MALARIA VECTOR INCORPORATION IN THREE RURAL RIVERINE VILLAGES IN THE BRAZILIAN AMAZON

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Abstract. Vector incorporation studies were conducted from April 2003 to February 2005 at three riverine villages 1.5 km to 7.0 km apart, along the Matapi River, Amapá State, Brazil. A total of 113,117 mosquitoes were collected and placed in pools of ≤7 mosquitoes (19,883 pools) and tested for species-specific circumsporozoite protein (CSP) of P. falciparum, P. vivax VK210, and P. vivax VK247 using the enzyme-linked immunosorbent assay (ELISA). A subset of 63,330 mosquitoes (12,191 pools) was tested for P. malariae. Anopheles darlingi and An. marajoara had the highest proportion of circumsporozoite protein positives for human malaria parasites compared with An. nuneztovari, An. triannulatus, and An. intermedius. Anopheles darlingi and An. marajoara had the highest entomological inoculation rates (EIR) and were considered to be the most important malaria vectors in the study. Anopheles nuneztovari was also an important vector. Differences in entomological inoculation rates were more dependent on mosquito abundance than on sporozoite rates.

INTRODUCTION

Approximately 99% of all malaria cases in Brazil are reported from the Amazon region. The vastness and inaccessibility of the region, diverse ecosystems, anthropogenic change, human migration, and vector heterogeneity hamper control efforts. Vector heterogeneity is extensive in the Amazon Basin, where more than 15 anopheline species are implicated as potential vectors of malaria. Intra- and inter-specific variability in anthropophilism, biting behavior (endophagic versus exophagic), biting activity (crepuscular versus nocturnal), larval habitat selection, and vector competency vary and must be considered when examining the role of anopheline species as malaria vectors. Multiple vector species may be involved in transmission in many regions. However, we know very little about the contribution of individual species to malaria prevalence in heterogeneous environments. The situation is more complex by the presence of three different species of human malaria in the Amazon region (Plasmodium falciparum, P. vivax, and P. malariae). Besides the classic P. vivax VK210, two variant epitopes of the circumsporozoite protein of P. vivax VK247 and P. vivax-like are present. Plasmodium vivax is the most common human malaria parasite in the region, followed by P. falciparum. Plasmodium malariae is important locally and is significantly underreported. The complex nature of vector-parasite interactions and interspecific variability in life histories create a challenge for malaria researchers and control programs.

Vector incorporation is a prerequisite for understanding the role of anophelines in malaria transmission and has been used to determine which species are the most important vectors. The entomological inoculation rate (EIR—average number of infective bites per person per unit time), is used as an index of malaria intensity and endemicity. It also is used to compare the contribution of individual species to overall malaria transmission. However, in the Amazon Basin the EIR is seldom used as an indicator of malaria intensity or to determine the contribution of individual vector species to malaria transmission.

In this study we present the results of parasite identifications from 22 consecutive months of collections of potential vectors and subsequent EIR estimates in a malaria endemic region of the Brazilian Amazon where multiple vectors (Anopheles darlingi, An. marajoara, An. nuneztovari, An. triannulatus, and An. intermedius) and multiple malaria parasites (P. falciparum, P. vivax VK210, P. vivax VK247, and P. malariae) occur. This article is one of a series that analyzes the contribution of different vectors to malaria transmission in heterogeneous environments.

MATERIALS AND METHODS

Study sites. Three communities separated by 1.5 km to 7.0 km, along the Matapi River, Amapá State, Brazil, were selected for study. São Raimundo (00°00' N; 051°15' W), São João (00°02’ N; 051°14’W), and Santo Antônio (00°05’N; 051°12’W) are rural riverine villages where the primary economic activities are fishing, small-scale agriculture, extraction of forest-products, and day labor at neighboring water buffalo and cattle farms. A census conducted in July 2003 indicated 62, 87, and 34 residents in the villages, respectively. The houses were built on stilts (1–1.5 m high) and made of wood with occasional holes between the wood slats. Houses have several unscreened windows with or without shutters. The climate is hot and humid (mean relative humidity 85%) with temperatures ranging from 22–32°C. The rainy season extended from January to July (mean rainfall 2100 mm), and the dry season from August to December (mean rainfall 178 mm).

