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Host preferences of phlebotomine sand flies at a hypoendemic focus of canine leishmaniasis in central Italy

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Abstract

A survey was carried out on phlebotomine sand flies and their feeding habits at a hypoendemic focus of *Leishmania infantum* in Macerata province, central Italy. During two consecutive years (2000–2001), 1465 sand fly specimens (42.5% of which were males) were collected from a variety of diurnal resting sites in the municipality of Camerino. The most prevalent species was *Phlebotomus perniciosus* (76.6%), followed by *P. papatasi* (10.4%), *Sergentomyia minuta* (9.1%), *Phlebotomus perfiliewi* (3.3%) and *P. mascittii* (0.5%). Among the 842 females collected, 578 (68.6%) were blood-fed. Based on the results of blood meal analyses, *P. perniciosus* fed on man, dogs, equines, sheep and birds; *P. perfiliewi* on dogs, equines, sheep and birds; *P. papatasi* on dogs, equines and birds. Two specimens of *P. mascittii* fed on equines. Forage ratios (FRs) and host selectivity indices gave different results for the large domestic animals. More than 95% of the specimens collected inside a stable, dog kennel, sheep pen and chicken house were found to have fed on the animals housed in the respective shelters. In addition, at one collecting site where almost all the hosts mentioned above were present simultaneously, both *P. perniciosus* and *P. perfiliewi* were found to have fed on all five species, indicating that host choice was probably related to its availability (i.e. number and size) rather than specific attractiveness. The feeding habits of the two *Leishmania* vectors may have implications for the epidemiology of leishmaniasis in urban and peri-urban areas, where sand fly females deprived of other vertebrate hosts (particularly the larger species) may begin to bite humans and dogs more frequently.

Keywords: Canine leishmaniasis; Vectors; Sand fly species; *Phlebotomus perniciosus*; Feeding habits; Central Italy

1. Introduction

In the Mediterranean regions, the proven vectors of human, visceral (VL) and cutaneous (CL) and canine (CanL) leishmaniases are *Phlebotomus* spp. belonging to the subgenus *Larroussius* (Diptera, Psychodidae). In Italy, *Phlebotomus perniciosus* and *Phlebotomus perfiliewi* are proven vectors of the protozoan parasite *Leishmania infantum* (Bettini et al., 1986; Maroli et al., 1987, 1988, 1994a), the former species being the more competent vector based on laboratory (unpublished data) and epidemiological observations. Entomological surveys have shown that *P. perniciosus* is able to colonise rural, peri-urban and

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urban areas (Corradetti, 1962; Biocca et al., 1977; Maroli and Bettini, 1977; Bettini et al., 1991). Resting adults are found during the day in both domestic and silvatic sites, including houses and animal shelters, wall crevices, tree holes, animal burrows, etc. (Maroli et al., 1994b; Ascione et al., 1996; Maroli and Khoury, 1998).

Traditionally, only the coastal areas and islands of the Tyrrhenian and Ionian seas were considered to be stable endemic foci of CanL (Pampiglione and Bettini, 1981; Pozio et al., 1985; Bettini and Gradoni, 1986). However, since 1990, new stable foci have appeared throughout the country, both in areas previously known to be endemic and non-endemic in Northern Italy. Among the endemic regions, new CanL foci have been recorded in Emilia Romagna (Baldelli and Di Francesco, 1997; Baldelli et al., 2001), Tuscany (Pedonese et al., 2000), Umbria (Corradetti et al., in press) and Abruzzo (Dalla Villa and Ruggeri, 1999). Two CanL macro-foci were identified in the Veneto (Poglayen et al., 1997) and Piedmont regions (Ferroglio et al., 2002a; Rossi et al., 1999) of Northern Italy.

The spread and increasing prevalence of CanL in Italy is probably linked to climatic factors influencing the behaviour and the abundance of vectors as well as the practice of keeping pet dogs. This has resulted in an increased dog population and transportation of infected animals from CanL–endemic regions to new areas, including towns, where *Leishmania* vectors already exist.

