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**Species composition, activity patterns and blood meal analysis of sand fly populations
(Diptera: Psychodidae) in the metropolitan region of Thessaloniki, an endemic focus of
canine leishmaniasis.**

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Abstract

Species composition, activity patterns and blood meal analysis of sand fly populations were investigated in the metropolitan region of Thessaloniki, North Greece from May to October 2011. Sampling was conducted weekly in 3 different environments (animal facilities, open fields, residential areas) along the outskirts of the city in areas of increased canine leishmania transmission. Six sand fly species (*Phlebotomus perfiliewi*, *Phlebotomus tobbi*, *Phlebotomus simici*, *Phlebotomus papatasi*, *Sergentomya minuta* and *Sergentomya dentata*) were identified using both classical and molecular techniques. DNA barcodes were characterized for the first time for two (*P. simici* and *S. dentata*) of the six recorded species. Phylogenetic analysis based on the COI gene sequences confirmed the grouping of *P. tobbi*, *P. perniciosus* and *P. perfiliewi* (subgenus *Larrousius*) and the monophyly of *P. simici* (subgenus *Adlerius*). By far the most prevalent species was *P. perfiliewi*, followed by *P. simici* and *P. tobbi*. The largest populations of sand flies were collected from animal facilities, followed by residential areas and open agricultural fields. Peak activity of sand flies overall occurred mid-August to mid-September and then declined sharply in October. Blood meal analysis showed that *P. perfiliewi* and *P. simici* feed preferentially on humans (88% & 95%, respectively) but also feed on chickens and goats. When designing a control strategy to alleviate sand fly nuisance in the region of Thessaloniki the following conclusions can be reached from this study: a) August and September are high risk months due to increased sand fly activity levels, b) animal facilities within or adjacent to urban settlements are high risk areas and may act as a maintenance and amplification foci for the vector as well as the parasite, and c) the abundance, ubiquity and feeding behavior of *P. perfiliewi* and *P. simici* establishes them as potentially important vectors of *Leishmania* in the region.

Introduction

Phlebotomine sand flies [Diptera: Psychodidae] are primary vectors of human and canine leishmaniasis and affect the lives of people in more than 90 countries (WHO 2010). In the Mediterranean Basin visceral (VL) and cutaneous leishmaniasis (CL) are endemic. Greece is one of the Mediterranean countries affected by the disease, with VL being the predominant form reported in nearly all regions of the country and CL occurring sporadically (Ntais et al. 2013). The most common etiological agent for leishmaniasis in Greece is *Leishmania infantum*, responsible for VL and CL cases in humans and visceral disease in dogs (CanL). In the recent years, even though sporadic human cases are reported annually (in total 644 cases were reported from 2004-2014, KEELPNO 2015) humans are not implicated as an important reservoir of *Leishmania*. The disease is very common in dogs with 20-24% prevalence of infection reported in several studies (Athanasίου 2012, Ntais et al. 2013, Sideris et al. 1999).

Ten sand fly species have been detected in Greece (Ivovic et al. 2007). Of these, the species *Phlebotomus perfiliewi* and *P. tobbi*, important vectors of leishmaniasis in the Mediterranean, had the widest geographical distribution. In their study, eighteen different areas were surveyed across the country, excluding the metropolitan region of Thessaloniki. Thessaloniki is the 2nd largest city of Greece (1 million citizens), a major Mediterranean port and popular tourist destination, with well-developed agricultural and farming industries. Thessaloniki is endemic to CanL with highest frequency of transmission around and along the outskirts of the city (Kontos 1986), where most agricultural and farming activities are concentrated. No organized sand fly monitoring and control programs are in place and no information is available on the temporal and spatial distribution of sand fly species, their blood feeding preferences, and their role on *Leishmania* transmission.