The ecosystem is a mixed flooded forest (várzea)-marsh habitat with many small streams (igapós) draining into the Pirativa River and the Matapi River systems. The freshwater

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level rises and falls with the tides, floods the forest floor during high tide, particularly during the rainy season. Malaria is endemic in the area and *P. falciparum, P. vivax VK210, P. vivax VK247, and P. malariae* were observed in mosquitoes by ELISA and serology of human blood in all three communities (Arruda, unpublished data). The predominant malaria parasite recorded at the local health post was *P. vivax.*

**Mosquito collection.** All night landing catches of mosquitoes were made monthly in the peridomestic environment within 5–10 m of 4–5 houses for nine consecutive days (three successive days in each village) from April 2003 to January 2005. Moon phases have been shown to affect the adult behavior of anophelines so collections were centered around the new moon, when moonlight influence on catch was expected to be minimal. There were two 6.5-hour collection periods from 5:30 PM–12:30 AM and 12:30 AM–6:30 AM. Different collectors were used for each collection period and collectors changed positions each night to prevent any bias. Mosquitoes were aspirated as they landed on one exposed leg of the collector and placed in unwaxed screened 0.5L ice cream cartons modified as cages. Mosquitoes were placed in a Styrofoam® container with wetted paper towels and transported to the field laboratory every hour until 10:30 PM and thereafter in the morning, killed with ethyl acetate, and identified to species using the key of Consoli and Lourenço de Oliveira.

Landing catch comparisons were made between inside and outside collections at one house in each village in June 2005 and at the same house in São João in August 2005. We followed the all night landing catch procedures as indicated earlier.

Use of human subjects was approved by the University of Florida Institutional Review Board (437-2002) and the Brazilian National Ethics Commission for Research (CONEP-1280/2001).

**Sporozoite rate.** Based on the known anopheline sporozoite rates (SPR) from preliminary studies carried out at this site (expected SPR of < 2.0%, CI of 1.0%), a maximum of 784 females per species collected monthly from each site was tested for species-specific CSP using standard protocols for the enzyme-linked immunoabsorbent assay (ELISA). The head and the thorax of individual mosquitoes were separated from the body using a scalpel (cleaned with alcohol after each dissection) and put in small plastic film vials with silica gel and stored in a freezer at −4°C. This procedure reduced the probability of detection of CS antigen from other parts of the body. Preliminary SPR results demonstrated that low infection rates were common in the study area, so we tested pools of ≤ 7 mosquitoes to insure a < 1.0% probability of > 1 infected mosquito per pool (expected SPR of < 2.0%). The CSP rate was used as an approximation of SPR calculated from ELISA results using the percentage of CSP positive mosquito pools.

**Entomological inoculation rate.** The EIR was calculated by multiplying the human biting rate (HBR)(number of anophelines per day/person) × the proportion of infected anophelines). Annual EIRs were calculated by multiplying by 365. Seasonal differences in EIRs were not analyzed in this study and will be compared in a subsequent article using a larger data set (32 months).

**Malaria cases.** Passive case detection was routinely carried out at the local health post in São Raimundo using thick blood smear stained with Giemsa. No distinction between *P. vivax VK210* and *P. vivax VK247* was possible by this method. We collected the data for this study for the three villages from January 2003 to December 2005. Using population census data for each village from July 2003, annual malaria incidence was calculated as the number of reported malaria cases per population at risk. The village populations remained stable during the study.

**Statistical analysis.** The proportion of mosquitoes infected (SPR) was first analyzed for significant effects of village (*N = 3*), and vector species (*N = 5*) and for each malaria parasite species and variant (*N = 4*) by maximum likelihood categorical analyses of contingency tables using the SAS CATMOD procedure. If no significant variation was detected among villages, data from the three villages were summed for each vector species by malaria parasite/variant and tested for significant differences among vector and *Plasmodium* spp./variant. Vector X parasite interactions were also tested to determine if the pattern of dependence on species was in turn dependent on which *Plasmodium* was being considered. If the maximum likelihood (ML) ANOVAs detected significant variations among villages or mosquito species, pairwise comparisons of infection rates were performed with ML contrasts to identify the sources of significant effects, applying Bonferroni corrections at the experimental-wise error rate of 5% because of non-orthogonality (more tests than degrees of freedom).

