fly species in selected sampling sites (A-E).**

**Tab 4. Differences in host preference within the most abundant sand fly species caught in site E.**

**4. Discussion**

Our study aimed to assess the presence of natural *Leishmania* infection in wild-caught sand fly species common in the Mediterranean area; also, the identification of blood meals in fed females was performed to gain evidence on sand flies' feeding habits allowing the identification of potential/alternative reservoir hosts for *Leishmania*.

Four out of five sand fly species analysed in this study are proven vectors of human leishmaniosis. Among *Phlebotomus* genus, *P. perniciosus* was the most abundant species, which agrees with other entomological surveys conducted in Southern Italy [12,17,27,38], and the presence of *Leishmania donovani* complex DNA in *P. perniciosus* herein reported confirms the primary role of this species in the maintenance and spread of leishmaniosis in the Mediterranean area. Only a *P. pernicious* (0.2%; no=594) scored positive to *L. donovani* complex. To the best of our knowledge, the sole data on the prevalence infection for *L. infantum* in *P. perniciosus* in Sicily came from a study conducted in the city of Catania where a higher infection rate (i.e. 11%; 8/72) has been detected [12]. This difference may be related with the high

endemicity of leishmaniosis in the area of Catania [10,12] and suggest as the risk of transmission of *Leishmania* can be high even in urban areas.

*Phlebotomus neglectus* and *P. perfiliewi* specimens were occasionally collected in suburban and rural environments, and *P. sergenti*, the sole vector able to transmit *L. tropica*, was also sporadically collected, being its presence limited to the eastern side of the Island [11-13].

The detection of *L. donovani* complex DNA in a *S. minuta* unfed female herein recorded, spurs to better investigate the potential role of this species in the circulation and eventually transmission of the parasite [39]. The positivity of this sand fly species may be correlated to its feeding behaviour and the large availability of mammalian reservoir hosts positive to *Leishmania* in endemic foci [17]. Although *Sergentomyia* species mainly feed on cold-blooded vertebrates, its sporadic/opportunistic anthropophilic feeding behaviour has been already suggested [4] and, accordingly, a higher human blood preference has been herein statistically demonstrated. Scientific evidence on the competence of *S. minuta* in transmitting *Leishmania* spp. to warm-blooded mammals are not available so far; however, it has recently been demonstrated that *L. donovani, L. infantum* and *L. major* are not able to develop late-stage infections in *Sergentomyia schwetzi* sand fly species [40].

*Sergentomyia* spp. are widespread Mediterranean sand fly species, and proven vectors of reptile *Leishmania* species (e.g., *L. tarentolae*) non-pathogenic to humans. The positivity of *S. minuta* to *L. tarentolae* herein recorded (2.1%; 26/1264) confirms that this species feeds on cold-blooded animals, being this protozoan parasite widespread in Gekkonidae species [41]. Nevertheless, experimental studies suggest the potential of *L. tarentolae* to infect/develop in human phagocytic cells [42-43].

In Italy, the presence of *Trypanosoma* in sand flies has been poorly reported. In particular, *Trypanosoma platydactyli* was described in *S. minuta* [44], and the infection by a *Trypanosoma* belonging to *Trypanosoma theileri* group with very high homology to other trypanosomes detected in European cervids was recently reported in *Phlebotomus perfilewi* [45]. In the Mediterranean area, *Trypanosoma nabiasi*, a rabbit trypanosome, and its co-infection with *L. infantum* was found in *P. perniciosus* female sand flies caught in the context of human leishmaniosis outbreak in Madrid [46], and natural infection of sand flies by trypanosomes of lizards, amphibians, birds and rodents has been already reported mainly in the American continent and Asia [47-50]. Despite *Trypanosoma* spp. are protozoa parasites transmitted by hematophagous insects [51-52] the potential of phlebotomine sand flies in transmitting trypanosomes is still unclear. The findings herein obtained, suggest that *S. minuta* might be potentially vector of both *Leishmania* and *Trypanosoma* parasites in southern Italy. Nevertheless, the detection of the trypanosomatid DNA is not enough to incriminate the species as competent vector since it may have been taken during blood meal without undergoing further in development and/or transmission. The detection of *L. donovani* complex DNA and, in addition, the unexpected detection of *Trypanosoma* DNA within *S. minuta* indicates that more attention is required when identifying parasitic organisms by PCR within sand fly vectors in areas where leishmaniosis is endemic. As matter of fact, it is important to highlight that when *Trypanosoma* and *Leishmania* species are present in the same geographical area, mixed infections could appear within the same host and/or vectors representing a challenge to their diagnosis [53-55].

Regarding the identification of blood meal sources in this study, several vertebrate hosts have been recognized as blood sources in the most abundant sand fly species (*P. perniciosus, S. minuta*), confirming the opportunistic feeding behaviour of these hematophagous insects [4,19-20].

Knowledge of the host preferences of sand flies under natural conditions is essential to understand how host choice and blood-feeding behaviour of the sand fly influence their vectorial capacity in leishmaniosis foci. Even though *Canis lupus familiaris* is regarded as the main domestic reservoir host of zoonotic leishmaniosis, it seems not to be the preferred

species on which they make their blood meals [4,19]. In this regard, the results gathered from the current study showed that despite three out of the five selected sites were shelters with high availability of dogs (i.e. from 300 to 500 dogs per site), canine DNA was not detected in the engorged *P. perniciosus* specimens caught in any of them. This finding could be biased by the large use of insecticides and repellents in dogs as preventive measures against sand flies bite and *Leishmania* transmission. In reality, during the sand fly sampling period, about a third of all dogs hosted in the three shelters were treated with repellents active against sand flies.

The 85% of total engorged females employed in this study were caught in a farm located in a rural area far away from human settlements where a large variety of vertebrate hosts were available, being dogs and rabbits (either domestic and sylvatic) the most abundant species. In this site, wild rabbits represented the most preferred mammal species for *P. perniciosus*, which agrees with what has already been reported in Spain [21-22,25]. In fact, high levels of anti-*Phlebotomus perniciosus* saliva antibodies in wild rabbits suggested their exposure and attractiveness to sand flies [56]. The large availability of lagomorphs could contribute to the maintenance of high density of *P. perniciosus* in areas where these mammal species are abundant [56] and, the role of lagomorphs in sustaining sylvatic *Leishmania* cycle independently from the domestic one has been strongly suggested [23,26].

The results achieved in the current study showed a different blood feeding pattern of *S. minuta* compared to *P. perniciosus*, suggesting that the two sand fly species do not compete for the same vertebrate host. It is well-known that *S. minuta* feeds on cold-blooded reptiles; noteworthy, reptile DNA was not amplified in engorged females herein analysed, and only DNA of warm-blooded vertebrates was amplified. Interestingly, *S. minuta* caught in site E mainly fed on humans, though the presence of this vertebrate host inside the farm is limited to a few hours during the day, and no inhabitations were present nearby.

## 5. Conclusions

The current survey describes *Leishmania* infection rate in *P. perniciosus* and *S. minuta* and documents, for the first time in Sicily, *Trypanosoma* DNA presence in *S. minuta* blood-fed females.

The findings herein reported, highlight the primary role of *P. perniciosus* as competent vector of *L. infantum* in the Mediterranean area; also, the identification of blood meal sources suggests the importance of wild rabbits in the maintenance of *P. perniciosus* and their potential role as sylvatic reservoirs of *Leishmania*. Noteworthy, in view of the anthropophilic feeding-behaviour of *S. minuta* found in this study, a role of this species in the spread of pathogenic *Leishmania* spp. to humans and/or other mammals could be hypothesized.

The role of wild animals in the epidemiology of leishmaniosis as well as the role of *S. minuta* as a vector of *Leishmania* spp. to humans is worthy of future investigations to achieve efficient control strategies.

**Fig 1.** Geographical characteristics of sand fly sampling sites and availability of vertebrate hosts.


| Sand fly collection sites | Traps | Collection season | Environment | Vertebrate hosts available in 100 meters radius |
|---|---|---|---|---|
| Site A | Laika/ CLT | 2017 | Suburban | Dogs, horses, humans |
| Site B | CLT | 2018 | Suburban | Cats, dogs, humans, pigs |
| Site C | CLT | 2018 | Suburban | Cats, dogs, humans |
| Site D | CLT | 2018 | Suburban | Dogs, humans |
| Site E | CLT | 2018 | Rural | Cats, cows, dogs, goats, hens, horses, humans, rabbits, rusters, pigs |

**S1 Table.** PCR protocols performed for detection and characterization of Trypanosomatidae DNA and blood source identification.