The Marche region of central Italy is known to be an unstable hypoendemic VL focus. Only three VL cases occurred between 1997 and 2000, with a very low cumulative morbidity (0.2/100,000 population), compared with that of other VL endemic foci such as Campania, where a morbidity of 3.6/100,000 was registered during the same period. However, based on the results of a recent serological survey on canine reservoirs in Macerata province, which showed 13.7% to be IFAT-positive (unpublished data), there is the risk that CanL will soon spread within Marche if competent vectors are present. Although there is no published information on the presence of sand fly species that transmit *Leishmania* in the region, the late Prof. E. Biocca collected 43 *P. perfiliewi* in 1970 (unpublished data). Thus, an entomological survey was carried out to study the sand fly fauna and the insects' feeding habits. The present note reports the results of collections made during two sand fly seasons (2000–2001) in the municipality of Camerino. Preliminary results on species composition were already presented at the Fourth International Symposium on Phlebotomine Sandflies and at the 22nd National Congress of Italian Society of Parasitologists (Bongiorno et al., 2002a,b).

2. Materials and methods

2.1. Study area

Marche forms the eastern seaboard of central Italy and is bound by Emilia Romagna to the north and Abruzzo to the south. From the relatively narrow coastal plains, the land rises sharply to the peaks of the Apennines, which form a natural boundary with Umbria and Tuscany to the west. Although the coastal areas are heavily populated, the inland countryside is sparsely inhabited. The inland mountainous zones consist principally of limestone and are characterized by bare peaks, fast-flowing rivers, gorges and many complexes of caves. In contrast, the areas nearer the coastal plain are well known for their fertile rounded hills topped by ancient fortified towns. The total population of the region is around 1.5 million, with an average density of less than 150 inhabitants/km^2. The region covers just less than 10,000 km^2.

The entomological survey was carried out in an inland mountainous area centred on the municipality of Camerino, at 500–600 m a.s.l. This hill town is situated on a ridge between two river valleys, on the border between central Marche and Umbria.

2.2. Description of collecting sites

Seven collecting sites, containing a variety of sand fly diurnal resting sites, were selected throughout Camerino, one site being located in Mecciano village and the others around Varano locality. Characteristics of each site, collecting methods used and the results of a domestic animal census within a 50 m radius are presented in Table 1. Sand fly specimens were collected twice weekly during a 4-month period (June–September), for two consecutive years (2001–2002). After capture, the flies were transported to the laboratory and anaesthetised with low

Table 1
Characteristics of the collecting sites: locality, resting site monitored, collecting methods and list of available hosts

No.	Collection site	Locality	Habitat	Methods	Hosts (number)
1	Kennel	Mecciano	Ex chicken house within the kennel	H[a]	Dogs (>50), cats (>5), humans (>10)
2	Horse stable	Varano	Stable	H	Horse (1), dogs (3), cats (3), humans (4)
3	Hen house	Varano	Chicken house	H	Dogs (3), cats (2), chickens (15) humans (>10)
4	Sheep pen	Varano	Sheep pen	CDC[b]	Sheep (>50), dogs (2), cats (2), humans (3)
5	Multi-species pen	Varano	Chicken, rabbit, horse and sheep pen	H, CDC	Sheep (>15), dogs (5), cats (5), horses (3), pigeons (>20), chickens (10), ducks (6), turkeys (4), geese (4), rabbits (12), humans (2)
6	Kennel	Varano	Stone drainage water collector close to a kennel	H	Dogs (>100), mice (?), lizards (?)
7	Drainage openings in walls	Varano	Drainage openings in walls	ST[c]	Dogs (3), cats (2), humans (>10), mice, lizards

[a] Hand collection.
[b] CDC miniature light traps.
[c] Sticky traps.

temperature; blood-fed females were then removed and stored at −20 °C until blood meal analysis could be performed. Male and unfed female specimens were cleared prior to identification to species level. Specimens were identified by their morphological characteristics, according to Theodor (1958) and Léger et al. (1983).

2.3. Testing of blood meals

Before testing, blood-fed females were identified to species level by removing the head and the terminal segments of the abdomen containing the spermathecae. Specimens were then classified into freshly fed, partially fed and late fed according to the amount and colour of the blood in the intestine.

