COMPARATIVE VECTOR BIONOMICS AND MORPHOMETRICS OF TWO GENETICALLY DISTINCT FIELD POPULATIONS OF *AN. DARLINGI* ROOT FROM BELIZE, CENTRAL AMERICA AND ZUNGAROCOCHA, PERU, SOUTH AMERICA

by

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ABSTRACT

Comparative vector bionomics and morphometrics of two genetically distinct field populations of *An. darlingi* Root from Belize, Central America and Zungarococha, Peru, South America

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Anopheles darlingi Root, a dominant vector species for malaria in Central and South America, has a broad distribution spanning from southern Mexico to northern Argentina and from the eastern side of the Andes Mountains to the Atlantic and Caribbean Coastline. It has not been reported in Nicaragua or Costa Rica. Variability in genetics, morphology and behavior has been reported across its range. It has been suggested that *An. darlingi* is a cryptic species. A deep divergence, detected by the nuclear *white* gene, separates the species into a northern lineage (Belize, Guatemala, Colombia, Venezuela and Panama) and a southern lineage (Amazonia and southern Brazil). It is unknown if these lineages confer differences to epidemiologically relevant behaviors. Variation in study methodologies do not allow for direct comparison of behavior across the range of *An. darlingi*. The objectives of this research were to compare 1) house entry, house exit, and host preference behavior and 2) wing morphology

between field populations of *An. darlingi* representing the two genotypes using a standard methodology that can allow for a statistical comparison.

Experimental hut sites were established in Cayo District, Belize (northern lineage) and Zungarococha, Peru (southern lineage). The Belize population exhibited bimodal entrance with a major peak entry occurring between 7:00-8:00 pm and a minor peak entry at sunrise and peak exiting occurring between 7:00-8:00 pm. The Peru population exhibited unimodal entrance with peak entry occurring between 10:00-11:00 pm and peak exiting occurring between 11:00-12:00 am. Entrance and exit behavioral patterns were significantly different between the Belize and Peru populations of *An. darlingi* [log-rank (Mantel-Cox) $P<0.001$]

Host Preference was evaluated using experimental huts containing either a pig host or a human host. In Belize, there was a significantly greater abundance of *An. darlingi* collected from the human host hut than the pig host hut ($P=0.025$). In Peru, no host preference was detected.

To confirm the genotype of the collected *An. darlingi,* an enzyme restriction assay was developed. All samples of *An. darlingi* collected in Belize were confirmed to be of the northern lineage and all samples from Zungarococha were confirmed to be of the southern lineage.

Wing shape variation within these samples was quantified and visualized using geometric morphometrics. Discriminant analysis of wing shape showed a significant amount of differentiation between the two populations with a positive classification rate of 79%. This study was a first step in trying to characterize and compare phenotypic differences in genetically distinct populations of *An. darlingi*.

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CHAPTER 1: Introduction

MALARIA ENTOMOLOGY AND DOMINANT VECTOR SPECIES

There are an estimated 219 million cases of malaria worldwide. About 80% of these cases are reported from Africa and about 0.5% reported from the Americas (35% of cases from the Americas are attributed to *Plasmodium falciparum*). There are an estimated 660,000 deaths due to malaria, 90% from Africa and about 0.2% from the Americas (188).

Malaria is caused by the protozoan parasite *Plasmodium*. There are five species of *Plasmodium* that can infect humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi* of which *P. falciparum* causes the most severe illness (189). Only the first three *Plasmodium* species listed are found in the Americas (188). Female mosquitoes of the genus *Anopheles* transmit *Plasmodium* parasites. The portion of the parasite life cycle within the mosquito host is called the sporogonic cycle and lasts approximately 8-15 days depending on temperature and *Plasmodium* species. Sporozoites are the infective stage of the life-cycle and are injected into a human host upon mosquito feeding (189).

Malaria control includes the diagnosis and treatment of malaria cases and vector control. Methods of vector control are primarily the use of insecticide-treated bed nets and indoor residual spraying (IRS) and also include larval control, repellents, and house modification that prevent entry of mosquitoes. The most important tool in prevention of malaria transmission is a detailed understanding of the ecology and behavior of local vector populations (189).

To incriminate a vector species you need to know the abundance and proportion of infected mosquitoes, age or parity of the mosquitoes, and the feeding behavior, including where and when a mosquito bites and what host is preferred. This information can be used to calculate vectorial capacity: an index defined as the capacity of a vector population to transmit malaria. Obtaining the measurements for this calculation is difficult and assumptions need to be made, but it is an important concept in the theoretical study of epidemiology and control of malaria (189).

There are about 465 formally recognized species of *Anopheles* mosquitoes, of which, about 70 have the potential to transmit human *Plasmodium* spp. and 41 are considered to be dominant vector species or species complexes (170). Factors that define a dominant vector species or primary vector are: 1) have wide geographical distribution, 2) have high local abundance (often seasonal), 3) have good dispersal and colonizing ability, 4) have adaptability to exploit different man-made environments, 5) have strong preference to feed on human blood, and 6) have high susceptibility to human pathogens (49; 123). In South America, particularly in the Amazon basin, the primary vector species is *Anopheles darlingi* Root, 1926, which exhibits each of the previously mentioned factors.

ANOPHELES DARLINGI ROOT

Anopheles darlingi is classified under the *Argyritarsis* section of the subgenus *Nyssorhynchus* (56) and was first characterized in 1926 by Francis Metcalf Root. Previous to this it was frequently misidentified as *Anopheles argyritarsis* (67; 98). The species has an extensive geographic distribution and can be found from southern Mexico to northern Argentina and from the eastern side of the Andes mountains to the Atlantic

and Caribbean coastline (66; 97; 121; 151). The species has not been identified in Nicaragua and Costa Rica. Recently *An. darlingi* was collected in Panama where it was previously never recorded (102). It is found mainly in regions < 500 m above sea level with temperatures between 21-28°C and high relative humidity (123). It is unable to survive in dry or arid areas. The variable having the highest relative influence (36.31%) on the presence of *An. darlingi* is maximum precipitation (171). Because *An. darlingi* populations are found over such a wide geographic range it is more likely to have a greater habitat diversity (specimens have been reported at altitudes above 800 m (171) and exhibit variations in bionomics across its distribution (27).

History and re-emergence of *Anopheles darlingi* and malaria risk in selected countries

Anopheles darlingi is capable of high transmission even when adult densities are relatively low (66; 83). Wild *An. darlingi* populations within the interior rainforest are capable of transmitting and maintaining malaria among remote and widely scattered settlements and homes indicative of a permanent reservoir with considerable flight range (27). In British Guiana, *An. darlingi* was observed to spread into newly inhabited areas suggesting a level of adaptability within the confines of its tolerable ecological range (66) and it was predicted that human intervention and traffic would propagate invasion into *An. darlingi* naïve areas (68).

In Brazil

The first recorded adult anti-malaria efforts in Brazil occurred in 1905 when Carlos Chagas, investigating an outbreak, noted the endophilic tendencies of the local anopheline species (a characteristic very commonly associated with *An. darlingi* in early

observations) and burned sulfur indoors in an effort to drive out the mosquitoes (48). In 1922 a group from the Rockefeller Foundation came to Brazil to study the epidemiology of malaria in tropical environments to determine if the successful eradication efforts used in the United States could be employed in these areas. Part of this group was Francis Metcalf Root from the School of Hygiene and Public Health at Johns Hopkins University who conducted a thorough survey of the Brazilian subgenus *Nyssorhynchus* and in 1926 first described *Anopheles darlingi*, which had previously been confused with other species and was considered the most efficient vector in the greater part of Brazil (48; 143).

The National Malaria Service of Brazil was created in 1941 and by 1950 was responsible for malaria control in all of Brazil (except for Sao Paulo) (48; 143). Success had been found in the eradication of *An. gambiae*, imported from African ships, decreased incidence of "bromeliad malaria" transmitted by the subgenus *Kerteszia* which breeds in bromeliad plants, and sharp decreases in incidence in most of the northeast and coastal plains. The National Malaria Service became the Malaria Eradication Campaign and eradication was believed to be realistic and close, especially armed with dichloro-diphenyl-trichloroethane (DDT) and chloroquine (48; 143). The only major area where malaria was still endemic was in the Amazon where anti-malaria efforts were logistically harder to carry out due to access issues, longer transmission cycles, poor housing construction negating the potential effectiveness of DDT, and a roughly 40% refusal rate for DDT indoor residual spraying (IRS) (29; 36). Because eradication seemed so real, research efforts began to decrease and focus on other tropical diseases affecting the

country. By 1970 malaria incidence in Brazil was at an all time low, including the Amazon region (48).

By the mid-1960s, many development projects began in the Amazon region funded by the Brazilian government (as well as private interests) including ranching, mining, and small to medium scale farms (36; 43; 45; 178). By the 1970s these projects led to massive deforestation, colonization efforts, highway construction, and the construction of hydroelectric dams (43; 178). Population in the Amazon increased over 2.5-fold between 1970-1996 (45) (up 30-fold from the 1940s (105)). With the increase in population came an increase in malaria incidence, with a notable increase in *P. falciparum* incidence (22). From 1997-2006, Amazonia Brazil had on average over 500,000 annually confirmed cases of malaria. Between 1999-2002, around 12,000-20,000 km^2 of forest was destroyed annually (138). The new settlements being established were occurring at the forest fringes. It has been shown that *An. darlingi* is found more in areas of recent deforestation due to increased availability of larval habitats, changes in feeding sources, and proximity of new settlers (48; 138). The resurgence in incidence in these frontier settlements showed a similar transmission pattern and was known as "frontier malaria".

Frontier malaria occurs in new development areas, which are closest to unaltered forest. There is very little infrastructure, the development of new communities is unplanned, sites might have high turnover due to agricultural failures, and there are high rates of migration of non-immune settlers living in close proximity to each other in temporary or semi-permanent shelters (37; 38; 45; 48). Growing anti-malarial drug resistance (36) and a *Plasmodium* reservoir amongst asymptomatic carriers also

contributed to increased incidence in this environment (37). The pattern of frontier malaria begins with an initial drastic increase of incidence when non-immune settlers arrive at the forest fringes (38). This continues for about 6-8 years, which is about the time for the community to establish itself and maintain a more permanent population center where an organized infrastructure can be established (urban expansion, access to healthcare, more permanent housing, etc.). After about 10 years, malaria levels drop or become low-level endemic (38; 45; 138; 178). Though the Amazon region accounts for about 60% of Brazil's land area it contains about 11% of Brazil's total population, and in the late 1980s this region contributed to 99% of Brazil's total malaria incidence (48; 105).

Early anopheline surveys pointed to *An. darlingi* as the primary vector of malaria in the interior, specifically the Amazon basin. In a survey conducted by Lourenco-de-Oliveira (1989) in Rondonia State, the authors noted a change in anopheline distribution as well as behavior (105). Where about 40 years previously *An. darlingi* accounted for only 26.7% of adults captured and non-*Nyssorhynchus* 14.7%, in 1989 *An. darlingi* accounted for 77.7% and non-*Nyssorhynchus* 0.1% (105). These *An. darlingi* were now observed feeding primarily outdoors or exiting from indoors immediately after feeding where previous surveys noted their endophily (29; 105). In Belem Brazil, *An. darlingi* was considered eradicated in 1968 (based on monthly collections) then was collected again beginning in 1992. Mosquito diversity was increasing in addition to densities. In the 1930s only 2 species of subgenus *Nyssorhynchus* were collected in Belem, in the 1940s it was up to 6 species collected, and in the 1990s, 10 species were collected. The authors indicate that the reappearance of *An. darlingi* and the changing fauna of

Nyssorhynchus might be a result of the urban expansion of Belem into the surrounding forest area introducing anthropogenically altered environments suitable for breeding (144). The same thing happened in Manaus, where *An. darlingi* was considered eliminated from the city by 1976 and was collected in the city and in the peri-urban area again in 1988. The authors noted a clear change in mosquito density and diversity before and after human alterations (177).

In Peru

The greatest malaria burden in Peru occurs in the Amazon Basin, in particular the departments of Loreto and Madre de Dios. The re-emergence of malaria occurred in the mid-1990s with a major epidemic focused in the rural villages surrounding the urban center of Iquitos (8; 39). Endemic *P. vivax* was first reported in April 1991 from the villages of Rumococha and Zungarococha and *P. falciparum* in November 1994 from the village of Padrecocha (8). Iquitos was founded in 1757 and by 1842 was a village with about 200 inhabitants increasing to about 140,000 in the early 1900s as a result of the rubber boom (113; 184). After WWI and the end of the rubber boom, cattle ranching and logging became major economic sources, which turned to petroleum and coca toward the 2nd half of the 20th century (113; 184). During the 1990s, population migration to Amazonia Peru was encouraged through public policies and enhanced by civil unrest in the Andes due to the Shining Path. The rural population of Loreto is approximately 474,000 and current economic activities include subsistence scale agriculture (plantain and cassava root), fishing, hunting, lumber milling, canning, and poultry, cattle and fish farming (8; 184). As a result of government active settlement policies, new industries dependent on natural resources, population influx, and the Iquitos-Nauta highway

construction, deforestation in this region increased to a rate of about 4257 hectares/year between 1983-1995 and the population along this new road increased at a rate of 7.4% as compared with 3.4% in the city of Iquitos (113; 184). Mining activities in the Madre de Dios department attracted non-immune workers beginning in the late 1990s where the majority of confirmed malaria cases occurred in a large gold mining camp. Currently, massive population migration is underway with the construction of the Transoceanic Highway, which will connect the Pacific Coast of Peru to the Amazon region introducing a new malaria-naïve population to a malaria-endemic region (39).

The Peruvian Amazon region was one of the last areas to report a re-emergence of epidemic malaria (8). After the eradication campaign of the 1960s, with only 641 cases (123 by *P. falciparum*) in 1961, the malaria burden in Peru fell and remained low for about 20 years. Malaria prevalence began to increase with 121,268 cases in 1997 (>54,000 by *P. falciparum*, including 85 deaths), and reached a peak in 2005, with *P. falciparum* accounting for about 30% of cases in 2004 (8; 57; 139). From 1992-1997 cases of malaria increased 50-fold in the district of Loreto compared to a 4-fold increase for the rest of Peru resulting in Peru having the 2nd highest prevalence of malaria in South America (behind Brazil) (8) and by 2008 Peru had the 3rd highest number of malaria cases in South America (behind Brazil and Colombia) (139).

The re-emergence of malaria prevalence, in particular *P. falciparum* incidence, is linked to the re-introduction of *An. darlingi* into the region (8). Prior to 1991, *An. darlingi* had not been recorded in or around Iquitos and in 1996, Fernandez et al. (1996) (as reviewed by (167)) reported *An. darlingi* in the vicinity of Iquitos for the first time (8; 167). Schoeler et al. (2003) conducted an extensive survey of anophelines in the Loreto

and Ucayali districts of Peru and found that *An. darlingi* was well established even further west in the Amazon Basin than previously reported. The author postulates that these populations may have always been present, but below detection levels and the discontinuation of vector control programs in conjunction with population expansion could explain the current abundance of *An. darlingi* populations and resultant increase in malaria prevalence (167).

Vittor (2006; 2009) examined biting rates and breeding sites at multiple sites representing different levels of deforestation along the Iquitos-Nauta highway, which is considered a high malaria transmission zone (8; 184; 185). Results showed a human biting rate about 278 times higher in deforested areas versus predominantly forested areas (after controlling for presence of humans) (184) and found that larvae of *An. darlingi* are more likely to be found in areas of secondary growth, which is the type of vegetation that would be present approximately 15 years after deforestation (185).

Though the impact of deforestation on malaria prevalence and vector composition and density has been documented, multiple transmission dynamics are likely depending on regional environmental factors. A study by Parker et al. (2013) showed extremely high levels of malaria transmission in remote areas of human occupation. Sites along the Mazan River, utilized by many laborers to fish, extract wood, and harvest palm leaves used in thatched roof construction, showed entomological inoculation rates (EIR) as high as 5.3 infective bites/person/night (EIRs showed a seasonality). In addition, of those workers tested for malaria, 88% of positive cases were asymptomatic at the time of sampling. These EIR rates are much higher than those of other peri-Iquitos studies focusing on deforested areas, bringing attention to potential malaria hot spots and

transmission from frequently exposed asymptomatic workers to families and communities located in areas with much lower transmission rates (139). This reinforces the potential danger for the spread of malaria if *An. darlingi* further increases its range and density in conjunction with population expansion.

In French Guiana, Guyana, Suriname and Venezuela

The pattern for these four countries is very similar. The DDT house spraying campaigns in the 1940s and 1950s were successful in eradicating *An. darlingi* in the coastal areas where the vector was endophilic and anthropophilic and where target communities were accessible (82; 140). Eradication of *An. darlingi* and malaria in the interior rainforest regions could never be obtained. Reasons for lack of success include native *An. darlingi* exhibiting more zoophilia and exophilia, remote and inaccessible local communities, socio-culturally related refusal for control measures in some areas, housing structures not conducive to IRS methods, and mining activities attracting naive migratory populations, temporary camps, ecological alterations and frequent travel among workers (13; 70; 111; 126; 159).

In Argentina

Before the DDT campaign, early malaria programs (prior to 1947) focused on larval control, engineering, free distribution of drugs. Indoor residual spray (IRS) efforts eliminated malaria in the northeast part of the country where *An. darlingi* was considered the main vector. Beginning in 1982, many control efforts were eliminated and incidence levels increased owing mostly to imported cases from Bolivia. Occupational migration, family visitation, and smuggling contribute to border traffic, which initiated the epidemics accounting for the majority of malaria recorded for the country (40-70%). In

addition, the need for sustainable energy resulting in the construction of multiple dams is creating new breeding habitats for *An. darlingi*, leading to a resurgence of malaria along the Argentina-Paraguay-Brazil border (44).

In Colombia

In Colombia, political conflict and socio-economic factors have lead to decreased surveillance in areas of malaria transmission limiting information and insight into vector dynamics and control efforts (123). Diagnosis and treatment of malaria is inefficient and vector control efforts are inconsistent especially in areas of armed conflict where public safety is of major concern resulting in epidemic outbreaks (18; 58; 123). *Anopheles darlingi* is considered a major malaria vector in many regions of Colombia, especially of *P. falciparum*, and resistance to DDT and the pyrethroid lambda-cyhalothrin has been reported from specific municipalities further complicating control efforts (58; 174). Mass migration, environmental changes due to new development projects, the presence of urban transmission in specific areas, and increased species diversity have renewed concerns of an impending malaria incidence increase (18; 123). The Colombian Social Protection Ministry has responded by increasing surveillance and implementing a broad-based control strategy (123).

In Belize

Anopheles darlingi was first identified from British Honduras in 1939 (98). Previous malaria surveys conducted in 1924-1925 yielded samples of "*An. argyrtarsis*" which were subsequently identified as *An. darlingi* (as this species was not characterized

until 1926). The species had not yet been documented north of Venezuela. Mr. Ivan Sanderson collected 9 (8 female, 1 male) mosquitoes that appeared to be *Anopheles argyritarsis*. The male was found to be *An. darlingi*. The discovery was brought to the attention of the Director of Gorgas Memorial Laboratory in Panama, the Chief Health Officer of the Panama Canal, and the General Manager of the Medical Department of the United Fruit Co. and in 1940 an expedition was arranged to survey the area for evidence of *An. darlingi*. Larvae were collected in Silk Grass Creek and adult females collected in the sleeping quarters of the Forest Reserve camp. The identification of the collected samples as *An. darlingi* was confirmed by Dr. Henry W. Kumm of the International Health Division of the Rockefeller Foundation (98). Dr. Kumm came to British Honduras between July 26 and August 12, 1940 to conduct a quick survey where *An. darlingi* was collected from the Toledo and Stann Creek districts (98; 99). The species comprised 71.8% of anophelines caught in houses and 27.3% of those collected from horse bait (99).

In Panama

Anopheles darlingi was officially documented in the Darien Province of eastern Panama for the first time in 2008 (102). Previously there was a discontinuity in its range in Costa Rica, Nicaragua, and Panama. Though the species may have been present for some time, it was never collected or misidentified. There were a reported 5,095 malaria cases in 2004, which represented a 6-fold increase in prevalence since 2001. There is a history of malaria cases, with an almost exclusive incidence of *P. falciparum* (including drug-resistant strains) in eastern Panama where *An. albimanus* is not as prevalent. Over 70% of this region is still covered in forests, which provide ideal breeding habitats for

An. darlingi. It is proposed that the presence of *An. darlingi* is the result of a recent introduction from Colombia that was brought about by environmental disturbance from deforestation and landscape changes due to increases in tourism to the area. Future environmental pressures resulting from increased tourism and migration of refugees fleeing armed conflict in Colombia could possibly expand the range of this efficient vector to other areas within Panama (102).

Though the situations are unique to each country's demographic, environmental, and political circumstances, with the exception of Belize, there is a common pattern to the distribution and re-emergence of *An. darlingi*. Early malaria control efforts using IRS with DDT were successful in coastal and urban areas. Less success was found in the Amazon Basin where natural populations continued to thrive. Amazon frontier expansion increased anthropogenically driven ecological changes and mass population migration. Established *An. darlingi* populations were able to adapt and thrive under these new environments increasing malaria transmission. *Anopheles darlingi* is an adaptable species, at least within the confines of its ecological boundaries, and was able to flourish in these environments spreading malaria as it did. It has been suggested that the wild *An. darlingi* populations inhabiting the interior rainforest and the peripheral populations inhabiting the coastal areas are cryptic species (66). Multiple research efforts have addressed this theory.

Incrimination

Anopheles darlingi is considered the dominant vector in South America, in particular the Amazon basin, and is among the most efficient vectors in the Neotropical

region (41; 83; 170). It is considered to be the primary vector of malaria in multiple locations throughout Brazil (47; 62; 145), French Guiana (53; 173), Suriname (158), and Venezuela (111; 127). Populations of *An. darlingi* across is geographical distribution have been found naturally infected with 3 *Plasmodium* spp. (see Table 1 for summary). The infection rates in natural mosquito populations are used to calculate the entomological inoculation rate (EIR). The EIR is a measure of transmission intensity that estimates the average number of infective bites received by a person per unit time. The EIR for *An. darlingi* varies across its range.

Larval habitats

In general, *Anopheles darlingi* prefers to breed in clear natural bodies of water that have relatively stable currents (see Table 2 for examples). These habitats are associated with floating debris, are partially shaded, and maintain a neutral pH (27; 61; 83; 159; 171). *Anopheles darlingi* has been found in many bodies of water to include lagoons, lakes, swamps, and slow flowing rivers or streams (83; 171).

Often larvae are associated with floating debris or detritus patches (pieces of wood, dead leaves, flowers & seeds) as well as submersed vegetation such as *Cabomba* in Belize and *Pistia* and *Eichornia* in Brazil (115; 171). These formations are key determinants in Belize for identifying *An. darlingi* larval habitats (99; 115; 143). Achee et al. (2006) found that the presence of detritus accounts for a significantly higher density of *An. darlingi* larvae and Grieco et al. (2007) observed that the overall survival rate of *An. darlingi* decreased when larvae were exposed to atypical habitats (4; 78).

Anopheles darlingi has been shown to exploit man-made breeding habitats resulting from deforestation, mining, and aquaculture which suggests a level of

adaptation which increases its contact with humans (171). In Brazil, *An. darlingi* larvae have been collected in small and large temporary ponds in an area where a hydroelectric dam was under construction (124) and from irregular excavation pits associated with manual brick manufacturing (40) and gold extraction (63). Even in these temporary man-made habitats, *An. darlingi* is only found in unpolluted water with neutral pH (40; 63; 123). Charlwood (1996) reports of manmade pools resulting from poorly made culverts that are exploited by *An. darlingi* (27). In Iquitos, Peru, Vittor et al. (2009) found that bodies of water with a larger circumference, usually attributed to fish farms, were positively correlated with the presence of *An. darlingi* larvae (185). Maheu-Giroux et al. (2010) followed up on this finding and observed a significant relationship between the density of fishponds and the presence of *P. vivax* malaria (112).

Levels of deforestation have been implicated in recent formation of breeding ground for *An. darlingi*. Vittor et al. (2009) found that a significant determinant for larval habitats is the presence of secondary growth, which occurs about 15 years after deforestation (185). The study also determined higher odds of finding *An. darlingi* in bodies of water with algal mats and within a 500 m radius of people (185). Proximity to humans was also a factor found for breeding sites in Roraima State, Brazil in deforested or forest fringe areas. The other 2 main factors for habitat suitability in these areas are in line with general *An. darlingi* preferences including 1) increased number of "microdams", which are limbs or debris that block the flow of water creating more stable conditions and 2) shade (133).