| Target gene | Primer sequence (5'-3') | Amplicon size | Reaction setup | Thermocycling conditions | Reference |
|---|---|---|---|---|---|
| *cytB*[a] | 1st PCR<br>Fw: AGCGGAGAGRARAGAAAAGG<br>Rev: CTACAATAAACAAATCATAATATRCAATT | 919 bp | 25 µl reaction: 5 µl of DNA; 0.8 µM of each primer; 12.5 µl of NZYTaq 2 x Green Master Mix | 95 ºC - 3 min; 45 cycles [94 ºC - 1 min; 48 ºC - 1 min; 72 ºC - 1 min]; 72 ºC - 5 min | (Pereira et al., 2019) |
| | 2nd PCR<br>Fw: GGTGTAGGTTTTAGTYTAGG<br>Rev: GYTCRCAATAAAATGCAAATC | 568 bp | 25 µl reaction: 5 µl of 1st PCR product[b]; 0.8 µM of each primer; 12.5 µl of NZYTaq 2 x Green Master Mix | 95 ºC - 3 min; 45 cycles [94 ºC - 1 min; 48 ºC - 1 min; 72 ºC - 1 min]; 72 ºC - 5 min | |
| *cytB*[b] | Fw: CCATCCAACATYTCADCATGATGAAA<br>Rev: GCHCCTCAGAATGATATTTGKCCTCA | 360 bp | 25 µl reaction: 5 µl of DNA; 0.6 µM of each primer; 12.5 µl of NZYTaq 2 x Green Master Mix | 94 ºC - 5 min; 40 cycles [94 ºC – 1 min; 55 ºC – 1 min; 72 ºC – 1 min]; 72 ºC - 7 min | (Maia et al., 2015) |
| *ITS*-rDNA | Fw: CTGGATCATTTTCCGATG<br>Rev: TGATACCACTTATCGCACTT | 311 bp | 25 µl reaction: 2.5 µl of DNA; 0.4 µM of each primer; 12.5 µl of NZYTaq 2 x Green Master Mix | 95 ºC - 2 min; 32 cycles [95 ºC - 20 sec; 53 ºC - 30 sec; 72 ºC – 1 min]; 72 ºC - 6 min | (El Tai et al., 2000) |
| *SSU*-rDNA | Fw: GAAACAAGAAACACGGGAG<br>Rev: CTACTGGGCAGCTTGGA | 930 bp | 25 µl reaction: 5 µl of DNA; 0.4 µM of each primer; 12.5 µl of NZYTaq 2 x Green Master Mix | 95 ºC - 1 min; 40 cycles [95 ºC – 1 min; 55 ºC – 1 min; 72 ºC – 1 min]; 72 ºC - 10 min | (Kato et al., 2011) |

[a]Trypanossomatidae kinetoplast DNA
[b]Host mitochondrial DNA
[c]PCR product was previously diluted 1:50 in nuclease-free water
Abbreviations: *cytB,* cytochrome *b*; *ITS*-rDNA, ribosomal internal transcribed spacer DNA; *SSU*-rRNA, small subunit ribosomal DNA; bp, base pars

**Table 1.** Total sand flies collected during the two entomological surveys.

| **Site** (Year) | **Sand fly species** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Sergentomyia minuta* | | *Phlebotomus perniciosus* | | *Phlebotomus sergenti* | | *Phlebotomus perfiliewi* | | *Phlebotomus neglectus* | |
| | Females | Males | Females | Males | Females | Males | Females | Males | Females | Males |
| Site A (2017) | 62 | 48 | 157 | 141 | | | | | 1 | 2 |
| Site B (2018) | 154 | 99 | 54 | 81 | | | | | | 2 |
| Site C (2018) | 153 | 172 | 1 | 22 | 1 | | | | | |
| Site D (2018) | 248 | 162 | 9 | 78 | 3 | 5 | | | | 2 |
| Site E (2018) | 647 | 144 | 373 | 239 | | | 3 | | | 27 |
| **Total** | 1,264 | 625 | 594 | 561 | 4 | 5 | 3 | | 1 | 33 |

**Table 2.** Female sand flies sampled and molecularly analyzed.

| **Site** (Year) | **Female sand fly species** | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Sergentomyia minuta* (no=1,264) | | | *Phlebotomus perniciosus* (no=594) | | | *Phlebotomus sergenti* (no=4) | | | *Phlebotomus perfiliewi* (no=3) | | | *Phlebotomus neglectus* (no=1) | | |
| | Unfed | Blood fed | Gravid | Unfed | Blood fed | Gravid | Unfed | Blood fed | Gravid | Unfed | Blood fed | Gravid | Unfed | Blood fed | Gravid |
| Site A (2017) | 60 | 1 | 1 | 153 | 4 | | | | | | | | 1 | | |
| Site B (2018) | 121 | | 33 | 43 | 6 | 5 | | | | | | | | | |
| Site C (2018) | 137 | 11 | 5 | 1 | | | 1 | | | | | | | | |
| Site D (2018) | 239 | 4 | 5 | 7 | | 2 | 2 | 1 | | | | | | | |
| Site E (2018) | 587 | 21 | 39 | 223 | 128 | 22 | | | | 2 | | 1 | | | |
| **Total** | 1,144 | 37 | 83 | 427 | 138 | 29 | 3 | 1 | | 2 | | 1 | 1 | | |

**Figure 2.** Maximum likelihood phylogenetic tree based on unambiguous multiple-*Leishmania* cytB sequences alignment, using the GTR+G+I model of evolution. At specific branch nodes bootstrap values (from 1000 random replicates of the original dataset) ≥75% are shown. The size bar indicates the number of nucleotide substitutions per site. The tree was rooted using as an outgroup a reference sequence of *Trypanosoma brucei brucei* (M94286). The sequences obtained in this study are identified with "*" and their respective accession numbers underscored.

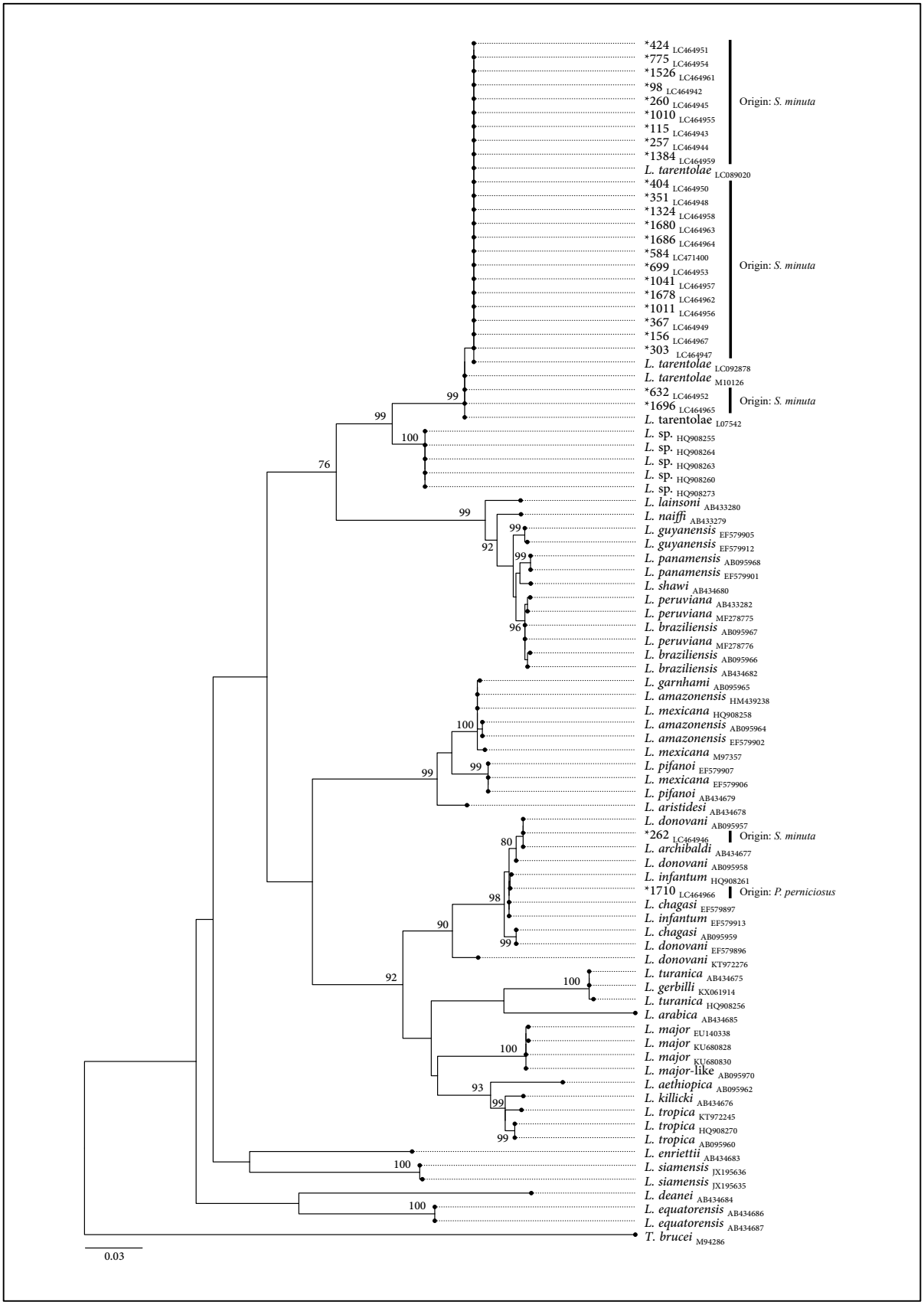

**Figure 3.** Maximum likelihood phylogenetic tree based on unambiguous multiple-*Trypanosoma* SSU-rDNA sequences alignment, using the GTR+G+I model of evolution. At specific branch nodes bootstrap values (from 1000 random replicates of the original dataset) ≥75% are shown. The size bar indicates the number of nucleotide substitutions per site. The tree was rooted using as an outgroup a reference sequence of *Trypanosoma vivax* (U22316). The sequences obtained in this study are identified with "*" and their respective accession numbers underscored.

