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Amazonian malaria: Asymptomatic human reservoirs, diagnostic challenges, environmentally-driven changes in mosquito vector populations, and the mandate for sustainable control strategies

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Abstract

Across the Americas and the Caribbean, nearly 561,000 slide-confirmed malaria infections were reported officially in 2008. The nine Amazonian countries accounted for 89% of these infections; Brazil and Peru alone contributed 56% and 7% of them, respectively. Local populations of the relatively neglected parasite *P. vivax,* which currently accounts for 77% of the regional malaria burden, are extremely diverse genetically and geographically structured. At a time when malaria elimination is placed on the public health agenda of several endemic countries, it remains unclear why malaria proved so difficult to control in areas of relatively low levels of transmission such as the Amazon Basin. We hypothesize that asymptomatic parasite carriage and massive environmental changes that affect vector abundance and behavior are major contributors to malaria transmission in epidemiologically diverse areas across the Amazon Basin. Here we review available data supporting this hypothesis and discuss their implications for current and future malaria intervention policies in the region. Given that locally generated scientific evidence is urgently required to support malaria control interventions in Amazonia, we briefly describe the aims of our current field-oriented malaria research in rural villages and gold-mining enclaves in Peru and a recently opened agricultural settlement in Brazil.

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1. Introduction

After decades of systematic control and attempted eradication, malaria continues to keep a firm grip and remains endemic in 21 countries in the Americas and the Caribbean. Nearly 561,000 slide-confirmed infections, with 89 deaths, were reported by the Pan American Health Organization (PAHO) in 2008 (PAHO, 2009). Cases in the nine Amazonian countries (Bolivia, Brazil, Colombia, Ecuador, Guyana, French Guyana, Peru, Suriname, and Venezuela) accounted for 89.3% of these infections. These figures, which are derived from yearly reports from the Ministries of Health, are likely to be underestimated, due to limitations in the coverage of malaria notification and diagnosis in many malarious areas, but there is no consensus regarding the most appropriate strategy to adjust for subnotification. For example, the World Malaria Report 2008 (World Health Organization, 2008) derived estimates of global malaria burden by adjusting the reported number of malaria cases in 2006 for health facility reporting rates, care-seeking behavior for fever and the extent to which suspected cases are confirmed with laboratory tests. Using these criteria, the reported number of malaria cases in the Americas was multiplied by 2.6, with 2,000–3,000 malaria-related deaths in 2006. However, the resulting figures were considered by PAHO to overestimate the malaria burden in the Americas (PAHO, 2009), and no further attempts at adjusting reported numbers of malaria cases were made in subsequent years.

Although transmission of both *Plasmodium falciparum* and *P. vivax* occur across the Amazon Basin (with rare *P. malariae* infections), *P. vivax* currently accounts for 77% of the malaria burden in Amazonia (PAHO, 2009). As with many other tropical and mainly rural infectious diseases, malaria in the Americas disproportionately affects people in the lower socioeconomic strata, with difficult access to health care.

1.1 Malaria in Brazil

The annual incidence of malaria in Brazil increased >10-fold since 1970 following massive human migration and the establishment of frontier agricultural settlements and open mining enclaves in the Amazonian rainforest (Cruz Marques, 1987). Nearly 315,000 slide-confirmed infections were recorded in this country in 2008, 99.9% of them in the Amazon Basin; these figures represent 56.1% of all slide-confirmed malaria episodes diagnosed in the Americas and the Caribbean in 2008 (PAHO, 2009). The Program for Malaria Control in the Amazon Basin (known as PCMAM after the Portuguese acronym), launched in 1989 and financed with US$73 million from the World Bank (Barat, 2006), had a clear short-term impact: malaria morbidity decreased by 60% between 1989 and 1996 (Oliveira-Ferreira et al., 2010). The PCMAM strategy, focused on early diagnosis and treatment of malaria cases to reduce transmission and mortality, was proposed as being more cost-effective than widespread house spraying with residual insecticides (Akhavan et al., 1999). The existing network of malaria outposts was reinforced and expanded across Amazonia to provide early and free diagnosis (based on thick smear microscopy) and free treatment of slide-confirmed malaria episodes (with standardized drug regimens), while house spraying was gradually phased out (Roberts et al., 1997). However, the gains were not sustained over the next years, and the number of malaria cases increased by 34% between 1998 and 1999 (Loiola et al., 2002). Further periods of intensification of malaria control through early diagnosis and treatment once again resulted in substantial decreases in malaria incidence between 2000 and 2002 and after 2007 (Oliveira-Ferreira et al., 2010). DDT likely was critically important in reducing malaria incidence in South American after World War II (Loiola et al., 2002), and its use in fact was intensified in 2000 through the “Action Plan for Intensification of Malaria Control in the Amazon” established by the Brazilian government in concert with the WHO-initiated Roll Back Malaria initiative. Nonetheless, malaria has remained intractable to control efforts in Brazil and elsewhere in Amazonia. In 2009, the manufacture and use of DDT in Brazil was legally prohibited, despite well-articulated pleas to continue its use given

lack of demonstrable human toxicity and environmental damage when use is limited to household spraying (Roberts et al., 2000).

Malaria transmission hotspots in Brazil, as in the rest of South America, are intermingled with areas of low or moderate risk (Figure 1). Most malaria transmission typically occurs in mining and logging camps and new farming settlements (da Silva-Nunes et al., 2008; de Castro et al., 2006; de Castro et al., 2007). These settlements not only induce massive environmental changes, such as deforestation (Figure 2), that alter vector biology and favor malaria transmission, but also cluster large number of non-immune migrants close to natural and man-made vector breeding sites (de Castro et al., 2007; Norris, 2004; Vittor et al., 2006; Vittor et al., 2009). Not surprisingly, malaria cases cluster in the sectors of most recent occupation in frontier settlements, where ongoing land clearing favors an increase in the abundance of the main malaria vector in the region, *An. darlingi* (da Silva et al., 2010).

