Seasonal variation and natural infection of *Lutzomyia antunesi* (Diptera: Psychodidae: Phlebotominae), an endemic species in the Orinoquia region of Colombia

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Lutzomyia antunesi *has been commonly reported in outbreaks of cutaneous leishmaniasis (CL) in the Orinoquia region of Colombia. The bionomics of this species were studied in the municipality of Villavicencio (Meta, Colombia). Sandflies were captured over the course of one week per month for one year in intradomiciliary, peridomiciliary and extradomiciliary housing areas. The captures were performed from 06:00 pm-06:00 am using CDC light traps and the females were processed for polymerase chain reaction (PCR) to detect* Leishmania *spp. A total of 22,097 specimens and 19 species were captured of which* Lu. antunesi *(89%) and* Lutzomyia walkeri *(5%) were the most abundant. Other species recognised as anthropophilic (*Lutzomyia panamensis, Lutzomyia gomezi, Lutzomyia flaviscutellata *and* Lutzomyia fairtigi*) were present in very low abundance (< 2%). Natural infection with* Leishmania *spp was detected using PCR in* Lu. antunesi, Lu. panamensis *and* Lu. flavicutellata, *showing infection rates of 1%, 4.8% and 7.5%, respectively. The present paper provides information on various ecological aspects of* Lu. antunesi. *An analysis of seasonality shows that this species increases in abundance in the hottest months (December, January and February), directly correlating with the maximum temperature and inversely correlating with precipitation. The natural infection rate is associated with the peaks of highest abundance.*

Key words: sandflies - seasonal variation - natural infection - *Lutzomyia antunesi* - leishmaniasis - Colombia

The number of cases of American cutaneous leishmaniasis (ACL) per year in the department of Meta (ME) of Colombia has increased during the last decade (139 in 2005 to 1,062 in 2012) (SIVIGILA 2012). Since 2005, several outbreaks have been recorded in different municipalities and are mainly related to new human settlements in rural areas that have been affected by different social factors (Velez et al. 2001); peridomiciliary transmission has been reported (INS 2012). This epidemiological profile has been associated with legal or illegal urbanisations in deforested areas of Villavicencio.

Lutzomyia antunesi (Coutinho, 1939) is likely the primary ACL vector in ME. This fly is naturally infected with *Leishmania (Leishmania) chagasi* in Brazil (Ryan et al. 1984) and it has been determined that this species plays an important role in the transmission of *Leishmania (Viannia) lindenbergi* (Silveira et al. 2002). The first report of leishmaniasis infection in Colombia involved the observation of undetermined flagellates in an ACL focus in a case of intradomiciliary and peridomiciliary

transmission (Ferro et al. 1997). Natural infection with *Leishmania* spp was recently confirmed (Vásquez-Trujillo et al. 2008).

Preliminary studies have been conducted in Colombia to assess the existing sandfly fauna associated with ACL transmission areas (Montoya-Lerma & Ferro 1999, Barreto et al. 2000, Bejarano 2006). Recent studies investigating *Lu. antunesi* have determined its relative abundance, vector-human contact, the presence of *Leishmania* parasites and sandflies in the same geographical area and the detection of these parasites in insects and domestic animals (Vásquez-Trujillo 2006, Vásquez-Trujillo et al. 2008), thereby fulfilling some criteria for vector incrimination (Young & Lawyer 1987, Killick-Kendrick 1990). However, environmental determinants and their relationship with seasonal abundance and infection have not yet been studied. This study aimed to increase the knowledge of *Lutzomyia* species and to determine the effects of seasonal variations in *Lu. antunesi* abundance and infection with *Leishmania* spp in an ACL-endemic area of Villavicencio.

MATERIALS AND METHODS

Study area - The study was conducted in a rural area near Villavicencio, northern ME, a subregion of the Eastern Plains (Llanos Orientales) region on the border of the western Andean foothills of Colombia, which together form part of the Colombian Orinoquia ecoregion. Samples were collected in three rural areas: La Re-

forma (4º45'81''N 73º28'34''W), Barcelona (4º39'05''N 73º35'68''W) and Cocuy (4º24'94''N 73º36'36''W), located 12 km, 4 km and 7 km away from Villavicencio, respectively (Fig. 1). These sites have similar environmental conditions, including an elevation 380 m above sea-level, an average temperature of 25ºC, 85% relative humidity (RH) and 3,600 mm annual rainfall according to the Colombian Institute of Hydrology, Meteorology and Environmental Studies (IDEAM). Sampling was conducted from June 2008-May 2009, encompassing both the wet and dry periods. The area is characterised as premontane humid tropical forest and has some elements of humid tropical forest (Holdrige 1967).

