Preliminary Biological Studies on Larvae and Adult *Anopheles* Mosquitoes (Diptera: Culicidae) in Miraflores, a Malaria Endemic Locality in Guaviare Department, Amazonian Colombia

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ABSTRACT In the malaria endemic municipality of Miraflores in southeastern Amazonian Colombia, several aspects of the biology of local *Anopheles* species were investigated to supplement the limited entomological surveillance information available and to provide baseline data for malaria prevention and vector control. *Anopheles darlingi* Root, 1926 was the most abundant species (95.6%), followed by *Anopheles braziliensis* (Chagas) (3.6%) and *Anopheles oswaldoi* s.l. (Peryassu) (0.7%). During the dry season, exophagic activity was prevalent only between 1800–2100 hours; after this (2100–0600 hours) only endophagy was encountered. In contrast, during the rainy season, both endophagy and exophagy occurred throughout the collection period. The human biting rate for *An. darlingi* was 8.6. This species was positive for *Plasmodium vivax* VK210 with a sporozoite rate = 0.13 (1/788). Breeding sites corresponded to stream ($n = 7$), flooded excavations ($n = 4$), flooded forest ($n = 1$), wetlands ($n = 2$), and an abandoned water reservoir ($n = 1$). *An. darlingi* predominated in these sites in both seasons. Based on these data, *An. darlingi* is the main local malaria vector, and we recommend that local prevention and control efforts focus on strengthening entomological surveillance to determine potential changes of species biting behavior and time to reduce human–vector interactions.

KEY WORDS *Anopheles darlingi*, malaria, Amazonia, Colombia

Colombia consistently reports the second highest annual malaria incidence in Latin America after Brazil (World Malaria Report 2011). Overall, in Colombia, *Plasmodium vivax* (Grassi and Feletti) is responsible for $\approx 74\%$ of malaria incidence, and *Plasmodium falciparum* (Welch) for the remaining 26%. More than 85% of Colombia's land area has the potential for endemic malaria transmission (Mendoza et al. 2000). The highest risk zones have been identified as the Pacific Coast, the Cauca River Valley, and the Urabá, Orinoquía, and Amazonia regions (Padilla and Peña 2002). The major malaria vectors in Colombia, all in subgenus *Nyssorhynchus*, are *Anopheles darlingi* Root, 1926, *Anopheles albimanus* Wiedemann, 1920, and *Anopheles nuneztovari* Gabaldón, 1940 (Montoya-Lerma et al. 2011).

The Department of Guaviare is situated in the Amazonia region of eastern Colombia and has an area of 42,327 km². It extends from 0° 32' to 3° 09' N, and from 69° 47' to 73° 47' W (Fig. 1) and includes several municipalities, e.g., San José del Guaviare, El Retorno,

Calamar and Miraflores (Instituto Amazónico de Investigaciones Científicas [SINCHI] 2000). According to the World Malaria Report (2011), this is a region of high transmission, with an API (annual parasite index; number of malaria cases per 1,000 inhabitants) of 10–50. Malaria is endemic in Guaviare, and during 2010 it had the highest API in Colombia (58.8; Instituto Nacional de Salud [INS] 2010). In 2010, the municipality of Miraflores had the highest API (56.4) in Guaviare, reporting 66.1% *P. vivax*, 19.5% *P. falciparum*, and 4.27% mixed *P. falciparum* and *P. vivax* cases (INS 2010). This level of malaria contributes to unstable endemic transmission throughout Colombia (Padilla et al. 2011). Furthermore, for the year 2010, the number of reported cases by state suggested a situation of epidemic malaria (Chaparro et al. 2013). The current study was performed in periurban Miraflores, and is the first entomological report for this municipality.

Materials and Methods

Study Site. Miraflores is situated in southeastern Amazonian Colombia (Fig. 1) at 213 m, and has an average annual temperature of 27°C, average relative humidity of 86%, and average annual rainfall of 2692.3 mm/yr. Tropical wet rainforest is the dominant local Holdridge life zone of Amazonian Colombia (SINCHI

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Fig. 1. Map of Colombia depicting location of four municipalities, including Miraflores, Guaviare Department where anopheline study was carried out in 2010.

Fig. 2. A detailed map of periurban Miraflores, Guaviare, Colombia, 2010. Five immature anopheline breeding site types and the wild adult female collection locality are depicted. Carrera 7 is the main road through the town of Miraflores. Nearby Vaupés River and two tributary streams, Picho and Umari, are labeled.

