PERSISTENT TRANSMISSION OF MALARIA IN COASTAL PAPUA NEW GUINEA: EFFECT OF VECTOR BITING PATTERNS AND HOST SELECTION

By

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ABSTRACT

PERSISTENT TRANSMISSION OF MALARIA IN COASTAL PAPUA NEW GUINEA: EFFECT OF VECTOR BITING PATTERNS AND HOST SELECTION

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In Papua New Guinea where malaria is endemic, nationwide vector control campaign based on free distribution of insecticidal bed nets is presently ongoing to alleviate the burden of the disease. In the north coast of Madang province, prevalence of malaria infection in human populations in 2006 was 42.1% *Plasmodium falciparum* and 41.7% *Plasmodium vivax*. After the commencement of the bed net campaign in this region in 2008, infection prevalence dropped for both *P. falciparum* (9.0% in 2014) and *P. vivax* (12.7% in 2010). Human biting rates, the number of *Anopheles* bites per person-night, dropped from a range of 10–60 in 2009 to 3.0–18 in 2011. Infectious biting rates, the number of *Plasmodium* spp. sporozoite-infective *Anopheles* bites per person-year, dropped from a range of 20–350 in 2009 to 0–15 in 2011. The research described in this dissertation investigated if the ongoing efforts to intensify the bed net program has succeeded in reducing malaria transmission further or whether further reduction is hampered by vector-related ecological factors which enable transmission to persist. Barrier screen and human landing catch mosquito sampling, active-case malaria detection, and bed net survey were conducted in 2016–2017 in the north coast villages of Madang. PCR methods were used to identify *Anopheles* species and their blood-meal host, and to test for malaria infection in humans and mosquitoes. Human blood meals were fingerprinted by microsatellite genotyping to identify the individuals bitten. The results show that despite high bed net usage (> 80% of villagers reportedly slept under a net) in all villages, the overall infection prevalence rebounded to 18% *P. falciparum* and 14% *P. vivax* in 2016–2017. The human biting rates increased to a range of 9.0–

31 bites per person-night. The infectious biting rates also increased to a range of 6.5–159.7 infective bites per person-year. *Anopheles* species richness ranged from four to six species per village, but relative abundance was highly uneven within and between villages, and community composition was generally dissimilar among villages. The vectors did not exhibit strict anthropophagy but were rather opportunistic in their host selection and utilized non-human hosts when access to humans was limited by the bed nets. The frequency of blood meals obtained from different human individuals was not random but rather clustered, and a few individual villagers were utilized as hosts more frequently than others. The human biting frequency was also clustered spatially and was high in few locations and low in most locations, causing the basic reproduction rate of malaria to increase by multiplicative factors ≥ 1.6. Most (29–50%) of the bites taken on humans occurred before 22:00 hr when 75–90% of people were awake and unprotected by the bed nets and 67–83% of the bites occurred before nearly everyone retired to bed at 2:00 hr. Most (> 50%) of the bites taken on humans occurred outdoors where the risk of exposure to the bed nets was low. These results show that in the north coast of Madang, malaria transmission has increased in recent years and continues to persist at high levels despite ongoing efforts to intensify the vector control program. The diverse *Anopheles* species, their high abundance and great dissimilarity in species composition among villages indicate that the bed net is failing to affect the vectors, allowing vector populations to revert to pre-bed net condition. The opportunistic host utilization and outdoor biting behavior of the local vectors enable them to evade the indoor-deployed bed nets by feeding on alternative hosts to supports their population size and continue to transmit malaria by feeding on humans outdoors and early in the evening when people are active and unprotected by the bed nets. By increasing the basic reproduction rate, the heterogeneity of bites on humans causes transmission to persist rather than dissipate.

This dissertation is dedicated to my son Jerome and daughter Jerolyn who witnessed me go through this studious academic pursuit as they transitioned from childhood to teenagers.

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KEY TO ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
CI	confidence interval
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
GLM	generalized linear model
HBR	human biting rate
HLC	human landing catch
IBR	infectious biting rate
LT	light trap
NDOH	National Department of Health
OR	odds ratio
PCoA	principal coordinates analysis
PCR	polymerase chain reaction
PNG	Papua New Guinea
qPCR	quantitative polymerase chain reaction
RC	resting collection
rRNA	ribosomal ribonucleic acid
SR	sporozoite rate
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1. Overview

This chapter presents a literature review of relevant materials as background information for the topics that are discussed in subsequent chapters. It begins with the most fundamental aspects of the biology of malaria parasites and their mosquito vectors and how these are linked to the process of transmission of the disease. This is followed by the global epidemiology of malaria, which includes its geographic distribution and the distribution of the primary mosquito vectors, the categorization of malaria epidemiology into different endemicity classes and the public health burden and control of the disease. The discussion then switches from a global to local scale by focusing on the epidemiology of malaria in Papua New Guinea (PNG). The chapter ends with the objective and statement of ethical approval of the research reported in this dissertation.

1.2. Biology of malaria parasites and mosquito vectors

1.2.1. *Plasmodium* life cycle

Malaria is a vector-borne infectious disease of humans. It is caused by infection of the liver and red blood cells by microscopic unicellular protozoan parasites in the genus *Plasmodium*. The disease is transmitted from person to person by the bites of female mosquitoes in the genus *Anopheles*. There are many species of *Plasmodium* but only four are known obligate parasites of humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*. They are called human malaria because they are not known to naturally infect and cause disease in other mammalian, avian or reptilian hosts (Antinori, et al. 2012; Gilles 1993; Schlagenhauf 2004). A fifth species, *Plasmodium knowlesi*, is a simian malaria but it can also

infect humans if humans are exposed to the bites of the mosquitoes that transmit this parasite among the simian hosts (Cox-Singh, et al. 2008; White 2008).

Transmission of the malaria parasites is directly linked to their life development cycle, therefore, a review of the fundamental aspects of this complex biological system is relevant. Although the development stages of the four *Plasmodium* species vary in fine details, they all undergo the same general life cycle which consists of two parts: a sporogonic and merogonic cycle. The sporogonic cycle includes all the sexual development stages, which take place in the mosquito vector (definitive host) whereas the merogonic cycle includes all the asexual development stages, which occur in the human host (intermediate host). The sporogonic cycle begins when an uninfected female *Anopheles* bites an infected person and ingests a blood meal containing the haploid stage of the parasite called the gametocyte. If both forms of gametocyte—microgametocyte (male gamete) and macrogametocyte (female gamete)—are present in the blood meal, sexual fusion takes place inside the mosquito midgut about an hour after the blood meal uptake to form a diploid cell called a zygote. About a day after the sexual fusion, the zygote transforms into a motile, partially elongated stage called an ookinete. The ookinete exits the mosquito midgut lumen by traversing the midgut epithelium and affixes itself on the outer side (the hemocoel side) of the gut. There the ookinete transforms into a round, immobile stage called an oocyst. Each oocyst undergoes sporogony, a 10–14 days process where the parasite undergoes multiple fission forming thousands of sporozoites—a motile, elongated, haploid stage of the parasite. Upon rupture of an oocyst, thousands of sporozoites are released into the hemolymph (insect blood) and migrate to the mosquito head and thorax where they inhabit the salivary glands. When the mosquito takes subsequent blood meals from humans, the sporozoites are

inoculated along with the mosquito saliva into those individuals, thereby infecting them (Ghosh, et al. 2000; Smith and Jacobs-Lorena 2010; Vlachou, et al. 2006).

The merogonic cycle begins when a person is inoculated with the sporozoites. One to several hours after inoculation, the sporozoites leave the inoculation site and travel through the blood stream to the liver where they invade and infect hepatocytes (Mota, et al. 2001; Yamauchi, et al. 2007). Inside the liver cells, each sporozoite undergoes schizogony, a multiple fission process which gives rise to thousands of haploid parasite stage called merozoites. When released from the liver into the blood stream, each merozoite invades a red blood cell (erythrocyte) and undergo schizogony again, producing thousands of merozoites in the blood cell. Upon rupture of the infected erythrocyte, thousands of merozoites are released into the blood stream where they further invade uninfected erythrocytes and repeat the schizogony process. It is the invasion, destruction and depletion of the red blood cells that directly or indirectly cause mild and severe forms of clinical illnesses observed in malaria patients. During the blood stage schizogony, some of the schizonts differentiate into gametocytes which circulate in the blood stream and where they can be picked up by a mosquito and undergo the sporogonic cycle (Roberts and Janovy 2005; Tuteja 2007).

1.2.2. *Anopheles* life cycle and their role as malaria vectors

There are over 3,500 known species of mosquitoes distributed over 49 different genera, but only some species in the genus *Anopheles* are known to transmit malaria (Foster and Walker 2019). Basic understanding of the life history of anophelines is necessary to understand their role in transmitting malaria. Like all mosquitoes, *Anopheles* are holometabolous insects and thus undergo four distinct stages of development: eggs, larvae, and pupae, which are aquatic stages,

and imago or winged adults which is non-aquatic (Romoser and Stoffolano 1998). Adult females are hematophagous, which means that they feed on blood of vertebrate hosts, primarily mammals including humans, to nourish and produce viable eggs (Black and Kondratieff 2005; Lehane 2005). After an adult male and a female *Anopheles* have mated, the inseminated female searches for a vertebrate host, obtains a blood meal and undergoes the egg development process (gestation). After 3–4 days of gestation it then goes in search of a suitable water body (larval habitat) to lay a batch of several hundred eggs. The eggs then hatch into first instar larvae, which undergo three more instars and a pupal stage development in the water before emerging as winged adults. The period between egg-laying and emergence of winged adults takes about 12–15 days. Two to three days after emergence, the adult mosquitoes are ready to mate and repeat the life development cycle (Eldridge 2005; Foster and Walker 2019).

While an adult male mosquito can participate in multiple mating events, a female mates only once in its lifetime, but the sperm it receives is sufficient to fertilize several batches of eggs. Every batch of eggs that a female *Anopheles* lays during its lifetime is preceded by a blood meal. The period between a blood meal and laying of an egg batch is called the mosquito's gonotrophic cycle (Eldridge 2005; Foster and Walker 2019). The need for blood meals and multiple gonotrophic cycles are the key factors that enable the transmission of the malaria parasites. When a female *Anopheles* obtains a blood meal from an infected human it also picks up the parasites, which undergo sporogonic development inside the mosquito. When that mosquito obtains blood meals from uninfected humans during subsequent gonotrophic cycles those individuals become infected. That is how malaria is transmitted from person to person.

1.3. Global epidemiology of malaria

1.3.1. Geographic distribution of the parasite species

Malaria is endemic in the tropical region of the world, particularly in developing countries (Hay, et al. 2004). Its restriction to the tropics is determined mostly by scantiness of the *Anopheles* vectors in the cold temperate regions and by inhibition of the sporogonic development in environments with temperatures < 15 °C (Dutta and Dutt 1978). However, the four malaria species vary in their geographic distribution within the tropical region. *P. falciparum* is the most widespread as it is found in nearly all malaria endemic regions of the world (Battle, et al. 2015; Guerra, et al. 2006; Hay, et al. 2009). Infection prevalence (proportion of surveyed humans who were infected with the parasites) of this species in children < 10 years old vary greatly from < 1% to > 70% depending on local endemicity (Battle, et al. 2015; Hay, et al. 2009). *P. vivax* distribution closely resembles that of *P. falciparum* in all continents except Africa where it is restricted to Madagascar and East African countries (Battle, et al. 2015; Guerra, et al. 2006; Howes, et al. 2016). Its absence in most parts of Africa is due to lack of *P. vivax*-specific Duffy binding receptor on the surface of the red blood cells of indigenous Africans which prevents the parasite from binding and infecting the red blood cells (Howes, et al. 2011; Rosenberg 2007). However, a recent study show that *P. vivax* is now spreading into other parts of Africa because of introduction of the Duffy-positive alleles into the human populations (Howes, et al. 2015). The infection prevalence of *P. vivax* in individuals of all ages vary from under 0.5% to above 10% depending on local endemicity (Battle, et al. 2015; Battle, et al. 2019; Howes, et al. 2016). *P. malariae* has been observed in most of the malaria endemic areas of the world but unlike *P. falciparum* and *P. vivax*, it appears patchy in its distribution with infection prevalence that rarely exceeds 2% (Cabrera and Arambulo 1977; Ghosh and Yadav 1995; Gordon, et al. 1991; Haworth

1988; Kawamoto, et al. 1999; Scopel, et al. 2004). Although higher prevalence of this species (15–30%) has been observed in some parts of sub-Saharan Africa and South-West Pacific (Anthony, et al. 1992; Boudin, et al. 1991; Browne, et al. 2000; Genton, et al. 1995a; Mueller, et al. 2007; Trape, et al. 1994). *P. ovale* is traditionally believed to be restricted to tropical Africa, the Island of New Guinea, Indonesia and Philippines (Collins and Jeffery 2005; Lysenko and Beljaev 1969). However, it has also been reported in the Indian subcontinent (Jambulingam, et al. 1989) and some parts of South East Asia (Baird, et al. 1990; Fuehrer, et al. 2010; Win, et al. 2002). In the areas where *P. ovale* was reported, its infection prevalence did not exceed 5% (Baird, et al. 1990; Collins and Jeffery 2005; Fuehrer, et al. 2010; Jambulingam, et al. 1989; Lysenko and Beljaev 1969; Mueller, et al. 2007; Win, et al. 2002).

1.3.2. Geographic distribution of the primary malaria vector species

There are over 40 species of *Anopheles* that are confirmed or suspected vectors of malaria worldwide. These vectors are not globally ubiquitous, rather each species is confined within a defined geographic range (Kiszewski, et al. 2004; Sinka, et al. 2012). Thus, the same malaria species is transmitted by different vector species in different parts of the world. For instance, the vector species *Anopheles gambiae* (*sensu lato*) (*s.l.*), *Anopheles funestus* and *Anopheles arabiensis* are the primary vectors in sub-Saharan Africa and are not found outside this continent (Fontenille and Lochouarn 1999; Kiszewski, et al. 2004; Meillon 1951; Sinka, et al. 2012). Interestingly, *An. gambiae* is more abundant than *An. arabiensis* in areas of sub-Saharan Africa where *P. falciparum* is prevalent and *P. vivax* is absent or less prevalent. However, *An. gambiae* is less abundant than *An. arabiensis* in Madagascar and the east African countries where *P. vivax* is present and more prevalent than *P. falciparum* (Fontenille and Lochouarn 1999; Kiszewski, et

al. 2004). The vectors *Anopheles stephensi*, *Anopheles culicifacies* (*s.l.*) and *Anopheles fluviatilis* (*s.l.*) are the main vectors confined to the Indian subcontinent (Kiszewski, et al. 2004; Sinka, et al. 2012). Those confined to South America are *Anopheles darlingi*, *Anopheles albitarsis* (*s.l.*), *Anopheles nuneztovaris* (*s.l.*) and *Anopheles aquasalis* (Kiszewski, et al. 2004; Sinka, et al. 2012). The vectors *Anopheles dirus* (*s.l.*), *Anopheles minimus* (*s.l.*), *Anopheles sinensis* and *Anopheles balabacensis* are some of the many species that are restricted to South-East Asia (Kiszewski, et al. 2004; Sinka, et al. 2011; Sinka, et al. 2012). Members of the *Anopheles punctulatus* (*s.l.*) species complex are important vectors confined to Indonesia, Australia and the Pacific island countries (Kiszewski, et al. 2004; Sinka, et al. 2011; Sinka, et al. 2012). Those confined to North America, Central America, Middle East, Europe, Western Asia and North Africa are listed elsewhere (Kiszewski, et al. 2004; Sinka, et al. 2012).

1.3.3. Malaria endemicity

The epidemiology of malaria classically holds to four general categories of endemicity: holoendemic, hyperendemic, mesoendemic and hypoendemic (Boyd 1949; Bruce-Chwatt 1980; MacDonald 1957; Nkumama, et al. 2017; Spencer 1963). These classes have traditionally been bounded by the so-called “spleen rate” which is the relative proportion of children found to have distended spleens by external palpation, the former having the highest and the latter the lowest. Because the spleen produces reticulocytes to replace red blood cells; and malaria parasites destroy red blood cells; spleens have to work harder in malaria-infected people and as a result become enlarged. They are easiest to palpate in children, and children are the most vulnerable to the ill effects of malaria infection as a rule. More modern methods involve estimation of the prevalence of infection in different age groups of people as done here, by detection of parasites

in the blood stream using such tools as microscopic examination of thick and thin blood smears, rapid diagnostic tests for parasite specific antigens circulating in blood, or use of standard or quantitative PCR methods.

Areas of the world where malaria is currently holoendemic, hyperendemic, mesoendemic or hypoendemic have been mapped and presented (Dalrymple, et al. 2015; Hay, et al. 2004). However, these epidemiological categories have much more profound implications than merely to fit into categories based on enlarged spleens or prevalence of infection. In the epidemiologic settings of any of these four categories, malaria transmission persists but its relative "stability" which is manifested in the tendency to show a stable age-specific infection prevalence profile of a certain characteristic, varies across these categories. In the holoendemic settings malaria transmission is intense and is without seasonal variation, and prevalence of infection in children ≤ 10 years old is > 75%. The vector population remains stable with constant high human biting rate (the frequency of vector bites per person per unit time) throughout the year (Hay, et al. 2004; Hay, et al. 2008; Spencer 1963).

Hyperendemicity differs slightly from holoendemicity in that although transmission in this category is as intense as in the holoendemic settings, seasonal variation exists and infection prevalence in children ≤ 10 years old range from 51–75%. In this endemicity, vector population size and thus the human biting rate vary seasonally. Thus, infection prevalence levels vary depending on the time of the year the survey was conducted; high prevalence is observed when transmission is in full swing and low during quiet season (Hay, et al. 2004; Hay, et al. 2008; Spencer 1963).

Common to both holoendemicity and hyperendemicity is that all human individuals are continually exposed to infectious *Anopheles* bites from birth and throughout their life. Mortality

rate is highest in infants and children under 5 years old due to lack of protective immunity in this age group compared to the older individuals (Baird 1998). Those that survive death in their childhood develop partial immunity, which is boosted throughout their adult life by repeated exposure to infective vector bites (Baird 1998; Doolan, et al. 2009). As a consequence of age-dependent protective immunity in these endemicities, the infection prevalence under usual conditions (i.e., no disease intervention implemented) is highest in the younger individuals (0–5 years in holoendemicity and 0–10 years in hyperendemicity) and dramatically declines with age (Baird 1998; Doolan, et al. 2009). Immunity is lost or wanes when individuals migrate out of the high transmission area for long period of time or when vector-human contact is interrupted long term by factors such as bed nets.

In mesoendemic settings, transmission intensity is moderate with more pronounced seasonal variation, and infection prevalence in children ≤ 10 years ranges from 11–50%. Vector population density is low most of the time and only increases during the rainy season. Unlike the two preceding endemicities, immunity in adults is weakly developed and thus age-specific prevalence of infection and risk of disease is shifted into even older age groups but mortality is still higher among children (Hay, et al. 2004; Hay, et al. 2008; Spencer 1963).

In hypoendemic settings, atmospheric temperature is either low (< 15 °C), such as high altitudes, or high (> 30 °C), which prevents transmission most of the time (Dutta and Dutt 1978; Spencer 1963). Epidemics occur only occasionally, particularly during heavy rainfall. The infection prevalence in children ≤ 10 years can reach as high as 10% but is usually 0%. The vectors are difficult to find and occasionally increase in abundance during heavy rainfall. In this endemicity, all age groups are more or less equally susceptible to infection and disease attack due to lack of immunity (Hay, et al. 2004; Hay, et al. 2008; Spencer 1963).

1.3.4. Public health burden and control of malaria

P. falciparum is not only widely distributed and highly prevalent but is also the most virulent of all the malaria species. Most infections by this species result in mild clinical illnesses such as high fevers and headaches. However, a small proportion of the infected individuals develops severe clinical conditions such as placental malaria, severe anemia, acidosis, respiratory distress, shock, renal failure, pulmonary edema, and cerebral pathology with convulsion and coma (Mendis and Carter 1995; Milner 2018). The other three species afflict intermittent high fevers, headaches and other mild illnesses but are rarely fatal (Mendis and Carter 1995; Milner 2018). As previously discussed, *P. ovale* and *P. malariae* have patchy or limited geographic distribution with consistently low (< 5%) prevalence of infection. In contrast, *P. vivax* is widely distributed with relatively high prevalence of infection and constitutes a significant amount of morbidity in humans. Thus, *P. falciparum* and *P. vivax* are both important malaria species from the standpoint of public health.

According to the World Health Organization (WHO), 40% of the world's population is at risk of malaria infection (Toure and Oduola 2004). The most vulnerable demographic groups are children under five years old, pregnant women, immunocompromised individuals, non-immune immigrants, mobile populations, and travelers. The most recent data from WHO show that in the year 2018 alone, a total of 228 million infection cases and 405,000 deaths due to malaria were reported worldwide. Most of the reported cases of infection (93%) and deaths (94%) were from the continent of Africa. Of the 405,000 reported deaths, 67% were children under 5 years old. Most of the infection cases were caused by *P. falciparum*; only 3.3% were by *P. vivax* (WHO 2019).

Like many other diseases, it is likely that the most effective way to control or eliminate malaria would be by means of a vaccine. However, no malaria vaccine is available at present and research is still ongoing in many laboratories around the world to develop one. Although antimalarial drugs are available, the primary means for controlling malaria in many endemic countries is through the use of insecticides that target the mosquito vectors. The insecticide-based approach for controlling the vectors is implemented in two ways: indoor residual spray and insecticidal bed nets. The indoor residual spray method involves spraying surfaces inside houses with insecticides so that mosquitoes that enter the house to feed on humans are killed when they contact the treated surfaces. The insecticidal bed nets serve as barriers, protecting humans who sleep under one from being exposed to the mosquitoes. Mosquitoes that contact the nets are killed by insecticides impregnated in the bed net fabric. By reducing mosquito abundance and frequency of bites taken on humans, the insecticide-based vector control methods interrupt malaria transmission.

Although the insecticide-based methods have reduced the worldwide burden of malaria significantly (Eisele, et al. 2010; Lengeler 2004; Pluess, et al. 2010), emergence of physiological resistance to the insecticides in vector populations around the world (Quinones, et al. 2015; WHO 2016) has impeded malaria elimination and paved the way for malaria rebound. In response to this problem, novel vector control methods such as population suppression by *Wolbachia*-induced cytoplasmic incompatibility (Alphey 2014; Bourtzis, et al. 2014), population suppression by release of mosquitoes with dominant lethal genes (Alphey 2014; Valdeza, et al. 2011) and population replacement of wild-type mosquitoes with pathogen-refractory strains through gene-drive systems (Alphey 2014; Sinkins and Gould 2006) are being developed. Although some of these methods have proven to be successful against non-anopheline vectors of

arboviruses (Carvalho, et al. 2015; Harris, et al. 2012), they have not yet been demonstrated to work effectively against natural populations of *Anopheles* vectors of malaria. Until the novel methods are available, malaria control in many parts of the world will continue to rely on the insecticide-based methods.

1.4. Malaria in Papua New Guinea

1.4.1. Landscape and geo-climatic description

PNG is a tropical country constituting the eastern half of the Island of New Guinea in the South-West Pacific. The Western half of the Island (Irian Jaya) is a province of Indonesia. The mainland of PNG is divided into northern and southern lowland (0–1,200 m altitude) plains by massive, connected mountain ranges which originate from the middle of the PNG-Irian Jaya border in the west and extends to the southeastmost tip of the mainland PNG (Figure 1.1). These ranges rise beyond 2,400 m above sea level. The northern plain consists mainly of dense tropical rainforests, lowland river valleys and flood plains of large rivers with several smaller mountain ranges. The climate there is hot and wet all year round. The southern plain consists of two geo-climatic regions. The first is a large area of continually hot and wet climate condition that begins at the foot of the mountain ranges and extends towards the south coast. The vegetation and landscape there are similar to the northern plain. The second is open monsoonal savannah which occupy thin strips of areas at the southwest coast and southeastmost tip of PNG (Figure 1.1). Between the northern and southern lowland plains are highland areas (> 1,200 m altitude). The climate condition there is mostly wet with temperate to cold temperatures (Beebe and Cooper 2002). Scattered over the northern and eastern seas of PNG is a group of islands that constitute the Bismarck Archipelago. In the southeastern sea is another group of islands that constitute the

Louisiade Archipelago. Rainfall in most of the lowland areas, including the savannah and the islands range from 1,300 to 7,000 mm annually (Muller, et al. 2003). Because PNG is situated close to the equator, temperature is generally stable throughout the year and differs only with respect to altitude. Temperature range from 30 °C at sea level and drops with increasing altitude to as low as 0 °C beyond 2,400 m above sea level.

The entire landscape of PNG consists of a patchwork of many different ecological zones clumped within an area of 460,000 km^2. These ecological zones are represented in the sea by coral reefs, offshore atolls and islands. On the coast are swamps, marshes, mangroves, lakes, grasslands, and plains. Further inland are dense rainforests, mountain ranges, plateaus and valleys. Cutting across this land divide are fast-flowing rivers with flood plains and deltas that open into the sea. Not only are these ecological zones inhabited by species-rich communities of flora and fauna that form complex ecosystems, but human populations of exceptionally diverse cultural and linguistic groups. Human populations are concentrated in the highlands (above 1,200 m altitude) and in the coastal areas (below 600 m), including the islands, with sparse distribution in the intermediate zones (Muller, et al. 2003).

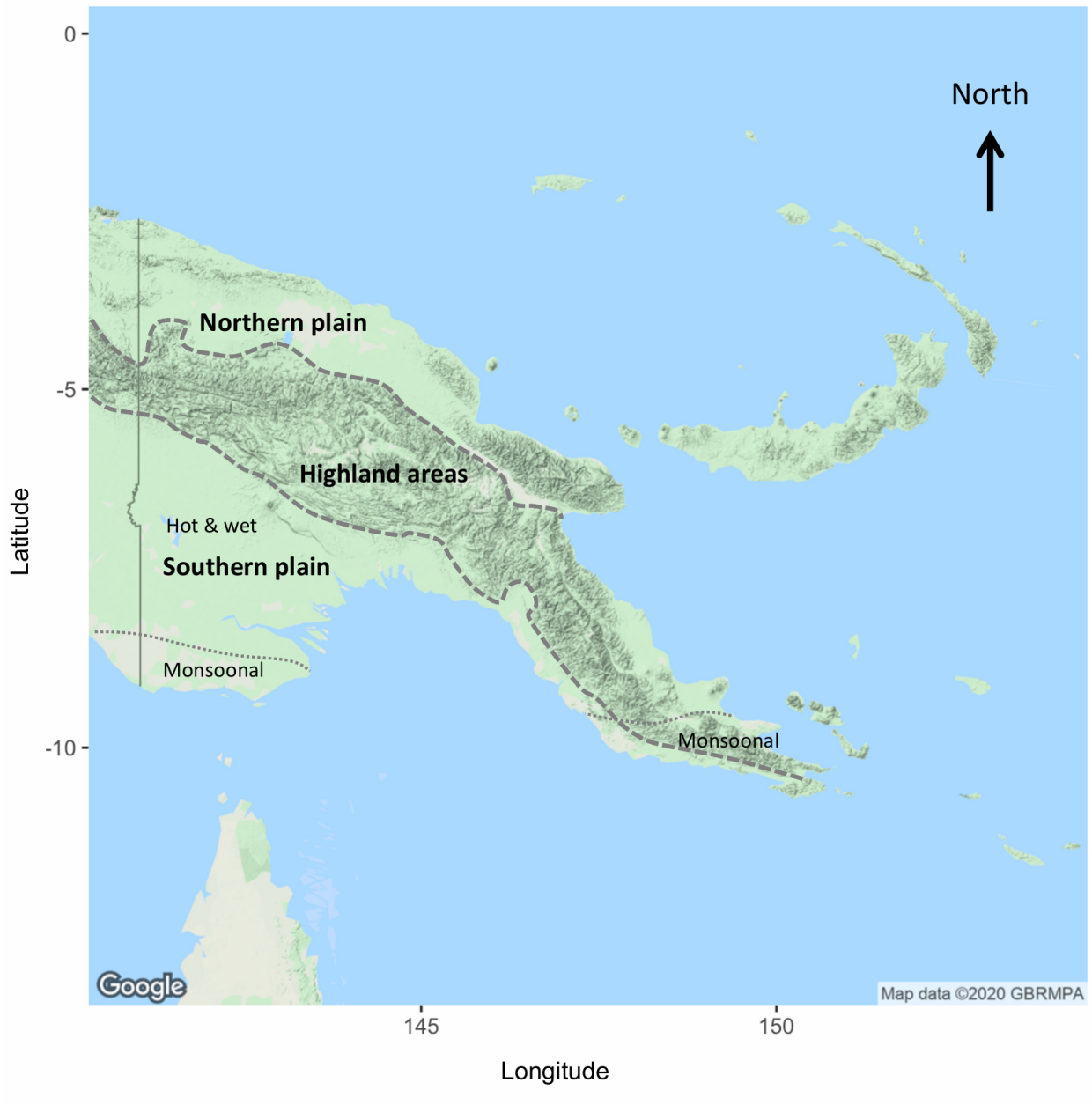

Figure 1.1. Map of Papua New Guinea showing three geo-climatic regions separated by grey dashed lines.

1.4.2. *Anopheles* vectors

Anopheles species that are common in the Eastern part of Indonesia, Northern Australia and the South West Pacific region where PNG is located are the members of the *An. punctulatus* (*s.l.*) species complex, which consists of 13 phylogenetically related cryptic species (Beebe, et al. 2000; Beebe, et al. 2015; Beebe and Saul 1995). Although most of them transmit malaria (Cooper, et al. 2009), only five—*Anopheles punctulatus* (*sensu stricto*) (*s.s.*), *Anopheles koliensis*, *Anopheles farauti* (*s.s.*), *Anopheles hinesorum* (formerly *Anopheles farauti* no. 2) and *Anopheles farauti* no. 4—are the primary vectors driving most of the transmission in PNG (Benet, et al. 2004; Burkot, et al. 1990; Burkot, et al. 1988; Hetzel, et al. 2016; Hii, et al. 2001; Reimer, et al. 2016; Thomsen, et al. 2017). Two non-members of the punctulatus group—*Anopheles longirostris* and *Anopheles bancroftii*—are also present in PNG and have been incriminated as minor vectors (Cooper, et al. 2009).

Although these vectors can be found in sympatry, each species exhibits a defined ecogeographic distribution, which makes them main vectors in some areas but less so in other areas. The larvae of *An. farauti* (*s.s.*) can tolerate high-salinity water bodies, which gives this species a competitive advantage over the other species to occupy and breed in brackish habitats (Charlwood, et al. 1986; Sweeney 1987). As a result of this adaptation, this species is most abundant in areas within 2 km from the coastline and rapidly diminishes in numbers beyond this boundary. Because of its ability to also breed in freshwater bodies, it is also found sporadically in inland lowland areas beyond 10 km from the coastline (Cooper, et al. 2002). *An. punctulatus* (*s.s.*) utilizes small, shallow, freshwater puddles that are open to direct sunlight as larval habitats. It rapidly colonizes transient water puddles shortly after they are formed either by anthropogenic activities such as tire ruts along roadsides, logging, mining, road constructions, dams, or gardens,

or by natural means such as stagnant riverine puddles (Charlwood, et al. 1986). *An. punctulatus* (*s.s.*) distribution is opposite to *An. farauti* (*s.s.*) in that it is abundant in inland lowland and decreases in numbers towards the coast. It is also found sporadically in the temperate environment of the highland region. Interestingly, *An. punctulatus* (*s.s.*) is widely distributed in the northern plain but in the southern plain this species is concentrated in areas to the east and is rare in areas to the west, particularly the monsoonal savannah areas (Cooper, et al. 2002). *An. koliensis* also utilizes the same larval habitats as *An. punctulatus* (*s.s.*) but it most commonly breeds in semi-permanent water bodies such as watercourses, ground pools and water-filled bomb craters (Charlwood, et al. 1986; Cooper, et al. 2002). Its geographic distribution is similar to that of *An. punctulatus* (*s.s.*), but it is more widespread than *An. punctulatus* (*s.s.*) in the northern plain (Cooper, et al. 2002). *An. farauti* no. 4 occupies the same habitats as *An. punctulatus* (*s.s.*), particularly stagnant water puddles in the flood plains of large rivers. However, unlike *An. punctulatus* (*s.s.*), it is sporadically distributed in the inland areas of the northern plain and only occasionally found in the southern plain (Cooper, et al. 2002). *An. hinesorum* is less restrictive and utilizes all the larval habitat types used by the other species. Although it is more abundant in the southern than northern plain, this species is the most widely distributed of all the members of the *An. punctulatus* (*s.l.*) complex (Cooper, et al. 2002).

1.4.3. Malaria epidemiology

PNG is arguably the most malaria endemic country outside the African continent. All four malaria species are found with *P. falciparum* and *P. vivax* the most prevalent species (Dijk and Parkinson 1973; Genton, et al. 1995a; Genton, et al. 1995b; Mehlotra, et al. 2002; Muller, et al. 2003). Prior to the implementation of a nationwide malaria control campaign (Hetzel 2009;

Hetzel, et al. 2014a; Hetzel, et al. 2012), malaria in the lowland areas (below 600 m altitude) of PNG where over 60% of the country's population (8.6 million people based on 2018 census; https://sdd.spc.int/pg) reside was holoendemic or hyperendemic (Cattani, et al. 1986a; Cattani, et al. 1986b; Dijk and Parkinson 1973; Genton, et al. 1995a; Genton, et al. 1995b; Mehlotra, et al. 2002; Muller, et al. 2003; Parkinson 1973). Malaria was hypoendemic with occasional epidemic outbreaks in the colder areas of the highland region where 40% of the population reside (Mueller, et al. 2005; Muller, et al. 2003). In some pockets of the most holoendemic parts of Madang and East Sepik provinces, infection prevalence of *Plasmodium* spp. among children under 10 years old reached as high as 78% and accounted for as much as 17% of deaths in children (Muller, et al. 2003). The rate of transmission of *Plasmodium* spp. was as high as 1,000 sporozoite-infective *Anopheles* spp. bites per person-year (Burkot, et al. 1988; Reimer, et al. 2016).

To alleviate the public health and socioeconomic burden of malaria in PNG, the National Department of Health embarked on a nationwide malaria control campaign, supported by the Global Fund and Rotary Against Malaria (Hetzel 2009). This program, which began in 2004 and intensified in 2006, focused primarily on free distribution of long-lasting insecticidal bed nets in rural villages throughout PNG and was supplemented with artemisinin-based antimalarial drugs and rapid antigen diagnostic test made available in local health centers (Hetzel 2009; Hetzel, et al. 2014a; Hetzel, et al. 2014b). By 2014, the national average of household bed net ownership (i.e., proportion of surveyed households owning at least one bed nets) and usage (proportion of interviewed individuals who reportedly sleep under a bed net) surpassed the target coverage of 80% compared to < 1% before the campaign. The number of people seeking antimalarial

treatments also increased from < 1% in 2009 to 78% in 2014, surpassing the target of 60% coverage (Hetzel, et al. 2014a).

As a consequence of the disease control campaign, considerable reduction in malaria was observed in PNG. Nationally, the overall (all ages) prevalence of infection for *Plasmodium* spp. was reduced from an average of 11% in 2009 to 0.9% in 2014 (Hetzel, et al. 2017). In the north coast of Madang province where malaria was holoendemic, the overall prevalence of infection for *P. falciparum* was reduced from 42.1% in 2006 to 9% in 2014. *P. vivax* also dropped from 41.7% in 2006 to 12.7% in 2010 but rebounded to 19.7% in 2014. In children below 10 years of age, the infection prevalence dropped from > 60% in 2006 for both malaria species to 13% *P. falciparum* and 30% *P. vivax* in 2014 (Koepfli, et al. 2017). The rate of transmission of *Plasmodium* spp. in the north coast of Madang dropped from a range of 20–350 sporozoite-infective *Anopheles* spp. bites per person-year in 2009 to 0–15 infective bites per person-year in 2011. In the East Sepik province, the rate of transmission dropped from 175–1,050 in 2009 to 0–25 infective bites per person-year in 2011 (Reimer, et al. 2016).