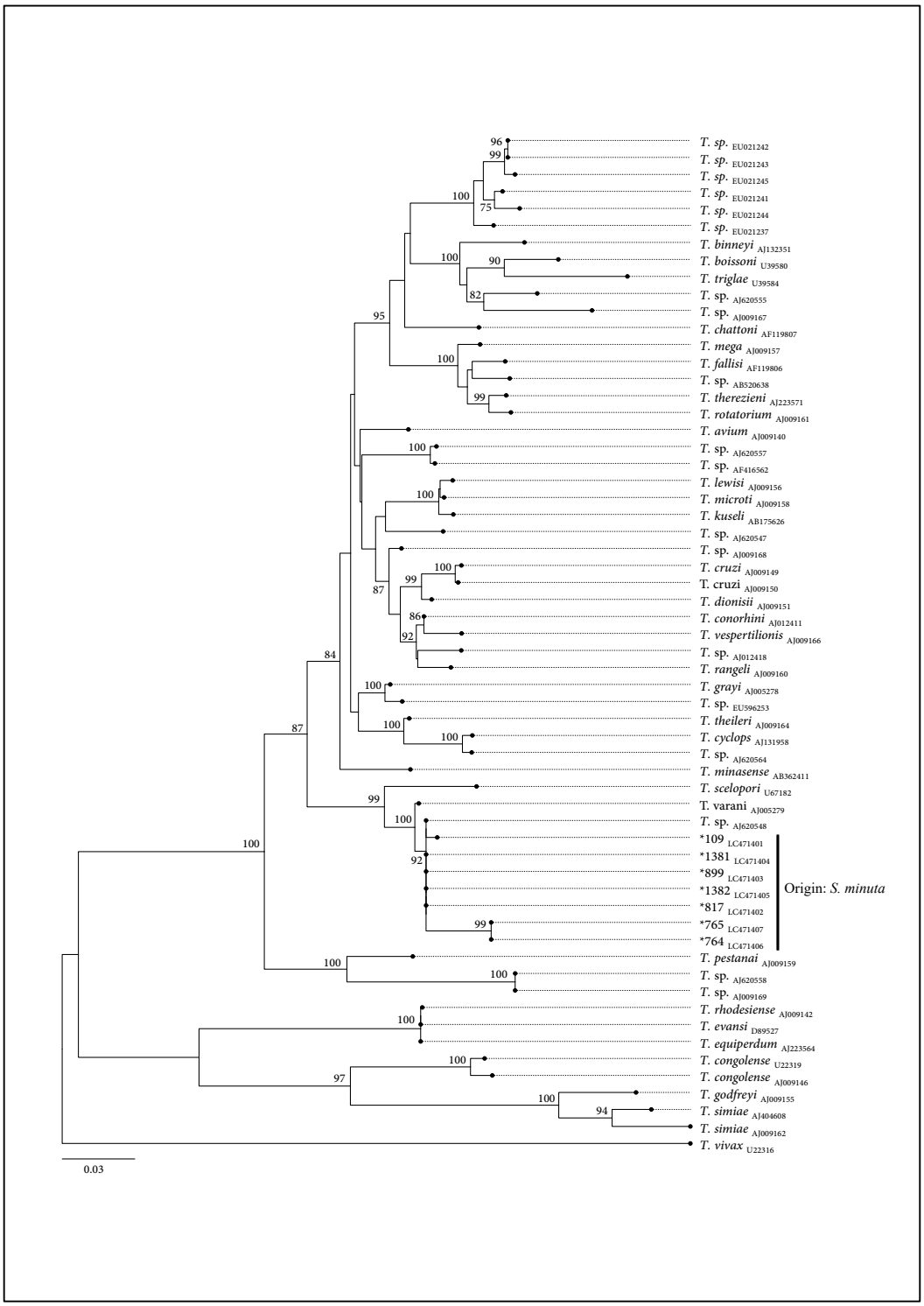

**Table 3.** *CytB* sequences analysis for each sample, percentages of BLAST identity and sequences accession numbers.

| **Blood meal source** (scientific name) | **Sand fly species** | | | **Biotype** | | BLAST analysis | |
|---|---|---|---|---|---|---|---|
| | *Phlebotomus perniciosus* | *Sergentomyia minuta* | *Phlebotomus sergenti* | Periurban | Rural | Identity (%) | Accession number |
| Rabbit (*Oryctolagus cuniculus*) | 27 | 1 | | | 28 | 99-100 | A |
| Human (*Homo sapiens*) | 8 | 16 | | 11 | 13 | 99-100 | B |
| Goat (*Capra hircus*) | 16 | | | 1 | 15 | 99-100 | C |
| Horse (*Equus caballus*) | 11 | 2 | | 4 | 9 | 99-100 | D |
| Pig (*Sus scrofa*) | 7 | 2 | | 2 | 7 | 99-100 | E |
| Dog (*Canis familiaris*) | 8 | 1 | | 1 | 8 | 99-100 | F |
| Rooster (*Gallus gallus*) | 3 | | | | 3 | 99-100 | G |
| Cow (*Bos taurus*) | 1 | 2 | | 2 | 1 | 99-100 | H |
| Cat (*Felis catus*) | 1 | | | | 1 | 99-100 | I |
| Donkey (*Equus asinus*) | | 1 | | | 1 | 99-100 | L |
| Rat (*Rattus norvegicus*) | | | 1 | 1 | | 99-100 | M |
| **Total** | 82 | 25 | 1 | 22 | 86 | | |

**A**: LC469805; LC469817; LC469821; LC469832; LC469834; LC469836; LC469839; LC469842-44; LC469846-48; LC469850-51; LC469853-56; LC469863; LC469865; LC469871; LC469875-76; LC469878; LC469882; LC469887. **B**: LC469829; LC469837; LC469840-41; LC469852; LC469857; LC469859-62; LC469867; LC469872; LC469874; LC469880-81; LC469891-93; LC469896; LC469898; LC469900-03. **C**: LC469807-10; LC469813-16; LC469818-20; LC469822-26. **D**: LC469806; LC469811; LC469827-28; LC469830; LC469833; LC469835; LC469869; LC469877; LC469884, LC469890; LC469897; LC469908. **E**: LC469838; LC469845; LC469849; LC469858; LC469868; LC469870; LC469873; LC469883; LC469899. **F**: LC469885-86; LC469888-89; LC469905; LC469907; LC469910-12. **G**: LC469812; LC469906; LC469909. **H**: LC469831; LC469894; LC469904. **I**: LC469866. **L**: LC469879. M: LC469895