**RESULTS**

**Mosquito infection rates.** A total of 113,117 mosquitoes were captured from April 2003 to January 2005, placed in 19,883 pools and analyzed for *P. falciparum, P. vivax VK210,* and *P. vivax VK247* CSP. A subset of 63,330 mosquitoes (12,191 pools) was tested for *P. malariae* from April 2003 to March 2004 because no monoclonal antibodies were available for ELISA testing of this malaria parasite species after March (Wirtz, personal communication). Five vector species tested positive for CSP (Table 1). Four other species, *An. braziliensis, An. oswaldoi, An. matugrossensis, and An. peryassui* were collected in low numbers (1606, 21, 9, and 7, respectively) and all pools of these species (*N = 325*) were negative for CSP. *Anopheles darlingi* and *An. marajoara* were recovered infected one or more times with all malaria parasite species in all villages and the other three potential vector species were less frequently infected (Table 1). Monthly sporozoite rates varied throughout the study and ranged from 0.0%–4.35%.

*Anopheles darlingi* and *An. marajoara* had the highest percent of CSP positive mosquitoes for each of the malaria parasites (Figure 1). The ML ANOVA showed no significant village effects for each of the vectors and *Plasmodium* species (Table 2). Therefore, each vector species was pooled across villages and tested for differences among vector and *Plasmodium* species (Table 1). *Plasmodium malariae* was analyzed separately because mosquitoes were tested only during the first 12 months of the study. The ML ANOVA for proportion infected by *P. falciparum, P. vivax 210,* and *P. vivax 247* showed significant differences among vector species (*P < 0.001*) and a significant vector species x parasite interaction (*P < 0.0111*), but no significant differences among parasite species (*P = 0.1197*). The contrast ML analysis showed no dif-
ferences in infection rates between An. darlingi and An. marajoara (Table 3). Anopheles nuneztovari did not differ significantly from An. triannulatus in infection rates for all Plasmodium spp and An. nuneztovari was infected significantly less than all other species. The proportion of An. marajoara and An. darlingi infected was also higher than An. nuneztovari at the 5% experiment-wise error rate following Bonferroni corrections.

Plasmodium vivax VK210 was significantly different from P. vivax VK247 (P = 0.0479), but the infection rates of these two variants were not different from that of P. falciparum (P = 0.1569 and P = 0.5008, respectively). This result was attributed to the differential infection of An. nuneztovari with P. vivax VK210 (P = 0.0007).

The ML ANOVA for P. malariae demonstrated a significant difference in infection rates among vector species (P = 0.0054). The ML contrasts showed that An. darlingi, An. marajoara, and An. nuneztovari did not differ in infection rates with this parasite (Table 3). They were found infected with P. malariae significantly more than An. intermedius and An. triannulatus, but only An. darlingi had a significantly higher infection rate than An. triannulatus at the 5% experiment-wise error rate following Bonferroni corrections.

Bonferroni corrections for multiple comparisons at the 5% experiment-wise error rate showed fewer significant differences between species in the proportions infected with P. malariae compared with the other human malaria parasites (Table 3). This was most likely attributable to the low P. malariae infection rates in this study (Table 1).

Entomological inoculation rates. The inoculation rates for the five major species collected varied among species and village. The HBR influenced the EIR more than the SPR in most cases (Table 4). Anopheles darlingi had the highest inoculation rates for all parasites and in all villages, except for P. falciparum and P. vivax VK247 in São Raimundo, where An. marajoara had the highest inoculation rates. Anopheles marajoara had a lower HBR, but higher SPRs for P. falciparum and P. vivax VK247 than did An. darlingi. The inoculation rates for An. nuneztovari were higher than An. marajoara in São João and were attributed to the higher mean densities of this species compared with An. marajoara. The cumulative EIRs for the four parasites in the three villages indicated that the malaria intensity was highest in Santo Antônio followed by São João and São Raimundo. Annual EIRs ranged from 45.26 for P. falciparum in São Raimundo to 790.59 for P. vivax VK210 in Santo Antônio.

Peridomestic versus inside collections. In June 2005, 36.8% of the total mosquitoes collected (N = 2752) were from inside houses (41% in São Raimundo, 42.4% in São João, and 35.6% in Santo Antônio). A total of 2024 mosquitoes were collected in the August comparison in São João with only 20 mosquitoes (1.0%) collected inside the house sampled.