Lutzomyia capture sites, collection and identification - A preliminary study was conducted on all properties in La Reforma (21), Barcelona (82) and Cocuy (44) to establish a rough composition of the populations of *Lutzomyia* species present in these areas and to determine which properties had the highest abundance of sandflies. Those properties with the highest abundance were then selected as monthly follow-up units (MFUs) for further longitudinal sampling. The preliminary study used CDC-type light traps placed 1.5 m above the ground in peridomiciliary areas, 15 m away from the houses; collections were performed between 06:00 pm-06:00 am.

Seasonal sampling was then conducted at three dwellings selected as MFUs according to the following selection criteria: (i) the houses were a minimum distance of 200 m away from the forest, (ii) there was a recent registry of confirmed cases of ACL in humans or canines and (iii) the inhabitants provided informed consent. Intradomiciliary, peridomiciliary and extradomiciliary sites (forests adjacent to the dwellings located a minimum of 200 m away) were sampled as capture habitats. The sampling lasted for one week per month over a 12-month period, simultaneously in the three rural areas, from June 2008-May 2009.

The sandflies collected were transferred to the Laboratory of Entomology of Department of Health of Meta, sacrificed at 0ºC and stored in Eppendorf tubes with 70% ethanol at 4ºC until taxonomic identification was conducted. A clarifying process was performed to identify male sandflies according to the methodology reported by Maroli et al. (1997). Female taxonomic classification was based on the following characteristics: fifth palp length, scutum pigmentation, flagellomere length and alpha and beta veins in the wings and spermathecae. For males, all taxonomic characteristics were evaluated after the clarifying process. The classification used was based on Young and Duncan (1994).

Determination of natural infection - Natural infection was determined by polymerase chain reaction (PCR). DNA was extracted using the phenol-chloroform protocol described by Golczer and Arrivillaga (2008) and amplified by the protocol standardised by Cabrera et al. (2002) using the *Leishmania* genus-specific OL1 and OL2 universal primers, which target kinetoplast minicircle DNA (Romero et al. 2001). This test was only performed for *Lutzomyia* species that had a vector-related background. The *Lutzomyia* species were tested in pools with a maximum of 30 insects classified by capture habitat (intradomiciliary, peridomiciliary and extradomiciliary) and by capture month. The head and thorax of the insects were used in an attempt to obtain infective forms only from the upper digestive tract.

Statistical analysis - Seasonal variation was evaluated first by transforming the variable "abundance" into its natural logarithm plus one [$\log_n (y+1)$] and a one-way ANOVA was performed, followed by Tukey's multiple comparison test (95% confidence level). Spearman's rank correlation non-parametric test (ρ) was used to evaluate the correlations between the abundance and environmental determinants. Data regarding the daily maximum, minimum and average temperatures, maximum, minimum and average daily rainfall and daily RH were obtained from the weather station closest to the sampling sites in the IDEAM weather station network (Unillanos station: 3503507, main climate type). Data were also obtained for one, two and 15 days prior to sampling to determine the relationship between the abundance and preceding daily climatic variables.

RESULTS

A total of 14,553 *Lutzomyia* specimens were collected during the preliminary sampling. The most abundant species was *Lu. antunesi* (63%, 86% and 93.3% of the total specimens captured in Barcelona, Cocuy and La Reforma, respectively), followed by *Lutzomyia walkeri* (35%, 17% and 6%)*, Lutzomyia gomezi* (0.4%, 0% and 2.5%) and *Lutzomyia panamensis* (1%, 0.4% and 1%). For the selected MFUs, 4,356 *Lutzomyia* per trap/night were obtained from a dwelling in the Barcelona rural area, 3,413 *Lutzomyia* per trap/night from the Cocuy rural area and 1,034 *Lutzomyia* per trap/night in the La Reforma rural area.