The blood origin was determined by a direct ELISA on nitro-cellulose membrane. Each female was homogenised in 50 μl of phosphate buffered saline pH 7.2 (PBS). Of this blood eluate, 1 μl was coated onto nitro-cellulose membrane and left to dry at room temperature for 15 min. For blocking the remaining binding sites, membranes were incubated in a 1% solution of powdered milk for 1 h on a plate shaker. Membranes were then washed three times with PBS containing 0.05% Tween 20 (PBS–Tw20). The blood origin was detected by the addition of peroxidase labelled anti-animal sera (Sigma) (purified IgG fraction, raised to the whole molecule), namely anti-human (A-8667), anti-dog (A-6792), anti-chicken (A-9046), anti-horse (A-9292), anti-rabbit (A-6154), anti-sheep (A-3415), and anti-mouse (A-4416). The anti-cat serum was provided by a colleague. The peroxidase conjugates were diluted (1:2000) in PBS–Tw20 supplemented with 0.2% of each of the heterologous serum in order to control for possible cross-reactivity. Conjugate preparations were incubated on the nitrocellulose membranes for 1 h on the plate shaker. After three washings, peroxidase substrate (4-chloro-1-naphthol) 15 mg diluted in 5 ml of methanol, and 20 ml of Tris–HCl 0.05 M pH 6.8 plus H_2O_2 (added just before usage) was added and the membranes incubated for 30 min in the dark. The enzyme reaction was stopped by washing with tap water. Samples were considered positive if dark blue spots developed on the antigen dots. In each test, negative and positive controls were included: as negative controls laboratory-reared *P. papatasi* males and blood from the heterologous animals at a dilution of 1:200 were used. As positive controls dilutions (1:500, -1×10^{-6}) of the homologous serum were applied.

2.4. Data analysis

Forage ratios (FRs) were calculated to determine the preferences of sand flies for the different vertebrate hosts present at the collecting sites, by

dividing the percentage of females feeding on a given host by the percentage which that host represented in the total census of animals and humans at the collecting site (Hess et al., 1968). According to the method described by these authors, an FR of 1.0 indicates neither preference nor avoidance of a given host animal, FRs significantly >1.0 indicate selective preferences and values <1.0 indicate avoidance in favour of other hosts.

Since the FR technique does not consider relative body mass of the host, we also calculated the host selectivity index (HSIx) using the method of Agrela et al. (2002). This takes into account the estimated average weight of each individual animal present at the collection site. The available biomass for each host species was calculated by multiplying the number of such hosts counted in the census by its estimated average weight. HSIx was then calculated by dividing the number of sand flies that fed on a given host by the available biomass of such hosts.

3. Results

3.1. Sand fly fauna composition

All collecting sites inspected were positive for sand flies. The number of sand fly specimens collected and the prevalence of species identified at each site are shown in Table 2. During the 2 years of study, 1465 sand flies were caught (42.5% male) belonging to the genera *Phlebotomus* and *Sergentomia*. Among the members of the subgenus *Larroussius*, *P. perniciosus* (76.6%) was the most abundant in kennels, stable and sheep pens, while *P. perfiliewi* (3.3%) was present in five out of seven sites monitored, predominating over the other species (18.5%) only in sheep pens. *P. papatasi* (10.4%) was recorded mainly in chicken houses. *P. mascittii* (0.5%) was very rare, being recorded at only two sites while *S. minuta* (9.1%) was the prevalent species in wall crevices.

3.2. Blood meal identification

Only collecting sites 1–6 produced blood-fed females of *Phlebotomus* species, and only unfed *S. minuta* were caught at site 7, represented by wall crevices. Of the 578 engorged sand flies, 515 (89.1%) were *P. perniciosus*, 42 (7.3%) were *P. papatasi*, 19 (3.3%) were *P. perfiliewi* and 2 (0.3%) were *P. mascittii*. Blood meal identification was possible in 557 (96.4%) of the females tested; undetermined blood meals were probably due to the sand fly taking only a small amount of blood from its host or having time to digest most of the meal before capture.

Specimens of both *P. perniciosus* and *P. perfiliewi* with two types of host blood were found; at the kennel (site 1), 28 *P. perniciosus* and 2 *P. perfiliewi* had recently fed on both dog and human and at site 5, 5 *P. perniciosus* showed the following feeding patterns: one fed both on humans and dogs, one on dogs and birds, one on equines and birds and two on equines and sheep.