1.5. Research rationale and objective

1.5.1. Rationale

Along the malaria endemicity continuum from holoendemic to hypoendemic, the overall population prevalence of infection declines as transmission rates are lower. In addition, the epidemic potential of malaria increases with this continuum as the extent of herd immunity declines as well. That is to say, malaria becomes less and less stable and more prone to epidemics with this continuum. The variations in stability of malaria are important considerations for the process of malaria control. Paradoxically, implementation of malaria

control such as through insecticidal interventions against vector populations particularly in the holoendemic and hyperendemic settings destabilizes malaria epidemiologically, increases the potential for epidemics, and cause changes in age-specific prevalence and parasite-host associations that result in higher risk of disease and death in certain age groups. For example, chronic anemia declines in children as parasite density goes down with fewer and fewer inoculations of parasites annually, but the frequency of severe neurologic outcomes such as cerebral malaria increases due to waning clinical immunity (Nkumama, et al. 2017). Attack rates increase in older children during their primary formative years in school (Nkumama, et al. 2017). Productive labor of the older age classes is more affected (Nkumama, et al. 2017). Therefore, malaria control implemented in malaria-stable populations does not necessarily follow the Hippocratic dictum "first do no harm" whether considered from the individual patient or that of the community, especially when it does not ensure complete elimination of the disease or prevent it from being reintroduced into the vulnerable population.

The reduction of malaria transmission rate and infection prevalence after the vector control campaign in the north coast of Madang (Koepfli, et al. 2017) indicates that the epidemiology of malaria in this region shifted from stable (holoendemic) to unstable (mesoendemic) state. This means that immunity has waned in previously immune individuals, creating a condition where individuals of all ages are now vulnerable to malaria infection and disease attacks then before the bed net campaign when only the children (and pregnant women) were vulnerable. To minimize disease attacks in the vulnerable populations, the bed net distribution program was intensified and extended to the present by the Rotarians Against Malaria (https://ram.rawcs.com.au/) to further suppress the malaria transmission rate to accomplish near zero prevalence of infection. However, an important challenge facing this goal

is that the relationship between transmission as measured by the infectious biting rate or a surrogate (the entomological inoculation rate) is nonlinear such that large declines in transmission rate result in small changes in prevalence of infection (Smith et al. 2005). Accordingly, greater effort is required to reduce transmission rate to low levels meaningfully and sustainably, and to consolidate those gains to prevent a spring-like rebound and even epidemic (Cohen et al. 2012). Indeed, a stark lesson of malaria control programs is that a failure to consolidate gains in reduction in prevalence can increase the potential energy for rebound from such as hypoendemic or mesoendemic conditions to hyperendemic or holoendemic conditions (Cohen et al. 2012).

Based on most recent observation, the rate of malaria patients visiting the local health centers in Madang province remained high despite the ongoing efforts to intensify the bed net campaign in this region. This observation points to the plausibility that further reduction of malaria transmission is being hampered and is most likely caused in large part by ecological factors that enable the mosquito vectors to evade the bed nets and continue to transmit the disease. Emergence of physiological resistance to the insecticides in the bed net fabric is one potential factor. However, this trait has not been observed in the vector populations in PNG (Katusele, et al. 2014; Keven, et al. 2010; Koimbu, et al. 2018), which implies that factors other than insecticide resistance are limiting the effectiveness of the bed nets.

The situation where malaria transmission continues to persist after it was reduced by a vector control method such as bed nets is termed “residual transmission” (Killeen 2014). Residual transmission indicates the natural or biological limit of the impact of a vector control method on malaria reduction such that once the limit is reached further effort to scale up or intensify the control method will yield negligible or no further impact. At this juncture,

incorporation of complementary vector intervention methods is necessary to help reduce malaria further. The importance of residual transmission is its potential to cause disease resurgence when the vector control program is weakened or withdrawn as was the case for many endemic countries in the past (Cohen, et al. 2012).

1.5.2. Objective

The objective of the research work described in this dissertation was to address the overarching hypothesis that in PNG further reduction of malaria transmission by ongoing bed net intervention program is hampered by ecological factors of the local vector populations that enable the mosquitoes to evade the bed nets. Thus, malaria transmission is currently persisting at higher rates compared to recent estimates. This objective was addressed by investigating five specific hypotheses outlined below.

Hypothesis 1 (addressed in chapter 2). The local *Anopheles* mosquitoes have high abundance and species diversity despite the presently ongoing insecticidal bed net program, and the mosquito communities are highly dissimilar in their species composition even between those communities that are in close spatial proximities to each other.

Hypothesis 2 (addressed in chapter 3). As a result of high vector abundance and species diversity (hypothesis 1), and ecological factors which enable the local vectors to evade the bed nets (hypotheses 3–5), the rates of transmission and prevalence of infection of malaria have rebounded rather the declined further in some areas in PNG.

Hypothesis 3 (addressed in chapter 4). Host-seeking mosquitoes evade the bed nets by entering the village and feeding on human hosts in the evening when people are active and disproportionately outdoors unprotected by the bed nets, thus the risk of contacting the indoor-deployed bed nets is minimal.

Hypothesis 4 (addressed in chapter 5 and 6). The primary vector species in PNG feed unbiasedly on humans and non-human (pigs and dogs) hosts rather than exhibit strict anthropophagy. As a result of the opportunistic utilization of the blood hosts, the vectors are able to avoid contact with the bed nets by feeding on non-humans to support vector abundance and transmit malaria by feeding on humans outdoors and during the periods when they are awake and unprotected by the bed nets.

Hypothesis 5 (addressed in chapter 4 and 7). Within villages, the per-person frequency of vectors bites clusters spatially and among human individuals rather than being randomly distributed among individuals, causing transmission to persist rather than dissipate by increasing the basic reproduction rate of malaria.

1.6. Research ethics approval

All research activities described in this dissertation were reviewed for compliance with ethical requirements by appropriate authorities. The study involved consented recruitment of human volunteers to (i) donate capillary blood samples (250 μl) for PCR detection of malaria infection and human microsatellite genotyping or DNA profiling, (ii) obtain information on their age, sex, household geolocation and possession of domestic animals, (iii) interview for whether they did

or did not sleep under bed nets, and (iv) obtain antimalarial chemoprophylaxis to serve as a mosquito collector. The protocols for acquisition of individual consent, collection of blood samples, and questionnaire survey were approved by the institutional review board (IRB) of PNG Institute of Medical Research (IRB No. 1715), PNG Medical Research Advisory Committee (IRB No. 16.08) (https://www.pngimr.org.pg/about/institutional-review-board/) and Michigan State University (IRB No. 17-1603) (https://hrpp.msu.edu/contacts/about/irb-office.html). The letter of approval from the Michigan State University IRB has been filed with the university's graduate school as part of the dissertation submission and approval process.

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CHAPTER 2: SPECIES COMPOSITION, ABUNDANCE AND NOCTURNAL ACTIVITIES OF FEMALE *ANOPHELES* IN MADANG, PAPUA NEW GUINEA: ASSESSMENT WITH BARRIER SCREEN SAMPLING

Publication

Keven JB, Katusele M, Vinit R, Koimbu G, Vincent N, Thomsen EK, Karl S, Reimer LJ, Walker ED. 2019. Species abundance, composition, and nocturnal activity of female *Anopheles* (Diptera: Culicidae) in malaria-endemic villages of Papua New Guinea: assessment with barrier screen sampling. Mal. J. 18:96

2.1. Abstract

Community composition of *Anopheles* mosquitoes, their abundance and peridomestic behavior, are important factors affecting malaria transmission. In this study, barrier screen sampling was used to investigate species composition, abundance, and nocturnal activity of *Anopheles* populations in villages of PNG. Mosquitoes were sampled between 18:00 hr–6:00 hr in seven villages from 2012 to 2017. The barrier screens were positioned between the village houses and the perimeter of villages where cultivated and wild vegetation ("the bush") grew thickly. Female *Anopheles* that rested on either village or bush side of the barrier screens, as they commuted into and out of the villages, were captured. Relative species abundance within villages and their compositional similarity among villages were assessed. Mosquitoes captured on village and bush sides of the barrier screens were sorted by feeding status and by hour of collection, and their numbers were compared using negative binomial generalized linear models. Females of six *Anopheles* species were present in the sample. Species richness ranged from two to six species

per village, but relative abundance was highly uneven within and among villages, and community composition was generally dissimilar among villages. More blood-fed mosquitoes were captured on the side of the barrier screens facing the village than bush whereas more unfed ones were captured on the bush than village side, suggesting commuting behavior of unfed host-seeking females into the villages from nearby bush and commuting of blood-fed females away from villages towards the bush. For six of 12 *Anopheles* populations studied, the majority of host-seeking mosquitoes arrived in the village before midnight when people were active and unprotected from the mosquitoes by bed nets. The uneven distribution of *Anopheles* species among villages, with each site dominated by different species, even among nearby villages, emphasizes the importance of vector heterogeneity in local malaria transmission and control. The nocturnal activity patterns of village entry and host-seeking predominantly occurring before midnight increases the risk of human exposure to *Anopheles* bites.

2.2. Introduction

Sampling adult female *Anopheles* mosquitoes is crucial for studies of bionomics, to estimate population parameters, quantify malaria and filarial parasite transmission, and evaluate vector-targeted disease interventions (Mboera 2005; Service 1977; WHO 1975). Three common sampling methods are i) the human landing catch (HLC), ii) baited or unbaited light trap (LT), and iii) resting collection (RC). These can be conducted in various locations such as within human or animal domiciles or amongst vegetation. The HLC method involves human volunteers luring and capturing host-seeking mosquitoes as they land on exposed legs (WHO 1975). The method provides direct estimates of the human biting rate (see chapter 3) and infectious biting rate (see chapter 3), and provides a means for characterizing such important bionomic properties

as nocturnal periodicity of the biting cycle (Service 1977; WHO 1975). The LT method involves the use of battery-powered suction devices fitted with light bulbs and/or artificial host odors to attract and trap host-seeking adult mosquitoes. The method provides estimates of relative mosquito density, indirect estimates of the infectious biting rate, and can be used to assess species diversity, community composition, relative abundance, and distribution (Amusan, et al. 2005; Cooper, et al. 2006; Dear, et al. 2018; Eisen, et al. 2008; Hii, et al. 2000; Mboera 2005; Sriwichai, et al. 2015). The RC method involves search and capture of endophilic mosquitoes settled inside human houses or animal sheds, and exophilic mosquitoes resting in the surrounding vegetation or in intentionally placed resting shelters (Mboera 2005; Odiere, et al. 2007; WHO 1975). It may involve spraying insecticides that rapidly knock down mosquitoes in indoor spaces (Odiere, et al. 2007). Such collections are used to study mosquito resting habits, recover blood-fed mosquitoes for analysis of host selection (see chapter 6), to analyze distribution in space and time, and to evaluate the effect of residual insecticide treatments on endophilic vectors (McCann, et al. 2017; WHO 1975).

All the mosquito sampling methods described above have been used to study PNG vectors. Many species of *Anopheles* are found in PNG (Cooper, et al. 2009; Cooper, et al. 2002; Cooper, et al. 2006), and most of them have been incriminated as vectors of malaria (Cooper, et al. 2009). However, seven of these species, namely *An. farauti* (*s.s.*), *An. hinesorum*, *An. farauti* no. 4, *An. punctulatus* (*s.s.*), *An. koliensis*, *An. longirostris* and *An. bancroftii*, are important vectors of malaria in lowland areas of PNG, including Madang province, where malaria is highly endemic (Burkot, et al. 1988b; Hii, et al. 2001; Reimer, et al. 2016; Thomsen, et al. 2017). The first five are major vectors whereas the last two play a minor role in the transmission of malaria in PNG (Burkot, et al. 1990; Burkot, et al. 1988b; Cooper, et al. 2009; Hetzel, et al. 2016; Hii, et

al. 2001; Reimer, et al. 2016; WHO 2011). Various aspects of these vector species including the human biting rate, infectious biting rate, nocturnal biting cycle, dispersal range, and survival rate have been studied using one or combinations of the sampling methods described above (Burkot, et al. 1988b; Charlwood, et al. 1985a; Charlwood, et al. 1985b; Hetzel, et al. 2016; Reimer, et al. 2016; Thomsen, et al. 2017). Studies of host selection relied on indoor and outdoor RC to recover blood-fed mosquitoes (Burkot, et al. 1988a; Charlwood, et al. 1985b; Hii, et al. 2001), but this approach has serious limitations in the PNG setting. All of the vector species in PNG tend to be exophagic (Sinka, et al. 2011), thus few individuals (mostly human-fed ones) are found resting indoors, resulting in insufficient and biased samples. The wide dispersal range of some of these species along with thick tropical vegetation makes the outdoor resting search for mosquitoes in peri-domestic environments a laborious task, often resulting in very few mosquitoes that also do not adequately represent the population (Charlwood, et al. 1985b).

The barrier screen sampling method was developed as an alternative to the methods discussed above (Burkot, et al. 2013; Davidson, et al. 2018). It involves the use of agricultural shade cloth positioned vertically around villages and imposing a physical barrier suitable for temporary landing and resting of *Anopheles* mosquitoes as they commute into and out of the villages. Mosquito collectors visit the barrier screens at specific intervals throughout the night to capture the resting mosquitoes. Unlike the indoor resting collection, both anthropophilic and zoophilic mosquitoes may be intercepted as they rest on the barrier screen. By sampling the mosquitoes as they commute into and out of a village throughout the night, the barrier screen method overcomes the laborious task associated with outdoor resting collection. The flexibility in screen placement allows sampling in various locations which reduces bias associated with particular sampling locations. The effectiveness of this method to produce a sample of blood-fed

mosquitoes for estimating host selection of *Anopheles* vectors of malaria in PNG is reported in chapter 6. The aim of the study comprising this chapter was to use the barrier screen method to analyze species composition, abundance and nocturnal movement patterns of *Anopheles* species.

2.3. Methods

2.3.1. Study sites

This study was conducted in seven malaria-endemic rural villages (Bulal, Dimer, Kokofine, Matukar, Megiar, Mirap and Wasab) in Madang province of PNG (Figure 2.1) in 2012–2017. These villages are located in three ecogeographic environments of the malaria-endemic region of northern PNG (coastal plain, hilly inland terrain, and inland alluvial plain) known to be inhabited by various *Anopheles* species, including the seven vector species listed above (Burkot, et al. 1989; Burkot, et al. 1988b; Charlwood, et al. 1985b; Charlwood and Graves 1986; Charlwood and Graves 1987; Hii, et al. 1997; Hii, et al. 2001; Reimer, et al. 2016; Thomsen, et al. 2017). The villages Matukar, Megiar and Mirap share similar features; all are situated on the coastal plain along the northern coastline just above sea level. Land cover consists of coconut plantation, secondary forest, vegetable gardens, brackish swamps, houses, foot trails, and exposed soil. The villages border sand beach and the shore of the Pacific Ocean. Bulal, Dimer and Wasab villages are situated several km inland from this coastline, on elevated hilltops about 150 m above sea level, with land cover and topography of steep-sided, forested hills and numerous streams flowing to rivers in nearby valleys. The seventh village, Kokofine, is situated on the alluvial plain of the Ramu river, 39 km from the coast and 400 m above sea level. Land cover there consists primarily of swamps, cocoa plantations and secondary forests. All villages have the same tropical climate condition of hot and wet with average atmospheric temperature of 28 °C.

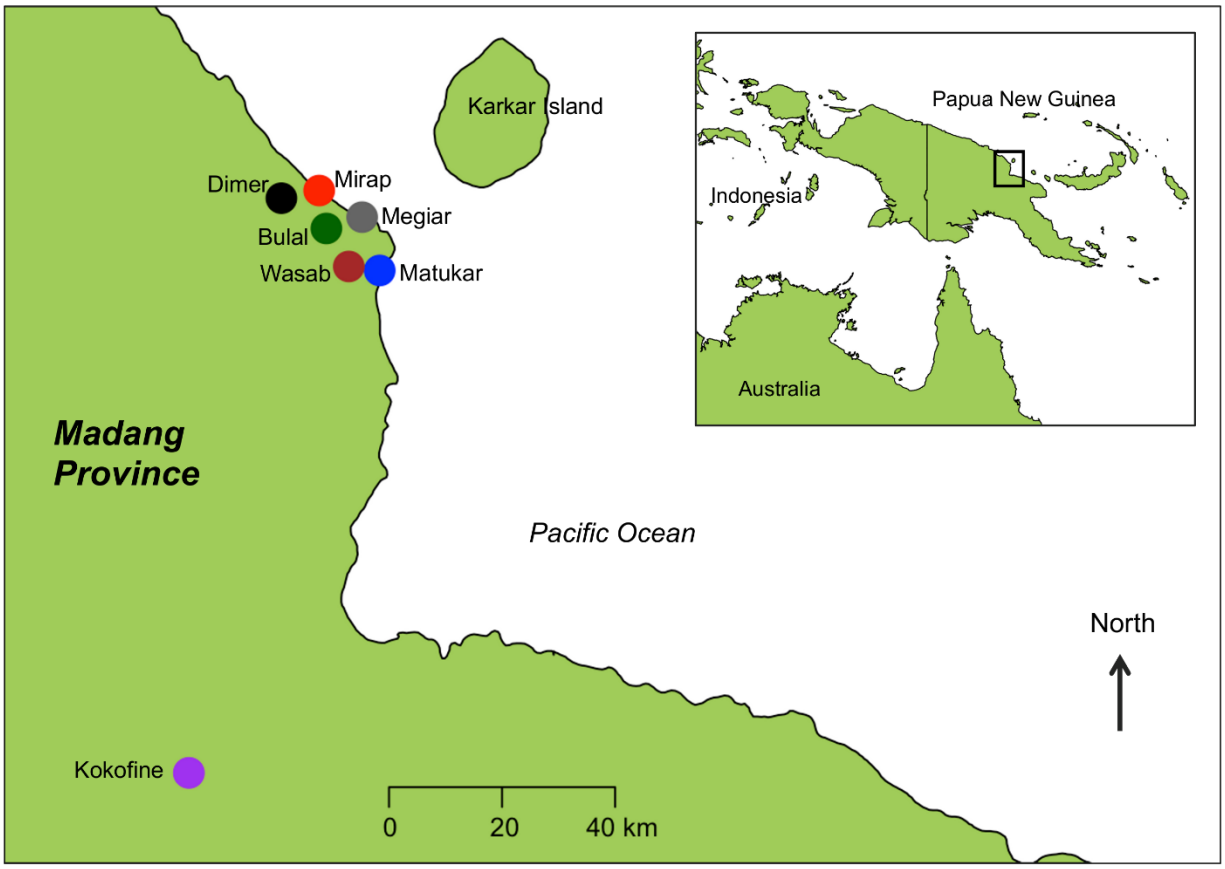

Figure 2.1. Map showing locations of the study villages. The villages are identified by names and colors: Bulal (green), Dimer (black), Kokofine (purple), Matukar (blue), Megiar (grey), Mirap (red) and Wasab (brown) in Madang Province, PNG. Areas shaded green represent land masses and unshaded represent the ocean.

2.3.2. Mosquito sampling

The structure and set up of barrier screens were similar to those described elsewhere (Burkot, et al. 2013). In this study, each barrier screen consisted of a 20 m long and 2 m wide polyethylene shade cloth (forest green, 70% shading grade) fastened on wooden poles or metal reinforcement bars and erected vertically to a height of 2.15 m (Figure 2.2a). The length of the barrier screen was chosen for efficiency of setting up the screen and the amount of time spent searching the barrier screen surface for resting mosquitoes. The height of the barrier screen was consistent with the ability of the collectors to reach for mosquitoes just above their average height (160 cm). The

barrier screens were placed between the village perimeter and surrounding environment or bush. Each barrier screen was positioned with one side of the screen facing the village, hereafter referred to as "village side", and the other facing away from the village, hereafter referred to as "bush side" (Figure 2.2a). The barrier screen intercepted host-seeking and blood-fed mosquitoes as they commuted into and out of the village. The intercepted mosquitoes were captured as they temporarily rested on the barrier screens (Figure 2.2b).

Figure 2.2. Barrier screen setup. (a) Barrier screen erected vertically at the edge of a village with one side facing the village and the other facing the bush. (b) Mosquito collector capturing resting mosquitoes with the aid of a mouth aspirator and a flashlight.

Mosquitoes were sampled using two, eight or ten barrier screens per village per night (see Table S2.1, Appendix 2.1 for the number of barrier screens deployed in a particular night in each village). Each barrier screen was assigned two trained mosquito collectors. One collector worked from 18:00 hr in the evening to midnight and was replaced by the second collector who worked from midnight to 6:00 hr in the morning. The collectors, who were stationed 20 m from the screen, visited both sides of the screen three times within each hour with approximately 20

minutes sampling interval, which involved five minutes commuting between the barrier screen and collector station, another five minutes searching and aspirating resting mosquitoes on the barrier screen, and 10 minutes break before next visit to the barrier screen. As this study was among the first to test the barrier screen method, no prior information was available to guide the sampling strategy. Mosquitoes were believed to rest only temporarily on the barrier screen, thus searching the barrier screen three times per hour was intended to maximize the number of mosquitoes collected. The collector walked along the barrier screen and collected resting mosquitoes with the aid of a flashlight and a mouth aspirator (Figure 2.2b). The collectors were provided with and instructed to apply mosquito repellents on their bodies to deter mosquitoes from biting them. Mosquitoes were sampled for six nights in Bulal, seven in Matukar, six in Megiar, 64 in Mirap, 63 in Wasab, 12 in Dimer and six in Kokofine during the years 2012, 2013, 2015, 2016 and 2017 (see Table S2.1, Appendix 2.1). When only two barrier screens per village per night were used, each barrier screen was changed to a new location on each night of sampling to minimize sampling biases associated with limited sampling locations.

Captured mosquitoes were placed in screened paper cups labelled with the hour of the night and the side of the barrier screen (i.e., bush or village side) on which the mosquitoes were captured. Information for each mosquito, including date and hour of collection, which side of the screen it was collected and the blood-engorgement status (blood-fed or unfed) were recorded. With the aid of a light microscope, mosquitoes were sorted by sex. Males, which were very few (*ca.* 1% of the mosquito sample), were identified and discarded. Female *Anopheles* were identified to their morphological species (Belkin 1962; Lee, et al. 1987), assigned a unique serial number, and stored dry on silica gel desiccant. In the laboratory, DNA was extracted from the abdomen (blood-fed ones) or head + thorax (unfed ones) of each mosquito using DNeasy Blood

& Tissue Kit (Product number: 69582; Qiagen, Valencia, CA, USA). Mosquitoes morphologically identified as members of the *An. punctulatus* (*s.l.*) complex were subjected to a PCR assay (Beebe and Saul 1995) to identify the species.

2.3.3. Data analyses

Variation among years in the relative abundance of different *Anopheles* species in a village was tested by chi-square test. Variation among villages in the number of *Anopheles* spp. (i.e. regardless of species) captured nightly on a barrier screen (i.e., vector abundance) was tested by Kruskal-Wallis test. Both tests were computed using the function *chisq.test* and *kruskal.test* of the package *stats* in R software version 3.4.1 (https://www.R-project.org/).

Similarity in species composition between pairs of villages was estimated by performing Bray-Curtis index analysis (Bray and Curtis 1957) on two mosquito abundance matrices (Table 2.2 and 2.3). The resulting Bray-Curtis dissimilarity index matrices (Table S2.2 and S2.3, Appendix 2.2) were used in principal coordinates analysis (PCoA) to produce ordination plots of the study villages. The plots were further modified as biplots of villages and *Anopheles* species by *a posteriori* projection of the mosquito species onto the PCoA axes based on their weighted average scores. The weighted average score of a species at PCoA axis 1 and axis 2 was calculated by averaging the product of its abundance (see Table 2.2 and 2.3) and the PCoA axis (1 or 2) score for all villages (Borcard, et al. 2011). Villages clustered together in the biplots are similar whereas those further apart are dissimilar in their species composition. The position of a village in the PCoA biplot was influenced by the abundance of the *Anopheles* species closest to it (Borcard, et al. 2011). The Bray-Curtis index matrix and PCoA biplot were computed using the R functions *vegdist*, *cmdscale* and *wascores* of the R package *vegan* (Oksanen, et al. 2018).

In this dissertation a vector population is defined as mosquitoes of a particular species from a specific location (village) and time (year). For each of 12 *Anopheles* populations encountered in this study, generalized linear model (GLM) with negative binomial distribution was used to compare number of mosquitoes in collections at each of the 12 hourly periods of the night and from the village and bush side of the barrier screen. In the GLM regression equation $\ln(\mu) = \beta_0 + \beta_1(Time) + \beta_2(Side) + \ln(S)$, the expected mosquito number μ was modelled as a linear function of the categorical predictor variables *Time*, with 12 levels representing the 12 hourly periods of the night and *Side*, with two levels representing the two sides of the barrier screens. Number of barrier screen-hours *S* was included as the offset term. The GLM was performed using the R function *glm.nb* from the package *MASS* (Venables and Ripley 2002). Significance level for all statistical tests was based on type I error rate of 5%.

2.4. Results

2.4.1. Species composition and abundance

A total of 13,897 female *Anopheles* mosquitoes of six different species namely *An. bancroftii*, *An. farauti* (*s.s.*), *An. farauti* no. 4, *An. koliensis*, *An. longirostris* and *An. punctulatus* (*s.s.*) were collected, including 4,379 (31.5%) blood-fed and 9,094 (68.5 %) unfed. Distribution of the mosquitoes of each of the *Anopheles* species over the study villages is shown in Table 2.1 (for all years combined), Table 2.2 (for April–December 2012) and Table 2.3 (for August 2016–March 2017). Data in Table 2.2 and 2.3 were used as mosquito abundance matrices in the Bray-Curtis species composition similarity analysis (see data analyses section).

Table 2.1. Number of mosquitoes collected in each of seven villages for all years combined.

Species	Bulal	Dimer	Kokofine	Matukar	Megiar	Mirap	Wasab
An. bancroftii	0	11	0	0	0	363	3
An. farauti (*s.s.*)	8	6	0	141	1120	4502	93
An. farauti no. 4	0	0	1624	0	0	3	0
An. koliensis	90	9	32	2	19	2232	1759
An. longirostris	2	16	9	28	0	189	361
An. punctulatus (*s.s.*)	34	179	67	19	0	160	816

Table 2.2. Number of mosquitoes collected in each of five villages in April–December 2012.

Species	Dimer	Kokofine	Matukar	Mirap	Wasab
An. bancroftii	11	0	0	127	3
An. farauti (*s.s.*)	6	0	141	1775	4
An. farauti no. 4	0	1520	0	0	0
An. koliensis	9	0	2	11	13
An. longirostris	16	0	28	45	275
An. punctulatus (*s.s.*)	179	0	19	60	147

Table 2.3. Number of mosquitoes (excluding the species *An. farauti* no. 4) collected in each of four villages in August 2016–March 2017.

Species	Bulal	Megiar	Mirap	Wasab
An. bancroftii	0	0	140	0
An. farauti (*s.s.*)	8	1120	945	15
An. koliensis	90	19	2193	1150
An. longirostris	2	0	112	21
An. punctulatus (*s.s.*)	34	0	79	431

The relative *Anopheles* species abundance expressed in terms of mean proportion ± standard error collected on barrier screens per night in each village is shown in Figure 2.3. The results presented in Figure 2.3 and Tables 2.1–2.3 show that the *Anopheles* species were not evenly represented within and among the villages. Figure 2.3a shows the relative proportion of mosquitoes in villages where sampling was done within a single year: Dimer (2012), Matukar (2012), Bulal (2017) and Megiar (2017). In Dimer, where five different species were found, *An. punctulatus* (*s.s.*) constituted 66.5 ± 9% of the mosquito sample. In Bulal, where four species were found, *An. koliensis* constituted 66.6 ± 7% of the sample. *An. farauti* (*s.s.*) constituted 69.0 ± 7% of samples consisting of four species in Matukar, and 98.0 ± 1% of samples consisting of two species in Megiar.

Mosquitoes were sampled in more than one year in Kokofine (two years), Wasab (four years) and Mirap (four years). In all three villages and sampling years, the *Anopheles* species were not evenly represented; one or more species predominated the samples whereas others were rare or not detected (Figure 2.3b–d). In Kokofine where four species were encountered (Table 2.1), the species composition changed from solely *An. farauti* no. 4 in 2012 to a more diverse community of four species in 2016, although *An. farauti* no. 4 still constituted most (49.0 ± 8%) of the 2016 sample (Figure 2.3b). The relative abundance of these four species in 2012 compared to 2016 was statistically different (chi-square test: $\chi^2 = 825.8$, df = 3, $P < 0.0001$). In Wasab, five species were encountered (Table 2.1) and their relative abundance over the four years (Figure 2.3c) varied statistically (chi-square test: $\chi^2 = 2041$, df = 12, $P < 0.0001$). In 2012, *An. longirostris* was the most abundant species (63.4 ± 6%) followed by *An. punctulatus* (*s.s.*) (32.0 ± 6%). In 2013, *An. farauti* (*s.s.*) increased in numbers, making it third most abundant species (26.3 ± 10%) after *An. punctulatus* (*s.s.*) (31.8 ± 12%) and *An. longirostris* (35.5 ± 13%). A

notable shift in the relative abundance was observed in 2015 where *An. longirostris* dropped to third place (12.3 ± 7%) whereas *An. koliensis* (28.7 ± 6) and *An. punctulatus* (*s.s.*) (48.3 ± 8%) rose to second and first place respectively. By 2016, *An. koliensis* was the most abundant species in Wasab, constituting 78.0 ± 2% of the sample, followed by *An. punctulatus* (*s.s.*) at 20.0 ± 2% (Figure 2.3c). The most striking trend observed in Wasab was the monotonic drop in *An. longirostris* from the most abundant species in 2012 to the third most abundant in 2016, and the rise of *An. koliensis* from the third most abundant species in 2012 to the most abundant in 2016 (Fig. 2.3c). In Mirap, six species were encountered (Table 2.1) and like Kokofine and Wasab, the relative abundance of these species over the four years (Figure 2.3d) differed statistically (chi-square test: $\chi^2 = 3678.6$, df = 15, $P < 0.0001$). However, in the first three years, this variation was not as visually dramatic as in Wasab because the change in relative species abundance happened only in the less abundant species (i.e. those < 14%), while the proportion of the predominant species *An. farauti* (*s.s*.) over the three years remained above 76% (Figure 2.3d). A dramatic shift occurred in 2017 when *An. koliensis* whose abundance was below 2% in the preceding years rose to dominance, constituting 63.1 ± 2% of the sample, followed by *An. farauti* (*s.s.*) at 27.0 ± 2%.

Anopheles density, measured as mean number of mosquitoes captured per barrier screen-night, varied among villages. For villages where mosquitoes were collected in the year 2012, Kokofine had the highest density (190 *Anopheles* per screen-night), followed by Mirap (36), Matukar (14), Wasab (11), and Dimer (10) (Figure 2.4a). This variation was statistically significant (Kruskal-Wallis test: $\chi^2 = 26.9$, df = 4, $P < 0.0001$). For villages where mosquitoes were collected in the year 2016–2017, Mirap had the highest density (72), followed by Megiar

(24), Wasab (18), and Bulal (3) (Figure 2.4b). This variation was also statistically significant (χ^2 = 21.1, df = 3, $P < 0.0001$).

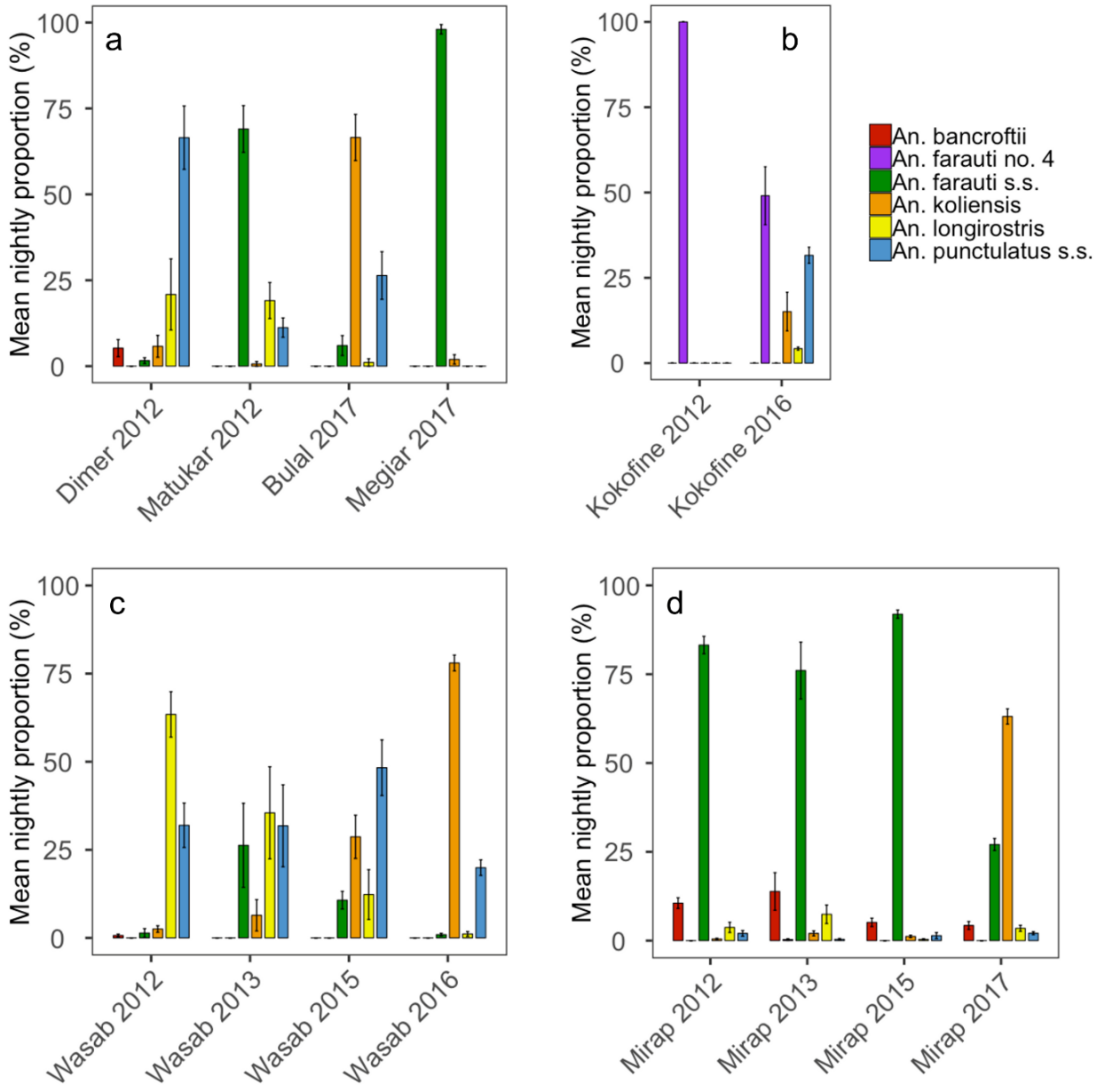

Figure 2.3. Relative species abundance of *Anopheles* mosquitoes. Each bar represents mean proportion (with standard error bar) of each *Anopheles* species captured on barrier screens per night in a village. (a) Relative abundance of *Anopheles* in four villages where mosquitoes were sampled in a single year. (b) Relative abundance of *Anopheles* in Kokofine in two different years. (c) Relative abundance of *Anopheles* in Wasab in four different years. (d) Relative abundance of *Anopheles* in Mirap in four different years.