Recent data also reveal changes in the relative contribution of different malaria parasite species to the total malaria burden in Brazil. Whereas transmission of *P. falciparum*, which predominated between 1985 and 1990, decreased steadily, that of *P. vivax* maintained an upward trend throughout the 1990s. *Plasmodium vivax* now accounts for >80% of the malaria burden in Brazil (Oliveira-Ferreira et al., 2010), mirrored in Peru as well although with lower absolute incidence. Overall *Plasmodium malariae* transmission remains low and focal (Scopel et al., 2004; da Silva-Nunes et al., 2008; Ladeia-Andrade et al., 2009; da Silva et al., 2010), and this species accounts for less than 1% of all slide-confirmed malaria infections in Brazil (Oliveira-Ferreira et al., 2010). As in other parts of the world (Sattabongkot *et al*., 2004), the relatively limited success in reducing malaria incidence in Brazil over the past decade (Ferreira and Da Silva-Nunes, 2010) has substantially resulted from failure to prevent *P. vivax* infections, providing a compelling stimulus for malaria vivax-oriented research in Amazonia.

1.2 Malaria in Peru

Malaria in Peru is transmitted in diverse settings including coastal, mountainous and jungle regions, with an important epidemic beginning in the mid 1990s (Aramburu Guarda et al., 1999; Roper et al., 2000) and with nearly 36,900 cases recorded in 2008. Most transmission is recorded in Loreto and Madre de Dios Departments, both in the Amazon Basin (Figure 3). Ecological changes favored the spread of the competent vector *Anopheles darlingi* in Loreto in the early 1990s (Schoeler et al., 2003) and over the following years led to major outbreaks of malaria in rural villages surrounding Iquitos, a major port city with 450,000 inhabitants (Aramburu Guarda et al., 1999; Bautista et al., 2006; Roper et al., 2000; Roshanravan et al., 2003). Additional sociodemographic factors are also likely to be responsible for the reemergence of malaria as a public health problem in Loreto. A major livelihood of people living in the rural areas surrounding Iquitos is based on agriculture, fishing and timber extraction. Men and women farm small plots of land for sugar cane, fruit, harvest wood for charcoal, and raise livestock for sale. These activities often occur on land located away from permanent dwellings, resulting in a group of people who camp in the fields during the work week and return to their houses in villages on weekends. Such activities provide the opportunity for people from different malaria-endemic regions to congregate and admix parasites. If this hypothesis is correct, people with such epidemiological characteristics (rural travelers, immigrants) would be major targets for control strategies since this might be a major driver for mobilizing parasite diversity among populations.

Since the late 1990s, malaria-naïve migrants, mostly from the Andean region of Peru, have been attracted to mining sites across the Madre de Dios river and its tributaries (Figure 4). Most confirmed malaria cases in the area, all due to *P. vivax*, came from Huepetuhe, the large gold mining camp located in the southwest of the Madre de Dios Department in

southeastern Peru. Malaria-endemic Madre de Dios Department is also currently affected by massive migration and drastic environmental changes induced by the construction of the Transoceanic Highway, which connects Amazonian Brazil to the Pacific Coast of Peru.

1.3 Transmission dynamics of *Plasmodium vivax*

Populations of *Plasmodium vivax*, the predominant local malaria parasite, are extremely diverse genetically and geographically structured in the Amazon Basin of both Brazil and Peru, with significant genetic divergence between populations separated by relatively short distances (Ferreira et al., 2007; Orjuela-Sánchez et al., 2010; Van den Eede et al., 2010). When comparing sympatric parasites of each species, much more microsatellite diversity is found in *P. vivax* than in *P. falciparum* populations from Amazonian Brazil (Ferreira et al., 2007; Orjuela-Sánchez et al., 2009a). The latter populations display a fast haplotype replacement rate over time, as revealed by genotyping parasites recovered from the same community over 12–24 months of follow-up (Ferreira et al., 2007; Orjuela-Sánchez et al., 2009b; Van den Eede et al., 2011). Such a large spatial-temporal genetic diversity may severely delay the development of the variant-specific component of naturally acquired immunity to *P. vivax* in Amazonians (Bastos et al., 2007; Souza-Silva et al., 2010). However, such an extensive genetic diversity paradoxically coexists with low meiotic recombination rates, with a strong linkage disequilibrium between genetic markers that map to different chromosomes (Ferreira et al., 2007; Orjuela-Sánchez et al., 2009b; Van den Eede et al., 2010; Van den Eede et al., 2011) and a slow decay in linkage disequilibrium with increasing physical distance between markers along the same chromosome (Orjuela-Sánchez et al., 2010).

Plasmodium vivax recurrences are often seen, in both Brazil and Peru, 1–6 months after treatment with standard doses of chloroquine and primaquine (Orjuela-Sánchez et al., 2009b; da Silva et al, 2010; Van den Eede et al., 2011). Because malaria in the Amazon Basin is transmitted year-round, recrudescences, relapses, and new infections may all originate these recurrences. Multilocus microsatellite typing was recently used to compare haplotypes in pairs of consecutive *P. vivax* infections diagnosed in cohorts of rural Amazonians in Brazil and Peru; in all studies, haplotypes that had not been detected in primary infections predominated in recurrences, consistent with new infections or relapses due to the reactivation of heterologous hypnozoites (Orjuela-Sánchez et al., 2009b; Van den Eede et al., 2011). Whether emerging resistance to chloroquine (de Santana Filho et al.2007) and perhaps to primaquine (the only drugs currently in use to treat *P. vivax* infections) plays a major role in post-treatment late recurrences in these countries remains unclear (da Silva-Nunes and Ferreira, 2010); subtherapeutic primaquine doses, however, are commonly used and clearly associated with relapses (Duarte et al., 2001).