There were multiple methodologies used to assess the determinants of breeding habitats. Descriptions were made about sites where greater numbers of *An. darlingi* were

found (99). Other studies measured relative abundance of larvae using a defined protocol and recorded different site characteristics, for example, surrounding vegetation, light incidence, pH, shade, turbidity, current, etc. (5; 11; 185), and calculated correlations between these observed characteristics and the presence of *An. darlingi* larvae. Observations should be considered carefully; for example, in Belize overhanging bamboo had been associated with preferred larval habitats. Achee et al. (2006) evaluated this indicator experimentally and determined that the bamboo itself was not attractive, but that it most likely aided in formation of detritus patches by blocking water flow (4). Maheu-Giroux et al. (2010) determined that fishpond density in Peru contributed to increased malaria risk using a retrospective cohort study, however, no larval collections were made to verify if these were confirmed breeding areas (112). Variation not only exists in the breeding habitats of *An. darlingi*, but in the experimental designs aimed at describing and quantifying these habitats as well.

Biting behavior

Throughout its range, *An. darlingi* is endophagic frequently entering houses to feed. Early publications emphasized its domesticity including Giglioli (1948) in British Guiana, Vand der Kuyp (1954) in Suriname, and Komp (1941) in British Honduras. This feeding preference contributed to initial success of the DDT campaign (27). Variation has been shown in endophagic and exophagic tendencies (84; 131; 175). In certain areas, even though *An. darlingi* is the prominent, if not the only, species collected indoors, the total density collected outside is much greater (70; 105).

There is heterogeneity found in temporal biting patterns for *Anopheles darlingi* across its wide distribution as well. Many factors can influence biting cycles including

mosquito density, seasonality (rainy or dry season), distance to breeding sites, host availability, mosquito age composition, and moon phase (27; 177). Temporal trends and densities vary according to geographic location and season, but the consistency of these temporal feeding trends within each geographic location season after season suggest an endogenous genetic factor as well (27).

In general, *An. darlingi* show either crepuscular or nocturnal trends with prominent peaks (though not always). When peaks are prominent they have been characterized as unimodal, bimodal and in some instances trimodal. These biting trends are varied throughout *An. darlingi's* range (see Table 3 for summary). Unimodal biting patterns usually occur later at night and examples can be found in Brazil (149; 186), Bolivia (80), Colombia (54), Suriname (84), and Venezuela (160). Bimodal patterns usually are crepuscular and examples can be found in Belize (6), Brazil (92; 151), and Venezuela (126). Trimodal patterns are not as common and usually result from crepuscular biting with a third peak in the middle of the night and examples can be found in Brazil, French Guiana, and Peru (154; 191). There are also patterns that cannot be determined, which can sometimes be attributed to low mosquito or seasonal densities. These patterns generally consist of continual biting through the night with no prominent peaks. For example, in Boa Vista, Roraima State, Brazil there were seasonal differences in biting patterns, but biting occurred throughout the night with no prominent peaks (40). Likewise in 3 villages located in the Upper-Maroni river of French Guiana, maximum activity was reached between 2130-2330 and remained high throughout the night with slightly elevated activity again between 0130-0330 showing no prominent peaks (70).

Peak seasonal densities in relation to rainy or dry season vary throughout *An. darlingi's* range as well. For example, in Suriname alone, Rozendaal (1987) found that one site had a peak density during the rainy season while another had higher densities in the dry season most likely a result of the geomorphology of the river systems where, during high rains, one river system would create flood plains, an ideal breeding habitat for *An. darlingi*, and during the dry season the river banks would create another ideal breeding habitat for *An. darlingi* (156).

Differences in mosquito population and seasonal density can cause variation among individual sites making the data harder to interpret. Forattini (1987) (as reviewed in Zimmerman 1992) suggested that distinct populations do exist, but because we have no method that can link these behaviors with any genetic polymorphism we cannot know the reason behind the variation. Essentially we are looking at individual snapshots in time (191).

Time, funding, personnel, and logistical constraints can be key determinants in field study designs. These factors can determine the amount of time that is spent on data collection, restricting the ability to perform long-term studies and contributing to the collection of individual snapshots in time. Caution needs to be taken when attempting to compare temporal peak activity. First, the number of consecutive collection nights should be considered. Examples of variation include a single 12-h collection night/month (175), 3 12-h collection nights/month (186), 2 consecutive nights bimonthly (11), and 12 consecutive nights (80). *Anopheles darlingi* abundance can vary from night to night; therefore peak activity found in a specific population might not be entirely representative of natural behavior. Second, the initiation time of collections should be looked at closely.

Most studies begin at sunset or 1800/1830. Since mosquito behavior is driven by photoperiod, time of sunset should be confirmed with the start time of the collection period. Third, attention needs to be placed on how mosquitoes were collected. Earlier studies were measured by human biting collections (54), which has been replaced by human landing collections (HLC). Though, this is a good estimator of biting, it is possible that total numbers of mosquitoes might be affected if the mosquito population does not always bite after landing/probing. When comparing HLC/biting to window interception traps, peak activity times will most likely be off as house entry (in the case of indoor HLC collection) precludes landing and biting. These factors demonstrate the need for long term year round data collection using similar collection methodologies to allow for direct comparison between geographical populations of *An. darlingi*.

Host preference

Host preference of a mosquito vector is driven by intrinsic genetic tendencies to respond to particular host cues and is heavily influenced by extrinsic environmental factors including host availability and abundance (14; 19; 27; 107; 192). Currently, there is minimal insight into how the genetic make-up of a species drives this behavior. It has been shown that carriers of certain chromosomal arrangements and karyotypes in the *An. gambiae* complex are more associated with human habitations and human blood (14). Comparative studies on host preference between the recently defined nuclear *white* gene northern and southern lineages of *An. darlingi* are lacking.

There are multiple methodologies and indices to observe mosquito feeding behavior. Traditional approaches include 1) analysis of mosquito blood meals: indicative of host selection (pattern of natural feeding at a particular place and time) and 2)

collection rates using a specific host type as bait: indicative of host preference (attraction to one particular host over other equally available hosts) (17). Because these approaches are really only measuring the endpoint (in a particular place and time) of a complex genetic and environmental interaction, inherent shortcomings exist for both methodologies that result in an incomplete picture of "true" host preference (14).

Blood meal analysis

To analyze mosquito blood meals, engorged resting mosquitoes are collected from a target area, usually inside and outside houses and in nearby vegetation, crushed, and then subjected to an ELISA (or alternative diagnostic assay) to determine the source of the blood meal. There are multiple indices to describe the results, two of these are 1) human blood index (HBI) and 2) foraging ratio (FR). The HBI is calculated as the number of positive feeds on humans divided by the total number of mosquitoes that tested positive for any host and can be used as an indicator to the degree of anthropophily (189). This metric only considers human hosts and does not take into account the relative abundance and availability of alternate blood sources. This metric is an important component of calculating vectorial capacity of malaria transmission (19; 65), but does not fully characterize the host preference of the target species. The FR index represents host preference more so than HBI as it is the proportion of positive blood meals of a particular host in respect to all available hosts in the study area where a value > 1 indicates a preference for that host type (76).

The first challenge of blood meal analysis is the acquisition of resting mosquitoes. Accurate interpretation of either index requires an unbiased sample of engorged females (64). *Anopheles darlingi* has been found resting indoors, on the outside of houses, and in

vegetation near domiciles (27; 192), but no preferred resting spots have been defined. With recorded flight ranges of 7.2 km (28) and 800 m (3) of re-captured *An. darlingi* from release points, it is very possible that preferred resting sites might lie outside of the study area (of which generally has no defined boundaries). The second challenge is conducting a census of animal abundance and diversity to ascertain all available host choices. It is only really possible to take a census of the domestic and larger animals in the area. Limitations in diagnostic assays should also be taken into account. Only specific animal species can be observed and identified as blood meal hosts. In addition, considering flight range again, the foraging area of *An. darlingi* might be much more vast than the immediate study site.

Animal attraction studies

The second approach is to collect target mosquito species directly from the host (or en-route to the host if using interception traps) to compare differences in mosquito abundance and diversity. This method does not represent natural foraging tendencies of the target mosquito species as it is creating an artificial feeding opportunity. It compares feeding preference for one host species over another. Challenges for this approach include minimizing variables that could bias results, mainly through a consistent study design. Hosts should be of similar body mass (it has been shown that *An. darlingi* has a preference for larger mammals (27)), there should be a consistent design for the way hosts are presented and mosquitoes are collected, interference in host cues due to presence of human collectors should be minimized, alternate host choices, at least from the direct collection area, should be minimized, and any potentially confounding factors that can be controlled for, should be (14).

Host feeding in An. darlingi

Variation occurs in the host preference of *An. darlingi*. The species is associated with high anthropophilic and anthropophagic tendencies, supported by noted endophilic and endophagic tendencies (11; 27). From the earliest reports characterizing vector behavior, *An. darlingi* was the predominant, if not the only, *Anopheles* species found biting and/or resting indoors (66; 98). Abundance of *An. darlingi* collected outdoors away from human dwellings and closer to the forest fringes is lower than that of intra- and peri-domiciliary collections (179). Opportunistic qualities of *An. darlingi* have been reported, particularly in the Brazil interior. As reviewed in Giglioli (1956), in the interior rainforest of Brazil, at the center of dispersal, *An. darlingi* exhibits more zoophilic and exophilic behavior than in the coastal districts, at the periphery of dispersal, where *An. darlingi* exhibits more anthropophilic and endophagic behavior (a contributing factor as to why IRS with DDT was less successful in the Amazon Basin). It should be noted that this behavior was not an effect from control measures as the same pattern, including *An. darlingi* resting on the exterior walls of homes, was observed in areas where DDT had not been used (66).

Blood meal analysis of *An. darlingi* was conducted in large cage experiments carried out in Belem, Brazil. Inside the cage were potential host choices: horse, cow, dog, chicken and human. Results showed that 46% of captured *An. darlingi* fed from a human host, 29% from a cow host and 13% from a horse host (27). Host preference would have been affected by the relative sizes of each host and perhaps the location of each host within the cage, however, the study design did control for host availability. In Amapa State, Brazil, Zimmerman et al. (2006) performed a blood meal analysis of *An.*

darlingi collected resting from underneath houses and in vegetation behind houses in three separate villages. Of the total number of *An. darlingi* that tested positive for any blood meal host for all villages combined, 13.1% (HBI 0.131) were positive for human blood. These data varied from 1.7% (HBI 0.017) – 40.5% (HBI 0.405) depending on the village and the site (house vs. vegetation) collected from. The most abundant blood meal source were bovines at 63.5% of total positive samples, which were predominantly collected from the vegetation at one village. A census of total bovine abundance was not feasible as the cattle were not in or near the villages. Following bovine hosts, 17.7% of positive blood meals were from pigs, 13.5% from dogs, 4.5% from rats, and 1.4% from chickens. Based on concurrent adult surveys, the authors expected a higher proportion of mosquitoes to be positive for human blood. This could imply that mosquitoes travelled further than the collection area for resting (192). Though humans ranked 4th in terms of positive blood meals, of other *Anopheles* species collected, *An. darlingi* demonstrated the second highest HBI. These results indicate that host availability plays in important role in host preference for *An. darlingi*. Analyzing the FR, in Belize, Grieco et al. (2002) used manual aspiration inside and outside houses, backpack aspiration of vegetation, and a mobile truck trap to collect *Anopheles* species. Only 10 *An. darlingi* were collected (1.0% of total anophelines collected) and an FR of 7.69 was found for humans inside the house and 5.2 outside the house (as one mosquito collected outside had fed on a cow) (76). Though these numbers are low and do not necessarily represent biological significance it is worth mentioning to re-enforce the anthropophilic tendencies of this *An. darlingi* population and to highlight that FR for humans and HBI will be higher when mosquitoes are collected within and around houses.

Using a non-illuminated bovine-baited trap, in Brazil, Klein et al. (1991) collected approximately 80% of *An. darlingi* from a human host and 20% from a the bovine host. The human hosts collected mosquitoes that landed on their exposed feet and legs and then from a non-illuminated bovine-baited trap during a 13-hour collection night (93). Sampling bias could be present as the bovine host was underneath a netted trap, which introduces an additional physical barrier and could also affect the emanation of host cues from the bovine host. Also in Brazil, Oliveira-Ferreira et al. (1992) performed comparative captures from an animal (always a cow with the exception of a single collection using a horse) and human host situated approximately 4 m apart in open terrain. Collections were performed from 1800 – 2100. *Anopheles darlingi* was the predominant species collected from the human host (71.5%) and was collected more frequently from the human host (65%) as compared with the animal host (46). It is unclear from the literature who performed the animal host collections and where they were situated. Each host stood only 4 m from each other, which could interfere with host cues. In addition, collections were performed during 3 hours at sunset. Though this might be the peak biting time for *An. darlingi* in this location, it might not give a clear picture of feeding tendencies throughout the night.

The overall results show that *An. darlingi* feeds on and is attracted to humans, especially in relation to other *Anopheles* species, host abundance and availability play a major role in host choice and should be factored into data interpretation, and studies looking at host feeding behavior of *An. darlingi* are few and variable. The inconsistency in methodologies makes it difficult to compare these behaviors across *An. darlingi*'s range. Standardized methods, ideally utilizing both approaches discussed above, would

be beneficial for comparative host preference studies of *An. darlingi* to better understand malaria transmission

EXPERIMENTAL HUTS

Experimental huts have been used since the 1940s when Haddow et al., in an effort to minimize confounding variables present in local houses, constructed custom standardized huts for use in mosquito collection in Kenya (137). Experimental huts mimic local housing structures and therefore represent conditions that exist in actual local communities. They allow the researcher to control variation resulting from household size, house construction materials, poorly constructed homes, distance to breeding sites, and the internal environment of the house, for example, presence of furniture (137). The huts are designed to allow for window, eave, and veranda traps for mosquito interception either entering or exiting the structure (132; 172) and can be easily modified to address a range of hypotheses. Experimental huts are ideal and instrumental in field studies examining the effect mosquito control strategies such as the effects of IRS, Push-Pull, house screening, and use of insecticide treated bed nets have on the behavior of local mosquito populations (25; 132). They can also be used untreated to measure baseline data important to understanding general bionomics of local vectors such as, indoor and outdoor biting behavior, portals of entry, entrance and exit patterns, and indoor resting behavior (25; 75; 151; 157). This data can then be used to assess the efficacy of selected treatments. Achee et al. (2005) used a portable hut design to measure the recapture rate at different distances of marked *An. darlingi* released from a fixed point (3). In Thailand, Suwonkerd et al. (2006) evaluated the flight behavior of *Aedes aegypti* in a mark-release-recapture study in response to human, dog, and no bait (176). Gillies in, 1964, used an

experimental hut divided into two rooms for separate hosts to induce a rapid change in host selection on two variants of *An. gambiae (69)*. These three examples demonstrate the versatility in experimental hut design and use. In summary, experimental huts are an effective tool in the study of medically important anthropophilic mosquito vectors.

GEOMETRIC MORPHOMETRICS

Morphometry is the measurement of external shape or form. Morphometrics, as defined by Bookstein (1996), is the "quantitative study of biological shape variation" (15). The (co)variation can be within and between populations of samples and/or with other (a)biotic variables (187). Whereas traditional morphometrics uses multivariate statistical analyses on sets of variables such as measurements of length, angles, and ratios, geometric morphometrics uses statistical analyses on shape configurations defined by specific landmarks (7; 87). A major advantage of the latter method is that the shape configurations are preserved throughout statistical analysis so that differences in shape between samples and groups can be visualized. Results of statistical analyses are designed to identify and quantify location and direction of variation (122; 187). The landmark configurations contain descriptions of size as well as shape and allow researchers to compare phenotypes of individuals, populations, and species.

Geometric Morphometrics "Revolution"

In the 1980s there was a paradigm shift in the field of morphometrics that was termed a "revolution in morphometrics" (7). Previous to this, there were two approaches to describing shape: 1) multivariate analysis of arbitrary measurements of distances, angles, and ratios which were visualized by scatter plots and numeric matrices and therefore lost the original shape of the object and 2) deformation grids, classically

associated with D'Arcy Thompson, which were hand-drawn graphical representations of shape changes between two objects visualized as distortions at specific Cartesian coordinates which lacked quantitative variables to allow for comparisons and analyses of variation in a sample (15; 122). What was needed was the combination of both disciplines so that shape variation could be statistically analyzed across a sample set while at the same time visually represented to glean biological significance of the results. The major obstacle was that multivariate methods could not apply to landmark data and maintain the original configurations as the analyses occurred in a separate abstract space from which could not be returned to visualize the results on the original configurations (15; 110). In addition, whole landmark configurations needed to be transformed into unique variables in order to be effectively analyzed by multivariate methods (15). Three independent concepts developed at the same time that overcame these obstacles: 1) David Kendall's theory of shape space, a Riemannian manifold where configurations of landmarks are represented as single points and multivariate methods can be applied to these shapes in a space tangent at the point of the average shape, 2) multivariate statistical methods that can be applied in the tangent space at the average form, and 3) the thin-plate spline which allows for the computational realization of D'Arcy's deformation grids (15; 16; 122). These new methods, in conjunction with capabilities of computers, have made it possible to analyze and visualize shape variation, defined by landmark configurations, across large data sets.

Landmarks

Landmarks are distinct points that are homologous on all samples that are being analyzed and will define the shape configurations (87; 187). In addition, any shape

variation that may occur can be visualized at each individual landmark (87). All analyses, observations, and conclusions are based on these configurations so landmark points should be chosen thoughtfully and should be easily identifiable. There are two types of landmarks: 1) Type I landmarks occur at discrete defined positions such as wing vein intersections and 2) semilandmarks which lie on boundaries or the curvature of an object and also incorporate extrema or distal points (110). Landmarks are raw coordinates that first need to be placed in a common frame of reference before any shape analysis can be applied.

Generalized Procrustes Analysis (GPA) (aka Procrustes superimposition, Procrustes fit, Generalized Least Squares)

In order to compare shape differences between two or more landmark configurations you must first put them into a common coordinate system. This is achieved by removing the effects of size, orientation, and location on the raw coordinate configurations. The procedure is known as Generalized Procrustes Analysis (GPA) (87; 187). First the centroid size of each sample is calculated. Centroid size is the square root of the sum of squared distances from each landmark to the centroid or geometric center of each landmark configuration. Centroid size is generally used as the measurement for global size of each sample. Each sample is then (generally) scaled to a centroid size of unit 1 and lined up so that the centroids are at the origin (0, 0) of a new coordinate system. The landmark configurations are then rotated around the centroid so that the sum of squared deviations between each equivalent landmark is minimal. The new coordinates created from this process are called Procrustes shape coordinates and can be used in further statistical analysis (7; 15; 122; 152; 187). These coordinates are

considered shape variables as they have been corrected for size, position, and orientation. The landmark configuration that is the average of the new Procrustes shape coordinates is called the consensus configuration. Differences in distance between equivalent landmarks in the consensus configuration and the samples are called Procrustes residuals, while differences in distance between equivalent landmarks of two different samples are called Procrustes distances, both are measurements of shape variation (95; 122; 152; 153). These new shape coordinates can now be statistically compared and represented visually. General Procrustes Analysis satisfies multiple purposes. First it removes size, orientation, and position from a group of comparable shape configurations and creates a new set of coordinates that can be utilized for direct comparison. Second, it creates a mean consensus configuration that serves as a reference point to measure variability in shape. Third, by superimposing all samples against this consensus configuration it allows the visualization of differences at specific landmark positions (95; 152).

Shape space

The process of superimposition places these shapes in a new shape space called Kendall's shape space. After scaling, reorienting, and aligning, shapes now occupy a space that has a 2p-4-dimensional (for 2D objects) Riemannian manifold (also called a morphosphere or hypersphere) where p equals the number of landmarks. Points within this shape space correspond to a complete landmark configuration. The distances between these points represent the difference in shape between two configurations. Because the shape space is non-linear (non-Euclidean), points must be projected onto a linear Euclidean space that is tangent to Kendall's shape space in order to be subjected to multivariate statistical analyses. Points and distances between points in the tangent space

are a good approximation of distances in Kendall's shape space. Because a linear approximation will be optimal when the point of tangency is as close as possible the points being analyzed, the point of tangency is the point that represents the mean consensus configuration (122; 152; 153; 187).

Geometric morphometrics applied to entomology

Geometric morphometrics is appearing more frequently in the literature (7). It has been used to evaluate shape and size variation in the wings of medically important species. This information can be used to discriminate between and among species and species complexes, assess environmental influences on size and shape, contribute to phylogenetic relationships, and quantify phenotypic variation.

In Triatominae

Geographic morphometrics was first introduced to the examination of *Triatominae* in a study which supported that *Rhodnius robustus* and *R. prolixus*, suggested to be a single species with separate ecotypes (sylvatic & domestic), are indeed two separate species and should be targeted separately in elimination interventions (183). From there it was shown that a single wing from a single individual could be assigned to its correct parental line using geometric morphometrics and these methods could be used to detect and identify species and population structures in the aim of identifying sources of re-infesting species (50; 51). Wing shape data also support that speciation could be the result of hybridization of two ancestral taxa (33). Studies on the temporal influence of variation showed that seasonal variations occurred in size, whereas, variation in shape was more prominent after multiple years suggesting a greater genetic influence on shape (165). Size is influenced more by environmental factors, whereas shape is influenced

more by genetic factors. Data showed that *Rhodnius pallascens* reared at a higher density and increased food availability, representing domestic species, demonstrated a faster development time and a smaller size. *Rhodnius pallascens* reared at lower density with lower food availability, representing sylvatic species, demonstrated a slower development time and larger size (148).

***In* Aedes aegypti**

Geometric morphometrics on wing shape was able discriminate between 4 Thai laboratory lines of *Aedes aegypti*, though size contributed significantly to variation. Size is strongly influenced by environmental factors and it was indicated that there was a decrease in size with successive generations under laboratory conditions. The cause of the variation was not clear. In a later study, data supported that the cause of shape variation in separate lab lines created from parents collected at the same place, time, and developmental stage was most likely due to genetic drift (88; 89). In another study, geometric morphometrics did not demonstrate divergence, while divergence was demonstrated using microsatellites. It was suggested that shape did not change significantly because it is stabilized by selective pressure (182). Other data shows that size and shape are positively correlated with Relative Humidity at the embryonic stage of development (125) and that size is negatively correlated with larval density and positively correlated with food availability. This data indicates a possible danger in just reducing vector larval density, as it could increase adult size of survivors and therefore vector potential, and supports control methods aimed at the elimination of organic material from breeding sites (90).

In other insect genera

Geographic morphometrics has been used to discriminate, via shape variation, between geographically separated or isolated species of insects. This has been demonstrated in *Glossina palpalis gambiensis* (tsetse fly) between 3 populations (2 mainland and one island population) (24), in *Apis mellifera* (bees) where landmark configurations from a single radial cell could discriminate between 3 racial groups (USA Italian bees, German Carniolan bees, and Africanized honey bees) and between Africanized bees from Brazil in 1968 and Africanized bees from Brazil present day (59; 60). The methodology was also used in honey bee species (*Apis* spp.) native to Thailand where landmark configurations from the forewing were able to successfully discriminate between 4 different species (147). In *Drosophila subobscura*, temperature was shown to affect wing size as well as variation in specific landmarks suggesting that shape may not be independent of environmental pressures (86).

In evolutionary developmental biology (evo-devo)

Geometric morphometrics can be valuable when trying to assess evolutionary development of molecular mechanisms that affect phenotypes dependent on natural selection. Shape, for example, is influenced by many genes located throughout the genome. One study target is fluctuating asymmetry in the shape of insect wings. Fluctuating asymmetry is the result of small random variations between the left and right side. Since the difference occurs on the same individual that has one genome and has been exposed to the same environmental influences, they would most likely occur in developmental processes. How and when would selective pressures trigger genomic variation to affect phenotypic variation and how have these processes evolved (94; 95)?

In Neotropical mosquito species

Geometric morphometrics was demonstrated to be a useful and cost-effective method for discrimination between closely related species in Colombia and Brazil. Discriminant analysis correctly classified 97% of females in the Albimanus Section and 86% in the Argyritarsis Section and allowed for the correct identification of 3 sympatric species from Putomayo which have been difficult to identify in the adult female stage (23). Similarly in *Kertszia* species in Brazil, geometric morphometrics correctly assigned *An. cruzii, An. homunculus*, and *An. bellator* to the appropriate species 78-88% of the time (104). Again in Colombia, a study on members of the *Albitarsis* complex found 2 molecular operational taxonomic units (MOTUs) using COI DNA bar-coding; *An. albitarsis* I and *An. albitarsis* F. Neither analyses using the nuclear *white* gene, ITS2 sequences, nor wing geometry supported the MOTU differentiation. In this case, geometric morphometrics supplements molecular data to demonstrate that these two MOTUs are in fact a single species and perhaps just mitochondrially differentiated populations or perhaps the character of wing shape is not effective at distinguishing between these species of the *Albitarsis* Complex. This was the first study to evaluate these MOTUs (72). There was one study that looked at wing shape variation in *Anopheles darlingi* in 5 ecoregions of Brazil. The authors show that certain populations are more similar to others, but these populations do not correlate with geographic location. The results support that *Anopheles darlingi* represents a meta-population in the studied areas of Brazil (129).