2000). Miraflores has an area of 12,756 km² and is designated as an Amazonian forest reserve within the National Wildlife Reserve Nukak (Ministerio del Medio Ambiente 1989). It is ≈150 km from San José del Guaviare, the capital of Guaviare, and is accessible only by river (≈10 d along the Vaupés River from Calamar municipality) or by air (1.5 h from San José del Guaviare). The economy is based on agriculture and logging. Miraflores is located on the northeastern margin of the Vaupés River. The rural area is occupied by more than 10 ethnic groups such as *Nukak Makú*, *Tucanos*, and *Carijonas*. At least five of these groups are also found in the periurban area, as a result of displacement from armed conflict (Miraflores, Guaviare, 2013).

The urban area is occupied by settlers who have arrived as migrants attracted by economic booms including the exotic skin trade and coca cultivation. It is the major regional commercial center for food, clothing, and medicine. Local institutional capacity is weak, influenced by groups involved in armed conflict. In general the population self-medicates and has poor nutritional status. The risk factors inherent in this situation favor malaria endemicity (CORPES 2004).

Mosquito Collections. In March (dry season) and September (rainy season) 2010, mosquito collections

were conducted during three consecutive nights in the periurban neighborhood of La Paz (01° 19' 0.01" N, 71° 57.2' 39" W; Fig. 2), where malaria cases were previously reported. The house where mosquitoes were collected has wooden walls and few bricks with only one entrance for both entry and exit. The roof is made of wood, with a dirt floor, and the only bedroom is completely made of wood without windows. The kitchen is open without walls or windows; it was connecting directly to the courtyard where vegetation is found. Sampling was carried out using human landing catch (HLC) indoors and outdoors from 1800 to 0600 hours, by a team of four collectors. Collectors worked in pairs, one collector indoors and one outdoors, and rotated every two hours to avoid collector bias (World Health Organization [WHO] 1975). Live females were placed in plastic bottles labeled with collection information and provided with a 10% sugar solution. They were transported to the Laboratory of Entomology, Faculty of Agronomy, Universidad Nacional de Colombia (UNC)—Bogotá, blood fed on *Mus musculus* L. (protocol identification below) and identified using the keys of Faran and Linthicum (1981), González and Carrejo (2007), and Rubio-Palis (2000). After the wild-caught females oviposited (or died), they were maintained over desiccant

for subsequent identification of *Plasmodium* species circumsporozoite (CS) protein using enzyme-linked immunosorbent assay (ELISA). Isofamilies were obtained by forced mating (one leg and wing removed) after which each female was lightly anesthetized with ethyl acetate and placed in water for oviposition (Estrada et al. 2003). Wild females and their isofamilies were used for taxonomic identification, and in both cases one leg and wing of each was mounted on a slide with Canada balsam and retained as a voucher. The number of mosquitoes caught per day per person (human-biting rate—HBR; WHO 1975) was estimated for the most abundant species, *An. darlingi*.

Plasmodium Detection. Only heads and thoraces were tested for the CS protein of *P. falciparum* and *P. vivax* 210 and 247 by ELISA, following standard protocols (Wirtz et al. 1987a,b, 1991, 1992). Mosquitoes were tested in pools of five specimens by collection method and date, to ensure that the probability of detecting more than one infected mosquito per pool is <1% (Rubio-Palis et al. 1992, Galardo et al. 2007, Magris et al. 2007). Negative controls were from the *An. albimanus* colony, Buenaventura strain, maintained at the Laboratory of Entomology, Faculty of Agronomy, UNC. The number of CS-positive *Plasmodium* pools was equivalent to the number of CS-positive mosquitoes; therefore, the sporozoite rate was estimated as the percentage of CS-positive mosquitoes divided by the total number of mosquitoes assayed.

Anopheline Larval Collections. Breeding sites in periurban Miraflores were surveyed to detect and collect *Anopheles* larvae, using 500-ml ladles every 5 m, for a total of 50 dips per breeding site. The number of individual larvae collected per dip was recorded. Each collection was georeferenced, and hydrological parameters (temperature, depth, pH, apparent color, current, shade) and associated vegetation cover and type were recorded for subsequent analysis (not included in this manuscript). At least 10 breeding sites could not be sampled due to the probable presence of landmines.