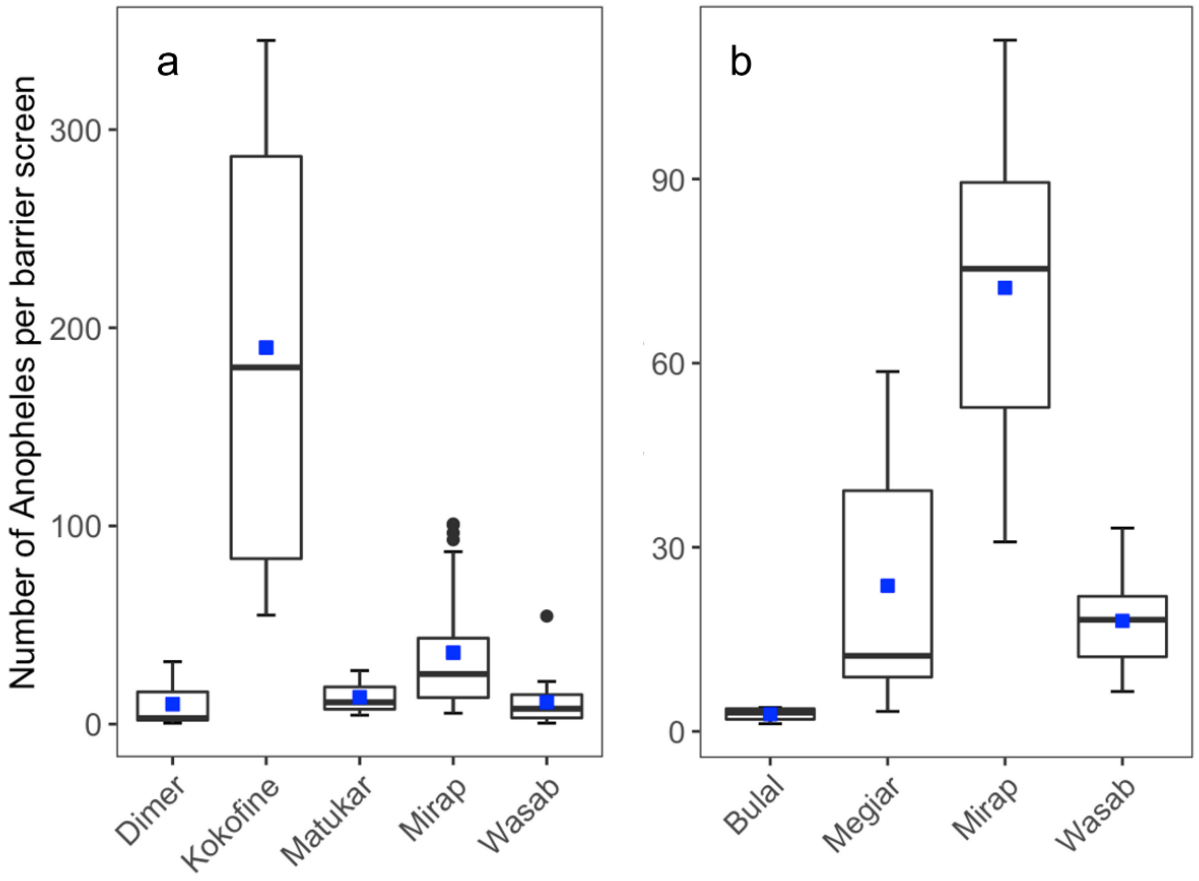

Figure 2.4. Box plots showing the distribution of *Anopheles* spp. numbers collected nightly per barrier screen. (a) Distribution of *Anopheles* spp. collected in five villages in April–December 2012. (b) Distribution of *Anopheles* spp. collected in four villages in August 2016–March 2017. Blue shaded squares are the means and black horizontal lines are the medians.

Ordination of the villages based on the Bray-Curtis dissimilarity matrix (Table S2.2, Appendix 2.2) derived from the analysis of April–December 2012 mosquito abundance data (Table 2.2) showed that Dimer and Wasab closely clustered together in the PCoA biplot; Matukar and Mirap also clustered together but less closely; and Kokofine was separate from the other villages (Figure 2.5a). The close grouping of the two inland villages Dimer and Wasab was influenced by the similar presence and abundance of *An. koliensis*, *An. punctulatus* (*s.s.*) and *An. longirostris* in the year 2012 (other years were excluded from the analysis due to lack of sampling in some villages in those years). Similarly, the grouping of the two coastal villages

Matukar and Mirap was influenced by the similar abundance and dominance of *An. farauti* (*s.s.*) in both villages. However, their cluster was not as tight as the two inland villages because of *An. bancroftii*, which was present and abundant in Mirap, but absent in Matukar. Kokofine differed greatly from the other villages, owing to the presence of *An. farauti* no. 4, which was the most abundant and exclusively found in that village (Figure 2.5a). In contrast, ordination of villages based on Bray-Curtis dissimilarity matrix (Table S2.3, Appendix 2.2) derived from the analysis of August 2016-March 2017 data (Table 3.3) showed lack of clustering (Figure 2.5b), which indicates high dissimilarity of species composition among the villages.

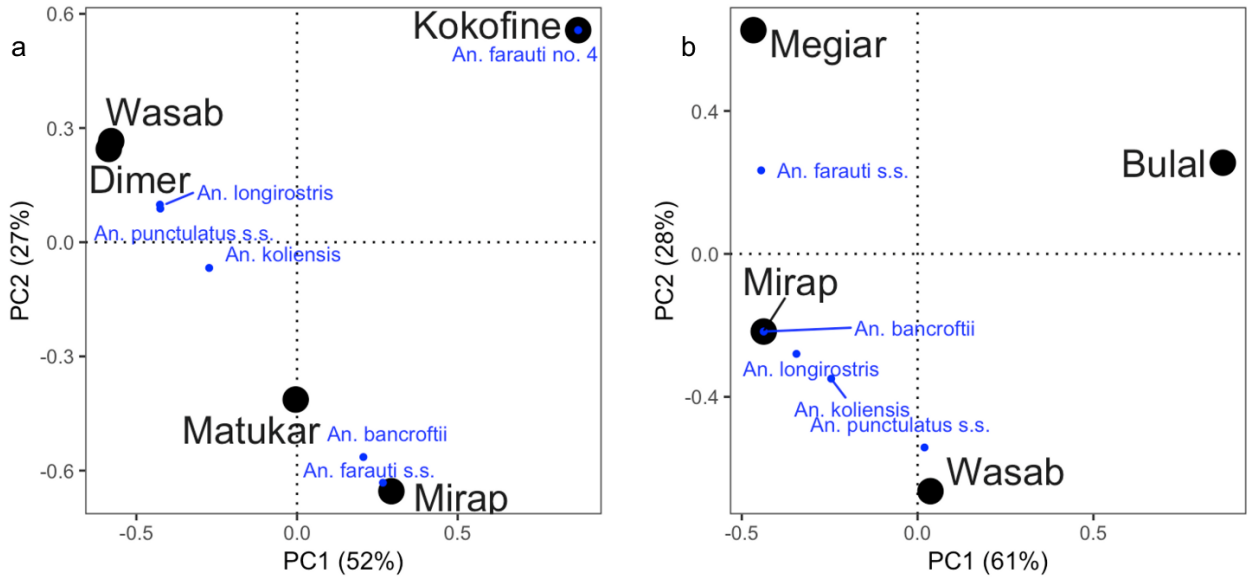

Figure 2.5. PCoA biplots of villages (black dots) with *Anopheles* species (blue dots) projected on the plot based on their weighted average scores. (a) PCoA biplot derived from the Bray-Curtis analysis of species abundance data of five villages between April–December 2012 (see Table 2.2). (b) PCoA biplot derived from data of four villages between August 2016–March 2017 (see Table 2.3). The two axes PC1 and PC2 were the principal coordinates with the highest proportion (percentage) of variation.

2.4.2. Nocturnal activities of mosquitoes

The mean number ± standard error of blood-fed and unfed mosquitoes captured on the bush and village side per barrier screen-night was plotted for each of the 12 hourly periods of the night for 12 *Anopheles* populations: five (four *An. farauti* (*s.s.*) and one *An. koliensis*) in Mirap (Figure 2.6a–e), one (*An. farauti* (*s.s.*)) in Megiar (Figure 2.6f), five (two *An. koliensis*, two *An. punctulatus* (*s.s.*) and one *An. longirostris*) in Wasab (Figure 2.7a–e) and one (*An. farauti* no. 4) in Kokofine (Figure 2.7f). Their corresponding GLM statistical results are shown in Figure 2.8 and 2.9. Except for Wasab-2015 *An. koliensis* (Figure 2.9a) and Wasab-2016 *An. punctulatus* (*s.s.*) (Figure 2.9e) which had statistically similar number of mosquitoes (both blood-fed and unfed) collected throughout the night, the other 10 populations had statistically different numbers of mosquitoes (either blood-fed, unfed or both) for one or more of the hourly periods of the night compared to 19:00 hr (Figure 2.8 and 2.9). Unfed mosquitoes for the three *An. farauti* (*s.s.*) populations in Mirap (Figure 2.8a–c), one *An. farauti* (*s.s.*) population in Megiar (Figure 2.8f), and one *An. koliensis* and one *An. longirostris* population in Wasab (Figure 2.9b and f) had statistically higher number of mosquitoes in the hourly periods before midnight and less mosquitoes in the hours after midnight, after controlling for side of barrier screens. This trend was also expressed by the blood-fed mosquitoes in some of these population but not as strongly as their unfed counterparts. For all 12 populations, the blood-fed mosquitoes captured on the village side of the screens were either statistically greater than (Figure 2.8a–d and Figure 2.9b, d and f) or equal to (Figure 2.8f and Figure 2.9 a, c and e), but not less than the bush side. In contrast, the unfed mosquitoes captured on the village side were either statistically less than (Figure 2.8b, c and f) or equal to (Figure 2.8a, d and e, and Figure 2.9a, b, c, e and f) but not

greater than the bush side for all populations except one (Wasab-2015 *An. punctulatus* (*s.s.*); Figure 2.9d).

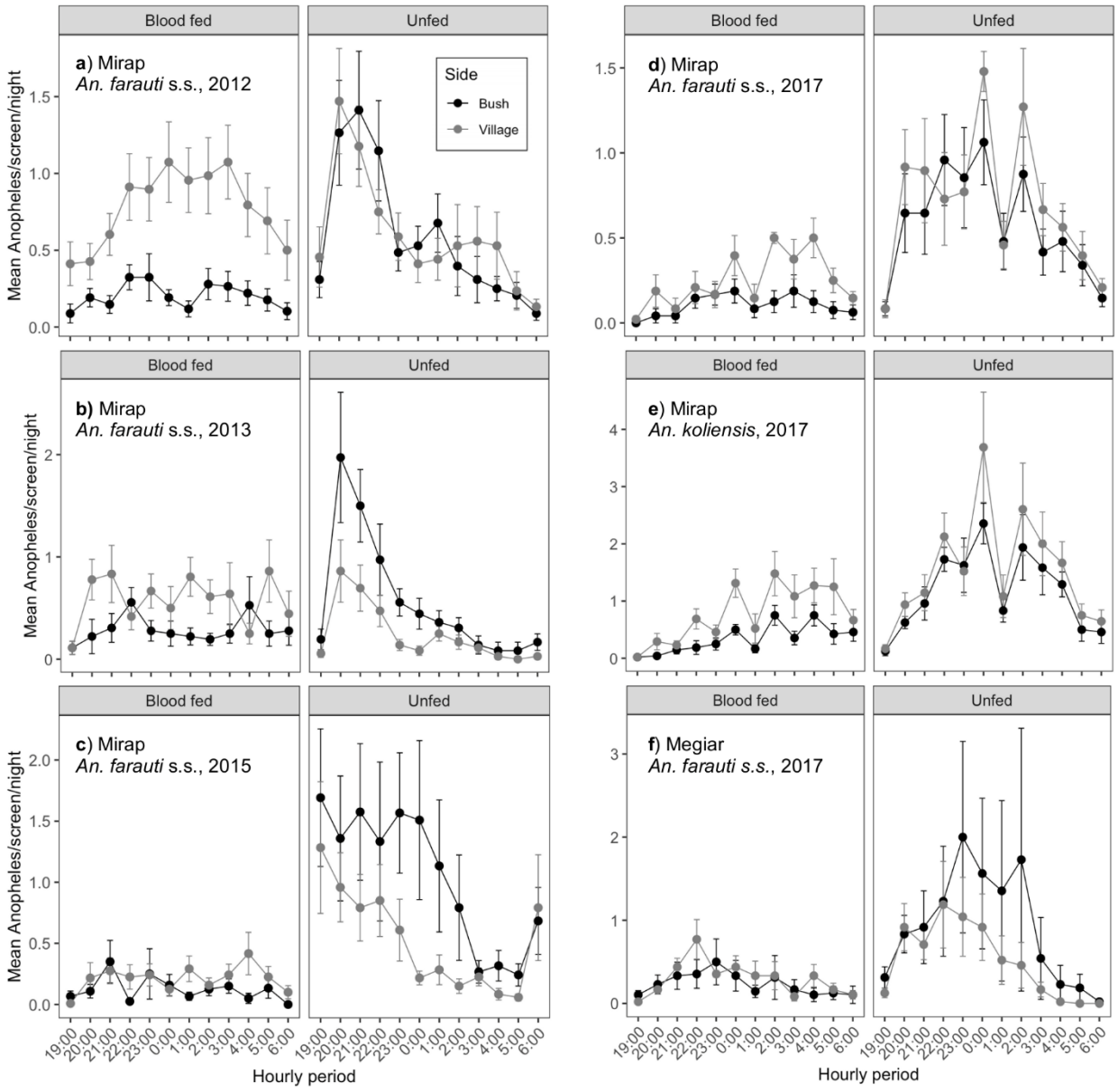

Figure 2.6. Mean number (dots) of blood-fed and unfed mosquitoes collected per barrier screen per night (y-axis) at each of the 12 hr periods of the night (x-axis, in 24 hr format). (a–e) Plot of five *Anopheles* populations in Mirap. (f) Plot of an *Anopheles* population in Megiar. Vertical bars are the standard error of the mean. Color black represents mosquitoes collected on the bush side of the barrier screen and grey represents those collected on the village side.

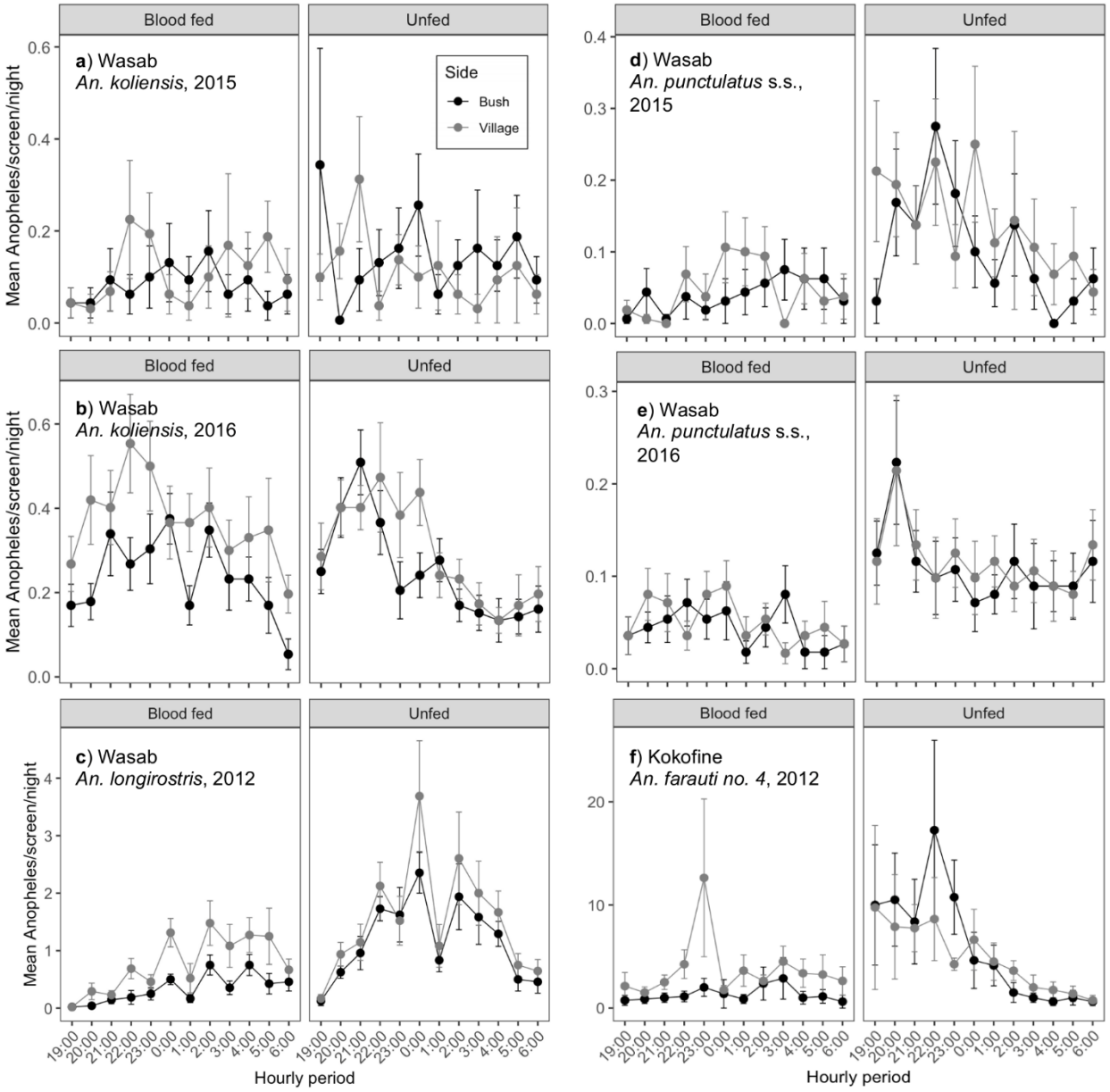

Figure 2.7. Mean number (dots) of blood-fed and unfed mosquitoes collected per barrier screen per night (y-axis) at each of the 12 hr periods of the night (x-axis, in 24 hr format). (a–e) Plot of five *Anopheles* populations in Wasab. (f) Plot of an *Anopheles* population in Kokofine. Vertical bars are the standard error of the mean. Color black represents mosquitoes collected on the bush side of the barrier screen and grey represents those collected on the village side.

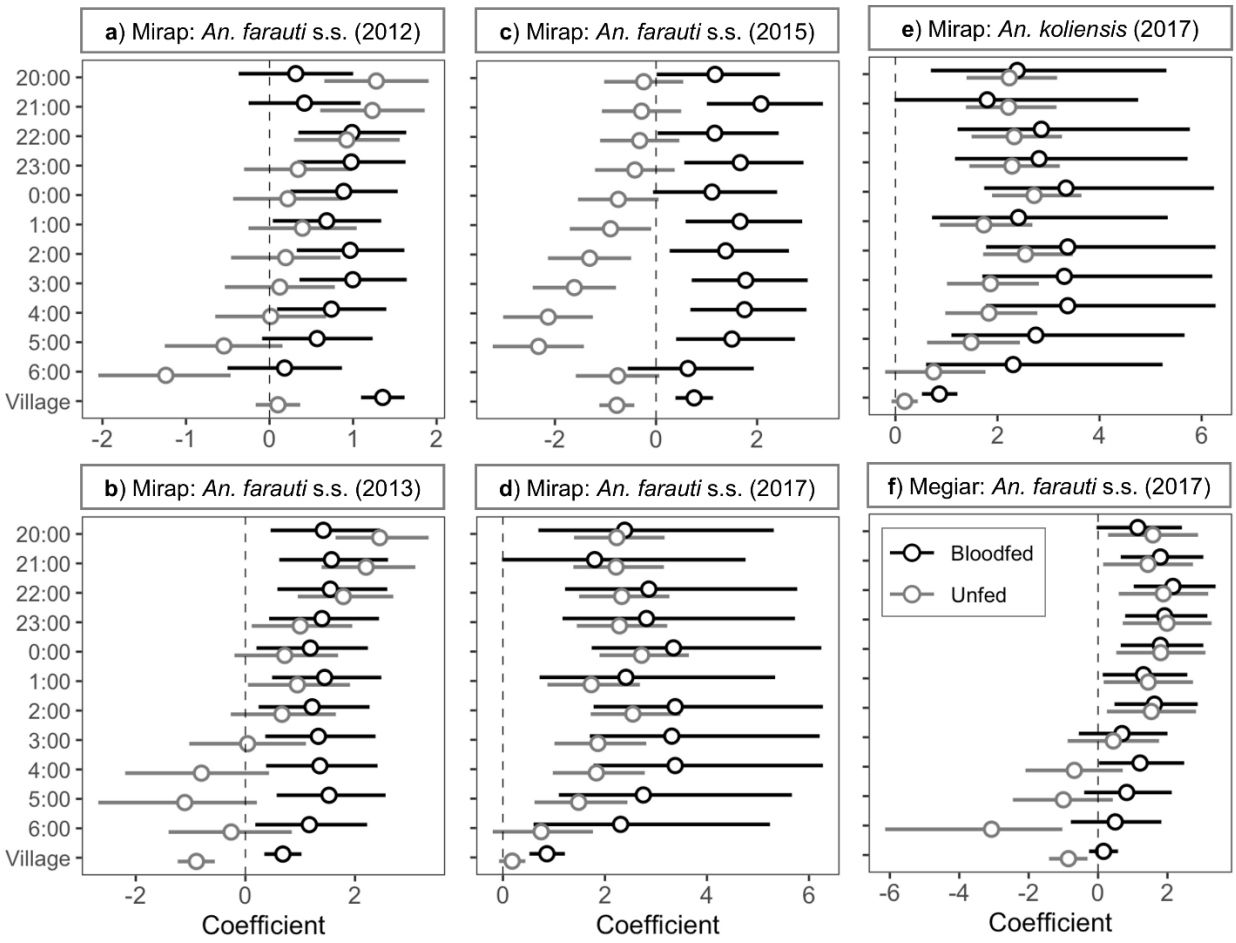

Figure 2.8. Plot of GLM coefficients (open circle with 95% confidence interval bars) showing the comparison of mosquito numbers captured in each of 11 hourly nocturnal periods compared to 19:00 hr (represented by the broken vertical line centered at 0) as the reference variable and on village side of the barrier screen compared to the bush side (represented by the broken vertical line) as the reference variable. (a–e) GLM coefficients of five *Anopheles* populations in Mirap. (f) GLM coefficients of one *Anopheles* population in Megiar. The covariate levels (y axis) include eleven of the twelve hourly periods of the night (expressed in 24 h format) and village side of the barrier screen. The GLM coefficients (x axis) are in logarithmic scale.

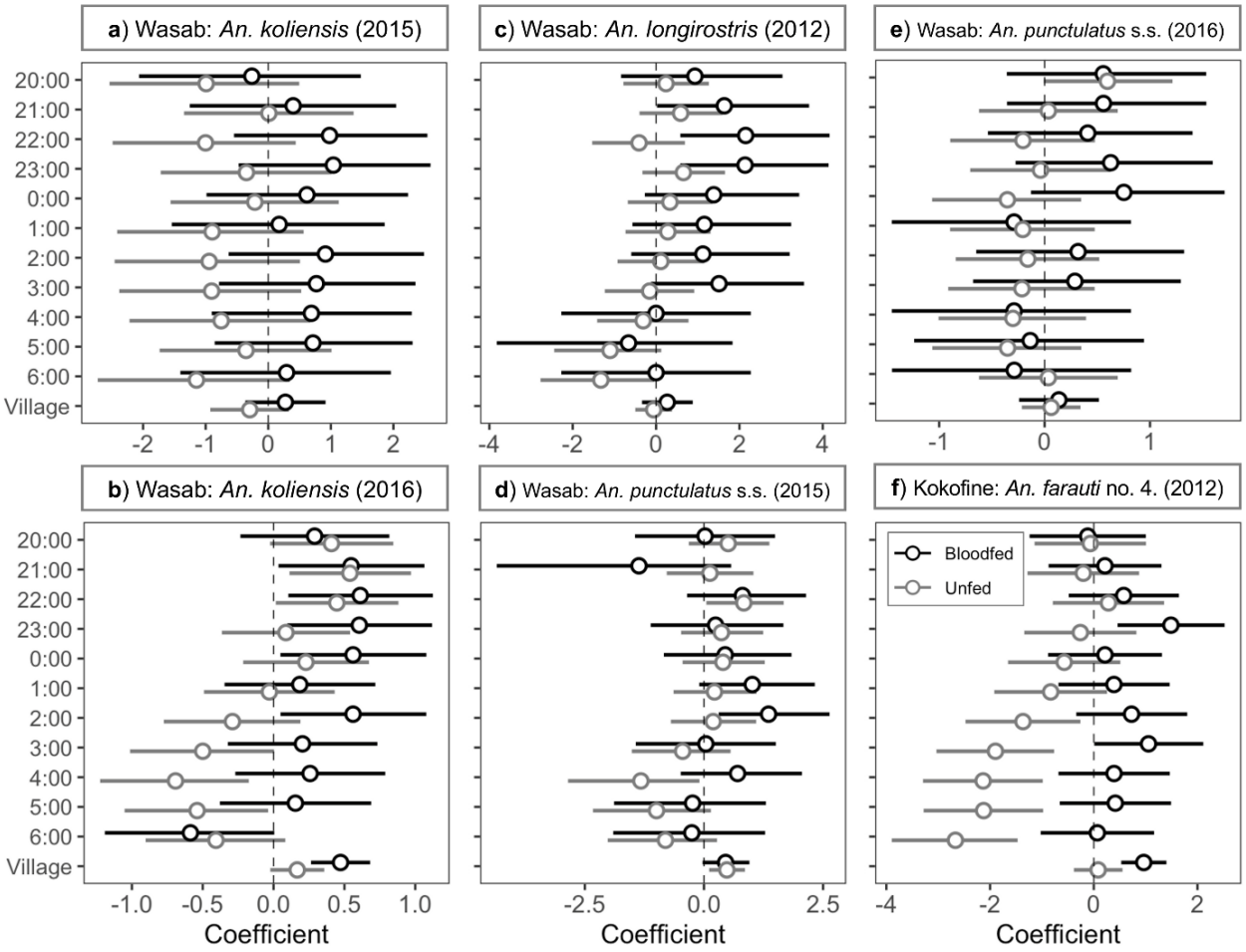

Figure 2.9. Plot of GLM coefficients (open circle with 95% confidence interval bars) showing the comparison of mosquito numbers captured in each of 11 hourly nocturnal periods compared to 19:00 hr (represented by the broken vertical line centered at 0) as the reference variable and on village side of the barrier screen compared to the bush side (represented by the broken vertical line) as the reference variable. (a–e) GLM coefficients of five *Anopheles* populations in Wasab. (f) GLM coefficients of one *Anopheles* population in Kokofine. The covariate levels (y axis) include eleven of the twelve hourly periods of the night (expressed in 24 h format) and village side of the barrier screen. The GLM coefficients (x axis) are in logarithmic scale.

2.5. Discussion

The effectiveness of the barrier screen method to produce an adequate and unbiased sample of blood-fed mosquitoes for estimating host selection tendencies of mosquito vectors, and to study

timing of host-seeking and infer flight behavior, has been reported for several *Anopheles* species of the southwest Pacific, including PNG in chapter 6 and elsewhere (Burkot, et al. 2013; Davidson, et al. 2018). The *Anopheles* fauna encountered in the seven villages using this method of sampling was limited to six species, but species composition and dominance varied considerably, with certain species dominant in some villages (e.g. *An. farauti* no. 4 in Kokofine, *An. farauti* (*s.s*.) in Megiar and Mirap) while completely absent (*An. farauti* no. 4) or present but relatively less dominant or common (*An. farauti* (*s.s*.) in Dimer and Wasab) in other villages. These findings are consistent with, and extend those, of two other studies using other sampling methods (Hii, et al. 1997; Reimer, et al. 2016). In particular, the dominance of *An. farauti* (*s.s.*) in the coastal villages was reversed by *An*. *punctulatus* (*s.s.*), in the nearby inland villages, although the latter was not always the most abundant species at the inland sites (Figure 2.3). *Anopheles longirostris* was present across all sites (except Megiar) at least in some years and typically a less common species, but was dominant in samples from Wasab for two of the four years (Figure 2.3c). These findings show that the *Anopheles* communities of these villages show particular patterns of dominant species yet with shifting composition over time. These shifts may be due to seasonal effects, conditions established by control efforts such as distribution of insecticidal bed nets programmatically, or random processes.

The observed diversity of the species combined with variation in their ecological attributes (Burkot, et al. 1988a; Charlwood, et al. 1985b; Charlwood and Graves 1986) such as outdoor and early-evening biting habits (chapter 4), and plasticity of host selection (chapter 6) can potentially attenuate single-intervention malaria vector control programmes such as insecticidal bed nets in PNG. For example, the host selection component of this study (chapter 6) showed that *An. farauti* (*s.s.*), *An. koliensis*, *An. longirostris* and *An. punctulatus* (*s.s.*) are

opportunistic (can feed unbiasedly on both humans and non-human hosts) in their host selection, whereas *An. bancroftii* was zoophagic (feeds excessively on non-human hosts). Thus, in villages like Wasab where vector diversity is high, and the species present include those that are highly flexible in their host choice, the effectiveness of bed nets on malaria transmission will be lessened. Such village level differences in species composition, even for those locations relatively close to each other, could explain the disparity in impact of vector control on malaria observed for a country-wide bed net campaign in PNG (Hetzel, et al. 2014). Notably, *An. koliensis* was the most affected species whose relative abundance was dramatically reduced immediately after the bed net distribution campaign (Hetzel, et al. 2016; Reimer, et al. 2016). The consistent rise of *An. koliensis* abundance in recent years in Wasab and to a lesser extent Kokofine suggests, among other potential causes, a decline in the effectiveness of the bed net campaign such as reduced bed net usage or increased flexibility in alternative host utilization.

For six of the 12 *Anopheles* populations analyzed (Mirap-2012 *An. farauti* (*s.s.*), Mirap-2013 *An. farauti* (*s.s.*), Mirap-2015 *An. farauti* (*s.s.*), Megiar-2017 *An. farauti* (*s.s.*), Wasab-2015 *An. koliensis* and Kokofine-2012 *An. farauti* no. 4) the number of host-seeking mosquitoes (i.e. the bush side, unfed mosquitoes) arriving in the villages peaked between 20:00 hr and 22:00 hr and declined towards morning (Figure 2.6 and 2.7). This relatively early arrival suggests that the adult resting sites and larval habitats for these populations were close to the villages, resulting in short commuting time between the habitats and the village. In contrast, the number of host-seeking mosquitoes in the other six populations was extended across the evening, midnight, and morning hours suggesting that the adult resting sites for these populations ranged in distances from nearby to further from the village. Interestingly, the *An. farauti* (*s.s.*) in Mirap exhibited early arrival behavior in the year 2012, 2013 and 2015 (Figure 2.6 a–c) but in 2017 the number

of host-seeking *An. farauti* (*s.s.*) as well as *An. koliensis* arriving in the village extended throughout the night (Figure 2.6d and e). Similarly, the *An. koliensis* mosquitoes in Wasab exhibited extended arrival in 2015 and early arrival in 2016 (Figure 2.7a and b). The change in arrival times in latter years may be caused by changes in the spatial distribution of mosquito breeding habitats around the villages. This observation reflects an earlier study in the north coast villages of Madang which found that blood-fed individuals of *An. farauti* (*s.l.*) flew < 50 m from the study villages before resting in the nearby vegetation, whereas *An. punctulatus* (*s.s*.) and *An. koliensis* dispersed widely (Charlwood, et al. 1985b). Those researchers attributed this variation in dispersal among the species to the proximity of their preferred larval habitats and resting sites to the villages. Similarly, in East Sepik province, biting rates of *An. farauti* (*s.l*.) and *An. longirostris* were highest in the evening and declined towards morning, whereas those of *An. koliensis* and *An. punctulatus* (*s.s.*) were lowest in the evening and peaked in the morning hours (Hii, et al. 1997). In Kokofine an early-evening biting pattern was observed for *An. farauti* no. 4 (Thomsen, et al. 2017). Generally, the primary host-seeking activity of most of these *Anopheles* populations coincides with evening activity of villagers who would, therefore, be unprotected by bed nets.

The higher proportion of blood-fed mosquitoes on the village than bush side of the barrier screen and higher proportion of unfed on the bush than village side of the screen is consistent with commuting behavior of mosquitoes. For exophilic mosquito populations like those investigated here, freshly blood-fed mosquitoes must exit the village towards their resting sites in the surrounding vegetations and are therefore likely to be intercepted by the village than bush side of the barrier screen. Similarly, unfed host-seeking mosquitoes must enter the village from

their resting sites to seek vertebrate hosts and are therefore likely to be intercepted by the bush than village side of the barrier screen.

This study has two potential limitations. First, the presence of the mosquito collectors near the barrier screens during mosquito collections was an unavoidable aspect of this sampling method but it also introduced potential sampling bias in favor of anthropophilic vectors. To minimize this bias, the collectors applied insect repellents on their bodies and positioned themselves further from the barrier screen and among the inhabitants of the hamlets when they were not visiting the barrier screen, although these measures were not completely effective as human DNA profiling analysis show that 22–31% of human blood meal samples were collectors' blood (see chapter 7). Second, for the villages where mosquitoes were collected for several years, the months during which mosquitoes were sampled within each village was not consistent over the years (see Table S2.1, Appendix 2.1). Also, mosquitoes were not collected at the same time in the different villages. As different species of *Anopheles* in PNG, particularly those within the *An. punctulatus* (*s.l.*) group, exhibit affinity for specific larval habitat types and the temporal distribution and abundance of these habitat types are associated with annual rainfall pattern (Charlwood, et al. 1986), these inconsistencies in sampling time may not capture or confound any observable temporal or spatial pattern in the species composition and abundance associated with annual rainfall season. For example, *An. farauti* no. 4 (whose bionomics is poorly understood but is believed to be associated with riverine puddles formed along the flood plains of Ramu River after flooding) was the only species collected in June of 2012 in Kokofine, but species richness rose to four in February 2016. Although it is possible that this change in species composition in 2016 could have resulted from a major ecological change after 2012, it is likely the result of annual seasonal variation, which was uncontrolled in this study.

2.6. Conclusion

In PNG, *Anopheles* species abundance and composition varied among sites, even those that are less than one km apart. Such local heterogeneity in species composition can complicate vector control efforts in PNG. For six of the 12 *Anopheles* populations, the majority of the host-seeking mosquitoes arrived in the village before midnight when most people were active and exposed to the mosquitoes. In areas where *Anopheles* exhibit this temporal host-seeking behavior, the bed net-based vector control will be ineffective against malaria. In addition to its effectiveness in sampling blood-fed mosquitoes for analysis of their host selection tendencies, the barrier screen sampling method is useful for analyzing other aspects of the vector populations. By serving as an interception device, the barrier screen sampling system permits inferences about local movement patterns including nocturnal activity of mosquito populations.

APPENDICES

Appendix 2.1. Dates of barrier screen collection in the study villages

Table S2.1. Dates of barrier screen mosquito sampling in each village in the years 2012, 2013, 2015, 2016 and 2017. Shaded cells represent the dates in which mosquitoes were sampled with the different colors correspond to each of the seven study villages. Numbers within each cell represents the number of barrier screens used to sample mosquitoes that night. Gradient shades represent villages where mosquitoes were sampled simultaneously.

	Year 2012																														
Dates	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	15th	16th	17th	18th	19th	20th	21st	22nd	23rd	24th	25th	26th	27th	28th	29th	30th	31st
January																															
February																															
March																															
April																2	2	2	2												
May																															
June				2	2	2	2								2	2	2	2								2	2	2	2		
July				2	2	2	2				2	2	2	2											2	2	2	2	2	2	
August							2	2	2	2					2	2	2	2	2	2											
September																															
October																															
November																										2	2	2	2	2	
December	2		2	2	2	2					2	2																			

Village color codes
Dimer
Kokofine
Mirap
Wasab
Matukar & Wasab (simultaneous sampling)
Dimer & Mirap (simultaneous sampling)
Bulal
Megiar

Table S2.1 (cont'd)

	Year 2013																													
Dates	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	15th	16th	17th	18th	19th	20th	21st	22nd	23rd	24th	25th	26th	27th	28th	29th	30th	31st
January																															
February																															
March																															
April																							2	2	2	2	2	2		2	
May	2	2	2	2																											
June																															
July																															
August																										2	2	2	2	2	2
September		2			2		2																								
October																															
November																															
December				2	2	2	2	2	2		2	2	2	2		2															

	Year 2015																													
Dates	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	15th	16th	17th	18th	19th	20th	21st	22nd	23rd	24th	25th	26th	27th	28th	29th	30th	31st
January																															
February																															
March																															
April																				2	2	2	2	2	2		2	2	2	2	
May	2	2																													
June																															
July															10	10															
August			10	10	10	10			10	10	10	10																			
September																															
October																															
November																															
December	2	2	2	2	2	2																									

	Year 2016																													
Dates	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	15th	16th	17th	18th	19th	20th	21st	22nd	23rd	24th	25th	26th	27th	28th	29th	30th	31st
January																															
February											2	2																			
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May																															
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July																															
August																		8	8												
September					8	8	8	8	8	8																					
October																															
November				8	8	8	8	8	8																						
December																															

Table S2.1 (cont'd)

	Year 2017																															
Dates	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	15th	16th	17th	18th	19th	20th	21st	22nd	23rd	24th	25th	26th	27th	28th	29th	30th	31st
January											8	8	8	8	8	8															
February				8	8	8	8	8	8																						
March																8	8	8	8	8	8										
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December																															

Appendix 2.2. Bray-Curtis index matrices

Table S2.2. Bray-Curtis dissimilarity index matrix based on species abundance data for April–December 2012 sampling period. Values closer to 0 indicate villages with similar species composition and values closer to 1 indicate dissimilar villages.

	Dimer	Kokofine	Matukar	Mirap	Wasab
Dimer	0.000	-	-	-	-
Kokofine	1.000	0.000	-	-	-
Matukar	0.791	1.000	0.000	-	-
Mirap	0.909	1.000	0.828	0.000	-
Wasab	0.460	1.000	0.832	0.900	0.000

Table S2.3. Bray-Curtis dissimilarity index matrix based on species abundance data for August 2016–March 2017 sampling period. Values closer to 0 indicate villages with similar species composition and values closer to 1 indicate dissimilar villages.