The objectives of our study were to a) identify sand fly species using morphology and DNA barcoding that are currently present in a leishmaniasis endemic region of North Greece, b) compare their relative abundance and activity patterns across 3 different habitats (animal facilities, agricultural fields, and residential areas) over a six-month period and c) investigate blood feeding preferences of the most dominant species exclusively in multi-species farms due to the broad vertebrate host community compared to single-species farms. This information may be useful for identifying periods and areas of increased sand fly activity and can be used for improving vector control programs by targeting the sand flies in a timely manner focusing on high priority areas conducive to sand fly breeding. Also, knowledge on the blood feeding preferences of the most prevalent species is of great ecological and epidemiological relevance and may provide an insight into their role in the zoonotic and anthroponotic transmission of leishmaniasis.

Materials & Methods

The survey area

The survey was conducted from May to October in 2011 in the metropolitan region of Thessaloniki, North Greece (40°40'26.49'' N, 22°56'51.53'' E). Thessaloniki is located on the northern coast of Thermaikos Gulf and is enclosed by the mountain Chortiatis on its southeast. The climate of the region is described as a humid temperate climate with cold winters and hot, humid summers, with rare rain events during the summer months. The mean annual temperature and precipitation is 16°C and 478 mm, respectively with 67% relative humidity (EMY 2015). All samplings occurred along the outskirts of the city and into the neighboring rural settlements. Three different habitats were targeted: animal facilities in proximity to residential areas (6 sites, 3 cattle farms, 2 kennels, 1 multi-species farm), open agricultural fields (3 uniform sites, a

mixture of rice-fields, cotton field, wheat & corn fields), residential areas (9 sites, human settlements along the edge of the city). The multi-species farm consisted of the following vertebrate hosts within a 50m radius from our collection point: horses (8), goats/sheep (25), chickens (40), pigeons (20), pigs (4), dogs (6) and cats (10). The maintenance personnel (5 technicians) responsible for animal husbandry lived on site during the sampling period.

Field sampling and identification techniques

Carbon -dioxide (dry ice) baited CDC light traps (John W. Hock, Gainesville, Florida, USA) were deployed weekly at 18 sites beginning 20 May 2011 (Figure 1) until the end of October 2011. Trapping was, also, conducted during August using sticky traps consisting of castor oil coated sheets of paper (21 x 29.5 cm). Approximately, total of 400 papers were used (200 in the residential and open field sites, and 200 in the animal facilities). The CDC traps were hung on either low trees or metallic posts (trap opening approximately 1m from the ground), whereas the castor oil coated sheets were placed in and around the animal dwellings and houses, in cracks and crevices on the structures/walls and in vegetation. Weekly sand fly catches were counted and identified morphologically down to species level following standard procedures, with the head and the terminal segments of the abdomen mounted on permanent microscope slides (Lewis 1982, Lane 1986, Leger et al.1986 1st & 2nd). Catches >200 specimens per light trap were identified using a random sample of 200 sand flies, so that total species numbers could be extrapolated to counts for the entire sample. Population dynamics of the most prevalent species over time was recorded. Identification was also confirmed by CO1 DNA barcoding in 30 randomly chosen samples. After morphological examination, the remaining part of the abdomen was transferred to 1.5 ml Eppendorf tube and stored in absolute ethanol at -20°C for DNA extraction. Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen,

Courtaboeuf, France) following the manufacturer's protocol. The CO1 region was amplified using the primers LCO1490 and HCO2198 as described by Folmer et al. 1994. Sequencing of the PCR products was performed on both strands by Genoscreen (Lille, France), using the PCR primers, applying the Big Dye Terminator v3.1 method on an AB 3730XL automated DNA sequencer. Sequences were edited using the software MEGA 6 (Tamura et al. 2013). Similarity search between these sequences and sequences in databases was performed using BLAST algorithm in the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST/>) and the Barcode of Life Data System Identification engine (BOLD-IDS, http://www.boldsystems.org/index.php/IDS_OpenIdEngine).

