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Research paper

Feline and canine leishmaniosis and other vector-borne diseases in the Aeolian Islands: Pathogen and vector circulation in a confined environment

Domenico Otranto[a,*], Ettore Napoli[b], Maria Stefania Latrofa[a], Giada Annoscia[a], Viviana Domenica Tarallo[a], Grazia Greco[a], Eleonora Lorusso[a], Laura Gulotta[c], Luigi Falsone[b], Fabrizio Solari Basano[d], Maria Grazia Pennisi[b], Katrin Deuster[e], Gioia Capelli[f], Filipe Dantas-Torres[a,g], Emanuele Brianti[b]

[a] Dipartimento di Medicina Veterinaria, Università degli Studi di Bari, Str.prov. per Casamassima km 3, 70010 Valenzano, Bari, Italy
[b] Dipartimento di Scienze Veterinarie, Università degli Studi di Messina, Polo Universitario Annunziata, 98168, Messina, Italy
[c] Ambulatorio Veterinario S. Lucia, Via F. Crispi 56, Lipari, 98055, Messina, Italy
[d] Arcoblu s.r.l., Milano, Italy
[e] Bayer Animal Health GmbH, Leverkusen, Germany
[f] Istituto Zooprofilattico Sperimentale delle Venezie, 35020 Legnaro, Italy
[g] Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, Brazil

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ABSTRACT

Vector-borne diseases (VBDs) are prevalently investigated in dogs. Studies on feline VBDs are scant, though feline leishmaniosis (FeL) is increasingly recognised as a disease of cats in endemic areas. Comprehensive investigations on the distribution of VBDs in populations of cats and dogs living in relatively small geographical areas, such as islands, are currently lacking. In this study the prevalence of *Leishmania infantum* and other VBD pathogens was assessed in cohorts of cats and dogs living in the Aeolian Islands.

Autochthonous animals (330 cats and 263 dogs) of different age and sex were sampled. Blood and conjunctival samples were collected from cats and dogs for serological and molecular testing. Eighty-five (25.8%) cats were positive for *L. infantum*, 13 (3.9%) for *Bartonella* spp. and 1 (0.3%) for *Hepatozoon felis*. One-hundred and ten dogs (41.8%) were positive for *L. infantum* and three (1.1%) for *Hepatozoon canis*. The incidence of *L. infantum* infection in cats positive after one season of exposure to sand fly was 14.7%. *Leishmania infantum* prevalence and year incidence were higher in dogs than in cats ($p = 0.0001$ and $p = 0.0003$, respectively). Thirty-four cats (10.3%) scored positive for ticks (mean intensity rate of infestation, 2.03 ± 1.4), which were identified to the species level as *Ixodes ventalloi* and *Rhipicephalus pusillus*. Conversely, *Rhipicephalus sanguineus* sensu lato (s.l.) was the only species identified in dogs (10.6%). A larger prevalence of infestation by *Ctenocephalides felis* was recorded in cats (n = 91; 27.6%) than in dogs (n = 33; 12.5%) ($p = 0.0001$). In addition, one female *Nosopsyllus fasciatus* (syn. *Ceratophyllus fasciatus*) and one male *Spilopsyllus cuniculi* were also identified in flea-infected cats. VBDs are endemic in the Aeolian Islands being *L. infantum* the most prevalent vector-borne pathogen circulating between cats and dogs. The overall seroprevalence of FeL herein recorded is higher than that assessed, only by IFAT, in populations of cats in Greece and in Spain. Because *L. infantum* and VBDs are more commonly associated with dogs, the recognition of cats as hosts of different vector-borne pathogens is of paramount importance towards a better management of these diseases in both animals and humans.

1. Introduction

Leishmaniosis and other vector-borne diseases (VBDs) are prevalent in dog populations worldwide being of increasing concern for their zoonotic potential (Otranto et al., 2009a; Otranto et al., 2009b). Conversely, epidemiology of feline VBDs (FeVBDs) are much less investigated resulting in scant data available (Otranto

* Corresponding author at: Dipartimento di Medicina Veterinaria, Università degli Studi di Bari, Str.prov. per Casamassima km 3, 70010 Valenzano, Bari, Italy.
E-mail address: domenico.otranto@uniba.it (D. Otranto).