Malaria incidence. Seventy-four P. vivax cases and 57 P. falciparum cases were reported by passive case detection at the health post in São Raimundo for the three communities studied. No P. malariae cases were reported at the health post. The majority of the P. vivax cases occurred in the dry season (August through December) in both years (N = 64).
There were two peaks of *P. falciparum*; one in Santo Antônio in April 2003 (*N* = 19) and in São Raimundo in June 2003 (*N* = 13) (Figure 2B). April and June 2003 accounted for 68% of the reported *P. falciparum* cases from the three villages and only three *P. falciparum* cases were registered after December 2004. The number of reported malaria cases (*P. vivax* + *P. falciparum*) was greater in Santo Antônio (*N* = 52) than in São Raimundo (*N* = 41) and São João (*N* = 38) and the annual malaria incidences were highest in Santo Antônio (Figure 3).

**DISCUSSION**

The SPR and EIR results of this study demonstrated that *An. darlingi* and *An. marajoara* were the two most important vectors. Results from a parallel study on host blood meals supports this conclusion. It was shown that *An. darlingi* and *An. marajoara* were much more anthropophilic than *An. nuneztovari, An. triannulatus,* and *An. intermedius,* indicating that human-vector contact would be higher for the first two compared with the latter species. *Anopheles darlingi* had similar SPR as reported in previously studies and is considered to be a more important vector than *An. marajoara* because of its higher EIR. *Anopheles marajoara* was recently reported as the most important vector of malaria (more important than *An. darlingi*) from research conducted approximately 50 km from our study area. The difference between the two studies may be due to the fact that we were working in stable rural villages, whereas Conn and others examined unstable peri-urban areas more affected by deforestation. The deforestation for agriculture and house construction may have favored *An. marajoara* through the destruction of shaded forest larval habitats of *An. darlingi,* while increasing open sunlit habitats preferred by *An. marajoara.* Also, we collected mosquitoes from 5:30 PM–6:30 AM because *An. darlingi* was active all night in our study area (unpublished data). Conn and others collected mosquitoes only from 7:00 PM–9:00 PM because their preliminary data indicated that *An. darlingi* was most active in the early evening, similar to *An. marajoara.* Biting periodicity differs geographically, and collection periods may impact the results of vector incrimination.

*Anopheles nuneztovari* is an important vector of *vivax* malaria in other parts of South America and was incriminated as a vector of *P. vivax* VK210 in the Brazilian Amazon and of *P. vivax* VK210 and VK247 and *P. malariae* in the State of Amapá. In our study it is considered an important vector of *P. vivax* VK210, *P. vivax* VK247, and *P. malariae* when its densities are high (e.g., São João; Table 4). Its role as a vector of *falciparum* malaria is little known. It was reported infected with *P. falciparum* in the States of Roraima, Amapá, and Pará and in the central Amazon state of Amazonas, Brazil. In our study area it had lower infection rates than *An. darlingi* and *An. marajoara,* but may be a potential vector of *falciparum* malaria when its densities are high. The reason for the significantly higher number of *An. nuneztovari* infected with *P. vivax* VK210 (*N* = 38) compared with *P.
vk247, and were very low and it is not much less susceptible to salivary invasion, and the authors considered it an occasional vector at high densities. Mosquito susceptibility to infection is an important component of the vector contribution to malaria transmission, and a comparison among the anophelines collected in this study is warranted.

The four other species collected in low numbers in this study, *An. brasiliensis*, *An. oswaldoi*, *An. matogrossensis*, and *An. nuneztovari*, were considered not important vectors in our study area. The vectorial capacity of *An. nuneztovari* was especially low. In contrast, *An. marajoara*, *An. intermedius*, and *An. triannulatus* are considered an important and a very important vector in the western Brazilian Amazonian State of Rondônia. *Anopheles triannulatus* was much less susceptible to salivary gland invasion, and the authors considered it an occasional vector at high densities. Mosquito susceptibility to infection is an important component of the vector contribution to malaria transmission, and a comparison among the anophelines collected in this study is warranted.
An. peryassui were found infected with malaria parasites in other studies.\textsuperscript{9,11,17,18} Of these, only An. oswaldoi is considered a primary vector of malaria in some sites,\textsuperscript{7} but not others.\textsuperscript{10} The EIRs for the species collected in this study are within the range of those reported for other anophelines\textsuperscript{18,32,34,45} but were much higher than those reported in Venezuela (combined annual EIRs for An. nuneztovari, An. albisetis [= An. marajoara], and An. oswaldoi for three villages of 7.1, 8.6, and 15.8, respectively)\textsuperscript{32} and in Roraima State, Brazil, where the monthly EIRs for An. darlingi ranged from 0.0–0.42; overall malaria infection rate of 0.085 and HBRs from 0.0–6.2.\textsuperscript{18}