POPULATION GENETICS

Population genetics is the study of allele frequency within and among populations of target species with the goal of assessing gene flow and demographic and evolutionary

history (49; 103). Understanding gene flow and identifying physical or ecological barriers to gene flow is essential for predicting spread of epidemiologically relevant genes (ex. insecticide resistance and parasite refractoriness) (49; 103). Demographic history of a population can give insight into colonization events and geographic distributions enabling researchers to interpret the effects that geological, climate, and anthropological changes might have on a specific population (103). Overall, population analysis could lead to a better understanding of malaria epidemiology and transmission dynamics (49; 103).

Briefly on cryptic species

In addition to assessment of gene flow, population genetics can be used to determine the presence of species complexes, which are comprised of cryptic species. Cryptic species are genetically distinct, reproductively isolated, morphologically indistinguishable, and can vary in vector status (155). Two examples of species complexes are 1) *An. gambiae* complex and 2) *An. albitarsis* complex

The *Anopheles gambiae* complex is comprised of 7 morphologically indistinguishable species that vary in their distribution, ecology, and ability to transmit malarial parasites. The fresh water species are *An. gambiae sensu stricto* (s.s.), *An. arabiensis*, and *An. quadriannulatus* and brackish water species are *An. bwambae*, *An. melas*, and *An. merus* and there was a later division of *An. quadriannulatus* into two species. *Anopheles gambie s.s.* and *An. arabienses* are the major contributors to malaria transmission with *An. gambiae* being one of the most highly anthropophilic mosquitoes known (71; 101). Differences in host preference exist within the complex with *An. quadriannulatus* being almost exclusively zoophagic. *Anopheles arabiensis* is an

opportunistic feeder preferring humans, but can be diverted to feed on animals. The other species are considered zoophagic, but will readily feed from humans (14).

The *Anopheles albitarsis* complex includes 6 species: *An. albitarsis, An. oryzalimnetes, An. marajoara, An. deaneorum, An. janconnae* and *An. albitarsis* F. Behavioral differences including endo- and exophily have been observed (134) and *An. deaneorum, An. marajoara,* and *An. janconnae* have been incriminated as vectors of malaria in Brazil. All except for *An. deaneorum* are morphologically indistinguishable from each other (117; 130).

Identification and discrimination between cryptic species is important for determining malaria transmission risk and developing successful cost-effective vector control strategies. Because of variation found in behavioral, ecological, morphological and genetic characteristics, it has been suggested that *An. darlingi* is a species complex.

Determining *An. darlingi*'s status as a cryptic species

In regards to the use of population genetics to determine the status of *An. darlingi* as a cryptic species or species complex, there has been a sort of evolution (see Table 4 for summary). Using peak biting behavior, iso-enzymes, and cuticular hydrocarbons, Rosa-Freitas et al. (1992) evaluated 3 mosquito populations from Brazil and determined that, even though 3 different biting patterns were observed, the populations could not be considered as separate species (154). In a similar study of multiple markers, Manguin et al. (1999) used isozymes, random amplified polymorphic DNA (RAPD-DNA), internal transcribed spacer 2 (ITS2), and morphologic characters on *An. darlingi* populations from 7 countries (Belize, Bolivia, Brazil, Colombia, French Guiana, Peru, and Venezuela). A deletion of CCC in the ITS2 sequences was found in the Belize population and the

distribution for wing types were found to be population dependent, but ultimately the results did not support *An. darlingi* as a cryptic species (116).

When Conn et al. (1999) used mitochondrial DNA restriction fragment length polymorphism (mtDNA RFLP) to analyze haplotype frequencies in *An. darlingi* from Bolivia, Brazil, and Venezuela, there was a significant correlation between genetic and geographic distances supporting isolation by distance (IBD) (31). In a later study using microsatellite markers, Conn et al. (2006) assessed population structure in *An. darlingi* from 7 locations in Brazil to look for evidence of a bottleneck in populations that had a history of insecticide use. No evidence was found for a population bottleneck, however, differences between locations north and south of the Amazon River were found, suggesting a degree of genetic isolation, which was again attributed to IBD (32).

Malafronte et al. (1999) used ITS2 to evaluate *An. darlingi* populations collected from 5 States within Brazil. The populations were almost identical except for a 4-5% sequence divergence observed from the Dourado population. Further studies need to assess the status of this population (114). In Iquitos, Peru, Pinedo-Cancino et al. (2006) used RAPD-PCR to analyze genetic variation in *An. darlingi* collected from areas with varying degrees of deforestation. Results fell within the range of intra-population variability and the purported populations were determined to be homogenous allowing for control efforts to treat them as a single unit (142).

Mirabello & Conn (2006) used mitochondrial cytochrome c oxidase 1 (mtCO1) to examine genetic relatedness and phylogeographic structure of populations from 19 localities throughout Central and South America. Two clusters were found: 1) South America and 2) Central America & northwest Colombia. Cluster 1 could be further

divided into: 1A) southern Amazon and southern South America and 1B) northern Amazon. Results showed little to no gene flow between Central America and northern Amazon suggesting that a barrier exists, which could be the mountain range crossing Costa Rica and western Panama (119). It was determined that a deep divergence existed in *An. darlingi* populations between genotype 1 (Amazonia and southern Brazil) and genotype 2 (Central America, Colombia and Venezuela) using the nuclear *white* gene (a singe copy protein-coding gene that is a homologue of the *Drosophila melanogaster* eye color gene), ITS, and CO1 genes. These results were interpreted as incipient speciation within *An. darlingi* (118). The divergence was confirmed using microsatellites (121).

It is unknown whether these two gentoypes confer differences in phenotypic expression that would affect the vectorial capacity of *An. darlingi*.

BRIEFLY ON SPECIATION

Speciation is the process by which populations, or a subset of a single population, become reproductively isolated (34; 169). Barriers to gene flow can be caused by divergent selection or evolution of specific traits that prevent gene exchange (34; 169). Divergent selection can be further characterized by ecological (sometimes referred to as natural) and sexual selection. Ecological speciation occurs when populations adapt to different environments fixing alleles that enable the population to thrive in that environment, but not necessarily in another environment (9; 166). Sexual selection arises as the result of reproductive success of the populations in question and is driven by the co-evolution of male signaling and female preferences (21; 34). There is currently a question of the strength that sexual selection has on speciation and the possibility that it may be a special case of natural selection (21; 163). It can be hard to distinguish the

roles between these two forms and most likely in many cases speciation is a combination of both factors, sexual selection affecting mating patterns and ecological selection affecting alternative traits involved in eventual speciation (21; 108).

The driving mechanisms for barriers to gene flow can be further categorizes as prezygotic and postzygotic. Prezygotic isolation mechanisms reduce the probability of populations interbreeding and include assortative mating and selection against migrants that would be at an ecological disadvantage. Postzygotic mechanisms occur after fertilization and include hybrid inviability and sterility (34; 166)

Spatial relationships of speciation can be defined as being primarily allopatric, parapatric, or sympatric. Allopatry occurs when populations are physically isolated from each other so that interbreeding does not occur, for example across geographical formations like mountain formation. Parapatric speciation occurs when populations are immediately adjacent to each other and only co-exist in a narrow contact zone. Sympatry occurs when populations inhabit the same geographic ranges (21).

Speciation in sympatric species is intriguing as it usually implies divergence in the presence of gene flow between populations. In these cases it is thought that divergent adaptations alone are not enough to drive and maintain separate species (21; 169). For allopatric species, the physical barriers to gene flow allow for weaker methods of isolation because chances of interbreeding are minimal, if they occur at all. Essentially, in allopatric speciation populations are first geographically separated which leads to reproductive isolation whereas in sympatric speciation the populations must first become reproductively isolated and then diverge into separate species (55). Therefore, sympatric speciation is believed to begin with sister or incipient species and then be achieved by

stronger methods of isolation which include ecologic divergence in multiple traits usually based on habitat suitability, assortative mating, and/ or selection against migrants and/or hybrids (181). In theory, sympatric speciation will show greater evidence of prezogytic isolation methods as opposed to postzygotic methods, which will be more apparent in allopatric speciation (55; 169; 181).

An accepted theory of sympatric speciation is that initially the divergent "speciation traits" will be clustered in smaller genomic regions that have lower levels of recombination, particularly in sex chromosomes, and will occur at an accelerated pace in comparison to allopatric speciation (9; 21; 135; 181). These locations are referred to as "genomic islands" of differentiation and would include genes that contribute to distinct adaptations and reproductive isolation (100; 135). Once populations have diverged, additional genetic incompatibilities will accumulate throughout the genome through mutation, genetic drift and ongoing selection and be will fixated and reinforced within the population (21; 34; 169; 181). When evaluating the evolution of a species it is important, yet sometimes extremely difficult, to determine the true sequence of which barriers to gene flow led to and which are a consequence of reproductive isolation (169; 181). When incipient species arise in sympatry there is an opportunity to study the evolution of their possible speciation without the interference of their evolutionary histories (55).

A current model of sympatric speciation in the presence of gene flow is *Anopheles gambiae* sensu stricto. The species is partially reproductively isolated and considered in the early stages of speciation (180). Differentiation between two molecular forms, defined as incipient species, has been identified using a polymorphism in the ribosomal DNA that is found near the centromere on the X chromosome (101). The

Mopti (M) and Savanna and Bamako (S) forms are otherwise indistinguishable at all life stages and are sympatric across most of their ranges in Western and Central Africa (90% of the range of M form overlaps with S form) (101; 180). The two forms are both anthropophilic, bite primarily at night, can be found resting indoors during the day, and contribute similarly to malaria transmission (101). Where the populations are sympatric, both forms have even been found resting in the same houses and flying in the same mating swarms (100).

In terms of ecological divergence, M forms can be found more in arid zones at higher latitutes while S forms predominate in humid lower latitudes (91; 101). This could be attributed to differences in their larval habitat adaptations. The S form of *An. gambiae* s. s. is associated with more temporary larval habitats free of predators and develops faster than the M form which is associated with more permanent larval habitats such as rice paddies and other irrigation reservoirs. The M form possesses better predator avoidance mechanisms, which enables it to survive a longer development time in these environments (100; 101). Most of the current knowledge surrounding the M and S forms is based on savanna populations. Savanna and Forest populations of M and S are genetically distinct. Recently in a sympatric forest region, where differences in larval habitat have not been yet been described, the M and S forms have been found to be segregated based on the degree of urbanization. Only the M form was present within a densely urbanized setting with larva found breeding in polluted water sources. This indicates even further divergence between and within the molecular forms based on local adaptation (91).

Lab crosses have shown successful hybridization without apparent inviability or sterility, though a relatively small percentage of hybrids can be found in the wild (100; 150). Assortative mating of the molecular forms has been described through the identification of sperm in wild-caught females (101; 150). In areas of sympatry, spatial swarm segregation has been found which could account for this reproductive isolation. In Mali, S forms were found above bare ground while M forms were found swarming in areas of dark centers within lighter areas (101). However, in Burkina Faso, mixed swarms were found suggesting a closer range way of identifying mates of the same molecular form (136; 150). Flight tones of tethered male-female partners of similar and different molecular forms demonstrated that the tethered pairs will match wing beat frequency. It is thought that when the harmonics are synchronized the air disturbance is minimized aiding in more successful copulation (141; 150). Frequency matching occurred significantly more often in same-form pairs than in mixed form pairs. This not only allows for mate recognition between molecular forms, but could possible play a role in swarm formation enabling swarm segregation between the populations (141). Despite evidence of assortative mating, there are limited areas where the percentage of natural hybridization is higher, even as high as 24%. Introgression and backcrossing specifically between M/S hybrids and S forms has been identified, further supporting that divergent selection between and within the M and S molecular forms is ongoing (136).

The variation in the genomes of the M and S forms of *An. gambiae* s.s. is homogenized with about 3% of differentiation found clustered in distinct regions, or "speciation islands", near the centromeres of all 3 chromosomes. It is suggested that the genes that contribute to ecological divergence and assortative mating would be found in

these regions (100; 101; 180). Recent studies using high-density arrays, however, have shown that the forms are differentiated at many more loci throughout the genome. Whether these sites include genes conferring reproductive isolation remains unknown (136). Clearly there is interbreeding between the two forms as evidenced by the minimal genome variability and existence of established hybridization in the wild. This suggests that divergence of the two forms was fairly recent and allows for *An. gambiae* s.s. to be an important model for sympatric speciation with gene flow (100).

Anopheles darlingi has been identified as an incipient species of two genotypes supported by evidence with mitochondrial *COI*, nuclear *white* gene, ribosomal ITS, and microsatellite sequence data (121). The divergence occurs between genotype 1 (Amazonia and southern Brazil) and genotype 2 (Belize, Guatemala, Colombia, Venezuela, and Panama). Significant genetic differentiation with little or no recurrent gene flow was found between the genotypes. Based on haplotype diversity and frequencies, it is believed that the populations found primarily in Brazil are older and more established and that the populations in Belize and Guatemala originated from Colombia (119). The levels of variation found using microsatellites was similar to that found between the M and S forms of *An. gambiae* s.s. (119; 121). The microsatellite loci have not been physically mapped to the polytene chromosomes (121). It is currently unknown if any of the molecular markers used are of genes under divergent selection or if they are found in a "speciation island" within the genome.

Knowledge of the evolutionary history of a vector species, particularly in regards to gene flow, is epidemiologically important for control strategies in terms of niche

habitats, insecticide resistance, susceptibility of disease agents, and any traits that could affect the species' vectorial capacity.

Malaria Vector Control

Prevention of malaria includes a combination of early case detection and confirmation diagnosis using microscopy and or rapid diagnostics tests, anti-malarial treatment using artemisinin combination treatment and chloroquine where appropriate, chemoprevention for the most vulnerable populations including pregnant women and infants, continued surveillance, and sustainable vector control strategies (188; 189).

The goals of prevention through vector control are to 1) reduce human-vector contact thereby protecting individuals from being bit by malaria infected mosquitoes and 2) reduce the average lifespan of the local mosquito population thereby lowering the intensity of malaria transmission at a community level (188; 189).

When an *Anopheles* mosquito bites an infected person it ingests the gametocyte form of the *Plasmodium* life cycle. In the mosquito gut, the gametocytes develop into sexually mature gametes. Male & female gametes unite to form a zygote, which develops into an active ookinete. The ookinete penetrates the wall of the mosquito midgut and becomes an oocyst. Nuclear division within the oocyst forms many sporozoites, which are the infective stage of *Plasmodium*. The oocyst will enlarge and burst, releasing the sporozoites, which migrate to the salivary glands. When a mosquito takes another blood meal, the sporozoites are injected into the human host. The life cycle within the mosquito host is called the sporogonic cycle and takes about 8-15 days depending on temperature, humidity and plasmodium species. By reducing mosquito lifespan, mostly through use of insecticides, you are reducing the likelihood that the

mosquito will live long enough for the *Plasmodium* parasite to develop into the infectious sporozoite stage (189).

Vector control can be focused on the larval or the adult stage of mosquitoes. Larval control includes habitat modification or application of larvicide to water sources used by mosquito species for breeding. For *Anopheles* mosquitoes, larval control usually occurs in urban and peri-urban settings where breeding sites are few, fixed, and findable, as high coverage is required to achieve a significant impact on vector densities (146; 188). Many species of *Anopheles*, including *An. darlingi*, breed in larger bodies of water that can be semi-permanent or seasonal where control of the larval stage is not feasible.

In these areas, primary focus of control methods is on adults. This is achieved through the use of insecticide treated bed nets (ITNs) and indoor residual spraying (IRS). Use of ITNs not only provides a physical barrier to protect individuals sleeping within them, but also acts on mosquito behavior in response to contact with the insecticides. This contact can either excite the mosquito to leave and seek another blood meal or, depending on the insecticide, dosage, and mosquito species, cause mosquito death. Widespread coverage within a community could lead to lower mosquito densities and survival and therefore lower malaria incidence, which has been demonstrated in specific studies (140). Success is dependent on nocturnal feeding patterns and other bionomics of local vector species. Universal access, primarily in malaria endemic African countries, is achieved through mass distribution campaigns and availability at antenatal and immunization clinics (188).

Indoor residual spraying is the application of residual (stable formulations) insecticides to the interior surfaces of homes. This targets endophilic mosquitoes and was the key method in the successful DDT eradication campaigns used in many countries (140; 146). Ideally, mosquitoes that would typically rest indoors after taking a blood meal would acquire either a lethal dose of insecticide and be killed or become excited/irritated by the insecticide and be forced to rest outdoors with a potential that lower temperatures might prolong or impede *Plasmodium* development in the mosquito host (140). This approach can reduce local malaria incidence and mortality provided that at least 80% of houses and structures in the target area are sprayed (188).

Other potentially successful interventions against adult mosquitoes include long-lasting insecticidal materials such as curtains, screens, and wall linings that have been impregnated with insecticides. Personal protection methods include repellent oils and creams, smoldering coils, and vaporizing mats (146), which can be effective for exophagic and earlier biting mosquito species.

Due to insecticide resistance and adaptive behavior in local vector species, changing malaria transmission dynamics including vector diversity and human migration, and inefficient and inappropriate vector control programs and insecticide use, integrated vector management (IVM) strategies are ideal. This includes a combination of methods applied simultaneously including use of insecticide treated materials (preferentially minimizing or at least alternating insecticides used), community education on transmission and control, environmental and housing structure modifications, etc. and requires collaboration of public and private entities with target communities (146).

Determining the optimal methods of control for any given location depends on an understanding of the local mosquito vector population. There is very little research on the direct impact of ITNs and IRS on *An. darlingi* populations throughout its geographic range.

OBJECTIVES

Considerable variation has been recorded in the bionomics and population genetics of *An. darlingi* across its range. Two genotypes have been defined for *An. darlingi* populations: a northern lineage (Belize, Guatemala, Colombia, Venezuela, Panama) and a southern lineage (Amazonia and southern Brazil). Comparative studies on the bionomics between the two lineages are lacking and, due to variation in study design, direct statistical comparison of any available information on *An. darlingi* is not possible. The objectives of this research were to compare 1) house entry, house exit, and host preference (epidemiologically relevant behaviors useful for vector incrimination) and 2) wing morphology (a measurable phenetic trait that is affected by genotype) between field populations of *An. darlingi* representing the two defined genotypes using a standard methodology that can enable a statistical comparison.

HYPOTHESIS AND SPECIFIC AIMS

Hypothesis: There will be differences in entrance and exit behavior, host preference, and wing morphology in *Anopheles darlingi* collected from geographically and genotypically separated populations

Specific Aim 1: Characterize and compare host-seeking behavioral differences between *Anopheles darlingi* collected in Belize and Peru

Specific Aim 2: Compare wing morphology between *Anopheles darlingi* collected in Belize and Peru and confirm the *white* gene lineage of each field population

STUDY SITES

In choosing study sites, the main objective was to include two locations that were known to have *An. darlingi* populations that would be representative of the two nuclear *white* gene lineages. A field laboratory and research team based in Orange Walk Town, Belize and a history of research and collaboration with the Belize Ministry of Health had already been established (2; 74). Similarly, collaborations between Uniformed Services University of the Health Sciences and the US Naval Medical Research Unit – 6 located in the city of Iquitos, Peru had already been established. An experimental hut site was previously instituted in the village of Zungarococha with the partnership of Laboratorio de Salud Publica de la DIRESA and the village council and inhabitants.

Belize (northern lineage)

The country of Belize is located in Central America and is bordered to the north by Mexico, to the west and south by Guatemala and to the east by the Caribbean Sea (see Table 5 for additional statistics).

Site establishment

Adult mosquito surveys were conducted between 2/3/10-3/29/10 to find a location with a higher density of *An. darlingi* where experimental huts could be established for data collection. Working off a purchased tourist Belize Travel Map (© Cubola Productions/Edigol Ediciones. S.A., 2006), adult mosquito populations were sampled along fresh water rivers found in the Toledo, Stann Creek, and Cayo Districts (see Figure

1 for locations surveyed). Collections would begin between 5:00-6:00 pm and continue until at least 9:00 pm (for sites where no mosquitoes were collected). The highest abundance of *An. darlingi* was found at the gatehouse to the Actun Tunichil Muknal (ATM) cave about 7 km from the village of Teakettle in Cayo District, Belize (17° 9'33.13"N, 88°50'34.62"W). The first collection night at the ATM site yielded 99 *An. darlingi,* which were the prominent mosquito species captured. There was a field next to the entrance house located about 30 m from Roaring River that was ideal to set up the experimental huts. Permission for use of the land was granted from the landowner. Experimental huts were transported from Golden Stream, reassembled at the new site, and entrance study collections began.

ATM

The site was flanked by farmland and across from an orange grove. The other side of the site was the river and the fringes of the Tapir Mountain Nature Reserve. Two experimental huts were set up in an area that was at river level and would flood when the river was high. The ATM gatehouse and a dormitory for the farm workers were on an elevated area approximately 60 m from the experimental huts (see Figure 2 for pictures).

Zungarococha, Peru (southern lineage):

Iquitos, Peru is the capital of the Loreto district. It is located where the Maranon and Ucayali Rivers combine and join the Amazon River and is only accessible by air and river travel (see Table 5 for additional statistics). The experimental hut study site was located in the village of Zungarococha located approximately 17.3 km outside of Iquitos down the Iquitos-Nauta Highway. Three experimental huts were located in a subsection of the village that was only accessible via a covered bridge. The site was approximately

1 km from the village center and surrounded by forest fringe. There were approximately 11 houses in the immediate vicinity with a central open area of white sand (see Figure 3 for pictures).