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and Dantas-Torres, 2010; Pennisi et al., 2015a). This lack of scientific data on FeVBDs apparently contrasts with the large number of cats living with families in Europe and in the US (about 90 and 66 million, respectively), and with the outdoor life-style of many of them (in Otranto, 2015). Though cats are exposed to a number of arthropods such as fleas, ticks and sand flies, and to the pathogens they may transmit (Maroli et al., 2007; Maia et al., 2010; Pennisi et al., 2013), their habits and behaviour (e.g. grooming) seem to minimize the risk for arthropod infection compared to dogs, resulting in scant information on the role of some ectoparasites, such as ticks, as vectors of pathogens to cats (Day, 2016). Thus, cats may be less susceptible than dogs to several pathogens, including vector-borne pathogens (VBPs) (Day, 2016). Over the last decade, studies on FeVBDs increased worldwide, especially those on feline leishmaniosis (FeL) (Pennisi et al., 2015a). Nonetheless, the infection by *Leishmania infantum* is more commonly associated with dogs, which are regarded as the main domestic reservoir of this protozoan. Sand flies, the natural vectors of *L. infantum*, may take their blood meals on cats (Maroli et al., 2009; Sales et al., 2015) and become infected after feeding on naturally infected cats (Maroli et al., 2007). Nevertheless, clinical diagnosis of FeL in endemic areas is not common, probably because of the subclinical infection occurring in most of the infected cats, or, merely because veterinary practitioners do not usually consider this disease in the list of differential diagnosis of their feline patients. The occurrence of FeL and other FeVBDs in cats has been reported in countries around the Mediterranean basin (Tabar et al., 2008; Solano-Gallego and Baneth, 2011; Vilhena et al., 2013; Silaghi et al., 2014), with prevalence generally well below those recorded in dogs (Poli et al., 2002; Cardoso et al., 2010). In addition, the large variability in prevalence data of FeL observed in cat populations (i.e. from 0.7 to 68.5%) has been attributed to the different sensitivity of diagnostic techniques employed and to the cut-off values set for the indirect immunofluorescence antibody test (IFAT) (Pennisi et al., 2015a). In addition, serological and molecular tests have been seldom combined for the diagnosis of FeL in the same animal population and often in a low number of cats (e.g. in Greece Chatzis et al. (2014a), Chatzis et al. (2014b); in Italy Pennisi et al. (2012); in Spain Ayllon et al. (2008), Sherry et al. (2011)), therefore limiting the overall information on the actual prevalence of the infection in cats.

Comprehensive investigations on the distribution of FeL and other VBDs in populations of cats living in confined environments, such as in small islands, are currently lacking. Under the above circumstances, the Aeolian Islands (Sicily), representing an environment isolated by the sea for definition, are featured by optimal conditions to study a well-defined population of animals, vectors and pathogens. Overall, Sicily is a region highly endemic for canine leishmaniosis (Brianti et al., 2014; Brianti et al., 2016) with an average of 31.5 notified cases/year in humans from 1987 to 1995 (Cascio et al., 1997).

Therefore, the aim of this study was to assess the prevalence of *L. infantum* infection (by molecular and serological techniques) in populations of cats living in the Aeolian Islands and to compare results with those of dogs from the same islands. A comprehensive analysis of the association among infection by *L. infantum* and other VBPs, anamnestic data and risk factors have also been provided in order to gain more information on the occurrence of these little known infections in cats.

2. Materials and methods

2.1. Ethical statement

This study was conducted in accordance with the principles of Good Clinical Practice (VICH GL9 GCP, 2000). For each animal included in the study the owner signed an informed consent form. The design and the experimental procedures used in this study were authorized by the Italian Ministry of Health (DGSA no. 0006088; 10/03/2015).

2.2. Study site

The study was carried out from January 2015 to June 2016, in Lipari and Vulcano, two of the main islands of the Aeolian archipelago, so named for the demigod of the winds Aeolus. For their beauty and nature, the Aeolian Islands (surface area of 114.7 km^2 in the Tyrrhenian Sea, province of Messina, Sicily, Italy, 38°32′N, 14°54′E) have a strong tourism vocation, with up to 260,000 visitors annually that increase the autochthonous population of nearly 15,000 inhabitants. Lipari has an area of 37 km^2 and is characterized by costal cliffs fronted by rocks, and the profile of the island is dominated by large central building of Monte S. Angelo (499 m a.s.l.). Lipari alternates very different landscapes with the western area being characterized by dry grass prairies with abundant presence of dwarf palm (*Chamaerops humilis*) and spring flowering of many species of orchids. In the highest part of the island prevails the Mediterranean maquis, featured by arbutus, heather, ash and aquiline ferns plants. Vulcano, with an area of 21 km^2, is located very close to Lipari; the two islands are indeed separated by a strait. The highest points of Vulcano are Monte Aria and Monte Saraceno (501 m a.s.l. each). Vulcano is mainly covered with thick bush, among which prevails genista (*Genista tyrrhena*) and cytisus (*Cytisus aeolicus*), two plants peculiar of the Aeolian archipelago. The climate of the Aeolian Islands is temperate, typical of the central Mediterranean area. The average temperatures vary from 10° C during winter to 27° C in summer, and are mitigated by marine breeze (source: Servizio Meteorologico Aeronautica Militare). Aeolian territory hosts a diverse fauna including bird species and wild mammals such as the Garden dormhouse (*Eliomys quercinus*, subspecies *liparensis*) and the widespread European wild rabbit (*Oryctolagus cuniculus*). The occurrence of the infection by *L. infantum* and other VBDs has been reported in the Aeolian archipelago in some symptomatic dogs and cats (Pennisi et al., 2015b; Persichetti et al., 2016).

