CHARACTERISTICS OF PHLEBOTOMINE SANDFLIES IN SELECTED AREAS OF SRI LANKA

SASC Senanayake[1], W Abeyewicreme [2], EM Dotson[3] and ND Karunaweera[1]

[1]Department of Parasitology, Faculty of Medicine, University of Colombo;
[2]Department of Parasitology, Faculty of Medicine, University of Kelaniya, Sri Lanka;
[3]Entomology Division , Center for Disease Control and Prevention, Atlanta, USA

Abstract. Cutaneous leishmaniasis (CL) is an endemic disease in Sri Lanka. Studies on vector aspects, although important for better understanding of disease transmission dynamics, are still limited. The present study describes the species distribution and behavioral patterns of sandflies within selected disease- prevalent zones in the country. Adult sandflies were collected from several field sites over a two-year duration in Sri Lanka using cattle-baited net traps, CDC light traps and manual methods. Species identification was performed using standard keys. *Leishmania donovani* and source of blood meal in blood-fed female sandflies DNA were identified using PCR-based methods. Aggregation period of adult sandflies during overnight collections was also noted. The collected sandflies were identified as *Phlebotomus argentipes glaucus* (previously known as morphospecies A) and a non-vector species, *Sergentomyia zeylanica*. Presence of *L. donovani* DNA was found in 2/634 female sandflies. The parasite ITS1 region of SSU rDNA had 99% sequence similarity with *L. donovani* from Bangladesh and India. The peak aggregation period of sandflies within cattle-traps was between 8:00 PM to 11:00 PM, indicating that vector control strategies could be conducted during this time period. As *Sergentomyia zeylanica* is likely to be merely a biting nuisance and showed more of an anthropophilic behavior, whereas the probable vector of CL in Sri Lanka (*P. argentipes glaucus*) demonstrated zoophilic behavior, has implications for the planning of future vector control strategies.

Keywords: *Phlebotomus argentipes glaucus* anthropophilic, cutaneous leishmaniasis, vector, zoophilic, Sri Lanka

INTRODUCTION

Leishmaniasis is a vector-borne parasitic disease. It is endemic in more than 90 countries and occurs in three different clinical forms (visceral, mucocutaneous and cutaneous) (Alvar *et al*, 2012). Two to four hundred thousand new cases of visceral leishmaniasis (VL) are diagnosed across the world every year, with 0.7-1.2 million cases of cutaneous leishmaniasis (CL) (Alvar *et al*, 2012). Between 20,000-40,000 deaths occur annually in association with leishmaniasis, and the majority is due to VL, mainly in India, Afghanistan and a number of Middle Eastern countries (Alvar *et al*, 2012).

Although Sri Lanka is situated in

Correspondence: SASC Senanayake, Department of Parasitology, Faculty of Medicine, University of Colombo, Sri Lanka.
Tel: 094 2 695300 ext 180
E-mail: senanayakesanath@yahoo.com; sanath@parasit.cmb.ac.lk

close proximity to India where both VL and CL are prevalent, the former country was considered free of both forms of the disease until 1992, when an indigenous case of CL from the southern part of the country was reported (Athukorale *et al*, 1992). Since then, the number of cases has increased, and CL has been an established disease to the present day (Siriwardana *et al*, 2007; Karunaweera, 2009). A locally acquired case of VL also has been reported from a north-central province in 2007 (Abeygunasekara *et al*, 2007), with a number of similar cases documented subsequently (unpublished). The threat posed by a newly established disease in a community that is non-immune no doubt justifies more detailed studies.

The causative organism of Sri Lankan CL has been identified as *Leishmania donovani* Mon 37, an organism known to cause visceral disease elsewhere (Karunaweera *et al*, 2003). Human *Leishmania* parasites are transmitted by sandflies that belong to the genera *Phlebotomus* and *Lutzomyia*. There are about 30 known species of sandflies that are capable of transmitting the disease around the world (Lane, 1993). Several species of sandflies that belong to two genera, *Phlebotomus* and *Sergentomyia*, have been reported in Sri Lanka (Lewis, 1978; Ozbel *et al,* 2011). Of these, *P. argentipes* is the only proven vector of leishmaniasis (Lane, 1993).

