

## ENTOMOLOGICAL AND PARASITOLOGICAL STUDY ON PHLEBOTOMINE SANDFLIES IN CENTRAL AND NORTHERN ALBANIA

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### Summary:

An entomological survey was carried out in two districts of central (Kruje) and northern (Lezhe) Albania. Six collecting sites, showing a variety of diurnal resting sites, were monitored for adult sandflies from June through October 2002. Flies were collected with CDC miniature light traps, sticky traps and mechanical or hand aspirators in peri-domestic sites, in bedrooms and inside cow barns, chicken coops and pigpens. All collecting sites monitored were found positive for sandflies. A total of 849 specimens were caught (29.2 % males) belonging to five *Phlebotomus* species. *Phlebotomus neglectus* (75.6 %) was the most abundant species followed by *P. perfiliewi* (14.4 %), *P. papatasi* (4.6 %), *P. tobbi* (3.6 %) and *P. similis* (1.8 %). The first adult of *P. neglectus* appeared on June 11 and the last one was collected on October 16. The highest density for this species was observed at the end of July. A total of 111 blood-fed females were caught from the two areas studied. *P. neglectus* was the only species found blood fed in Lezhe and the same species was prevalent (56.1 %) in Kruje followed by *P. perfiliewi* (30.3 %), *P. tobbi* (10.6 %); *P. papatasi* was represented by only two specimens. Blood meal origin was determined in 45/66 (68.2 %) of the females tested from Kruje district. *P. neglectus* was found fed on four hosts, showing the following feeding patterns: cow (71.4 %), dog (17.1 %), chicken (5.7 %) and human (5.7 %); *P. perfiliewi* was found fed on cow (80.0 %) and chicken (20.0 %), *P. tobbi* on cow (50.0 %), chicken (25.0 %) and dog (25.0 %). One specimen of *P. papatasi* was found fed on cow. When such prevalences were analysed by the available biomass for each host present at the collecting site, *P. neglectus* resulted to be an opportunistic feeder rather than exhibiting preferences for any specific animal. PCR analysis of 39 *P. neglectus* from the Lezhe district gave negative results for the presence of *Leishmania* DNA.

**KEY WORDS :** phlebotomine sandfly, feeding habit, *Leishmania*, PCR, Albania.

### Résumé : ENQUÊTE ENTOMOLOGIQUE SUR LES PHLÉBOTOMES DANS DEUX DISTRICTS ALBANAIS

Les auteurs rapportent les résultats d'une enquête entomologique conduite dans deux districts albanais, Kruje et Lezhe. Dans la période juin-octobre 2002, six stations de piégeage contenant de nombreux lieux de repos diurne de phlébotomes ont été retenues. Ce sont des sites péri-domestiques, des chambres, des étables, des poulaillers et des porcheries. Les phlébotomes ont été capturés à l'aide de pièges lumineux miniatures CDC, de pièges adhésifs et de captureurs mécaniques et à bouche. Toutes ces stations ont été positives. 849 phlébotomes ont été capturés parmi lesquels 29,2 % de mâles. Cinq espèces appartenant au genre *Phlebotomus* ont été identifiées. *Phlebotomus neglectus* (75,6 %) est l'espèce la plus abondante, suivie dans l'ordre décroissant par *P. perfiliewi* (14,4 %), *P. papatasi* (4,6 %), *P. tobbi* (3,6 %) et *P. similis* (1,8 %). Le premier imago de *P. neglectus* est apparu le 11 juin et le dernier a été capturé le 16 octobre. Pour cette espèce, la densité la plus abondante a été observée à la fin de juillet. 111 femelles gorgées ont été capturées dans les deux sites prospectés. *P. neglectus* est la seule espèce trouvée gorgée à Lezhe et la plus abondante à Kruje (56,1 %), suivie dans cette station par *P. perfiliewi* (30,3 %), *P. tobbi* (10,6 %) et *P. papatasi* représenté seulement par deux exemplaires. L'origine du repas de sang a été déterminée sur 45 femelles (68,2 %) du district de Kruje; 35 *P. neglectus* se sont nourris sur quatre hôtes différents avec les préférences suivantes: vache (71,4 %), chien (17,1 %), poule (5,7 %) et homme (5,7 %); *P. perfiliewi* s'est nourri seulement sur vache (80 %) et poule (20 %). *P. tobbi* se gorge sur vache (50 %), poule (25 %) et chien (25 %). Lorsque nous corrigeons ces nombres en estimant la biomasse disponible pour chaque hôte dans la station de capture, il semblerait que *P. neglectus* soit un opportuniste. Les 39 femelles de *P. neglectus*, testées par la technique de PCR, ne présentent pas d'ADN de *Leishmania*.