Table 1. Summary of *An. darlingi* Incrimination

INCRIMINATION						
Country	**Location**	**Date**	**Infection Rate**	***Plasmodium* spp.**	**Notes**	**References**
Belize	Toledo and Stann Creek	1940	3.57%			Komp 1941
Brazil	Bahia	1931	28.70%	not determined		Davis & Kumm 1932
	4 sites in Rondonia State	1985-1988	0.48%	*P. falciparum* *P. vivax*	used immunoradiometric assay (IRMA)	de Oliveira-Ferreira et al. 1990
	15 locations throughout Brazilian Amazon region	1994-1998	1.82%	*P. falciparum* *P. vivax*		Tadei & Thatcher 2000
	Boa Vista Roraima State	1996-1998	8.50%	P. falciparum P. vivax VK210 P. vivax VK247	EIR 0-5 infective bites/person/year	da Silva-Vasconcelos et al. 2002
	Boa Vista Roraima State	2001-2002	2.10%	*P. falciparum* *P. vivax* VK210 *P. vivax* VK247		Povoa et al. 2006
	3 communities along Matapi River, Amapa State	2003-2005	1.83%	*P. falciparum* *P. malariae* *P. vivax* VK210 *P. vivax* VK247		Galardo et al. 2007
	Vila Candelaria and Bate Etaca, Rondonia State	2001-2004	0.26%		EIR for Vila Candelaria 2 and 10 infective bites/person/year indoors & outdoor respectively	Gil et al. 2007
	Municipality of Careiro Amazonas State	2008-2010			strong correlation between human biting rate (HBR) of *An. darlingi* and malaria incidence rate (MIR)	Martins-Campos et al. 2012
Colombia	Cordoba State	2007-2008	1.45%	*P. vivax* VK247		Gutierrez et al. 2009
	Antioquia Department	2008-2010	0.09%	*P. vivax* VK210	EIR 3.7 infective bites/person/year	Naranjo-Diaz et al. 2013
French Guiana	3 villages in Upper-Maroni basin	2000-2002	0.10%	*P. falciparum* *P. vivax* *P. malariae*	EIR 21.6 infective bites/person/year data for 3 villages combined	Girod et al. 2008
	2 villages along Maroni River	1998-1999	0.06%	*P. falciparum*	EIR 5-10 infective bites/person/year	Fouque et al. 2010
	4 villages on Maroni	2004-2005	0.01%	*P. falciparum* *P. vivax* *P. malariae*	EIR 8.7-66.4 infective bites/person/year	Hiwat et al. 2010
	3 villages along Maroni and Oyapok Rivers	2003-2006	0.17% & 0.06%	*P. falciparum*	EIR 5.7 & 8.7 infective bites/person/year	Girod et al. 2011

French Guiana cont'd	11 sites along coast and Maroni and Oyapock valleys	2006-2011	0.22%	*P. vivax* VK210	*An. oswaldoi, An. intermedius* & *An. nuneztovari* also positive	Dusfour et al. 2012
Peru	75 sites in depts of Loreto, Ucayali, Madre de Dios & San Martin	2001-2002	0.98%	*P. falciparum* *P. vivax* VK210 *P. vivax* VK247	*An. bennarrochi*: All three species (0.14%)	Flores-Mendoza et al. 2004
	outside Iquitos	unpublished	0.08%	*P. falciparum* *P. vivax*	unpublished data	Turell et al. 2008
	Periurban, rural, forest village and forest in vicinity of Iquitos, Peru	1996-1997	0.30%	*P. falciparum*	EIR 0-12.0 infective bites/person/month	Reinbold-Wasson et al. 2012
	21 sites along Mazan River in dept of Loreto	2008-2009	1.40%	*P. falciparum* *P. vivax* VK210 *P. vivax* VK247	EIR high of 5.13 infective bites/person/night	Parker et al. 2013
Venezuela	Ocamo Amazonas State	1994-1995	0.76%	*P. falciparum* *P. vivax* VK247 *P. malariae*	EIR 128.6 infective bites/person/year	Magris et al. 2007
	Sifontes Bolivar State	1999-2000	0.82%	*P. falciparum* *P. vivax* VK210 *P. vivax* VK247	EIR 2.21 infective bites/person/year	Moreno et al. 2007
	Sucre and Cedeno Bolivar State	2008-2009	0.18%		EIR 9.81 infective bites/person/year	Rubio-Palis et al. 2013

Table 2. Examples of breeding habitats

EXAMPLES OF BREEDING HABITATS			
Country	**Location**		**Reference**
Belize	General	Mainly in detritus patches in clear flowing streams or rivers in shaded or partially shaded areas	Manguin et al. 1996; Achee et al. 2006; Grieco et al. 2007
Brazil	Roraima State	Irregular excavation pits associated with manual brick manufacturing	da Silva-Vasconcelos 2002
	Roraima State	1) contact with humans 2) increased presence of "microdams" 3) shade	Nagm et al. 2007
	Boa Vista Roraima State	1) presence of shade 2) proximity to human dwellings	Barros et al. 2010
	Amapa State	Abandoned gold mining excavation pits	Galardo et al. 2013
	Rondonia State	Small and large temporary and permanent ponds along the future extension line for a new hydroelectric dam	Morais et al. 2012
French Guiana	Oyapock/Camopi and Maroni Rivers	Forested zones are flooded at the end of long rainy season providing productive breeding habitats	Girod et al. 2011
Peru	Iquitos	Greater likelihood to be found in fish farms	Vittor et al. 2009; Maheu-Giroux et al. 2010
		Greater likelihood to be found in areas with secondary growth	Vittor et al. 2009
Suriname	General	1) creeks: under canopy, between rock and fallen trees 2) river edges: in shaded patches of floating debris and 3) pools: formed in or near the river bed when river levels dropped; never found with in the tidal zone	Rozendaal 1990; 1992
	Aseli Kamp	River edge in mats of water hyacinth and water fern and seasonally-flooded forest between grass stems and floating debris	
Venezuela	General	Majority found in rivers and streams. Then from smaller pools and ponds. 67% found in shade/partial shade	Gabaldon 1949
	Amazonas State	Permanent lagoons filled with overflow from river about 400 m from dwellings	Magris et al. 2007

Table 3. Summary of biting trends I & II

BITING TRENDS I						
Country	**Location**	**Date**	**Trend**	**Peak**	**Notes**	**Reference**
Belize	Cayo District	2002-2003	bimodal	~3 hrs after sunset ~1 hr before sunrise		Achee et al. 2006
Brazil	Ituxi River Amazonas State	1979-1980	bimodal	sunset sunrise	daytime biting	Roberts et al. 1987
	""	""	unimodal	1800-2200	used entrance traps	""
	Ariquemes Rondonia State	1986-1988	unimodal	1800-2400		Lourenco-de-Oliveira et al. 1989
	Costa Marques Rondonia State	1986-1987	bimodal	1800-2000 0500-0600		Klein & Lima 1990
	Belem and Aura Para State		trimodal	sunset 2400 sunrise	Deane 1948	reviewed by Rosa-Freitas et al. 1992
	BR174 Manaus Boa Vista		unimodal	2000-2400 & 2400-0300	Hayes & Charlwood 1979	reviewed by Rosa-Freitas et al. 1992
	Highway, km 137, Para State		unimodal	2000-2100	Charlwood & Hayes 1978	reviewed by Rosa-Freitas et al. 1992
	Uauaris Roraima State		unimodal	2400-0200	Charlwood & Hayes 1978	reviewed by Rosa-Freitas et al. 1992
	Porto Velho Rondonia State		trimodal	sunset 2400 sunrise	Deane 1948	reviewed by Rosa-Freitas et al. 1992
	Jaru Rondonia State		trimodal	sunset 2000-2100 sunrise	Charlwood & Alecrim 1989	reviewed by Rosa-Freitas et al. 1992
	Aripuana Mato Grosso State		bimodal	2000 sunrise	Charlwood & Hayes 1978	reviewed by Rosa-Freitas et al. 1992
			bimodal	sunset sunrise	Charlwood & Wilkes 1979	reviewed by Rosa-Freitas et al. 1992
	Dourado Sao Paulo State		bimodal	sunset sunrise	Forattini 1987	reviewed by Rosa-Freitas et al. 1992
	Juturnaiba	1989	unimodal	2000-0100		Rosa-Freitas et al. 1992
			bimodal	1800-2200 major 0400-0600 minor	Tadei 1988	reviewed by Zimmerman 1992
	Boa Vista Roraima State	1996-1998	unimodal	sunset	only found during 1 collection time; generally biting throughout night	da Silva-Vasconcelos et al. 2002
	Santana Amapa State	1999-2000	unimodal	5-hr post sunset	trends varied, but generally elevated through middle of night	Voorham et al. 2002
	Rondonia State	2004	unimodal	2100-2200		Cruz et al. 2009
	Negro River basin Amazonas State	2003-2004	bimodal	1800-1900 0400-0500	Only at 1 site; other 3 sites had too low densities to see trends	Suarez-Mutis et al. 2009

Brazil cont'd	Boa Vista Roraima State	2003-2004	none	none	no hourly differences	Barros et al. 2010
	Acre State	2008-2009	unimodal bimodal	1st half of night sunset and sunrise	trends vary by time of year	Moutinho et al. 2011
	Manso dam Mato Grosso State	2005-2006	unimodal	1800-1900		Ribeiro et al. 2013

BITING TRENDS II						
Country	**Location**	**Date**	**Trend**	**Peak**	**Notes**	**Reference**
Bolivia	Bolivian Amazon	2003	unimodal	1900-2200		Harris et al. 2006
Colombia	El Pescado	1965	unimodal bimodal unimodal	2200-0100 2000-2100 & 0200-0300 1900-2000	high density moderate density low density	Elliott 1968
	Cordoba and Antioquia States	2007-2008	no trend	active 1900-0300		Gutierrez et al. 2009
	Amazonas State	2003-2004	bimodal	1800 - 2400 sunrise	indoors	Rodriguez et al. 2009
French Guiana			trimodal	1800-1900 0100-0200 0700-0800	Pajot 1979	reviewed by Zimmerman 1992
	3 villages in Upper-Maroni basin	2000-2002	no trend	max activity 2130-2330 remained constant max activity 0130-0330	outdoor biting behavior	Girod et al. 2008
Honduras			unimodal	1800-2300	Rivera & Nelson in Zimmerman & Rangel	reviewed by Zimmerman 1992
Peru			trimodal	sunset 0200 sunrise	Elliott 1972	reviewed by Rosa-Freitas et al. 1992
	8 sites along Iquitos-Nauta Highway	2000-2001	unimodal	2100 - 2300		Vittor et al. 2006
	outside Iquitos	1996-1997	unimodal	1800-2000		Turell et al. 2008
	Periurban, rural, forest village and forest in vicinity of Iquitos, Peru	1996-1997	unimodal	~ 2 hrs post-sunset	3.8% collected during the daytime; no differences in indoor:outdoor	Reinbold-Wasson et al. 2012
	21 sites along Mazan River in dept of Loreto	2008-2009	unimodal	2100-2200		Parker et al. 2013
Suriname	Aseli Kamp Lawa River	1979-1981	unimodal	2100-2300	varied slightly at 2 sites	Hudson 1984
	Drietabiki	2006-2007	unimodal	slow increase until 0600	These are outdoor trends. Indoors was similar but not as prominent.	Hiwat et al. 2012
	Jamaica		unimodal	0100-0200		
Venezuela			bimodal	1800-2000 0500-0600	pers comm. From Berti & Zerpa	reviewed by Zimmerman 1992

	Ocamo Amazonas State	1994-1995		begins around 2400 and remains elevated until sunrise		Magris et al. 2007
	Sifontes Bolivar State	1999-2000	bimodal	2300-2400 0300-0400	minor peaks active throughout night mostly exophagic	Moreno et al. 2007
	Sucre and Cedeno Bolivar State	2008-2009	unimodal	sunset		Rubio-Palis et al. 2013

Table 4. Overview of population genetics

POPULATION GENETICS STUDIES ADDRESSING *AN. DARLINGI* AS CRYPTIC SPECIES				
Country	**Marker(s)**	**Findings**	**Meaning**	**References**
Brazil	Cuticular hydrocarbons Isoenzmes Behavior	Within levels of intraspecific variation Within levels of intraspecific variation Polymorphic	Single species	Rosa-Freitas et al. 1992
7 countries	Isozymes RAPD-PCR ITS2 Morphology	High degree of similarity Within levels of intraspecific variation No variation Within levels of intraspecific variation	Single species	Manguin et al. 1999
Bolivia, Brazil, and Venezuela	mtDNA RFLP	Significant correlation between genetic and geographic distances	Populations are genetically isolated by distance	Conn et al. 1999
5 states within Brazil	ITS2	Identical except for a 4-5% sequence divergence in Dourado population	Dourado population stands out. Separate species?	Malafronte et al. 1999
19 localities in Central and South America	COI	3 clusters: 1A) southern Amazon and South America 1B) northern Amazon & 2) Central America and northwest Colombia	Starting to see division in populations; though not due to isolation by distance	Mirabello & Conn 2006
7 localities in Brazil	microsatellites	Found genetic difference in populations north and south of Amazon River	Populations are genetically isolated by distance	Conn et al. 2006
Peru	RAPD-PCR	Iquitos populations homogenous	Single population in Peru	Pinedo-Cancino 2006
31 localities	microsatellites and nuclear *white* gene	microsatellites show 5 population clusters 1 from Central America and the remaining 4 from Amazonia (population structure) the *white* gene divides the Central American populations from the Amazonian populations	Deep divergence represented by *white* gene and COI (from above) interpreted as incipient species	Mirabello et al. 2008

Table 5. Brief country summary statistics

Brief Country Summary Statistics		
	Belize	**Peru**
Ave Annual Temp	79.2°F: Int'l Airport	79°F: Iquitos
Ave Annual RH	79.5	81
Ave Annual Rainfall	77.9 in: Roaring Creek	113.4 in: Iquitos
Sunset	Nov 5:16 pm - July 6:32 pm	Oct 5:44 pm - Feb 6:15 pm
Daylight Hours	Dec 11:05 hrs - June 13:10 hrs	June 11:54 hrs - Dec 12:20 hrs
Cultures	Mestizo, Creole, Garifuna, Maya, Indian, Mennonite, Chinese	Amerindian, Mestizo, White, Black, Japanese, Chinese
Population	333,200	29,849,303: country 474,000: rural population outside Iquitos
% in High Transmission	0	5 in total country 10-50 confirmed cases/1000 found in Loreto District
% in Low Transmission	69	12
% Malaria-free	31	84
P. falciparum	3%	11% Found in Amazonian Districts
P. vivax	97%	89%
Major *Anopheles*	*An. albimanus* *An. darlingi*	*An. darlingi* *An. pseudopunctipennis* *An. albimanus*

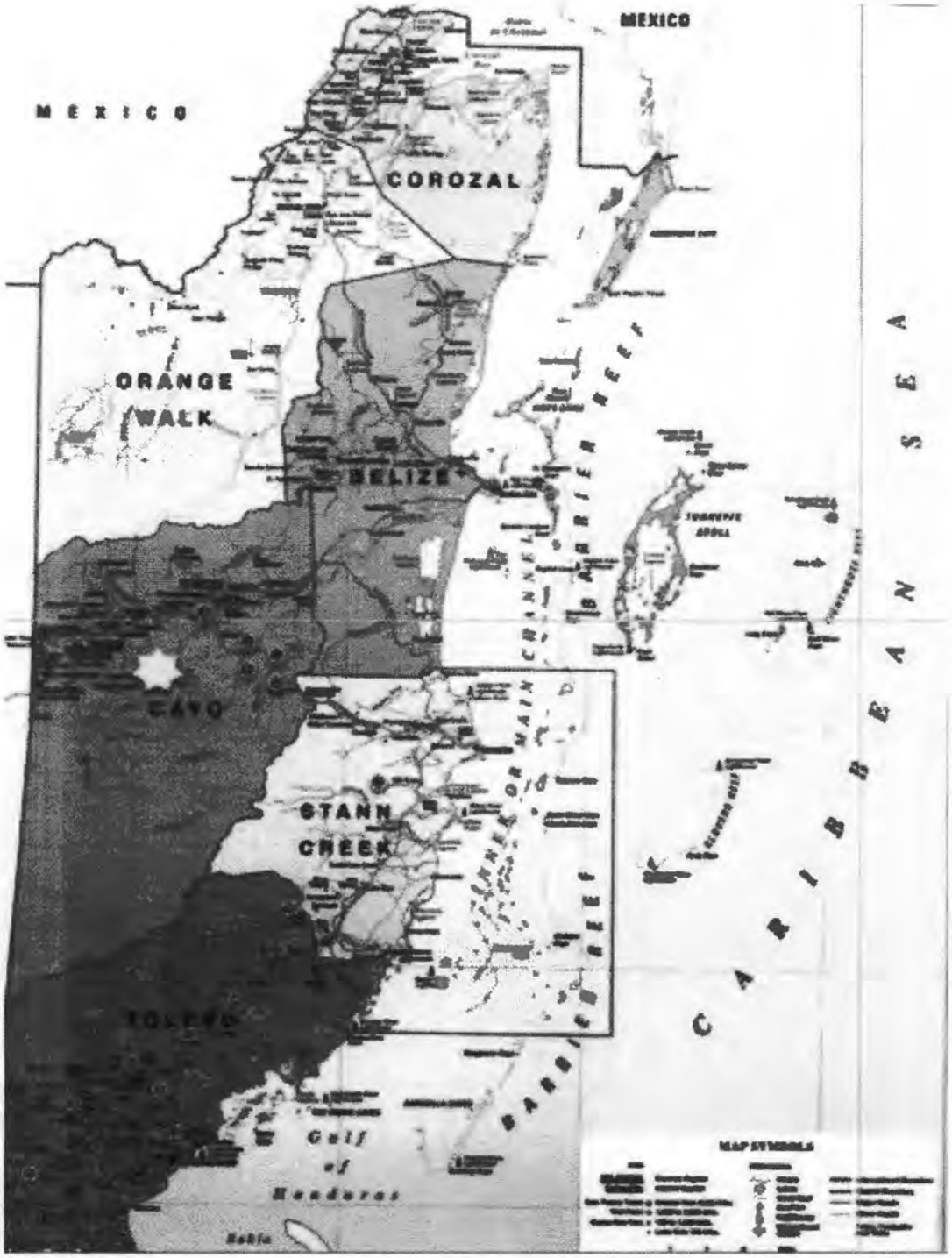

Figure 1. Map of adult collection survey sites conducted in Belize

Figure 2. Actun Tunichil Muknal (ATM) study site
A. Roaring River, B. Slower flowing pool of Roaring River, C. Flanking field and orange grove across street, D. Experimental huts

Figure 3. Zungarococha, Peru study site
A. Covered bridge leading to site, B. White sand in center of village, C. Experimental hut, D. Lagoon

CHAPTER 2: Comparison of experimental hut entrance and exit behavior between *Anopheles darlingi* from the Cayo District, Belize and Zungarococha, Peru

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ABSTRACT

Anopheles darlingi Root is a major vector for malaria in Central and South America. Behavioral, ecological, genetic, and morphologic variability has been observed across its wide distribution. Recent studies have documented that two distinct genotypes exist for *An. darlingi:* a northern lineage (Belize, Guatemala, Colombia, Venezuela and Panama) and a southern lineage (Amazonia and southern Brazil). In order to determine if these genotypes exhibited different behavioral traits, entrance and exit movement patterns between two field populations of *An. darlingi* that represented each genotype were evaluated using experimental huts. The Belize population exhibited bimodal entrance with peak entry occurring between 7:00-8:00 p.m. and 5:00-6:00 a.m. and peak exiting occurring between 7:00-8:00 p.m. The Peru population exhibited unimodal entrance with

peak entry occurring between 10:00-11:00 p.m. and peak exiting occurring between 11:00-12:00 a.m. with a secondary smaller peak at 2:30 a.m. Entrance and exit behavioral patterns were significantly different between the Belize and Peru populations of *An. darlingi* [log-rank (Mantel-Cox) $P < .001$]. Information from the present study will be used in the future to determine if there is a correlation between genotype and host-seeking behavior and can be used in the present for regional vector risk assessment.

INTRODUCTION

Understanding behavior that drives vector-human contact for medically important insect vectors is necessary to determine risk of pathogen infection. For species like *An. darlingi*, that prefer to breed in flowing rivers (4; 115) and larger bodies of water (185), control efforts focus on the adult female. In order to successfully target the adult populations, vector control programs require accurate information pertaining to the house-frequenting behavior of the target vector species.

Anopheles darlingi has been implicated as a major vector for malaria throughout Central and South America (41; 47; 57) and has been implicated in the resurgence of malaria in Brazil (29) as well as Amazonia Peru (8). *Anopheles darlingi* has a very wide distribution spanning from southern Mexico to northern Argentina, with a discontinuity in Nicaragua and Costa Rica (121; 170). More recently, however, *An. darlingi* was collected in Panama where it previously was never recorded (102).

In general, *An. darlingi* is an anthropophilic and endophagic species and has a high tendency for endophily across its distribution (6; 27; 41; 83; 151; 156). Temporal biting trends have been reported as unimodal, bimodal, and trimodal. Unimodal trends have been observed in Brazil at sunset (151) and between 8:00-12:00 a.m. (164), in

Colombia between 10:00-1:00 a.m. (54), in Suriname between 9:00-11:00 p.m. (85) and 11:30-1:30 a.m. (156), and in Peru between 8:00-10:00 p.m. (179). Bimodal patterns are commonly reported with peak of biting at sunset (or shortly after), continuous biting throughout the night, and then a second peak of biting at dawn (6; 92; 151; 177). Trimodal patterns observe peak densities at sunset, sunrise and anywhere between 10:00-2:00 a.m. (154).

Few studies have been conducted to examine house entry and exit behavior using interception traps as it relates to *An. darlingi*. In Brazil, Roberts et al. (1987) recorded a peak entrance of unfed female *An. darlingi* during and immediately after sunset with a gradual decline occurring throughout the remainder of the night. Engorged and gravid female *An. darlingi* exited the experimental house at sunrise (151).

Variability in *An. darlingi* populations across its geographic range has also been documented based on cuticular hydrocarbons, isoenzymes and alloenzymes (116; 128; 154), morphologic markers (116), and different genetic markers (32; 116; 119). The behavioral and genetic variations of *An. darlingi* along with the species' wide distribution have led many in the scientific community to suggest (and debate) that *An. darlingi* is a species complex. Supporting this idea, data have shown that geographical populations of *An. darlingi* fall into two genotypes. Genotype 1 (southern) is found in Amazonia and southern Brazil and Genotype 2 (northern) is found in Belize, Guatemala, Colombia, Venezuela and most recently Panama (102; 119; 121). It is unknown whether these two genotypes demonstrate different behavioral characteristics, which may alter their contact with human hosts thereby increasing the potential for malaria transmission.

Despite reported behavioral heterogeneity of *An. darlingi*, studies employing the same sampling design to control for differing collection methodologies in multiple geographic locations are lacking. The goal of the current study was to evaluate and compare temporal entrance and exit behavior patterns of both northern and southern lineage *An. darlingi* populations. The information will be useful when evaluating regional malaria risk and determining successful intervention strategies.

MATERIALS & METHODS

Behavioral studies were conducted in two locations that represent northern and southern *An. darlingi* genotypes. In Belize, a total of 11 entrance and 9 exit collections were conducted between May and June 2010. In Peru, 15 entrance and 10 exit collections were conducted between March and April 2011 based on peak seasonal *An. darlingi* densities (6; 179). Both entrance and exit collections were conducted from 6:00 p.m. until 6:00 a.m. Each collector was equipped with a flashlight and mouth aspirator to facilitate mosquito capture. The collectors rotated positions at the end of each shift and changed huts and shifts each night in a Latin Square rotation to reduce collector bias. Though the number of persons in each experimental hut was standardized, the number of persons in the surrounding homes was variable which might have an impact on the intensity of mosquito behavior, but would not affect overall time trends for entry and exit. HOBO® H8 family data loggers (Onset Computer Corporation, Bourne, MA) were positioned inside each hut to record temperature, relative humidity and dew point.

Study sites

Belize (northern genotype): Two experimental huts were located near the entrance to the Actun Tunichil Muknal (ATM) cave located adjacent to the Roaring River

(17° 9'33.13"N, 88°50'34.62"W) in the Cayo District, Belize, Central America (Fig. 4). Huts were positioned 30 m from the margin of Roaring River on a plot of land that was also occupied by two dormitory style living quarters (approximately 60 m from the huts) that housed anywhere between 5 – 15 individuals to include the park rangers, land guardians and agricultural workers. The site was flanked by open farmland and an orange grove.

Peru (southern genotype): Three experimental huts were positioned on a sparsely inhabited plot of land approximately 1 km from the center of Zungarococha village located approximately 17 km from the city of Iquitos in Amazonia Peru (3°49'32.39"S, 73°21'1.43"W) (Fig. 4). The site was surrounded by forest and was located about 300 m from a lake that is fed by numerous tributaries of varying depth and flow based on the seasonal rainfall. Eleven houses with 2-6 inhabitants each were located in the direct vicinity (approximately 3-20 m) of the experimental hut.

Hut Construction: Huts were constructed to mimic local housing styles using local building materials (Fig 5). In Belize, 2 identical huts measured approximately 3 m wide x 3 m long x 2.5 m high and were elevated 30 cm off the ground. Four walls were constructed from untreated plywood. Roofs were corrugated zinc laid with an approximate 10° pitch away from the front of the huts. Each hut contained 3 windows and 1 door with each wall containing one portal of entry. In Peru, 3 identical huts measured approximately 4.5 m wide x 3 m long x 2.5 m high and were elevated 45 cm off the ground. Four walls were constructed from untreated 2 x 4 wood panels. Roofs were corrugated zinc and sat about 3.5 m high at the apex in the center of the huts. Each hut contained 4 windows, one on each sidewall and two on the front wall flanking the

door. At both sites the huts were positioned approximately 30 m from each other. All windows measured 1 m x 1 m in dimension and doors measured 2 m x 1 m in dimension. Window interception traps were approximately 1 m^3 and were constructed using green polyester mesh netting attached to an internal frame (either metal or wood) and included a sleeve on two sides of the trap to allow for the introduction of a mouth aspirator for collection and removal of mosquitoes. Interception traps were attached to either the interior or exterior frame of the windowsill to allow for collecting mosquitoes as they entered or exited the huts, respectively. The window facing side of each trap was constructed into a funnel ending in a letterbox style opening to facilitate mosquito entry into the trap. Any cracks or openings were closed with typical mattress foam to increase the probability of mosquito movement through windows and doors.