2.3. Animal populations, sampling procedures and pathogens investigated

All animals sampled in this study (330 cats and 263 dogs) were referred to the only veterinary clinic in the Aeolian archipelago (Ambulatorio Veterinario Santa Lucia, Lipari), owned by one of the authors (LG), and were selected based on the owners' willingness to have their pet included in the survey. Cats and dogs were mostly housed in Lipari and Vulcano islands and in Salina, Filicudi, Stromboli, Alicudi, and Panarea, in descending order, of different age, sex and living outdoor or having constant outdoor access. Data about age, sex, breed, and antiparasitic treatments were collected. Systemic signs (e.g. loss of weight, fever, pale or icteric mucous membranes, peripheral lymphadenomegaly, hepatomegaly, splenomegaly, bleeding), as well as skin (e.g. ulcers, papules, nodules, crusts, haemorrhagic blisters, scales, alopecia/hypotrichosis) and ocular disorders (e.g. blepharitis, conjunctivitis, keratitis, uveitis or panophthalmitis) suggestive of VBDs were recorded in each animal's file along with data on the presence of ticks and fleas.

Dogs and cats were examined for the presence of ticks and fleas by thumb counting. For each dog, the number of ectoparasites and/or developmental stages of ticks detected were recorded in a separate form, ticks were counted and the infestation categorized on the basis of their number into the following four classes: low ($\leq$10); medium (10 > x $\leq$ 20); high (20 > x $\leq$ 30); very high (>30).

Fig. 1. A cat positive by multiple tests supporting *Leishmania infantum* infection and displaying clinical signs of feline leishmaniosis.

Fleas and ticks were identified according to morphological keys (Berlinguer, 1964; Manilla, 1998).

Conjunctival swabs were obtained for the molecular diagnosis of *L. infantum* infection. Blood was collected for serological, cytological and molecular testing (see below). In particular, from each animal, one EDTA tube (1 mL) and one clot-activator tube (5 mL) were filled. Blood and buffy coat smears were prepared at the time of bleeding and after centrifugation of the blood in capillary tubes, respectively.

Leishmania infantum infection was assessed by IFAT and by quantitative PCR (qPCR) on blood and conjunctival swabs. Infection by other VBDs (i.e. *Anaplasma* spp., *Babesia* spp., *Ehrlichia* spp., *Hepatozoon* spp. and *Bartonella* spp.) was assessed by cytological evaluation of blood and buffy coat smears and/or by conventional PCR (cPCR) on blood samples. cPCR and nested PCR on blood samples were used for Feline leukaemia virus (FeLV) and Feline immunodeficiency virus (FIV) detection, respectively. Conjunctival swabs, whole blood and serum samples were stored frozen (−20 °C) until analysis.

2.4. Serological testing

Serum samples from cats and dogs were tested for anti-*L. infantum* antibodies by IFAT as described elsewhere (Otranto et al., 2010). Importantly, for cats, the positive control serum was obtained from a cat with clinical illness (Fig. 1) also found with a high number of amastigotes of *L. infantum* at the cytological examination of skin lesion. Samples were scored as positive when they produced a clear cytoplasmic and membrane fluorescence of promastigotes from a cut-off dilution of 1:40 and 1:80 for cats and dogs, respectively (Otranto et al., 2010; Spada et al., 2013), by using conjugates specific for dogs (anti-dog IgG, Sigma-Aldrich, St. Louis, Missouri, USA) and cats (anti-cat IgG; Sigma-Aldrich, St. Louis, Missouri, USA). Positive sera were titrated until they gave negative results.

2.5. Cytological examination

Blood and buffy coat smears were prepared as described above and stained using May-Grünwald-Giemsa quick stain (Bio-Optica, Milan, Italy). Intracellular inclusions or free forms of pathogens were searched in each smear by examining the entire stained area at low magnification (×100) and representative areas at high magnification (×1000) for 10 min.

2.6. PCR

Genomic DNA was extracted from blood and conjunctival swabs using the QIAamp DNA Micro Kit (Qiagen, Milan, Italy), following the producer's recommendations. DNA of *Anaplasma* spp., *Babesia* spp., *Ehrlichia* spp. and *Hepatozoon* spp. were molecularly detected by cPCR using primers and run protocols described elsewhere (Olmeda et al., 1997; Inokuma et al., 2002; Harrus et al., 2011). Molecular diagnosis of *Bartonella* spp. DNA was carried out by PCR targeting a 775 bp fragment of the 16S–23S internal transcribed spacer (ITS) region using primers 325 s and 1100as (Diniz et al., 2007). FeLV and FIV proviral DNAs were searched using primers and protocol previously described (Endo et al., 1997; Stiles et al., 1999).