**Figure 4.** Vertebrate hosts identified per each sand fly species in selected sampling sites (A-E).

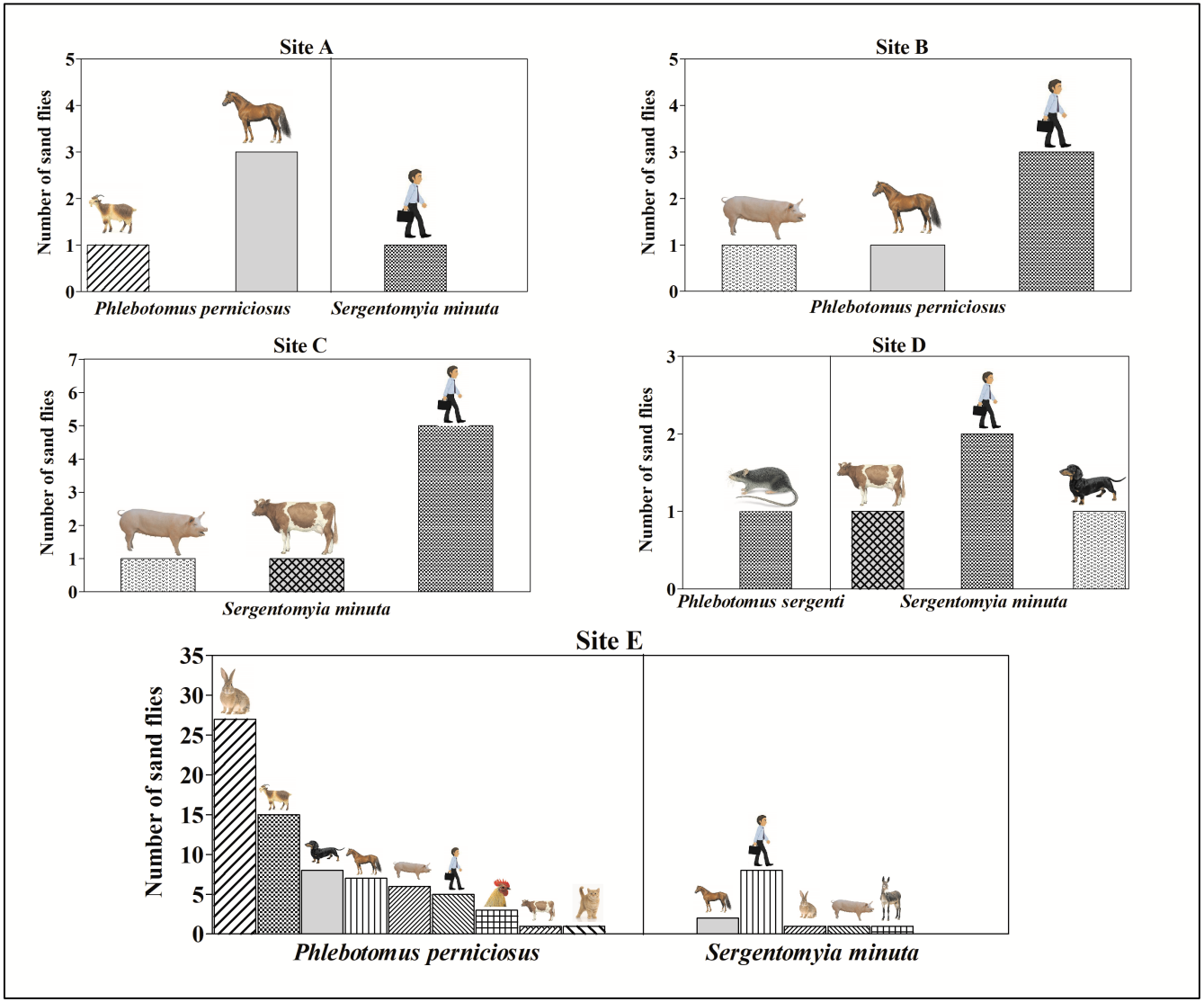

**Table 4.** Differences in host preferences within the most abundant sand fly species caught in site E.

| ***Phlebotomus perniciosus* blood meal sources** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Capra hircus* | *Canis familiaris* | *Equus caballus* | *Sus scrofa* | *Homo sapiens* | *Gallus gallus* | *Bos taurus* | *Felis catus* |
| *Oryctolagus cuniculus* | **$\chi2 = 4.8$ P = 0.01** | **$\chi2 = 13.6$ P = 0.0001** | **$\chi2 = 15.3$ P <0.0001** | **$\chi2 = 17.3$ P <0.0001** | **$\chi2 = 19.4$ P <0.0001** | **$\chi2 = 15.3$ P <0.0001** | **$\chi2 = 24.2$ P <0.0001** | **$\chi2 = 24.2$ P <0.0001** |
| *Capra hircus* | | $\chi2 = 2.5$ P = 0.06 | **$\chi2 = 3.4$ P = 0.03** | **$\chi2 = 4.5$ P = 0.01** | **$\chi2 = 5.8$ P = 0.008** | **$\chi2 = 9.1$ P = 0.001** | **$\chi2 = 24.2$ P <0.0001** | **$\chi2 = 13.8$ P = 0.0001** |
| *Canis familiaris* | $\chi2 = 2.5$ P = 0.06 | | $\chi2 = 0.07$ P = 0.39 | $\chi2 = 0.32$ P = 0.29 | $\chi2 = 0.8$ P = 0.19 | $\chi2 = 2.5$ P = 0.06 | **$\chi2 = 5.8$ P = 0.008** | **$\chi2 = 5.8$ P = 0.008** |
| *Equus caballus* | **$\chi2 = 3.4$ P = 0.02** | $\chi2 = 0.07$ P = 0.39 | | $\chi2 = 0.08$ P = 0.38 | $\chi2 = 0.36$ P = 0.27 | $\chi2 = 1.7$ P = 0.09 | **$\chi2 = 4.7$ P = 0.01** | **$\chi2 = 4.8$ P = 0.01** |
| *Sus scrofa* | **$\chi2 = 4.5$ P = 0.01** | $\chi2 = 0.32$ P = 0.29 | $\chi2 = 0.8$ P = 0.40 | | $\chi2 = 0.09$ P = 0.37 | $\chi2 = 1.1$ P = 0.15 | **$\chi2 = 3.7$ P = 0.02** | **$\chi2 = 3.8$ P = 0.03** |
| *Homo sapiens* | **$\chi2 = 5.8$ P = 0.008** | $\chi2 = 0.8$ P = 0.19 | $\chi2 = 0.36$ P = 0.27 | $\chi2 = 0.09$ P = 0.37 | | $\chi2 = 2.7$ P = 0.04 | **$\chi2 = 2.7$ P = 0.04** | **$\chi2 = 2.8$ P = 0.04** |
| *Gallus gallus* | **$\chi2 = 9.1$ P = 0.001** | $\chi2 = 2.5$ P = 0.06 | $\chi2 = 1.7$ P = 0.09 | $\chi2 = 1.1$ P = 0.15 | $\chi2 = 0.5$ P = 0.2 | | $\chi2 = 1.3$ P = 0.15 | $\chi2 = 1.0$ P = 0.15 |
| *Bos taurus* | **$\chi2 = 13.8$ P = 0.0001** | **$\chi2 = 5.8$ P = 0.008** | **$\chi2 = 4.7$ P = 0.01** | **$\chi2 = 3.7$ P = 0.02** | **$\chi2 = 2.7$ P = 0.04** | $\chi2 = 1.3$ P = 0.15 | | $\chi2 = 0.9$ P = 0.50 |
| ***Sergentomyia minuta* blood meal sources** | | | | | | | | |
| | *Equus asinus* | *Equus caballus* | *Homo sapiens* | *Sus scrofa* | | | | |
| *Oryctolagus cuniculus* | $\chi2 = 0.9$ P = 0.5 | $\chi2 = 0.4$ P = 0.3 | **$\chi2 = 8.3$ P = 0.02** | $\chi2 = 0.9$ P = 0.5 | | | | |
| *Equus asinus* | | $\chi2 = 0.4$ P = 0.3 | **$\chi2 = 8.3$ P = 0.02** | $\chi2 = 0.9$ P = 0.5 | | | | |
| *Equus caballus* | $\chi2 = 0.4$ P = 0.3 | | **$\chi2 = 5.9$ P = 0.007** | $\chi2 = 0.4$ P = 0.3 | | | | |
| *Homo sapiens* | **$\chi2 = 8.3$ P = 0.02** | **$\chi2 = 5.9$ P = 0.007** | | **$\chi2 = 8.3$ P = 0.02** | | | | |

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**Chapter 3**

Section 3.1

## *Leishmania infantum* in wild animals in endemic areas of southern Italy

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**Abstract**

*Leishmania infantum* infection in wildlife is increasingly reported in Europe, but scant data are available in Italy so far. This study aimed to investigate the circulation of *L. infantum* among sylvatic hosts in Sicily (southern Italy), a highly endemic area for canine leishmaniosis, through serological and molecular tools. Target tissues (skin, spleen, lymph nodes) collected from 71 European rabbits, 2 European hares, 7 red foxes, 11 European wildcats and 1 pine marten, were qPCR analysed for the detection of *L. infantum* DNA. Additionally, 40 rabbits, older than one year, were serologically screened for specific anti-*Leishmania* antibodies. *Leishmania infantum* was molecularly diagnosed in 5.4% (n=5) of the examined animals (3/71 European rabbits, 2/7 red foxes). In many of the qPCR positive animals (4/5), the parasite DNA was more prevalent in visceral than cutaneous tissues. None of the positive animal showed signs of disease and/or macroscopic alterations of organs; low parasitic burden in all positive tissue samples was also recorded. Only one rabbit serum (i.e., 2.5%) tested positive for anti-*Leishmania* antibodies. The seropositive rabbit was in good health status and no amastigotes were observed in lymph-node aspirate and blood smears.

This study provides first evidence of *L. infantum* infection in wild animals from Sicily (southern Italy). Despite the low prevalence of infection here reported, the circulation of the *Leishmania* in wild reservoirs in Sicily remains worthy of future investigations for a better understanding of their role in the epidemiology of the disease as well as to fine-tune control strategies in the area.

**KEYWORDS:** *Leishmania infantum*; Leporidae; red fox; qPCR; serology; Sicily.

## 1. Introduction

Leishmaniosis is one of the most important vector-borne diseases worldwide caused by obligate intracellular protozoans of the genus *Leishmania* (Kinetoplastida, Trypanosomatidae) that infects cells of the monocyte-macrophage lineage of several mammal species, including humans [1]. Infection is transmitted by the bite of female sand flies of the subfamily Phlebotominae, being *Phlebotomus perniciosus* the most relevant biological vector in Europe [2]. *Leishmania infantum* is the causative agent of zoonotic visceral (VL) and cutaneous leishmaniosis (CL) in humans, and of generalized canine leishmaniosis in dogs. Approximately 875 human autochthonous cases are reported each year in the Mediterranean basin only, and at least 2.5 million dogs are infected [3-6]. Though typically endemic in Mediterranean countries, leishmaniosis is northward spreading into previously non-endemic areas of Europe [2,7,8].

In most of the areas where *L. infantum* is endemic, infected and/or diseased dogs are recognized as the main epidemiological reservoir, although other domestic and wild mammals have been found infected with *Leishmania* and proposed as secondary or alternative hosts [9,10]. Serological and molecular detection of *L. infantum* infection among European wildlife has been reported in carnivores, lagomorphs and rodents [11,12]. Nevertheless, the ability to act as competent reservoir, i.e., infecting sand flies while taking blood meal, has only been confirmed in hares (*Lepus granatensis*), rabbits (*Oryctolagus cuniculus*) and black rats (*Rattus rattus*) [13-15]. Particularly, the epidemiological role of lagomorphs as reservoir hosts of *L. infantum* has been widely elucidated in Spain during an outbreak of human leishmaniasis in the area of Madrid [13, 14, 16-18]. On that occasion, the infection rate recorded in dogs of the area was only of 1.6-2%; thus, other animal species were screened, including a population of lagomorphs recently introduced in a park bordering the villages where human cases occurred [16,18]. Surprisingly, a high seroprevalence of positive samples was found among hares (i.e., 74.1%), and wild rabbits (i.e., 45.7%) of the park [18]. Also, the high exposure of lagomorphs to *P. perniciosus* bites has been showed by the detection of high anti-saliva antibody levels [17], and the identification of blood meals of sand flies caught in the context of the human leishmaniasis outbreak in Spain, confirmed lagomorphs as the preferred food source of *P. perniciosus* [19]. Therefore, apparently healthy hares were incriminated as the main source of human infection, playing a primary role as reservoir in the sylvatic *Leishmania* transmission cycle [13,18].