P. argentipes is generally considered as a species complex (Illango, 2010). Initially they were classified into morphospecies A and B, depending on the length of a sensory structure, sensilla chaetica, situated in the second segment of the flagellomere of the antenna (Surendran *et al*, 2005). Recent reassessment of *P. argentipes* complex has indicated the presence of three varieties, or sibling species (Illango, 2010).

A feature of morphospecies B that was reported from the northern parts of Sri Lanka is compatible with the description of *P. argentipes* var. *annadalei* Sinton (Illango, 2010). However, sandflies that belong to *P. argentipes* complex analyzed from elsewhere in the country have been identified as *P. argentipes* var. *glaucus* (Ranasinghe *et al*, 2012), which was previously known as morphospecies A (Surendran *et al*, 2005). Recent studies on sandflies from Jaffna peninsula in the northern province also have revealed the presence of morphologically different sibling species within the *P. argentipes* complex (Gajapathy *et al*, 2011). Furthermore, *L. donovani* transmission by *P. argentipes* complex recently has been confirmed (Gajapathy *et al*, 2013).

This study was designed as a descriptive cross-sectional analysis to investigate the prevalence and distribution of sandfly species in selected disease endemic areas within Sri Lanka. Aggregation behavior of the Sri Lankan sandflies was determined with a view to obtaining an insight into the peak aggregation period. In addition, blood meal analysis of wild caught female sandflies was performed to identify host preference of the vectors, details of which would be important in the design of future vector control strategies.

MATERIALS AND METHODS

Collection of sandflies for species identification and distribution

Sandflies were collected over a period of two years (June 2006 to May 2008) from selected sites within the districts of Kurunegala, Matara, and Hambantota, located in the northwestern and southern provinces of Sri Lanka (Fig 1), where CL is known to be prevalent (Epidemiological Unit, 2012). A database maintained at

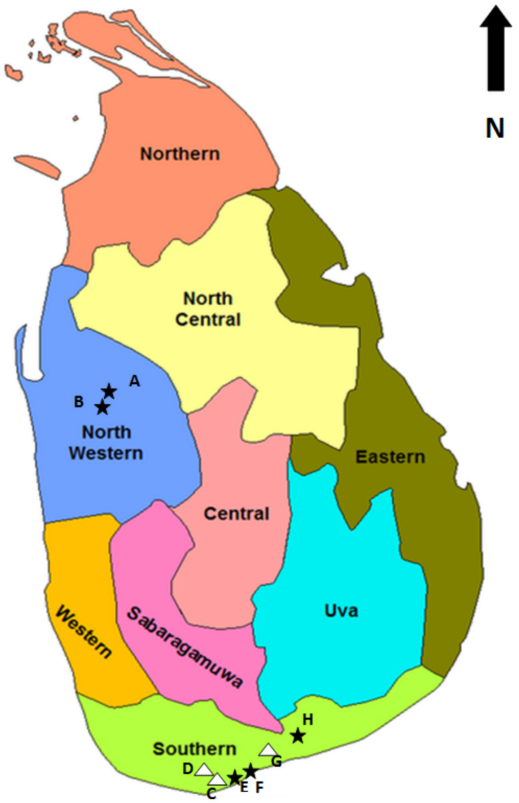

Fig 1–Provincial map of Sri lanka showing study sites used for the collection of adult sandflies. Study sites used for the collection are marked from A - H (A, Udawela; B, Pannala; C, Nilwella; D, Dickwella; E, Pahajjawa; F, Moraketiara; G, Walasmulla; H, Mamadala). ★ Sites where all three trapping methods were used. △ Sites where cattle-baited net traps and manual methods were used.

the Department of Parasitology, Faculty of Medicine, University of Colombo, Sri Lanka on leishmaniasis patients from year 2000 was also used to select the locations. Sites within Pannala, Udawela, Moraketiara, and Pahajjawa (Fig 1) were visited monthly during the study period. Mamadala South and Mamadala North (Fig 1) were visited once in every two months. The other three sites (Nilwella, Walasmulla and Dickwella) were visited only once. The sandfly collections were performed in each site within a radius of approximately 50 m in distance from the locations where there were newly confirmed cases of CL. Attempts were made to collect as many of sandflies as possible throughout the period.