The number of reported malaria cases (Figure 2) and the malaria incidence was highest in Santo Antônio (Figure 3) and may be due to high EIRs in this village compared with the other two villages (Table 4). However, the large number of potential infected bites would suggest a greater risk of transmission than is shown by the case incidence data. Low numbers of reported cases are common in the Amazon region and may be due to a high proportion of asymptomatic cases\textsuperscript{46,47} and inadequate diagnosis through microscopy, especially for P. malariae.\textsuperscript{47} The failure to report P. malariae in endemic areas is not surprising because the only method for malaria diagnosis in Brazil is the microscopic examination of thick blood smear stained with Giemsa. In this procedure it is not possible to assess the morphology of infected red blood cells and parasite-altered shape could lead to a mistaken identification of P. malariae as P. vivax. Recently, a significant number of P. malariae cases were encountered in the Amazon region when blood samples were also evaluated by polymerase chain reaction (PCR).\textsuperscript{22,48} These difficulties could have contributed to the low frequency of reported cases and an underestimation of malaria incidence in our study. There is also the possibility that the simian malaria parasite, P. brasilianum occurs in the region because it is found in the neighboring state of Para\textsuperscript{49} and in French Guiana.\textsuperscript{50} The monoclonal antibodies directed against a species-specific surface protein (CS protein) of P. malariae (6B10) used in immunoassays

\textbf{FIGURE 2.} Numbers of reported (A) P. vivax and (B) P. falciparum malaria cases from April 2003 to January 2005 in the three study villages.
have a common epitope with *P. brasiliensis* sporozoites. Monkeys are implicated as reservoir hosts of *P. malariae* and *P. brasiliensis* in Brazil. Both parasites exhibit low parasitemia and are not detected by malaria microscopists, complicating diagnosis. Further, *P. malariae* and *P. brasiliensis* may be the same species. Monkeys were not found in the vicinity of the villages but were heard at times in outlying forests. Further research is warranted to clarify the possibility that a zoonotic cycle of *P. brasiliensis* occurs along the Matapi River and impacts the pattern of human malaria.

In Brazil, anophelines are normally collected concurrently inside and outside houses. However, the biting behavior of anophelines in the Amazon Basin of Brazil appears to have shifted from primarily inside to outside. Preliminary data on endophagy in our study area indicated that the vast majority of anophelines bite outside rather than inside houses. This was also the case in a recent study conducted within 50 km of our study site. However, we noticed that blood-fed mosquitoes were resting inside a food store in São João in early 2005. The two one-night comparisons demonstrated that there may sometimes be more mosquitoes biting inside houses than presumed. The EIRs from the peri-domestic area may have overestimated the true EIRs. The EIRs would be impacted by site of collection if the June 2005 results were representative or there existed seasonal differences in exophagic and endophagic behavior of anophelines. Most villagers were inside after 10:00 PM (Galardo, personal observation) and therefore a more realistic EIR may be one that is calculated from biting collections in areas where human activity is taking place. Other factors such as house construction and use of mosquito nets also would impact the EIR. Well-constructed houses tend to have fewer mosquitoes, and mosquito nets may reduce vector-host contact, perhaps increasing the risk of transmission outside houses.

In conclusion, all four human malaria species and variants known from the Americas were circulating in the mosquitoes in the study area. There were only slight intra-population (village) differences in infection rates. The EIRs were dependent more on human biting rate than the SPR and influenced the ranking of importance of the vectors. *Anopheles darlingi* and *An. marajoara* were the most important malaria vectors in this study. These data reconfirm *An. darlingi* as the principal malaria vector in the Amazon region of Brazil and support the conclusion of Conn and others that *An. marajoara* is an important vector in this region of Brazil. We regard *An. nuneztovari* as a vector of malaria transmission of at least *P. vivax* 210, *P. vivax* 247, and *P. malariae* based on the results of the EIR and its history as a vector in other regions of Brazil and South America. The low EIRs and zoophilic feeding habits of *An. triannulatus* and *An. intermedius* suggest that they are not important vectors at this study site. An analysis of the contribution of individual vectors to the EIR over a longer interval and vector susceptibility and vector longevity studies would further clarify the role of individual vectors in this heterogeneous environment.

**FIGURE 3.** Annual malaria incidence rates (*P. vivax + P. falciparum*) for the three villages ■ São Raimundo, ◆ São João, ＄ Santo Antonio.

**REFERENCES**