Sicily (southern Italy) is considered highly endemic for canine leishmaniosis with a mean annual incidence of 39.4% in unprotected dogs [20,21]. Approximately 47% of the Sicilian human population live in areas at risk for *Leishmania* infection, consisting in suburban and rural areas where competent sand fly species are abundant [22]. An epidemiologic survey conducted between 1987 and 1995 in Sicily revealed that the annual incidence of VL in humans was 6 cases per 1,000,000 residents, whereas in 2002 the incidence peaked 9.4 cases, representing 27% of the national disease burden [22,23].

The increasing incidence of leishmaniosis and the consequent failure of control strategies have been repeatedly put in relationship with the existence of vertebrate reservoirs of infection other than dogs [11]. Nevertheless, few studies in Italy have investigated the epidemiological role of sylvatic hosts in the maintenance of *Leishmania* infection in endemic foci [24-29].

This study aims to assess *L. infantum* infection in sylvatic animals by quantitative-PCR analysis of target tissues (i.e., skin, spleen, lymph-nodes) and by serology in rabbits kept in an en-plain air system, in order to increase the knowledge on the role of these animals in the epidemiology of leishmaniosis in Sicily.

## 2. Materials and methods

*2.1 Animals and tissues collection*

Carcasses of sylvatic animals occasionally found dead or regularly hunted in Sicily from 2015 to 2017 were used in this study. Carcasses were transported to the Department of Veterinary Sciences (University of Messina) and individually necropsied. Upon necropsy the presence of signs of disease or pathological alterations of organs was evaluated in each carcass and tissues (i.e., skin, spleen, popliteal lymph-nodes) were sampled and stored frozen (-20 C°) until molecular analyses.

*2.2 Molecular analyses*

Genomic DNA was extracted from each sample of skin, spleen and popliteal lymph node using the QIAamp Blood and Tissue kit (QIAGEN, Hildem, Germany) according to the manufacturer's instructions. Extracted DNA was re-suspended in 50 µl of elution buffer and frozen at −80 °C until analysis. A fragment (120 bp) of the *L. infantum* minicircle kinetoplast DNA (kDNA) was amplified by qPCR as described elsewhere [30-32]. For all PCR tests, positive (DNA of pathogen-positive sample) and negative (no DNA) controls were included.

*2.3 Serological analyses*

Blood samples were collected from a peripheral vein (auricular) of rabbits older than one year and kept in outdoor cages in different *en plain-air* breading systems located in areas known to be highly endemic for *L. infantum* infection [20]. Blood samples were collected in 2.5 ml cloth activator sterile tubes and maintained at 4°C until the arrival in laboratory. Each blood sample was centrifuged and the serum immediately tested to detect antibodies anti-*Leishmania donovani* complex, using the rk39 immunochromatographic test (Leishmania Dipstick rapydtest®, APACOR, England) which use in naturally *Leishmania* infected hares and wild rabbits has been largely validated in previous studies [13,14]. Lymph-node aspirate and whole blood samples were also collected in rabbits that scored seropositive in order to detect the presence of *L. infantum* amastigotes by cytological observation of smears.

**3. Results**

*3.1 Animals and tissues collection*

A total of 92 wild animal carcasses (n=73 lagomorphs and n=19 carnivores) were collected and used in this study. Particularly, target tissues (i.e., skin, spleen, popliteal lymph nodes) of 71 European rabbits (*Oryctolagus cuniculus*), 2 European hares (*Lepus europaeus*), 11 European wildcats (*Felis silvestris silvestris*), 7 red foxes (*Vulpes vulpes*) and 1 pine marten (*Martes martes*) were sampled for *L. infantum* DNA detection. Animals were hunted and/or found dead in six different provinces of Sicily. Specifically, 32 out of the 71 rabbits were from the Aeolian islands (Messina), or from the province of Ragusa (n=17), Caltanissetta (n=11) and Enna (n=11). The two hares were hunted in the province of Enna. The European wildcats were found dead in the area of the Etna Regional Park (province of Catania), while the foxes and the pine marten were found road-killed in the province of Palermo (Fig. 1).

*3.2 Presence of Leishmania DNA in target tissues*

Overall, from the 92 analysed animals, 5 (5.4%) were q-PCR positive, including 4.2% European rabbits (3/71) and 28.6% red foxes (2/7). However, agreement among qPCR results in different tissues was low and only a red fox tested positive in both skin and organs (i.e., spleen and popliteal lymph-nodes), while in the other positive fox, *L. infantum* DNA was detected only in the spleen. Among wild rabbits, 2 out of 3 were q-PCR positive to spleen samples, and one was positive

only to skin sample. PCR threshold cycles (Ct) in positive fox samples ranged from 24.11 to 36.66, while Ct values in PCR-positive wild rabbits were higher, ranging between 32.74 and 36.73 (Table 1).

*3.3 Seroprevalence in rabbits*

Forty rabbits (n=36 New Zealand and n=4 wild European) kept in outdoor cages in en plain-air breading systems were blood sampled and serologically tested (Fig. 1). Only one serum sample (1/40; 2.5%) scored seropositive for the detection of *Leishmania*-specific antibodies. The positive rabbit, a New Zealand white male 36-month-old, was in apparently good health status and no amastigotes were detected in lymph-node aspirate and/or blood smears.

**4. Discussion**

The present study provides data of *L. infantum* circulation in wild lagomorphs and carnivores in Sicily. Overall, from the 92 analysed animals, 5 (5.4%) were qPCR positives, including European rabbits (3/5) and red foxes (2/7). Among positive animals, the ratio of skin/organs was 1/2 in rabbits and 1/2 in foxes. This finding suggests, for both animal species, a higher visceral than cutaneous localization of the parasite, and agrees with data reported in literature for foxes [25,26], but not for rabbits, where a predominant skin rather than visceral PCR positiveness has been reported in previous surveys [33,34]. Experimental studies demonstrated that parasite visceralisation and disease development is regulated by the host's immune response and associated with different *Leishmania* species [35]. Therefore, the parasite's visceral tropism herein observed could be explained by the *Leishmania* species involved (i.e., *L. infantum*) as well as by the type of immune response mounted by the host. PCR threshold cycles in fox positive samples ranged from 24.11 to 36.66, while Ct values in PCR-positive wild rabbit samples were higher, ranging from 32.74 to 36.73, indicating a lower parasitic load in the lagomorph species. Detection of low *Leishmania* burden is a common finding in wildlife, and may suggest the capability of these animal species to better control the infection reflecting a kind of natural resistance in developing of the disease [11,36]. Indeed, none of the *Leishmania* infected animals herein reported showed clinical signs and/or macroscopic alterations of organs [5]. Absence of macroscopic lesions consistent with leishmaniosis is also a common feature of wild infected animals; In fact, only minimal microscopic lesions in few tissues, e.g. presence of macrophages with Leishmania amastigotes without any other inflammatory reaction, have been revealed though histopathological investigation in lymph nodes of *L. infantum* infected hares and rabbits [37].

Among European wildlife, red fox received most attention in leishmaniosis surveys due to its taxonomic relationship with the domestic dog and also because of its abundance in rural and peri-urban areas [11]. Nevertheless, few studies have been carried out in foxes in Italy, and molecular prevalence rates came from only two surveys carried in Campania region (southern Italy) and one in Tuscany (Central Italy) in which the rate of prevalence ranged from 20.8% to 52.2% [25,26,28]. Higher prevalence of infection has been reported in Spain, where up to 74% of PCR tested foxes scored positive to *L. infantum* [36, 38].

As regards to serological results herein reported, the finding of only one seropositive rabbit out of the forty tested (2.5%) confirms the susceptibility of the species to *Leishmania* infection but also suggests that parasite is not significantly spread in the screened animal population. Nevertheless, serological methods lack in sensitivity especially with asymptomatic carriers and tend to underestimate *Leishmania* infection [37,39]. Indeed, in apparently healthy animals, it is likely that the cellular immune response is greater than the humoral, justifying the absence of clinical signs and low or undetectable presence of antibodies [35]. As matter of facts, results of a recent study conducted in the contest of a human leishmaniosis outbreak in Spain, suggested the concomitant use of different diagnostic techniques (e.g., histopathology, DFA assay,

IFAT, qPCR) to increase the sensitivity and truthfulness of the *Leishmania* infection in wild asymptomatic lagomorphs [37]. Despite the low seroprevalence here observed, it is interesting to note that in the same farm where rabbits have been sampled for the current study, *Oryctolagus cuniculus* was detected as the preferred host species by *P. perniciosus* according to blood meal identification [40]. This feeding preference is consistent with what has already been demonstrated in Spain [14,20], and suggests as rabbits may contribute to the maintenance of high density of sand fly species when they are abundant in number like in Sicily.

In Italy, wild brown hares captured in five protected areas were tested by indirect immunofluorescence antibody test (IFAT) to detect antibodies against *Leishmania* spp., and a total of two out of 222 animals (0.9%) scored positive to infection with a titre of 1:20 [27]. In the same areas brown hares were also molecularly investigated for the detection of *Leishmania* DNA showing a prevalence of infection of 9.8% [29]. Again, also for this Leporidae species serological tools lack in sensitivity and a combination of diagnostic techniques is mandatory for a trustful estimation of *Leishmania* circulation in the examined population.

Our survey resulted in low infection rates of the screened sylvatic animals suggesting a minimal circulation of the parasites and a marginal involvement of the tested species in the epidemiology of leishmaniosis in Sicily. It is also true that the intrinsic constrains in diagnose *Leishmania* infection in wild animals coupled with the limited availability of these animals for studies do not definitively allow neither to confirm nor to exclude the hypothesis of their involvement as alternative sylvatic hosts in the epidemiology of *L. infantum* in Sicily. The circulation of the *Leishmania* in wild reservoirs in Sicily remains, therefore, worthy of future investigations for a better understanding of their role in the epidemiology of the disease as well as to fine-tune control strategies in the area.