Adult sandflies were collected using two or three methods: a) cattle-baited net traps, b) CDC miniature light traps, and c) manual collection with aspirators. Cattle-baited net traps and light traps were used at every collection sites, and manual collections of sandflies from surrounding houses of the area were conducted less frequently with the consent of the residents.

Cattle-baited net traps were set up with a single cow hired from a farmer of the village. Cattle were kept within the net trap between 6:00 PM to 6:00 AM of the following day. Sandflies were collected at 10:00 PM and 6:00 AM using manual aspirator within the cattle-baited net traps. Both male and female sandflies were collected, and all samples were transferred to screw-capped tubes containing 80% ethanol. CDC light traps were set up outdoors and left over-night, and the trapped sandflies were separated from other insects and preserved as described.

Sandflies were collected using aspirators under a light source from selected indoor and outdoor locations that are likely to be sandfly-resting sites. These included walls of living and bed rooms, store rooms, cattle sheds, wood stores (outdoor), and bushes and trees around the houses. Indoor collections were made between 8:00 and 10:00 PM. The collected sandflies were preserved as described.

Collection of sandflies to determine aggregation behavior

Sandflies were collected by the cattle-

traps on an hourly basis for three consecutive days from Pannala and Udawela during two visits each. Traps were set up with a cow inside and left overnight for a period of 12 hours (from 6:00 PM to 6:00 AM of the following day). Sandflies were collected using manual aspirators at the end of each hour. Each collection was labeled and subsequently identification for sex and species.

Collection of blood-fed sandflies for analysis of blood meal

Blood-fed female sandflies caught using both light traps and manual methods from the surroundings of the study sites were stored separately. Blood-fed females found within the cattle traps were excluded from the analysis of blood meal to avoid misinterpretation due to host availability.

Sandfly species identification

Male and female flies were separated in the laboratory by observing the genitalia under a dissecting microscope. Identification of subspecies within the *P. argentipes* complex was performed using their distinguishing features (Illango, 2010). Both male and female sandflies were treated with KOH and mounted in Berlese's medium. Dissections of heads of both sexes were carried out to reveal cibarium structures. Presence of cibarial teeth was used as the key factor to differentiate *Phlebotomus* and *Sergentomyia* at the genus level (Lewis, 1978). Female sandflies were dissected also to detect spermathecal structure, which is a key feature to distinguish between species (Lewis, 1978). The length of the flagellomere second segment of the antenna was compared with the length of the sensilla chaetica, which helps in the differentiation between sibling species of *P. argentipes* (Illango, 2010).

Detection of *Leishmania* spp DNA

Individual female sandflies were crushed, and DNA was extracted using Gentra Puregene Blood Kit® (QIAGEN, New Delhi, India) according to the manufacturer's instructions and stored in a final volume of 25 µl. DNA was extracted from 634/823 female sandflies identified as *P. argentipes*. DNA was PCR amplified using R221 and R332 primers, which target *Leishmania* spp ribosomal DNA (Lachaud *et al*, 2001). PCR thermocycling conditions consisted of 95°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 1 minute, with a single final heating at 72°C for 10 minutes. Amplicons were stored at 4°C until sequenced in 3130X sequence analyzer (Applied Biosystems, Foster City, CA) equipped with 3130x data collection v.3.0® software.