PCR products were examined on 2% agarose gels stained with GelRed (VWR International PBI, Milano, Italy) and visualized on a GelLogic 100 gel documentation system (Kodak, New York, USA). The amplicons were purified and sequenced, in both directions using the same primers as for PCR, employing the Taq Dye Deoxy Terminator Cycle Sequencing Kit (v.2, Applied Biosystems) in an automated sequencer (ABI-PRISM 377). Sequences were compared with those available in GenBank. A fragment (120 bp) of the *L. infantum* minicircle kinetoplast DNA (kDNA) was amplified by qPCR as described elsewhere (Francino et al., 2006; Dantas-Torres et al., 2011). For all PCR tests, positive (DNA of pathogen-positive blood sample) and negative (no DNA) controls were included.

2.7. Statistical analysis

Differences of *L. infantum* prevalence between cats and dogs and in relation to data of animals were calculated using Pearson Chi-square or Fisher's exact test, as appropriate. The prevalence was also calculated in relation to the number of seasons of exposure to sand fly, which was assessed based on their age at the time of sampling and considering animals positive at serology and/or PCR. The year "incidence of exposure" to *L. infantum* was calculated including animals exposed to one sand fly season only (i.e. June-October) (Gaglio et al., 2014). All the statistical analyses were performed using SPSS for Windows, version 13.0. (SPSS Inc., Chicago, US).

3. Results

The prevalence of the infection for *L. infantum* and other pathogens in cats and dogs calculated by serology and/or molecular tests is reported in Tables 1–3 . In particular, 85 (25.8%) autochthonous cats were positive for *L. infantum* by IFAT and by one or more molecular diagnostic test. In addition, 13 (3.9%) cats were positive for *Bartonella* spp. DNA of which nine were identified as *B. henselae* and two as *B. clarridgeiae*. One cat (0.3%) scored PCR positive for *Hepatozoon felis* with a 99% nucleotide identity with a reference sequence deposited in GenBank (AN: KC138534). Overall, seven and two cats were positive for FeLV (2.1%) and FIV (0.6%), respectively. Among cats serological positive to *L. infantum*, 75.3% had an antibody titre of 1:40, whereas in the remaining the titre varied from 1:80 to 1:640. None of the target pathogens was microscopically observed in the stained smears.

Table 1
Prevalence of infection by vector-borne pathogens detected by indirect immunofluorescence antibody test (IFAT) and/or PCR on blood or conjunctival swabs (c.s.) in autochthonous cats and dogs. Significant differences of *Leishmania infantum* prevalence between dogs and cats are marked with equal letters (upper-case = p < 0.01).

Animals	*Leishmania infantum*				*Hepatozoon* spp.	*Bartonella* spp.	*B. henselae*	*B. clarridgeiae*
	IFAT Pos/N (%)	qPCR blood Pos/N (%)	qPCR c.s. Pos/N (%)	Total Pos/N (%)	cPCR blood Pos/N (%)	cPCR blood Pos/N (%)	Pos/N (%)	Pos/N (%)
Cat	85/330 (25.7)	7/330 (2.1)[A]	6/330 (1.8)[B]	85/330 (25.7)[C]	1/330 (0.3)	13/330 (3.9)	9/330 (2.7)	2/330 (0.6)
Dog	91/263 (34.6)	32/263 (12.2)[A]	24/263 (9.1)[B]	110/263 (41.8)[C]	3/263 (1.1)	–	–	–

– = not detected.

Table 2
Association between variables: age, gender and animal origin of cats and the serological and molecular positivity for pathogens. Significant differences of *Leishmania infantum* prevalence are marked with equal letters (upper-case = $p < 0.01$).

Variables		*Leishmania infantum*				*Hepatozoon* spp.	*Bartonella* spp.
Age	N	IFAT N (Titre)	qPCR blood Pos (%)	qPCR c.s. Pos (%)	Total Pos (%)	cPCR blood Pos (%)	cPCR blood Pos (%)
<1 year	99	14 (1:40)	–	1 (1.01)	14 (14.1)[A]	–	10 (10.1)
1 < 2 years	101	18 (1:40); 3 (1:80); 1 (1:160); 1 (1:640)	3 (3.0)	1 (1.0)	23 (22.8)[B]	1 (1.0)	1 (1.0)
>2 years	130	32 (1:40); 9 (1:80); 4 (1:160); 2 (1:320); 1 (1:640)	4 (3.1)	4 (3.1)	48 (36.9)[A,B]	–	2 (1.5)
Gender							
Male	151	33 (1:40); 8 (1:80); 4 (1:160)	2 (1.3)	1 (0.7)	45 (29.8)	1 (0.7)	6 (4.0)
Female	179	31 (1:40); 4 (1:80); 1 (1:160); 2 (1:320); 2 (1:640)	5 (2.8)	5 (2.8)	40 (22.3)	–	7 (3.9)
Geographical origin*							
Lipari	282	48 (1:40); 12 (1:80); 4 (1:160); 2 (1:320); 2 (1:640)	7 (2.48)	6 (2.12)	68 (24.1)[E]	1 (0.3)	12 (4.2)
Vulcano	44	16 (1:40); 1(1:160)	–	–	17 (38.6)[E]	–	1 (2.3)

* 4 cats sampled in Salina (n = 2), Panarea and Stromboli (n = 1 each) scored negative for any parasite.