Ethics. HLCs were conducted using an informed consent agreement under a protocol and collection procedures that were reviewed and approved by the Ethics Committee of the Faculty of Medicine of UNC and by the Institutional Review Board of the Wadsworth Center, NY State Department of Health (NYSDOH), Albany, NY protocol no. 02-028. Blood feeding of the mosquito females followed the vertebrate management protocols of the Faculty of Veterinary Medicine of UNC, the Ethics Committee on Animal Care of the National Institutes of Health—Office of Laboratory Animal Welfare (assurance no. A5791-01), and Institutional Animal Care and Use Committee (IACUC) committee protocol no. 11-421, NYSDOH.

Results

In total, 828 female anopheline mosquitoes representing four species were collected using HLC in 36 h

indoors and outdoors, during March and September. The most abundant species was *An. darlingi* ($n = 789$), followed by *Anopheles braziliensis* (Chagas) ($n = 28$), and *Anopheles oswaldoi* s.l. (Peryassu) ($n = 6$). The mean biting rate for *An. darlingi* was 8.6, 1.3 for *An. braziliensis*, and 0.7 for *An. oswaldoi* s.l. The biting activity of *An. darlingi* was analyzed to consider exposure during three periods determined by habits of the human population (Fig. 3). The only time Miraflores has electricity is during the first period (P1), 1800–2200 hours; during the second period (P2), 2200–0300 hours, people are in their homes; and during the last period (P3), 0300–0600 hours, people begin daily activities (Fig. 3). During the dry season, exophagic activity was confined to P1; endophagy was the only biting behavior during P2–P3. In contrast, during the rainy season, both feeding behaviors occurred throughout the night (P1–P3). All 828 mosquitoes, representing 166 pools, were assayed by ELISA. Only *An. darlingi* was positive for *P. vivax* 210, with a sporozoite rate of 0.13% (1 of 788). The infected mosquito was captured in March between 2400–0100 hours indoors.

The main breeding sites in Miraflores were grouped into stream ($n = 7$), flooded excavations ($n = 4$), flooded forest ($n = 1$), wetlands ($n = 2$), and an abandoned water reservoir ($n = 1$; Fig. 2). During the rainy season, water levels of the Vaupés River rise and flood part of the municipality, forming two major anopheline breeding sites (flooded forest and flooded excavation; Table 1). Three species were collected in these breeding sites: *An. darlingi* (64.5%) was the most abundant, followed by *An. oswaldoi* s.l. (25.8%). *An. braziliensis* was present, but uncommon. A significant number of larvae ($n = 38$) could not be identified owing to high mortality of early instars during transportation to Bogotá.

Discussion

An. darlingi has long been associated with a rainforest environment (Faran and Linthicum 1981, Consoli and Lourenço-de-Oliveira 1994), and it is considered the main Amazonian malaria vector (Zimmerman 1992, Lounibos and Conn 2000, Rubio-Palis 2000, Montoya-Lerma et al. 2011). In Miraflores, *An. darlingi* was caught throughout the night showing mainly exophagy (Fig. 3). Exophagic behavior is common in *An. darlingi*, but it also feeds endophagically, depending on environment and host availability (Hiwat and Bretas 2011, Moutinho et al. 2011). Low densities were due to limited sampling effort; therefore, we could not obtain adequate data to define biting patterns for this species in the study area.

An. darlingi was found naturally infected with *P. vivax*, and the peak seasonal, indoor–outdoor biting times (Table 2) were related to human activities (Fig. 3). However, because of the small sample size ($n = 1$ infected specimen), *An. darlingi* is a presumed vector in this locality, but additional studies should be undertaken for confirmation. The behavior of the vector

Fig. 3. Percentage of adult female anopheline mosquitoes (y-axis) collected per hour, between 1800 and 0600 hours (x-axis) by collection location (indoor, black vertical bar; outdoor, white vertical bar) and season in Miraflores, Colombia, in 2010. Upper graph shows results from March—dry season; lower graph shows results from September—rainy season.

species influences the local epidemiological pattern, and the same populations of *An. darlingi* may show behavioral variation in response to environmental change in addition to seasonal fluctuation, thus increasing the complexity of malaria transmission dynamics (Charlwood 1996, Voorham 2002, Santos et al. 2009). Even with low *An. darlingi* capture rates, transmission by this species has been recorded in the Amazon region (Lanelli et al. 1998, Tadei et al. 1998, Conn et al. 2002).

The Miraflores municipality may be in a permanent state of malaria outbreak. Possible reasons for this

include migration of members of indigenous communities from high malaria transmission areas, uncontrolled movement of gold miners, the presence of a local army force where members also rotate among high and low transmission areas, and movement of regional traders. Furthermore, drastic changes in the ecosystem, such as the cut and burn method of the forest clearing for crops and livestock, have likely contributed to risk factors for local *Plasmodium* transmission.