**MOTS CLÉS :** phlébotome, préférence trophique, *Leishmania*, PCR, Albanie.

## INTRODUCTION

Phlebotomine sandflies (Diptera: Psychodidae) are responsible for the transmission of *Leishmania infantum*, the protozoan agent of human

and canine leishmaniasis in the Mediterranean area. Review data on the phlebotomine sandfly fauna of Albania show that *P. neglectus* is the most abundant species being widespread in almost all Albanian territories (Adhami & Murati, 2000; Velo *et al.*, 2003).

Recent results from a retrospective analysis of human visceral leishmaniasis (VL) cases occurred in Albania, have shown an increase in the disease morbidity, resulting 20-40-fold higher than in other southern European countries (Velo *et al.*, 2003). During the period 1997-2001, 867 parasitologically confirmed VL cases have been recorded in 35 out of 36 Albanian districts

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with an average of 173 cases/year and a cumulative morbidity of 2.8/10,000 population. VL cases have almost doubled during the past 10 years and a high proportion of patients (67.6 %) was represented by children below five years of age. An entomological study was carried out during June–October 2002 in two Albanian districts most involved in this VL recrudescence, with the aim to study sandfly fauna composition, the insect feeding habits and natural *Leishmania* infections.

## MATERIALS AND METHODS

### STUDY AREA

The entomological survey was carried out in the districts of Kruje and Lezhe, where high VL morbidity levels (5.1 and 12.5/10,000 population, respectively) were recently reported. The two districts, located in central and northern part of Albania (Fig. 1), respectively, consist of hilly territories (Kruje 600–658 m a.s.l., Lezhe 180–208 m a.s.l.). Kruje district, which has a surface of 333 km<sup>2</sup> and a density of 193.3 inhabitants/km<sup>2</sup> is located in the inland part of Albania. The annual rainfall in this district averages 900–1,700 mm and temperatures 5–16° C. Lezhe district, which has a surface of 142.3 km<sup>2</sup> with a density of 142.3 inhabitants/km<sup>2</sup>, is very close to the Adriatic coast, with an average annual rainfall of 1,500–1,800 mm and an



Fig. 1 – Map of Albania showing the location of Lezhe and Kruje districts.

average annual temperature of 8–25° C. Both districts are in a geographical zone that belongs to the mesolitic layer. The sediments of this layer are composed mainly of lime rock, carbon and magmatic rocks.

### COLLECTING SITES

Six collecting sites were selected, three in Kruje and three in Lezhe, showing a variety of phlebotomine sandfly diurnal resting sites. Characteristics of each site, collecting methods used and presence of domestic animals within a range of 50 m, are presented in Table I.

### SANDFLY COLLECTION

Adult sandfly collections were carried out three times a month from June through October 2002. CDC miniature light traps, sticky traps and mechanical or hand aspirators were used outside peri-domestic sites, and inside cow barns, chicken coops and pigpens. Mechanical or mouth aspirators were used in diurnal collections inside bedrooms and cattle sheds in Kruje. An average of 10 sticky traps were used in each station for two consecutive nights. After capture, living flies were transported to the laboratory and anesthetized with low temperature; blood-fed females were then isolated and stored at -20° C pending further analysis. Males and unfed females were cleared prior to identification to species level. Specimens were identified by their morphological characteristics, according to Theodor (1958) and Léger *et al.* (1983).