Table 3
Association between variables: age, gender and origin of dogs and the serological and molecular positivity for pathogens. Significant differences of *Leishmania infantum* prevalence are marked with equal letters (lower-case = p < 0.05).

Variables		*Leishmania infantum*					*Hepatozoon* spp.	*Bartonella* spp.
Age	N	IFAT Pos (%)	N (Titre)	qPCR blood Pos (%)	qPCR c.s. Pos (%)	Total Pos (%)	rPCR blood Pos (%)	rPCR blood Pos (%)
<1 year	23	6 (26.1)	6 (1:80)	0	1 (4.3)	7 (30.4)	–	–
1 < 2 years	41	11 (26.8)	10 (1:80); 1 (1:160)	2 (8.7)	2 (4.9)	12 (29.3)	–	–
>2 years	199	74 (37.2)	64 (1:80); 9 (1:160); 1 (1:320)	30 (15.1)	21 (10.5)	91 (45.7)	3 (1.5)	–
Gender								
Male	147	46 (31.3)	41 (1:80); 4 (1.160); 1 (1:320)	17 (11.6)	12 (8.2)	61 (41.5)	1 (0.7)	–
Female	116	45 (38.8)	38 (1:80); 6 (1.160)	15 (12.9)	11 (9.5)	50 (43.1)	2 (1.7)	–
Geographical origin								
Alicudi	1	1	1 (1:80)	–	–	1(100)	–	–
Filicudi	9	7 (77.8)[a]	6 (1:80); 1(1:160)	4 (44.5)	4 (44.5)	9 (100)	–	–
Lipari	175	62 (35.4)[a]	52 (1:80); 8 (1:160); 1(1:320)	18 (10.3)	14 (8)	73 (41.7)	1 (1.3)	–
Salina	22	10 (45.5)	10 (1:80)	2 (9.1)	2 (9.1)	10 (45.5)	2 (9.1)	–
Stromboli	1	1	1 (1:80)	–	–	1 (100)	–	–
Vulcano	55	10 (18.2)	9 (1:80); 1 (1:160)	6 (10.9)	5 (9.1)	13 (23.6)	–	–

The majority of cats positive for *L. infantum* were more than two years old (n = 48/85, 56.5%), of which 32 (66.6%) showed IFAT titres of 1:40, followed by 16 cats (33.3%) with titres ranging from 1:80 to 1:640 (Table 2). Fourteen cats (16.5%) younger than one year old had IFAT titres of 1:40 (Table 2).

Overall, nine cats (2.7%) scored molecularly positive in blood and/or conjunctival swabs. All these animals were also IFAT positive with the following titrations: 1:640 (2); 1:320 (1); 1:160 (2); 1:80 (2); 1:40 (2). Co-infections by *L. infantum* and *Bartonella* spp. were molecularly detected in three cats, with two cats positive for *L. infantum* and *B. henselae* and one for *L. infantum* and *B. clarridgeiae*. No difference was observed in relation to the positivity of *L. infantum* and *Bartonella* spp. and the cat gender, whilst a higher rate of *L. infantum* infection (38.6%) was observed in Vulcano ($\chi^2 = 29.670$ $p < 0.01$) (Table 2). Any of the four animals sampled in Salina (n = 2), Panarea and Stromboli (1 animal each island) scored positive to FeL or other VBDs (data not included in Table 2). Three cats tested positive for FIV proviral DNA, while only one cat was infected by FeLV. Of these, two FIV-positive and the single FeLV-positive cats were co-infected by *L. infantum* (data not shown). Two (one FIV positive and one FeLV positive) of the three co-infected cats showed some clinical signs suggestive of FeL, such peripheral lymphadenomegaly, loss of weight, skin disorder (crusts) and ocular disorder (conjunctivitis).

In cats positive by one or more diagnostic tests for *L. infantum*, the following clinical signs and lesions were observed: peripheral lymphadenomegaly (48.7%), skin disorders (41%) (i.e. scales, crust and alopecia), splenomegaly (23.1%), and ocular disorders

Table 4
Prevalence of cats and dogs positive for *Leishmania infantum* (serology at any titres and/or PCR) according to the seasons of exposure to sand fly. Significant differences are marked with equal letter (uppercase = $p < 0.01$).

Season of exposure	Examined cats/dogs	*Leishmania* pos cats/dogs	% cats/dogs
1	136/37	20/10	$14.7^{A}/27.0^{B}$
2	64/27	17/9	26.6/33.3
3 or more	130/199	48/91	$36.9^{AC}/45.7^{BC}$
Tot	330/263	85/110	$25.7^{D}/41.8^{D}$

(8.9%) (i.e. blepharitis, conjunctivitis, panophthalmitis and keratitis). Moreover, statistical significant differences in frequency of peripheral lymphadenomegaly ($\chi^2 = 12.195$; $p = 0.0022$) and skin disorders ($\chi^2 = 14.864$; $p = 0.0006$) between *L. infantum* infected and negative cats were observed.