Entrance collections

Two experimental huts were used for entrance behavior collections in Belize and 3 experimental huts were used in Peru. Window interception traps were positioned inside the huts with the funnels directed towards the interior of the hut to facilitate capturing entering mosquitoes. Doors to the experimental huts remained closed for the duration of the collections. A 2-person collection team entered each hut just before the collection began and remained inside for the duration of the sampling period (12 h). One collector aspirated mosquitoes from the entrance traps during two 3-h shifts in Belize (i.e. 6-9 p.m., 9-12 a.m., 12-3 a.m., or 3-6 a.m.) or one 6-h shift in Peru (i.e. 6-12 a.m. or 12-6 a.m.) while the second collector rested. Mosquitoes were aspirated from window traps for the first 20 minutes of each half hour (i.e. 6:00-6:20 p.m., 6:30-6:50 p.m., etc.). All mosquitoes from a sampling period were placed into collection cups labeled with the hut

number, date, and time of collection. At the Belize site the mosquitoes were immediately killed in the field using acetone vapors and identified to species using morphological characteristics (190). In Peru, collection cups were transported to the Laboratorio de Salud Publica de la DIRESA in insulated coolers where they were freeze-killed for species identification (56) and subsequently dissected to observe parity rates (10). Identified samples were sorted into individually labeled Eppendorf vials accordingly.

Exit collections

Two experimental huts were used for exit behavior collections in Belize and 2 experimental huts were used in Peru. Window interception traps were positioned outside the huts with the funnels directed towards the exterior of the hut to facilitate capturing exiting mosquitoes. Doors to the experimental huts remained partially open (about 1 ft) for the duration of the collections. One collector entered each hut at the beginning of each shift and remained indoors underneath an untreated bed-net for the duration of their shift. The second collector for each experimental hut remained at a separate "resting station" and only approached the outside of the hut when sampling mosquitoes from the interception traps. The resting station in Belize was a tent set about 60 m away from the experimental huts. The resting station in Peru was the 1 hut that was not being used for mosquito collections (approximately 30 m from each other). The second collector aspirated mosquitoes from the exit traps during two 3-h shifts in Belize (i.e. 6-9 p.m., 9-12 a.m., 12-3 a.m., or 12-6 a.m.) or one 4-h shift in Peru (i.e. 6-10 p.m., 10-2 a.m., or 2-6 a.m.). Mosquitoes were aspirated from window traps for the last 10 minutes of each half hour (i.e. 6:20-6:30 p.m., 6:50-7:00 p.m., etc.). Mosquitoes were processed in the same way as those from the entrance collections.

Statistical analyses

The total number of mosquitoes captured from all huts was averaged per hour per night at each study location to show hourly entrance and exit behavioral trends. During the course of the entrance behavior experiments the time of sunset in Belize ranged from 6:24-6:30 p.m. and in Peru from 5:56-6:05 p.m. For the exit behavior experiments, the time of sunset in Belize ranged from 6:30-6:32 p.m. and in Peru from 5:51-5:55 p.m. This would represent an approximately 25-40 minute difference between results at each site in regards to mosquito responses to photoperiod. The present study averaged the total number of mosquitoes captured per hour per night so that our first collection period (6:00-7:00 p.m.) encompassed the time of sunset for each site. The graphs and discussion label this first collection point as time point 0 as this is when sunset occurred. Any time points beyond point 0 represents hour post-sunset (time point 1 is 7:00-8:00 p.m. or 1 hour post-sunset, etc.). A Kaplan-Meier survival curve was plotted on the total number of *An. darlingi* that entered or exited over 12 time points to assess the entrance and exit behavioral patterns for each location. The time points were estimated for when 25% (ENT_{25}), 50% (ENT_{50}), and 75% (ENT_{75}) of the total number of *An. darlingi* entered and for when 25% (EXT_{25}), 50% (EXT_{50}), and 75% (EXT_{75}) of the total number of *An. darlingi* exited the experimental huts over the course of the night (Table 6). The patterns of entrance and exit behavior were analyzed and p-values determined using a log-rank (Mantel-Cox) test with p-values below 0.05 considered statistically significant. Correlations between indoor temperature, relative humidity, and dew point recorded by HOBO® H8 family data loggers (Onset Computer Corporation, Bourne, MA) were

analyzed using the Pearson correlation coefficient. All analyses were performed using IBM® SPSS® Statistics 20.

RESULTS

Mosquitoes collected

Anopheles darlingi was the most abundant anopheline species collected at both sites (Table 7), accounting for over 99% of the anophelines collected in entrance studies in Peru (1977/1991) and exit studies in Belize (1553/1559) and Peru (697/704) and 86.5% of the anophelines collected in entrance studies in Belize (1032/1193). Of the total number of anophelines collected for all studies combined (5447), *An. darlingi* accounts for over 96% (5259). In Belize, other species collected were *An. vestitipennis* Dyar and Knab (131 entrance, 3 exit), *An. albimanus* Wied. (16 entrance, 2 exit), *An. punctimacula* Dyar and Knab (10 entrance, 1 exit), and *An. pseudopunctipennis* Theobald (4 entrance, 0 exit). In Peru, other species collected were *An. benarrochi* Gabaldon (9 entrance, 1 exit), *An. mattogrossensis* Lutz and Neiva (3 entrance, 1 exit), *An. mediopunctatus* Lutz (1 entrance, 1 exit), *An. oswaldoi* Peryassu (1 entrance, 0 exit), *An. shannoni* Davis (0 entrance, 2 exit), *An. peryassui* Dyar and Knab (0 entrance, 1 exit), and *An. triannulatus* Neiva and Pinto (0 entrance, 1 exit).

Entrance behavior

In Belize, *An. darlingi* exhibited bimodal entrance with a primary peak occurring at time point 1 and entering numbers dropping dramatically in the following sample period and remaining low for the remainder of the night (Fig. 6A). A smaller secondary entrance peak occurred at time point 11. In Peru, *An. darlingi* exhibited unimodal entrance, with a peak occurring at time point 4 (Fig. 6A). Unlike Belize, the entrance

activity of the Peruvian *An. darlingi* population decreased gradually over the remainder of the night.

The Kaplan-Meier curve (Fig. 7A) shows the estimated percentage of entering *An. darlingi* collected over a 12-h time period. Overall, there were statistically different entrance patterns ($P < .001$) between the *An. darlingi* population in Belize and the *An. darlingi* population in Peru. In Belize, ENT_{50} occurred at time point 3 (9:00-10:00 p.m.), indicating an estimated 50% of the total number of *An. darlingi* collected has entered before 10:00 p.m. (Table 6). In Peru, ENT_{50} occurs at time point 5, 2 hours later than the Belize population.

Inside the huts in Belize, temperatures ranged from 89-99°F (average 94°F) and 47-77% (average 56%) relative humidity at the beginning of the collection night to 77-80°F (average 78°F) and 81-88% (average 84%) relative humidity at the end of the collection night. Inside the huts in Peru, temperature ranged from 80-91°F (average 86°F) and 54-79% (average 65%) relative humidity at the beginning of the collection night to 72-77°F (average 75°F) and 79-96% (average 89%) relative humidity at the end of the collection night. There was no significant correlation between the numbers of *An. darlingi* collected in either Belize or Peru and indoor temperature or relative humidity.

Exit Behavior

In Belize, there is one prominent peak of *An. darlingi* exiting the experimental huts at time point 1 followed by a gradual decline of exiting until approximately time point 8 (Fig. 6B). In Peru, the primary peak of exiting occurred at time point 5, with a minor peak occurring at time point 8 (Fig. 6B).

The Kaplan-Meier curve (Fig. 7B) shows the estimated percentage of exiting *An. darlingi* collected over a 12-h time period. Overall, there were statistically different exit patterns ($P < .001$) between the *An. darlingi* population in Belize and the *An. darlingi* population in Peru. In Belize EXT_{50} occurs at time point 3 and in Peru at time point 5 (Table 6); similar to the respective entrance patterns for each population.

Inside the huts in Belize, temperatures ranged from 88-97°F (average 91°F) and 42-68% (average 55%) relative humidity at the beginning of the collection night to 73-78°F (average 76°F) and 85-92% (average 88%) relative humidity at the end of the collection night. There was a moderate positive correlation between the total number of *An. darlingi* collected from the exit window interception traps in Belize and the indoor temperature (Pearson correlation coefficient 0.420; $P < .001$) and a moderate negative correlation between the total number of *An. darlingi* collected from the exit window interception traps in Belize and the indoor relative humidity (Pearson correlation coefficient -0.402; $P < .001$). Inside the huts in Peru, temperature ranged from 83-90°F (average 86°F) and 55-74% (average 67%) relative humidity at the beginning of the collection night to 73-75°F (average 76°F) and 85-92% (average 90%) relative humidity at the end of the collection night. There was no significant correlation between the numbers of *An. darlingi* collected in Peru and indoor temperature or relative humidity.

Parity

A total of 675 (34%) and 268 (38%) of *An. darlingi* captured at the Peru site were dissected for parity observations from entry and exit evaluations, respectively (Fig. 8). Of these, 163 (24.1%) from entry evaluations and 128 (47.8%) from exit evaluations were determined to be nulliparous while the remaining 512 (75.9%) and 140 (52.2%)

were identified as parous, respectively. The percentage of nulliparous females gradually increased throughout the course of the study (Fig. 8). To determine parity, mosquitoes were pooled over a single 12-hour collection night and a random sample of females were selected from that pool for dissection. Future studies should include a more thorough analysis of parity rates, including rates for each individual collection period, to determine the effect mosquito reproductive age has on entrance and exit behavior.

DISCUSSION

Variability in biting behavior of *An. darlingi* has been recorded and observed across its distribution (6; 151; 156; 179). Though these studies all used mainly human landing/biting catches, the collection times and set-ups were varied. In order to be able to statistically compare entrance and exit movement patterns of *An. darlingi* from two distantly separated locations, a common collection methodology was employed in the present study.

The present entrance behavior study evaluated numbers of *An. darlingi* collected from window interception traps and observed time trends for house entry. Although previous studies in Belize have shown bimodal trends with a primary peak occurring in the hours after sunset and a secondary peak immediately before sunrise using human landing/biting catches (6), the primary peak occurs about 3 hours after sunset which is about 1-2 hours later than reported in the current study. These results are not unexpected as house entry would preclude landing and biting occurring indoors, however, the previous data show that there was no marked delay in landing/biting times between collectors positioned outdoors and collectors positioned indoors (6). The differences in peak collection time could also be attributed to specific study sites, distance to breeding

sites, and seasonal densities. In Amazonia Peru, peak biting density, as evaluated by human landing/biting catches, was shown to occur between 8:00-10:00 p.m. and gradually declined through sunrise (179). The indoor biting trend was very similar to the outdoor biting trend, but showed about a 1-h delay in peak density (about 9:00 p.m. for outdoor and 10:00 p.m. for indoor). These data are very similar to the present study, which shows a unimodal peak density between 10:00-11:00 p.m. and a gradual decrease through sunrise.

Results from exit evaluations conducted as part of the current study are difficult to compare to previous reports. This is because earlier evaluations focused on describing exit patterns in relation to physiological status (i.e. blood fed or unfed) and resting time and location (151). Collectors positioned indoors for our study were within untreated bed-nets, therefore, there was no opportunity for those *An. darlingi* that entered the huts to feed. Roberts et al. (1987) reported in Amazonia Brazil that the majority of mosquitoes exited the house after 4:00 a.m. The majority of these females were late fed or Sella Stage 2 and above (151). In the current study, 50% of *An. darlingi* from Belize had exited the hut by time point 3 and 75% by time point 5. In Peru, 50% exited before time point 5 and 75% by time point 8. None of the mosquitoes collected from the exit traps were blood-fed. Since host-seeking females were unable to acquire a blood meal, there could have been a bias towards mosquitoes exiting the huts prematurely in search of an available host.

Data from the current study suggests that not only do *An. darlingi* collected in Belize have an earlier entrance peak (one hour post sunset) but the population has an earlier and more gradual entrance pattern. The majority (75%) of mosquitoes are

estimated to have entered by time point 7, therefore, the majority of *An. darlingi* are entering over the span of 7 h (7:00-2:00 a.m.). The *An. darlingi* in Peru had a later peak entrance time than in Belize (time point 5) but they also enter later at night and not as gradually as the Belize population, as the majority (75%) of mosquitoes are estimated to have entered by time point 8 over the span of 4 h (11:00-3:00 a.m.). These observations suggest that the greatest burden of potential infectious bites occurs earlier in the evening in Belize when many individuals are still active in and around the house. In addition to bed-net usage, alternative interventions, for example, mosquito coils, vaporizer mats and emanators might be useful as supplemental interventions against *An. darlingi* in Belize. However, studies will be required to determine behavioral efficacy of these alternative interventions to the vector populations.

In Belize, entrance and exit peaks overlapped (time point 1), suggesting that almost immediately after entering and being unsuccessful in acquiring a blood meal, *An. darlingi* will exit. As there is a moderate correlation between total numbers of *An. darlingi* captured exiting the huts in Belize and indoor temperature and relative humidity, it is also possible that mosquitoes were excited or agitated by the internal environmental conditions and as a result exited prematurely. In Peru, there is a 1-h difference between peak entrance (time point 4) and peak exit times (time point 5). This suggests that the *An. darlingi* population from Peru has a propensity to rest prior to searching for a host or initiating biting behavior. In Mato Grosso, Brazil, Charlwood (26) observed mosquitoes resting near the host for up to 10 min before biting. Also, in Suriname, Hudson (85) observed that *An. darlingi* rested near the collector 7.7 min (range of 1-35 min) before biting. The observations were made over 2 nights with 52 unfed females. If it were the

case that *An. darlingi* in Peru rested for a longer period before seeking a host, then this would have operational implications for adult control. For instance, indoor residual spraying (IRS) might have a greater effect as there would be a greater likelihood that mosquitoes would rest on chemically treated walls. Although this in no way suggests that IRS has no role in the Belize populations of *An. darlingi*, it might suggest that other interventions could function to supplement IRS to exploit this difference in behavior. Further studies are required in both populations to elucidate indoor resting behavior, to include time and location in relation to biting responses.

Data from the present study suggest that *An. darlingi* collected from Belize and Peru exhibit distinct and significant differences in epidemiologically relevant behaviors. Though biting and house entrance and exit times are variable in different geographies across the species' range, the consistency of time trends over generations of *An. darlingi* in each specific geography suggests a genetic influence (27). The present study is a first step to describe behavioral differences in genetically different populations of *An. darlingi*. Ongoing studies are being conducted to describe the genetic and morphometric differences between these study populations in an attempt to correlate host-seeking behaviors with genotype.

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Table 6. Estimated time (time point) when 25%

Table 6

Estimated time (time point) when 25%, 50%, and 75% of total *An. darlingi* entered (ENT) or exited (EXT) experimental huts in Belize and Peru

	Entrance						Exit					
	ENT_{25}		ENT_{50}		ENT_{75}		EXT_{25}		EXT_{50}		EXT_{75}	
Belize	8:00 PM	(1)	10:00 PM	(3)	2:00 AM	(7)	8:00 PM	(1)	10:00 PM	(3)	12:00 AM	(5)
Peru	10:00 PM	(3)	12:00 AM	(5)	3:00 AM	(8)	9:00 PM	(2)	12:00 AM	(5)	3:00 AM	(8)

Table 7. Total number and species of mosquitoes collected from each experiment

Table 7

Total number and species of mosquitoes collected from each experiment

	Entrance				Exit			
	Belize		Peru		Belize		Peru	
Anopheles darlingi	1032	86.50%	1977	99.30%	1553	99.62%	697	99.01%
Anopheles vestitipennis	131	10.98%	-		3	0.19%	-	
Anopheles albimanus	16	1.34%	-		2	0.13%	-	
Anopheles punctimacula	10	0.84%	-		1	0.06%	-	
Anopheles pseudopunctipennis	4	0.34%	-		0		-	
Anopheles benarrochi	-		9	0.45%	-		1	0.14%
Anopheles mattogrossensis	-		3	0.15%	-		1	0.14%
Anopheles mediopunctatus	-		1	0.05%	-		1	0.14%
Anopheles oswaldoi	-		1	0.05%	-		0	
Anopheles shannoni	-		0		-		2	0.28%
Anopheles peryassui	-		0		-		1	0.14%
Anopheles triannulatus	-		0		-		1	0.14%
Totals	1193		1991		1559		704	

"-" indicates that this species is not found at the specific location

% indicates the percentage of the total mosquitoes collected for that study

Figure 4. Study site locations.
Site Actun Tunichil Muknal (ATM) is located in the Cayo District, Belize, Central America and site Zungarococha is located in the Loreto Department, Peru, South America.

A

B

Figure 5. Experimental hut construction
(A) Cayo District, Belize, Central America and (B) Loreto Department, Peru, South America.

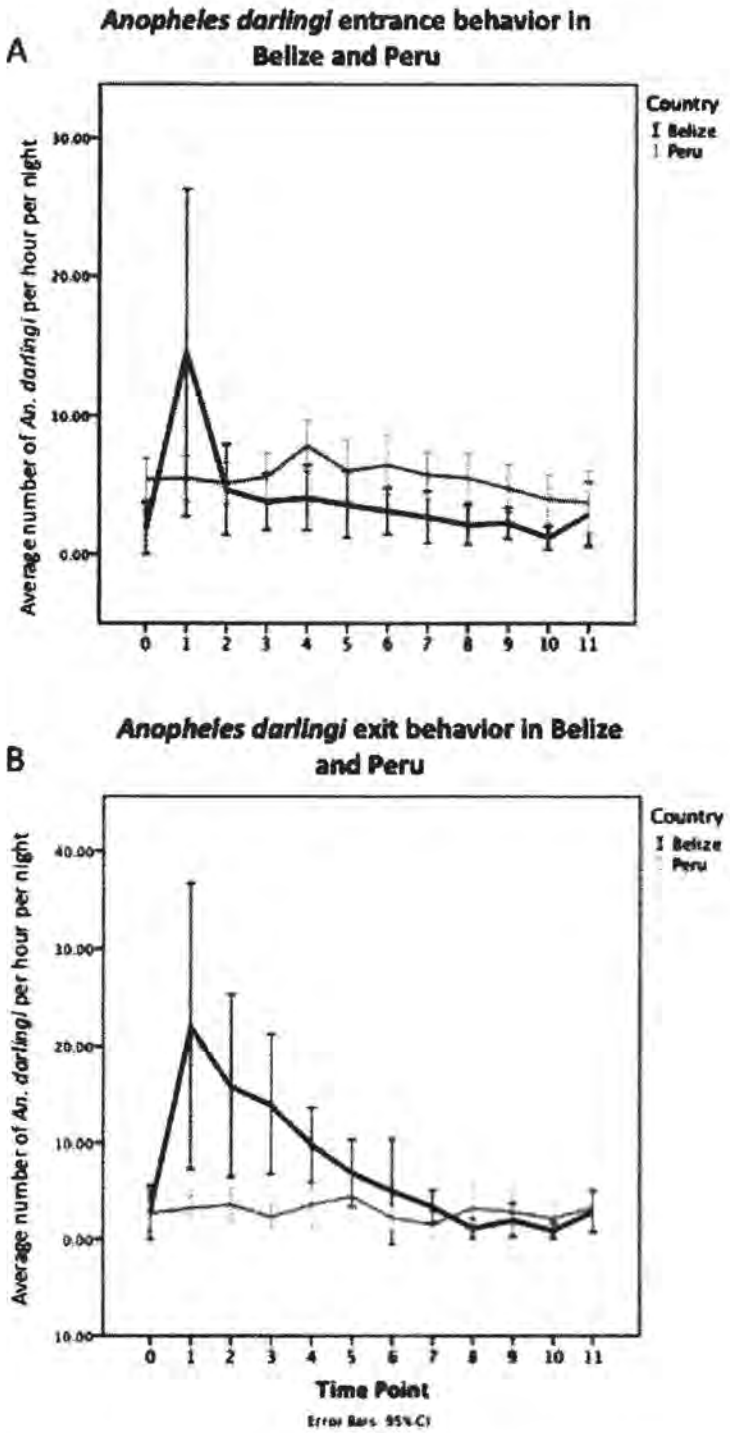

Figure 6. Average number of *An. darlingi* collected each hour (A) hut entry evaluations in Cayo District, Belize, Central America (n=11) and Loreto Department, Peru, South America (n=15) and (B) hut exit evaluations in Cayo District, Belize, Central America (n=9) and Loreto Department, Peru, South America (n=10).

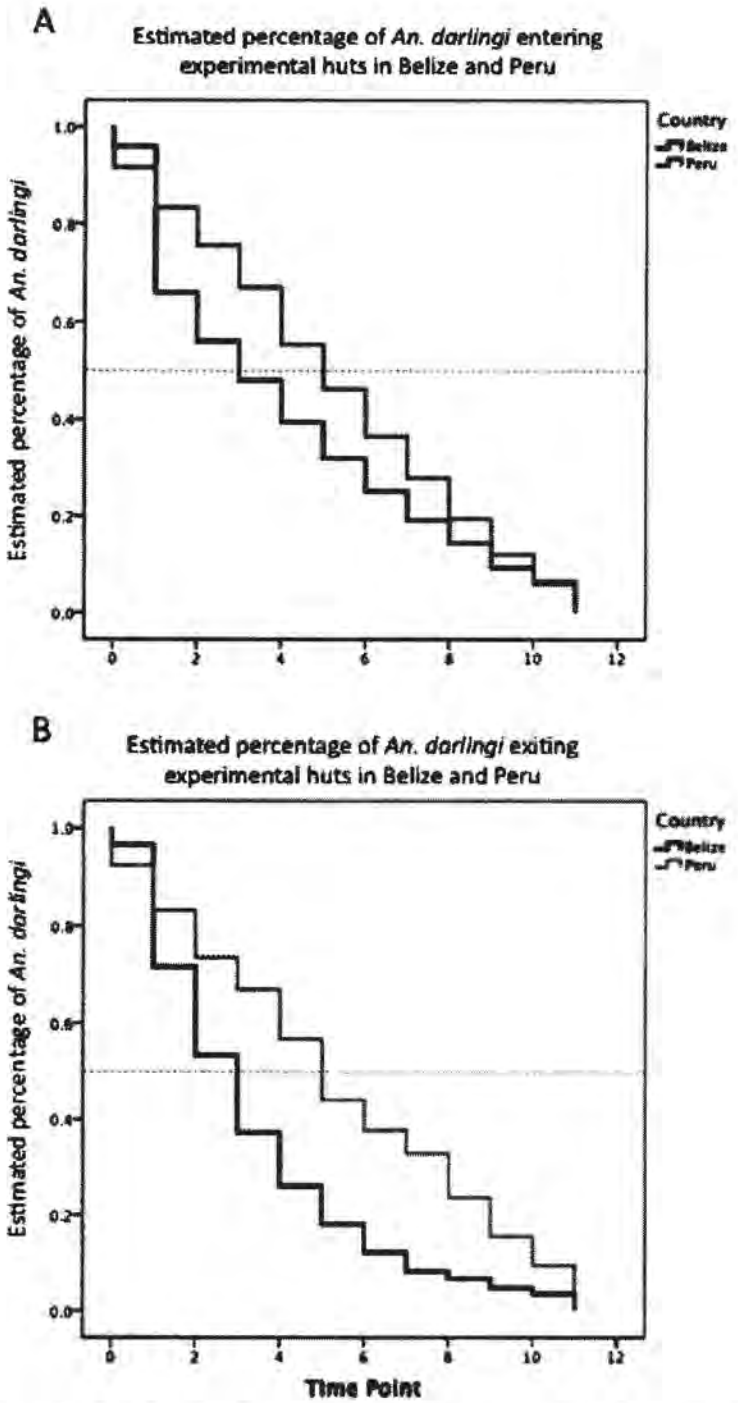

Figure 7. Kaplan-Meier survival analysis over 12 time points for estimated percentage of mosquitoes

(A) entering experimental huts in Cayo District, Belize, Central America (n=11) and Loreto Department, Peru, South America (n=15) [log-rank (Mantel-Cox) $P < .001$] and (B) exiting experimental huts in Cayo District, Belize, Central America (n=9) and Loreto Department, Peru, South America (n=10) [log rank (Mantel-Cox) $P < .001$].