2. Asymptomatic parasite carriage

At a time when malaria elimination is again on the public health agenda of several endemic countries (Feachem and Sabot, 2008; Mendis et al., 2009), it remains unclear why malaria has proved so difficult to control across the Amazon Basin, where transmission rates remain far below those recorded in tropical Africa. To provide scientific evidence that can be translated into effective interventions for malaria control in the region, we are currently investigating the clinical and public health significance of asymptomatic parasite carriage. We hypothesize that asymptomatic infections represent a major source of infective gametocytes for the increased vector population found in recently deforested areas across the Amazon Basin.

2.1 Asymptomatic malaria in frontier settlements

Unstable and hypo- or mesoendemic malaria transmission usually seen in Amazonia typically fails to elicit the status of clinical immunity seen among adults exposed to holoendemic malaria in rural Africa. The entire population is vulnerable to infection and nearly all slide-positive infections are followed by clinical disease (Camargo et al., 1999; Camargo et al., 1996; Prata et al., 1988). However, the longstanding dogma that immunity rarely develops in areas of low malaria endemicity (MacDonald, 1956, 1957) has been challenged by studies of Javanese transmigrants in Papua New Guinea (Baird et al., 1991) and, in more recent observations, in frontier settlements and riverine communities across the Amazon Basin of Brazil (Alves et al., 2002; da Silva et al., 2010; da Silva-Nunes et al., 2008; Ladeia-Andrade et al., 2009; Suarez-Mutis et al., 2007), where subclinical infections with very low parasite loads, most of them detected only by polymerase chain reaction (PCR), are quite common. After five to eight years of continuous exposure to low to moderate levels of malaria transmission, the prevalence or incidence of both infection and disease decreases steadily, suggesting that Amazonian populations acquire not only anti-disease immunity but also some degree of anti-parasite immunity (Ladeia-Andrade et al., 2009). Native and migrant populations exposed to malaria seem to develop immunity, but the immunological correlates of clinical protection remain largely unexplored. Asymptomatic malaria parasite carriage is also commonly seen among inhabitants of rural villages surrounding Iquitos, in Peru, with very recent exposure to epidemic malaria (Branch et al., 2005; Parekh et al., 2007; Roshanravan et al., 2003).

2.2 A role for aggressive active case detection?

Malaria infections across the Amazon Basin are routinely identified through active or passive case detection (ACD and PCD, respectively). According to the classical definitions from the malaria eradication era (Pampana, 1963), cases are found through PCD when febrile subjects visiting malaria diagnosis outposts have a blood sample tested positive for malaria parasites. ACD implies periodic visits to households, with collection of thick blood smears from every person having had fever since the last visit (Najera, 2001). A major limitation of ACD and PCD is that both strategies target only symptomatic infections (classically defined as “fever cases”); as a result, asymptomatic infections go undetected and untreated (Coura et al., 2006), extending the average duration of parasite carriage to around 270 days (Freeman et al., 1999). Because the clinical spectrum of symptomatic malaria in semi-immune Amazonians ranges from a very mild illness to a full-blown disease with periodic fever paroxysms, ACD and PCD face a heterogeneous disease in which cyclical paroxysms with fever, chills and profuse sweating are not necessarily prominent features (da Silva-Nunes and Ferreira, 2007).

The periodic population-wide screening for malaria parasites, irrespective of any clinical symptoms, is known as aggressive active case detection (AACD; Macauley, 2005), mass blood survey or mass blood examination (Pampana, 1963). Alternatively, AACD may be carried out during the epidemiological investigation of a focus, targeting persons living in the neighborhood of a positive case (Pampana, 1963). Although AACD can potentially detect asymptomatic infections and supplement ACD and PCD in areas where the sources of infection cannot be sufficiently screened by using fever as a criterion (Pampana, 1963), whether AACD should be implemented as a public health strategy in Amazonia remains a matter of debate (Alves et al., 2002; Alves et al., 2005; Coura et al., 2006; da Silva et al., 2010; da Silva-Nunes et al., 2008; Ladeia-Andrade et al., 2009; Macauley, 2005; Roshanravan et al., 2003; Vinetz and Gilman, 2002). To evaluate the cost-effectiveness of population-wide AACD, the relative role of asymptomatic infections in maintaining malaria transmission in Amazonia must be quantified. Mathematical models identified

asymptomatic infections as a crucial target for malaria eradication efforts in Africa (Aguas et al., 2008), but no similar analysis are available for other endemic areas.

2.3 Gametocyte carriage and infectivity in asymptomatic infections

The prevalence and average duration of gametocytemia in asymptomatic infections remain largely unknown. Little is known on infectiousness of asymptomatic carriers of low-grade parasitemias (Alves et al., 2002; Alves et al., 2005). In addition, it remains unclear whether people living in malaria-endemic regions develop immunity against the sexual stages of malaria parasites that infect mosquitoes, the so-called naturally acquired transmission-blocking immunity. If this were demonstrated to be the case, the presence of naturally acquired transmission-blocking antibodies would provide the basis of novel approaches to the development of malaria transmission-blocking strategies. We have experimentally infected local *Anopheles darlingi* mosquitoes with *Plasmodium vivax* directly obtained from malaria patients in the Iquitos region of Peru and found that only about half of patients with acute *P. vivax* malaria were able to infect mosquitoes (this observation has held up with more than 200 such feedings using blood from acute vivax malaria patients; JMV, unpublished observations). There was no relationship between the presence of gametocytes (the transmission stages) and infectivity of mosquitoes, suggesting that some clinical factor was impairing parasite transmission from humans to mosquitoes. Based on these results, we hypothesize that patients who inefficiently infect mosquitoes have developed acquired immunity in the form of antibodies against protein antigens expressed by the transmission stages of the malaria parasite.