A total of 22,094 *Lutzomyia* specimens were captured during the seasonal sampling. The evaluation of the richness observed at each habitat revealed seven species captured in the intradomiciliary areas, 18 in the peridomiciliary areas and 19 in the extradomiciliary areas. The most abundant species was *Lu. antunesi* (89%), followed by various species with vector backgrounds: *Lu. panamensis* (2.3%), *Lutzomyia flaviscutellata* (1.05%), *Lu. gomezi* (0.8%) and *Lutzomyia fairtigi* (0.6%) (Table I). The seasonal variation analysis obtained by ANOVA

Fig. 1: geographic location of the study area in Colombia and in department of Meta compound of the villages Barcelona, Cocuy and La Reforma. 1: Meta; 2: Casanare; 3: Arauca; 4: Vichada.

TABLE I

The number of *Lutzomyia* specimens collected in an endemic area of the municipality of Villavicencio, department of Meta, Colombia, from June 2008-May 2009

Species	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	I ♀	I ♂	P ♀	P ♂	E ♀	E ♂	Total	(%)
Lu. antunesi	764	261	950	552	923	386	3,073	2,840	3,272	513	5,560	604	420	304	3,208	2,481	5,952	7,333	19,698	89.087
Lu. aragaoi	-	-	-	-	-	-	1	2	2	1	2	11	-	-	2	-	2	15	19	0.086
Lu. barrettoi barrettoi	-	-	-	-	-	-	2	-	6	1	1	-	-	-	1	-	5	4	10	0.045
Lu. barrettoi majuscula	-	1	-	-	8	2	9	-	5	13	7	5	-	-	-	1	5	44	50	0.226
Lu. begonae[a]	3	2	1	-	4	-	-	-	1	2	-	-	-	-	2	6	1	4	13	0.059
Lu. chagasi	-	-	-	-	1	-	1	-	1	-	1	-	-	-	-	1	-	3	4	0.018
Lu. fairtigi	1	5	8	2	41	1	61	-	1	4	11	4	-	1	11	13	19	95	139	0.629
Lu. flaviscutellata	21	11	1	24	12	12	31	28	33	23	30	8	-	-	18	12	128	76	234	1.058
Lu. gomezi	2	8	5	9	9	12	10	41	56	10	22	6	8	1	29	14	55	83	190	0.859
Lu. micropyga	0	-	-	-	-	-	-	-	1	-	-	-	-	-	-	1	-	-	1	0.005
Lu. nematoducta[a]	2	1	4	1	2	1	-	2	-	-	4	-	4	1	-	-	12	-	17	0.077
Lu. panamensis	21	16	50	42	32	18	70	82	55	15	35	73	3	33	61	137	54	221	509	2.302
Lu. preclara	1	1	2	-	-	-	2	-	1	-	17	-	-	-	-	-	18	6	24	0.109
Lu. punctigeniculata[a]	2	1	3	6	6	-	-	-	1	-	3	-	-	-	2	3	5	12	22	0.099
Lu. saulensis	-	-	2	1	-	-	1	1	1	1	1	-	-	-	1	-	2	5	8	0.036
Lu. sordellii	5	1	-	-	-	1	3	3	9	3	1	0	1	1	14	2	7	1	26	0.118
Lu. vattierae	1	-	1	57	-	1	4	2	1	1	10	-	1	-	4	-	14	59	78	0.353
Lu. walkeri	295	43	14	20	26	15	42	155	207	99	95	38	34	35	163	462	221	134	1,049	4.744
Lu. yuilli	-	-	1	-	-	-	1	-	-	-	-	-	-	-	-	-	2	-	2	0.009
Lu. (Sciopemyia) spp	-	-	-	-	-	-	-	-	-	9	-	9	-	-	3	-	15	-	18	0.081
Environmental determinants (average)																				
Rainfall (mm)	14.4	13.7	9.19	16.4	12.3	12.3	3.4	4.24	1.78	8.3	15.5	11.7	-	-	-	-	-	-	-	-
Maximum temperature (ºC)	30.6	30.8	32.4	31.8	31.4	33.2	31.6	32.8	33.2	34.2	33.2	32.6	-	-	-	-	-	-	-	-

a: new *Lutzomyia* species record for Meta; E: extradomiciliary; I: intradomiciliary; P: peridomiciliary.

showed that only total *Lu. antunesi* (F = 4.99; p < 0.001) and *Lu. antunesi* at extradomiciliary sites (F = 5.99; p < 0.001) exhibited significant seasonal variation. These groups had different rates of abundance during the sampling months of December (t = 2.212; p = 0.0367), January (t = 2.237; p = 0.0257), February (t = 2.343; p = 0.0277) and April (t = 3.162; p = 0.004) and these months exhibited the highest abundance according to Tukey's multiple comparison test.