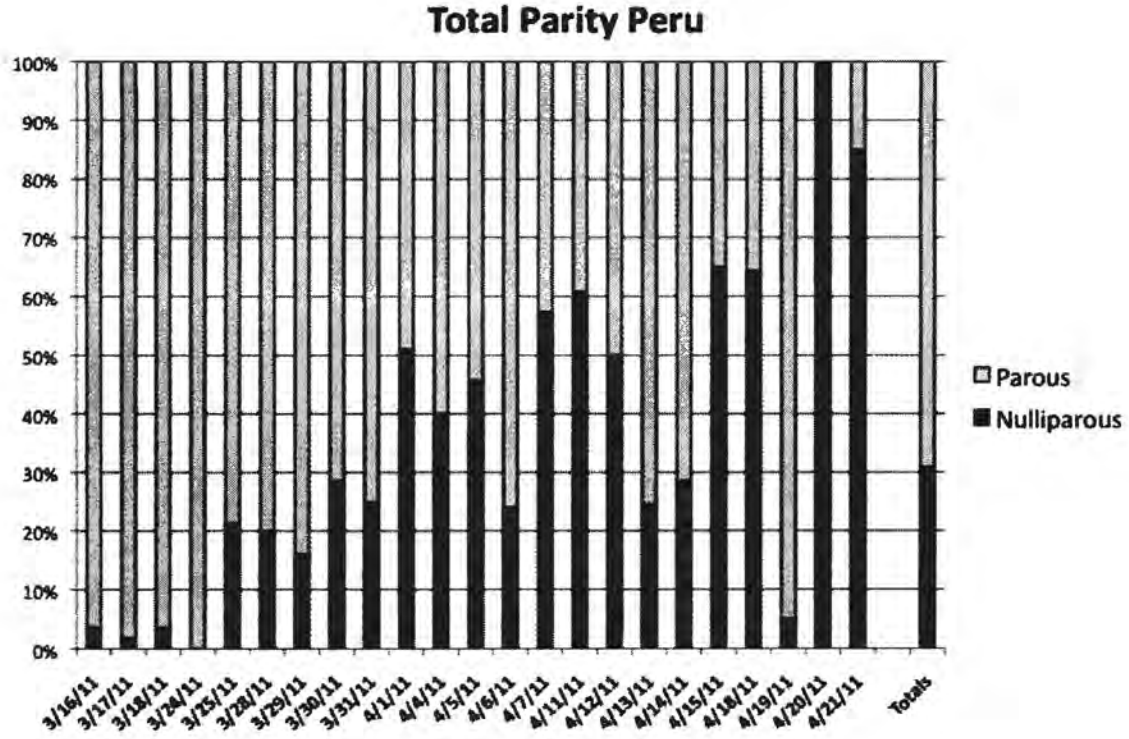

Figure 8. Percent of parous and nulliparous *An. darlingi* Collected during hut entrance (3/16/11-4/7/11) and hut exit (4/11/11-4/21/11) evaluations from Loreto Department, Peru, South America as determined by tracheal distention of the ovaries.

CHAPTER 3: Host feeding preference of *Anopheles darlingi* from Cayo District, Belize and Loreto District, Peru

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ABSTRACT

Anopheles darlingi, a major vector for malaria in Central and South America, has been shown to have two distinct genotypes: a northern lineage and a southern lineage. Variability in host feeding preference has been observed across its geographic distribution. It is unknown if the different genotypes confer differences in host-seeking behavior. Studies were conducted in Cayo District, Belize and Zungarococha, Peru representing northern and southern *An. darlingi* genotypes, respectively. Mosquitoes were collected from window interception traps positioned on experimental huts containing either a human or a pig host over a period of 12 hours. A subset of collection nights in Peru was used to evaluate differences in mosquito abundance before and after

the application of an untreated bed net on the pig host. In Belize, there was a significantly greater density of *An. darlingi* collected from the hut containing the human host than from the hut containing the pig host ($P = .025$). Alternatively, there was a significantly greater density of *An. albimanus* collected from the hut containing the pig host than from the hut containing the human host ($P = .002$). In Peru, there was no significant difference in the density of *An. darlingi* collected from the huts containing either host species. The study also showed that there were more *An. darlingi*, *Culex* spp., and total mosquitoes collected from the traps before using an untreated bed net on the pig host than after ($P < .01$ for each) suggesting an interruption or inhibition of host cues.

INTRODUCTION

Anopheles darlingi Root has a very wide distribution spanning from southern Mexico to northern Argentina, with a discontinuity in Nicaragua and Costa Rica (97; 121; 151; 170) and has been universally recognized as being highly anthropophilic with endophilic tendencies (6; 27; 151; 192). It has been implicated as a major vector for malaria throughout Central and South America (41; 42; 47; 57; 77). *Anopheles darlingi* has been defined by two genotypes: the southern genotype is found in Amazonia and southern Brazil and the northern genotype is found in Belize, Guatemala, Colombia, Venezuela, and most recently Panama (102; 119-121). It has not been determined if the different genotypes confer differences in host-seeking behavior that influence vector potential for malaria transmission.

One important aspect of understanding the vector potential in the epidemiology of malaria transmission is to identify the vector's preference for a human blood meal (1; 17; 76; 109). This factor, in addition to the mosquito's longevity and susceptibility, will

define the probability and intensity of malaria transmission (19; 93; 109). Host preference of a mosquito vector is influenced by intrinsic genetic tendencies as well as extrinsic environmental factors including host availability (19; 27; 107; 192).

Host feeding preference of *An. darlingi* has been evaluated using several sampling strategies. One way to analyze host-feeding preference of a mosquito species is to collect resting mosquitoes and determine their blood meal type. In Brazil, Zimmerman et al. (2006) collected *An. darlingi* from resting sites under houses and in nearby vegetation in three different villages. Of the percentage of mosquitoes that were positive for taking a blood meal, those that tested positive for a human blood meal ranged from 1.7 – 40.5%, depending on the village and the sample site, with an overall total of 13.1% of positive *An. darlingi* that fed on human hosts (192). The same study reported that 63.5% of positive *An. darlingi* fed from bovine hosts, 15.7% from pig hosts, 13.5% from dog hosts, 4.5% from rat hosts, and 1.4% from chicken hosts (percentages include multiple host blood meals). The availability of bovine hosts compared with other host species could not determined because cattle were not found in the villages where mosquitoes were collected (192). As reviewed in Charlwood (1996), another study in Brazil utilized a large cage, which contained horse, cattle, dog, chicken and human hosts. Blood meal types were determined from collected blood fed *An. darlingi*. The study showed 46% fed from human hosts, 29% from cattle hosts and 13% from horse hosts (27). In Belize, Grieco et al. (2002) used manual aspiration inside and outside houses, backpack aspiration of vegetation, and a mobile truck trap to look at the foraging ratio (FR) of *Anopheles* spp. The FR looks at the proportion of blood meals for each host in respect to all possible hosts in the collection area. A value > 1 represents host preference. Though

only 10 (1.0% of total anophelines collected) *An. darlingi* were collected, an FR of 7.69 for humans was found inside the house and 5.2 outside the house as one mosquito collected outside had fed on a cow (76).

Another way to look at host feeding preference of mosquitoes is to compare the density and species of mosquitoes collected directly from human and animal hosts essentially intercepting host-seeking tendencies. In Brazil, Klein et al. (1991) looked at host preference of *Anopheles* mosquitoes in an urban and a rural site. Humans collected natural populations of mosquitoes landing on their exposed feet and legs and then from a non-illuminated bovine-baited trap during a 13-hour collection night. Of the total number of *An. darlingi* collected for both sites about 80% of *An. darlingi* were collected off a human host and about 20% from the non-illuminated bovine-baited trap. There could be a bias as the bovine host was under a netted trap, whereas the human host was not (93). Also in Brazil, Oliveira-Ferreira et al. (1992) collected from a cow and a human about 4 m apart in open terrain. *Anopheles darlingi* was collected from the human (65%) more frequently than the cow (46).

The variability in sampling methods does not allow for direct comparison of either host species or geographically different populations of *An. darlingi*. To address the question of whether the two genotypes convey differences in host feeding preference, the current study used an identical sampling design in two locations that represented each of the genotypes. This is the first known study that attempts to link host feeding preference of *An. darlingi* with genotype.

MATERIALS AND METHODS

Studies were carried out for 16 nights between May-June 2012 in Belize and for 15 nights between April-May 2011 in Peru based on peak seasonal *An. darlingi* densities (6; 179). Collections were conducted from 6:00 p.m. until 6:00 a.m. HOBO® H8 family data loggers (Onset Computer Corporation, Bourne, MA) were positioned inside each hut to record temperature, relative humidity and dew point.

Study sites

Belize (northern genotype): Two experimental huts were positioned near the entrance to the Actun Tunichil Muknal (ATM) cave located adjacent to the Roaring River (17° 9'33.13"N, 88°50'34.62"W) in the Cayo District, Belize, Central America (Fig. 9). Huts were located 30 m from the margin of Roaring River on a plot of land that was also occupied by two dormitory style living quarters that housed approximately 5 – 15 individuals to include the park rangers, land guardians and agricultural workers. The site was flanked by open farmland and an orange grove. Animals in the immediate vicinity of the study site included approximately 5 dogs, a variety of bird species, small field rodents, frogs and snakes.

Peru (southern genotype): Three experimental huts were positioned on a sparsely inhabited plot of land approximately 1 km from the center of Zungarococha village located approximately 17 km from the city of Iquitos in Amazonia Peru (3°49'32.39"S, 73°21'1.43"W) (Fig. 1). The site was surrounded by forest and was located about 300 m from a lake that is fed by numerous tributaries of varying depth and flow based on the seasonal rainfall. Eleven houses with 2-6 inhabitants each were located in the direct vicinity. Animals in the immediate area of the study site included about 10 dogs, chickens, a variety of bird species, rodents, iguanas and snakes.

Experimental huts

Huts were constructed to mimic local housing styles using local building materials. In Belize, two identical huts measured approximately 3 m wide x 3 m long x 2.5 m high and were elevated 30 cm off the ground. Four walls were constructed from untreated plywood. Roofs were corrugated zinc laid with an approximate 10° pitch away from the front of the hut. Each hut contained 3 windows and 1 door with each wall containing one portal of entry. In Peru, 3 identical huts measured approximately 4.5 m wide x 3 m long x 2.5 m high and were elevated 45 cm off the ground. Four walls were constructed from untreated 2 x 4 wood panels. Roofs were corrugated zinc and sat about 3.5 m high at the apex in the center of the huts. Each hut contained 4 windows, one on each sidewall and 2 on the front wall flanking the door. At both sites the huts were positioned approximately 30 m from each other. All windows measured 1 m x 1 m in dimension and doors measured 2 m x 1 m in dimension. Window interception traps were approximately 1 m^3 and were constructed using green polyester mesh netting attached to an internal frame (either metal or wood) and included a sleeve on two sides of the trap to allow for the introduction of a mouth aspirator for collection and removal of mosquitoes. Interception traps were attached to the interior frame of the windowsill to allow for collecting mosquitoes as they entered the huts. The window facing side of each trap was constructed into a funnel ending in a letterbox style opening to facilitate mosquito entry into the trap. Any cracks or openings were closed with foam to increase the probability of mosquito movement through windows.

Hosts

A single animal host, a pig, was compared with a human host at both study sites. At each site, one pig was used throughout the study. Each pig was approximately 45 kg at the beginning and approximately 65 kg at the end of the study period and was temporarily positioned within a portable corral inside a hut during mosquito collections. In Belize, two human hosts and in Peru, three human hosts ranging from approximately 70-85 kg in weight rotated collection nights in the human host hut.

A portable corral was constructed at each location that could be placed inside and moved between each hut. The portable corrals were constructed from wood planks with a tarp or plastic bottom to allow for filling with dirt and to facilitate cleaning and maintenance. When the pig was not located in the portable corral it remained in its normal outdoor corral setting.

Mosquito sampling

Two experimental huts, each containing a single host (pig or human) were used at each location. The doors were closed in each but the windows remained open. Entrance interception traps were placed on all windows to capture entering mosquitoes while creating a mosquito free space inside. Interception traps were monitored for mosquitoes at three collection points throughout the night; 10:00 p.m., 2:00 a.m., and 6:00 a.m. In Belize a fourth collection point was added at 8:00 p.m. to capture the earlier peak of host seeking activity found in the Belize population (162).

A subset of collection nights in Peru was used to observe the difference in mosquito density and composition before and after application of an untreated bed net on the pig host. For the first 3 nights of collections in Peru, the pig host did not utilize a bed net. On the fourth night and for the rest of the study (12 nights) an untreated bed net was

applied over the portable corral. The human host utilized an untreated bed net for the entirety of the study.

Each night one person remained inside the designated hut to provide human host cues and collect from the window interception traps. Two additional collectors were positioned at a mosquito processing station 30 m from the nearest experimental hut. These collectors entered the hut containing the pig host at designated sampling points to capture mosquitoes from the window interception traps. Each collector was equipped with a flashlight and mouth aspirator. The huts remained the same with respect to human or pig host for 3-4 consecutive nights. During the movement of the corral, the huts were cleaned and well ventilated for at least 48 hours before collections resumed to minimize potential residual odors.

All mosquitoes were aspirated into plastic cups labeled with either human or pig, the date, and the collection time. In Belize, mosquitoes were transported live to a field processing station. Throughout the collection nights and the mornings following, the mosquitoes were knocked down with acetone fumes, identified to species (190), counted, and dissected to observe parity rates (10). In Peru, the cups were transported back to the Laboratorio de Salud Publica de la DIRESA. There they were freeze killed, identified to species (56), and counted. All identified samples (not dissected) were sorted into Eppendorf vials accordingly.

Statistical Analyses

A Wilcoxon signed rank test was used to analyze differences in mosquito density between host types for each species of mosquito captured. General Linear Model was used to analyze differences in *An. darlingi* densities between hosts and sampling points.

A Kruskal Wallis test was used to analyze differences in *An. darlingi* densities captured at each sampling point. A Mann-Whitney test was used to analyze differences in densities for each mosquito species between those captured before and after bed net application on the pig host in Peru. Correlations between indoor temperature, relative humidity, and dew point recorded by HOBO® H8 family data loggers (Onset Computer Corporation, Bourne, MA) were analyzed using the Pearson correlation coefficient. All analyses were performed using IBM® SPSS® Statistics 20 (IBM Corporation, Armonk, NY).

RESULTS

Belize

A total of 879 mosquitoes were collected from the experimental huts in Belize. *Anopheles darlingi* was the most abundant mosquito species collected (539), accounting for 61.3% of the total density of mosquitoes collected and the most abundant species of mosquito collected for each host (349 human, 190 pig) (Table 8). Following *An. darlingi* in total density collected were *Culex* spp. (182 human, 136 pig), *An. albimanus* Wied. (1 human, 15 pig), *An. vestitipennis* Dyar and Knab (2 human, 2 pig), and *An. punctimacula* Dyar and Knab (0 human, 2 pig).

In Belize, there was a significantly greater density of *An. darlingi* collected from the human host hut than the pig host hut ($P = .025$) (Fig. 2). There were also a significantly greater total density of mosquitoes collected from the human host hut than the pig host hut ($P = .024$), though this is most likely because *An. darlingi* accounted for over 61% of the total number of mosquitoes collected.

The total density of *An. albimanus* collected from the huts, however, was statistically greater for the pig host than the human host ($P = .002$) (Fig. 10). The total numbers collected were low with 16 mosquitoes collected over 16 nights. Fifteen mosquitoes were collected from the pig host hut and 1 mosquito was collected from the human host hut.

Using General Linear Model there was a significant difference in density of *An. darlingi* collected from each host ($P = .036$), but not for host by time. This suggests that even though *An. darlingi* was collected more frequently from the human host hut, the pattern of mosquitoes collected at each sampling point was the same for each host. In other words, temporal trends in house entry time were similar regardless of host.

Using Kruskal Wallis to analyze differences in total mosquitoes caught per time point, there was a significantly lower number of *An. darlingi* caught between 2:00 and 6:00 a.m. than caught between 6:00 and 8:00 p.m. ($P = .009$) and 8:00 and 10:00 p.m. ($P = .005$) (Fig. 11). This suggests that greater densities of *An. darlingi* are entering the huts earlier in the night (before 10:00 pm) than in the early morning.

A total of 236 *An. darlingi* collected in Belize were dissected to observe parity from the tracheal distention of the ovaries (10). Of these mosquitoes, 157 (66.5%) were determined to be nulliparous while the remaining 79 (33.5%) were determined to be parous.

Inside the human host huts in Belize, temperatures ranged from 77-86°F (average 81°F), relative humidity ranged from 70-89% (average 79%), and dew point ranged from 71-76°F (average 74°F) at the earliest collection point to temperatures of 71-78°F (average 75°F), relative humidity of 88-95% (average 91%), and dew points of 68-74°F

(average 72°F) at the latest collection point. Inside the pig host houses, temperatures ranged from 77-85°F (average 81°F), relative humidity ranged from 72-88% (average 79%) and dew point ranged from 71-76°F (average74°F) at the earliest collection point to temperatures of 70-78°F (average 75°F), relative humidity of 88-95% (average 92%) and dew points of 68-75°F (average 72°F) at the latest collection point.

There was a medium to high negative correlation between relative humidity and the number of *An. darlingi* collected from the human hut (Pearson correlation coefficient -0.599, $P < .001$) and a medium positive correlation between temperature and *An. darlingi* collected from the human hut (Pearson correlation coefficient 0.438, $P = .001$). No significant correlation was found between temperature, relative humidity, or dew point and collection time point or between temperature, relative humidity, or dew point and host types in Belize. This would suggest that differences in total numbers of *An. darlingi* collected in human and pig houses are not due to internal house environments.

Peru

A total number of 1,231 mosquitoes were collected from the experimental huts in Zungarococha. The density of *An. darlingi* at the study site during the time period of this experiment was low. In descending order of total mosquitoes collected is *Culex* spp. (539 human, 576 pig), *Mansonia* spp. (9 human, 36 pig), *Aedeomyia squamipennis* Lynch Arribalzaga (28 human, 5 pig), *An. darlingi* (15 human, 16 pig), *An. shannoni* Davis (1 human, 5 pig), and *Psorophora* spp. (0 human, 1 pig).

There were no significant differences between any of the mosquito species collected from the human host hut and the pig host hut (Fig. 12). There were also no

significant differences between *Anopheles darlingi* collected between any of the time points.

Inside the human host huts in Peru, temperatures ranged from 75-85°F (average 80°F), relative humidity ranged from 65-84% (average 76%) and dew point ranged from 68-75°F (average 72°F) at the earliest collection point to temperatures of 71-77°F (average 74°F), relative humidity of 77-92% (average 88%), and dew points of 66-73°F (average 70°F) at the latest collection point. Inside the pig host houses, temperatures ranged from 74-84°F (average 80°F), relative humidity ranged from 69-86% (average 78%) and dew point ranged from 69-75°F (average72°F) at the earliest collection point to temperatures of 71-77°F (average 74°F), relative humidity of 77-95% (average 87%), and dew points of 67-73°F (average 70°F) at the latest collection point.

There was a medium negative correlation between relative humidity and the number of *An. darlingi* collected from the human and pig huts (Pearson correlation coefficient -0.483, $P = .001$ and -0.357, $P < .05$, respectively). There was also a medium negative correlation between dew point and the number of *Culex* spp. collected from the human and pig houses (Pearson correlation coefficient -0.311 and -0.368, $P < .05$, respectively). There is a commonality of the negative correlation between relative humidity and numbers of *An. darlingi* collected at both study sites. Further studies should be conducted focusing on the effects of indoor relative humidity and temperature on the numbers of entering *An. darlingi*. No significant correlation was found between temperature, relative humidity, or dew point and collection time point or between temperature, relative humidity, or dew point and host types in Peru.

Before and after bed net application

A total number of 1,171 mosquitoes were collected from the pig host house over 15 collection nights, 532 of which were collected before applying the bed net and 639 collected after applying the bed net (Table 9). The quantities of each species of mosquitoes collected over 12 nights after applying the bed net are reported in the previous section as the portion of the study where the pig slept under an untreated bed net was directly compared to the data from when the human slept under an untreated bed net. The quantities of each species of mosquitoes in descending order collected over the 3 nights before applying the bed net were *Culex* spp. (461), *An. darlingi* (57), *Mansonia* spp. (10), *An. oswaldoi* (2), *An. shannoni* (1), and *Psorophora* spp. (1). Whereas after bed net usage *An. darlingi* was the third most abundant species collected from the pig host representing 2.5% of the total mosquitoes, before the bed net was applied *An. darlingi* was the second most abundant species collected representing 10.7% of the total mosquitoes.

The density of *An. darlingi*, *Culex* spp., and total mosquitoes collected from the pig host in Peru was significantly greater before applying the bed net than after applying the bed net ($P < .01$ for each) (Fig. 13). The data show that a significantly lower number of *An. darlingi* entered the pig host hut after the bed net was implemented (average of 19 per night before and 1.33 per night after) as well as for *Culex* spp. (average of 154 per night before and 48 per night after).

Discussion

The current studies did not evaluate natural host tendencies of *An. darlingi* as the experimental design employed only two host types, human and pig. This study evaluated

a reasonable alternative host to a human host in the form of a pig, which can commonly be found in villages near both study sites. Further research using multiple host choices can help to understand the host preferences and attractions of *An. darlingi* in specific locations throughout the species' range.

In Belize, there was a significant preference of *An. darlingi* for the human host as about 65% of the total density collected was from the human host hut. In contrast, there was a significant preference of *An. albimanus* for the pig host hut in Belize. This species has been shown to be zoophilic using blood meal analysis (76; 106). When collected outdoors in the vegetation in Belize, the percentage of *An. albimanus* with pig blood meal was 18% with an FR of 2.0 while for humans was 11% with an FR of 0.73, though the mosquitoes preferred cows to all other animals (in outdoor collections) (76). Data was very similar in Mexico with *An. albimanus* slightly favoring pigs over humans, but cow hosts to both (106). These studies, however, need to factor in host availability and total body mass. The present study only examined the number of mosquitoes found entering a structure containing either a human or a pig. Further studies will need to be carried out in areas with higher *An. albimanus* densities and multiple host species to elucidate the host preference of this species.

In Belize, the total number of *An. darlingi* collected per time point was statistically significant for the collection period of 2:00–6:00 a.m. The number of mosquitoes collected at this time point was significantly lower than the number of mosquitoes collected between 6:00-8:00 p.m. and between 8:00-10:00 p.m. This suggests that the majority of *An. darlingi* were collected before 10:00 pm. This is consistent with results from an entrance behavior study previously carried out using the

same study sites (162). This data reinforce that there is a greater burden of potentially infected mosquitoes entering houses earlier in the evening when individuals may still be active in and around the house. In these communities, alternative intervention methods supplementing bed nets might be effective. There was no difference between host and time. Though *An. darlingi* was collected more frequently from the human host, the pattern of mosquitoes collected at each collection time was the same for each host.

In Belize, approximately one week before the studies were carried out, the river flooded due to excessive rain likely washing out any breeding spots for *An. darlingi*. This could explain the younger population of *An. darlingi* throughout this study as suggested by ovary dissection. The nightly totals for *An. darlingi* began increasing about 2 weeks post-flooding as well.

There was no significant preference for human host or pig host for *An. darlingi* collected in Peru. Low mosquito densities at the time of this study were a contributing factor. Further studies need to be conducted at the Zungarococha, Peru site to ensure that trends observed in this study remain true under higher mosquito densities.

The second study in Peru evaluated the impact of using an untreated bed net. The data show that a significantly lower number of *An. darlingi* entered the pig host house after the bed net was implemented (average of 19 per night before and 1.33 per night after) as well as for *Culex* spp. (average of 154 per night before and 48 per night after). Most studies evaluating the effects of untreated bed nets were conducted in areas where malaria prevalence is high. Focus is appropriately on reduction of *Plasmodium* burden in human and mosquito populations. In The Gambia, the use of an untreated bed net in good condition was associated with a significantly lower prevalence of *P. falciparum*

(51% protection) and significantly reduced morbidity (30). A retrospective analysis study in southern Tanzania showed use of an untreated bed net reduced malaria transmission 4.2-fold while adding a longer-lasting insecticide treated bed net was associated with an additional 4.6-fold reduction in malaria transmission (161). In Papua New Guinea, Burkot et al. (1990) implemented untreated bed nets and reported a significant reduction in man-vector contact (by looking at the percentage of mosquitoes that fed on humans) as well as sporozoite antigen rates for *Plasmodium falciparum*, but this reduction was not enough to significantly affect parasite rates in the human population or in mosquito density (20). This same study found no significant difference in mosquito density from outdoor or indoor landing after bed net usage (20).

In the present study, a significant reduction of mosquitoes even entering the houses when untreated bed nets were used was observed suggesting perhaps an inhibition of important host cues conferred by bed net usage. The bed nets used were untreated but freshly opened from plastic packaging. There is a possibility that somehow the bed net emanated odors from the packaging that was unattractive to mosquitoes. The number of collection nights was limited (3 nights before bed net usage) and the densities of *An. darlingi* were low. Other environmental factors could have contributed to the reduction of entering mosquitoes as well. Additional studies need be conducted to look at the efficacy of untreated bed nets against this medically important species.

Though *An. darlingi* is most commonly collected in inhabited areas and is known to be an anthropophilic species, it has also been collected in uninhabited areas suggesting that this species can utilize nonhuman blood to maintain high densities in areas or at times with few or no human inhabitants (158). It has been shown in other species that

having a host preference (or specializing in a particular host) can confer fitness (107; 168). An overview of these fitness studies shows that when a specific vector feeds from a preferred host, whether animal or human, certain attributes that affect overall fitness are more successful (such as fecundity) (107). Whether human blood confers any fitness advantages for *An. darlingi* cannot be determined from the current study but should be evaluated in future efforts.