2.4 Diagnostic challenges

Laboratory methods appropriate for large-scale use, such as microscopy and rapid diagnostic tests (RDTs), are not sensitive enough to detect low-grade, asymptomatic infections. A highly sensitive PCR-based diagnosis, which is between 2.7-fold and 8.6-fold more sensitive than conventional microscopy in detecting malaria parasites in apparently healthy Amazonians (da Silva et al., 2010), has been suggested as a public health tool for AACD in Peru (Roshanravan et al., 2003), but its large-scale use remains constrained by its high cost and complexity. RDTs are not a feasible alternative alternative to PCR for population-based screening of asymptomatic infections because of low parasitemia during asymptomatic infection. Most commercially available RDT kits use the Histidine Rich Protein 2 (HRP2) as the target antigen (Moody, 2002) and antibodies to HRP2 may also cross react with another member of the HRP gene family, HRP3. Our recent evaluation of RDTs revealed a substantial number of malaria parasites in Peru and Colombia with a deletion of the *HRP2* and *HRP3* genes. In Peru, the *HRP2* and *HRP3* genes were missing in 41% and 70% isolates of *P. falciparum*, respectively; both genes had been deleted in 22% of these isolates (Gamboa et al., 2010). Similar studies are underway in Brazil. These deletions of HRP2 and HRP3 may compromise the ability of RDTs based on these antigens to detect the presence of parasites in patient samples, especially in asymptomatic carriers of low-grade parasitemias.

2.5 Asymptomatic infections and risk of subsequent clinical disease

Recent evidence suggests that long-term asymptomatic carriage of malaria parasites protects against subsequent disease in Tanzania (Bereczky et al., 2007) and Senegal (Males et al., 2008), possibly by reducing the risk of superinfection with more virulent strains. These results imply that eradicating asymptomatic infections can increase the risk of clinical malaria over the next transmission season. The biological bases for this phenomenon remain largely unknown, but data from experimental rodent malaria models suggest that ongoing blood-stage infection, once a minimum parasite load is reached, may arrest the development of subsequently inoculated sporozoites in the liver. Such an inhibition of superinfection

seems to be mediated by the iron regulatory hormone hepcidin, produced in response to blood-stage parasitemia (Portugal et al., 2011). Because of their major public health implications, these findings require validation, with more extensive adjustment for potential confounders such as cumulative exposure to malaria and acquired immunity (Gosling, 2008), in epidemiologically diverse endemic settings, such as the Amazon Basin.

3. Vector biology and control

One of the main contributors to continued malaria transmission in the Amazon is deforestation that alters breeding sites (Vittor et al., 2006, 2009; Monteiro de Barros et al., 2011), and settlement by susceptible humans near new frontier agricultural and open mining projects in rainforests (da Silva-Nunes et al., 2008; de Castro et al., 2006). Another important factor is the presence of asymptomatic parasite carriers, a potential source of gametocytes for infecting mosquitoes (Alves et al., 2005). The other critical piece of this transmission picture is the species of local malaria vector and its ecology, behavior, and population structure (de Castro et al., 2006; Lounibos and Conn, 2000; Vittor et al., 2009).

3.1 Local vector species

The World Health Organization's strategic framework for vector control calls for "an evidence-based decision-making approach which involves the adaptation of strategies and interventions to local vector ecology, epidemiology and resources that are guided by operational research and subject to routine monitoring and evaluation." However, malaria vector biology remains understudied in Amazonia, and much less is known about species other than *Anopheles darlingi* that contribute to the maintenance of local transmission (but see Zimmerman et al., 2006; Galardo et al., 2007, 2009). In particular, species misidentification remains a serious issue in Amazonian vector biology (Marrelli et al., 2005; Sallum et al., 2008; Matson et al., 2008; Cienfuegos et al., 2011). By combining male genitalia analysis, progeny broods, and molecular sequences, puzzling anomalies can be resolved (Ruiz et al., 2005; Sallum et al., 2008).

Anopheles (*Nyssorhynchus*) *darlingi* is the most anthropophilic and efficient malaria vector in Latin America, capable of transmitting *P. falciparum*, *P. vivax* and *P. malariae* (Galardo et al., 2007; Lourenco-de-Oliveira et al., 1989; Magris et al., 2007). It is broadly distributed in the Neotropics, and has recently been detected for the first time in eastern Panamá (Loaiza et al., 2009). The larval habitats of *An. darlingi* are typically natural water bodies, particularly slow-moving margins of streams or rivers with shaded, clear water, or lagoons that remain following flooding (reviewed in Charlwood 1996; Rejmánková et al., 1999; Sinka et al., 2010). In addition, *An. darlingi* larvae can be found in human-modified habitats, such as fish ponds (Lounibos and Conn, 2000; Vittor et al., 2009).

In much of Loreto Department in Amazonian Peru, *An. darlingi* has become synonymous with malaria transmission, but only since its spectacular emergence around Iquitos in the 1990s (Aramburu Guarda et al., 1999; Fernández et al., 1996; Roberts et al., 1997) and subsequent spread through much of Loreto (Schoeler et al., 2003). Whereas in 1991, several hundred malaria cases were reported in Loreto (Roper et al., 2000), by 1997, the number had risen to tens of thousands (Schoeler et al., 2003). The region therefore faced epidemic malaria, mainly linked to deforestation and the invasion of this aggressive vector (Aramburu Guarda et al., 1999; Vittor et al., 2006). Currently, in sites around Iquitos, as well as in nearby Puerto Almendra (Turell et al., 2008), *An. darlingi* is the primary vector, transmitting mainly *P. vivax*.