Confirmation of *L. donovani* in sandfly

Positive samples were amplified by nested PCR using IR1 and IR2 (Cupolillo *et al*, 1995) and ITS1F and ITS2R primers (Parvizi *et al*, 2008), which amplify ITS1 region of small sub-unit ribosomal DNA (SSU rDNA) of *L. donovani*. Another fragment of *L. donovani* ITS2 region of SSU rDNA also was amplified using ITS2R and ITS2R4 primers (Parvizi *et al*, 2008). PCR thermocycling conditions consisted of 95°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 55°C for 30 seonds, and 72°C for 1 minute, with a final step of 72°C for 10 minutes. Amplicons were kept at 4°C until sequenced as described above. Sequences obtained were compared with available GenBank database (National Center for Biotechnology Information, Bethesda MD).

Blood meal identification

DNA from blood-fed female sandflies were extracted using a commercial available

Table 1
Total collection, males, females and species distribution of sandflies in Sri Lanka.

Site	*n*	*P. argentipes glaucus*		*S. zeylanica*	
		Female	Male	Female	Male
Pannala (Kurunegala District)[a]	1,216	123	942	17	134
Udawela (Kurunegala District)[a]	2,091	245	1,520	55	271
Pahajjawa (Matara District)[a]	914	40	329	43	502
Moraketiara (Matara District)[a]	1,274	0	0	122	1,152
Mamadala North (Hambantota District)[a]	2,351	278	2,056	3	14
Mamadala South (Hambantota District)[a]	987	116	871	0	0
Walasmulla (Hambantota District)[b]	118	6	112	0	0
Nilwella (Matara District)[b]	26	7	19	0	0
Dickwella (Matara District)[b]	94	8	86	0	0
Total	9,071	823	5,935	240	2,013

[a]Sites visited regularly during the study period. [b]Sites visited once during the study period.

Table 2
Sandflies collected from selected study sites in three districts in Sri Lanka using cattle-baited net traps.

Site	*n*	*P. argentipes glaucus*		*S. zeylanica*	
		Female	Male	Female	Male
Pannala (Kurunegala District)[a]	1,001	93	831	5	72
Udawela (Kurunegala District)[a]	1,078	104	808	21	145
Pahajjawa (Matara District)[a]	324	14	183	11	116
Moraketiara (Matara District)[a]	115	0	0	4	111
Mamadala North (Hambantota District)[a]	1,457	142	1,308	0	7
Mamadala South (Hambantota District)[a]	870	83	787	0	0
Walasmulla (Hambantota District)[b]	115	6	109	0	0
Nilwella (Matara District)[b]	26	7	19	0	0
Dickwella (Matara District)[b]	87	5	82	0	0
Total	5,073	454	4,127	41	451

[a]Sites visited regularly during the study period. [b]Sites visited once during the study period.

kit (Promega, Madison, WI). PCR was performed using mammalian specific primers MAMAF and MAMFR (Molaei *et al*, 2006). A 23.5-µl reaction contained 2 µl of genomic DNA, 0.5 µl of 25 mM $MgCl_2$, 1.25 µl of primers, 2 µl of dNTPs, 0.2 µl of *Taq* DNA polymerase, 5 µl of 5X PCR buffer and 11.55 µl of distilled water. PCR thermocycling conditions consisted of 95°C for 2 miutes, followed by 36 cycles of 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 1.5 minutes, with a final step of 72°C for 7 minutes. Amplicons products were separated by 0.7% agarose gel-elec-

Table 3
Sandflies collected from selected study sites in Sri Lanka using light traps.

Site	*n*	*P. argentipes glaucus*		*S. zeylanica*	
		Female	Male	Female	Male
Pannala (Kurunegala District)[a]	61	9	33	3	16
Udawela (Kurunegala District)[a]	37	3	9	11	14
Pahajjawa (Matara District)[a]	23	2	9	7	5
Moraketiara (Matara District)[a]	198	0	0	33	165
Mamadala North (Hambantota District)[a]	27	12	9	2	4
Mamadala South (Hambantota District)[a]	15	7	8	0	0
Walasmulla (Hambantota District)[b]	3	0	3	0	0
Nilwella (Matara District)[b]	0	0	0	0	0
Dickwella (Matara District)[b]	7	3	4	0	0
Total	368	33	75	56	204

[a]Sites visited regularly during the study period. [b]Sites visited once during the study period.