**Declaration of interest**

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Authors declare to have no commercial or financial interests to disclose.

**Fig. 1.** Species and geographical origin of animals tested in the study.

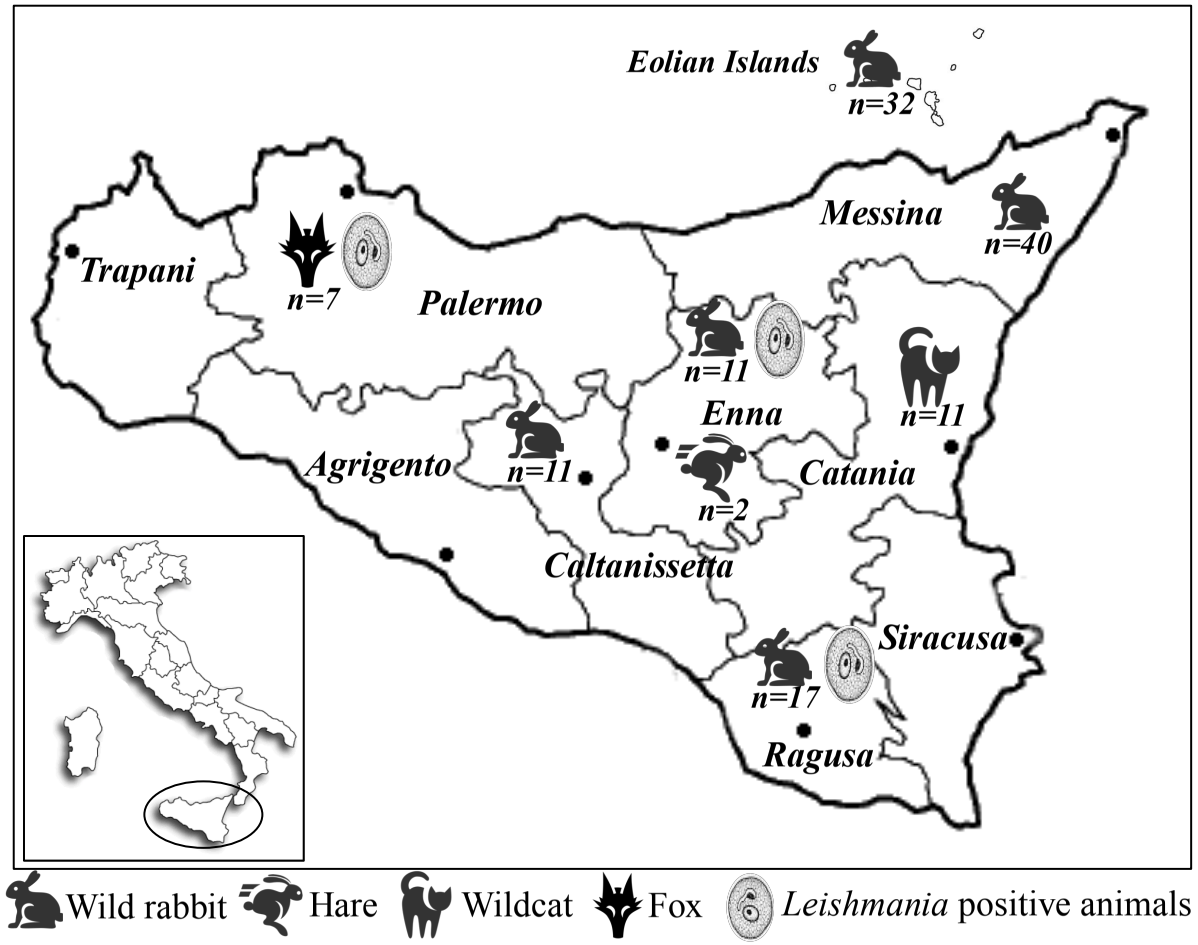

**Table 1.** *Leishmania infantum* qPCR positive target tissues and cycle threshold (Ct) values.

| Species | Prevalence of infection | Provenience of positive animals | Results of qPCR of target tissues (Ct values) | | |
|---|---|---|---|---|---|
| | | | Skin | Lymph-nodes | Spleen |
| *Oryctolagus cuniculus* | 3/71 (4.2%) | Ragusa (RG) | Positive (36.73) | Negative | Negative |
| | | Pietraperzia (EN) | Negative | Negative | Positive (34.79) |
| | | Pietraperzia (EN) | Negative | Negative | Positive (32.74) |
| *Vulpis vulpis* | 2/4 (50%) | Palermo (PA) | Positive (30.19) | Positive (24.66) | Positive (24.11) |
| | | Palermo (PA) | Negative | Negative | Positive (36.66) |

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**General discussion and conclusions**

This doctoral thesis provides novel information on three main research lines of leishmaniosis in southern Italy, namely domestic animal hosts, vectors - including proved and suspected vectorial capacity of species and the study feeding sources - and, finally, the potential role of wild animal hosts as reservoirs for infection.

In **Chapter 1** the primary role of dog as reservoir of *Leishmania infantum* in Mediterranean basin is highlighted and a novel effective strategy for simultaneously prevention of canine leishmaniosis (CanL) and other vector-borne diseases such as canine dirofilariosis is proposed.

Geographical distribution of vector-borne infections is closely related to the distribution and expansion of their vectors, and climatic changes and human activities lead to shifts in environmental conditions and indirectly influenced disease transmission and spreading (Pantchev et al., 2015). As result, some diseases regarded in the past as "tropical" or "exotic" can occur or even emerge in continental Europe (Di Luca et al., 2017). Under this scenario, dogs are continuously exposed to a wide plethora of vector-borne diseases and, CanL and canine dirofilariosis are among the most common diseases throughout Europe (Genchi and Kramer, 2017; Maia et al., 2016; Maia et al., 2015; Trotz-Williams et al., 2003). In particular, in several European countries the distribution of CanL and canine hearthworm disease (CHD) overlaps, and the spread and zoonotic potential of these diseases make their simultaneous prevention mandatory (Papadopoulos et al., 2017; Baneth et al., 2016; Hamel et al., 2016; Maia et al., 2016; Maia et al., 2015; Tabar et al., 2013; Otranto et al., 2010). Because of this growing risk of vector-borne pathogen transmission, adoption of effective and large spectrum preventative strategies is strongly advocated especially in dogs as they may serve as reservoirs for human infections. The study proposed in Section 1.1 addressed this need by investigating the efficacy and safety of a spot-on combination containing fipronil 6.76% w7v /permethrin 50.48% w/v in association with a chewable formulation containing afoxolaner 1.9%w/w /milbemycin oxime 0.4% w/w for the simultaneous prevention of *Leishmania* infection, dirofilariosis and other vector-borne diseases in naturally exposed dogs through a 6-month field study. The study provided for the first time evidence-base data on the efficacy and safety of this association which is now proposed as a comprehensive prevention treatment in dogs. As matter of fact, this study was conducted in a hyper-endemic area for CanL (Brianti et al., 2014), and also endemic for VBD transmitted by ticks (Brianti et al., 2012; Giannetto et al., 2007), and neither new seroconversion to vector-borne pathogens, nor blood microfilariae were detected in dogs at the end of transmission season (Abbate et al., 2018). Therefore, the concomitant use of these two antiparasitic products may be regarded as a safe and effective prevention strategy for dogs, particularly in geographical areas where the risk of CanL and CHD overlaps.

In Section 1.2, a case of feline leishmaniosis (FeL) with long-term follow-up data is reported to provide more information on clinical signs, clinical-pathological abnormalities, diagnosis and treatment of this scantly documented disease of feline patients (Brianti et al., 2019). Feline leishmaniosis is increasingly reported in endemic foci, although less frequently than CanL (Colella et al., 2019; Pennisi and Persichetti, 2018; Pennisi et al., 2015). Nevertheless, its real occurrence might be underestimated since little information on clinical features are available in literature and because of its poorly consideration as causative agent of disease in this animal species (Otranto et al., 2017; Iatta et al., 2019). Generally, cats are infected by the same *Leishmania* species affecting dogs, human and other mammals, being *L. infantum* the species most frequently isolated (Brianti et al., 2019; Metzdorf et al., 2017; Can et al., 2016; Pennisi et al., 2015). *Leishmania* affected cats with overt clinical signs have high blood parasitemia (Brianti et al., 2019; Pennisi and Persichetti, 2018; Migliazzo et al., 2015) and may participate in sustaining the parasite cycle and in the spreading of the disease, as competent vectors were found infected after feeding on cat with FeL (Maroli et al., 2007). As a consequence, the role of

cat as secondary domestic host of infection has recently gained attention, whereas its role in the epidemiology of visceral leishmaniosis (VL) is still poorly understood (Gramiccia et al., 2011; Maia and Campino, 2011; Millán et al., 2011). Because of the above, cats living in *Leishmania* endemic area need to be protected from the risk of becoming infected and the protection becomes mandatory when a "One Health" approach of *L. infantum* infection control is adopted (Courtenay et al., 2017; Pennisi et al., 2015; Da Silva et al., 2010; Maroli et al., 2007). As in dogs, the most effective preventative measures target sand flies and are finalized in the reduction of infective bites by means of repellent products of proven anti-feeding effect against *Leishmania* vectors (Brianti et al., 2017). Also, as for dogs, treatment of *L. infantum* infected cats is a key factor in reducing the parasitic load, resulting in a lower infectivity to sand flies (Brianti et al., 2019) and, therefore, must to be adopted in all diseased animals.