Sequence alignment and phylogeny

Twenty-two sequences were analyzed at the CO1 locus. Fourteen sequences were produced in this study (Genbank accession numbers KU519497 to KU519510) and eight sequences of *Phlebotomus*, *Sergentomyia* and *Lutzomyia* (*L. umbralis* defined as the outgroup) were obtained from Genbank database. Sequences were aligned using the multiple alignment algorithm MUSCLE (Edgar 2004) executed in MEGA6. The evolutionary relationships between taxa were inferred using MrBayes 3.1.2 (Huelsenbeck et al. 2001). Bayesian trees were constructed using the evolutionary model (GTR+I+ Γ) obtained with MODELTEST (Posada and Crandall 1998) executed in PAUP 4.0b10 (Swofford 2000) using PaupUp graphical interface (Calendini & Martin 2005) under the hierarchical likelihood ratio tests (hLRT). Two simultaneous runs of 4 million generations were performed, and convergence was maximized by ensuring the average standard deviation of split frequencies fell below 0.002 and potential scale reduction factor approached 1.0. The first 25% of each run was discarded as burnin for the estimation of the consensus topology and the posterior probability of each node. Alternatively,

phylogenies obtained by Maximum Parsimony (MP) and Maximum likelihood (ML) using the GTR+I+ Γ evolutionary model were executed in MEGA6. MP trees were obtained using Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). Nodal support for MP and ML topology was estimated using parametric bootstrapping with 500 replicates.

Blood meal analysis

Out of the 15,000 sand flies trapped in total from the multi-species farm, 50 engorged females were randomly selected and subjected to blood meal analysis. Females were identified to species level using morphology and CO1 DNA barcoding as described above. DNA was extracted from the abdomen to maximize the host DNA concentration in each sample; however, detection of miniscule amounts of host DNA in a blood meal still remained challenging. Our experimentation suggested that at least 40 ng, but usually 100-150 ng, of template DNA from blooded sand fly abdomens was necessary for providing a concentration of the targeted PCR product suitable for direct sequencing. For the identification of the source of blood meals, a 359-bp conserved region of the vertebrate host mitochondrial cytochrome b gene (cyt b) was amplified exactly as described by Es-Sette et al. (2014), except for the polymerase and buffer which was the HotStarTaq plus master mix kit (Qiagen). DNA of a male sand fly was included in each batch of reactions as a negative control. Amplified products were sent in Genoscreen (Lilles, France) for sequencing on both strands using the PCR primers. Sequencing method, editing, alignment and identification of host species sequences were performed as described above.

Results & Discussion

Sand fly species identification, relative abundance, & activity patterns across 3 different habitats

A total of 78,069 sand flies were collected. CDC light trap collections accounted for 77,762 specimens (99.6%), representing the majority of the total sand flies caught, whereas sticky trap collections accounted for only 307 specimens (0.4%). Sand flies collected belong to 6 species: *P. perfiliewi*, *P. simici*, *P. tobbi*, *P. papatasi*, *Sergentomyia minuta*, *S. dentata*. *Plebotomous perfiliewi* and *P. simici* were the most abundant and widely distributed species followed by *P. tobbi* and representing 77.6%, 16.8% and 5.3% of the total population, respectively. *Sergentomyia* species were rarely sampled (<0.2% of the total population) and only two *P. papatasi* specimens were collected throughout the sampling season. From the six species recorded only the two most prevalent species (*P. perfiliewi* and *P. simici*) were sampled with sticky traps. Baited CDC light traps are known to outperform other non-baited trapping methodologies in sand fly collections such as sticky traps, (Veronesi et al. 2007, Bernier et al. 2008), however, the small sand fly sample collected in this study with the sticky traps is attributed more to the limited sampling and less to the inherent weaknesses of the trapping method.