More than 95% of the sand fly specimens collected inside the stable, kennel, sheep pen and chicken house were found to have fed on the animals

Table 2
Prevalence of sand fly species identified from seven collecting sites in Marche region (Italy) during 2 years of entomological survey (2000–2001)

No.	Collection site	Total	*P. perniciosus* (%)	*P. perfiliewi* (%)	*P. papatasi* (%)	*P. mascittii* (%)	*S. minuta* (%)
1	Kennel (Mecciano)	425	91.5	5.2	1.4	0.0	1.9
2	Horse stable	557	87.4	2.2	9.0	1.1	0.4
3	Hen house	119	20.2	0.0	78.2	1.7	0.0
4	Sheep pen	27	77.8	18.5	0.0	0.0	3.7
5	Multi-species pen	133	92.5	6.0	1.5	0.0	0.0
6	Kennel (Varano)	85	94.1	1.2	1.2	0.0	3.5
7	Drainage openings in walls	119	0.0	0.0	0.0	0.0	100.0
Total (%)		1465	76.7	3.3	10.4	0.5	9.1

Table 3
Percentage of *P. perniciosus* reacting with each anti-species reagent and the corresponding forage ratio (FR) and host selectivity index (HSIx) at a variety of collecting sites

No.	Collecting site	Tested	Index	Human	Equine	Dog	Avian	Ovine
1	Kennel (Mecciano)	213	%	11.7	0.0	88.3	0.0	0.0
			FR	0.7	0.0	1.1	0.0	0.0
			HSIx	0.1	0.0	0.2	0.0	0.0
2	Stable	225	%	3.1	96.9	0.0	0.0	0.0
			FR	0.1	7.7	0.0	0.0	0.0
			HSIx	0.0	0.4	0.0	0.0	0.0
3	Chicken house	2	%	0.0	0.0	0.0	100.0	0.0
			FR	0.0	0.0	0.0	1.9	0.0
			HSIx	0.0	0.0	0.0	0.0	0.0
4	Sheep pen	14	%	0.0	0.0	0.0	0.0	100.0
			FR	0.0	0.0	0.0	0.0	1.1
			HSIx	0.0	0.0	0.0	0.0	0.0
5	Multi-species	44	%	11.4	31.8	9.1	13.6	34.1
			FR	2.6	4.8	0.8	0.3	1.0
			HSIx	0.1	0.0	0.0	0.1	0.0
6	Kennel (Varano)	6	%	0.0	0.0	100.0	0.0	0.0
			FR	0.0	0.0	1.0	0.0	0.0
			HSIx	0.0	0.0	0.0	0.0	0.0
Cumulative		504	%	8.5	45.2	40.1	2.4	3.8
			FR	0.8	21.1	1.3	0.1	0.1
			HSIx	0.1	0.1	0.3	0.1	0.0

occupying these respective shelters. At site 5, where all hosts tested were present at the same time, both *P. perniciosus* and *P. perfiliewi* were found to have fed on almost all of them. In summary, the identification of single and multiple blood meals showed that the majority of *P. perniciosus* reacted with anti-equine (45.2%) and anti-dog (40.1%) sera. The remaining females were positive for anti-human (8.5%), anti-sheep (3.8%) and anti-bird (3.8%) sera. The percentages of *P. perniciosus* blood meals reacting with each species reagent and the corresponding FRs and HSIx at the different collecting sites are shown in Table 3.

Among the sand fly species analysed (Table 4), *P. perfiliewi* exhibited cumulative reacting percentages of 36.8, 36.8, 10.5, 10.5 and 5.3, respectively, for equines, dogs, humans, avian and ovine, while *P. papatasi* took most avian blood (70.6%), followed by equine (26.5%)

Table 4
Cumulative percentages of *P. perfiliewi* and *P. papatasi* reacting with each anti-species reagent and the corresponding forage ratio (FR) and host selectivity index (HSIx)

Species	Tested	Index	Human	Equine	Dog	Avian	Ovine
P. perfiliewi	19	%	10.5	36.8	36.8	10.5	5.3
		FR	1.0	17.3	1.2	0.5	0.2
		HSIx	0.0	0.0	0.0	0.0	0.0
P. papatasi	34	%	0.0	26.5	3.0	70.6	0.0
		FR	0.0	12.4	0.0	3.3	0.0
		HSIx	0.0	0.0	0.0	0.2	0.0

Two *P. mascittii* fed on equines.

and dog (3.0%). The two specimens of *P. mascittii* were found to have fed on equines.