An analysis of Spearman's correlation revealed that rainfall was the variable that best explained the total abundance of *Lutzomyia* species (ρ = -0.704; p < 0.0001). *Lu. antunesi* (the most abundant species) had a similar pattern and was similarly affected by rainfall (ρ = -0.688; p < 0.0001) (Fig. 2). Other environmental determinants, such as the maximum temperature one day before sampling and the minimum temperature two days before sampling, positively correlated with the *Lu. antunesi* abundance in all capture habitats. The maximum temperature (29-31ºC; ρ = 0.627; p < 0.0001) was the variable that most significantly correlated with *Lu. gomezi*, whereas the maximum temperature one day before sampling (ρ = 0.419; p = 0.0110) and rainfall were the main environmental variables (ρ = -0.408; p = 0.0136) that correlated with *Lu. flaviscutellata* and *Lu. panamensis*, respectively (Table II).

A total of 10,760 female *Lutzomyia* were assessed by PCR to investigate the rate of natural infection. The evaluated species were *Lu. antunesi, Lu. panamensis, Lu. gomezi and Lu. flaviscutellata*. The monthly infection rates obtained primarily correlated with the months that had the least rainfall (November-February) and those months for which the high abundance was recorded (December, January and April). *Lu. antunesi* was found to be an important species, as it was the most abundant,

and also had the most consistent monthly infection rates. The importance of *Lu. flaviscutellata, Lu. panamensis* and *Lu. gomezi* as vectors was also confirmed, as they were found to be naturally infected despite very low abundance rates compared to *Lu. antunesi* (Table III).

DISCUSSION

Of the total species identified in this study, *Lutzomyia begonae, Lutzomyia nematoducta* and *Lutzomyia punctigeniculata* were identified for the first time in ME. *Lu. begonae* has been identified in other departments that have different ecological characteristics, such as Guaviare (Cabrera et al. 2009), and the species is always associated with wooded areas. *Lu. nematoducta* and *Lu.*
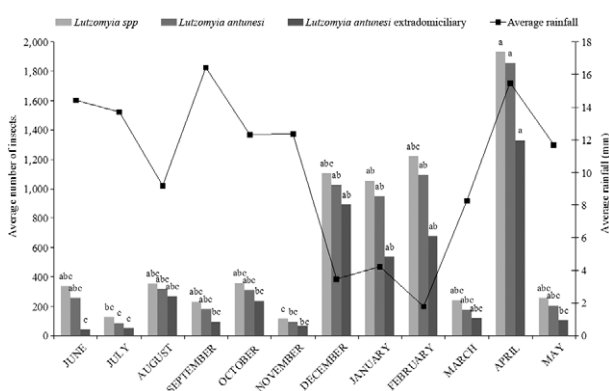

Fig. 2: average monthly total of *Lutzomyia, Lutzomyia antunesi* and *Lutzomyia antunesi* extradomiciliary population and monthly rainfall reported for the endemic area of the municipality of Villavicencio, department of Meta, Colombia, from June 2008-May 2009. There were no significant differences for groups having the same letter.