Among dogs positive for *L. infantum* at serology and/or molecular test (n = 110; 41.8%), 91 (82.7%) were more than two years old and showed an IFAT titre ranging from 1:80 to 1:320. Nineteen dogs were positive only by qPCR (6 in conjunctival swabs and 16 in blood samples), of which three were positive to both conjunctival swabs and blood samples. All animals scoring positive to both qPCR (14.2%) and IFAT, displayed an IFAT titre of 1:80. Three of the sampled dogs (1.1%) tested positive at qPCR on blood samples for *Hepatozoon canis*. All the *H. canis* positive dogs were also co-infected by *L. infantum*; in addition, two of these animals scored positive at cytology on buffy coat smears for *H. canis*. No other animals were positive at cytology.

Overall, *L. infantum* prevalence in dogs (41.8%) was significantly higher than in cats (25.8%) ($\chi^2 = 16.4$, $p = 0.0001$). The *L. infantum* year incidence, assessed by the seasons of exposure to sand flies, was considerably higher in dogs (27%; 10/37) than in cats (14.7%; 20/136) ($\chi^2 = 12.907$; $p = 0.0003$) (Table 4).

Thirty-four cats (10.3%), living in Lipari and Salina, scored positive for ticks with a mean intensity rate of infection of 2.03 ± 1.4. Of the 69 ticks collected, 49 were identified as *Ixodes ventalloi* (42 females, 6 males and one nymph) and 20 as *Rhipicephalus pusillus* (7 females and 13 males). None of the cats from Vulcano, Panarea and Stromboli were infested by ticks. Twenty-eight dogs (10.6%) scored positive for ticks; twelve (42%) were harbouring less than 10 ticks whereas 46% of the infested dogs had >30 ticks. All the ticks (n = 161) collected from dogs were identified as *Rhipicephalus sanguineus* s.l. (i.e. 64 females, 89 males and 8 nymphs). Cats and dogs scored positive for *Ctenocephalides felis* with a larger prevalence of infestation in cats (n = 91; 27.6%) than in dogs (n = 33; 12.5%) ($\chi^2 = 19.089$, $p = 0.0001$). In addition, one male *Nosopsyllus fasciatus* (syn. *Ceratophyllus fasciatus*) and one male *Spilopsyllus cuniculi* were identified in two cats from Lipari (Fig. 2).

4. Discussions

VBPs are endemic in cats and dogs of the Aeolian Islands, with *L. infantum* being the most prevalent agent diagnosed. Based on the current literature (reviewed in Pennisi et al., 2015a), the population of cats herein examined was the largest sampled in a limited period of time, in a relatively small and remote geographical area such islands, using both serological and molecular tools to diagnose *L. infantum* infection. The seroprevalence of FeL recorded herein (25.8%) is higher than that assessed only by IFAT in cats from Greece (21.6%, cut off 1:64) (Huebner et al., 2008) and Madrid, Spain [3.7%, cut off 1:50 (Ayllon et al., 2012) and 3.2%, cut off 1:100 (Mirò et al., 2014)], and similar to that recorded in endemic areas of southern Spain (28.3%) using the same 1:40 cut off (Martín-Sánchez et al., 2007). The antibody prevalence here recorded is higher than that previously reported in a large study performed in regions of southern Italy in which a higher cut off (1:80) was however used (i.e. Calabria and Sicily, 6.9% Pennisi et al., 2012), but it is lower compared to a past study where the same cut off (1:40) was used (59.1%, Pennisi et al., 1998). In this latter study, anti-*Leishmania* antibodies were investigated in a large group of FIV positive cats (57 individuals) and an association between *Leishmania* and FIV antibody positivity was found (Pennisi et al., 1998). Conversely, the low number of FeLV and FIV positive cats tested herein does not allow to make assessments about retroviral and *Leishmania* co-infections.

The comparison of prevalence data in literature, resulting from different methodologies (e.g. IFAT and ELISA) and populations of animals, is somehow troublesome because of the nature of sampled cats, which are mostly client owned animals referred to clinical facilities from diverse geographical areas, and due to the small number of animals tested in the majority of published studies (Pennisi et al., 2015a). In addition, serological diagnostic methods differ in protocols, including different cut-off values for IFAT. For example, though several studies reported 1:40 as the cut-off for the IFAT (Pennisi et al., 1998; Poli et al., 2002; Vita et al., 2005; Martín-Sánchez et al., 2007; Duarte et al., 2010; Spada et al., 2013; Chatzis et al., 2014b) and a definitive validation study has never been published, 1:80 has been recently suggested as cut-off for the diagnosis of FeL on the basis of results from cats of non-endemic areas (Pennisi et al., 2012).