	Bulal	Megiar	Mirap	Wasab
Bulal	0.000	-	-	-
Megiar	0.958	0.000	-	-
Mirap	0.926	0.582	0.000	-
Wasab	0.847	0.975	0.503	0.000

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CHAPTER 3: PERSISTENT MALARIA TRANSMISSION IN HUMAN POPULATIONS WITH HIGH BED NET USAGE IN MADANG, PAPUA NEW GUINEA

3.1. Abstract

In the absence of disease intervention, malaria infection prevalence in human populations in Madang province equilibrates between 35–45%, of which a large portion are children under 10 years old. As a result of the nationwide bed net-based vector control campaign, malaria dropped considerably in Madang. In this study, malaria infection prevalence in humans and human biting rates (*HBR*) and annual infectious biting rates (*IBR*) of vectors were quantified in villages on the north coast of Madang. Bed net survey, active case malaria detection, and human landing catch mosquito sampling of female *Anopheles* were conducted in the villages. A PCR method was used to test for malaria infection in humans and mosquitoes. The results show that in all villages, the proportion of villagers who reportedly slept under a bed net was > 80%. Overall (all villages combined) infection prevalence for *P. falciparum* rebounded two-fold from 9% in 2014 to 18% in this study. In contrast, *P. vivax* decreased from 19.7% in 2014 to 14% in this study. For both malaria species, older individuals were equally or more likely to be infected than children under 5 years old. The mean *HBR* ± standard error of *Anopheles* spp. among the villages ranged from 2.7 ± 0.6–31 ± 2.7 bites per person-night. The mean *IBR* for *P. falciparum* ranged from 6.5 ± 1.3–159.7 ± 13.7 and for *P. vivax* ranged from 0.0 ± 0.0–22.8 ± 1.96 infective *Anopheles* spp. bites per person-year. The current *HBR* and *IBR* were higher than most recent estimates. These results show that in Madang, and most likely other parts of PNG, malaria transmission is persisting at high rates despite ongoing efforts to intensify the bed net program.

3.2. Introduction

Two epidemiological quantities are often used to assess the magnitude of malaria transmission: (i) prevalence of infection in the human population, often by age group; and (ii) annual infectious biting rate or *IBR* (aka annual entomological inoculation rate or *EIR*). Infection prevalence is the proportion of surveyed human individuals that were tested positive for malaria infection. *IBR* is the average number of sporozoite-infected *Anopheles* bites that an individual receives per year. It is quantified by the formula $IBR = HBR * SR * 365$, where *HBR* (human biting rate) is the average number of *Anopheles* bites per person-night, *SR* (sporozoite rate) is the proportion of *Anopheles* mosquitoes tested positive for sporozoite infection and 365 is the number of days in a year (Mboera 2005; Smith, et al. 2005).

PNG is arguably the most malaria endemic country outside Africa. According to 2011 national census data from the PNG National Statistics Office nearly 61% of the country's population live in the lowland areas (0–1,600 m above sea level) where malaria is holoendemic or hyperendemic and 39% occupy highland areas (> 1,600 m above sea level) where malaria is hypoendemic (Muller, et al. 2003). All four human malaria species are found in PNG, but *P. falciparum* and *P. vivax* are the most widespread and are responsible for most of the infection cases (Genton, et al. 1995a; Genton, et al. 1995b; Mehlotra, et al. 2002; Mehlotra, et al. 2000; Mueller, et al. 2005; Muller, et al. 2003). Numerous *Anopheles* species are found in PNG and most are known to transmit malaria (Cooper, et al. 2009). Five of these vectors namely *An. farauti* (*s.s.*), *An. farauti* no. 4, *An. hinesorum*, *An. koliensis* and *An. punctulatus* (*s.s.*) are the most widespread and are responsible for most of the transmission (Burkot, et al. 1989; Burkot, et al. 1990; Burkot, et al. 1988; Hii, et al. 2001; Reimer, et al. 2016; Thomsen, et al. 2017).

Active case malaria detection surveys in villages of Madang and East Sepik provinces in the years 1980–2001 showed that infection prevalence in these holoendemic populations (all ages) equilibrates at 35–45% but can reach as high as 70% or as low as 10% in some villages (Burkot, et al. 1990; Cattani, et al. 1986a; Cattani, et al. 1986b; Genton, et al. 1995a; Mehlotra, et al. 2002; Mehlotra, et al. 2000). As expected for holoendemic malaria settings, a large proportion of the infection cases in those villages were children < 10 years old who were immunologically susceptible; most adults were immune to the disease and thus were less likely to be infected (Burkot, et al. 1990; Cattani, et al. 1986a; Cattani, et al. 1986b; Genton, et al. 1995a; Mehlotra, et al. 2002; Mehlotra, et al. 2000). Malaria-associated mortality occurred mostly in children, accounting for 4–17% of deaths in children, but less so in adults mainly due to the acquired immunity in individuals of older ages (Genton, et al. 1995a; Moir, et al. 1989). With regard to transmission rates, a study conducted from 1983–1987 in 11 Madang villages located within an area of 20 km radius found that *IBR* ranged from 79–526 infective *Anopheles* spp. bites per person-year for *Plasmodium* spp., 44–293 infective bites per person-year for *P. falciparum* and 0–233 infective bites per person-year for *P. vivax* (Burkot, et al. 1988).

To alleviate the burden of malaria in PNG, the National Department of Health (NDOH), with funding from the Global Fund to Fight AIDS, Tuberculosis and Malaria, embarked on a nationwide-wide malaria control campaign based on mass distribution of free long-lasting insecticidal bed nets between the years 2004 and 2009. The rationale for the insecticidal bed nets as a malaria control method is that by sleeping under it, vector-human contact is interrupted, resulting in reduced transmission of the disease. Treatment of the bed nets with insecticides is intended to kill vectors that come in contact with them, thereby reducing the vector population size and survival rate, and eventually the *HBR*. By the end of the NDOH-led campaign in 2009,

65% of households nationwide owned at least one bed net, but this figure was below the target of 80% household coverage (Hetzel, et al. 2012). Extension of the bed net distribution campaign by Rotary Against Malaria (https://ram.rawcs.com.au/) from 2009 to the present further increased the coverage to an estimated 82% of households nationwide (Hetzel, et al. 2014a). Further, to complement the bed nets, increased supplies of artemisinin-based antimalarial drugs and rapid diagnostic test kits were made available at public hospitals and local health centers to diagnose and treat malaria (Hetzel, et al. 2014b).

Studies conducted before and after the bed net campaign showed considerable malaria decline in PNG. Nationally, *Plasmodium* spp. infection prevalence which was at 11% in the 2008–2009 national malaria survey, dropped to 5.1% in the 2010–2011 survey and further down to 0.9% in the 2013–2014 survey (Hetzel, et al. 2017). In the holoendemic north coast of Madang where bed net distribution began in 2008, the infection prevalence for *P. falciparum* dropped from 42.1% in 2006 to 9% in 2014. *P. vivax* also dropped from 41.7% in 2006 to 12.7% in 2010, but rebounded to 19.7% in 2014 (Koepfli, et al. 2017). The *IBR* for *Plasmodium* spp. in six villages in Madang north coast ranged from 20–350 infective *Anopheles* spp. bites per person-year in 2009 but was reduced to a range of 0–15 infective *Anopheles* spp. bites per person-year in 2011. In five villages in East Sepik province, the *IBR* for *Plasmodium* spp. ranged from 175–1,050 in 2009 but was reduced to a range of 0–25 *Anopheles* spp. infective bites per person-year in 2011 (Reimer, et al. 2016). Although the levels of infection prevalence and *IBR* were lower after the bed net campaign than they were before the campaign, they were still high from the perspective of public health. The aim of the study comprising this chapter was to investigate if malaria transmission has been further suppressed in recent years by the ongoing vector control campaign or whether the disease has persisted or rebounded.

3.3. Methods

3.3.1. Study sites and mosquito sampling

This study was conducted in the four Madang villages of Bulal, Megiar, Mirap and Wasab whose geographic locations (see Figure 2.1) and ecogeographic environments were described in detail in chapter 2. In brief, Megiar and Mirap are situated on the coastal plain along the seacoast where the brackish water-tolerant vector *An. farauti* (*s.s.*) is known to be predominant (Beebe and Cooper 2002; Charlwood, et al. 1986; Sweeney 1987). Bulal and Wasab are situated several km inland from the coastline, on elevated hilltops where the vectors *An. punctulatus* (*s.s.*) and *An. koliensis* are known to be predominant (Beebe and Cooper 2002; Charlwood, et al. 1986).

Mosquitoes were sampled using the human landing catch method, which involved human volunteers luring host-seeking mosquitoes to land on exposed parts of their legs. The mosquitoes were captured immediately using a mouth aspirator aided by a flash light before they had time to pierce the skin and obtain a blood meal (WHO 1975). Twenty-four (Megiar, Mirap and Wasab) or 20 (Bulal) households were randomly selected to participate in mosquito sampling. For each village, 12 (Megiar, Mirap and Wasab) or 10 (Bulal) of the selected households were randomly chosen to participate in indoor collection (i.e., mosquitoes were collected inside the houses) whereas the other 12 or 10 households participated in outdoor collection.

Mosquitoes were collected from 18:00 hr to 6:00 hr each night for four consecutive nights in Bulal (March 16th–19th, 2017), six in Megiar (February 4th–7th and March 2nd–3rd, 2017) and Mirap (January 11th–16th, 2017), and 12 in Wasab (September 5th–10th and November 4th–9th, 2016). Each household participated in mosquito sampling for two nights (Megiar, Mirap and Bulal) or four nights (Wasab) only. Two members (18–70 years old) of each household were consented to collect mosquitoes inside or outside their own house. The collectors were also

consented to take antimalarial prophylaxis before participating in the mosquito collection. In each house, one person collected mosquitoes for six consecutive hours (18:00 hr–0:00 hr) before being replaced by the second person who continued for the next six hours (0.00 hr–6:00 hr). Mosquitoes were captured by a mouth aspirator and transferred into screened paper cups labelled with the hour of the night, the household number, and the location (indoor or outdoor) where the mosquitoes were collected. With the aid of a light microscope, mosquitoes collected each night were separated into their respective genus the next morning. Male mosquitoes were discarded, and female *Anopheles* were identified to species based on morphological keys (Belkin 1962; Lee, et al. 1987). Each female mosquito was placed in a 2 ml microcentrifuge tube, assigned a unique serial number, and metadata (date, time, house location) were recorded. The mosquitoes were kept on silica gel desiccant in the field for up to 7 days and transported to the laboratory where they were stored at -20 °C.

3.3.2. Human blood sampling and bed net survey

An active case malaria detection survey was implemented a few days before the commencement of the mosquito sampling in each village. Capillary blood samples (250 μl) were collected from each consented individual (ages 0.5–85 years old; consent for minors was obtained from their parents or guardians) and placed in a microtainer blood collection tube, which contained the anticoagulant potassium EDTA. The samples were kept on ice packs in the field and transported to the laboratory where they were stored in freezers. To estimate the frequency of bed net usage, consented individuals who participated in the active case detection survey were also asked whether they slept under a bed net the night before the interview. To estimate household

ownership of bed nets, a senior member of each household was asked for the number of bed nets owned by their family.

3.3.3. PCR detection of malaria in humans and mosquitoes

Malaria parasite infection in individual human and mosquito samples was assessed as follows. A multiplex quantitative PCR (qPCR) assay containing two fluorescent-labelled TaqMan probes targeting the 18S rRNA gene of *P. falciparum* (forward primer: ATT GCT TTT GAG AGG TTT TGT TAC TTT; reverse primer: GCT GTA GTA TTC AAA CAC AAT GAA CTC AA; probe: FAM-CAT AAC AGA CGG GTA GTC AT-MGB) and *P. vivax* (forward primer: GCA ACG CTT CTA GCT TAA TCC AC; reverse primer: CAA GCC GAA GCA AAG AAA GTC C; probe: VIC-ACT TTG TGC GCAT TTT GCT A-MGB) was optimized for sensitivity (detected as low as one target gene copy/µl sample) and amplification efficiency (> 90%). The primers and probes were previously tested to be specific to their target DNA locus (Kamau, et al. 2013). Positive DNA controls were used for the assay optimization. Optimum reaction mixture (10 µl volume) consisted of 1x TaqMan Multiplex Master Mix (Catalog number: 4481882; Thermo Fisher Scientific, Waltham, MA, USA), 0.6 µM of each primer, 0.4 µM of each probe and 2 µl of DNA sample. PCR reactions were performed on a QuantStudio 7 Flex instrument (Applied Biosystems, Foster City, CA, USA) with fast cycling consisting of 1 cycle of 95 °C for 20 seconds followed by 40 cycles of 95 °C for 1 second and 60 °C for 20 seconds.

Using sterile technique, the abdomen of each *Anopheles* mosquito was separated from the rest of the body. DNA was extracted from the abdomen-detached body part (i.e., head + thorax) of each mosquito and human blood sample (100 µl) using a DNeasy Blood and Tissue Kit (Catalog number: 69582; Qiagen, Valencia, CA, USA). Mosquitoes morphologically identified

as members of the *An. punctulatus* (*s.l.*) complex were analyzed in a PCR assay (Beebe and Saul 1995) to identify them to the species level. Both the human and mosquito samples were subjected to the qPCR method described above to test for *P. falciparum* and *P. vivax* infection. Samples with amplification threshold cycle (C_q) ≥ 38 were inconclusive and thus considered negative. As the mosquito DNA was isolated from the part of the body anterior to the abdomen, it was considered to be devoid of oocysts and other blood stages of the *Plasmodium* parasites which inhabit the mosquito midgut. Thus, the PCR positive samples were considered infected with the sporozoite stage of the parasite which inhabit the salivary glands in the head and thorax of mosquitoes and data used to estimate salivary gland, sporozoite infection rate.

3.3.4. Data analyses

Bed net ownership was expressed as the proportion of surveyed households that owned one or more nets. Bed net usage was expressed as the proportion of interviewed individuals who slept under a net the night before they were interviewed. Malaria infection prevalence was expressed as the proportion of surveyed individuals who tested positive by qPCR. Comparison of the odds of *P. falciparum* or *P. vivax* infection between human groups used Fisher's exact test of contingency tables or logistic regression of binary data. Both tests were performed using the functions *fisher.test* and *glm* from the package *stats* in R software. Significance level for all statistical tests was based on type I error rate of 5%.

A vector population is defined here as mosquitoes of a particular species sampled from a specific location and time. The *SR*, *HBR* and *IBR* were estimated for each vector population as follows: The *HBR* was calculated for each sampling night by dividing the total number of *Anopheles* collected by landing catch in a night by the number of collectors on that night. It was

assumed that rate of landing catch equated to rate of biting, i.e., all landing mosquitoes were assumed to have bitten a human host had they been provided the opportunity before capture. The mean *HBR* was then estimated from these data points with night as unit of replication, rationalized because these nights were close together in time. The *IBR* was estimated for each night (see formula under section 3.2) and the mean was calculated with night as unit of replication.

3.4. Results

3.4.1. Bed net ownership and usage

Census survey conducted during the study identified 97 households and 587 people living in Bulal, 172 households and 992 people in Megiar, 220 households and 1,283 people in Mirap, and 76 households and 395 people in Wasab. The proportion of the households that participated in the bed net survey was 88% in Bulal, 77% in Megiar, 58% in Mirap and 72% in Wasab. The proportion of the surveyed households that owned at least one bed net based on verbal response from the head of households was 100% in Bulal, 99% in Megiar, 100% in Mirap and 98% in Wasab. Mean number (± standard error) of reported bed nets per household was 4.3 ± 0.23 in Bulal, 4.3 ± 0.16 in Megiar, 3.3 ± 0.13 in Mirap and 3.6 ± 0.23 in Wasab. The proportion of interviewees who reportedly slept under a bed net (i.e., bed net usage) the night before they were interviewed was 93% (n = 357 interviewees) in Bulal, 81% (n = 478) in Megiar, 94% (n = 630) in Mirap and 90% (n = 256) in Wasab.

3.4.2. Malaria infection prevalence

The proportion of the human population that consented to participate in the malaria survey was 65% (383/587 people) in Bulal, 44% (434/992) in Megiar, 34% (441/1,283) in Mirap, and 72% (285/395) in Wasab. Age range for the study participants was 1–84 years in Bulal, 1–82 years in Megiar, 0.5–79 years in Mirap and 1–66 years in Wasab. Sex ratio (male:female) of the study participants was 1:1.2 in Bulal, 1:1.6 in Megiar, 1:1.1 in Mirap and 1:1 in Wasab. The infection prevalence for *Plasmodium* spp. was 32% in Bulal, 36% in Megiar, 51% in Mirap, 9% in Wasab and 34% for all villages combined. Infection prevalence according to species (i.e., for *P. falciparum* and *P. vivax* separately) is shown for each village in Figure 3.1, and for all villages combined was 18% *P. falciparum*, 14% *P. vivax* and 2.5% mixed infection. The odds of infection with *P. falciparum* was statistically the same as that of *P. vivax* in the two inland villages—Bulal and Wasab (see Figure 2.1, chapter 2 for mapped location of these villages). In the two coastal villages (see Figure 2.1, chapter 2) the odds of infection was greater for *P. falciparum* than *P. vivax* in both Megiar (Fisher's exact test: odds ratio (OR) = 1.69; 95% confidence interval (CI): 1.16–2.46; $P = 0.005$) and Mirap (OR = 1.59; 95% CI: 1.15–2.22; $P = 0.005$). The prevalence of both malaria species in each of four age groups (0–5, 6–10, 11–15 and >15 years old) is shown in Figure. 3.2. The odds of infection in the three older age groups were statistically the same or higher but not lower than the 0–5 years age group (reference group) for both malaria species in all four villages (Figure 3.3). In Bulal, Megiar and Mirap the odds of *P. falciparum* infection increased with age, but this trend was not observed for *P. falciparum* in Wasab or *P. vivax* in all villages (Figure 3.3). There was no statistical difference in the odds of infection among the two human sexes for any malaria species, even by age groups.

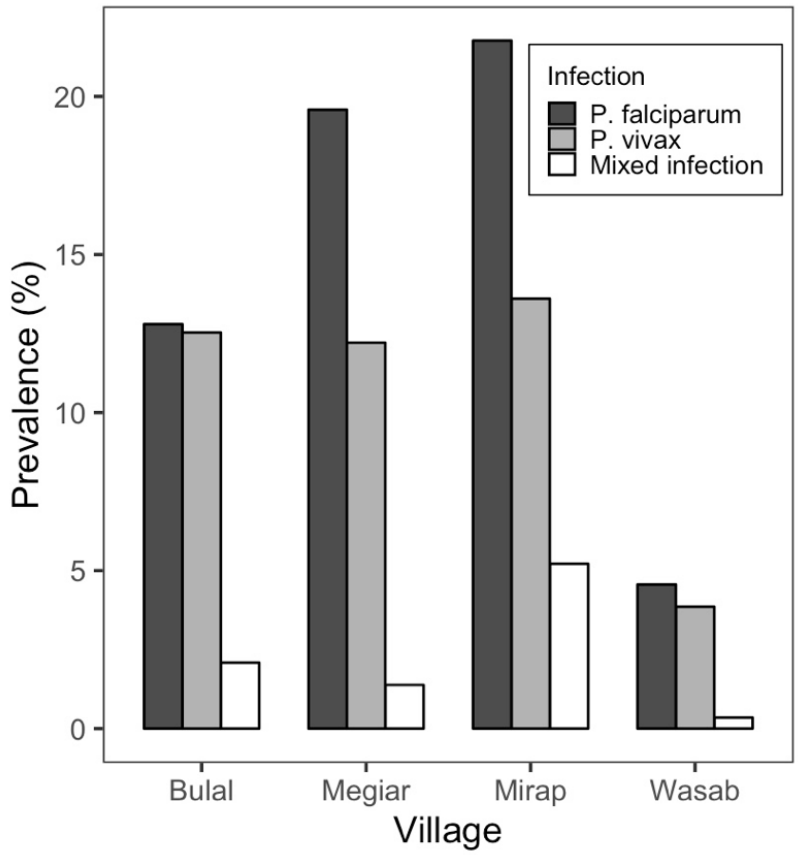

Figure 3.1. Prevalence of infection (all ages and sexes) of *P. falciparum*, *P. vivax* and mixed species in four villages. The number of consented human individuals tested for malaria infections was 383 in Bulal, 434 in Megiar, 441 in Mirap and 285 in Wasab.

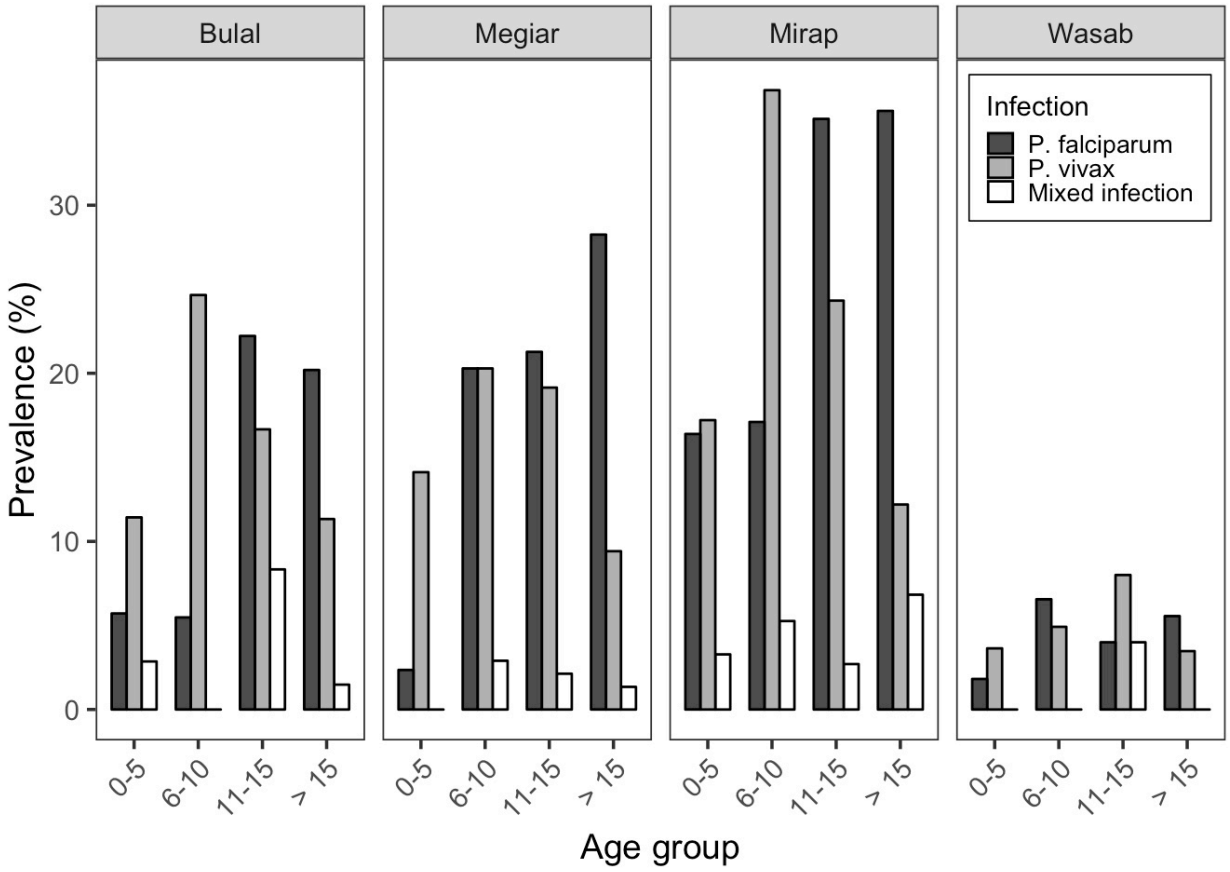

Figure 3.2. Prevalence of malaria infection in four human age groups in four villages. The number of human individuals tested for malaria infections in each age group in each village was (in order of lowest to the highest age groups) as follows: Bulal (70, 73, 36, 203), Megiar (85, 69, 47, 223), Mirap (122, 76, 37, 205), Wasab (55, 61, 25, 144). One individual from Bulal, 10 from Megiar and one from Mirap who were also tested were excluded from the data due to unknown age.

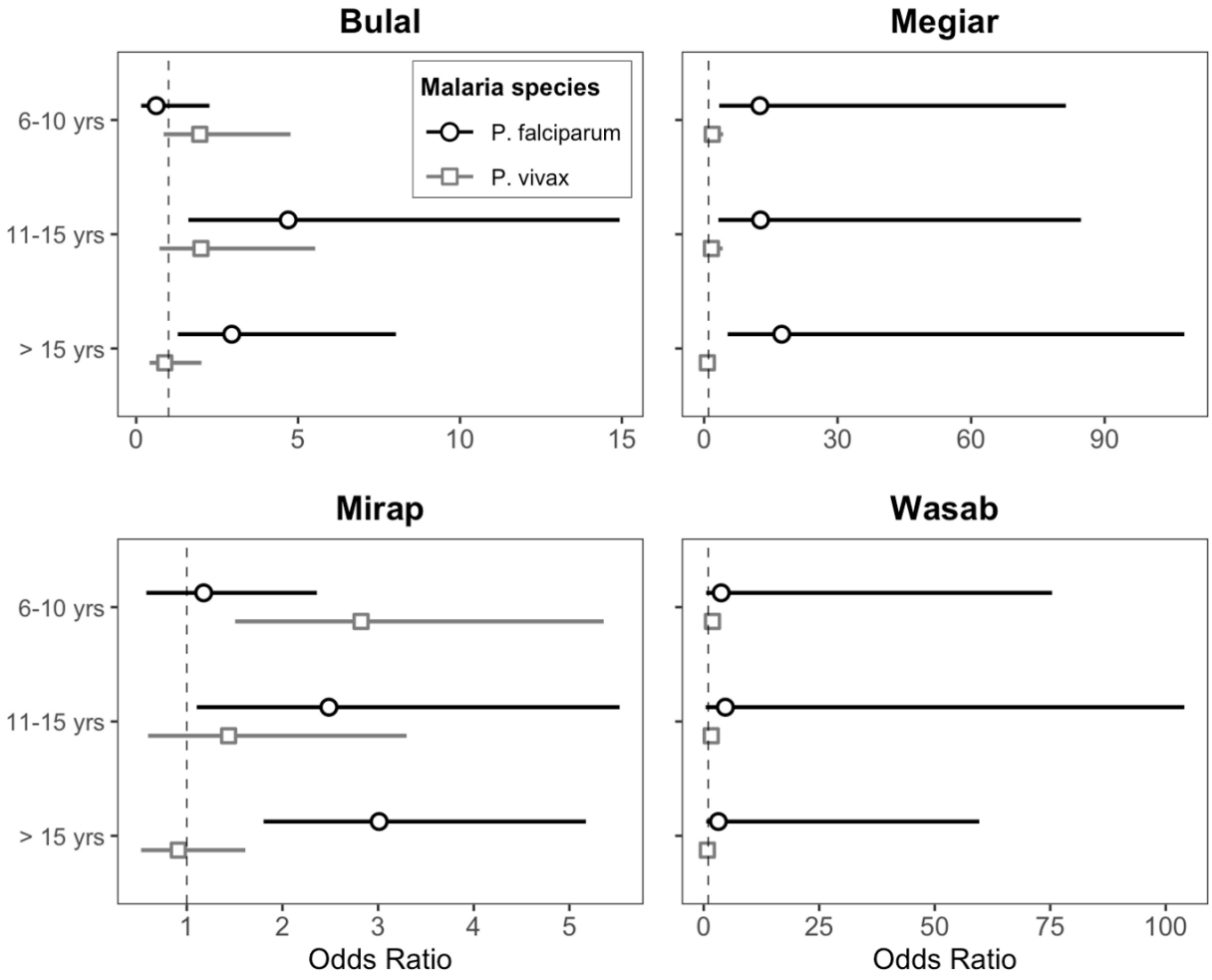

Figure 3.3. Plot of logistic regression odds ratio (open circles or boxes) with 95% confidence interval bars for *P. falciparum* (black) and *P. vivax* (grey) infection in four human age groups in four villages. The covariates plotted on the y axis are three age groups, each was compared with 0–5 years group (not plotted) as the reference group, represented by the broken vertical line passing through odds ratio 1.0.

3.4.3. *Anopheles* vectors

A total of 3,013 female *Anopheles* of four species were collected. Although more than one species was encountered in each village, their relative abundance varied (Figure 3.4). In Bulal, where three species were encountered, *An. koliensis* comprised most (81%) of the sample. In Megiar, where two species were encountered, *An. farauti* (*s.s.*) dominated (96%) the sample. Of

three species encountered in Mirap, *An. farauti* (*s.s.*) (32%) and *An. koliensis* (65%) were the abundant ones, and in Wasab where four species were represented in the sample, *An. koliensis* (77%) and *An. punctulatus* (*s.s.*) (19%) were the two most abundant ones. *An. farauti* (*s.s.*) was more abundant in the coastal villages than in the inland villages. In contrast, *An. punctulatus* (*s.s.*) was more abundant in the two inland than coastal villages. *An. koliensis* was the most common species in all villages except Megiar where *An. farauti* (*s.s.*) was more common.

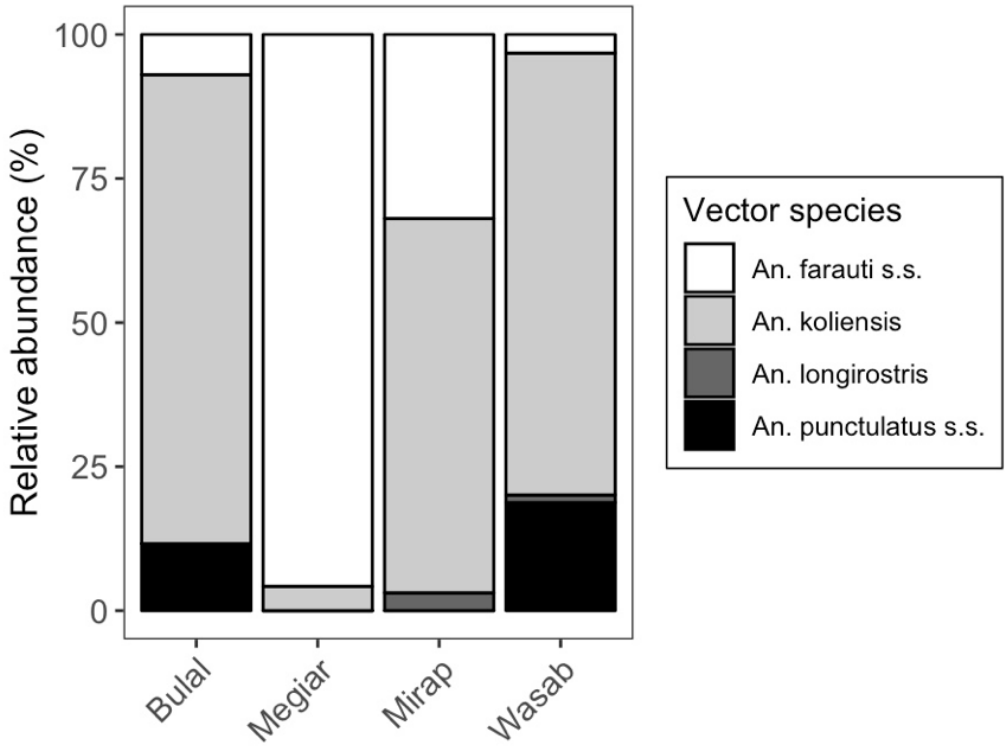

Figure 3.4. Relative abundance of four *Anopheles* species in four villages. The mosquito sample size was 86 in Bulal, 473 in Megiar, 1493 in Mirap and 961 in Wasab.

3.4.4. Sporozoite rates

The *P. falciparum* and *P. vivax SR* for the 12 vector populations investigated in this study are presented in Table 3.1. The *SR* for *Anopheles* spp. in each village was also calculated and presented in Table 3.2. Both *P. falciparum* infected and *P. vivax* infected mosquitoes were detected in all villages, except in Bulal where no *P. vivax* infected mosquito was found (Table 3.2).

Table 3.1. *SR* of 12 vector populations (values outside parentheses are number of sporozoite-positive mosquitoes and inside are the *SR*).

Village	Vector species	Number tested*	*P. falciparum*	*P. vivax*
Bulal	*An. farauti* (*s.s.*)	6	0 (0)	0 (0)
	An. koliensis	70	2 (0.0286)	0 (0)
	An. punctulatus (*s.s.*)	10	0 (0)	0 (0)
Megiar	*An. farauti* (*s.s.*)	453	2 (0.0044)	2 (0.0044)
	An. koliensis	20	0 (0)	0 (0)
Mirap	*An. farauti* (*s.s.*)	477	2 (0.0042)	2 (0.0042)
	An. koliensis	970	19 (0.0196)	1 (0.001)
	An. longirostris	46	0 (0)	0 (0)
Wasab	*An. farauti* (*s.s.*)	30	0 (0)	0 (0)
	An. koliensis	737	2 (0.0027)	0 (0)
	An. longirostris	13	0 (0)	1 (0.0769)
	An. punctulatus (*s.s.*)	180	0 (0)	0 (0)

* Number of mosquitoes tested for sporozoite infection

Table 3.2. *SR* of *Anopheles* spp. in four villages (values outside parentheses are number of sporozoite-positive mosquitoes and inside are the *SR*).

Village	Number tested*	*P. falciparum*	*P. vivax*
Bulal	86	2 (0.0233)	0 (0)
Megiar	473	2 (0.0042)	2 (0.0042)
Mirap	1493	21 (0.0141)	3 (0.002)
Wasab	960	2 (0.0021)	1 (0.001)

* Number of mosquitoes tested for sporozoite infection

3.4.5. Human biting rates

The mean *HBR* ± standard error for the 12 vector populations are shown in Figure 3.5. Within villages, the *HBR* varied among the vector species. In Bulal, *An. koliensis* had the highest biting rate (2.2 ± 0.5 per person-night) than the other two species which had < 0.3 bites per person-night. In Megiar, more of the bites were from *An. farauti* (*s.s.*) (9.4 ± 2.5) than *An. koliensis* (0.4 ± 0.2). In Mirap, where three species were present, *An. koliensis* had the highest *HBR* (20.2 ± 1.8) followed by *An. farauti* (*s.s.*) with two-fold less (9.9 ± 1.1) than the former species, followed by *An. longirostris* with < one bite per person-night. Most of the bites in Wasab were delivered by *An. koliensis* (6.6 ± 1.5) compared to the other three species which had < 2 bites per person-night. *HBR* was also calculated for *Anopheles* spp. in each village and the results (Figure 3.6) showed great variation among the villages. Mirap had the highest *HBR* (31 ± 2.7); Megiar (9.9 ± 2.7) and Wasab (8.6 ± 1.7) had similar *HBR* and were three-fold less than Mirap, followed by Bulal with 2.7 ± 0.6 bites per person-night.