Animal facilities were the most prolific habitat accounting for 93% of the total population, followed by residential areas (6.98%) and open fields (0.02%). The mean weekly numbers of sand flies collected per/CDC trap at each site from May- October and the male/female activity ratio are shown in Figures 2 and 3, respectively. Traps located in open field areas collected very few sand flies presumably due to the absence of appropriate vertebrate hosts for blood meal acquisition. It is well known that animal shelters provide a suitable environment for sand fly breeding (Killick-Kendrick 1999) and we suspect that animal facilities associated

with residential areas, such as in the case of our study, may be facilitating *Leishmania* transmission due to the overlap of vector and susceptible host habitat.

Sand fly population size started increasing in July and peaked in August and September in animal facilities, where as in residential areas one population peak was observed in July and population size decreased sharply in September. No activity trends could be observed in open field areas due to the small sand fly sampling size. Females were more dominant in the trap catches accounting for more than 78% of the total population (61,194 females, 16,875 males). Male relevant abundance seemed to be higher in the middle of the season (July) with a sharp decline towards the end of the summer (August), with the only exception of animal facilities where male population became more abundant during August. Our study was the first aimed to determine species composition and activity patterns with respect to season, landscapes, and blood feeding sources of sand flies in the metropolitan region of Thessaloniki. Activity patterns of sand flies can vary from region to region and are strongly correlated to environmental factors such as climatic conditions. Our findings come in agreement with studies conducted in regions with similar climate, where maximum sand fly abundance was observed between July and September (Kasap et al. 2008, Janini et al. 1995).

The relevant abundance by habitat (animal facilities, residential areas, open field) of the 3 most prevalent species is shown in Fig. 4. Overall, *P. perfiliewi* was the most dominant species in animal facilities and residential areas but not in open field environments where *Sergentomyia* species predominated followed by *P. tobbi*. In animal facilities and residential areas *P. perfiliewi* had the highest relevant abundance later in the season (August-October), whereas *P. simici* predominated during July. *P. tobbi* showed a similar activity pattern in both animal facilities and

residential areas with highest relevant abundance early in the season (May-June) and a gradual decline towards the end of the summer.

DNA barcoding unambiguously assigned the examined samples to species for which DNA barcodes were already available in the GenBank database i.e. *P. perfiliewi* (KJ481175, Bounamous et al. 2014), *P. tobbi* (KT634317, Giantsis et al. 2016) and *S. minuta* (KP828566, Depaquit et al. 2015), with 96%, 99% and 99% similarity respectively. There was no barcode sequence corresponding to *P. simici* and *S. dentata* in Genbank or BOLD, thus, the COI sequences that we deposited (Genbank accession numbers KU519497-KU519501 and KU519509), represent the first species-specific DNA barcodes for *P. simici* and *S. dentata*, respectively.

Phylogenetic analysis

Recently, Maia et al. (2015) evaluated the utility of the CO1 gene in phylogeny of sand flies, by performing an extensive phylogenetic analysis of more than twenty different *Phlebotomus* and *Sergentomyia* species. However, sequences from two of the three most predominant species surveyed in our study namely *P. tobbi* and *P. simici* were not part of their dataset. Phylogenetic relationships among taxa analyzed in this study are depicted in the tree of Fig. 5. Our results confirmed the clear phylogenetic distinction reported by Maia et al., (2015) between *Sergentomyia* and *Phlebotomus*. We obtained respectively five, two and five haplotypes for *P. perfiliewi*, *P. tobbi* and *P. simici*. All haplotypes formed monophyletic clusters supported by high posterior probability and bootstrap values corresponding to their respective species. Our results also strongly supported the monophyletic clade grouping *P. tobbi*, *P. perniciosus* and *P. perfiliewi*, which corresponds to the subgenus *Larrousius* (Depaquit 2014, Maia et al. 2015), (Fig. 5). In addition, CO1 phylogenetic analysis supported the close relation of *P. tobbi* and *P.*

perniciosus, similarly to results obtained with nuclear elongation factor alpha, cyt b, 16-18S rRNA genes (Aransay et al. 2000) and salivary proteins analyses (Vlkova et al. 2014). *P. simici* was found to be an outgroup to the subgenus *Larrousius* in agreement with its current subgenus taxonomic rank *Adlerius* (Fig. 5). The phylogenetic analysis confirmed *S. minuta* and *S. insularis* to be sister species, and suggested *S. dentata* to be considered as sister taxa to the clade *S. minuta-S. insularis*.