In the present study, all the IFAT positive cats younger than 1 year of age showed a titre of 1:40, while in elder animals the titre was up to 1:640. This finding could reflect the dynamic of FeL infection in cats with an initial exposure to the protozoan (antibody titres 1:40), followed by an increase of antibody titres in the elder animals due to the settlement of the infection and/or to continuous infections in the further transmission seasons (Table 2). Also, there is a lack of information regarding the transmission of maternal anti-*Leishmania* antibodies from the queen to kittens, and for how long this passive immunity could persist.

Interestingly, the prevalence of PCR positive animals at blood (2.1%) or conjunctival swabs (1.8%) is quite similar and lower than that recorded testing the same tissues from cats of a close area in South Italy (i.e. 7.8% in blood and 16.7% in conjunctival swabs, Pennisi et al., 2012) or in Greece (13% in blood and 3.1% in conjunctival swabs, Chatzis et al., 2014a). Again, the molecular methodologies employed for the diagnoses of FeL are quite diverse and, the results, difficult to compare. However, there are no other studies comparing molecular testing from these two tissues in cats but conjunctival swabs seem to be a promising non-invasive sampling method for the molecular detection of *Leishmania* DNA in cats as well as it is in dogs (Lombardo et al., 2012). The difference in prevalence of leishmaniosis between cats (i.e. 25.8%) and dogs (i.e. 41.8%) was significant, and in accordance with other studies (Poli et al., 2002; Cardoso et al., 2010). However, the relatively high prevalence of infection in cats indicates that these animals are as exposed as dogs to the risk of infection. Nonetheless, a larger prevalence of *L. infantum* infection in dogs than in cats has also been put in relationship to the difference in the immune system of these two species and to a more efficient Th1 immune response in cats compared to dogs (Day, 2016).

The presence of qPCR positive dogs, which were negative by IFAT, may indicate the presence of resistant animals and/or animals in the early stage of the infection.

Though no significant correlation was found between *Leishmania* infection, in cats and dogs, and sex, cats older than 1 year displayed a much higher prevalence (22.8%) than younger ones (14.1%) and the difference was statistically significant ($p = 0.0025$). This is different from the picture of dogs where prevalence of infection is almost similar in all classes of age (Table 3).

According to the collection sites, the prevalence of *L. infantum* infection in cats in Vulcano (38.6%) was higher than in Lipari (24.1%), where in dogs it was much larger (41.7%). Conversely, in

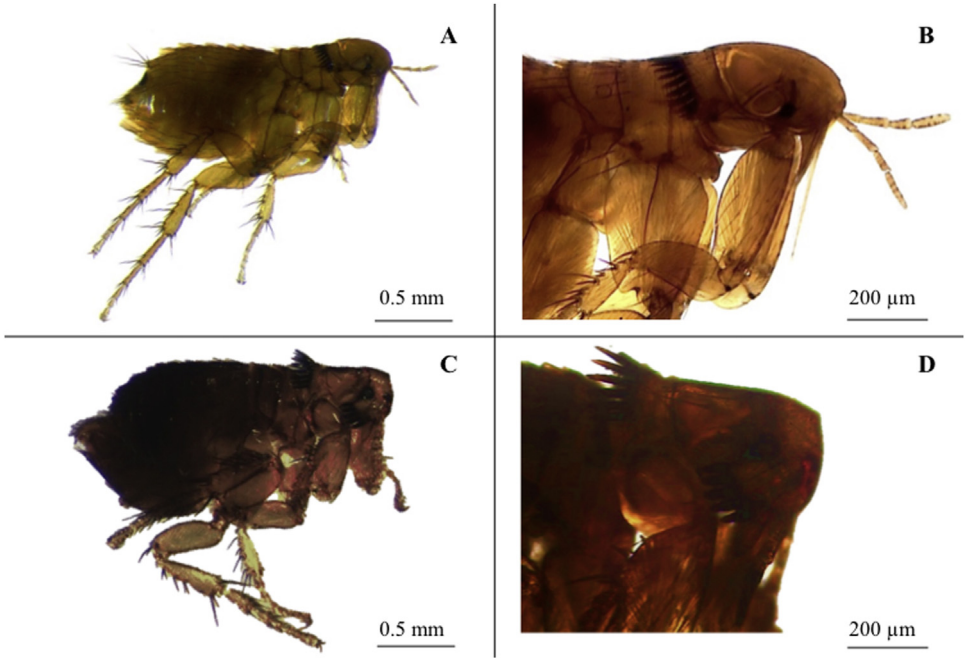

Fig. 2. Male of *Nosopsyllus fasciatus* (A and B) and of *Spilopsyllus cuniculi* (C and D).

Vulcano the situation was completely inverted, being the prevalence of FeL (38.6%) higher than that in dogs (23.6%) (Tables 2 and 3). This finding, however, could be related to the differences in the populations under examination. In fact, the cat population examined in Vulcano was mainly composed of animals living in colonies with a low level of care and treatments (e.g. regular use of ectoparasiticides). A remarkable difference was seen in relation to the positivity of dogs to different pathogens and geographical origin (Table 3), though the low number of animals from different islands does not allow any definitive conclusion.