TABLE II

Environmental determinants correlated with the most abundant *Lutzomyia* species in the municipality of Villavicencio, department of Meta, Colombia

	Rainfall (mm)		Maximum temperature (°C)		Maximum temperature < 1 day (°C)		Minimum temperature < 2 days (°C)	
	ρ	p	ρ	p	ρ	p	ρ	p
Total	**-0.704**	< 0.0001	0.26	0.1256	**0.378**	0.0232	**0.431**	0.0086
Lu. antunesi	**-0.688**	< 0.0001	0.257	0.1296	**0.382**	0.0216	**0.441**	0.0071
Lu. antunesi (I)	**-0.507**	0.0016	0.21	0.2201	**0.346**	0.0385	**0.337**	0.0446
Lu. antunesi (P)	**-0.509**	0.0015	0.227	0.1829	**0.55**	0.0005	0.18	0.2939
Lu. antunesi (E)	**-0.579**	0.0002	**0.364**	0.0291	0.266	0.1171	**0.527**	0.0010
Lu. flaviscutellata	**-0.384**	0.0209	0.307	0.0688	**0.419**	0.0110	**0.356**	0.0333
Lu. panamensis	**-0.408**	0.0136	**0.378**	0.0230	**0.342**	0.0410	0.326	0.0525
Lu. gomezi	-0.122	0.4779	**0.627**	< 0.0001	**0.383**	0.0211	**0.335**	0.0457
Lu. gomezi (E)	-0.143	0.4059	**0.566**	0.0003	0.307	0.0683	**0.334**	0.0462

significant Spearman's coefficient of correlation (ρ) values regarding an alpha threshold = 0.05 are shown in bold. E: extradomiciliary; I: intradomiciliary; P: peridomiciliary.

punctigeniculata were found in both peridomiciliary and extradomiciliary areas, though they exhibited higher abundance in extradomiciliary habitats. *Lu. antunesi, Lu. panamensis* and *Lu. gomezi* were the most abundant species in all the capture habitats, demonstrating their adaptation to anthropophilic settings where blood meal sources and possible breeding sites can be found. Therefore, dwellings and their surrounding areas are high-risk areas for acquiring ACL (Pardo et al. 2006, Santamaría et al. 2006), which is evident from previous studies conducted in the area (Vásquez-Trujillo et al. 2008).

The ecological behaviour of an ACL focus encompasses different characteristics: its niche, its reservoirs, the vector and its seasonal abundance pattern, which is influenced by environmental determinants. Molina et al. (2008) showed that *Lutzomyia fairtigi* underwent a seasonal variation in the department of Casanare that was mainly determined by increased rainfall (ρ = 0.675; p < 0.001), whereas no changes in abundance or any association with environmental determinants were recorded for *Lu. antunesi* throughout the year. In contrast, the present study showed that *Lu. antunesi* exhibited a seasonal variation in abundance (F = 4.990; p < 0.001) that was determined by rainfall (ρ = -0.688; p < 0.001), with abundance increasing during the hottest months and in the absence of rain (maximum temperature 1 day before sampling) (ρ = 0.55; p < 0.001). These differing results demonstrate that a particular species can behave differently depending on the local environmental conditions, its niche adaptation, food sources, soil and other factors that dominate and thereby affect *Leishmania* transmission in the area.

The increased *Lutzomyia* abundance determined by temperature (maximum temperature 1 and 2 days before sampling) could be explained by increased physiological lifecycle activity (Killick-Kendrick 1978, Ready & Croset 1980), primarily consisting of nocturnal activity patterns and biting behaviour (Morrison et al. 1995, González et al. 1999, Souza et al. 2002). Conversely, reduced rainfall abundance could be related to the reduced physiological activity of fourth-instar larvae (Killick-Kendrick 1978) or mechanical effects on breeding sites (wash out).

Importantly, the opposite effect was observed during the months with the highest precipitation, specifically September and April. This result is expected for September based on the correlation model obtained, which demonstrates an inverse effect between rain and abundance (Table II). However, in April, the abundance of *Lutzomyia* species increased despite the high rainfall (Fig. 2), which can be explained by the transition from summer to winter and its effect on the soil conditions, low water table, low saturation and high temperatures. These factors benefit the development of the larval stage, as opposed to September when rains were continuous and the temperature was two degrees lower (Table I).

With regard to *Lu. gomezi*, the increased abundance observed during the dry months (December, January and February) agreed with previous studies conducted in Venezuela (Feliciangeli 1987). In contrast, the behaviour of *Lu. panamensis* was different from a previous study by González et al. (1999), reporting that *Lu. panamensis* abundance increased dramatically with rainfall.