In the mining camps in southeastern Amazonian Peru, limited available data suggest a more complex picture. In Madre de Dios Department, near the Peru-Bolivia-Brazil border, adult

An. darlingi was the most abundant species captured biting humans indoors and outdoors in two of four localities; however, *An. benarrochi* was the most abundant in the other two localities (Tineo et al., 2003). In a newer study in the same region, *An. benarrochi* is hypothesized to be an important regional vector (Flores-Mendoza et al., 2004). *An. benarrochi* has been reported as a primary vector in western Loreto and Ucayali (Aramburu Guarda et al., 1999; Fernández et al., 1996; Schoeler et al., 2003). Despite being much more abundant in West Loreto and Ucayali than *An. darlingi* (71% vs. 24%), *An. benarrochi* was infected with both *P. falciparum* and *P. vivax* at much lower rates (Flores-Mendoza et al., 2004), suggesting that *An. benarrochi* is less competent. These data are consistent with a recent study in southern Colombia that described a new species, *An. benarrochi* B (Ruiz et al., 2005), and found that despite its anthropophily and high prevalence (66.1% of 2,445 anophelines tested), no *An. benarrochi* B were positive for *Plasmodium* by ELISA (Quinones et al., 2006). Furthermore, by comparing ITS2 sequences and male genitalia of voucher specimens Quinones et al.(2006) determined that both *An. benarrochi* s.s. and *An. benarrochi* B occur in Peru. Detection of a morphological variant and ITS2 PCR-RFLP differences in *An. benarrochi* collected from Loreto and Ucayali in Peru (Matson et al., 2008) may support these findings. It is critically important to accurately identify the main vector(s) in the mining areas of Madre de Dios Department to evaluate the risk of malaria transmission and the effectiveness of current control methods.

Anopheles oswaldoi has been identified as a malaria vector in Brazil (de Arruda et al., 1986; de Oliveira-Ferreira et al., 1990), playing a major role in malaria transmission in the northwestern state of Acre, bordering with Bolivia and Peru (Branquinho et al., 1993). However, the species originally identified as *An. oswaldoi* s.s. in Acre is now believed to be one of two new species in the *An. oswaldoi* or *An. benarrochi* complexes (Sallum et al., 2008). *An. oswaldoi* was described as a complex (Flores-Mendoza et al., 2004; Marrelli et al., 1999) and one of the member species is a vector in Colombia (Quinones et al., 2006; Rodriguez et al., 2009). *An. deaneorum*, a member of the Albitarsis complex (Klein et al., 1991a; Rosa-Freitas, 1989; Wilkerson et al., 1995a, 1995b) was also implicated, though it was, and remains, much less abundant (Branquinho et al., 1993). This species is competent for *P. falciparum* and *P. vivax* in a laboratory setting (Klein et al., 1991a, 1991b, 1991c), and it has been found infected in field studies (Branquinho et al., 1993), assuming this identification is confirmed (Sallum et al., 2008). Because the anopheline collections for Branquinho et al.'s 1993 study were done in 1990–1991, a long time ago in a dynamic frontier malaria area (da Silva-Nunes et al., 2008), we hypothesize that major shifts in species composition have occurred in this region.

3.2 Environmental change and vector populations

As a settlement becomes stable, there is generally a plateau of malaria cases (de Castro *et al*. 2006). However, with subsequent expansion and additional habitat alteration, a new cycle of increased mosquito-human contact can be initiated. Deforestation and other anthropogenic activities often create diversity in larval habitats, resulting in changes in mosquito species composition and increased abundance (Povoa et al., 2003; Tadei et al., 1998; Vittor et al., 2006; Moutinho et al., 2011). In the areas studied by Tadei et al. (1998), anopheline abundance was estimated to be approximately five times greater in disturbed compared with undisturbed habitats. The significant correlation between deforestation and human biting rates of *An. darlingi* along the Iquitos-Nauta road (Vittor et al., 2006, 2009) is an important finding; breeding sites of *An. darlingi* were detected in areas with 24.1% forest cover, in contrast to 41% coverage areas where this species was absent (Vittor et al., 2009). A new study that analyzed multiple forested and deforested sites along a river in Roraima state, Brazil, found the highest abundance of *An. darlingi* larvae in the forested-deforested transition zones during the dry season, and in natural microdams where the river current was

obstructed (Monteiro de Barros et al., 2011). Shade and nearby human dwellings were also important correlates. Taken together, these data suggest that *An. darlingi* is most strongly associated with some forest cover in diverse types of habitats significantly altered by human activities, especially in rural settings (Moutinho et al., 2011).

The unprecedented invasion into the Iquitos region of *An. darlingi* resulted in major increases in its abundance in most localities (Aramburu Guarda et al., 1999). It also bore the molecular signature of an invasive species, with limited diversity, as seen in (Pinedo-Cancino et al., 2006) using RAPDs, and subsequently confirmed in some localities by microsatellite analysis (Mirabello et al., 2008). We postulate that a similar pattern of *An. darlingi* expansion, and increased malaria transmission, will take place in the rural settlements near Iquitos and in the mining camps in Madre de Dios Department, both in Peru, as well as in the recently colonized agricultural projects in Amazonian Brazil (Moutinho et al., 2011). A better understanding of the dispersal of species into newly altered habitats is vital to reduce human-vector contact.

4. A role for insecticide-treated bednets (ITNs) for malaria control in Amazonia?

Long-lasting insecticide-treated nets (ITNs) have emerged in the 1990s as one of the great hopes for controlling malaria worldwide. Their efficacy has been clearly demonstrated in different endemic areas, especially in Africa, where they reduce anemia incidence and overall mortality (Curtis et al., 2006). In a systematic literature review of *Plasmodium falciparum* endemic settings, it is assumed the impact of IRS is equal to that of ITNs on reducing malaria-attributable mortality in children (Eisele et al, 2010). When ITNs are provided free of charge and a high population coverage is achieved, their impact on malaria transmission in Africa is comparable to that in the best house spraying projects (Lengeler and Snow, 1996). In a review paper of Pluess and collaborators (2010), some limited data suggested that ITNs give better protection than IRS in unstable areas, but more trials are needed to compare the effects of ITNs with IRS, as well as to quantify their combined effects. In this review, ITNs appeared to provide better protection against any infection compared to IRS in India (Misra, 1999). In another review, using the Entomological Inoculation Rate (EIR) to assess the impact of vector control on malaria transmission, two different studies with separate ITN and IRS intervention groups, (in Tanzania and Solomon Islands) showed that in the second year of the Tanzania study, EIR was 90% lower in the ITN community and 93% lower in the IRS community, relative to the community without intervention, and that the ITN and IRS effects were not significantly different. In contrast, in the Solomon Islands study, EIR was 94% lower in the ITN community and 56% lower in the IRS community (Shaukat et al., 2010).