Table 4
Sandflies collected from resting places using manual method in selected study sites of Sri Lanka.

Site	*n*	*P. argentipes glaucus*		*S. zeylanica*	
		Female	Male	Female	Male
Pannala (Kurunegala District)	154	21	78	9	46
Udawela (Kurunegala District)	976	138	703	23	112
Pahajjawa (Matara District)	567	24	137	25	381
Moraketiara (Matara District)	961	0	0	85	876
Mamadala North (Hambantota District)	867	124	739	1	3
Mamadala South (Hambantota District)	102	26	76	0	0
Total	3,627	333	1,733	143	1,418

trophoresis, stained and visualized under UV light. Amplicons were sequenced and analyzed as described above.

RESULTS

The number of sandflies collected from all the sites using three different collection methods was 9,071 (8,008 males, 1,063 females). The numbers of sandflies collected from the sites visited regularly ranged from 914 (Pahajjawa, Matara District) to 2,351 (Mamadala North, Hambantota District) (Table 1). Collections made from sites that were visited only once during the study period ranged from 26 (Nilwella) to 118 (Walasmulla). Two species of sandflies belonging to genera *Phlebotomus* and *Sergentomyia* were found, with *P. argentipes (glaucus)* being the main species, except in Pahajjawa and Mora-

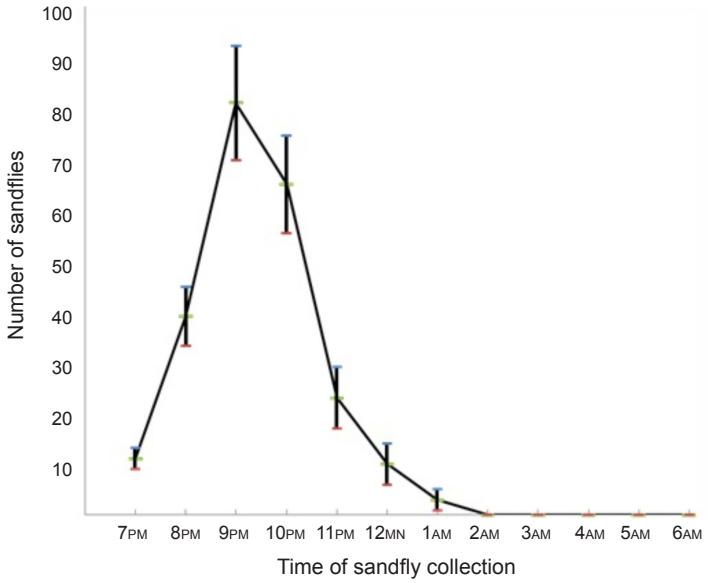

Fig 2–Average number of both male and female sandflies attracted overnight to the cattle-baited net traps on an hourly basis on three consecutive days from the sites in northwestern Sri Lanka. Results are presented as mean ± SE; 95% CI.

ketiara, both within the Matara District, where *S. zeylanica* predominated (Table 1).

The largest sandfly collection obtained was from cattle-baited net traps (n = 5,073) (Table 2), compared with the collection made using light traps (n = 368) (Table 3) and manual methods (n = 3,627) (Table 4). The numbers of sandflies collected using cattle-baited net traps ranged from 115 (Moraketiara, Matara District) to 1,457 (Mamadala North, Hambantota District) in sites visited monthly for a period of 2 years (Table 2). The highest number (2,327) of sandflies were collected from Mamadala area (1,457 from Mamadala North and 870 from Mamadala South). Collections using cattle-baited net traps from the sites that were visited once during the study period ranged from 26 (Nilwella) to 115 (Walasmulla) for a single night collection (Table 2). There was a distinct male predominance observed in the sandfly collections from cattle-baited net traps (male:female, approximately 10:1). The maximum number of sandflies caught in cattle-baited net traps in sites from the northwestern province of Sri Lanka during three consecutive nights was obtained between 8:00 and 11:00 PM, with highest number collected between 9:00 and 10:00 PM (Fig 2).