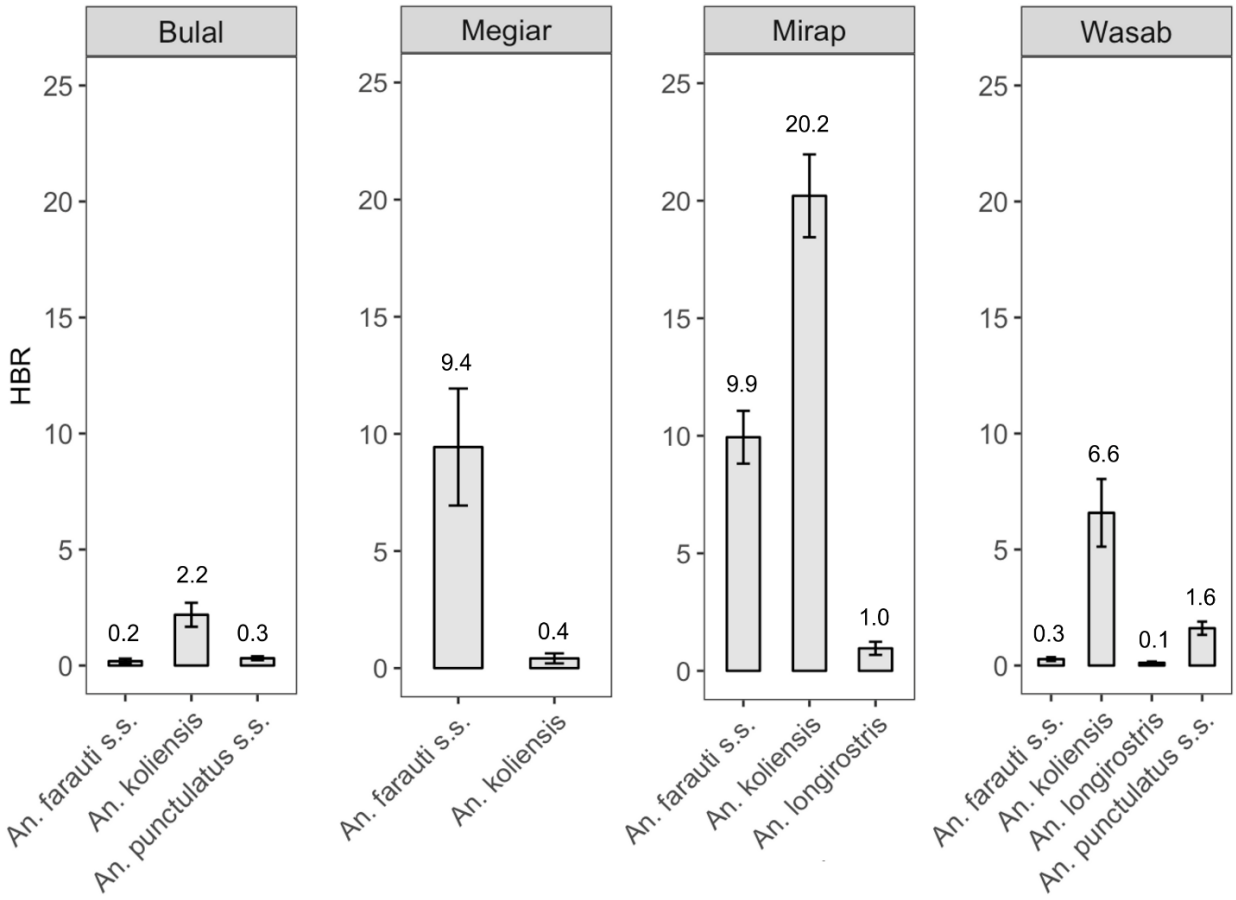

Figure 3.5. Bar plots of mean *HBR* (y axis) with standard error bar for 12 vector populations (x axis) across four villages. Values above the bars are the estimated means.

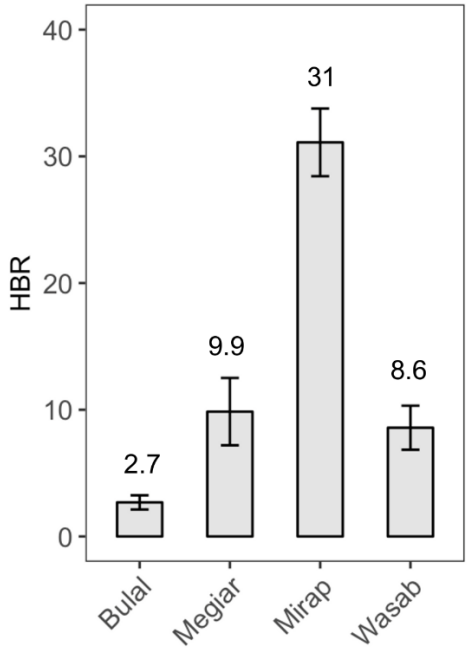

Figure 3.6. Bar plots of mean *HBR* with standard error bar (y axis) for *Anopheles* spp. in four villages (x axis). Values above the bars are the estimated means.

3.4.6. Annual infectious biting rate

Of the 12 vector populations, six had *IBR* > zero; the other six had zero *IBR* as a result of zero *SR* (Figure 3.7a). The mean *IBR* (± standard error) for *An. koliensis* in Bulal was 22.8 ± 5.4 for *P. falciparum* and zero for *P. vivax*. For *An. farauti* (*s.s.*) in Megiar, this quantity was 15.2 ± 4.0 for both malaria species. For the two populations in Mirap, *An. farauti* (*s.s.*) had 15.2 ± 1.7 for both malaria species and *An. koliensis* had 144.6 ± 12.6 for *P. falciparum* and 7.4 ± 0.6 for *P. vivax*. For the two populations in Wasab, *An. koliensis* had 6.5 ± 1.4 for *P. falciparum* and zero for *P. vivax*, and *An. longirostris* had 3.3 ± 1.6 for *P. vivax* and zero for *P. falciparum*. The mean *IBR* for *Anopheles* spp. ranged from zero to 22.9 for both malaria species across the four villages except for *P. falciparum* in Mirap where it was seven-fold higher (159.7) (Figure 3.7b).

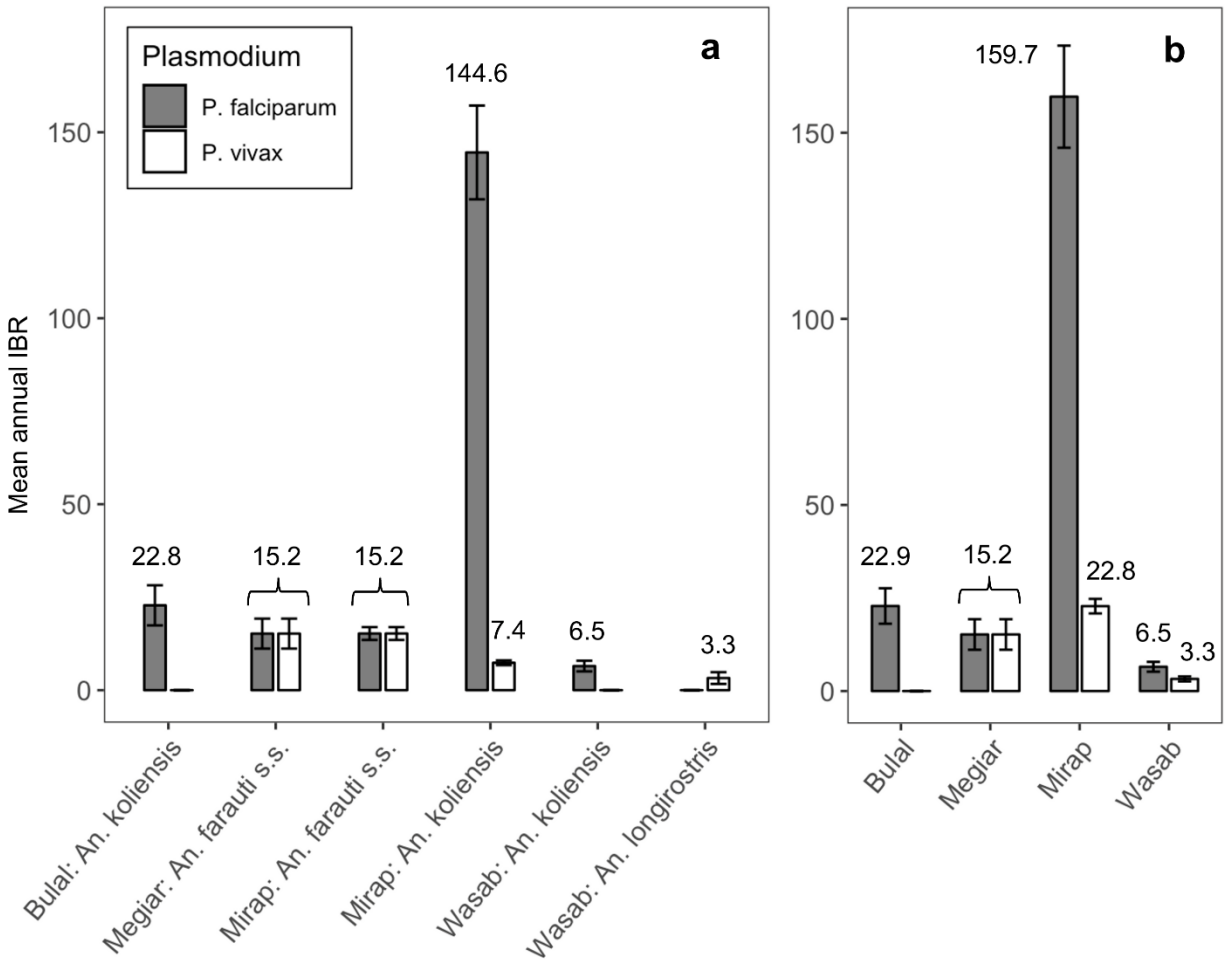

Figure 3.7. Bar plots of mean *IBR* with standard error bar (y axis) for *P. falciparum* and *P. vivax*. (a) Mean *IBR* for six vector populations (x axis). (b) Mean *IBR* for *Anopheles* spp. in four villages (x axis). Values above the bars are the means.

3.5. Discussion

The stability of malaria across the continuum from holoendemic to hypoendemic is described in detail in chapter 1 (section 1.3.3). The research presented in this chapter examines the dynamics of malaria stability, or lack thereof, after a long period of holoendemicity followed by approximately 15 years of bed net-based malaria intervention campaign. Prior to the bed net campaign, human communities living in the lowland areas of PNG exhibited the age-dependent

infection prevalence pattern which is typical for holoendemic settings in which infection rate is highest in children < 10 years old and declines monotonically with age (Burkot, et al. 1990; Cattani, et al. 1986a; Cattani, et al. 1986b; Genton, et al. 1995a; Mehlotra, et al. 2002; Mehlotra, et al. 2000). Data from nationwide longitudinal surveys conducted during and after the NDOH-led bed net campaign showed statistically high malaria infection (10–23%) in the younger age groups (< 10 years old) compared to the adults earlier in the campaign period (2008–2009), but continued to reduce through the course of the campaign nationally (Hetzel, et al. 2017). By 2014, the national infection prevalence had fallen below 1% and there was no statistical difference in the level of infection among age groups (Hetzel, et al. 2017). In villages on the north coast of Madang, the same region where the four villages in this study are located, the prevalence of infection was reduced substantially and became more homogeneous across age groups, particularly *P. falciparum* (Koepfli, et al. 2017).

This research uncovered continued high use of insecticidal bed nets, with current household bed net ownership (≥ 98% of households surveyed owned a net) and individual usage (≥ 81% of people slept under a net) surpassing the 80% operational target set by the campaign program authorities (Hetzel, et al. 2012). Yet, despite high bed net coverage and usage, very high infection prevalence was observed in the village populations. The overall (all villages and ages combined) infection prevalence for *P. falciparum* was 18%, which was two times greater than in 2014. *P. vivax* infection prevalence was 14%, which was 1.4 times lower than in 2014 which was at 19.7%, but 1.1 times greater than in 2010 which was 12.7% and the lowest post-bed net prevalence on record in the north coast of Madang (Koepfli, et al. 2017).

HBR and *IBR* were surveyed in three of the four study villages (Megiar, Mirap and Wasab) during the bed net campaign. In Mirap, the *HBR* for *Anopheles* spp. dropped from a

mean of 60 bites per person-night earlier in the campaign (2008–2009) to 18 bites per person-night later in the campaign (2010–2011). The *IBR* for *Plasmodium* spp. also dropped from a mean of 320 to 10 infective bites per person-year (Reimer, et al. 2016). Results of the current study showed that in this village the *HBR* has rebounded to a mean of 31 bites per person-night (two-fold increase) and the *IBR* to 183 infective bites per person-year (18-fold increase) in 2017 (Figure 3.6 and 3.7b) compared to the 2010–2011 results. In Megiar, the mean *HBR* dropped from 10 to five *Anopheles* spp. bites per person-night and *IBR* dropped from 150 to 10 infective *Anopheles* spp. bites per person-year (Reimer, et al. 2016). In the present study, the *HBR* has rebounded to a mean of 9.9 *Anopheles* spp. bites per person-night (two-fold increase) and *IBR* to 30.4 infective *Anopheles* spp. bites per person-year (three-fold increase) in 2017 (Figure 3.6 and 3.7b) compared to the 2010–2011 results. In Wasab, the mean *HBR* dropped from 11 to three bites per person-night and *IBR* dropped from 175 to 15 infective bites per person-year (Reimer, et al. 2016). Current results show that while the mean *HBR* rebounded by three-fold in 2016, the *IBR* remained low (Figure 3.6 and 3.7b). Although an entomological survey was not conducted in Bulal during the bed net campaign to compare with the result of the current study, it is likely that it also underwent the same transition as the other three villages.

The comparison of *HBR* and *IBR* results of the current study to that conducted during the bed net campaign focused only on the results for *Anopheles* spp. in general. This was because in the previous study, both indices were not calculated separately for each vector species. In the current study, the contribution of the different vector species to malaria transmission was assessed (Figure 3.5 and 3.7a). In the two inland villages (Bulal and Wasab), *An. koliensis* was the primary vector responsible for most of the transmission, whereas *An. punctulatus* (*s.s.*) played a supplementary role. In the two coastal villages, *An. farauti* (*s.s.*) was present and had

the same nightly biting rate in both villages however, while in Mirap *An. koliensis* was more abundant and contributed most of the transmission. In the earlier study (Reimer, et al. 2016), it was *An. punctulatus* (*s.s.*) in inland villages and *An. farauti* (*s.s.*) in coastal villages that were the primary vectors. *An. koliensis* was present but played only a supplementary role in malaria transmission. Furthermore, in Madang and many parts of PNG, this species was the most affected by the bed nets; its abundance relative to other species was reduced dramatically during the campaign (Hetzel, et al. 2016; Reimer, et al. 2016). Its rise to dominance in present times is indicative of recent changes in the ecology of *Anopheles* vectors which are causing them to be resilient to the bed nets and causing malaria resurgence in these villages.

There are two possible explanations for the high infection prevalence and *IBR* in the current study compared to the previous estimates. First, although malaria transmission was interrupted and reduced to low intensity by the bed nets in many parts of PNG including Madang (Hetzel, et al. 2017; Hetzel, et al. 2016; Reimer, et al. 2016), it may not have been interrupted in the four study villages. In contrast, it is possible that malaria transmission was reduced in these villages but has rebounded in recent years. The second case is more likely than the former based on the following: If transmission was never interrupted in these villages, the age-related immunity profile should be the same as the pre-campaign period (Hetzel, et al. 2017; Koepfli, et al. 2017) and the infection prevalence data in Figure 3.2 would show disproportionately higher infection in the younger age groups than the older ones. Instead, the data showed statistically similar prevalence across all age groups for *P. vivax* in all villages and higher prevalence in the older than younger age groups for *P. falciparum* in three of the four villages (Figure 3.2 and 3.3). This observation indicates that acquired immunity in all individuals waned as a result of long-

term interrupted transmission by the bed nets causing all individuals to become equally susceptible to infection.

There were important caveats that can potentially affect the *HBR* and *IBR* estimates in this study. Unlike the cross-section malaria infection survey in human communitites, mosquito data are prone to environmental perturbations. Events such as heavy rain, strong wind, bushfire, or changes in the availability of larval habitat types occurring at any point in time can affect the number and types (i.e., species) of mosquitoes at that time. These effects are usually minimized through longitudinal mosquito sampling at several intervals over a few months or a year. In the current study, mosquitoes were collected for only four to six consecutive nights in each village. As a consequence, many of the *Anopheles* populations had very low numbers, which can greatly increase the uncertainty of the *HBR* estimates. Also, the number of sporozoite-infected mosquitoes is notoriously low (usually < 1% of mosquito sample tested for malaria infection), even in high prevalence settings; it requires testing hundreds of mosquitoes to find an infected one. A vector population with zero *SR* will also have zero *IBR* despite having relatively high *HBR*. Thus, given that both malaria species were highly prevalent in all villages in this study, vector populations that had zero *IBR* for *P. falciparum*, *P. vivax* or both cannot be considered as non-vectors. Rather, a sufficiently large sample size is needed to increase the chance of detecting sporozoite-positive mosquitoes.

3.6. Conclusion

Increased bed net coverage is expected to reduce malaria levels. Therefore, the rise of bed net ownership and usage to > 80% in the villages investigated in this study should have resulted in further reduction of the infection prevalence, *HBR* and *IBR* relative to the levels observed in

2011. Instead, the results presented here showed clear evidence of malaria persistence or rebound despite the high bed net usage in all villages. As bed nets target the *Anopheles* vectors, the high malaria prevalence, *HBR* and *IBR* observed in this current study compared to those reported in recent studies indicate underlying ecological factors that rendered the vectors resilient to the bed nets. Some potential ecological factors that may underlie vector resilience include resistance to the insecticides in the bed net fabric, diversity and composition of vector species, tendency to seek and bite hosts outdoors more than indoors, tendency to bite earlier in the evening than later in the night, heterogeneity of human exposure to vector bites and opportunistic host selection behavior. Insecticide resistance has been investigated in *Anopheles* populations in PNG and no evidence of it has been found thus far (Katusele, et al. 2014; Keven, et al. 2010; Koimbu, et al. 2018); the other factors were investigated and are reported in the other chapters of this dissertation.

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CHAPTER 4: SPATIAL AND TEMPORAL HETEROGENEITY OF HUMAN EXPOSURE TO *ANOPHELES* BITES IN THE NORTH COAST VILLAGES OF MADANG PROVINCE, PAPUA NEW GUINEA

4.1. Abstract

Considerable gains in malaria reduction achieved by the ongoing nationwide insecticidal bed net campaign in PNG is threatened by recent resurgence of the disease in some parts of the country. This study examined the hypothesis that flexibility in mosquito host-seeking behavior enables them to access human hosts while simultaneously circumventing exposure to the insecticide in bed nets, mediated through patterns of vector-human contact in space and time. Host-seeking female *Anopheles* were sampled by the human landing catch method inside and outside several houses from dusk to dawn in four villages in Madang province. Blood samples were obtained from village residents and were tested for malaria infection by PCR. The results show that host-seeking behavior of *Anopheles* spp. (of four species total) was not homogeneous temporally or spatially. In all villages, 29–49.7% of the *Anopheles* catches occurred between 18:00 hr–22:00 hr) when 75–90% of people were awake and outside the bed nets, and 66.6–83% of catches occurred between 18:00 hr–2:00 hr before almost 100% of the residents retired to bed. Spatially, the frequency of outdoor catches was slightly higher than indoor catches. The number of human-biting *Anopheles* spp. among households was spatially heterogeneous, resulting in malaria basic reproduction rate values greater than the values under homogeneous condition by multiplicative factors ≥ 1.6. Despite clustering of mosquito catches, human malaria infection cases by location of home were not clustered. These results indicate that in Madang and most likely in other parts of PNG, the majority of the mosquito vectors are able to access humans and transmit malaria

without contacting bed nets, thereby rendering bed nets less effective as a malaria control method. The high basic reproduction rate causes malaria to persist in these villages with the potential for rebound.

4.2. Introduction

Insecticidal bed nets which target mosquito vectors are the mainstay of malaria control worldwide, particularly in endemic countries like PNG. The way bed nets work against malaria is that by sleeping under the nets, humans are barred from mosquito bites, thereby preventing or reducing the rate of transmission of the disease. By coming in contact with the nets, the mosquitoes are exposed to lethal dose of the insecticide, which results in (i) reduced population size of the vectors to levels insufficient to support transmission and (ii) reduction in the average age (i.e., longevity) of the vector populations below the duration required for the parasite to develop into the infectious stage (sporozoite) in the mosquitoes, thereby interrupting transmission of the disease (Darriet, et al. 1984; Lines, et al. 1987; Snow and Jawara 1987; Snow, et al. 1988).

Despite these seemingly effective strategies offered by the bed nets, there are some ecological aspects of vector populations that have the potential to render the bed net method ineffective in preventing malaria transmission. One such factor is physiological resistance to the insecticide. This happens when the mosquitoes acquire genetic mutations in the insecticide's target site which prevents the chemical compound from exerting its toxic effect, or when the metabolic detoxification of the insecticide is enhanced by duplication of genes that codes for detoxifying enzymes (Hemingway, et al. 2004; Hemingway and Ranson 2005). Although, physiological resistance has been reported for *Anopheles* populations in other parts of the world

(Quinones, et al. 2015), it has not been observed in the malaria vectors in PNG (Katusele, et al. 2014; Keven, et al. 2010; Koimbu, et al. 2018).

As bed nets are deployed inside houses, vector populations that exhibit endophagic (tend to bite humans inside houses) behavior are easy to control as they're likely to be exposed to the insecticides. Vector populations that exhibit exophagic (tend to bite humans outside houses) behavior are difficult to control as they are less likely to come in contact with the nets (Killeen 2014). Even if the vectors are endophagic, they must also seek human hosts late in the night and in the early morning hours when most people are asleep and protected by the bed nets in order for them to contact the nets; host-seeking in the evening when people are usually active and outside their bed nets reduces the vectors' chance of exposure to the insecticides (Killeen 2014).

Further, within a human community, human exposure to *Anopheles* bites can be spatially heterogeneous; that is, individuals in some locations of the village are more exposed to mosquito bites than those in other locations. This spatial heterogeneity can cause the basic reproduction rate (R_0) of malaria—a measure of disease persistence—to be greater than the values under random or homogeneous exposure. Thus, human biting rates under heterogeneous condition can challenge the outcome of bed net intervention by enabling the disease to persist, with potential for rebound (Dye and Hasibeder 1986; Hasibeder and Dye 1988; Woolhouse, et al. 1997).

In PNG, malaria prevalence and transmission intensity was reduced considerably to manageable levels nationally by ongoing nationwide bed net-based vector control program which commenced in 2004 (Hetzel, et al. 2015; Hetzel, et al. 2017; Hetzel, et al. 2016; Reimer, et al. 2016). However, research described in chapter 3 showed that in the north coast of Madang province, the infection prevalence and transmission intensity have both rebounded despite high bed net usage in the study villages. Prior research showed that insecticide resistance was nil in

the vector populations, eliminating that explanation for the resurgence (Katusele, et al. 2014; Keven, et al. 2010; Koimbu, et al. 2018). The aim of the research in this study was to investigate the temporal and spatial patterns of human exposure to *Anopheles* bites and how these may enable the vectors to evade bed nets and cause malaria transmission to rebound.

4.3. Methods

4.3.1. Study sites, mosquito sampling and active case malaria detection

This study was conducted in the same four villages (Bulal, Megiar, Mirap and Wasab) described in chapter 3. The geographic locations and ecogeographic description of these villages; the mosquito sampling design and procedure; mosquito species identification; and human malaria infection survey were as described in chapter 3, section 3.3.

4.3.2. Data analyses

Temporal and spatial heterogeneity of human exposure to *Anopheles* bites. The mean number of female *Anopheles* collected nightly by human landing catch at each of six bihourly periods of the night were calculated. Generalized linear model (GLM) with negative binomial distribution was used to compare the number of mosquitoes collected nightly at each bihourly periods. In the GLM equation $\ln(\mu) = \beta_0 + \beta_1(Time) + \ln(S)$, the expected mosquito number μ was modelled as a linear function of the categorical predictor variable *Time*, with six levels representing the six bihourly periods. Number of house-night *S* was included as offset term. The frequency distribution of mosquito numbers captured in different houses was tested for fit to the Poisson and negative binomial distribution models based on chi-square test of fit of expected and observed frequency distributions. Statistically supported fit to the Poisson model indicates

random distribution of mosquitoes among the households, whereas fit to the negative binomial model indicates heterogeneous or clustered distribution. For villages whose mosquito frequency fitted negative binomial, the effect of spatial heterogeneity on the R_0 of malaria was quantified using the formula $R_f = \sum_{i=1}^{m} \frac{{v_i}^2}{h_i}$, where v_i is the proportion of vectors in house i (where i = 1 to m and m is the number of households), and h_i is the proportion of humans in the house. This formula, derived by (Dye and Hasibeder 1986) and applied to empirical data by (Woolhouse, et al. 1997), estimates the multiplicative factor R_f. Whatever the true R_0 value may be under spatially random condition, when vectors exhibit spatial heterogeneity the R_0 increases by a multiplicative factor R_f, which is a value ≥ 1.0. Statistical difference in the number of *Anopheles* captured inside and outside houses in each village was compared using the Mann-Whitney U test, with house-night as the unit of replication.

Spatial distribution of malaria case households. The *K*-function, a spatial point pattern statistical analysis method, was used to test whether households with one or more human cases of malaria infections were clustered or randomly distributed in space. The theoretical basis of this statistical method is discussed elsewhere (Bivand, et al. 2013; Diggle and Chetwynd 1991; Diggle, et al. 2007). Generally, the *K*-function quantifies spatial intensity (number of spatial points located in a circle of radius *r*) of so-called point process data. In spatial epidemiology, disease cases are spatially clustered if the difference *D* of the *K* estimates of case and control households (i.e., $D = K_{\text{cases}} - K_{\text{controls}}$) is statistically greater than 0. In this study, the *D* statistic from *K* estimates of malaria infected (case) and uninfected (control) households was quantified to test for clustering.

Computation of the statistical tests. All statistical tests were performed in R software. The Mann-Whitney U test was performed using the function *wilcox.test* of the package *stats*. The

model fit test was computed using the function *fitdist* of the package *fitdistrplus*. The GLM analysis was performed using the functions *glm.nb* of the package *MASS*. Computation for the test of spatial clustering of malaria case households was accomplished via Monte Carlo statistical procedure using algorithms (written in R codes) described on page 205–206 in (Bivand, et al. 2013). Significance level for all tests was based on type I error rate of 5%.

4.4. Results

4.4.1. *Anopheles* vectors

A total of 3,013 female *Anopheles* of four different species, namely *An. farauti* (*s.s.*), *An. koliensis*, *An. longirostris* and *An. punctulatus* (*s.s.*) were collected over the sampling period. The distribution of these vector species and for *Anopheles* spp. (i.e., regardless of species) in each village is shown in Table 4.1. As expected, based on the findings in chapter 2, most (96%) of *An. farauti* (*s.s.*) were from the two coastal villages—Megiar and Mirap—and all (100%) of *An. punctulatus* (*s.s.*) were from the two inland villages—Bulal and Wasab (see map in Figure 2.1, chapter 2 for geographic location of the villages). Like *An. farauti* (*s.s.*), *An. koliensis* was present in all villages but unlike *An. farauti* (*s.s.*) it did not exhibit a bias for either coastal or inland sites. Like *An. punctulatus* (*s.s.*), *An. longirostris* was found in only two villages, but unlike *An. punctulatus* (*s.s.*), it did not exhibit a coastal-inland bias.

Table 4.1. Number of *Anopheles* mosquitoes according to species collected in four villages.

Vector species	Bulal	Megiar	Mirap	Wasab
An. farauti (*s.s.*)	6	453	477	31
An. koliensis	70	20	970	737
An. longirostris	0	0	46	13
An. punctulatus (*s.s.*)	10	0	0	180
Total *Anopheles* spp.	86	473	1,493	961

4.4.2. Temporal heterogeneity of exposure to *Anopheles* bites

The mean number of mosquitoes per person-night for a specific vector species or *Anopheles* spp. (indoor and outdoor mosquito data were combined for this analysis) was plotted for each of the six bihourly periods of the night for each village (Figure 4.1). In the corresponding GLM plot (Figure 4.2), the number of *Anopheles* spp. per person-night in each bihourly period of the night was statistically compared with the second period (20:00–22:00 hr) as the reference, because the mean mosquito number was the highest in this period in all villages, except Wasab (Figure 4.1). In Bulal, the number of *Anopheles* spp. collected in two morning periods 0:00–2:00hr and 4:00–6:00 was significantly lower than the reference period; the other periods were not different from the reference period (Figure 4.2). In Megiar, the number of *Anopheles* spp. in only the last two periods (2:00–4:00 hr and 4:00–6:00 hr) was significantly lower than the reference period (Figure 4.2). The number of *Anopheles* spp. in the first period in Mirap and the last period in Wasab were significantly lower than the reference period (Figure 4.2). Generally, the same trend for *Anopheles* spp. was observed for specific *Anopheles* species in all village (Figure 4.1 and 4.2).

The data were also evaluated in terms of the proportion of human-biting *Anopheles* spp. collected at each of three four-hourly nocturnal periods: evening (18:00–22:00 hr), late-night (22:00–2:00 hr) and morning (2:00–6:00 hr). In Bulal nearly half (46.5%) of the *Anopheles* sought humans in the evening; the other half sought humans almost equally between the late-night (28.0%) and morning (25.5%) periods (Figure 4.3). In Megiar, nearly half (49.7%) of the *Anopheles* sought humans in the evening whereas the other half sought humans unequally between the late-night (33.6%) and morning (16.7%) (Figure 4.3). Unlike Bulal and Megiar, the proportion of human-seeking *Anopheles* was generally the same in all three periods in Mirap and Wasab (Figure 4.3).

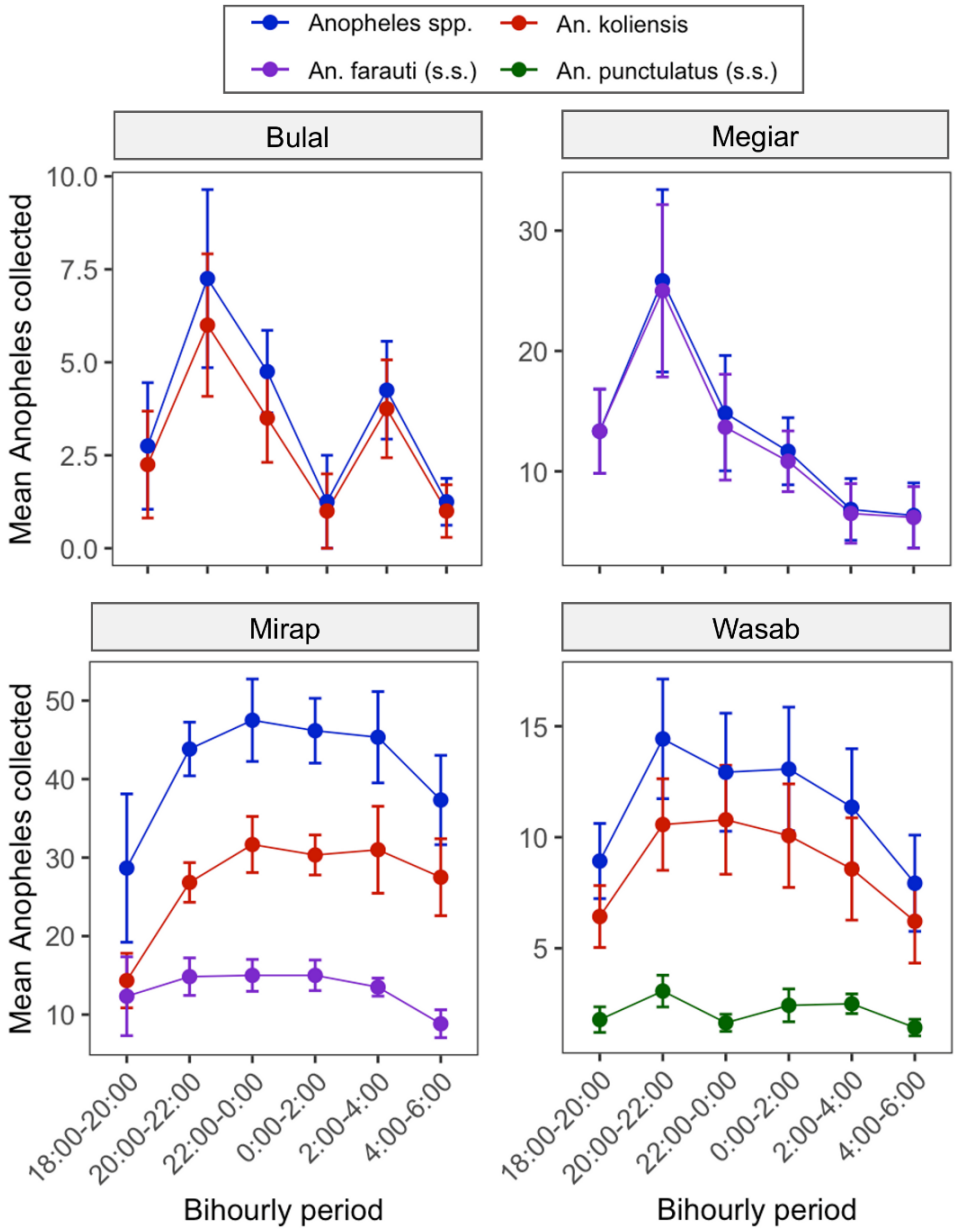

Figure 4.1. Mean number (colored dots) of mosquitoes collected per person-night at each of six bihourly periods of the night (expressed in 24 hr format) in four villages. Color purple red and green each represents a specific vector species whereas blue represents *Anopheles* spp. in general. Vertical bars are the standard error of the means.

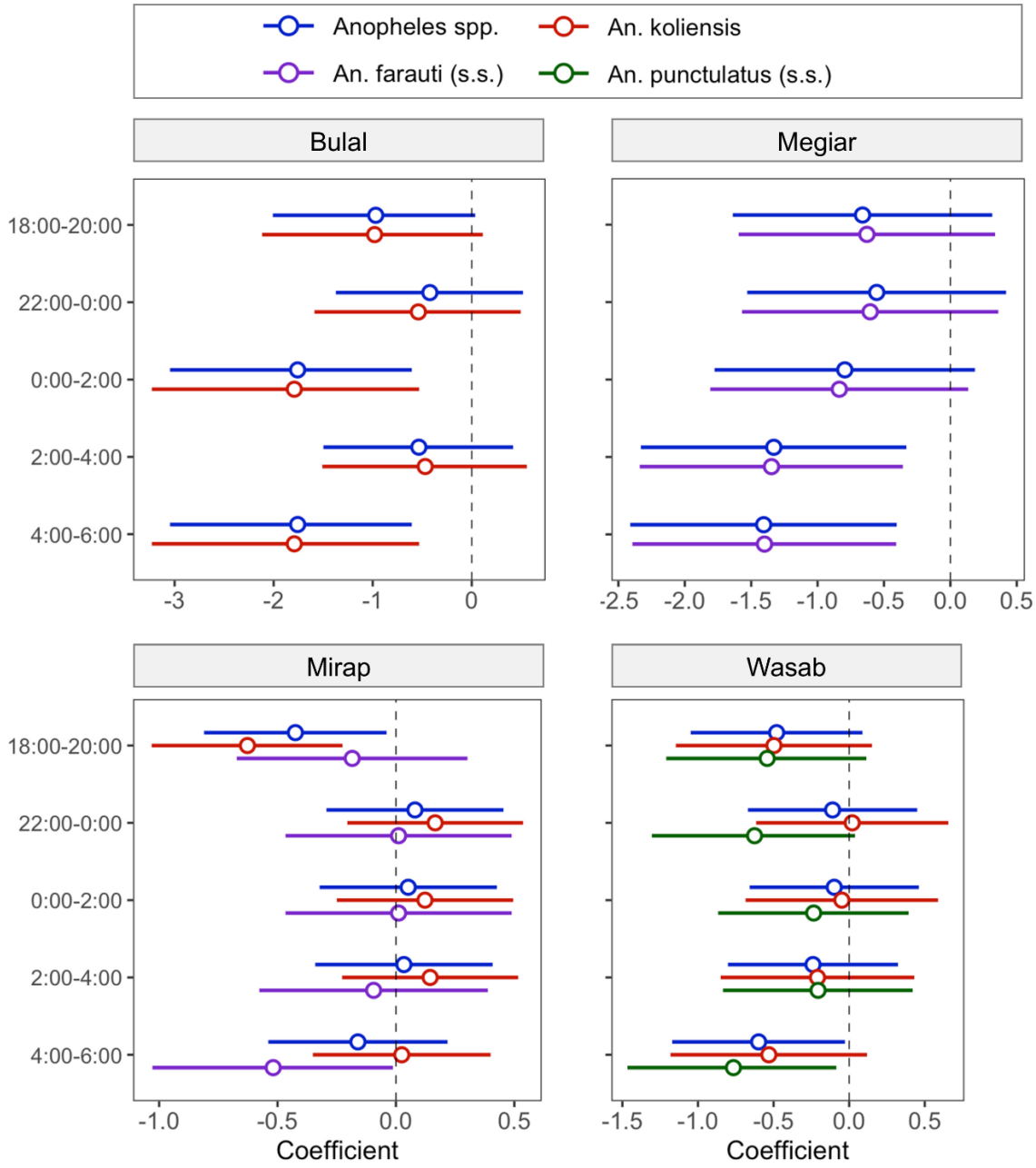

Figure 4.2. Plot of GLM coefficients (open circle) with 95% confidence interval bars of five variables (y-axis) of *Anopheles* in four villages. The variables include five of the six bihourly periods of the night (expressed in 24 hr format), each compared with the second period 20:00-22:00 hr (not plotted)—the reference variable (represented by the broken vertical line centered at zero). The colors purple, red, and green each represent a specific vector species whereas blue represents *Anopheles* spp. in general. The estimated coefficients (x-axis) are in logarithmic scale.