No blood meals were apparently taken on cats, and mice by any of the sand fly species tested.

4. Discussion

P. perniciosus is known to be very abundant only along the coastal regions of Italy at 200–300 m a.s.l.; the highest elevation at which this species has been reported in the country is 1070 m a.s.l. in Abruzzo (Maroli et al., 1991). The results of our study show for the first time that *P. perniciosus* populations could also reach significant densities inland. This species was prevalent and widespread in all the sites surveyed in the municipality of Camerino, located at 500–650 m a.s.l. Sand fly adults were active from middle of June until the first week of September, showing that the sand fly season at this altitude is shorter than that reported for the insects in Sicily and Campania (Ruta et al., in press; Ascione et al., 1996), but similar to that of northern Italian populations (Ferroglio et al., 2002b).

Knowledge on the host preferences of phlebotomine sand flies under natural conditions is essential to understand their vectorial capacity in different leishmaniasis foci. The results of blood meal tests show that, in general, the two proven vectors, *P. perniciosus* and *P. perfiliewi*, seem to be opportunistic feeders rather than exhibiting preferences for any specific animal. Similar feeding habits are also known for other *Leishmania* vectors, both in the Old and New World (Killick-Kendrick, 1999; Morrison et al., 1993; Agrela et al., 2002). In Spain, France and Italy, phlebotomine vectors have been observed to feed on a wide range of domestic animals, with varying degrees of anthropophilism (De Colmenares et al., 1995; Guy et al., 1984; Killick-Kendrick et al., 1977).

The observed percentage of flies (6.3%) with double blood meals is probably a consequence of the difficulties sand flies face in freely engorging on a single host due to host defensive movements, little or no exposed host skin or the difficulty to locate adequate skin blood capillaries.

In our study, more than 95% of fed females collected in the stable, kennel, sheep pen and chicken house were found to feed on the respective animals housed in these shelters. In addition, when almost all animal tested were present simultaneously, *P. perniciosus* and *P. perfiliewi* both fed on all of them, indicating that host choice is probably related to its availability (e.g. number and size) rather than to its specific attractiveness.

Some further observations can be made regarding *P. perniciosus*, of which a large number of specimens (504) have been analysed. The comparison of cumulative percentages did not show obvious differences between the number of females fed on dogs and equines. Nevertheless, when such numbers are adjusted based on the available biomass for each host (HSIx), this value was higher for dogs (0.3) than for horses and humans (0.1) (Table 3). FR values gave contrasting results with a very high value for equines (21.1) and relatively low values for dogs (1.3) and humans (0.8). Thus, according to the HSIx values, horses are not highly attractive to sand flies whereas the unadjusted FRs suggests that they are preferred in respect to other hosts. This emerges particularly from the data collected at site 5 where a large variety of hosts were present simultaneously.

In a previous study, Corradetti (1936) showed that *P. perfiliewi* in Abruzzo appeared not to feed on sheep or pig and preferred cattle and humans in domestic situations. Following an outbreak of VL in the region of Emilia Romagna, Killick-Kendrick et al. (1977) observed that under natural conditions this fly appears to be a catholic feeder with probable preferences for man, cattle, dog, rabbit and hare. Our data seem to confirm the previous observations on the attraction of *P. perfiliewi* to large animals, the FR value for equines (17.3) being very high.

With respect to *P. papatasi*, none of the females tested reacted with human blood; this species was fed on equines, birds and dog. However in Italy, the species is known to be highly endophilic and anthropophilic and it has been reported in domestic resting sites in urban areas (Maroli and Bettini, 1997). Nevertheless, its presence in chicken houses is becoming very common in many areas of Italy (Corradetti et al., in press; Romagnoli et al., 2002).

Observations on the host preferences of phlebotomines in the study area permit us to draw a tentative conclusion that should be confirmed by more detailed studies in other Italian VL and CanL foci. The feeding habits of the two *Leishmania* vectors, may have relevant implications for the epidemiology of leishmaniasis in urban and peri-urban areas, where

sand fly females, being deprived of other vertebrate hosts, particularly the larger species, may begin to bite humans and dogs more frequently. Evidence of such a shift in the feeding pattern is presented by the recent appearance of VL and CanL cases in urban areas, where infected dogs probably have been introduced during the last decade (Gradoni et al., 1993; Cascio et al., 1997).

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