**Chapter 2** focuses on phlebotomine sand flies as unique proven vectors able to transmit *Leishmania* protozoa (Maroli et al., 2013). As changes in vector distribution are associated with changes in the risk of pathogen transmission, the monitoring of arthropod vectors becomes of importance in surveillance programs. Developing novel and efficacious capture methodologies that monitor vector populations is valuable for better understanding entomological and epidemiological dynamics of vector-borne diseases (Alexander, 2000). Light traps are largely employed for the collection of these insects, but numerous advantages over the standard incandescent lamp favour the use of light- emitting diodes (LEDs) as an alternative and cheaper light source for capturing medically important insects in surveillance study (Gaglio et al., 2017; Muller et al., 2015; Mann et al., 2009; Cohnstaedt et al., 2008). Additionally, the use of LEDs of different colours seems to bias the power of attraction of lights traps (Silva et al., 2015; Hoel et al., 2007), thus influencing the estimation of sand fly population, as demonstrated for *Phlebotomus papatasi* (Hoel et al., 2007). In section 2.1, the attractiveness of light traps equipped with different LED colours (white, red, green, blue, UV) to phlebotomine sand fly species endemic in the Mediterranean is reported and compared to capture performance of a classic light trap (CLT) (Gaglio et al., 2018). Results obtained in the above study demonstrated that light traps equipped with UV LED possess a higher attractiveness to *P. perniciosus,* the main vector of human and canine leishmaniosis, compared to other coloured LED traps. Thus, UV LED trap may be employed in sand flies monitoring as a highly effective alternative technology to the conventional method with incandescent lamps (Gaglio et al., 2018). Moreover, LEDs provide a much longer battery life, overcoming one of the constraints in conventional CLT.

In the same Chapter, Section 2.2, MALDI-TOF mass spectrometry is described as an alternative quicker approach for sand fly species identification compared to the conventional morphological method (Arfuso et al., 2019). The in-house reference database generated for the most representative sand fly species (i.e. *P. perniciosus*, *Sergentomyia minuta*), showed consistent results with morphological identification. The application of the MALDI-TOF protocol designed in the survey produced distinct, consistent and reproducible species-specific protein spectra of the sand fly specimens analysed with no substantial differences between males and females of the respective species. In fact, the thorax of sand flies was used for mass protein spectra analysis, therefore the exclusion of the terminal body sections of tested specimens prior MALDI-TOF analysis, removed the most sex-specific proteins. The species identification by MALDI-TOF revealed consistent results with the morphological identification supporting the potential of this method for rapid, simple and reliable phlebotomine sand fly species identification. Its employment would facilitate entomological surveys improving the knowledge on geographical distribution and/or spread of these medically important vectors.

Finally, in Section 2.3 the detection of natural *Leishmania* infection in wild caught sand flies is reported, in order to assess infection rates and identify circulating *Leishmania* species in vectors (Abbate et al., 2019, *submitted*). Surveillance on vectors is crucial also because of the risk of introduction of non-endemic *Leishmania* species and in order to assess the

risk of transmission (Alten et al., 2016; Antoniou et al., 2013). In addition to the detection of *Leishmania* infection in caught sand files, blood source identification was also assessed in all blood fed females to gain information on vectors' feeding habits and to allow recognition of potential reservoir hosts for *Leishmania* (Abbate et al., 2019*, submitted*). Indeed, knowledge of the host preferences of sand flies under natural conditions is crucial to understand their vectorial capacity in different leishmaniasis foci.

In the study, twenty-eight sand flies (n=27 *Sergentomyia minuta*; n= 1 *Phlebotomus perniciosus*)*,* scored positive to *Leishmania* spp. DNA. The sequencing of positive purified amplicons revealed that the majority of *S. minuta* scored positive for *Leishmania tarentolae* (26/27), whereas *L. donovoni* complex DNA was isolated in 1 *S. minuta* and 1 *P. perniciosus* specimens. *Leishmania* infection rate reported in this study is consistent with previous data for Southern Italy (Latrofa et al., 2018). The presence of *L. donovoni* complex DNA in *P. perniciosus* confirms the role of this species in the maintenance and spread of leishmaniasis in Sicily.

Although, several surveys reported the finding of *Leishmania* spp. pathogenic to human and other mammals in *S. minuta* specimens, no scientific evidences are available about on its role as vector of infection so far (Latrofa et al., 2018; Pereira et al., 2017; Maia et al., 2015). Through the study in Section 2.3 we described, for the first time, the presence of *Trypanosome* sp. DNA in *S. minuta* from a hyper-endemic area of canine *Leishmaniosis* in Southern Italy. Interestingly, the sequencing of positive ITS-1 amplicons for *Leishmania* spp., revealed high similarity for *Trypanosoma* spp. sequences deposited in GenBank. Phylogenetic analysis carried out on *Trypanosoma* sp. sequences herein found in *S. minuta* specimens revealed that five *Trypanosoma* sequences share the same common ancestry of *Trypanosoma varani* and of a *Trypanosoma* sp. isolated from Gecko, forming together a monophyletic cluster supported by a high value of boodstrap (92); whereas 2 *Trypanosoma* sequences resulted different, sharing the same common ancestry of *T. varani*, but segregate independently and leading to a non-identification at species level. In Italy, there is no data on the vectorial role of *S. minuta* in the transmission of *Trypanosome* spp. to wild and domestic animals. In the Mediterranean area, rabbit trypanosome and its co-infection with *L. infantum* was found in *P. perniciosus* female sand flies caught in the contest of human leishmaniosis outbreak in Madrid (González et al., 2018). All these findings suggest that *S. minuta* might have a role in the transmission of *Leishmania* and *Trypanosome* parasites; however, more extensive molecular studies and phylogenetical analyses are advocated to better characterize the *Trypanosoma* species detected in the study reported in Section 2.3 as well as to better clarify its taxonomic position. Moreover, it is important to highlight that when *Trypanosoma* and *Leishmania* species are present in the same geographical area, mixed infections could appear within the same host and/or vectors posing a challenge to the diagnosis (Bastrenta et al., 2003, De Araújo et al., 2013, Díaz-Sáez et al., 2014).

As regards to the identification of blood meal sources, most of the investigations on blood meal preferences demonstrated that sand flies have an opportunistic feeding behaviour and obtain blood from a variety of domestic and/or wild animals and from humans as well (Bravo-Barriga et al., 2016; Maia et al., 2015; Rossi et al., 2008). Interesting, despite canine species is regarded as the main domestic reservoir of zoonotic leishmaniasis, it seems to be not the preferred for blood meal; indeed, three out of the five selected sites are shelters with high availability of dogs (i.e. from 300 to 500 dogs per site). This dislike, however, can be related with the massive use of chemical repellents in dogs as preventive measure against leishmaniasis in highly endemic region (Bravo-Barriga et al., 2016; Maia et al., 2015**).**

Results gained in the study reported in Section 2.3 demonstrate that wild rabbits represented the most preferred blood source for *P. perniciosus* and this finding agrees with what has already been demonstrated in Spain (Gonzalez et al., 2017; Gonzalez et al., 2015; Martín- Martín et al., 2014; Jimenez et al., 2013); whereas *S. minuta* tested in the study mainly fed

on humans. Besides these two-main species, several other vertebrate hosts have been recognized in both sand fly species, underlining the opportunistic feeding behaviour of these hematophagous insects.

The higher frequency of blood meals on wild rabbits suggests, at least for *P. perniciosus*, a sort of preference to this leporidae species and puts forward the hypothesis on its involvement in the epidemiology of leishmaniasis as sylvatic reservoir. Additionally, the detection of *L. infantum* and *Trypanosoma* sp. DNA in *S. minuta*, together with the anthropophilic feeding-behaviour observed, incites to clarify the ability of this species in the transmission of pathogens to humans and other warm-blooded animals. It is noteworthy that in southern Portugal a blood-fed *S. minuta* sand fly has also been found infected by *L. infantum,* and human blood was identified as meal source (Pereira et al., 2017). Therefore, further surveys and xenodiagnoses studies are needed to clarify whether *S. minuta* can be act as natural vector of *Leishmania* and *Trypanosoma* parasites.

Finally, the study reported in **Chapter 3**, investigated the circulation of *L. infantum* infection in wild mammals in Sicily as potential reservoirs in a hyper-endemic area of the Mediterranean basin. Throughout Europe, serological and molecular evidence of *L. infantum* infection among wildlife has been increasingly reported (Tomassone et al., 2018; Millàn et al., 2014), but scant data are still available in Italy so far (Rocchigiani et al., 2018; Ebani et al., 2016; Piantedosi et al., 2016; Verin et al., 2010; Dipineto et al., 2007; Mancianti et al., 1994). In the reported study, *Leishmania* infection was investigated by quantitative-PCR analysis of target tissues (i.e. skin, lymph nodes, spleen) in wild carnivores and lagomorphs occasionally found dead or regular hunted. Additionally, serological investigation was carried out in lagomorphs kept in an en-plain air system to detect specific anti-*Leishmania* antibodies (Abbate et al., 2019; *submitted*). Overall, from the 92 analysed wild animals (n= 73 lagomorphs; n= 19 carnivores), 5 were q-PCR positive, including 3 European rabbits and 2 red foxes. The low prevalence of infection (i.e. 5.4%) and the low parasitic loads recorded (Ct values: 24.11- 36.73) suggest that the parasite is not significantly spread in screened sylvatic animal population. In addition, none of the positive animals showed clinical signs and or macroscopic alteration lesions compatible with leishmaniosis. As matter of fact, a recent study suggests that only minimal histopathological alterations are associated to *L. infantum* in wild infected lagomorphs (Ortega-Garcìa et al., 2019). Detection of low parasitic burden and also the asymptomatic clinical course of the infection are common findings in wildlife, and could reflect the capability of these animal species to control the infection (Sobrino et al., 2008; Millàn et al., 2014).