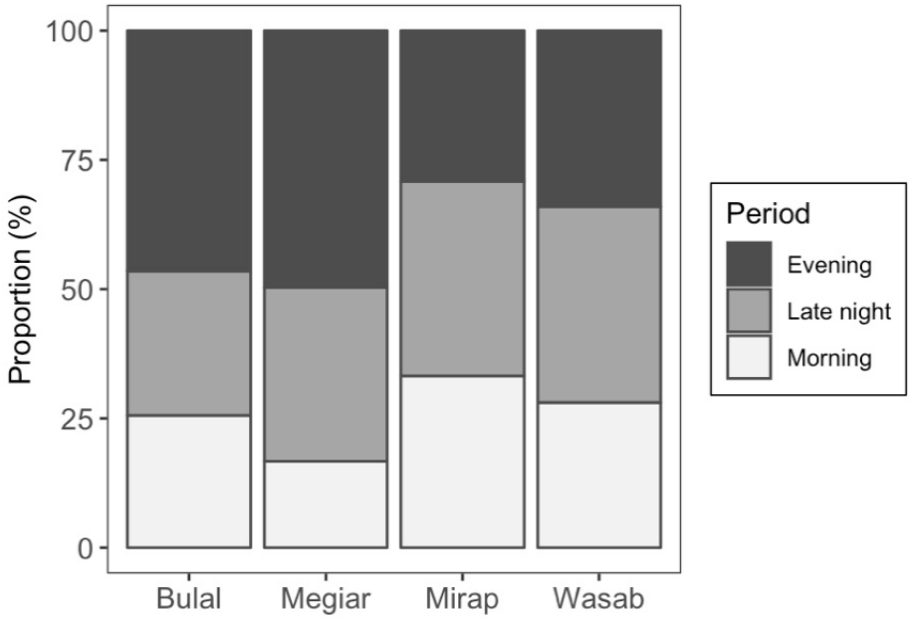

Figure 4.3. Proportion of human-seeking *Anopheles* spp. captured at three nocturnal periods in four villages. Mosquito sample size in each village are: Bulal (*n* = 86), Megiar (*n* = 473), Mirap (*n* = 1493) and Wasab (*n* = 961).

4.4.3. Spatial heterogeneity of exposure to *Anopheles* bites

The mean number of *Anopheles* spp. per house-night collected outdoor tended to be higher than indoor collections for all villages except Bulal, which was opposite, but numbers were low (Figure 4.4). However, these variations were not significantly different for all four pairs (Mann-Whitney U tests). The total number of *Anopheles* spp. collected in each household throughout the sampling period is displayed as bubble plots (Figure 4.5) for all villages except Bulal which was excluded due to low sample size (*n* = 86). For both indoor and outdoor collections in all three villages, the number of *Anopheles* varied among the households. The observed heterogeneity among the households was supported by fit of the frequency distribution of household *Anopheles* numbers, both indoor and outdoor collections, to the negative binomial and not Poisson model (Table 4.2). Although the *Anopheles* numbers varied among the households in Mirap, there was

no clear visual spatial trend associated with this heterogeneity as most of the houses with low *Anopheles* numbers were found in close spatial proximity to those with larger numbers (Figure 4.5). In contrast, the other two villages exhibited observable spatial trend. In Megiar, the number of mosquitoes was lower in the houses on the south-east end of the village but higher in houses on the north-west end (Figure 4.5). In Wasab outdoor collection households, the *Anopheles* numbers increased from north-east to south-west; indoor collection households did not show any clear spatial trend (Figure 4.5). Assessment of the effect of spatial heterogeneity on transmission potential show that the estimated values of R_0 multiplicative factor R_f were > 1.0 for both indoor and outdoor collections in all three villages (Table 4.3).

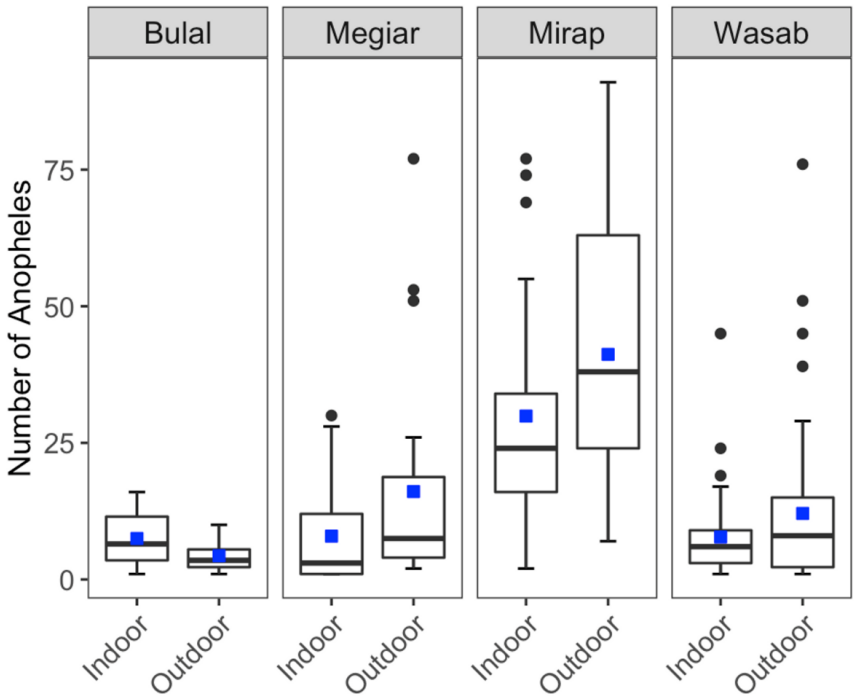

Figure 4.4. Boxplots showing the distribution of *Anopheles* spp. numbers captured nightly inside and outside of houses in four villages. Blue shaded squares are the means, black horizontal lines are the medians, and black dots are outliers.

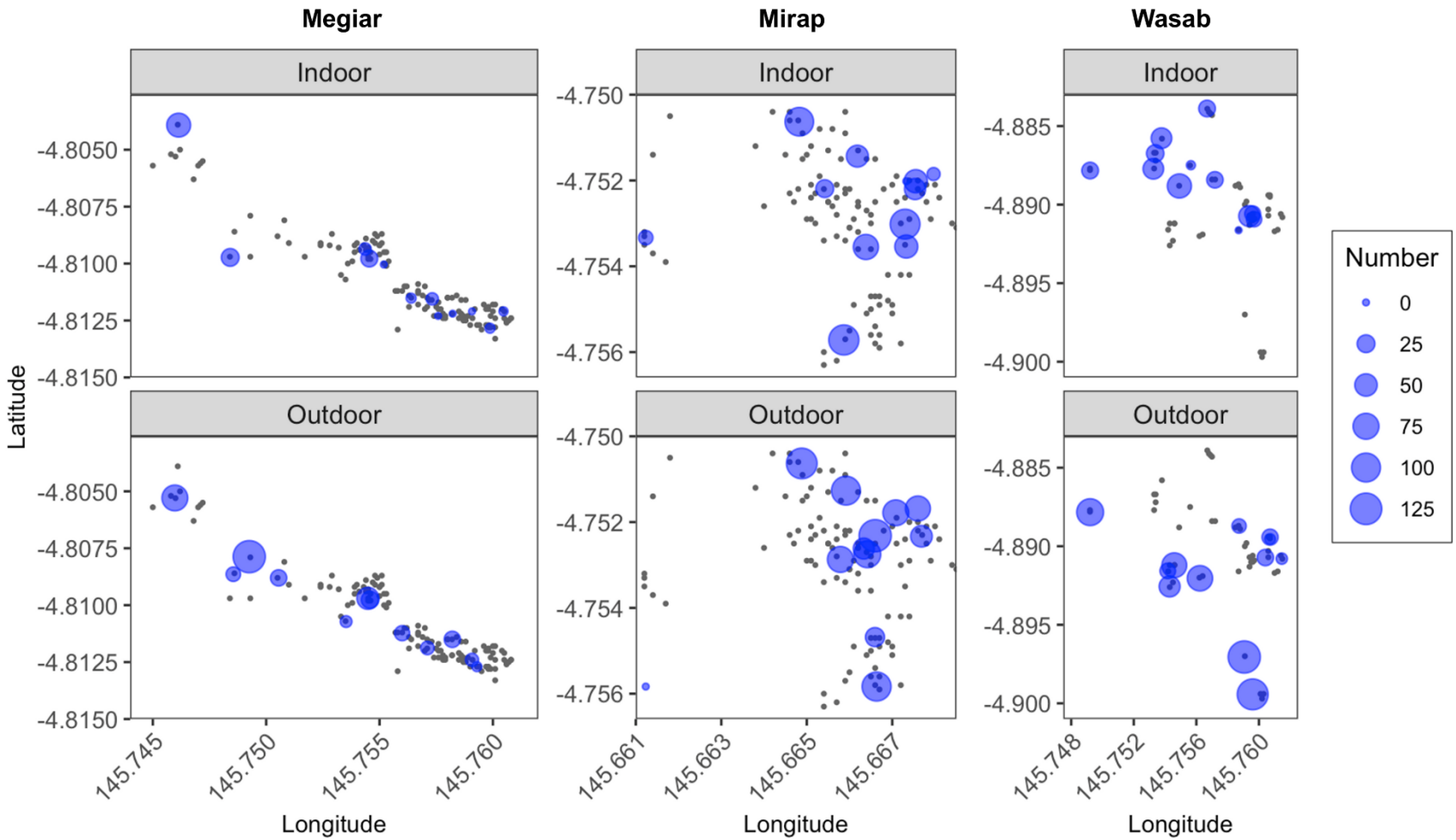

Figure 4.5. Map showing the location of houses where mosquitoes were sampled in Megiar, Mirap and Wasab villages. Blue shaded circles are sampled houses and grey points are unsampled houses. Data was plotted for both indoor (top row) and outdoor (bottom row) collections. Size of the blue circles correspond to the number of mosquitoes collected in the house

Table 4.2. Chi-square test of fit of negative binomial and Poisson models to frequency distribution of *Anopheles* numbers collected inside and outside houses in three villages.

Village	Location	*N*	Mean	Var	Model	χ^2	df	*P*
Megiar	Outdoor	12	29.5	1353	Neg. Binomial	3.36	1	0.07
					Poisson	2.5×10^5	2	< 0.0001
	Indoor	12	10.2	290	Neg. Binomial	1.51	1	0.22
					Poisson	1.9×10^4	2	< 0.0001
Mirap	Outdoor	12	72.1	1440	Neg. Binomial	1.62	1	0.20
					Poisson	1.6×10^4	2	< 0.0001
	Indoor	12	52.3	1458	Neg. Binomial	1.69	1	0.19
					Poisson	3.5×10^{10}	2	< 0.0001
Wasab	Outdoor	12	48.0	1802	Neg. Binomial	3.75	1	0.053
					Poisson	3.3×10^8	2	< 0.0001
	Indoor	12	25.4	279	Neg. Binomial	0.14	1	0.14
					Poisson	1.4×10^{20}	2	< 0.0001

Abbreviations: N, number of sampled households; Var, variance; df, degrees of freedom; *P*, p-value associated with the chi-square test.

Table 4.3. Estimated R_f for indoor and outdoor mosquito collections in three villages.

Village	House location	R_f
Megiar	Indoor	3.89
	Outdoor	4.59
Mirap	Indoor	1.96
	Outdoor	1.60
Wasab	Indoor	2.77
	Outdoor	1.68

4.4.4. Spatial distribution of malaria case households

Maps showing the geolocation of malaria infection case and non-case (negative) households in each of the four villages are presented in Figure 4.6–4.9. The results of the *K*-function test of spatial clustering of case households are also presented. None of the villages had *D* estimates significantly different from 0; the *D* estimates were within the 95% confidence interval limit (grey shaded area in panels b and d of Figure 4.6–4.9) at all distance *r*. These results indicate that the case households were randomly distributed in space like the non-case households rather than clustered.

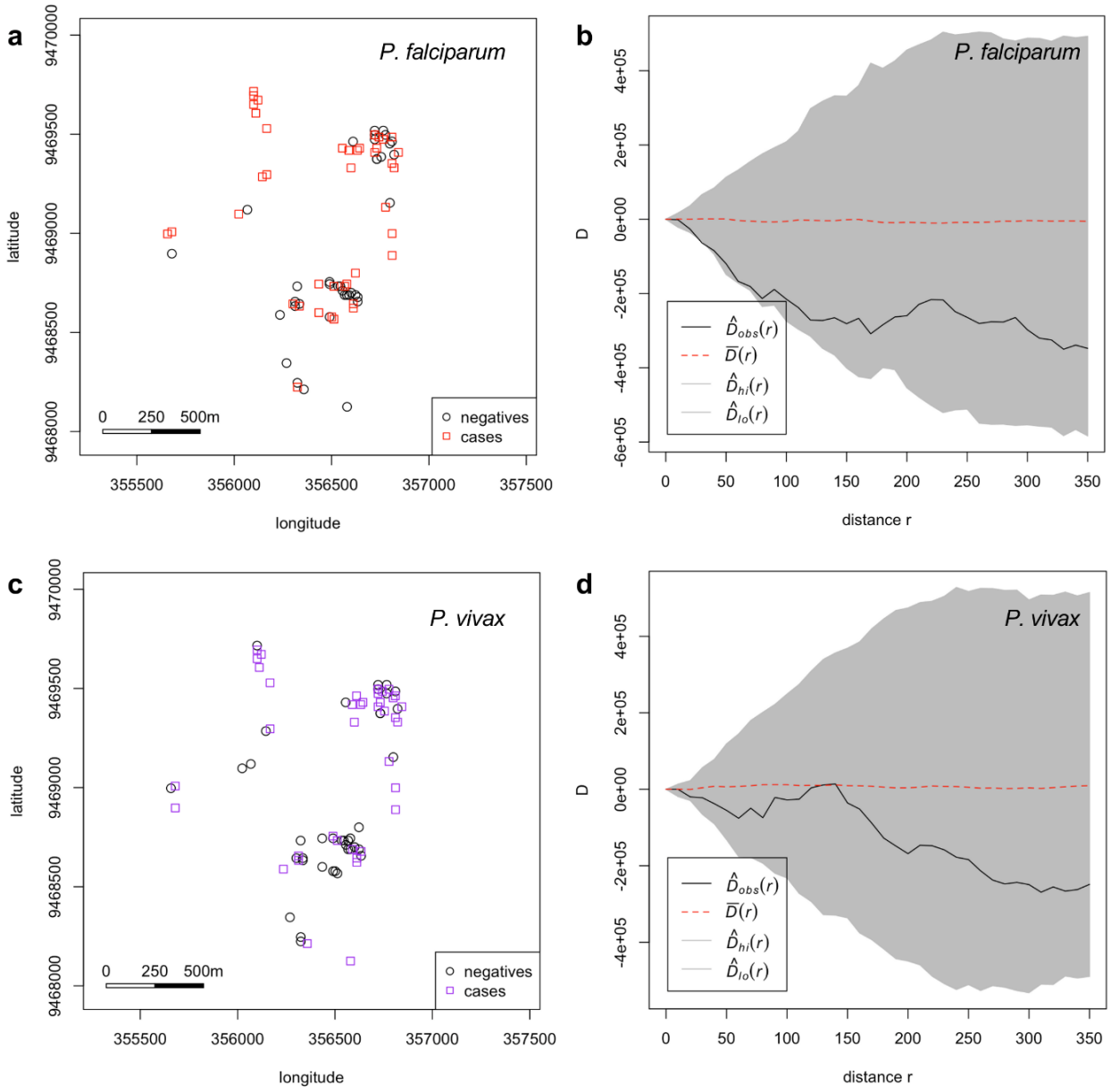

Figure 4.6. Geolocation of malaria case (colored squares) and non-case (black circles) households for *P. falciparum* (panel a) and *P. vivax* (panel c) in Bulal, along with their corresponding *D* statistic plot (panel b and d). In the *D* statistic plots, the broken red line is the null condition ($D = 0$ for all distance r in meters) which indicates random distribution of the case households, the black line indicates the observed *D* statistic (y-axis) at distance r in meters (x-axis), and the grey area is the 95% confidence interval of the observed *D* statistic.

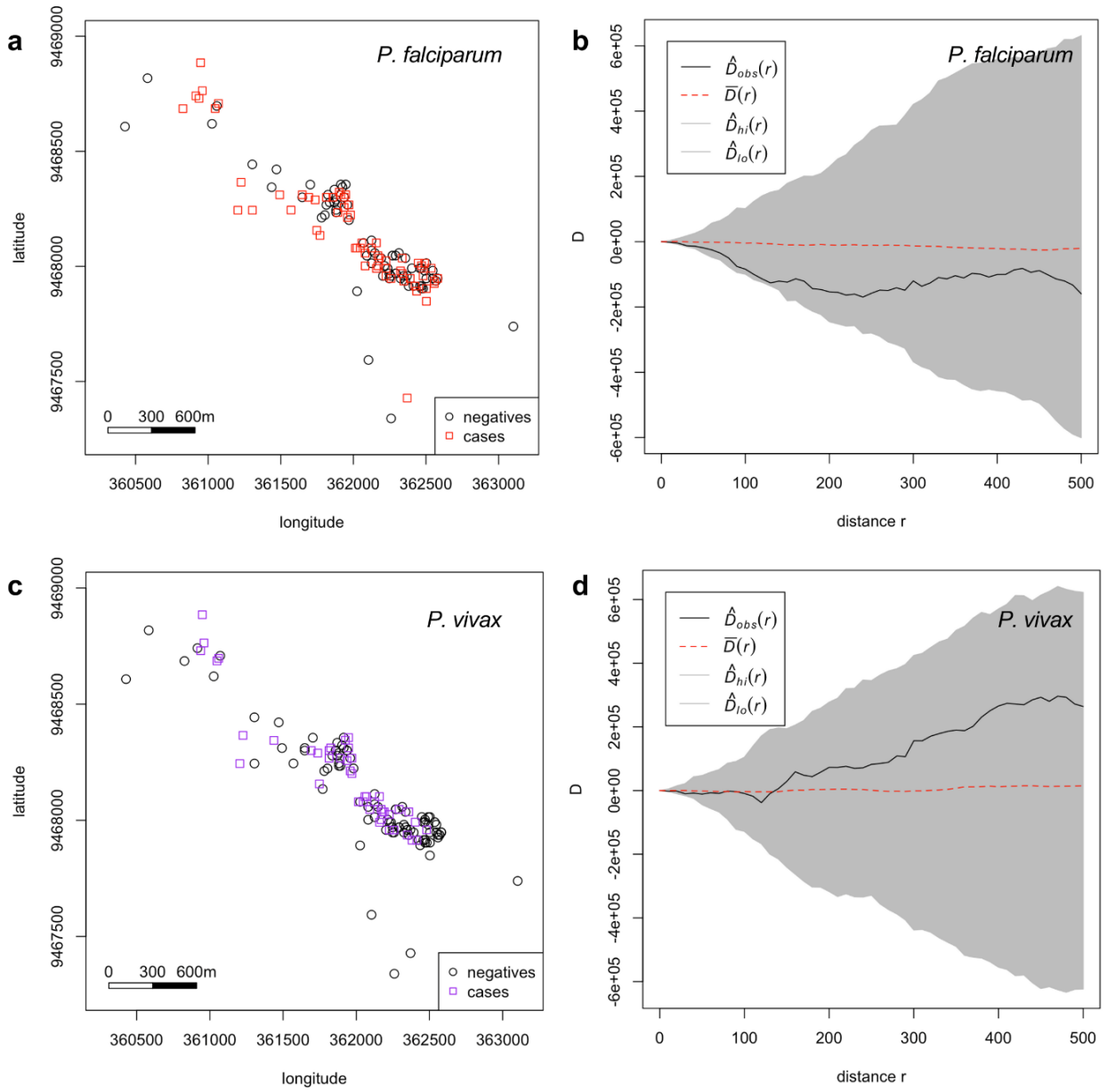

Figure 4.7. Geolocation of malaria case (colored squares) and non-case (black circles) households for *P. falciparum* (panel a) and *P. vivax* (panel c) in Megiar, along with their corresponding *D* statistic plot (panel b and d). In the *D* statistic plots, the broken red line is the null condition ($D = 0$ for all distance r in meters) which indicates random distribution of the case households, the black line indicates the observed *D* statistic (y-axis) at distance r in meters (x-axis), and the grey area is the 95% confidence interval of the observed *D* statistic.

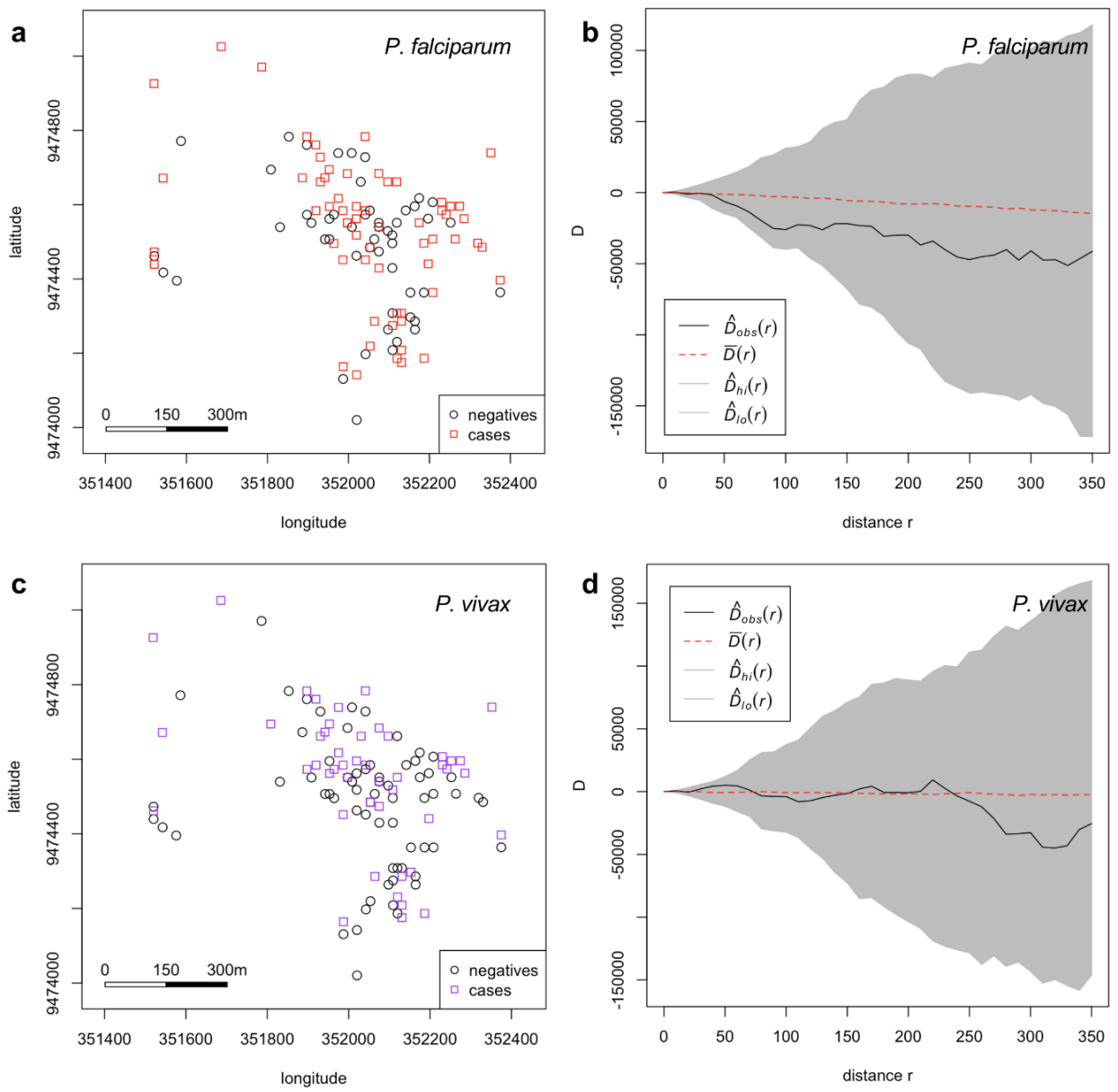

Figure 4.8. Geolocation of malaria case (colored squares) and non-case (black circles) households for *P. falciparum* (panel a) and *P. vivax* (panel c) in Mirap, along with their corresponding *D* statistic plot (panel b and d). In the *D* statistic plots, the broken red line is the null condition ($D = 0$ for all distance r in meters) which indicates random distribution of the case households, the black line indicates the observed *D* statistic (y-axis) at distance r in meters (x-axis), and the grey area is the 95% confidence interval of the observed *D* statistic.

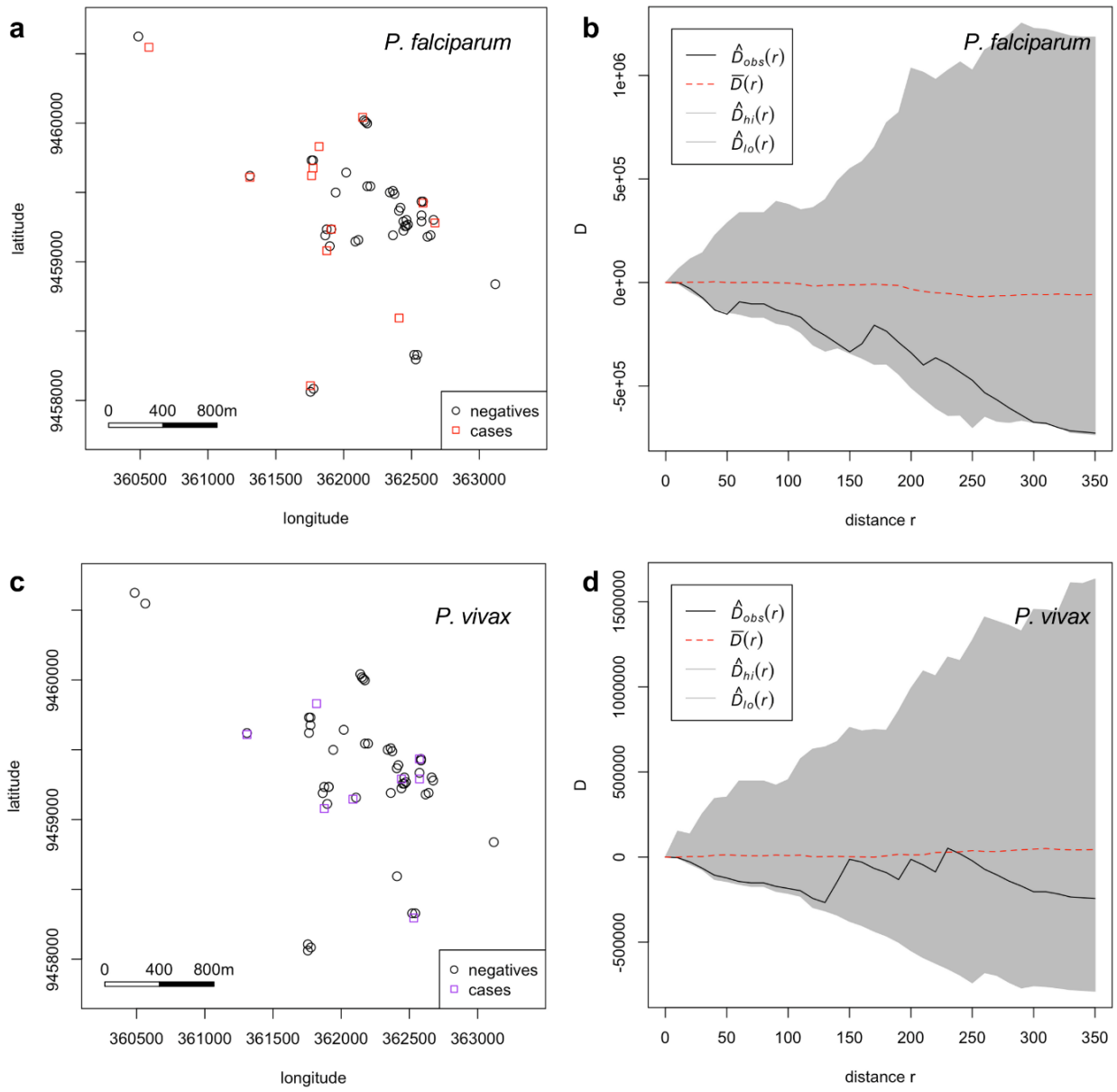

Figure 4.9. Geolocation of malaria case (colored squares) and non-case (black circles) households for *P. falciparum* (panel a) and *P. vivax* (panel c) in Wasab, along with their corresponding *D* statistic plot (panel b and d). In the *D* statistic plots, the broken red line is the null condition ($D = 0$ for all distance r in meters) which indicates random distribution of the case households, the black line indicates the observed *D* statistic (y-axis) at distance r in meters (x-axis), and the grey area is the 95% confidence interval of the observed *D* statistic.

4.5. Discussion

Two distinct nocturnal bihourly biting patterns exhibited by the *Anopheles* spp. were observed. The first was in Bulal and Megiar where the mosquitoes exhibited early biting activity which peaked in the second period (20:00–22:00 hr), followed by dramatic decline in the biting activity in the later periods, although in Bulal a secondary peak of activity occurred in the fifth period (2:00–4:00 hr). The second pattern was seen in Mirap and Wasab where the biting activity also peaked in the second period however, unlike the previous two villages, the biting rate did not decline but held steady for the subsequent periods of the night before dipping in the last period. As speculated from the barrier screen data (chapter 2), these nocturnal patterns were plausibly linked to the commuting time of the mosquitoes between their resting or larval habitats and the village, wherein mosquito populations whose habitats were in close proximity to the village arrived earlier in the night to seek hosts as exhibited by the *Anopheles* in Bulal and Megiar. Mosquito populations whose habitats were further from the village arrived late in the night and in the morning periods; the secondary peak in Bulal represent this condition. Mosquito populations whose habitats were randomly distributed in space, ranging from closest to furthest from the village, exhibited the nocturnal activity pattern displayed by the Mirap and Wasab populations.

The tendency of the mosquitoes to land on and attempt to bite humans during the period of the night when most people are active is an important factor that can render the bed nets ineffective as a vector control method (Killeen 2014; Killeen and Chitnis 2014). A survey by questionnaire of individuals in the four study villages showed that 75–90% of the people retired to bed after 22:00 hr and nearly 100% of people retired to bed after 2:00 hr (Rodriguez-Rodriguez et. al, unpublished; personal communication). In Bulal and Megiar 46.5–49.7% of the

Anopheles bites occurred in the evening when 75–90% of people were exposed, and 75–83.3% of the bites occurred before 2:00 hr before 100% of the people retired to bed. In Mirap and Wasab 29–34% of the bites occurred in the evening (75–90% of people exposed), and 66.6–72% of the bites occurred before 2:00 hr before 100% of the people retired to bed.

The tendency of the mosquitoes to bite humans more frequently outdoors than indoors is another important factor that limits the efficacy of the bed nets. By biting outdoor, mosquitoes can evade not just the lethal contact with the indoor-deployed insecticidal bed nets but the protective barrier to their human hosts (Killeen 2014). In this study, although there was no statistical difference in the number of *Anopheles* collected inside versus outside houses in all the study villages, more mosquitoes were collected outdoors than indoors and conservatively it can be concluded that frequency of landing (and presumably therefore, biting) was equivalent between the two locations. The malaria vectors of PNG simply have no preference for either indoor or outdoor biting and may not even distinguish the two locations. Importantly, however, the biting (as reflected by landing catches here) is clearly peridomestic. Further, although a significant proportion of the vectors landed on the human collectors inside houses, most of them may not contact bed nets in the case of human residents of those houses, because a majority of the vector-human contact occurred during the period of the night when the human hosts were active and not under the bed nets.

Assessment of spatial heterogeneity in the number of human-biting mosquitoes is important for two reasons: (i) to assess the transmission potential of malaria which is useful to guide disease intervention plans and (ii) to identify hotspots of transmission so that disease intervention can be targeted to the risk groups. One way to assess the transmission potential is through estimation of the R_0, which is the number of secondary malaria cases in fully susceptible

individuals that ensue from one primary case during its entire infectious life span. The magnitude of the difference in the true R_0 values under heterogeneous relative to homogeneous condition can be quantified in terms of R_f directly from the same data that were used to test spatial heterogeneity (Dye and Hasibeder 1986; Woolhouse, et al. 1997), as was applied in this study (see description of data analysis in Methods, this chapter). For malaria to persist (endemic), rebound, or spike (epidemic) the value of R_0 theoretically must be > 1.0; values ≤ 1.0 will eventually lead to malaria decline (Bailey 1982). Under heterogeneous condition, malaria persistence is strengthened as the R_0 increases by a multiplicative factor R_f. In the three villages that were investigated, the estimated R_f values ranged from 1.96–3.89 for indoor collections and 1.6–4.59 for outdoor collections (Table 4.3). The R_f values estimated in this study were similar to the value of 3.7 reported for *Anopheles* spp. in a village in East Sepik province of PNG (Woolhouse, et al. 1997). Thus, spatial heterogeneity is a strong contributing factor to resumption of high malaria transmission rates and rebound of human prevalence of infection described in chapter 3. The northwest end of Megiar and the southwest side of Wasab village where the vector-human contact rates were highest (Figure 4.5) can be considered hotspots of transmission. People in these areas are most likely to be infected (carriers) and serve as sources which amplify transmission and promote spread of infection. Thus, intervention programs such as distribution of free insecticidal bed nets and administration of antimalarial prophylactic drugs or transmission-blocking vaccines (Carter 2001), particularly in resource-limited countries like PNG, can be targeted to individuals living in those hotspots. This analysis begs the question of which is more important to inform malaria control: the locations of homes where people have malaria, which is randomly distributed, or the location where *Anopheles* bites on humans (as reflected by landing catches) aggregate spatially. The former requires a high target of universal

coverage, whereas the latter does not but the spatial structure of mosquito-human contact is not readily discernable at this stage of research.

Given the spatial heterogeneity in the number of human-biting mosquitoes, particularly in Megiar and Wasab where there was clear visual directional trend, it is logical to expect the spatial distribution of households with malaria infection cases to be non-random or clustered with respect to the non-case households. The case households should cluster in the areas of the village where the numbers of human-biting mosquitoes were high (i.e., hotspots of transmission), such as the northwest end of Megiar and southwest side of Wasab. Instead, the *K*-function tests show that all the four study villages exhibited random rather than clustered distribution (Figure 4.6–4.9). There are two plausible reasons why there was spatial heterogeneity in the number of human-biting mosquitoes but not human infection cases. First, there must be a threshold value of biting rates below which malaria transmission is interrupted that would lead to spatial heterogeneity of infection cases. The number of human-biting mosquitoes observed in this study might be higher than the threshold level, thus allowing infection cases to be spatially homogeneous. This assertion is supported indirectly by a study which showed that an extremely low infectious biting rate is required to achieve malaria infection prevalence below 5% (Beier, et al. 1999). Second, the results discussed here were based on mosquito data from a single time point; not from longitudinal sampling. Thus, the stationarity of the spatial heterogeneity is not known but may vary temporally so that areas with a low number of human-biting mosquitoes at one time point have higher numbers at a different time point. The temporal variation of spatial heterogeneity eventually results in spatially homogeneous level of exposure to *Anopheles* bites and hence spatially homogeneous infection cases.

4.6. Conclusion

The temporal and spatial patterns of vector-human contact reported in this study indicate that in Madang, and most likely other parts of PNG, bed nets may not be fully effective in preventing malaria transmission. Temporally, a large proportion of the human-biting activity of the vectors occurred in the evening, before midnight. As most people are active during this period, malaria transmission takes place before the people go to sleep under their bed nets. Spatially, a large proportion of host-seeking *Anopheles* bit humans outside houses where the risk of contacting the indoor-deployed insecticidal bed nets was negligible. For those that bit humans inside houses, most did so in the evening when people were not sleeping under their nets. These factors limit bed nets from fully reducing malaria transmission through suppression of vector abundance and reduction of vector longevity. The number of human-biting mosquitoes varied among households within the villages (i.e., spatial heterogeneity), resulting in R_f values that were ≥ 1.6, which indicates that the disease has the potential to persist. Finally, despite the spatial heterogeneity in exposure to vector bites, this did not lead to spatial clustering of human infection cases. This inconsistency can be explained by two hypotheses: (i) the current low number of human-biting mosquitoes was insufficient to interrupt transmission and/or (ii) the spatial heterogeneity is temporally unstable and changes over time, culminating in spatially homogeneous exposure to infectious bites. These ecological factors are potential determinants underlying malaria resurgence as discussed in chapter 3.