In terms of natural infection, *Lu. flaviscutellata, Lu. panamensis* and *Lu. gomezi* were confirmed as potential cutaneous leishmaniasis vectors in the rural areas of Villavicencio due to their constant infection rates, mainly during dry months (November-February), despite their very low abundance, particularly in wooded (extradomiciliary) areas (Table I). Studies conducted in the wild ar-

TABLE III

Monthly natural infection rate (%) regarding the most abundant *Lutzomyia* species
in the municipality of Villavicencio, department of Meta, Colombia, Colombia

	Lutzomyia	I	P	E	*Lu. antunesi*	*Lu. flaviscutellata*	*Lu. panamensis*	*Lu. gomezi*
June	0	0	0	0	0	0	0	0
July	0	0	0	0	0	0	0	0
August	1.92	4.8	9.3	0.92	0.44	16	19	0
September	0	0	0	0	0	0	4	66
October	0	0	0	0	0	0	0	0
November	8.2	42	10.1	3.7	4.7	57	25	40
December	1.06	0	2.2	0.85	0.42	5.8	10.5	33
January	4.9	64	0.51	3.7	4.7	5.5	7.6	15
February	0.27	0	0.35	0.26	0.12	12.5	0	0
March	0	0	0	0	0	0	0	0
April	0.12	0	0.4	0	0.13	0	0	0
May	0	0	0	0	0	0	0	0
Total	1.25	11.6	0.6	0.8	1.02	7.53	3.32	10.3
	135/10,760	56/479	24/3,606	54/6,675	99/9,707	11/146	15/452	9/87

E: extradomiciliary; I: intradomiciliary; P: peridomiciliary.

eas of the department of Guaviare have shown that *Lu. flaviscutellata* can act as a vector due to its anthropophilic behaviour (Cabrera et al. 2009). This fly predominates in the woods and savannahs of Brazil, has been implicated in the transmission of *Leishmania (Leishmania) amazonensis* and identified as a possible vector of ACL in the state of Bolívar, in Venezuela (González & Devera 1999, Ribeiro et al. 2007, da Silva et al. 2010).

Lu. gomezi and *Lu. panamensis* have been proposed as vectors for *Leishmania (Viannia) braziliensis* and *Leishmania (Viannia) panamensis* in Venezuela and Panamá (Christensen et al. 1983, Rodríguez et al. 1999). These species are widely distributed throughout Colombia and are present in 18 of 32 departments (Bejarano 2006). Furthermore, it has been demonstrated that these species have a great ability to adapt to environmental changes in their habitat (Travi et al. 2002). *Lu. gomezi* and *Lu. panamensis* were incriminated as vectors in a suburban focus of visceral leishmaniasis in Chicamocha Canyon (Sandoval et al. 2006) and natural infection with *L. (V.) panamensis* has been recently found in areas of human activity in the department of Boyacá (0.5-0.6% natural infection rates) (Santamaría et al. 2006).

Conversely, *Lu. antunesi* exhibited infection rates of 0.1-4.7%, which agree with the rates described in the literature (Travi et al. 1988). *Lu. antunesi* stands out as the dominant species in the study area, as it is present in both anthropophilic (intradomiciliary and peridomiciliary) and extradomiciliary areas in great abundance (85%, 85% and 91% for each habitat). Even though this species has not been considered to be medically important in Colombia, it has been identified in regions with high ACL incidence and comprises equal or higher population percentages compared to species that have already been incriminated, indicating that it could be present for disease transmission (Sandoval et al. 2006). Ryan et al. (1984) first identified the natural infection of *Lu. antunesi* in Brazil with the presence of flagellate forms in a focus of visceral leishmaniasis. The first epidemiological nexus in Colombia was registered in the department of Amazonas where *Lu. antunesi* is considered an important species because it was present in high abundance in rural and periurban intradomiciliary and peridomiciliary areas of the municipality of Leticia during an active focus (Ferro et al. 1997). Natural infection with *Leishmania* spp was recently identified in an ACL focus in the rural area of Villavicencio (1.6% infection rate) (Vásquez-Trujillo et al. 2008).

Increased *Lu. antunesi* abundance in anthropophilic areas and gallery forest, mainly during dry months, coupled with evidence of natural infection with *Leishmania* spp highlights the epidemiological importance of this endemic species in Colombia's Orinoquia region and the possible role it plays in parasite transmission in ME.

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