As regard to serology, the low seroprevalence found in screened rabbits (i.e. 2.5%; 1/40) suggests again a minimal spread of the parasite infection in the tested population and agrees with a previous study carried out in the centre of Italy in lagomorphs (Ebani et al., 2016). Nevertheless, the undetectable presence of antibodies in potentially exposed wild animals could be put in relationship with the prevalent cellular immune response than the humoral one that also justifies the absence of clinical signs (McCall et al., 2013). In fact, the employment of serological methods may underestimate *Leishmania* infection in asymptomatic lagomorphs, and, as recently suggested, the concomitant employment of different diagnostic techniques seems to represent a valid solution to increase the truthfulness of *Leishmania* prevalence of infection (Ortega-Garcìa et al., 2019, Martìn-Sánchez et al., 2001).

All together these findings confirm the importance of wildlife sanitary surveillance programs for the detection and monitoring of certain neglected pathogen agents that can affect wild and/or domestic animals and humans as well.

In conclusion, vector-borne zoonotic diseases represent a problem of rising importance, as they are related not only to the environment but also to human activities and a simple and definitive resolution of these diseases do not exist. Indeed, they are hard or even impossible to be definitively eradicated, since spreading patterns are permanently changing and many natural vectors and reservoir hosts exist.

Data presented in this doctoral thesis highlight the role of alternative domestic and wild animal reservoirs in leishmaniosis endemic areas and provide pivotal information on sand fly vectors useful in surveillance and control programs; Indeed, data on abundancy, identification and natural *Leishmania* infection and blood meal preferences of these hematophagous insects have been reported and discussed to elucidate the complex interaction between parasite-vector and hosts. Further researches should be devoted to assess the capability of wild animals to maintain leishmaniosis in sylvatic cycles and to better investigate the role of other sand fly species, as *S. minuta*, as vector able to transmit pathogens including *Leishmania* to humans and other worm-blooded animals. Furthermore, the impact of global warming should be considered to evaluate future trends in sand fly diversity and distribution and hence *Leishmania* occurrence.

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**About the Author**

Jessica Maria Abbate was born in Messina (ME) on January 16th 1991. She graduated in Veterinary Medicine, with the maximum score, in 2016; in the same year, she started a PhD course in "Public Health and Food safety" at the University of Messina under the supervision of Prof. Emanuele Brianti.

Apart from her primary interest in leishmaniosis, she collaborated on several scientific projects running in the Parasitology Unit of the University of Messina. Research activities of Jessica Abbate are focused on different topics of veterinary parasitology, including diagnosis, treatment, and control of parasitic diseases of domestic animals and lungworm infections in cats.

During the second year of the PhD course, she attended a one-year specialization course in Forensic Veterinary Pathology at the University of Veterinary Medicine of Napoli, Italy.

During the third year of PhD course, she carried out two scientific collaborations with foreign research groups: a three months and half internship at the Institute of Hygiene and Tropical Medicine New University of Lisbon (Portugal) focused on molecular identification of *Leishmania* DNA and blood sources in phlebotomine sand flies collected from endemic areas in Sicily; a two months and half internship at the University of Veterinary Sciences of Murcia (Spain), focused on the epidemiology of dermal filariosis in sylvatic ungulate species.

At the end of the PhD (September 30th 2019), she authored or co-authored 8 articles published in peer reviewed international journals, 2 articles submitted for publication, 1 dossier in national journal, 3 oral presentations, 3 abstracts and 1 poster at national conferences.

**List of publications:**

**International journals**

1. **Abbate JM**, Arfuso F, Napoli E, Gaglio G, Giannetto S, Latrofa MS, Otranto D, Brianti E. 2019. *Leishmania infantum* in wild animals in endemic areas of southern Italy. Comparative Immunology, Microbiology & Infectious Diseases. *Accepted*

2. Iaria C, **Abbate JM**, De Benedetto G, Mazzullo S, Ieni A, La Pietra A, Mazzullo G, Lanteri G. 2019. A rare case of thecoma in a dog. Biomedical journal of Scientific & Technical Research. 19 (issue 2): 14188-14192.

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4. Brianti E, Celi N, Napoli E, **Abbate JM**, Arfuso F, Gaglio G, Iatta R, Giannetto S, Gramiccia M, Otranto D. 2019. Treatment and long-term follow-up of a cat with leishmaniosis. Parasites & Vectors. 12:121.

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7. **Abbate JM,** Arfuso F, Gaglio G, Napoli E, Cavalera MF, Giannetto S, Otranto D, Brianti E. 2018. Larval survival of *Aelurostrongylus abstrusus* lungworm in cat litters**.** Journal of Feline Medicine and Surgery**.** Vol. 2018, p. 1-6. doi: 10.1177/1098612X18811168.

8. Gaglio G, Napoli E, Arfuso F, **Abbate JM**, Giannetto S, Brianti E. 2018. Do Different LED Colors Influence Sand Fly Collection by Light Trap in the Mediterranean? Biomed Research International. Vol. 2018, p. 1-7, ISSN: 2314-6133, doi: 10.1155/2018/6432637.

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**1.** **Abbate JM**, Maia C, Pereira A, Arfuso F, Gaglio G, Rizzo M, Caracappa G, Brianti E. 2019. *Leishmania* infection and blood feeding preferences of phlebotomine sand fly species common in the Mediterranean area. PLoS One. *Submitted*

*2.* Napoli E, Arfuso F, Gaglio G, **Abbate JM**, Giannetto S, Brianti E. 2019. Effect of different temperatures on survival and development of *Aelurostrongylus abstrusus* (Railliet, 1898) larvae. Journal of Helminthology. *Submitted*

**National journals**

1. **Abbate JM,** Gaglio G, Antognoni MT, Veronesi F, Brianti E. 2018. Aggiornamenti su ehrlichiosi e anaplasmosi. **SUMMA animali da compagnia,** vol. 2, p. 43-53, ISSN: 1828-5538.

**National Congress**

*1.* **Abbate JM**, Gaglio G, Arfuso F, Caracappa G, Giannetto S, Pereira A, Maia C, Brianti E. 2019. *Leishmania* infection and blood meal sources in phlebotomine sand flies from Sicily. In: **LXXIII Convegno SISVET**, 19-22/06/2019. Olbia (Italy). P.43. *Oral*

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3. **Abbate JM,** Napoli E, Arfuso F, Gaglio G, Giannetto S, Brianti E. 2017. Safety and efficacy of the concurrent treatment of dogs with Frontline Tri-Act® and NexGard Spectra®. In: **LXXI Congresso Nazionale SISVET,** 28/06-1/07/2017. Napoli (Italy). P.229. Oral

4. Arfuso F, Gaglio G, **Abbate JM**, Caracappa G, Lupia A, Napoli E, Giarratana F, Latrofa M, Giannetto S, Otranto D, Brianti E. 2019. Identification of phlebotomine sand flies through maldi-tof mass spectrometry and in-house reference database. In: **LXXIII Convegno SISVET**, 19-22/06/2019. Olbia (Italy). P.43. *Abstract*

5. Gaglio G, Napoli E, Arfuso F, **Abbate JM**, Giannetto S, Brianti E. 2018. Attractiveness of different colored LEDs for sand fly monitoring. In: **XXX Congresso SOIPA**, 26-29/06/2018. Milano (Italy). P.160. *Abstract*

6. Brianti E, Celi N, Napoli E, **Abbate JM**, Arfuso F, Gaglio G, Iatta R, Giannetto S, Gramiccia M, Otranto D. 2019. Treatment and long-term follow-up of a cat with leishmaniosis. In: **LXXIII Convegno SISVET,** 19-22/06/2019. Olbia (Italy). *Poster*

**Training courses and conferences attended**

1. 17-19.03.2017. International Congress SCIVAC "Malattie infettive e parassitarie nel nuovo millennio: dalla prevenzione alla diagnosi e terapia." (Verona, 17-19 March 2017).

2. 07.05.2017. Symposium MSD-AH «PREVENZIONE a 360°». "Prevenzione per il controllo delle malattie nell'interfaccia uomo/animale: nessuna speranza senza un approccio ONE HEALTH". (Catania, 7 May 2017).

3. 10-11.06.2017. Dermatological Cytology course. Officina Veterinaria, Misterbianco (CT).

4. 28.06-01.07.2017. LXXI Congresso SISVet. Università degli Studi di Napoli "Federico II". (Napoli, 28 June-1 July 2017).

5. 10.03-15.12.2017. One-year specialized course in Forensic Veterinary Pathology. Dipartimento di Medicina Veterinaria e Produzioni Animali dell'Università degli Studi di Napoli "Federico II".

6. 26.06-29.06.2018. XXX Congresso Nazionale di Parassitologia (SOIPA). Università degli Studi di Milano. (Milano, 26-29 giugno 2018).

7. 24.01-28.01.2018. Partecipazione al 12 Simposio invernale di medicina del cane e del gatto. (Andalo, TN 24-28 gennaio 2018).

8. 19-22.06.2019. LXXIII Convegno SISVET (Olbia, 19-22 June 2019).