The number of sandflies found within light traps was the lowest (368) (Table 3) compared to both the cattle-baited net traps (Table 2) and manual collections (Table 4). The highest number (198) of sandflies collected using light traps was made in Moraketiara, Matara District where only *S. zeylanica* was found. Male-to-female ratio of sandflies found within the light traps was 3:1 (*P. argentipes* 2.3:1, *S. zeylanica* 3.6:1).

Manual collection of sandflies from resting places was carried out only in sites where regular visits were made (Table 4). The collection ranged from 102 (Mamadala South, Hambantota District) to 976 (Udawela, Kurunegala District). Both *P. argentipes* and *S. zeylanica* were found, with slightly higher numbers of *P. argentipes*. The male-to-female ratios between two species were 5.2:1 for *P. argentipes* and 9.9:1 for *S. zeylanica*.

All insects identified as *P. argentipes* were identical in morphology, with the length of the sensilla chaetica of the flagellomere second segment exceeding half of the length of the flagellomere (data not shown). This finding is consistent with the subspecies *P. argentipes* variety

glaucus (morphospecies A) of *P. argentipes* complex.

Two out of 634 female sandfly extracts gave positive PCR results for the presence of *Leishmania* spp. DNA with R221 and R332 primers. Analysis of the amplified sequences (GenBank accession no. KJ512884) showed 99% compatibility with *L. donovani* (GenBank accession no. Ld GQ332356.1), *L. infantum* (accession no. Li GQ332359.1), and *L. chagasi* (accession no. Lc GQ332357.1) (Alam *et al*, 2009).

PCR targeting the ITS1 region of SSU rDNA of the positive samples (KJ512884) showed 100% compatibility with Asian *L. donovani* type G and H (Kuhls *et al*, 2005). Further sequence analysis of ITS2 region showed 99% compatibility with *L. donovani* from Bangladesh (Alam *et al*, 2009).

One hundred six blood-fed sandflies [*P. argentipes (glaucus*), $n = 44$; *S. zeylanica*, $n = 62$] collected through light traps and manual methods revealed *P. argentipes glaucus* contained higher samples with animal blood (cattle blood, $n = 28$; buffalo blood, $n = 6$) than human blood ($n = 10$) ($p < 0.01$). Whereas the majority (46/62) of blood meals from *S. zeylanica* were positive for human blood, and the remaining were negative for other types of mammalian blood.

DISCUSSION

Although six species of sandflies originally have been described in Sri Lanka (Lewis, 1978), with several other species reported subsequently (Gajapathy *et al*, 2011; Ozbel *et al*, 2011), only two species were identified in this study. This may be due to the limited number of geographic locations studied or due to types of collection methods used (only three methods were used for collection of adult sandflies). There are many methods described to collect sandflies, and there is no single method that would guarantee the collection of all species (Killick-Kendrick, 1987).

Most of the selected areas were dominated by *P. argentipes*, except two sites that were dominated by *S. zeylanica*. *P. argentipes* is the proven vector of VL in Bangladesh, India and Nepal (Lane, 1993) and is the only species out of the described species in Sri Lanka that has proven disease transmission capacity (Lane *et al*, 1990). Sandflies that belong to the genus *Sergentomyia* have never been reported as vectors of any kind of human leishmaniasis in any part of the world. Therefore, the confirmatory finding of the probable vector status of *P. argentipes* may not be surprising.