Knowledge of fauna composition and vector behavior in wild areas is essential for the adoption of po-

Table 1. *Anopheles* immature forms collected from each breeding site type in Miraflores, Guaviare, Colombia, March and September, 2010

Month	Species	Breeding site types				
		Stream (n = 7)	Flooded excavation (n = 4)	Flooded forest (n = 1)	Wetlands (n = 2)	Abandoned water reservoir (n = 1)
Mar.	<i>An. darlingi</i>	5	2	1	1	0
	<i>An. oswaldoi</i> s.l.	1	3	1	1	0
	<i>An. braziliensis</i>	0	1	0	0	1
Sept.	<i>An. darlingi</i>	5	1	3	1	1
	<i>An. oswaldoi</i> s.l.	1	0	1	0	0
	<i>An. braziliensis</i>	0	0	0	0	1

Table 2. HBR and numbers of adult female *An. darlingi* collected in Miraflores, Guaviare, Colombia, 2010, during three periods of nightly activity in both dry (March) and rainy (September) seasons

Period of night (P) and hour	HBR							
	Indoor				Outdoor			
	Mar.	Total	Sept.	Total	Mar.	Total	Sept.	Total
P1								
18–19	0.3	2	8.3	50	0.1	1	2.3	14
19–20	0.5	3	7.1	43	3	20	2.8	17
20–21	4.1	25	7.1	43	5.1	31	3.3	20
21–22	2.1	13	3.3	20	0	0	8.6	52
P2								
22–23	1.6	10	5	30	0	0	6.5	39
23–24	2.8	17	0.3	2	0	0	1.5	9
0–1	2.8	17	2.1	13	0	0	5.16	31
1–2	2.5	15	6.5	39	0	0	3.16	19
2–3	3.8	23	4.1	25	0	0	3.3	20
P3								
3–4	2.6	16	0.3	2	0	0	0.5	3
4–5	3.1	19	1.6	10	0	0	4.8	29

tential alternative vector control strategies aimed at preventing transmission. During the rainy season *An. darlingi* exhibited both exo- and endophagic biting behaviors, with exophagy more pronounced during P2–P3 compared with dry season behavior, as was previously observed in Roraima state, Amazonian Brazil (Barros et al. 2007), and in other parts of the Amazon forest (Lanelli et al. 1998, Sá et al. 2005, Hiwat and Bretas 2011).

The main breeding sites were natural water collections as expected for the ecological conditions of Miraflores, where people live on the fringes of rainforest, only occupying a small area associated with the aircraft landing strip, but are continually expanding into the forest. Breeding was associated with human-made excavations because people use gravel for multiple purposes such as mining or house construction. All of the breeding sites are well defined in the dry season, but during the rainy season, the Vaupés River floods the periurban area, forming two huge breeding sites. *An. darlingi* was found in all types of breeding sites, so in theory it can emerge from a breeding site, obtain a bloodmeal and then rest in vegetation, likely in the forest, where vector control is impractical. In this way, populations of *An. darlingi* can be maintained all year, contributing to both abundance and transmission.

Vector control is an important component of malaria control programs because it is one of the most efficient strategies to prevent transmission (Brochero and Quiñones 2008); however, forest malaria may be more difficult to control than nonforest malaria (Tadei and Dutary Thatcher 2000, Galarido et al. 2009, Santos et al. 2009). Forest vectors are often partially or wholly exophilic and exophagic and do not normally enter houses protected by indoor residual spraying (Enayati and Hemingway 2010). In Miraflores, it may be impractical to try to control *An. darlingi* immatures in breeding sites because this species appears to be locally ubiquitous. Furthermore, such a strategy in periurban and urban areas of Miraflores is impossible due to the presence of landmines and armed conflict. Health Authorities could try to implement long-lasting

insecticidal nets in all houses after considering local community attitudes, beliefs, and practices, and use the results to evaluate changes in abundance or behavior of *An. darlingi* populations.

Furthermore, biological characteristics of wild female *An. darlingi*, which are behaviorally plastic (Voorham 2002), often biting outdoors, combined with their nocturnal activity, suggest that multiple control strategies might be more successful. Prevention and control strategies could focus on improved entomological surveillance to detect potential changes in behavioral patterns and relative species incrimination in local transmission. The reduced diversity of *Anopheles* species detected in this preliminary study in Miraflores may be the result of the limited number and frequency of collections, suggesting that further studies should be undertaken for a more comprehensive understanding of local malaria transmission patterns.

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