### TESTING OF BLOOD MEALS

Blood meal origin could be determined only for females caught from Kruje. 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 according to the method previously described (Bongiorno *et al.*, 2003). Five peroxidase-labelled anti-animal IgG antibodies (Sigma) were tested, namely anti-human (A-8667), anti-dog (A-6792), anti-chicken (A-9046), anti-rabbit (A-6154), anti-bovine (A-7414). The anti-cat serum was a gift from the University of Camerino, Italy. Moreover, flies resulted negative to the above hosts were also tested for anti-sheep (A-3415), anti-mouse (A-4416) and anti-horse (A-9292) sera. An anti-pigeon serum was not available.

To determine the sandfly preference for hosts, forage ratios (FRs) were calculated by dividing the percentage of females feeding on a given host by the frequency at which that host was represented in the total census

No	Locality	Habitat	Methods*	Available hosts (number)
1	Kruje (L. Kala)	House, outside	CDC & ST	Humans (6), cat (1), rabbits (5), pigeons (25)
2	Kruje (L. Mezinaj)	Bedroom	MC	Humans (10), cats (1)
3	Kruje (L. Mezinaj)	Cows and chicken house	CDC & MC	Humans (15), dogs (8), cows (2), chickens (22), cats (6), rabbits (5)
4	Lezhe (Nora, Aida)	House, outside	CDC & ST	Humans (12), chickens (10), cats (2), rabbits (5), pig (1)
5	Lezhe (St iri)	Cows and chicken house	CDC & ST	Humans (6), dogs (5), cows (2), chickens (15), cats (3), rabbits (11)
6	Lezhe	Goat pen	CDC	Humans (11), dogs (5), chickens (10), cats (2), rabbits (12), goats (15)

\* CDC, miniature light traps; MC, mechanical and mouth aspirator; ST, sticky traps.

Table I. – Characteristics of the collecting sites: locality, resting site monitored, collecting methods and list of available hosts within a range of 50 m

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 method does not consider relative body mass of the host, the host selectivity index (HSIx) was also evaluated according to Agrela *et al.* (2002). This index estimates the 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.

#### DNA EXTRACTION AND AMPLIFICATION

Search for leishmanial DNA by PCR could be performed only for females caught from Lezhe. Sandfly species identification was carried out by removing only the genitalia. Genomic DNA was extracted from single female homogenized in 1.5 ml sterile tubes using a plastic pestle. 40 µl lysis buffer (100 mM TRIS-HCl, 100 mM NaCl, 25 mM EDTA, 0.5 % SDS, pH 8) was added and the homogenate was digested overnight at 37° C by 2 µg/µl of proteinase K (Promega). The DNA was extracted by phenol-chloroform and precipitated with 100 % ethanol then centrifuged for 30 min at 13,000 × g. The DNA pellet was resuspended in 50 µl of sterile water and stored at – 20° C until use. Small-subunit ribosomal DNA was amplified by PCR technique using the Kinetoplastida-specific primers R221 and R332 (van Eys *et al.*, 1992). Negative (no DNA) and two positive (*L. infantum* DNA and *Phlebotomus* plus *L. infantum* DNA) controls were used in all experiments. Finally, the PCR product was electrophoresed through a 1.5 % agarose gel and visualized under UV transillumination.

## RESULTS AND DISCUSSION

### SANDFLY FAUNA

All collecting sites monitored were positive for phlebotomine sandflies. The numbers of sandfly specimens collected and the prevalence of the species identified in the two districts are presented in Table II. Five species belonging to *Phlebotomus* genus were identified among the 849 caught sandflies. *Phlebotomus neglectus* was the most abundant species followed by *P. perfiliewi*, *P. papatasi*, *P. tobbi* and *P. similis*. The highest number of sandflies was collected in Kruje (79.1 %), where all the above species were recorded, while only *P. neglectus*, *P. perfiliewi* and *P. tobbi* were present in Lezhe, being *P. neglectus* the most abundant (92.6 %).