Nevertheless, Zimmerman and Voorham concluded, in their review published more than a decade ago, that “it would be premature to use insecticide-impregnated mosquito nets or other materials as a major component of an integrated malaria control program in the Americas at this time” and called for well-designed large-scale trials in this region (Zimmerman and Voorham, 1997)(see also (Kroeger et al., 1995; Kroeger et al., 1997)). This is partly because the biting behavior of Neotropical malaria vectors, especially *An. darlingi*, is predominantly exophagic (feeds outdoors) and often unimodal, during the early evening (Vittor et al., 2006; Voorham, 2002; Zimmerman and Voorham, 1997); but see (Moreno et al., 2007; Rosa-Freitas et al., 1992) when human activity peaks and people are generally not under mosquito nets (Loaiza et al., 2008). *An. darlingi* can present a marked early biting behavior and high outdoor-to-indoor biting ratio (Tadei et al., 1998) in some areas, although a late biting behavior and indoor preference have also been described

(Rozendaal, 1989). Very little is currently known about the biting behavior of other malaria vectors in Amazonia (Sinka et al., 2010).

Surprisingly, no large community-based randomized trials of ITNs have been carried out in the Americas since the 1997 review of Zimmerman and Voorham. The fact that the use of ITNs alone does not adequately reduce malaria rates in every region (but see Charlwood et al., 2005), may undermine their efficacy to impact malaria transmission throughout the Amazon Basin (Alexander et al., 2005; Harvey et al., 2008; Hill et al., 2007). One decade after the publication of Zimmerman and Voorham's review, we are still waiting for "well-conceived, large-scale trials at the community or regional level" that are "based on a thorough understanding of the dynamics of malaria transmission in the areas involved" (Zimmerman and Voorham, 1997). ITNs are currently recommended for use against malaria transmission by *An. darlingi* in Amazonian French Guiana (Girod et al., 2008) and, since 1999, the Peruvian Ministry of Health has been distributing ITNs in the Amazonian region for use against biting mosquitoes with some success (Harvey et al., 2008). In Brazil, only recently (2007–2010) has the Ministry of Health started to freely distribute ITNs in malaria-endemic areas. There are two studies in the Amazon region showing evidence of the effectiveness of the ITNs in this area. One in Amazonian Colombia showed that impregnated nets were associated with more than a 50% reduction in malaria relative to no net use (Alexander et al, 2005), although the advantage of impregnated over non-impregnated nets was not statistically significant. Furthermore, a recent randomized trial, in the Amazonas State of Venezuela, of lambdacyhalothrin-versus placebo-treated nets, found a protective efficacy of 55% (Magris et al, 2007). However, variation in terms of vector species (Tadei & Dutary-Tacher, 2000) and possibly of human behavior, mean that optimal policies may vary within the Amazon region.

4.1 Assessing ITN-based interventions

To predict the effectiveness of ITN-based malaria control strategies, it is critically important to monitor and evaluate peak anopheline biting activities for variation and plasticity of seasonal and temporal change, particularly in relation to ecosystem transformations that can dramatically increase malaria transmission (Gil et al., 2007; Moreno et al., 2007; Vittor et al., 2006). One of the main problems of the invasion of new vector species, such as *An. darlingi* into Iquitos, Peru, is the potential introduction and spread of genes conferring insecticide resistance, which could reduce the effectiveness of current vector control measures. Resistance to pyrethroids is increasing due to overuse and the lack of alternative insecticides (Chareonviriyaphap et al., 2003; Chouaibou et al., 2006; Singh et al., 2011). Unfortunately, the genetic potential to develop multiple insecticide resistance is common in *Anopheles* spp.(Casimiro et al., 2006; Chareonviriyaphap et al., 2003; Hargreaves et al., 2003). Periodic surveillance of insecticide susceptibility is necessary for adequate management of vector strategies but this is completely absent in Amazonia. Furthermore, in areas where different *Anopheles* species coexist, resistance evaluation is more complicated since each species may respond differently to the same insecticide (Hargreaves et al., 2003). *An. darlingi* resistance to DDT was first reported in Colombia (Suarez et al., 1990), and a recent paper shows some Colombian populations of *An. nuneztovari* resistant to pyrethroids and organophosphates (Fonseca-Gonzalez et al., 2009). Overall, there is a serious lack of accurate and current information about susceptibility levels in Amazonian malaria vectors.

One way to monitor the effectiveness of ITNs or insecticides (in use as indoor residual spray locally (e.g. Santos et al., 2007), in Amazonian Brazil) on the size of anopheline populations is to measure the effective population size (N_e), or simply put, the number of reproductive females, at distinctive temporal points (Pinto et al., 2002; Pinto et al., 2003). This measure is indirect, in that it depends on temporal variation in allele frequencies (*i.e.*, genetic drift) and population size (Wondji et al., 2005). Based on the heterogeneous, but generally small N_e

estimates of *An. darlingi* (92 – 202 females, in Amazonian Brazil, (Conn et al., 2006; Scarpassa and Conn, 2007); around Iquitos, 8 −1,786 females (Mirabello et al., 2008), there could be large fluctuations in this species in Peru and Brazil (Taylor et al., 1993). Diversity measures (observed and expected heterozygosities, mean number of alleles per locus, allelic and genotypic frequencies, and levels of differentiation between populations) are also informative in deducing any effects of ITNs and (or) insecticide in comparisons before and after applications, and also among local and regional populations (Wondji et al., 2005). The estimates of gene flow, especially among anopheline populations in malaria endemic areas, can provide baseline information for the tracking of genes that confer insecticide resistance (Lehmann et al., 2003).