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CHAPTER 5: PROBE-BASED MULTIPLEX QUANTITATIVE PCR IDENTIFIES BLOOD-MEAL HOSTS IN *ANOPHELES* MOSQUITOES FROM PAPUA NEW GUINEA

Publication

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5.1. Abstract

Determination of blood-meal hosts in blood-fed female *Anopheles* mosquitoes is important for evaluating vectorial capacity of vector populations and assessing effectiveness of vector control measures. Sensitive molecular methods are needed to detect traces of host blood in mosquito samples, to differentiate hosts, and to detect mixed host blood-meals. This paper describes a molecular probe-based qPCR for identifying blood-meal hosts in *Anopheles* malaria vectors from PNG. TaqMan oligonucleotide probes targeting specific regions of mitochondrial or nuclear DNA of the three primary *Anopheles* hosts—humans, pigs and dogs—were incorporated into a multiplex, qPCR which was optimized for sensitivity and specificity. Amplification of serially diluted DNA showed that the qPCR detected as low as 10^{-5} ng/µl of host DNA. Application to field-collected, blood-fed *Anopheles* showed that the qPCR identified the vertebrate hosts for 89% (335/375) of mosquitoes whereas only 55% (104/188) of blood-meal samples tested in a conventional PCR were identified. Of the 104 blood-fed *Anopheles* that were positive in both PCR methods, 16 (15.4%) were identified as mixed blood meals by the qPCR whereas only three (2.9%) were mixed blood meals by the conventional PCR. The multiplex qPCR described here is

sensitive at detecting low DNA concentration and mixed host DNA in samples and useful for blood meal analysis of field mosquitoes, in particular mixed-host blood meals.

5.2. Introduction

Identification of blood-meal hosts in blood-sucking arthropod vectors of human diseases forms an integral component of analysis of both vectorial capacity and effectiveness of vector control measures. For human malaria, analysis of female *Anopheles* mosquito blood meals is important for assessing the degree of feeding on humans (anthropophagy), usually expressed in terms of human blood index: the proportion of blood-engorged mosquitoes that fed on humans relative to non-human hosts. This index is used to evaluate epidemiological outcomes of vector-targeted malaria control programs since vectorial capacity, which is a measure of disease transmission potential of a vector population, varies with the human blood index (Garrett-Jones 1964a; Garrett-Jones 1964b). Blood meal analysis is also important for assessing host selection, which is the propensity of a mosquito population to feed more or less on a host species relative to other host species available to the mosquito (Boreham and Garrett-Jones 1973). Assessment of host selection is important when considering vector control methods such as insecticide-treated bed nets and zooprophylaxis which seek to divert mosquito feeding to non-human hosts.

Immunological methods such as the precipitin test, latex agglutination, and enzyme-linked immunosorbent assays were the earliest methods used in mosquito blood meal analysis (Beier, et al. 1988; Boorman and Mellor 1977; Burkot, et al. 1981; Weitz 1956). These methods have produced countless valuable data (Boreham and Garrett-Jones 1973; Boreham, et al. 1979; Burkot, et al. 1989; Burkot, et al. 1988; Edman and Downe 1964; Senior-White 1952; Shemanchuk, et al. 1963) but have been superseded in recent years by nucleic acid-based

methods. In cases where mosquito populations are known to feed on a wide range of vertebrate hosts or when the host range has not been characterized, sequencing of amplicons amplified with universal primers followed by a BLAST (Basic Local Alignment Search Tool) search against publicly available vertebrate host sequences has been used (Hamer, et al. 2011; Hamer, et al. 2009; Logue, et al. 2016; Molaei, et al. 2006; Molaei, et al. 2010). However, this approach is expensive and sequencing facilities are not available in many laboratories. In cases where mosquito populations are known to feed on a limited range of host species, less expensive methods such as restriction-fragment length polymorphisms (Meece, et al. 2005), DNA-DNA hybridization (Fritz, et al. 2013), conventional multiplex PCR with species-specific primers (Kent and Norris 2005) or qPCR with species-specific probes (Gunathilaka, et al. 2016; Hurk, et al. 2007) can be employed.

In PNG, the blood-meal host range for the *Anopheles* vectors of malaria is generally limited to humans, pigs and dogs (Burkot, et al. 1989; Burkot, et al. 1988; Keven, et al. 2017; Logue, et al. 2016). Thus, PCR methods with host-specific primers or probes are appropriate in this setting. Conventional multiplex PCR with primers specific to these three hosts is currently applied to mosquitoes originating from PNG (Keven, et al. 2017). However, with a large sample, the gel-electrophoresis step can be labor-intensive and time-consuming, resulting in accumulation of unanalyzed samples and delays in data availability. Furthermore, as shown in this present study, the conventional multiplex PCR has high rates of false-negative blood meals and is less sensitive at detecting mixed blood meals (see result section). The objective of this study was to develop a probe-based multiplex qPCR for identifying the common mammalian blood-meal hosts in female *Anopheles* mosquitoes originating from PNG.

5.3. Methods

5.3.1. Samples

Genomic DNA isolated directly from human and animal tissues were used in the optimization of the qPCR. Pig DNA was isolated from a piglet's tail (25 mg), removed as part of standard tail-docking routine at Michigan State University Swine Farm. Dog DNA was isolated from a blood sample (50 µl) by a laboratory that studies genetic diseases of dogs. Human DNA was isolated from a blood sample (50 µl) of a study participant in PNG during a cross-sectional malaria epidemiology survey by PNG Institute of Medical Research. Genomic DNAs isolated from abdomens of blood-fed *Anopheles* mosquitoes collected in a malaria-endemic village of PNG were used to test the new qPCR method. DNA extractions were performed using Qiagens DNeasy Blood & Tissue Kit.

5.3.2. Probe design

Nucleotide sequences for the primers and probes described in this study are shown in Table 5.1. The probes were designed within the mitochondrial cytochrome c oxidase subunit 1 gene (*cox*1) for pig, mitochondrial cytochrome b gene (*cytb*) for dog, and intron 1 of tyrosine hydroxylase gene (*th*) for human using the online program PrimerQuest accessible at the Integrated DNA Technologies website. Each probe was labelled at the 5'end with reporter dye FAM, VIC or ABY and at the 3' end with TaqMan quencher dye QSY (see Table 5.1). The primers and probes were obtained from Invitrogen and Applied Biosystems respectively through Thermo Fisher Scientific.

Table 5.1. Primers and probes for the three vertebrate host species.

Organism	Primer/probe name	Nucleotide sequence 5'-3'	Amplicon size (in bp)
Human	human*th*_F	GGCCTGTTCCTCCCTTATTT	101 [a]
	human*th*_R	TACACAGGGCTTCCGAGT	
	human*th*_P	FAM- ATGGAGTCTGTGTTCCCTGTGACC -QSY	
Pig	pig*cox*1_F	CTGACTACTTCCACCATCCTTC	108
	pig*cox*1_R	TGGGCTAAGTTTCCAGCTAAA	
	pig*cox*1_P	VIC- ATAGTAGAAGCCGGAGCGGGTACT -QSY	
Dog	dog*cytb*_F	TGGACAAAGCAACCCTAACA	103
	dog*cytb*_R	CCGGTTTCGTGTAGAAATAGGA	
	dog*cytb*_P	ABY- TCATCCTCCCTTTCATCATCGCAGC -QSY	

[a] Due to presence of a microsatellite repeat sequence (5'-AATG-3') within the human amplicon, the PCR product size varies between different human individuals. Presence of the repeat sequence does not affect PCR amplification as the primers and probe sequence do not extend into the repeat region.

5.3.3. Development of multiplex qPCR

For each target organism, the optimum qPCR reaction mixture was determined by testing different concentrations of the specific probe and primers in uniplex reactions (10 µl volume) containing 1x TaqMan Multiplex Master Mix (Catalog number: 4461882; Thermo Fisher Scientific), and 2 µl of serially diluted (10-fold dilutions) template DNA samples (10 ng/µl starting concentration). PCR reactions were performed on a QuantStudio 7 Flex instrument (Applied Biosystems) with Fast Cycling condition consisting of one cycle of 95 °C for 20 seconds followed by 40 seconds of 95 °C for one second and 60 °C for 20 seconds. Results were evaluated in software QuantStudio (version 1.3). Standard curves were evaluated and the reagent concentration that gave the highest percent amplification efficiency (measured as Efficiency = $(10^{(-1/slope)} - 1) * 100)$) and lowest threshold cycle or C_q (which corresponds to highest

sensitivity) was considered optimum and was used as the starting concentration for optimization of multiplex qPCR. For each of the three assays, multiplex qPCR mixture that gave the highest sensitivity and percent efficiency was considered optimum. The standard curve for each assay was derived from amplification results of triplicate dilution series (10-fold) of the target DNA.

5.3.4. Assay validation

Specificity of the probes and primers to their target DNA region was confirmed *in silico* by performing BLAST and primer-BLAST (Ye, et al. 2012) searches for potential matches to untargeted sequences in the National Center for Biotechnology Information database. Cross-reactivity of each probe to non-target organisms was also tested *in vitro* by subjecting DNA of the non-target organisms to uniplex qPCR containing the probe. For example, the human probe was tested for cross-reactivity to eight vertebrate species, namely pig, dog, cow, goat, horse, cat, rat and chicken; three *Plasmodium* species likely to be present in the blood meals, namely *P. falciparum*, *P. vivax* and *P. malariae*, and five species of *Anopheles* mosquitoes commonly found in PNG, namely *An. farauti* (*s.s.*), *An. koliensis*, *An. punctulatus* (*s.s.*), *An. longirostris* and *An. bancroftii*.

Ten-fold serially diluted DNA samples of humans, pigs, and dogs were amplified in the multiplex qPCR and the conventional, multiplex PCR (Kent and Norris 2005) and their results were compared for sensitivity. Primers for the single gene target of the mitochondrial *cytb* gene, reaction mixture, and cycling condition for the conventional PCR used in this study was as described in (Keven, et al. 2017). The *Anopheles* blood-meal DNA samples (n = 375) were analyzed in the qPCR to validate the applicability of the method to field mosquitoes. A subset of these qPCR-tested blood-meal samples (n = 188) was also analyzed in the conventional PCR and

its amplification success rate was compared with that of the qPCR. Amplification results with C_q value ≥ 38 were inconclusive and therefore considered negative.

For mosquitoes whose blood-meal hosts were successfully identified by the qPCR, the host DNA concentration was estimated using the formula

$$\log_{10}(Conc) = (C_q - Intercept)/Slope$$

where *Conc* is the DNA concentration in ng/µl. The slope and intercept are specific to each host species (see Table 5.2). For the single blood-meal mosquitoes, the mean host DNA concentration for those that were detected in the conventional PCR was statistically compared (two-sample t-test) with those that were undetected. Similarly, for the mixed blood meals, the mean DNA concentration for the hosts that was detected in the conventional PCR was compared with those that were undetected.

5.4. Results

The three blood-meal probes performed well together in multiplex reactions and were specific to their target organism. Optimum reaction mixture for the multiplex qPCR (10 µl volume) consisted of 1x TaqMan Multiplex Master Mix, 0.6 µM of each human primer, 0.5 µM of each dog and pig primer, 0.3 µM of human probe and 0.25 µM of pig and dog probes, and DNA template (concentration vary by samples). Standard curves and reaction efficiencies for the three assays are shown in Table 5.2. The assays were sensitive at detecting low DNA concentrations; the lowest detectable DNA concentration in the dilution series was 10^{-4} ng/µl for human and 10^{-5} ng/µl for pig and dog (Figure 5.1a-d). In contrast to the qPCR, the conventional one PCR did not detect DNA concentrations below 10^{-3} ng/µl for human, 10^{-4} ng/µl for pig and 10^{-2} ng/µl for dog (Figure 5.1a-d). In addition to the insensitivity of the dog primers to low DNA concentration,

amplification of the higher DNA concentration resulted in weak PCR bands compared to that of human and pig (Figure 5.1c and d). Also, for all three hosts, the PCR band intensity in mixed DNA samples (Figure 5.1d) was weaker than single host DNA samples (Figure 5.1a-c).

Table 5.2. Standard curve and reaction efficiency of the three qPCR assays.

Organism	Slope	% Efficiency	Y-Intercept	*R* squared	SE
Human	-3.36	98.4	25.6	0.997	0.21
Pig	-3.50	93.1	22.3	0.998	0.19
Dog	-3.51	92.7	23.7	0.996	0.22

Abbreviation: SE, standard error

Of the 375 *Anopheles* blood meals analyzed in the qPCR, 335 (89.3%) were positive for one or more of the three vertebrate hosts whereas only 40 (10.7%) were negative. Of the 335 positive samples, 225 (67.2%) were human blood meals, 18 (5.4%) were pigs, and 41 (12.2%) were dogs, with no other hosts detected in these single host results. The remaining 15.4% of positive samples were of mixed species, including 11 (3.3%) human + pig, 32 (9.55%) human + dog, four (1.2%) pig + dog, and four (1.2%) human + pig + dog. A random subset of these blood-meal samples (n = 188) were analyzed in the conventional, multiplex PCR and the results were compared with that of the qPCR by means of concordance-discordance frequency matrix (Table 5.3). Of 104 samples that were positive in both PCR methods, 16 (15.4%) were identified as mixed blood meals by the qPCR whereas only 3 (2.9%) were mixed blood meals by the conventional PCR. Twenty-four samples that were negative in the qPCR were also negative in the conventional PCR (i.e. 100% concordance). For those blood meals that were positive in the qPCR reactions (columns of Table 5.3), only 62.3% of human, 63.6% of pig, 50% of dog, 0%

human + pig mix, 20% of human + dog mix and 0% of pig + dog mix were concordant with conventional PCR results. The majority of the discordant blood meals were mosquitoes that were positive by the qPCR but turned out negative in the conventional PCR (n = 60); only a few (n = 16) were actually incongruent in the blood-meal types determined by the two methods. Of these, 13 were mosquitoes with mixed blood meals by qPCR but appeared as single blood-meal types in conventional PCR (Table 5.3).

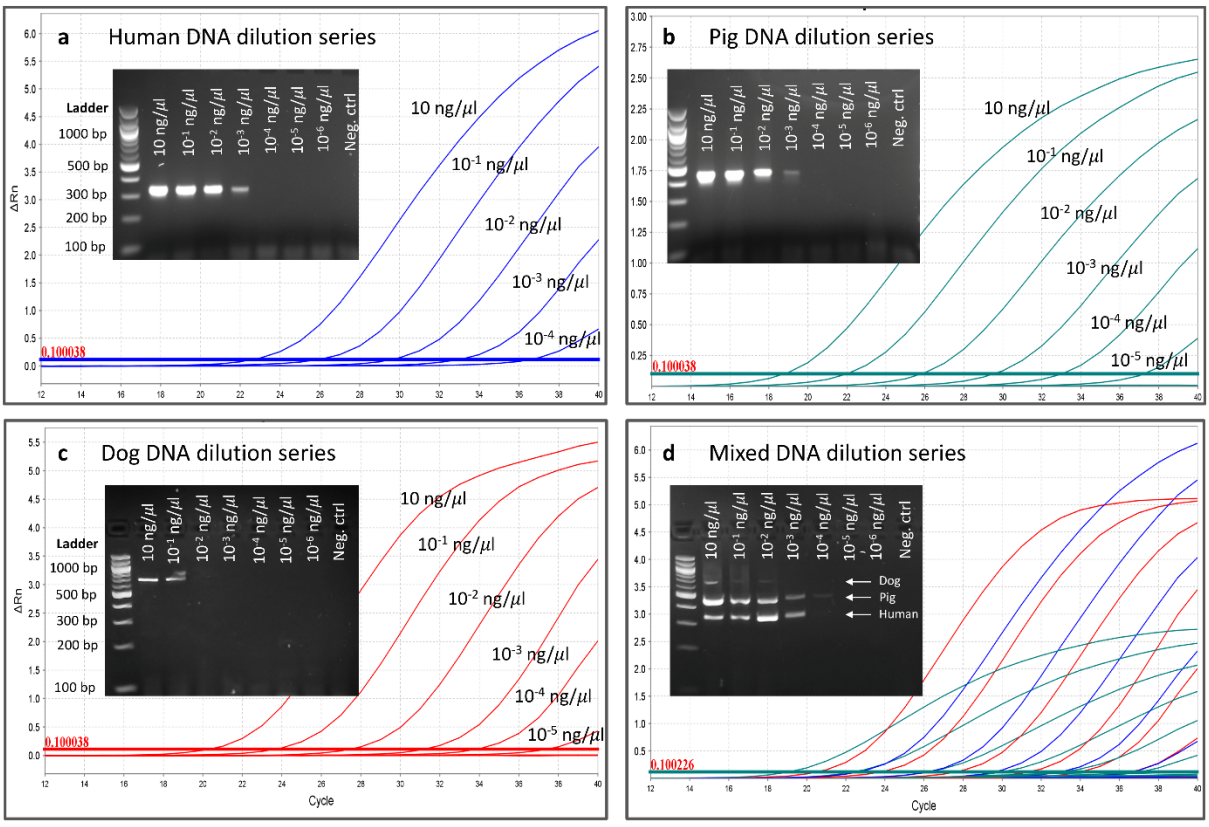

Figure 5.1. Results of conventional (the agarose gel images) and quantitative (the linear amplification plots) multiplex PCR both applied to the same 10-fold DNA dilution series. (a) PCR plot and gel image for human. (b) PCR plot and gel image for pig. (c) PCR plot and gel image for dog. (d) PCR plot and gel image for mixed sample. For the qPCR curves in panel d, color blue indicates human, green indicates pig and red indicates dog as in panel a-c. The mixed samples (panel d) contain equal DNA concentration (in ng/µl) of each host.

Table 5.3. Frequency of concordant and discordant *Anopheles* blood-meal results analyzed by quantitative versus conventional PCR.

Conventional PCR	Quantitative PCR						
	Human	Pig	Dog	Human/ Pig	Human/ Dog	Pig/ Dog	Negative
Human	66 (62)	0 (0)	2 (8)	2 (33)	9 (60)	0 (0)	0 (0)
Pig	1 (1)	7 (64)	0 (0)	1 (17)	0 (0)	0 (0)	0 (0)
Dog	0 (0)	0 (0)	12 (50)	0 (0)	1 (7)	0 (0)	0 (0)
Human/Pig	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Human/Dog	0 (0)	0 (0)	0 (0)	0 (0)	3 (20)	0 (0)	0 (0)
Pig/Dog	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Negative	39 (37)	4 (36)	10 (42)	3 (50)	2 (13)	2 (100)	24 (100)

Notes: Numbers outside of parenthesis are frequency of concordant (along diagonal) or discordant (off-diagonals) blood-meal types and inside parenthesis are percentage of column-wise total.

The results of the statistical comparison of the mean host DNA concentration of mosquito blood meals that were detected versus undetected in the conventional PCR are shown in Figure 5.2. As predicted, for mosquitoes with single blood-meal host (grouped according to host species), the mean DNA concentration of those that was detected was significantly higher than the undetected ones for human (t-test: $t_{(68)} = 2.15$, $P = 0.04$), pig (t-test: $t_{(8)} = 2.9$, $P = 0.02$) and dog (t-test: $t_{(17)} = 2.49$, $P = 0.02$) (Figure 5.2a). Similarly, for mosquitoes with mixed blood-meal (not grouped according to host species), the mean DNA concentration for those that were detected was significantly higher than the undetected ones (t-test: $t_{(18)} = 2.84$, $P = 0.01$) (Figure 5.2b). The mean DNA concentration for detected single blood meals (Figure 5.2a) was significantly higher for pig compared to human (Tukey HSD test: mean difference = 0.63; 95% family-wise confidence interval or CI: 0.40–0.86; adjusted $P < 0.0001$), higher for dog compared

to human (mean difference = 1.27; 95% family-wise CI: 1.09–1.45; adjusted $P < 0.0001$) and higher for dog compared to pig (mean difference = 0.64; 95% family-wise CI: 0.36–0.9; adjusted $P < 0.0001$). For undetected single blood meals, the mean DNA concentration (Figure 5.2a) were not significantly different between pig and human (mean difference = -0.008; 95% family-wise CI: -0.42–0.40; adjusted $P = 0.999$) but were significantly higher for dog compared to human (mean difference = 0.73; 95% family-wise CI: 0.48–0.97; adjusted $P < 0.0001$) and for dog compared to pig (mean difference = 0.74; 95% family-wise CI: 0.28–1.18; adjusted $P = 0.0007$).

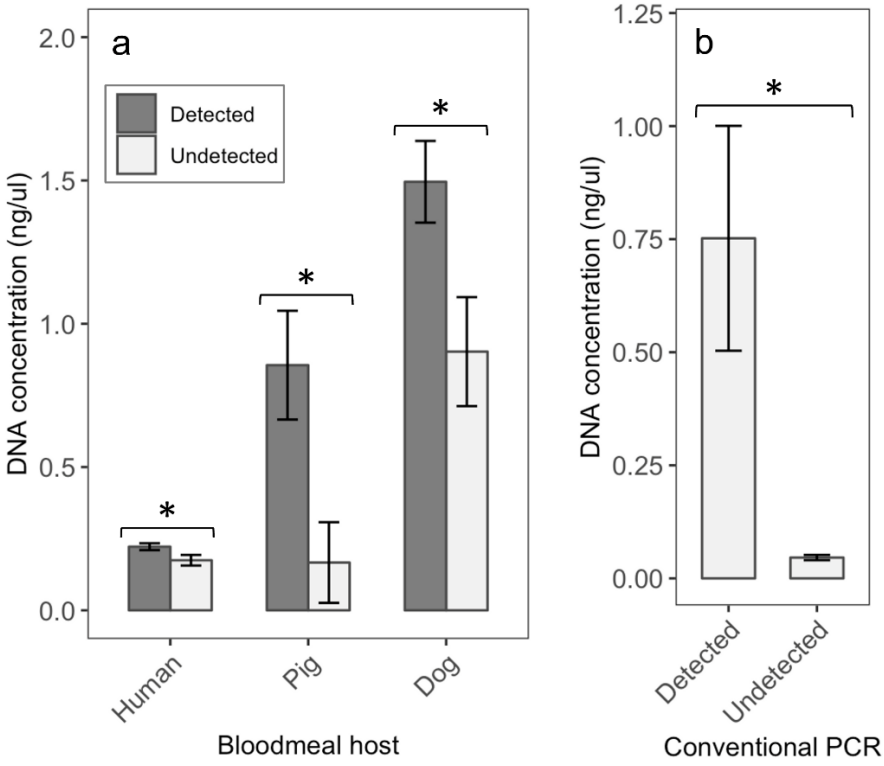

Figure 5.2. Bar plot showing mean DNA concentration (with standard error bar) for mosquito blood meals that were detected versus undetected by conventional PCR. (a) Comparison of mean DNA concentration for single blood meals grouped according to human (detected, $n = 6$; undetected, $n = 38$), pig (detected, $n = 7$; undetected, $n = 3$) and dog (detected, $n = 12$; undetected, $n = 10$) hosts. (b) Comparison for mixed blood meals (detected, $n = 19$; undetected, $n = 22$) not grouped according to host species. Asterisks (*) indicate significant difference ($P < 0.05$) between groups (Student's t-test).

5.5. Discussion

The qPCR method offers several advantages over conventional PCR. First, when analyzing large number of samples with conventional PCR, the post-PCR steps such as gel-electrophoresis and manual scoring of results are labor-intensive, delay availability of data, generate large quantities of waste, and increase risk of exposure to ethidium bromide, a mutagen. These concerns are eliminated when qPCR is used. Second, it may be desirable to quantify DNA template or copy number of the target gene in samples, which qPCR can accomplish. Third, while qPCR is not always more sensitive than conventional PCR (Bastien, et al. 2008), many studies including this current one have shown that the former method can detect much lower DNA template concentrations compared to the latter (Dagher, et al. 2004; Dworkin, et al. 2002; Hierl, et al. 2004; Mary, et al. 2004). Thus, sensitivity of detection is better, as was shown here.

Identification of arthropod blood meals by qPCR has been applied to sand flies, biting midges, kissing bugs, fleas and mosquitoes (Graham, et al. 2012; Gunathilaka, et al. 2016; Hurk, et al. 2007; Ibanez-Cervantes, et al. 2013; Sales, et al. 2015; Van-Der-Saag, et al. 2016; Woods, et al. 2009). Most of these were SYBR green-based systems; only three were probe-based. Of the three probe-based qPCR, one was for identifying Australian mammals in *Culex* mosquito blood meals and did not include humans, pigs or dogs (Hurk, et al. 2007), another was for identifying blood-meal hosts of biting midges and included humans and pigs but not dogs (Van-Der-Saag, et al. 2016), and another was for identifying flea blood meals and included humans and dogs but not pigs (Woods, et al. 2009). Notably, the human probe in the latter flea blood-meal study was tested here and found to cross-react with dog DNA. Surprisingly, a thorough Google Scholar search did not find a paper describing probe-based qPCR designed specifically for identifying mammalian hosts of *Anopheles* blood meals.

When evaluating the sensitivity of the qPCR assay from amplifications of 10-fold dilution series of target DNA samples, the lowest detectable concentration for human DNA (10^{-4} ng/μl) was ten-fold greater than for pigs and dogs which was 10^{-5} ng/μl. This difference was attributed to the copy numbers of the target DNA sequences; the pig and dog probes target mitochondrial genes which exist in multiple copies per cell, whereas the human probe targets a single-copy nuclear DNA sequence. Several human probes targeting various mitochondrial gene locus were designed and tested. However, they all exhibited non-specific amplification of the two nonhuman hosts *in vitro* despite appearing to be target specific by *in silico* test (Table S5.1, Appendix 5.1). Nevertheless, the detectable limit of human DNA concentration with the current probe (10^{-4} ng/μl) is sufficiently low for detecting mosquito blood meals. The lower detectable limit of qPCR compared to the conventional one, particularly dog, indicates a difference in the sensitivity of the two methods.

The results show that the blood-meal qPCR was more sensitive at detecting host DNA in mosquitoes (detection success rate of 89%) compared to the more commonly used conventional, multiplex PCR (detection success rate of 55%). It is possible that the 11% of mosquitoes whose blood-meal hosts were not identified by the qPCR could have fed on other host sources (e.g., chickens, cats). However, when subjected to two conventional PCR utilizing generic mammalian and avian primers, none showed a positive result, which was consistent with findings from our previous study (Keven, et al. 2017). Thus, the likelihood that host breadth was greater than the three hosts we targeted with probes here is low. A common observation in all of these unamplified blood meals was that they all contained traces of blood in their abdomens (< 0.3 μl), based on light microscopy examination of the mosquito abdomens 4–8 hours after they were collected. Given the non-nucleated status of mammalian red blood cells and disproportionately

low ratio of white to red blood cells, the small volumes of blood meal were likely insufficient to yield a detectable concentration of DNA.

The trace blood assertion was further supported by showing that the qPCR-quantified host DNA concentration of single blood meals was significantly higher in those samples that were detected in the conventional PCR than those that were undetected for all three hosts. The mean DNA concentration for the detected samples was expected to be statistically the same between the three hosts. However, the result showed otherwise; pig DNA concentration was significantly four-folds higher than humans and two-fold less than dog (Figure 5.2a). This heterogeneity in host DNA quantity could be caused by factors such as variation among the hosts in the number of white blood cells per unit volume of blood. However, a more plausible explanation is variation in the average quantity of blood mosquitoes obtain from each host as a result of variation in host sensitivity to mosquito bites. That is, humans are more sensitive than pigs followed by dogs to mosquito bites, causing them to quickly interrupt blood-feeding mosquitoes before they had time to acquire the maximum amount of blood. Despite the variation between the three hosts in their blood-meal DNA quantity, the mean DNA concentration for mosquitoes that were undetected in the conventional PCR was expected to be the same. The expectation is based on the reasoning that below a certain DNA concentration, the qPCR amplification becomes undetectable. However, the result showed that although the mean DNA concentration was statistically the same for humans and pigs as predicted, it was five-folds higher for dog (Figure 5.2a). This indicates that the dog conventional primers are less efficient and have higher sensitivity threshold than the other two hosts, which is consistent with its behavior observed in the amplification of DNA dilution series.

The conventional PCR did not detect mixed blood meals sensitively; most of the samples identified as mixed blood meals by qPCR were identified as single blood meals by the conventional method (Table 5.3). The inaccurate detection of mixed blood meals was attributed to low DNA quantity of one or more of the hosts in a mixed blood-meal sample and was supported by the result which showed statistically lower mean DNA concentration for undetected compared to detected hosts (Figure 5.2b). Thus, a primary outcome of this study is the sensitivity of the probe-based, qPCR method to detect blood from different mammal species in the same blood meal. This finding indicates that a significant proportion of unidentified blood-meal sources in studies that used the conventional, multiplex PCR (Barrera, et al. 2012; Bashar, et al. 2012; Burkot, et al. 2013; Busula, et al. 2015; Keven, et al. 2017; Liu, et al. 2011; Pappa, et al. 2011) may, among other factors, be due to the low sensitivity of this method. Furthermore, the proportion of mixed blood meals may be underestimated, and single blood meals overestimated in some published studies. At the very least, such findings indicate interrupted blood feeding, which is an important variable contributing to transmission of malaria (Tedrow, et al. 2019).

5.6. Conclusion

This work describes the development of a probe-based multiplex qPCR for identifying the common blood-meal hosts of *Anopheles* mosquitoes from PNG. The qPCR assay was sensitive at detecting smaller amounts of target DNA in samples compared to standard PCR, which makes it appropriate for analysis of mosquito blood meals that often contain low amounts of host blood. Its ability to detect mixed blood meals compared to standard PCR makes it appropriate for use in studies that investigate interrupted feeding habits of mosquitoes. Although the qPCR assay described here was intended for analysis of mosquito blood meals from PNG and other parts of

South West Pacific, addition of probes specific to vertebrate hosts of mosquitoes from other parts of the world (e.g., cows and goats in Africa) is possible and encouraged.

APPENDIX

Appendix 5.1. Non-specific human primers

Table S5.1. Human primers designed to target mitochondrial genes found to be human-specific by in silico test but were non-specific (also amplifies dog and/or pig DNA) by in vitro test.

Gene target	Accession	Primer/probe	Oligonucleotide sequence (5'-3')	length (bp)	T_m (°C)	%GC	5' dye	3' dye	Product size (bp)	Source
Cytochrome b	MG272719.1	forward primer	TCAATCGCCCACATCACTCG	20	60.46	55	N/A	N/A	168	Woods et al. 2009
		reverse primer	CAGGAGGATAATGCCGATGTTTC	23	59.5	47.83	N/A	N/A		
		probe	TGGCTGAATCATCCGCTACCTTCACGC	27	74.8	55.6	FAM	QSY		
NADH dehydrogenase subunit 1	NC_012920.1	forward primer	TCTCACCATCGCTCTTCTACT	21	62.28	47.6	N/A	N/A	99	This study
		reverse primer	AGGCTAGAGGTGGCTAGAATAA	22	62.25	45.45	N/A	N/A		
		probe	TAGGAGGCCTAGGTTGAGGTTGACC	25	68.27	56	FAM	QSY		
NADH dehydrogenase subunit 5	MG272719.1	forward primer	CTCCTAGACCTAACCTGACTAGAA	24	62	45.8	N/A	N/A	136	This study
		reverse primer	GGATGAGTGGGAAGAAGAAAGA	22	62	45.5	N/A	N/A		
		probe	ACAGCACCAAATCTCCACCTCCAT	24	68	50	FAM	QSY		
Cytochrome C oxidase 1	NC_012920.1	forward primer	TTTCACCTCCGCTACCATAATC	22	62	45.5	N/A	N/A	104	This study
		reverse primer	GCACTGCAGCAGATCATTTC	20	62	50	N/A	N/A		
		probe	TTTAGCTGACTCGCCACACTCCAC	24	68	54.2	FAM	QSY		

Note: N/A, not applicable

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CHAPTER 6: PLASTICITY OF HOST SELECTION BY *ANOPHELES* VECTORS OF MALARIA IN MADANG, PAPUA NEW GUINEA: IMPLICATIONS FOR PERSISTENT MALARIA TRANSMISSION

Publication

Keven JB, Reimer LJ, Katusele M, Koimbu G, Vinit R, Vincent N, Thomsen E, Foran DR, Zimmerman PA, Walker ED. 2017. Plasticity of host selection by malaria vectors of Papua New Guinea. Parasit. Vectors 10:95

6.1. Abstract

Host selection is an important indicator of malaria transmission potential of *Anopheles* vectors and their response to indoor-deployed insecticidal bed nets. Despite the ongoing nationwide bed net-based malaria control campaign in PNG, little is known about the host selection of the local vectors. This study investigated the host selection of *Anopheles* vector populations in Madang. Blood-fed mosquitoes were sampled using the barrier screen method and blood meals analyzed for vertebrate host source with PCR methods. The abundance of common hosts was estimated in census surveys and exact binomial test was used to determine if each host was selected in proportion to its abundance relative to the other host species. Four thousand one hundred and twenty-four blood-fed *Anopheles* females of six species were sampled across seven Madang villages. Of these, 3,554 (86%) yielded a definitive host source; all were human, pig, or dog. Results show that hosts were not selected in proportion to their abundance, but rather were under-selected or over-selected by the mosquitoes. Low mosquito anthropophagy was associated with high bed net usage. Five species, namely *An. farauti* (*s.s.*), *An. farauti* no. 4, *An. koliensis*,

An. longirostris and *An. punctulatus* (*s.s.*), exhibited plasticity in their host selection, likely in response to varying levels of bed net usage and were thus considered opportunistic in their intrinsic host preference. In contrast, *An. bancroftii* exhibited strong affinity for pig blood despite overwhelmingly high human abundance and was considered zoophilic. By feeding on alternative hosts the likelihood of these opportunistic species contacting the bed nets inside houses is lower, making them difficult to control. By maintaining high population size on non-human blood sources, the proportion that feed on humans outdoors can sustain transmission despite high bed net usage in the village.

6.2. Introduction

Host selection is an outcome of the combined effects of a mosquito's intrinsic (genetic) host preference for a particular host species modulated by extrinsic factors (Lyimo and Ferguson 2009; Takken and Verhulst 2013). That is, even though a mosquito may intrinsically prefer a particular host species due to genetic factors, environmental factors such as availability or accessibility of the preferred host may cause the mosquito to resort to an alternative one. Therefore, in mosquito-borne disease epidemiology host selection is an important determinant of vectorial capacity, because it influences the extent to which mosquitoes in populations feed predominantly on humans or non-humans (Dye 1986; Garrett-Jones 1964). Thus, an *Anopheles* population whose members intrinsically prefer humans are potential vectors of malaria. However, the vectorial capacity of the mosquito population depends on whether extrinsic conditions allow the mosquitoes to actually feed on humans.

Knowledge of host selection is not only important for evaluating the vectorial capacity of a vector population, but also for guiding vector-based malaria control programs, such as the

distribution of insecticidal bed nets. The implementation of bed nets is appropriate if we know that local vectors are sufficiently anthropophilic that bed nets will have the intended effect (Russell, et al. 2016). The inflexibility of anthropophilic species to utilize alternative hosts causes them to pursue humans inside houses and thus increases their likelihood of becoming exposed to the insecticides in the bed net fabric. The increased likelihood of contacting bed nets enables reduction of their population size to levels insufficient to support transmission. In contrast, if the mosquitoes are opportunistic and exhibit plasticity in host selection, then bed nets may have little effect because these mosquitoes can maintain high population size by feeding on non-human hosts outdoor. By maintaining high population size, the proportion of opportunistic vectors that feed on human individuals before they go under their bed nets can sufficiently sustain transmission in the community. Treating the alternative hosts with endectocides lethal to blood-feeding mosquitoes may be more appropriate for controlling such opportunistic vectors. By implementing both methods, the anthropophilic and opportunistic vectors might be successfully controlled.

Human malaria is endemic to PNG (Muller, et al. 2003) and the main vectors are members of the *An. punctulatus* (*s.l.*) group (Beebe and Cooper 2002; Beebe, et al. 2015; Beebe and Saul 1995), primarily *An. punctulatus* (*s.s.*), *An. koliensis*, *An. farauti* (*s.s.*), *An. farauti* no. 4, and *An. hinesorum* (Benet, et al. 2004; Burkot, et al. 1989; Burkot, et al. 1988b; Cooper, et al. 2009; Hetzel, et al. 2016; Reimer, et al. 2016; Thomsen, et al. 2017). *An. bancroftii* and *An. longirostris* are also vectors of malaria in PNG (Cooper, et al. 2009). Nationwide insecticidal bed net-based vector control campaign has been ongoing in PNG since 2004 (Hetzel 2009; Hetzel, et al. 2014a; Hetzel, et al. 2012) to help alleviate the burden of malaria. However, little is known about the host selection behavior of these vectors and their relationship with bed net usage. The

research described in this chapter addresses this knowledge gap and provides guidance on existing as well as new vector control strategies in PNG.

6.3. Methods

6.3.1. Study sites and mosquito sampling

This study was conducted in seven malaria-endemic rural villages (Bulal, Dimer, Kokofine, Matukar, Megiar, Mirap and Wasab) of Madang, PNG whose geographic locations and ecogeographic environments were described in detail in chapter 2, section 2.3. Mosquitoes were collected in each village using the barrier screen method with sampling design, procedure and time period described in detail in chapter 2, section 2.3. Only the blood-fed female *Anopheles* mosquitoes were used in this study.