Ongoing studies are planned to describe the genetic and morphometric differences using these *An. darlingi* study populations in an attempt to correlate host-seeking behaviors with specific genotype. If host-seeking behaviors of specific geographic *An. darlingi* populations can be linked to their respective genotype it will be instrumental in understanding and assessing the vector potential of regional populations in malaria transmission.

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Table 8. Total number and species of mosquitoes collected from each host at each site

Table 8

Total number and species of mosquitoes collected from each host at each site

Belize	Human	Pig	Total	%	Peru	Human	Pig	Total	%
An. darlingi	349	190	539	61.3%	*An. darlingi*	15	16	31	2.5%
An. albimanus	1	15	16	1.8%	*An. shannoni*	1	5	6	0.5%
An. vestitipennis	2	2	4	0.5%	*Ad. squamipennis*	28	5	33	2.7%
An. punctimacula	0	2	2	0.2%	*Mansonia* spp.	9	36	45	3.7%
Culex spp.	182	136	318	36.2%	*Psorophora* spp.	0	1	1	0.1%
					Culex spp.	539	576	1115	90.6%
Totals	534	345	879	100.0%	Totals	592	639	1231	100.0%

Table 9. Total number and species of mosquitoes collected from the pig host before and after bed net use in Peru

Table 9

Total number and species of mosquitoes collected from the pig host before and after bed net use in Peru

	Before bed net	%	After bed net	%	Totals	%
An. darlingi	57	10.7%	16	2.5%	73	6.2%
An. shannoni	1	0.2%	5	0.8%	6	0.5%
An. oswaldoi	2	0.4%	0	0.0%	2	0.2%
Ad. squamipennis	0	0.0%	5	0.8%	5	0.4%
Mansonia spp.	10	1.9%	36	5.6%	46	3.9%
Psorophora spp.	1	0.2%	1	0.2%	2	0.2%
Culex spp.	461	86.7%	576	90.1%	1037	88.6%
Totals	532		639		1171	

Figure 9. Study site locations.
Site Actun Tunichil Muknal (ATM) is located in the Cayo District, Belize, Central America and site Zungarococha is located in the Loreto Department, Peru, South America.

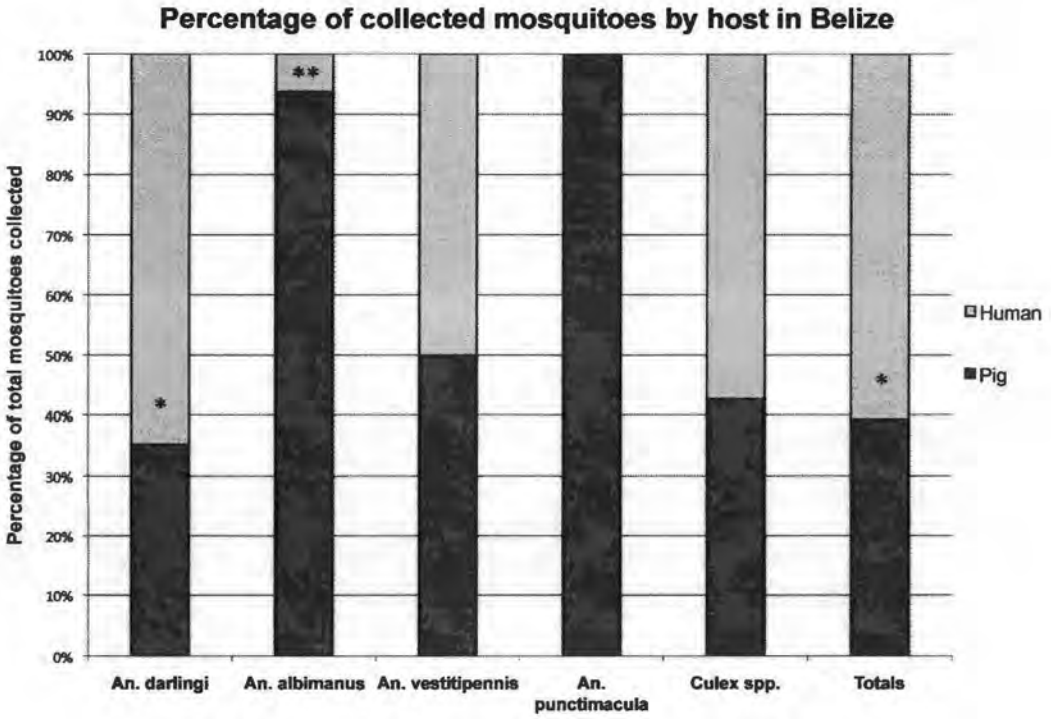

Figure 10. Total percentage of each species of mosquito
Collected from window interception traps from each host over 16 nights in the Cayo District, Belize. The "*" indicates that statistically greater numbers of mosquitoes were collected from the human host hut than the pig host hut ($P < .05$) and the "**" indicates that statistically greater numbers of mosquitoes were collected from the pig host hut than the human host hut ($P < .01$).

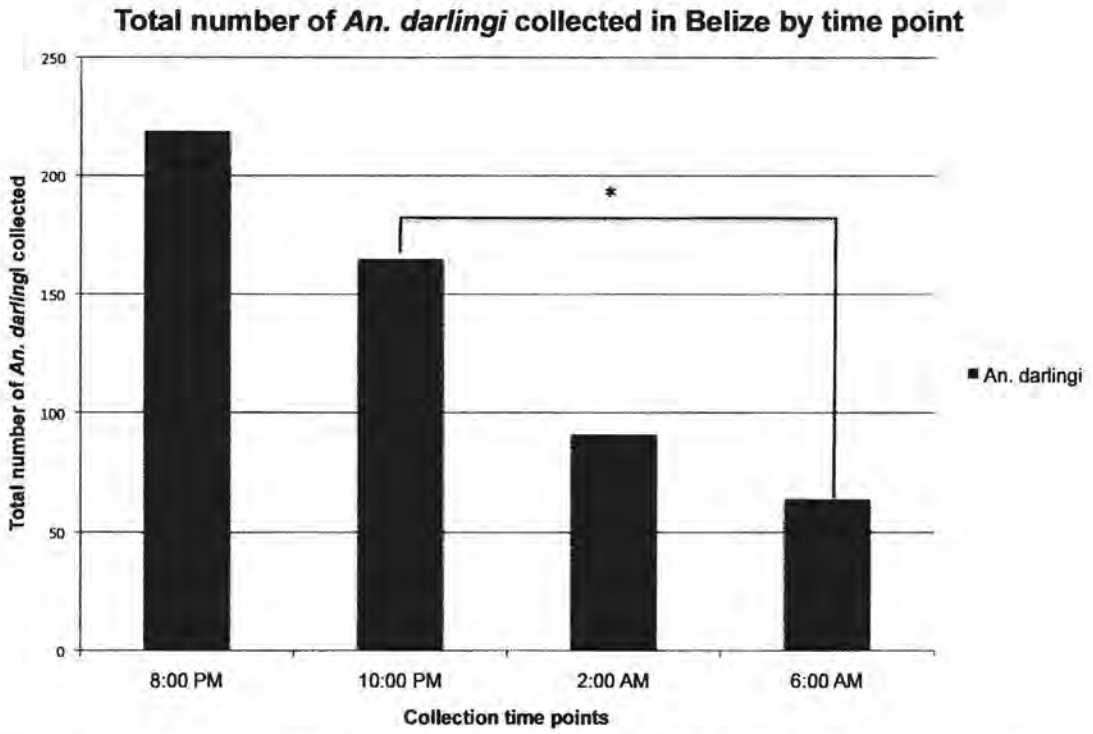

Figure 11. Total number of *An. darlingi* collected at each time point
From window interception traps from both hosts over 16 nights in the Cayo District, Belize. The "*" indicates that the total density of mosquitoes captured at the 6:00 a.m. time point is significantly lower ($P < .01$) than the density of mosquitoes captured at the 8:00 and 10:00 p.m. time points.

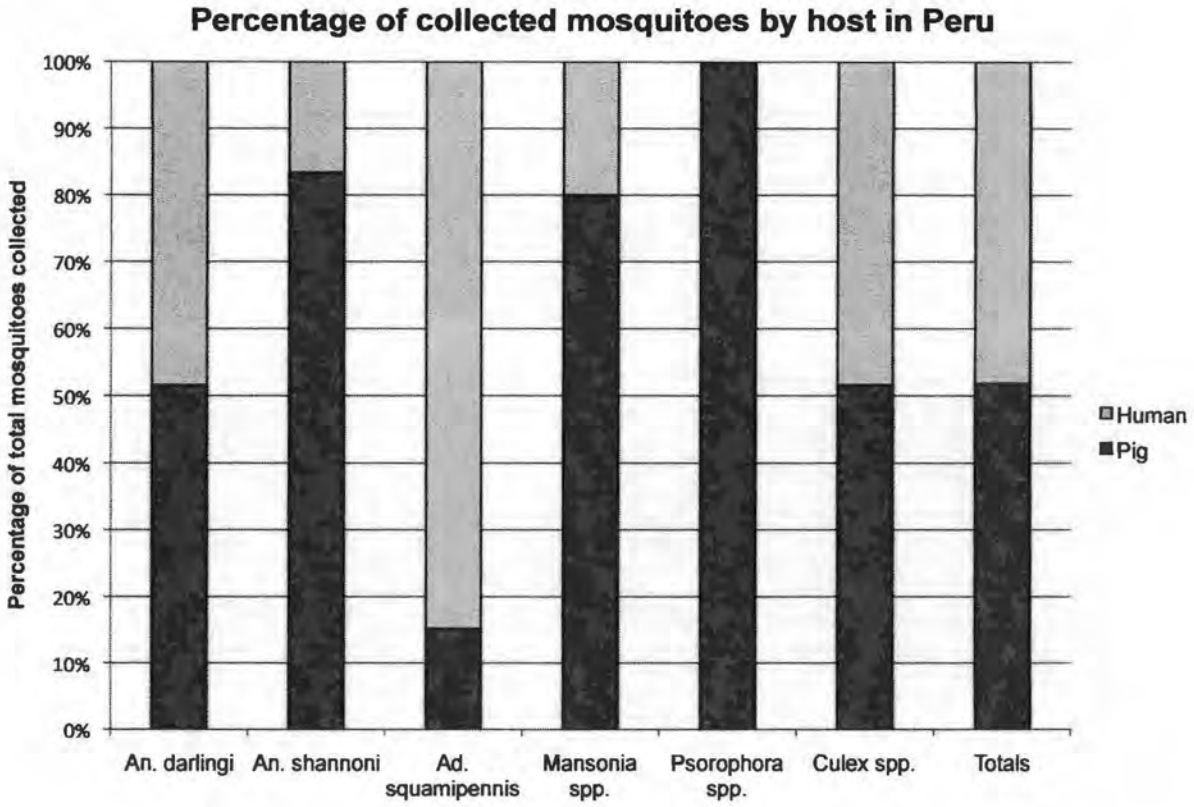

Figure 12. Total percentage of each species of mosquito
Collected from window interception traps from each host over 12 nights in Zungarococha, Peru.

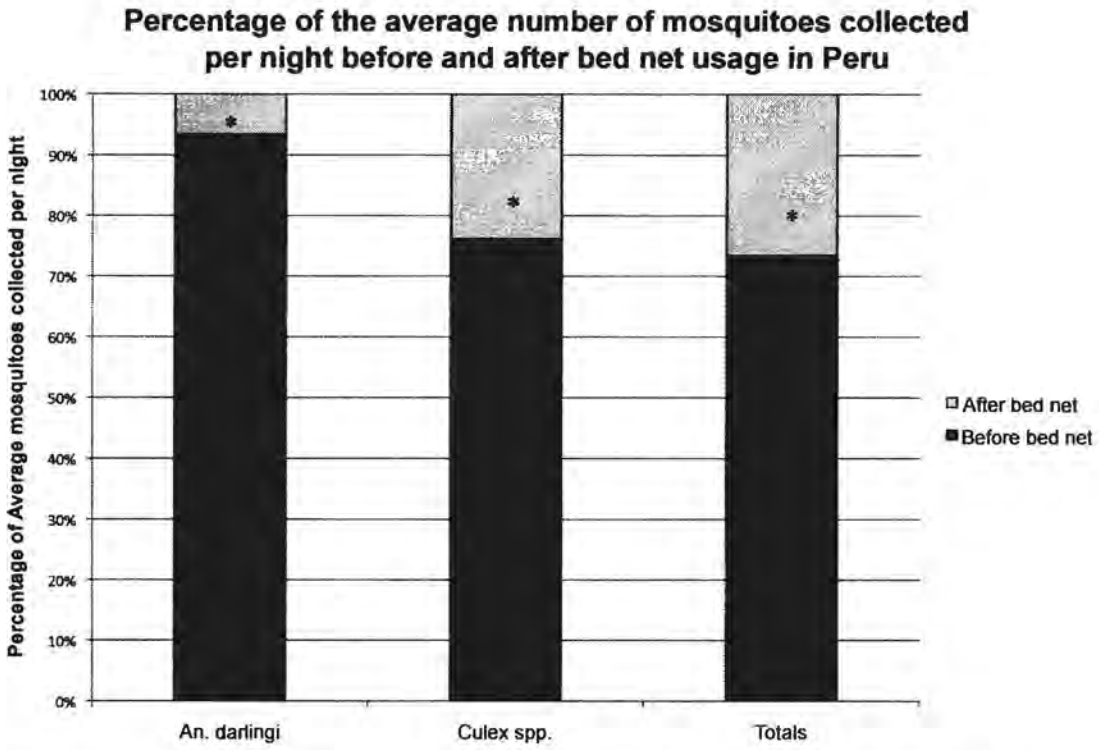

Figure 13. Percentage of the average number of mosquitoes collected per night For *An. darlingi*, *Culex* spp. and total of all species collected from window interception from the pig host over 3 nights before and over 12 nights after bed net usage in Zungarococha, Peru. The "*" indicates that the total density of mosquitoes collected before applying the bed net was significantly greater than after applying the bed net ($P < .01$).

CHAPTER 4: Differences in wing geometry between two genotypes of *Anopheles darlingi* from Cayo District, Belize and Iquitos, Peru

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ABSTRACT

Anopheles darlingi, a major vector for malaria in South America, exhibits behavioral, ecological, morphological, and genetic variation across its wide distribution. A deep divergence, based on the nuclear *white* gene, has described two lineages: a northern lineage (Belize, Guatemala, Colombia, Venezuela, and Panama) and a southern lineage (Amazonia and southern Brazil). An enzyme restriction of the nuclear *white* gene was used to characterize two *An. darlingi* populations collected in experimental hut behavioral studies from Cayo District, Belize and Zungarococha, Peru. Wing shape variation from these samples was analyzed using geometric morphometrics. *Anopheles darlingi* from Belize and Peru were characterized as northern and southern lineage, respectively. Discriminant analysis of wing shape showed a significant amount of differentiation between the two populations with a positive classification rate of 79%. This study is a first step in trying to characterize and compare phenotypic differences in genotypically distinct populations of *An. darlingi*.

BACKGROUND

Anopheles darlingi has been implicated as a major vector for malaria in South America, especially in the Amazon region (40; 41; 57), and has contributed to the resurgence of malaria in Brazil (29) and Amazonia Peru (8). The species has a very broad distribution spanning from southern Mexico to northern Argentina and from the eastern side of the Andes mountain range to the Atlantic and Caribbean coast (68; 171). The species has not been found in Nicaragua or Costa Rica. Barriers to gene flow have been indicated between populations in Central America and northern Amazon (119).

Variability in *An. darlingi* populations across its wide geographic range has been documented using cuticular hydrocarbons (154), isoenzymes, (116; 128; 154), morphology (116), and behavior (154), which has led to the suggestion that *An. darlingi* is a species complex. A deep divergence, as characterized by the nuclear *white* gene, has led to the identification of 2 lineages: 1) northern lineage: Belize, Guatemala, Colombia, Venezuela, and Panama and 2) southern lineage: Amazonia and southern Brazil. Phenotypic expression of the two genotypes has not been characterized.

Geometric morphometrics of wing shape can be used to quantify phenetic variation within or between mosquito populations (51). It has been used to discriminate between cryptic species of Triatominae (183), laboratory iso-female lines of *Aedes aegypti* (88), geographically separated or isolated species of *Glossina palpalis gambiensis* (24) and *Apis mellifera* (60), and closely related species in the Albimanus and Argyritarsis Section of Neotropical Anopheles *(23)*.

Using experimental huts, differences in entry and exit patterns between populations of *An. darlingi* from Cayo District Belize and Zungarococha, Peru were found (162). *Anopheles darlingi* samples collected from these experiments were used in the current analysis. The objective of this research was to 1) identify the samples as northern or southern lineage and 2) characterize the phenetic variation between these two populations.

METHODS

Mosquito sampling

Adult female *Anopheles darlingi* were collected in Cayo District, Belize (17° 9'33.13"N, 88°50'34.62"W) between May – June 2012 and Zungarococha, Peru (3°49'32.39"S, 73°21'1.43"W) between March – April 2011. Mosquitoes were collected from window interception traps from experimental huts used to determine house entrance and exit patterns (162) and host preference (unpublished). Adult female *An. darlingi* were morphologically identified using the keys of Wilkerson et al. (1990) in Belize and Faran and Linthicum (1981) in Peru (56; 190).

Nuclear *white* gene genotyping

Total DNA was extracted from mosquitoes using the "purification of total DNA from insects" protocol from the DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA). The *white* gene PCRs were performed using PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare Bio-Sciences, Pittsburgh, PA). Each 25 μl reaction contained 6 μl of 10μM primers WF and W2R (118), and 13μl of sample DNA. The PCR program included initial denaturation for 2 min. at 95°C and 35 cycles of 1 min. at 95°C, 1 min. at 57°C and 3 min. at 72°C then a final primer extension for 3 min. at 72°C. A subset of samples from Belize (n=13) and Peru (n=8) were sequenced by the Biomedical Instrumentation Center, Uniformed Services University of the Health Sciences and the Genomics & Bioinformatics Core Facility, University of Notre Dame and identified as the northern lineage and southern lineage, respectively. The 800 bp *white* gene includes conserved point mutations differentiating the northern and southern genotypes of *An. darlingi* allowing a restriction endonuclease to cleave these sites resulting in distinct banding patterns. The restriction endonuclease CviQI (New England BioLabs, Inc., Ipswich, MA) recognizes the site 5'-GTAC-3' and cleaves between G-T. Products from the digestion

were run on a 2.0% agarose gel at 75V to view banding patterns. The northern genotype shows bands of approximately 87 and 466 bp and the southern approximately 87 and 590 bp allowing for visual discrimination (Fig. 14).

Geometric Morphometrics

Wings from the aforementioned studies were dissected from female *An. darlingi* collected within the window interception traps. A random set of females was chosen for dissection and one or both wings from each specimen (depending on condition of the wing) were affixed to microscope slides using double-sided tape. Digital photographs of each wing were taken using the ProScope HR high-resolution handheld microscope (Bodelin Technologies, Lake Oswego, OR). The microscope was mounted on the stand it came with and each wing was photographed with a micrometer using the 100X lens. The microscope attached directly to a laptop using a USB port and the pictures were captured and saved using the Proscope HR software (Bodelin Technologies, Lake Oswego, OR). All photographs were digitized using tpsDig V2.16 (Copyright© 2010. F. James Rohlf, Ecology & Evolution, SUNY at Stony Brook). The scale for each picture was measured against the micrometer that was photographed with each wing. Fourteen total landmarks were chosen based on vein intersections (Fig. 15) (190).

A separate tps file was created for each site (Belize and Peru). Each set of digital photographs from each site was digitized 3 times by the same person deleting any photographs where the wings were damaged, folded, or could otherwise not be digitized. All 3 replicates for each site were aligned and only the samples present in all 3 replicates (those that were not discarded) were selected giving a final total of 285 specimens for Belize and 214 specimens for Peru that were used for analysis. For each site, the total set

of all 3 replicates that were chosen were subjected to General Procrustes Analysis (GPA). The new procrustes coordinates for each sample were then averaged across 3 replicates. The averaged procrustes coordinates were subjected to GPA for alignment and the procrustes residuals used for principal components analysis (PCA), canonical variates analysis (CVA), and discriminant function analysis (DFA). All analyses were carried out in MorphoJ 1.04a (96).

RESULTS

Enzyme restriction

The CviQi endonuclease restriction digest applied to the *white* gene product showed 100% (n=43) of Iquitos, Peru samples identified as northern genotype and 100% (n=37) of Belize samples identified as southern genotype.

Principal Components Analysis

The first two principal components (PCs) explain 33.5% of the total variation (PC1 20.1% and PC2 13.4%). A total of 11 PCs was required to recover 90% of the shape variability. Most of the variation occurs at landmarks 1, 10, 12, 13 and 14 (Fig. 16).

Canonical Variates Analysis

Canonical variates analysis determined that variation between *An. darlingi* samples from Belize and Peru was statistically significant ($P<0.0001$ permutation tests for Mahalanobis and Procrustes distances). Most of the variation between the populations occurs at landmarks 1, 12, 13, and 14 (Fig. 17).

Discriminant Function Analysis

Discriminant function analysis correctly assigned 79% of *An. darlingi* wings to the correct country of origin using leave-one-out cross validation (Table 10). Results are statistically significant ($P < 0.0001$ permutation tests for Mahalanobis and Procrustes distances).

Discussion

Anopheles darlingi samples collected from window interception traps from Cayo District, Belize and Iquitos, Peru were correctly identified as nuclear *white* gene northern lineage and southern lineage, respectively. In experimental hut studies, the Belize *An. darlingi* population exhibited a bimodal entrance pattern with an initial primary peak occurring between 7:00-8:00 pm and a minor secondary peak at sunrise as compared to the Peru population which exhibited unimodal entrance with a peak between 10:00-11:00 pm. Exit of unfed females from Belize peaked between 7:00-8:00 pm and from Peru peaked between 11:00-12:00 am. In addition, entrance and exit movement patterns between the two populations, as assessed using a Kaplan-Meier curve, were statistically different (162). This is the first time behavioral expression in confirmed populations of each genotype have been characterized.

Results of the principal components analysis are comparable to other similar studies. Baylac et al. (2003) investigating the status of a species complex of *Bassus* sp., contributed 90% of the shape variability to the first 11 axes (12), likewise, Francoy et al. (2009) attempting to discriminate between populations of *Apis mellifera* Linnaeus, 1758 separated by 34 years of Africanization found the first two PCs accounted for 35.72% of shape variation (60). There is overlap between the two countries on the scatter plot of PC

1 and PC2, but minor differentiation can be observed at the upper and lower limits of the PC axes.

The canonical variates analysis showed statistically significant separation between the two populations, particularly at landmarks 1, 12, 13, and 14, which were the prominent sites of variation found in CVA as well Discriminant analysis of the two populations also showed significant differentiation with 79% of *An. darlingi* being correctly assigned to its respective country of origin. This percentage is along the range of similar studies as well. In a study comparing differentiation between geographically separated populations of dengue vectors, Henry et al. (2010) found classification rates ranging from 50% to 80% after validation (81). Camara et al. (2006) looked at 3 populations of *Glossina palpalis gambiensis*, 1 isolated on an island and 2 located on the mainland and found the reclassification rates of 77% for the island population and 91% for the mainland populations (24).

The landmark sites where the variability occurs are located in the same area of the wing and occur where the subcosta meets the edge of the wing, the point where media-1 and media-2 converge, the interior end of R_{4+5}, and the point where R_2 and R_3 converge, respectively. Wing shape is less affected by environmental variance (52) and is species specific (35). Veins establish the rigidity of the wing and distribution of sensory organs required for flight. They also influence the melanin pattern, which can vary widely in closely related species (35). The landmarks chosen for this analysis lie along the venation edges and intersections, therefore shape, as we define it with the landmark configuration, will most likely be affected by venation. *Anopheles darlingi* populations from the northern lineage and southern lineage are shown to have barriers to gene flow

and are described as being incipient species (119). Manguin et al. (1999) compared variation in wing costal dark and pale spots of *An. darlingi* from 7 countries. Eight variants were identified and observed frequency distribution varied significantly and was population dependent. The population from Belize exhibited the least amount of variants suggesting a lower amount of gene flow. It was determined that levels of variation fell within intraspecific levels (116).