We anticipate that successful vector control for malaria in Amazonian Peru and Brazil can be accomplished with the accurate incrimination of the local primary malaria vector species, the assessment of the impact of ecological changes (deforestation and mining) on their population structure and behavior, and the evaluation of ITNs or insecticide treatment to control *An. darlingi* and other vectors.

5. Setting the stage for applied malaria research in Amazonia

There has been an explosion of information from large scale genomic, proteomic and gene expression profiling of multiple *Plasmodium* species. The whole genome sequence of *Homo sapiens* and the major malaria-transmitting vector mosquito, *Anopheles gambiae* have been published. A major challenge confronting the malaria community is how to apply such knowledge towards the amelioration of malaria at the field level, in the real world setting. There is an emerging consensus that “public health needs to be evidence-based if it is to be done correctly” (Eriksson, 2000), but translating scientific evidence into public health interventions may be particularly challenging.

Currently available tools and interventions may contribute to bringing down the overall malaria burden in Amazonia, but locally generated evidence is urgently needed to address major gaps in existing interventions. The external validity of well-designed controlled trials in Africa and Asia may be surprisingly limited when interventions deal with diseases with relatively complex causal pathways. For example, the varying biting behavior of local malaria vectors is a clear source of effect modification that affects the generalizability of ITN efficacy trials (Kroeger et al., 1999). Patterns of antimalarial drug resistance are also clearly regional, and a given therapy that has proven to be highly effective in some endemic settings may fail in others. Additional examples of major gaps in the malaria research agenda are the need for improved strategies to identify and treat asymptomatic reservoirs of disease and to monitor and prevent the emergence of resistance to several pesticides, especially pyrethroids, in the vector population. To address these and other gaps in our understanding of the scientific basis for malaria control, we designed field-oriented studies in areas with diverse epidemiology across the Amazon Basin of Brazil and Peru. The field sites include: (a) Remansinho, a typical frontier agricultural settlement in Amazonian Brazil with endemic malaria transmission, (b) rural villages close to Iquitos, a major city in Peru that became recently exposed to epidemic malaria, and (c) gold-mining enclaves near Puerto Maldonado, in Madre de Dios, Peru, with explosive malaria outbreaks due to *P. vivax.* Research in these sites is carried out in close collaboration with the malaria control teams of the Ministry of Health of both countries, with the aim of enhancing the partnership between researchers and decision-makers to face the challenges in malaria control in Amazonia.

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Highlights

- Transmission of malaria continues but is declining in the Americas
- Mortality rates are low with fewer than 600,000 annual cases regionally
- Amazonian countries are the main malaria-endemic regions
- Asymptomatic parasite carriage is common in Amazonia
- Environmental change affecting vector abundance contributes to malaria transmission