Overall, by adding new sand flies sequences, we confirmed that the CO1 barcode is appropriate for routine species level assignment in this group and also that phylogenetic analyses of CO1 do allow conclusive delineation of sand flies into individual species.

Blood feeding preferences

The blood meal source was successfully determined in 49 out of the 50 examined blood-fed female sand flies, all belonging to the 3 predominant species trapped in the farm (Table 1). Negative controls did not yield any products, while a single 359-bp band was observed in all blood-fed sand flies. The blood source was identified with >97% percentage of identity to the cyt b sequences of the vertebrate host species. Apart from two poultry (both *P. perfiliewi*) and two goat/sheep blood fed sand flies (one *P. perfiliewi* and one *P. simici*), all remaining flies fed on humans, despite the fact that collections were made in an animal facility with a large variety of vertebrate hosts (Table 1).

Vector species are generally considered opportunistic feeders, thus their host preferences should be determined taking into account the structure of the sampling habitat (Rossi et al. 2008). For instance *P. sergenti* populations from Sanliurfa (Turkey) were found to be ornithophilic (Svobodova et al. 2003), whereas the same species in Morocco exhibited an anthropophilic character (Es-Sette et al. 2014). However the engorged sand flies examined here,

were all collected from a multi-species animal facility, where several different blood sources were available (Table 1). Taken together, our results suggest that at least two of the three examined species (*P. perfiliewi* and *P. simici*) are highly anthropophilic. Although apparent abundance of a sand fly species is not in itself sufficient to incriminate it as a vector, the blood-feeding behavior, synanthropic distribution and abundance observed strongly suggest that *P. perfiliewi* and *P. simici* may contribute to the transmission of human and canine leishmaniasis in Greece.

When designing a control strategy to alleviate sand fly nuisance in the region of Thessaloniki the following conclusions can be reached from this study: a) August and September are high risk months due to increased sand fly activity levels, b) animal facilities within or adjacent to urban areas are high risk areas due to their high sand fly breeding potential, as they may act as a maintenance and amplification reservoir for the vector as well as the parasite, and c) the abundance, ubiquity and feeding behavior of *P. perfiliewi* and *P. simici* establishes them as potentially important vectors of *Leishmania* in the region.

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351

352 **Table 1.** Blood meal source of the three predominant sand flies species trapped in the multi-
353 species farm. Number of animals in the farm are shown in brackets

Sand fly host	<i>P. perfiliewi</i>	<i>P. simici</i>	<i>P. tobbi</i>
Horses (8)	0	0	0
Goats/sheep (25)	1	1	0
Chickens (40)	2	0	0
Pigeons (20)	0	0	0
Pigs (4)	0	0	0
Dogs (6)	0	0	0
Cats (10)	0	0	0
Humans (≥ 5)	22	19	4
Total number of blood meals/ vector species	25	20	4

Figure 1. CDC light trap network, Thessaloniki region (animal facilities, residential areas, open field)

Figure 2. Activity patterns of sand fly species in 3 different environments from May to October 2011.

399

Animal Facilities

A

400

Open Field

B

401

Residential Areas

C

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403

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405

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Figure 3. Activity patterns of male/female sand fly species from May to October

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A

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B

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C

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Figure 4. Relative abundance of *P. perfliewi*, *P. simici* and *P. tobbi* in 3 different environments from May to October

Figure 5. Bayesian phylogenetic analysis of partial cox1 sequences (655bp) amplified from phlebotomine sand flies collected in Greece. Bayesian Posterior probability values/Parsimony bootstrap values/ Maximum likelihood bootstrap values indicated support for clades. *Lutzomyia longipalpis* (JQ769143) was chosen as the outgroup