Inferences cannot be made as to whether the observed shape variation is genotype specific. As Manguin (1999) found, the differentiation could be population dependent. Wing venation is associated with flight characteristics, which would most likely be specifically adapted to the geographical area where *An. darlingi* inhabits, thus variation would exist between separate geographic populations suited for their local environment. More wing samples from across the distribution of both genotypes need to be analyzed in order to better understand the phenetic variability present in this species. This is just a first step in trying to characterize phenotypic expression of the recently defined nuclear *white* gene lineages.

CONCLUSION

Two *An. darlingi* populations identified from the northern and southern lineage have significant variation in wing morphology, which can be used to correctly classify them 79% of the time.

Table 10. Discriminant function results if wing shape in *An. darlingi*

	Belize	Peru	Total
Belize	**235**	50	285
Peru	54	**160**	214
Total			**499**

Figure 14. Banding patterns resulting from the nuclear *white* gene enzyme restriction using CviQI.

Samples run on 2.0% agarose at 75V, Lane 1: PCR negative control, Lane 2: *An. albimanus* captured in Belize, Lane 3: *An. benarocchi* captured in Peru, Lanes 4-8: *An. darlingi* captured in Belize, Lane 9: molecular mass marker, Lanes 10-14: *An. darlingi* captured in Peru. *An. darlingi* samples were sequenced and confirmed to be of their respective lineages.

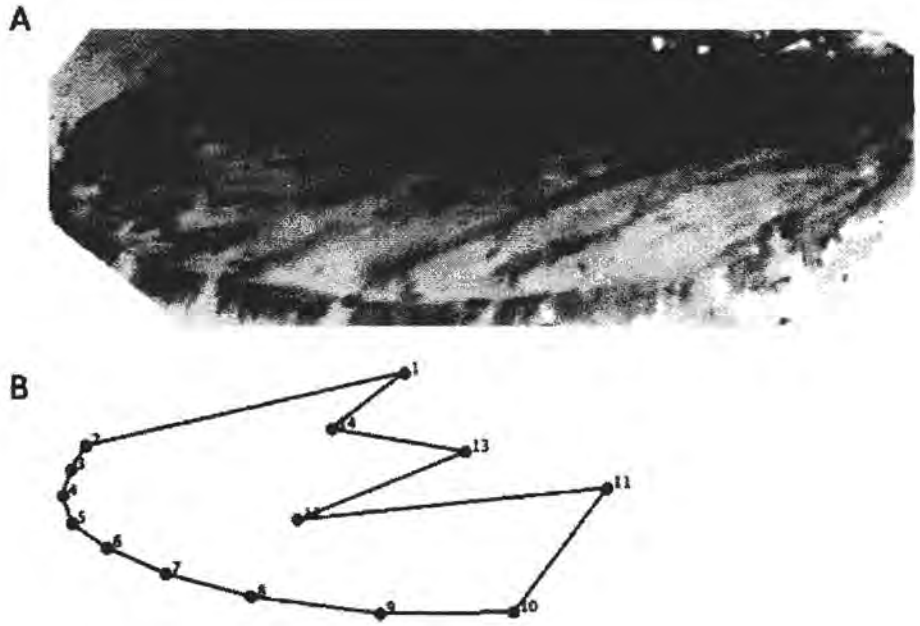

Figure 15. Landmark Configuration used for *An. darlingi* wing shape. A) wing of *An. darlingi* showing 14 landmarks B) wireframe representation of the landmarks developed in MorphoJ 1.04a

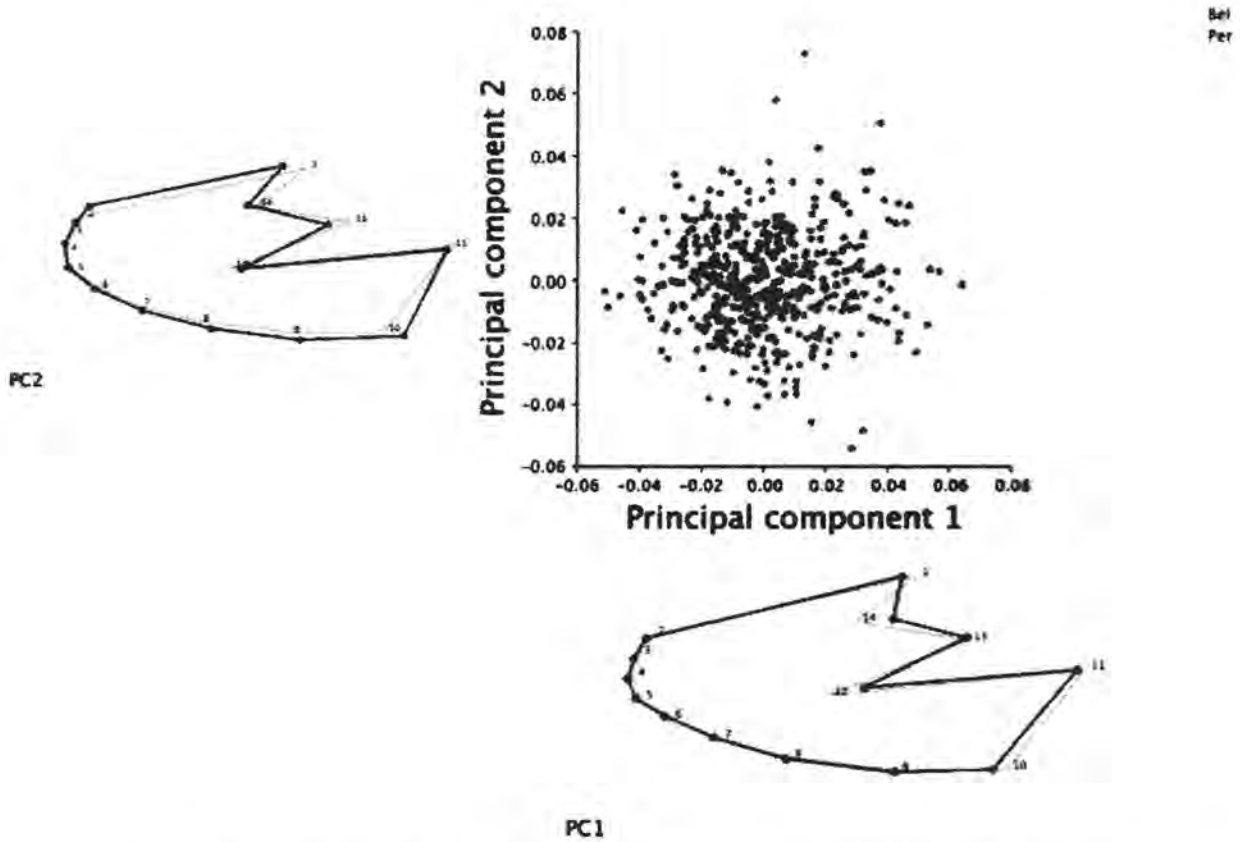

Figure 16. Scatter plot of the principal components representing the most shape variation. Principal component 1 (20.1%) and principal component 2 (13.4%) represent 33.5% of the total variation. Wireframe diagrams of shape variation.

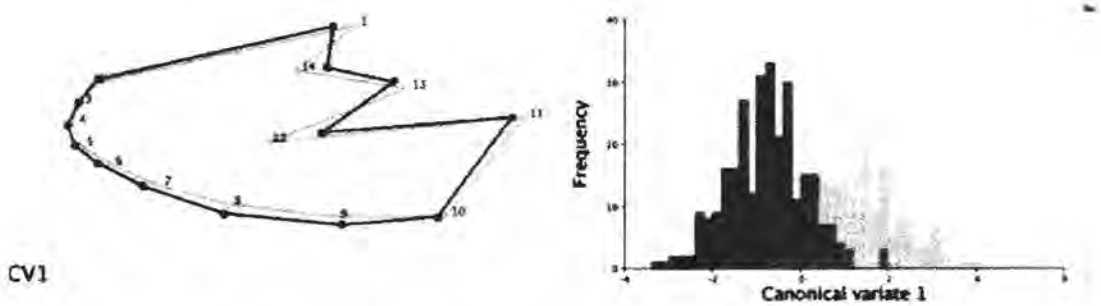

Figure 17. Canonical variates analysis
Wireframe showing where variation occurs. Histogram of CV scores. Results were statistically significant (P<0.000)

CHAPTER 5: Overall summary and discussion

The objective of this research was to use a standardized methodology to compare two field populations of *Anopheles darlingi* that represent each of the defined genotypes. Since the genotypes have been published, multiple papers have characterized local populations as being of either the northern lineage or the southern lineage, for example, when *An. darlingi* was first reported in Panama, the population was characterized as being of the northern genotype (102). In Colombia, surveyed *An. darlingi* were also found to be exclusively of the northern lineage (79). Each of these studies was genetically characterizing local mosquito populations. This is the first research that aims to link the separate genotypes to phenotypic expression that is epidemiologically relevant in terms of malaria transmission dynamics. Research was conducted in Cayo District, Belize where the northern lineage has been confirmed and in Zungarococha, Peru where southern lineage has been confirmed.

EXPERIMENTAL HUTS

Use of experimental huts had two advantages. First, they allowed for direct comparison of *An. darlingi* attraction to two different host species (human and pig). Because the huts were situated approximately 30 m from each other and because each host type was evaluated on the same dates, differences in the external environment (as it relates to climate) were not a factor. Hosts were rotated evenly between the two huts and no collection variation between the huts was apparent. Each host was situated in the center of the hut so that the internal environment was comparable, with the exception of when collectors entered the pig hut for mosquito collections. There may have been

unknown external factors surrounding each hut that affected total numbers of *An. darlingi*, but the experimental huts allowed us to control the conditions inside the hut and minimize additional confounding factors. Second, the experimental huts allowed for comparable data to be collected from two field sites located approximately 1800 miles from each other. Though hut construction was slightly different at each locality, the sampling methods were standardized, which allowed for the data to be relatable.

SUMMARY AND DISCUSSION OF CHAPTER 2

The objective of this study was to characterize and compare experimental hut entry and exit between two genotypically divergent populations of *An. darlingi*. Results showed a different peak in entrance and exit times and statistically significant differences in movement patterns throughout the night between the two study sites. A unique aspect of the presented data is the observation of overall temporal movement patterns that occur in house entrance and exit of *An. darlingi* throughout the night. The Kaplan-Meier survival curve allowed us to look at a 12-h period and assess the percentage of mosquitoes that were active in the house and the duration in time of which they were active. This needs to be analyzed cautiously as our exit results are only indicative of those female mosquitoes that were unable to acquire a blood meal. Blood fed females would most likely exhibit different exit patterns as they would be looking for resting sites to digest their blood meal and prepare to lay a batch of eggs.

These results represent only a snapshot in time, in particular, a snapshot of behavior at peak seasonal densities and might not be representative of temporal entrance and exit patterns at other times of the year. In Belize, Achee et al. (2006) noted differences in outdoor to indoor biting ratios over the course of 10 months representing

variation in feeding behavior. Longitudinal studies are required in order to determine if the peak seasonal entrance and exit patterns remain true throughout the year.

The genetic determinants for host-seeking behavior have not been determined. That peak activity times are fixed for each location as exhibited by similarity of results with previous, unrelated studies using different collection methodologies (6; 179), is an indicator of genetic influence. This is the first study to attempt to describe host-seeking behavior between two populations of the recently characterized nuclear *white* gene lineages.

SUMMARY AND DISCUSSION OF CHAPTER 3

The objective of this study was to compare the host preference of *An. darlingi* populations in Belize to populations in Peru. Belize populations exhibited a significant preference for the human host. Peru populations exhibited a more opportunistic host preference entering both host huts almost equally. This result could have been influenced by the low mosquito abundance in Zungarococha, Peru that occurred throughout the course of the study. The study would have to be repeated to determine if *An. darlingi* at times of greater abundance exhibits the same opportunistic patterns. Results from Belize reinforce the species' anthropophilic status in this area. Though *An. darlingi* demonstrated a preference for the human host hut, about 35% of *An. darlingi* in Belize were still attracted to the pig host hut implicating a level of opportunistic feeding potential.

The study design was chosen for a few reasons. There were a number of animal baited host preference studies on *An. darlingi* but most had been conducted during the DDT vector control campaigns and the few, more recent studies either did not report

enough detail regarding the study set-up or there was inconsistency in how each host was presented as a feeding source. For example, Klein and Lima (1991) presented a bovine host within a trap setting, but a human host was presented in the open (93), introducing a collection bias. Another reason was the infeasibility of conducting an animal census at our locations to calculate a foraging ratio. The site in Belize was pretty remote, located on the fringe of the Tapir Mountain Nature Reserve, and surrounded by farmland. The abundance and diversity of animal species would have been too great to count. The same is true in Zungarococha. Though this site was directly in the village and counts of the domestic animal species would have been feasible, it directly bordered the forest fringes where again abundance and diversity of available animals would be great. Human biting indices would have been feasible in both locations and overall proportions of host sources would have enhanced the host preference data. Future studies should include this analysis to provide a more complete picture of the overall feeding habits.

The last reason for choosing the baited host preference approach is that experimental huts were available and it was a perfect way to present two host sources in the same controlled manner. As mentioned previously, the internal environment of the experimental huts was standardized and rotating the hosts between huts enabled any potential unknown confounding factors that might have been present in any individual hut to be minimized. This study represented an artificial feeding opportunity for host-seeking *An. darlingi*. Though it does not represent natural foraging tendencies, it does represent a "choice" and a tendency to pursue one host type over another host type.

Ultimately the goal is to link phenetic expression to genotype. In the *An. gambiae* complex, certain karyotypes of *An. arabiensis* and *An. funestus* showed significant

associations with human blood (14) and a rapid change in host choice was induced in response to selection pressure between two host types (14; 69), indicating a polymorphism for host preference in this species. Still the genetic link is elusive. This would be the first study to try to differentiate host preference based on known genotypic variation in *An. darlingi*.

SUMMARY AND DISCUSSION OF CHAPTER 4

The objectives of this study were to 1) confirm the nuclear *white* gene lineage of the *An. darlingi* samples collected from the entrance, exit, and host preference studies conducted in Cayo District, Belize and Zungarococha, Peru and 2) compare wing shape variation of the same population. The nuclear *white* gene PCR product of a subset of samples from Belize and Peru were sequenced, confirmed to be of their respective genotypes using Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and aligned using the freely available alignment software SeaView v4 (73). The point mutations that differentiate the genotypes were identified and an endonuclease that would target these areas was determined. The documented banding patterns using the originally published enzyme restriction assay (118) was not observed between the samples acquired in the present studies. The newly identified endonuclease showed restriction fragments lengths at sizes they were calculated to be. Using this new assay, all samples from the Belize population were determined to be of the northern genotype and all Peru samples determined to be of the southern genotype. Thus confirming that the populations from the field studies were actually genotypically different populations.

Geometric morphometrics quantifies shape change. Wing shape is a phenotypic expression of the genetic makeup of a mosquito. The shape variation between the two

populations was great enough that the two populations could be significantly differentiated. The scatter plot of the first two principal components did not show two distinct populations. Canonical variance analysis and discriminant function analysis both reported significant differentiation and the landmarks that exhibited the most variation were similar to those that determine PC1, which represents 20.1% of the total variation. Caution should be taken when interpreting these results. Only 2 populations were used in the evaluation, therefore there was a 50% chance that they would be correctly identified. These statistical methods purposefully look for the maximum differentiation so it is possible that the 79% of samples correctly identified is not actually biologically relevant.

The landmarks demonstrating the greatest amount of variability are located in the same region of the wing. It is difficult to characterize the individual landmark differences in any more detail then that they exist. The landmark configuration used for the samples are essentially defined by wing venation, as all the points are located either at the ends of veins or at intersections of veins. Veins are instrumental in the elaboration of the wing melanin patterns, which can vary widely even between closely related species (35). Manguin et al. (1999) observed 8 different costal dark spot patterns on *An. darlingi* samples from 7 countries, with Belize populations exhibiting the least amount of frequency. It was determined that even though frequencies were population dependent, the levels of variation were intraspecific. Though it might be a leap to link wing shape and costal dark spots, both influenced by venation, it is possible that the morphometric differences found in these *An. darlingi* samples are also population dependent evolving from an ecological adaptation.

Wing rigidity and sensory organs, which are integral for the coordination and execution of flight, are also governed by the veins (35). Pennetier et al. (2010) found that during copulation, male-female pairs matched the frequency of wing beat harmonics, likely aiding the process by reducing the air flow turbulence between the partners (141; 150). Frequency matching of the M and S molecular forms of *An. gambiae* s.s. was statistically greater when like form mated with like form (M with M and S with S), suggesting that this trait enables assortative mating between the molecular forms (141; 150). No studies have been conducted on wing beat frequencies using *An. darlingi*. Future studies would need to be conducted to determine if there is a relationship first, between wing shape and wing harmonics and second, whether the 2 genotypes of *An. darlingi* in fact demonstrate differentiation in wing beat frequencies.

Shape is believed to be influenced more by genetic makeup than of environmental influences (as compared with size). However, there would most likely be specific habitat and niche differences that would influence each population's adaptation to their respective environments that could influence development and maintenance of a distinct morphology. Multiple *An. darlingi* populations spanning its distribution need to be evaluated in order to achieve a grander picture of wing shape variation. Expanding on this, it cannot be determined if the differentiation is on an individual population level that would be characterized as intraspecific variation or if it is on a genotypic scale supporting evidence for *An. darlingi* being an incipient species. Overall, what the data says is that two genetically different populations displayed significantly different phenotypes.

ADAPTIVE DIVERGENCE AND SPECIATION

The data put forth in this dissertation, in and of itself, neither acknowledges nor refutes that *An. darlingi* is an incipient species as it is only comparative between two distinct locations at specific points in time. Based on the data, there is a strong indication that *An. darlingi* from Belize and Peru are diverging populations. They exhibit differences in peak house entrance and exit times, differences in house entrance and exit movement patterns as analyzed using a Kaplan-Meier curve, and each population is genetically distinct based on nuclear *white* gene analysis.

Charlwood (1996) stated that species with broad geographic ranges are more likely to be separated based on physical and climate related factors, therefore, exhibiting a greater habitat diversity which could lead to cline development, in other words ecological divergence, and eventually speciation (27). Variability in gene frequency, morphology, and behavior can readily be found among and within different geographic populations of *An. darlingi*. It is likely that the heterogeneity found amongst certain markers is indicative of adaptive divergence within *An. darlingi*.

Barriers to gene flow between the two genotypes support the designation of *An. darlingi* as an insipient species (121). One major question is whether the nuclear *white* gene is a locus that is under divergent selection and would, therefore, represent the selective trait that would distinguish two separate molecular forms. There are still many unanswered questions. In *An. gambiae* s.s., the molecular forms of M and S can be differentiated using a polymorphism found in the rDNA. This locus has been mapped to a region of high differentiation near the centromere of the X chromosome (101). The genome of *An. darlingi* has only begun to be analyzed, therefore, no "speciation islands" of high differentiation between forms has been identified. These sites are where traits

responsible for ecological divergence are hypothesized to reside (180). To the author's knowledge, the nuclear *white* gene has not been mapped to the genome of *An. darlingi*. In addition, *An. gambiae* s.s. M/S hybrids have been found at varying proportions in wild population. Though no inviability or sterility of hybrids has been demonstrated (150), indicative of postzygotic isolation mechanisms, the low proportions suggest a level of assortative mating, which is a prezygotic isolation mechanism. Furthermore, assortative mating between the M and S forms has been demonstrated through analysis of inseminated wild-caught females, observation of segregated mating swarms, and demonstration of wing beat frequency matching, all supporting that like forms preferentially recognize and mate with like forms (141; 150). Nuclear *white* gene hybrids and evidence of assortative mating between the two genotypes of *An. darlingi* have not been reported. That is not to say that they do not exist, they just either have not been found or not been reported on yet. Areas where both the northern and southern genotypes co-exist have been referenced (118). Further research in these geographic areas will help to answer some of these questions.

Outside of the nuclear *white* gene and molecular analysis, purported adaptive behavioral divergence has been documented. Overall, *An. darlingi* continues to demonstrate highly anthropophilic and endophilic tendencies, especially in comparison to other *Anopheles* species. However, there is evidence of zoophilia as well as exophagia in certain populations. As mentioned previously, *An. darlingi* populations on the coast of Brazil displayed anthropophilic and endophilic tendencies, while in the interior displayed zoophilic and exophilic tendencies (66). It has been suggested that populations closer to the center of distribution, for example the Amazon rainforest, exhibit behaviors that are

more indicative of a "wild" population (66). These populations are quite adaptable and readily follow humans away from the center of their natural range. The human migration and habitat alteration are relatively recent. The populations that branched out could be becoming more established and as more time passes, and more differentiated from the reservoir "wild" populations demonstrating adaptive divergence.

In terms of malaria transmission, *An. darlingi* has been incriminated as a primary vector even when adult densities are low indicating that it is an efficient vector. However, malaria incidence in the Central America is much lower than in South America. It is possible that *An. darlingi* populations in Central America have a decreased susceptibility to *Plasmodium* parasites, but it is also possible that other factors including climate, environment, topology, habitat availability, human demographics and migration, and history of vector control contribute to the difference in incidence between these areas. For example, in Belize *An. darlingi* breeds in faster flowing clean rivers. These locations are usually inhabited by smaller villages and are easily accessible by roads. If malaria occurs, it is easier to control via localized treatment and vector control. In Peru, specifically the rural areas outside of Iquitos, *An. darlingi* breeds in lagoons and rivers which are much more abundant. There are many more villages with greater populations that surround these breeding sites. Typical occupations require travel into more remote areas where infectious *An. darlingi* are present. There is a level of resistance for these workers who then become asymptomatic carriers of malaria. This ultimately increases risk within the village, particularly in the homes of the workers, as they are a mobile stable reservoir for *Plasmodium* (39; 139). These types of transmission dynamics are not present in Belize. Comparative susceptibility studies between *An. darlingi* from different

geographic locations would provide insight as to whether adaptive geographic differentiation exists in parasite susceptibility.

A challenge for future research is to define the relationship between genotype and complex phenotype (14). Levels and patterns of gene flow are important when assessing and predicting the spread of genes that confer insecticide resistance and parasite susceptibility and refractoriness. Ongoing research is needed to understand how gene flow and genotypes affect behavioral traits responsible for disease transmission.

EPIDEMIOLOGICAL RELEVANCE

The most important tool in prevention of malaria transmission is a detailed understanding of the ecology and behavior of local vector populations (189). Though the present research may not answer the question of whether *An. darlingi* is an incipient species, it provides data that is important on a local scale to aid in the implementation of efficient and successful vector control measures. *Anopheles darlingi* populations from the Cayo District in Belize and Zungarococha, Peru demonstrate very different host-seeking behaviors. In Belize, *An. darlingi* enter houses in search of blood meals more actively earlier in the evening when individuals are most likely not asleep and protected by bed nets. Though bed nets are still an important control method as mosquitoes still enter houses throughout the night, alternate repellent methods would be instrumental in targeting the mosquito vector population at a time when it exhibits a greater burden. Mosquito coils, emanators, and insecticide treated materials should be evaluated to determine their efficacy. In Peru, peak house entrance of *An. darlingi* occurred later in the evening between 10:00-11:00 pm, when many individuals would be asleep and protected by bed nets. Distribution campaigns would be advantageous to ensure that

populations at risk have access to insecticide treated bed nets. In addition, exit data suggests that there is a time period when *An. darlingi* might remain in the house searching for a host. Again, alternative methods might be able to target these populations. Indoor residual spraying is the most obvious intervention, but use of insecticide treated materials might also provide supplemental protection.

In terms of host preference, both populations of *An. darlingi* have historically been shown to be anthropophilic. In the present studies, varying degrees of opportunistic feeding preferences were demonstrated. In both locations, it is unlikely that any type of zooprophylaxis would be successful to a degree that it would decrease malaria transmission.

FUTURE DIRECTIONS

Now that these two genotypes have been defined and accepted, more comparative studies of phenotypically expressed characters can be observed. In regards to the current research, host preference could be broadened to include multiple host types and the incorporation of blood meal analysis and experimental huts could be used to observe resting preferences. Outside of this research, purported differences in comparative susceptibility of malaria parasites, survival rates, overall general bionomics, and wing acoustics could be evaluated.

The presented research is useful in a direct comparison of two populations, but it is really meant to be the first of many sets of comparable measurements. These studies need to be repeated across the geographic range of *An. darlingi*. With standardized methodologies a multi-country analysis would be possible. Ideally studies would be longitudinal to factor in seasonal variations as well as monitor changes in vector

dynamics. Human migration, deforestation, and mosquito habitat and population expansion are constantly in flux and have been demonstrated to be key factors in the transmission of malaria in Central and South America. This study is an initial step in trying to quantify how genetic variation is expressed in traits important to the transmission of malaria.

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