All *P. argentipes* specimen collected from the study areas belonged to subspecies *P. argentipes* var. *glaucus (*morphospecies A). This species has not been reported from VL-endemic areas in India and is thus considered as a non-vector species (Surendran *et al*, 2005). Nevertheless, it is likely that it is indeed the vector of CL in Sri Lanka. A parallel situation appears to exist with regards to malaria vectors in Sri Lanka, with the principal vector species in the local setting: *Anopheles culicifacies* sibling species B, considered as an ineffective or vector with poor efficacy in India (Surendran *et al*, 2006).

The vast number of male sandflies found, especially in cattle-traps as observed in this study, is a well-documented phenomenon (Killick-Kendrick, 1987). This particular observation merely represents the behavioral pattern of male sandflies that become attracted in large numbers to locations with female sandflies (Lane, 1993). However, such differences in the ratios of male-to-female sandflies are

not apparent when they are maintained in laboratories (Killick-Kendrick, 1987).

The vector status of *P. argentipes glaucus* as the most probable vector that transmit leishmaniasis in Sri Lanka was confirmed with molecular identification of *L. donovani* genetic material from female sandflies and is in line with the recent observations made by others (Gajapathy *et al*, 2013). Although a small number of positive female sandflies (2/634) was detected, the finding is significant when compared to the similarly low positivity rate reported in wild caught sandflies studied from disease endemic areas elsewhere (Kumar *et al*, 2001).

The peak aggregation of sandflies was observed during the hours of 8:00 to 11:00 PM. However, a previous study conducted in the central part of Sri Lanka has shown a second peak during the early morning hours (Lane *et al*, 1990), which was not observed during this study. This could be due to environmental factors; the central areas of Sri Lanka are located at high altitudes with low average temperatures compared with the areas selected in this study.

The possible vector that was found in this study (*P. argentipes glaucus*) showed preference for blood of animals rather than humans. The vector of leishmaniasis in India, *P. argentipes* var. *sensu lato* (Illango, 2010); however, is anthropophilic (Lane *et al*, 1990). The observed variations in host preference may be due to the different characteristics of each subspecies within the *P. argentipes* complex. Studies in West Bengal using blood-fed *P. argentipes* has shown no preference towards humans or animals (Ghosh *et al*, 1990), although, similar studies in Bihar, India have confirmed their anthropophilic behavior (Garlapati *et al*, 2012).

Sandflies that belong to *P. argentipes glaucus* is the predominant species in most of the study areas investigated. In areas where *S. zeylanica* was the predominant species, very few sandflies were found within the cattle-baited traps. Cattle-baited net trap is the best method for collecting adult *P. argentipes glaucus*, although it gives a higher male-to-female ratio. Manual collection of sandflies from resting places is useful for trapping both *P. argentipes glaucus* and *S. zeylanica*. Although light traps gave the largest proportion of female sandflies, the total number of sandflies found within the traps were smaller; therefore, they are not very efficient.

In summary, this study describes the species distribution, aggregation behavior and host preference in wild-caught sandflies in selected areas of Sri Lanka. Although similar studies have been conducted elsewhere on a more limited numbers of sandflies (Lane *et al*, 1993; Surendran *et al*, 2005; Ozbel *et al*, 2011; Ranasinghe *et al*, 2012), this is the first study that investigated large numbers of sandflies from areas representing two provinces in the country. Moreover, as far as it is known, this is also the only study that gives details of blood meal analysis of wild-caught sandflies in Sri Lanka. *P. argentipes glaucus* was confirmed as the probable vector of CL in Sri Lanka. However, further entomological surveys and laboratory experimentation to demonstrate its infectivity are needed for a definitive confirmation of its vector status. The peak period of aggregation of sandflies observed has implication for planning vector control strategies and for the practice of better personal protection against sandfly bites. The probable vector of CL in Sri Lanka, *P. argentipes glaugus*, demonstrated zoophilic behavior, which has implications in the design of future control strategies. *S. zeylanica*, although a

non-vector species, could play an important role as a biting nuisance due to their anthropophilic nature. Further studies covering the entire island will provide more details on sandfly species distribution and vector habits, which would be useful in developing strategies for effective control of leishmaniasis in Sri Lanka.

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