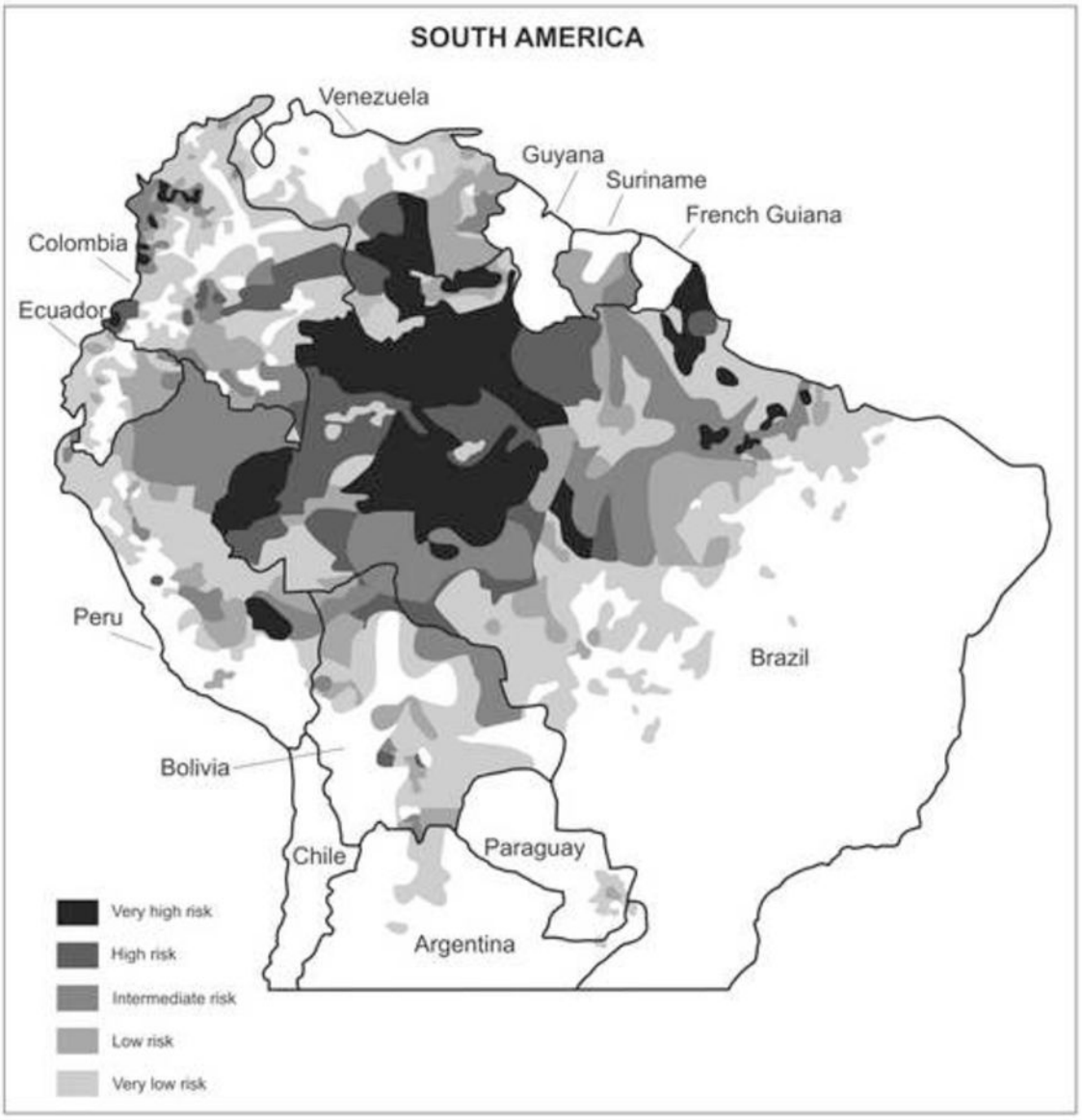

Figure 1.
Map of South America showing the malaria-endemic areas with different shadding pattern according to transmission levels in 2008. Source of data: Pan American Health Organization, 2009. Report on the Situation of Malaria in the Americas, 2008. Washington, DC. Available at: http://new.paho.org/hq/index.php?option=com_content&task=view&id=2459&Itemid=2049

Figure 2.
Deforestation for slash-and-burn agriculture (left) and dwellings surrounded by rain forest (right) in the agricultural settlement of Remansinho, near Acrelândia, Acre, northwestern Brazil (Pictures by Marcelo U. Ferreira).

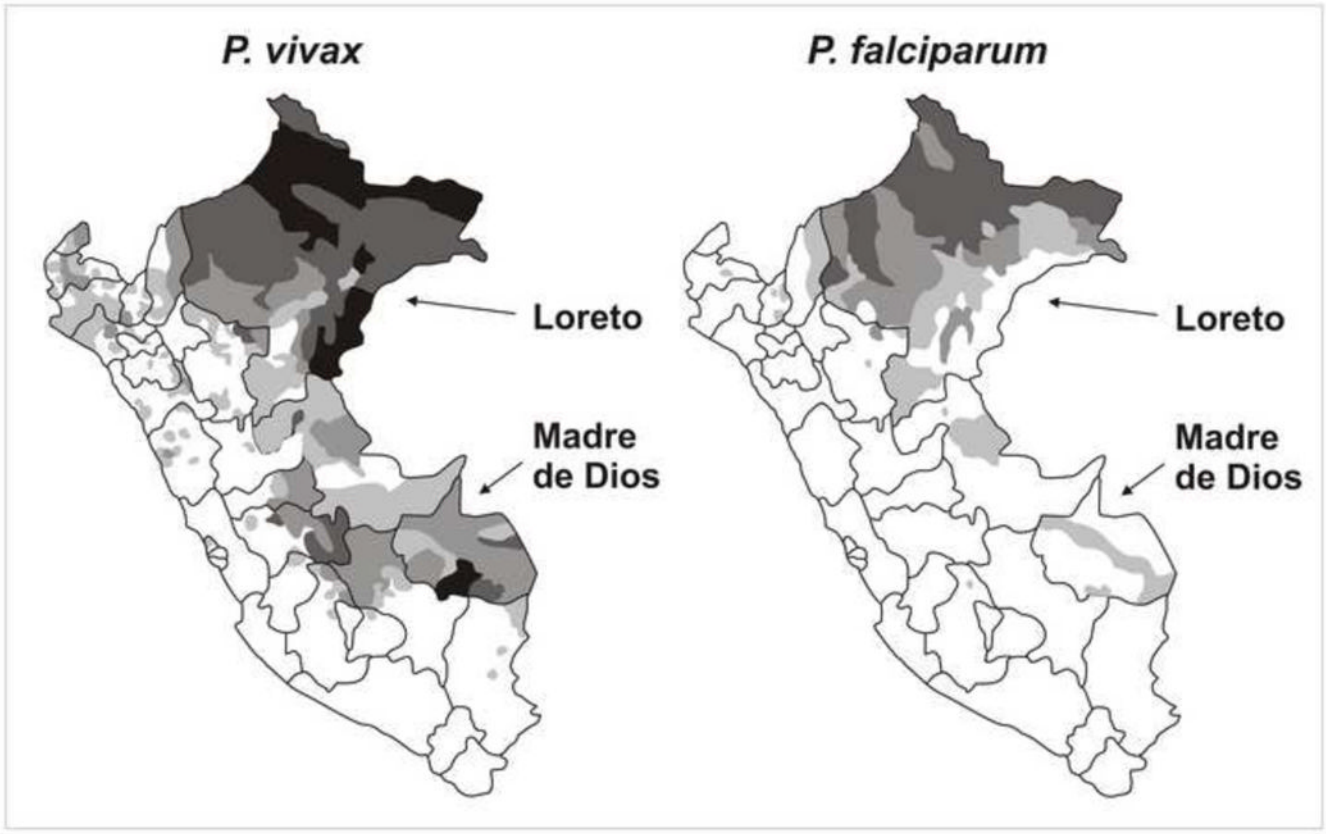

Figure 3.
Map of Peru showing the areas with highest levels of malaria transmission, the Departments of Loreto (where one of our field sites is located) and Madre de Dios (where our second Peruvian field site is located). The different shadding patterns (as in Figure 1) reflect different transmission levels in 2008. Source of data: Ministry of Health of Peru, 2009.

Figure 4.
Gold-mining enclave surrounding the rain forest in the Department of Madre de Dios, Peru (Picture by Marta Moreno).