In general, our survey confirms what previously observed on the sandfly fauna composition of Albania, and particularly on the distribution and abundance of *P. neglectus* (Adhami & Murati, 2000; Velo *et al.*, 2003). The three *Phlebotomus* (*Larrousius*) species, i.e. *P. neglectus*, *P. perfiliewi* and *P. tobbi*, could play a role in the transmission of VL in the area studied, being proven *L. infantum* vectors (Léger *et al.*, 1988, 2000; Maroli *et al.*, 1987).

As for the seasonal trend of adult phlebotomine fauna, it could be determined only for *P. neglectus*, the most widespread species. The first adult appeared on June 11 and the last one was collected on October 16, the highest number of specimens being collected at the end of July. A similar seasonal trend has been reported for *P. perniciosus* and *P. perfiliewi* in Italy (Maroli & Bettini, 1977).

### BLOODMEAL IDENTIFICATION

Among 66 fed females caught in Kruje district, *P. neglectus* was the prevalent species (56.1 %) followed by *P. perfiliewi* (30.3 %) and *P. tobbi* (10.6 %);

District	Sex	<i>P. neglectus</i>	<i>P. perfiliewi</i>	<i>P. papatasi</i>	<i>P. tobbi</i>	<i>P. similis</i>
<b>Kruje</b>	M	175 (89.3)	9 (4.6)	9 (4.6)	3 (1.5)	
	F	303 (63.6)	108 (22.7)	30 (6.3)	20 (4.2)	15 (3.2)
	Total (%)	672	M+F 478 (71.2)	117 (17.4)	39 (5.8)	23 (3.4)
<b>Lezhe</b>	M	50 (96.2)	0	0	2 (3.8)	0
	F	114 (91.2)	5 (4.0)	0	6 (4.8)	0
	Total (%)	177	M+F 164 (92.6)	5 (2.9)	0	8 (4.5)
Overtotal (%)	849	M+F 642 (75.6)	122 (14.4)	39 (4.6)	31 (3.6)	15 (1.8)

Table II. – Prevalence of the sandfly species identified in each district. M, male; F, female.

Species	Tested	Index	Human	Dog	Bovine	Chicken
<i>P. neglectus</i>	35	% reactors	5.7	17.2	71.4	5.7
		FR	0.116	1.35	22.5	0.164
		HSI	0.002	0.075	0.042	0.03
<i>P. perfiliewi</i>	5	% reactors	–	–	80.0	20.0
		FR	–	–	25.2	0.573
		HSI	–	–	0.007	0.015
<i>P. tobbi</i>	4	% reactors	–	25.0	50.0	25.0
		FR	–	1.969	15.75	0.716
		HSI	–	0.013	0.003	0.015

Table III. – Cumulative percentages of *P. neglectus*, *P. perfiliewi* and *P. tobbi* reacting with each anti-species reagent and the corresponding forage ratio (FR) and host selectivity index (HSI). A single *P. papatasi* specimen fed on bovine.

*P. papatasi* was represented by only two specimens. Table III shows the results of blood meal analysis. The identification was possible in 45/66 of females tested (68.2 %). Undetermined blood meals were due to small amount of fresh blood or to advanced blood digestion. By adjusting blood origin prevalence of *P. neglectus* according to FR and to HSI based on the available biomass, this species results in a quite opportunistic feeder 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 2002; Bongiorno *et al.*, 2003). In Spain, France and Italy, phlebotomine vectors have been observed to feed on a wide range of domestic animals, with varying degrees of anthropophily (De Colmenares *et al.*, 1995; Guy *et al.*, 1984; Killick-Kendrick *et al.*, 1977; Bongiorno *et al.*, 2003).

#### SEARCH FOR *LEISHMANIA* INFECTIONS

*P. neglectus*, collected in Lezhe, was the only species investigated for the search of leishmanial DNA by PCR technique. Of the 39 female specimens examined, 36 were blood fed, at different stages of blood digestion. None of them was found positive.

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