The incidence of *L. infantum* infection in cats positive after one season of exposure to sand fly (14.7%) represents a unique data in literature and indicates that the Aeolian Islands are highly endemic for *L. infantum* and that preventative measures are necessary in animals living in this area (Table 4) . Clinical signs such as lymphadenomegaly and skin lesions associated with FeL infection were observed probably also due to the high incidence and multiple exposure of cats to this pathogen.

Feline retroviruses (FeLV and FIV) were found to poorly circulate in the Aeolian cat population, FIV being detected at a lower frequency in comparison with previous studies conducted in Sicily (Pennisi, 1989; Bechtle et al., 1992; Pennisi et al., 2000). This is probably due to lower sensitivity of PCR compared with antibody detection, which is commonly used for diagnosing FIV infection in cats (Hosie et al., 2009).

Ctenocephalides felis is the most common species of fleas parasitizing domestic cats and dogs worldwide, followed by *Ctenocephalides canis*. Cats were significantly more infested by fleas than dogs. While *C. felis* was the flea species most frequently identified in cats (n = 91), the detection of other species, such as *N. fasciatus* and *S. cuniculi*, confirms what was already observed in previous studies on flea infestation in cats (Bond et al., 2007). The European rabbit flea, *S. cuniculi*, occurs in the ear of rabbits and, though they may be attached for long time to the host, adult fleas usually live in burrows of rabbits being the infestation mainly associated to the breeding seasons to young generations. *Spilopsyllus cuniculi*, the vector of myxomatosis, has also been often found on the edges of the ear of cats, and it has been indicated as a potential vector of *Bartonella* sp. (Márquez et al., 2009).

Though knowledge of natural history of *I. ventalloi* is limited, it has been implicated as a potential vector of some pathogens (e.g. *Anaplasma phagocytophilum*, *Rickettsia helvetica*, *Rickettsia monacensis*, *B. clarridgeiae*, and Eyach virus) (Chastel et al., 1984; Santos-Silva et al., 2006; Márquez, 2008; Hubálek and Rudolf, 2012; Otranto et al., 2014; Pennisi et al., 2015b). This tick primarily parasitizes the European wild rabbit at all its developmental stages, but may occasionally be found on cats, dogs, ground-dwelling birds and humans (Santos Dias and Santos-Reis, 1989; Santos-Silva et al., 2011; Otranto et al., 2014; Pennisi et al., 2015b). The affiliation of *I. ventalloi* to lagomorphs makes its distribution patchy (Manilla, 1998) and this tick species has been reported in France, Tunisia, Morocco, Spain, Portugal, Germany and Great Britain (Chastel et al., 1984; Petney and Maiwald, 1996; Jameson and Medlock, 2011; Santos-Silva et al., 2011; Estrada-Peña et al., 2014). In Italy, it has been reported in a human living in southern Italy (Otranto et al., 2014) and in cats from the Aeolian Islands (Pennisi et al., 2015b). Both *I. ventalloi* and *R. pusillus* have been already identified in cats in Lipari (Persichetti et al., 2016; Latrofa et al., 2016) whereas *R. sanguineus* s.l. was the only species identified in tick-infested dogs, but not in cats. The transmission of *I. ventalloi* from the rabbit to domestic or wild cats may occur, especially in areas like the Aeolian Islands, where large populations of wild lagomorphs and domestic cats coexist (Pennisi et al., 2015b). The occurrence of *S. cuniculi* in cats along with the large prevalence of *I. ventalloi* support scientific data about the presence of a large population of rabbits in the islands and the sympatry of these two animal species in the same environment. Whether the European wild rabbits, which actually are hunted during autumnal months in the Aeolian Islands, may act as reservoir of *L. infantum* playing a similar role to that of other lagomorphs in outbreak of human visceral leishmaniosis (Ruiz-Fons et al., 2013) deserves further investigations.

At the latitude of the studied sites up to three sand fly species, suitable vectors of *L. infantum*, have been detected (i.e. *Phlebotomus neglectus*, *Phlebotomus perniciosus* and *Phlebotomus perfiliewi*) being active from June to October (Gaglio et al., 2014). Though FeL is rarely diagnosed, cats are exposed to sand fly bites in endemic areas since they usually are free-roaming and enjoy nocturnal outdoor access particularly in the warm seasons. In addition, cats are usually not

treated like dogs with pyrethroid repellents, which are in most of the cases toxic to this species.

5. Conclusions

Aeolian Islands present suitable environments to understand the transmission dynamics of pathogens and vectors. Although in the Mediterranean basin *L. infantum* is mostly associated to dogs, the recognition of subsidiary reservoir animals in endemic areas is of paramount importance for the control of the associated disease in both animals and humans. Indeed, approximately 47% of the Sicilian population lives in areas at risk for visceral leishmaniasis (Cascio et al., 2002), making an early diagnosis coupled with vector control strategies in cat and dog populations necessary.

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