6.3.2. Identification of *Anopheles* species and their blood-meal hosts

The abdomen of each blood-fed mosquito was separated from the rest of the body using sterile technique and DNA was isolated from it using the DNeasy Blood and Tissue kit. Mosquitoes morphologically identified as member of the *An. punctulatus* (*s.l.*) group were further identified to their species using a PCR assay (Beebe and Saul 1995). As humans, pigs, and dogs are the main blood-meal sources of PNG mosquitoes, based on the findings in chapter 5 and elsewhere (Burkot, et al. 1989; Burkot, et al. 1988a; Logue, et al. 2016), the DNA samples were first tested for these three hosts using the multiplex qPCR described in chapter 5. Samples that did not amplify in the qPCR likely fed on other hosts (e.g., cats and chickens) and were subjected to two standard PCR with a generic mammalian primer pair (forward: 5′- CCA TCC AAC ATC TCA GCA TGA TGA AA-3′ and reverse: 5′- GCC CCT CAG AAT GAT ATT TGT CCT CA -3′) that

targets a 395 bp region of the mitochondrial cytochrome b gene (*cytb*), or an avian primer pair (forward: 5′- GAC TGT GAC AAA ATC CCN TTC CA -3′ and reverse: 5′- GGT CTT CAT CTY HGG YTT ACA AGA C -3′) which targeted a 508 bp region of avian *cytb* gene (Molaei, et al. 2006). The standard PCR reaction mixture consisted of *ca* 20 ng of DNA template added to a PCR tube (50 µl volume) containing 10 mM Tris at pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 1.0 mM dNTP, 0.5 units of *Taq* polymerase, and 50 pmol of each primer. Thermal cycle consisted of 1 cycle of 95 °C for 5 minutes followed by 35 cycles of 95 °C for 1 minute, 58 °C for 1 minute, and 72 °C for 1 minute, and 1 cycle of 72 °C for 7 minutes. PCR amplicons were run on 2% ethidium bromide-stained agarose gels and visualized under ultraviolet light. Amplicons of the PCR positive samples were purified using QIAquick PCR Purification Kit (Catalog Number 28106; Qiagen) and sequenced by direct sequencing. The DNA sequences were subjected to a BLAST search against vertebrate *cytb* sequences in the GenBank database. A subject sequence that had ≥ 99% sequence similarity to the query sequence was considered the likely host from which the mosquito fed.

6.3.3. Demographic survey

Surveys were conducted during the mosquito sampling period in each village in 2012 (Dimer, Kokofine, Matukar, Mirap and Wasab), 2016 (Mirap and Wasab) and 2017 (Bulal and Megiar). Heads of households were interviewed for information on number of people per household, their age, number of bed nets owned, and number and species of domestic animals owned by the household. Individuals were asked if they slept under a bed net the night before the interview.

6.3.4. Data analyses

The tendency of a vector population to feed on a particular host species compared to other available hosts, was quantified by performing two-tailed exact binomial test which compared the observed blood index (i.e., the proportion of blood-fed mosquitoes that fed on that host species) and the expected blood index (the proportion of that host relative to all other host species in village census count). Only single blood-meal data were used for this analysis; mixed blood meals were excluded. A host species was considered over-selected by a mosquito population if the observed blood index was statistically greater than the expected index, under-selected if the observed was less than expected, or fed on unbiasedly (i.e., feed on the host in proportion to its relative abundance) if there was no statistical difference. Each mosquito was assigned a binary value 1 if its blood meal was from a human or 0 if it was from a pig or dog and multiple logistic regression was used to evaluate the relationship between the probability of feeding on human blood meal and two predictor variables: i) bed net usage (proportion of interviewed villagers who reportedly slept under a bed net the night before the interview) and ii) mean number of household members per bed net (households as unit of replication). Both the binomial and logistic regression tests were computed using the R functions *binom.test* and *glm* of the package *stats*. Significance level for all tests was based on type I error rate of 5%.

6.4. Results

6.4.1. Mosquito blood-meal sources

A total of 4,124 blood-fed *Anopheles* mosquitoes of six different species were analyzed and the blood-meal hosts of 3,554 (86%) were identified. The 14% of the samples that did not was likely due to trace amounts of blood materials insufficient to yield a PCR-detectable DNA

concentration (see chapter 5 for more details). Human, pig, and dog were the only host species identified in the blood meals despite the presence of cats, chickens and ducks in all the villages. The exact number of mosquitoes of each *Anopheles* species that fed on a human, pig, or dog, or a mixture of these hosts (human + pig, human + dog, pig + dog or human + pig + dog) in each village are shown in supplementary Table S6.1–6.6 (Appendix). The blood-meal source data in the supplementary Table S6.1–6.6 were also expressed as relative percent proportion in Figure 6.1. In Figure 6.1, each bar represents the blood-meal sample of an *Anopheles* species in a village. In all 16 categories (i.e., 16 bars in Figure 6.1), there were more single than mixed blood meals; the highest proportion of mixed blood meals was in *An. koliensis* in Megiar (18.2%; Figure 6.1d). For single blood meals, the proportion of each of the three hosts in a sample (i.e., each bar in Figure 6.1) varied greatly. For example, Mirap *An. bancroftii* blood meals consisted of 12.8% humans, 76.9% pigs and 7.7% dogs (Figure 6.1a), and *An. punctulatus* (*s.s.*) in Wasab consisted of 71.9% humans, 10.4% pigs and 13.4% dogs (Figure 6.1f). Among villages, the proportions of each host in blood-meal samples of the same mosquito species varied among villages (Figure 6.1c-f). For example, the proportion of human blood meals in *An. longirostris* was 67.9% in Wasab but was only 15% in Mirap (Figure 6.1e).

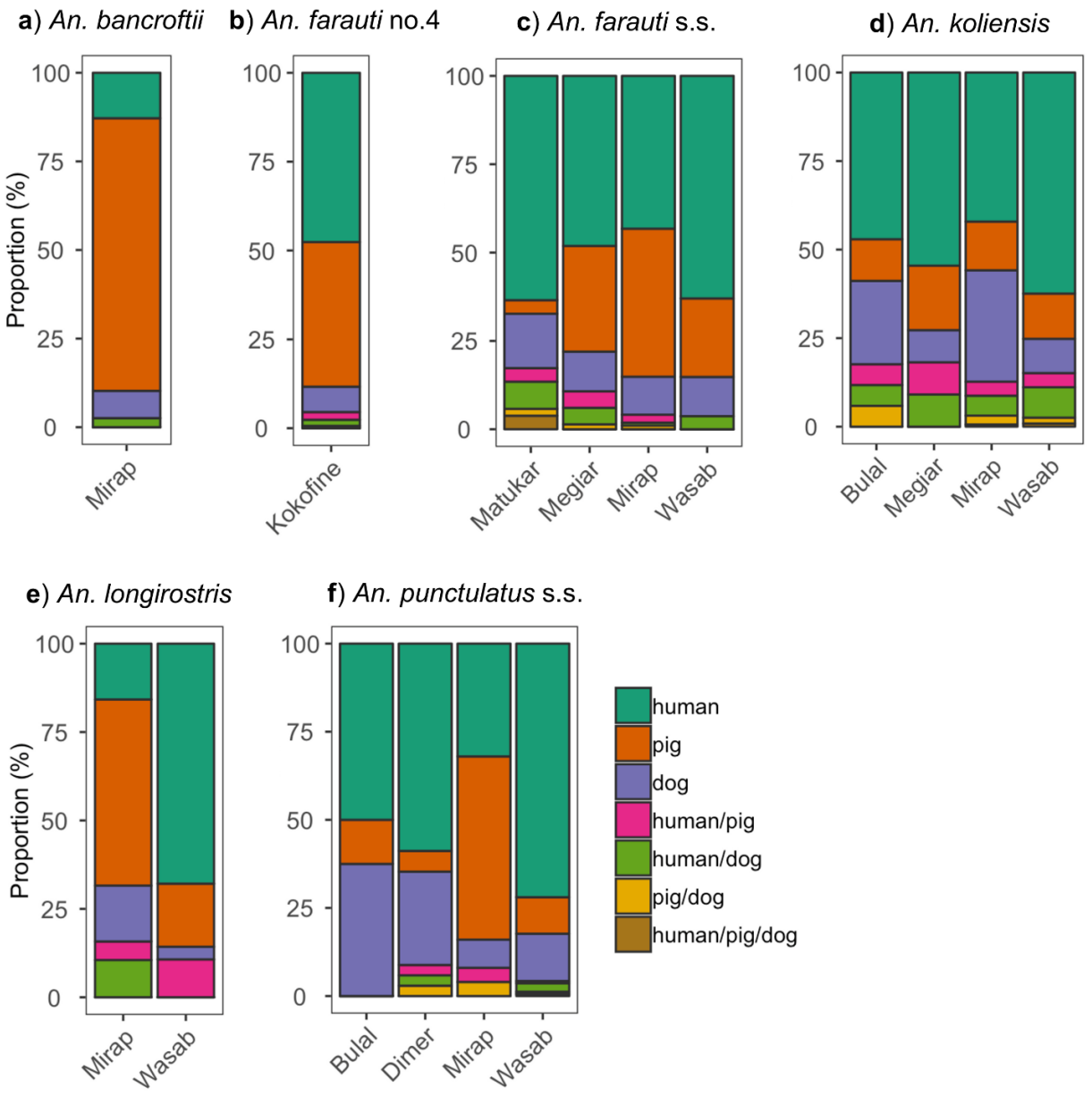

Figure 6.1. Proportion of blood-meal sources of mosquitoes sampled in study villages. (a) *An. bancroftii* in Mirap (n = 39). (b) *An. farauti* no. 4 in Kokofine (n = 462). (c) *An. farauti* (*s.s.*) in Matukar (n = 52), Megiar (n = 214), Mirap (n = 1,332) and Wasab (n = 27). (d) *An. koliensis* in Bulal (n = 34), Megiar (n = 11), Mirap (n = 480) and Wasab (n = 548). (e) *An. longirostris* in Mirap (n = 19) and Wasab (n = 84). (f) *An. punctulatus* (*s.s.*) in Bulal (n = 8), Dimer (n = 34), Mirap (n = 25) and Wasab (n = 164).

6.4.2. Mosquito host selection

A mosquito population was defined here as mosquitoes of a particular species collected from a specific location and time. The results of host selection tests are presented for two populations of *An. bancroftii* (Table 6.1), one population of *An. farauti* no. 4 (Table 6.2), five populations of *An. farauti* (*s.s.*) (Table 6.3), six populations of *An. koliensis* (Table 6.4), two populations of *An. longirostris* (Table 6.5) and six populations of *An. punctulatus* (*s.s.*) (Table 6.6). The results show that host selection varied among different populations of the same *Anopheles* species, except *An. bancroftii* and *An. farauti* no. 4; the former did not show variation in host selection and the latter consisted of a single population and comparison of host selection among populations could not be done for this species. The two *An. bancroftii* populations were from Mirap village but two different sampling periods (Table 6.1). Both over-selected pigs, under-selected humans and fed unbiasedly on dogs, although sample size was low for both populations ($n \leq 29$). Like *An. bancroftii*, the population of *An. farauti* no. 4 from Kokofine (generous sample size, $n = 441$) over-selected pigs but under-selected or fed unbiasedly the other two hosts (Table 6.2). Unlike *An. bancroftii*, the five *An. farauti* (*s.s.*) populations varied in their host selection (Table 6.3). The Matukar population fed unbiasedly on all three hosts, although the sample size was low ($n = 43$). For the Wasab population, none of the three hosts was over-selected; all were under-selected (pigs) or fed on unbiasedly (humans and dogs), although the sample size was also low ($n = 22$). The two Mirap and one Megiar populations where the sample sizes were modest ($n \geq 191$) all over-selected pigs and under-selected humans but varied in their selection of dogs. Of the six *An. koliensis* populations (Table 6.4), four (Bulal, Megiar, Mirap 2012–2013 and Wasab 2012–2013) fed unbiasedly on all three hosts, although the sample sizes of these populations were low ($n \leq 28$; see Table 6.4). For Mirap 2015–2017 population where

sample size was generous (n = 409), humans were under-selected whereas both non-human hosts were over-selected. In contrast, Wasab 2015–2016 population (generous sample size, n = 453) over-selected humans whereas the non-humans were under-selected or fed on unbiasedly. For the two *An. longirostris* populations (Table 6.5), the Mirap 2012-2013 population, pigs were over-selected whereas humans and dogs were under-selected or fed on unbiasedly, but the sample size was low (n = 12). In contrast, the Wasab 2012–2013 population with modest sample size (n = 73) over-selected humans, under-selected dogs and unbiasedly fed on pigs. For the six *An. punctulatus* (*s.s.*) populations (Table 6.6), except for Wasab 2015–2016 population which had a modest samples size (n = 100), the rest had low samples sizes ($n \leq 31$, see Table 6.6). Three populations, Bulal, Wasab 2012–2013 and Mirap 2012–2013 fed unbiasedly on all three hosts. The Dimer population over-selected dogs but fed unbiasedly on both humans and pigs. For Mirap 2015–2017 population, pigs were over-selected, humans were under-selected, and dogs were fed on unbiasedly. For Wasab 2015–2016 population, humans and dogs were over-selected whereas pigs were under-selected.

Table 6.1. Number of humans, pigs and dogs in village census and blood meals of two populations of *An. bancroftii* along with their host selection tendency based on exact binomial comparison of the expected (Exp) and observed (Obs) blood index.

Village	Period	Host	Census	Blood meals	Exp	Obs (95% CI)	*P*	Host selection
Mirap	2012–2013	humans	732	4	0.767	0.137 (0.039–0.317)	< 0.0001	Under-selected
		pigs	110	23	0.115	0.793 (0.603–0.920)	< 0.0001	Over-selected
		dogs	112	2	0.117	0.069 (0.008–0.228)	NS	Unbiased
Mirap	2015–2017	humans	1781	1	0.87	0.111 (0.003–0.482)	< 0.0001	Under-selected
		pigs	181	7	0.09	0.778 (0.400–0.972)	< 0.0001	Over-selected
		dogs	90	1	0.04	0.111 (0.003–0.482)	NS	Unbiased

Abbreviation: NS, not significant

Table 6.2. Number of humans, pigs and dogs in village census and blood meals of a population of *An. farauti* no. 4 along with their host selection tendency based on exact binomial comparison of the expected (Exp) and observed (Obs) blood index.

Village	Period	Host	Census	Blood meals	Exp	Obs (95% CI)	*P*	Host selection
Kokofine	2012–2013	humans	167	220	0.536	0.499 (0.451–0.547)	NS	Unbiased
		pigs	70	188	0.215	0.426 (0.380–0.474)	< 0.0001	Over-selected
		dogs	88	33	0.271	0.075 (0.052–0.103)	< 0.0001	Under-selected

Abbreviation: NS, not significant

Table 6.3. Number of humans, pigs and dogs in village census and blood meals of five populations of *An. farauti* (*s.s.*) along with their host selection tendency based on exact binomial comparison of the expected (Exp) and observed (Obs) blood index.

Village	Period	Host	Census	Blood meals	Exp	Obs (95% CI)	*P*	Host selection
Matukar	2012–2013	humans	432	33	0.751	0.767 (0.614–0.882)	NS	Unbiased
		pigs	67	2	0.117	0.047 (0.006–0.158)	NS	Unbiased
		dogs	76	8	0.132	0.186 (0.084–0.334)	NS	Unbiased
Megiar	2015–2017	humans	734	103	0.741	0.539 (0.466–0.611)	< 0.0001	Under-selected
		pigs	171	64	0.172	0.335 (0.269–0.407)	< 0.0001	Over-selected
		dogs	86	24	0.087	0.126 (0.082–0.181)	NS	Unbiased
Mirap	2012–2013	humans	732	230	0.767	0.473 (0.428–0.519)	< 0.0001	Under-selected
		pigs	110	216	0.115	0.444 (0.400–0.490)	< 0.0001	Over-selected
		dogs	112	40	0.117	0.082 (0.059–0.110)	0.016	Under-selected
Mirap	2015–2017	humans	1781	117	0.87	0.386 (0.331–0.444)	< 0.0001	Under-selected
		pigs	181	121	0.09	0.399 (0.344–0.457)	< 0.0001	Over-selected
		dogs	90	65	0.04	0.215 (0.170–0.265)	< 0.0001	Over-selected
Wasab	2015–2016	humans	295	16	0.52	0.727 (0.498–0.893)	NS	Unbiased
		pigs	207	3	0.37	0.136 (0.029–0.349)	0.026	Under-selected
		dogs	64	3	0.11	0.136 (0.029–0.349)	NS	Unbiased

Abbreviation: NS, not significant

Table 6.4. Number of humans, pigs and dogs in village census and blood meals of six populations of *An. koliensis* along with their host selection tendency based on exact binomial comparison of the expected (Exp) and observed (Obs) blood index.

Village	Period	Host	Census	Blood meals	Exp	Obs (95% CI)	*P*	Host selection
Megiar	2017	humans	734	6	0.741	0.667 (0.299–0.925)	NS	Unbiased
		pigs	171	2	0.172	0.222 (0.028–0.600)	NS	Unbiased
		dogs	86	1	0.087	0.111 (0.003–0.482)	NS	Unbiased
Mirap	2012–2013	humans	732	9	0.767	0.900 (0.555–0.997)	NS	Unbiased
		pigs	110	1	0.115	0.100 (0.003–0.445)	NS	Unbiased
		dogs	112	0	0.117	0.000 (0.000–0.308)	NS	Unbiased
Mirap	2015–2017	humans	1781	193	0.87	0.472 (0.423–0.522)	< 0.0001	Under-selected
		pigs	181	65	0.09	0.159 (0.125–0.198)	< 0.0001	Over-selected
		dogs	90	151	0.04	0.369 (0.322–0.418)	< 0.0001	Over-selected
Wasab	2012–2013	humans	211	11	0.639	0.917 (0.615–0.998)	NS	Unbiased
		pigs	71	1	0.215	0.083 (0.002–0.385)	NS	Unbiased
		dogs	48	0	0.145	0.000 (0.000–0.265)	NS	Unbiased
Wasab	2015–2016	humans	295	331	0.52	0.731 (0.687–0.771)	< 0.0001	Over-selected
		pigs	207	69	0.37	0.152 (0.120–0.189)	< 0.0001	Under-selected
		dogs	64	53	0.11	0.117 (0.089–0.150)	NS	Unbiased

Abbreviation: NS, not significant

Table 6.4 (cont'd).

Village	Period	Host	Census	Blood meals	Exp	Obs (95% CI)	*P*	Host selection
Bulal	2017	humans	489	16	0.719	0.571 (0.372–0.755)	NS	Unbiased
		pigs	92	4	0.135	0.143 (0.040–0.327)	NS	Unbiased
		dogs	99	8	0.146	0.286 (0.132–0.487)	NS	Unbiased

Abbreviation: NS, not significant

Table 6.5. Number of humans, pigs and dogs in village census and blood meals of two populations of *An. longirostris* along with their host selection tendency based on exact binomial comparison of the expected (Exp) and observed (Obs) blood index.

Village	Period	Host	Census	Blood meals	Exp	Obs (95% CI)	*P*	Host selection
Wasab	2012–2013	humans	211	57	0.639	0.781 (0.669–0.869)	0.014	Over-selected
		pigs	71	14	0.215	0.192 (0.109–0.301)	NS	Unbiased
		dogs	48	2	0.145	0.027 (0.003–0.095)	0.001	Under-selected
Mirap	2012–2013	humans	732	3	0.767	0.250 (0.055–0.572)	0.0002	Under-selected
		pigs	110	8	0.115	0.667 (0.349–0.901)	< 0.0001	Over-selected
		dogs	112	1	0.117	0.083 (0.002–0.385)	NS	Unbiased

Abbreviation: NS, not significant

Table 6.6. Number of humans, pigs and dogs in village census and blood meals of six populations of *An. punctulatus* (*s.s.*) along with their host selection tendency based on exact binomial comparison of the expected (Exp) and observed (Obs) blood index.

Village	Period	Host	Census	Blood meals	Exp	Obs (95% CI)	*P*	Host selection
Dimer	2012	humans	622	20	0.712	0.645 (0.454–0.808)	NS	Unbiased
		pigs	158	2	0.181	0.065 (0.008–0.214)	NS	Unbiased
		dogs	94	9	0.108	0.290 (0.142–0.480)	0.004	Over-selected
Mirap	2012–2013	humans	732	8	0.767	0.889 (0.518–0.997)	NS	Unbiased
		pigs	110	1	0.115	0.111 (0.003–0.482)	NS	Unbiased
		dogs	112	0	0.117	0.000 (0.000–0.336)	NS	Unbiased
Mirap	2015–2017	humans	1781	0	0.87	0.00 (0.000–0.232)	< 0.0001	Under-selected
		pigs	181	12	0.09	0.857 (0.572–0.982)	< 0.0001	Over-selected
		dogs	90	2	0.04	0.143 (0.018–0.428)	NS	Unbiased
Wasab	2012–2013	humans	211	24	0.639	0.800 (0.614–0.923)	NS	Unbiased
		pigs	71	4	0.215	0.133 (0.038–0.307)	NS	Unbiased
		dogs	48	2	0.145	0.067 (0.008–0.221)	NS	Unbiased
Wasab	2015–2016	humans	295	72	0.52	0.720 (0.621–0.805)	< 0.0001	Over-selected
		pigs	207	9	0.37	0.090 (0.042–0.164)	< 0.0001	Under-selected
		dogs	64	19	0.11	0.190 (0.118–0.281)	0.025	Over-selected

Abbreviation: NS, not significant

Table 6.6 (cont'd)

Village	Period	Host	Census	Blood meals	Exp	Obs (95% CI)	*P*	Host selection
Bulal	2017	humans	489	4	0.719	0.500 (0.157–0.843)	NS	Unbiased
		pigs	92	1	0.135	0.125 (0.003–0.527)	NS	Unbiased
		dogs	99	3	0.146	0.375 (0.085–0.755)	NS	Unbiased

Abbreviation: NS, not significant

6.4.3. Relationship between bed net usage and anthropophagy

The proportion of interviewed villagers that reportedly slept under a bed net and the mean number of household members per bed net in each village during one or two mosquito sampling periods are shown in Table 6.7. The results of multiple logistic regression which tested the association between the two predictor variables and the probability of obtaining a human blood meal by mosquitoes (i.e., anthropophagy) of *An. farauti* (*s.s.*), *An. koliensis* and *An. punctulatus* (*s.s.*) are shown in Table 6.8; the other three vector species were not assessed because of low sample size or a single population. For all of the three vector species tested, low anthropophagy was associated with high bed net usage in the village. This association was strongly significant for *An. farauti* (*s.s.*) and *An. koliensis* ($P < 0.05$), and showed the same trend but was "marginally significant" ($P = 0.096$) for *An. punctulatus* (*s.s.*). No statistical relationship was observed between anthropophagy and mean number of persons per net for all three vectors.

Table 6.7. Two bed net metrics in each village during one or more sampling periods.

Village (sampling period)	Proportion of people using a bed net	Mean number of people/bed net
Bulal (2017)	0.93	1.47
Dimer (2012)	0.46	1.41
Matukar (2012)	0.55	1.48
Megiar (2017)	0.81	1.16
Mirap (2012–2013)	0.84	2.45
Mirap (2015–2017)	0.94	1.81
Wasab (2012–2013)	0.58	1.89
Wasab (2015–2016)	0.90	1.56

Table 6.8. Results of multiple logistic regression which tested for association between mosquito anthropophagy and two predictor variables.

Species	*N*	variable	*β*	SE	*P*
An. farauti (*s.s.*)	1,537	Bed net usage	-3.58	0.82	< 0.0001
		Mean persons/net	-0.15	0.11	0.162
An. koliensis	920	Bed net usage	-15.87	3.71	< 0.0001
		Mean persons/net	-0.98	0.55	0.076
An. punctulatus (*s.s.*)	219	Bed net usage	-1.37	0.82	0.096
		Mean persons/net	0.66	0.66	0.322

Abbreviations: *N*, number of mosquitoes; *β*, regression coefficient; SE, standard error of the regression coefficient; *P*, p-value

6.5. Discussion

This study found that humans, pigs, and dogs were the primary blood-meal sources of the *Anopheles* vectors of malaria in these villages despite the presence of cats, chickens, and ducks in the villages. The narrow host range observed here is consistent with previous studies (Burkot, et al. 1989; Burkot, et al. 1988a; Charlwood, et al. 1985b; Hii, et al. 1997; Hii, et al. 2001; Logue, et al. 2016), although some previous studies also detected a small number of mosquitoes that had fed on cats, chickens, opossum and bats. This difference may be due to the use of different sampling methods such as outdoor resting collection by some previous studies, but those hosts were uncommon in all cases compared to the three primary hosts.

A number of studies (Burkot, et al. 1989; Burkot, et al. 1988a; Charlwood, et al. 1985b; Hii, et al. 1997; Hii, et al. 2001) have described the host preference of PNG vectors based on patterns of host selection using the human blood index. A mosquito species that had consistently high human blood index was classified as anthropophilic ("human loving"), whilst those with consistently low human blood index as zoophilic ("animal loving"); one with variable human

blood index was considered opportunistic (i.e. selects hosts indiscriminately). However, an important limitation with this approach is that it does not take into account the relative abundance of the different host species within the mosquito foraging habitat. Various measures including the forage ratio (Hess, et al. 1968), the feeding index (Kay, et al. 1979) and the Manly resource selection ratio (Hamer, et al. 2011; Hamer, et al. 2009), have been used to evaluate mosquito host selection. These measures are inter-related, and each requires some information of the range of available hosts, their relative abundance and related attributes. The Manly resource selection ratio is preferable because it provides a statistical test of departure from random host selection with confidence intervals. However, there is no upper limit to the range of values it can take. Therefore, in this study exact binomial test which also provides a statistical test of departure from randomness with confidence intervals and whose values are constraint between zero (lower limit) and one (upper limit) was used to quantify host selection.

The intrinsic host preference of the *Anopheles* species investigated here can be delineated based on their pattern of host selection. The tendency of the two *An. bancroftii* populations to feed on pigs despite overwhelming abundance of humans relative to pigs in the village indicates that this species is zoophilic in its host preference. Although the current data supporting this assertion is weak (low sample size and only two populations), this finding is consistent with a previous study (Hii, et al. 1997) which found *An. bancroftii* with very low human blood index even when the other species in the same village had high human blood index. Four species, namely *An. farauti* (*s.s.*), *An. koliensis*, *An. Longirostris,* and *An. punctulatus* (*s.s.*) can be considered opportunistic as they exhibited varying host selection among populations. Three of the five *An. farauti* (*s.s.*) populations exhibited a biased affinity for pig blood whereas the other two exhibited unbiased selection of the three hosts. Of the six *An. koliensis* populations, four

exhibited unbiased selection of the three hosts, one over-selected pigs and the other over-selected humans. Of the two *An. longirostris* populations, one over-selected humans and the other over-selected pigs. Three of the six *An. punctulatus* (*s.s.*) populations exhibited unbiased selection of the hosts and the other three exhibited biased affinity for humans, pigs or dogs. The plasticity of host selection by the four species observed here is consistent with previous studies, which found large variation in the human blood index among populations of these species (Burkot, et al. 1989; Burkot, et al. 1988a; Charlwood, et al. 1985b).

A range of factors and processes influence the host selection of opportunistic mosquitoes. These include host availability, host density, physical access to hosts, differential host attractiveness, behavior of hosts in response to mosquitos' attempts to feed, and a mosquito's intrinsic host preference (Lyimo and Ferguson 2009; Takken and Verhulst 2013). This study emphasized relative abundance of hosts as a determining factor and found that mosquito host selection was not merely a function of relative host abundance. Rather, the strong affinity for pig or dog blood meals by many of the mosquito populations, particularly the *An. farauti* (*s.s.*), despite overwhelmingly high abundance of humans relative to the non-humans in all villages indicates that other factors impinged on it. One likely factor causing the observed host selection bias was unavailability of many human hosts due to protective effects of bed nets. The opportunistic nature of their host preference allows them to respond flexibly to host utilization under varying levels of bed net usage. This contention was supported by the logistic regression test which showed that low anthropophagy exhibited by these species was associated with higher levels of bed net usage. Although *An. farauti* no. 4 was found in only one village and its host selection is reported here for the first time, marked overutilization of pigs indicates that it is also

an opportunistic species, responding flexibly to host utilization under high bed net usage (92%) in Kokofine.

The modulation of host selection by bed nets has been observed both in PNG and elsewhere. A study in Kenya showed that the majority of blood meals taken by *Anopheles funestus* and *Culex quinquefasciatus* changed from humans before permethrin-impregnated bed nets were distributed to non-humans after the bed net distribution (Bogh, et al. 1998). In a coastal village of Madang, PNG, the proportion of blood meals taken on humans by *An. farauti* (*s.l.*) dropped from 70% before permethrin-impregnated nets were distributed to 38% after the bed net distribution (Charlwood and Graves 1987). In the Wosera district of East Sepik province, PNG, increased use of insecticide-free bed nets resulted in a decline of the human blood index of *Anopheles* mosquitoes (Hii, et al. 2001).

The use of insecticidal bed nets for controlling mosquito vectors remains the primary malaria intervention method in PNG. The reduction of malaria incidence, prevalence (Hetzel, et al. 2015; Hetzel, et al. 2017; Hetzel, et al. 2016) and transmission intensity (Reimer, et al. 2016) in PNG in recent years have been attributed to the intensification of the nationwide bed net campaign over the last 1.5 decades (Hetzel 2009; Hetzel, et al. 2014a; Hetzel, et al. 2012; Hetzel, et al. 2014b). No resistance to pyrethroids has been detected in malaria vector populations in PNG (Katusele, et al. 2014; Keven, et al. 2010; Koimbu, et al. 2018), but transmission continues to persist and very recent data indicate malaria resurgence in four of the seven study villages (chapter 3), perhaps in large part because of the opportunistic host preference of the vectors described here whose lack of dependence on human blood allows them to escape the lethal effect of the insecticides in the bed net fabric. These vector species live in sympatry and co-transmit malaria in most of the endemic areas of PNG (Burkot, et al. 1988b; Charlwood, et al. 1985a;

Charlwood, et al. 1985b; Charlwood and Graves 1986; Hetzel, et al. 2016; Hii, et al. 2000; Hii, et al. 1997; Hii, et al. 2001; Reimer, et al. 2016; Thomsen, et al. 2017). Therefore, while bed nets may affect the more anthropophilic and vulnerable species, transmission is still sustained by the more opportunistic and behaviorally flexible species. This condition, when combined with increased outdoor and early-evening biting observed in some vector population of PNG ((Reimer, et al. 2016; Thomsen, et al. 2017); chapter 2 and 4), presents a challenge to the bed net program in PNG as well as the rest of the South West Pacific region where these opportunistic species are the primary regional vectors.

This study was not without limitations. First, although the statistical analysis of host selection was based on the total number of the three primary hosts in the village, there were variations in the relative number of these hosts among households. Therefore, the household-level variation can bias the host selection results because mosquitoes were sampled near a household throughout the night. This bias was minimized by relocating each barrier screen to a new location in the village every subsequent night to try and capture a good representation of the village (see chapter 2, section 2.3). Second, the mobility of domestic hosts, including humans, throughout the night while mosquitoes were being sampled on the barrier screen can change the actual availability of the hosts and bias the result of host selection. However, it was observed that although the animals roamed freely, dogs and pigs were found mostly close to their owner's house during the night. Third, readers may be concerned with bias associated with indoor resting mosquitoes not sampled by the barrier screen method. The effect of this bias is minimal as members of the *An. punctulatus* (*s.l.*) group are primarily exophilic even when they feed indoors (Sinka, et al. 2011).

6.6. Conclusion

This study investigated the host selection of different populations of six *Anopheles* species and delineated their intrinsic host preference based on their pattern of host selection. It was found that, with exception of *An. bancroftii* which was zoophilic, the other vector species were opportunistic blood feeders. The study also found that the opportunistic nature of the mosquito host preference causes them to flexibly divert blood feeding away from humans to the two non-human hosts when the level of bed net usage by the village inhabitants increases. The host selection plasticity of these opportunistic vectors can potentially limit the success of the bed net program in PNG and the Southwest Pacific region where these *Anopheles* mosquitoes are the primary transmitters of malaria. While malaria elimination by the bed net program is achievable from areas of the world where strict anthropophilic vectors are present, it is difficult for bed nets alone to achieve malaria elimination in PNG where most of the vectors are opportunistic. These opportunistic vectors will be resilient to bed nets and continue to transmit the disease. Thus, alternative vector control methods or strategies such as treatment of pigs and dogs with endectocides need to be developed and implemented alongside the bed net program in PNG.

APPENDIX

Appendix 6.1. Tabulated blood-meal sources of mosquitoes

Table S6.1. *An. bancroftii* blood-meal sources. Numbers outside parenthesis are mosquito counts (*n*) and inside parenthesis are percent of column-wise totals. Dashes ("-") along columns indicate villages with zero blood-meal samples for this mosquito species.

		Villages						
Blood-meal type	**Host**	**Bulal** *n* (%)	**Dimer** *n* (%)	**Kokofine** *n* (%)	**Matukar** *n* (%)	**Megiar** *n* (%)	**Mirap** *n* (%)	**Wasab** *n* (%)
Single	Human	-	1 (100.0)	-	-	-	5 (12.8)	-
	Pig	-	0 (0.0)	-	-	-	30 (76.9)	-
	Dog	-	0 (0.0)	-	-	-	3 (7.7)	-
Mixed	Human/Pig	-	0 (0.0)	-	-	-	0 (0.0)	-
	Human/Dog	-	0 (0.0)	-	-	-	1 (2.6)	-
	Pig/Dog	-	0 (0.0)	-	-	-	0 (0.0)	-
	Human/Pig/Dog	-	0 (0.0)	-	-	-	0 (0.0)	-

Table S6.2. *An. farauti* (*s.s.*) blood-meal sources. Numbers outside parenthesis are mosquito counts (*n*) and inside parenthesis are percent of column-wise totals. Dashes ("-") along columns indicate villages with zero blood-meal samples for this mosquito species.

		Villages						
Blood-meal type	**Host**	**Bulal** *n* (%)	**Dimer** *n* (%)	**Kokofine** *n* (%)	**Matukar** *n* (%)	**Megiar** *n* (%)	**Mirap** *n* (%)	**Wasab** *n* (%)
Single	Human	0 (0.0)	2 (100.0)	-	33 (63.5)	103 (48.1)	576 (43.2)	17 (63.0)
	Pig	0 (0.0)	0 (0.0)	-	2 (3.9)	64 (29.9)	558 (41.9)	6 (22.2)
	Dog	1 (100.0)	0 (0.0)	-	8 (15.4)	24 (11.2)	143 (10.7)	3 (11.1)
Mixed	Human/Pig	0 (0.0)	0 (0.0)	-	2 (3.9)	10 (4.7)	30 (2.3)	0 (0.0)
	Human/Dog	0 (0.0)	0 (0.0)	-	4 (7.7)	10 (4.7)	11 (0.8)	1 (3.7)
	Pig/Dog	0 (0.0)	0 (0.0)	-	1 (1.9)	3 (1.4)	14 (1.1)	0 (0.0)
	Human/Pig/Dog	0 (0.0)	0 (0.0)	-	2 (3.9)	0 (0.0)	0 (0.0)	0 (0.0)

Table S6.3. *An. farauti* no. 4 blood-meal sources. Numbers outside parenthesis are mosquito counts (*n*) and inside parenthesis are percent of column-wise totals. Dashes ("-") along columns indicate villages with zero blood-meal samples for this mosquito species.

		Villages						
Blood-meal type	**Host**	**Bulal** *n* (%)	**Dimer** *n* (%)	**Kokofine** *n* (%)	**Matukar** *n* (%)	**Megiar** *n* (%)	**Mirap** *n* (%)	**Wasab** *n* (%)
Single	Human	-	-	220 (47.6)	-	-	-	-
	Pig	-	-	188 (40.7)	-	-	-	-
	Dog	-	-	33 (7.1)	-	-	-	-
Mixed	Human/Pig	-	-	10 (2.2)	-	-	-	-
	Human/Dog	-	-	8 (1.7)	-	-	-	-
	Pig/Dog	-	-	3 (0.7)	-	-	-	-
	Human/Pig/Dog	-	-	0 (0.0)	-	-	-	-

Table S6.4. *An. koliensis* blood-meal sources. Numbers outside parenthesis are mosquito counts (*n*) and inside parenthesis are percent of column-wise totals. Dashes ("-") along columns indicate villages with zero blood-meal samples for this mosquito species.

		Villages						
Blood-meal type	**Host**	**Bulal** *n* (%)	**Dimer** *n* (%)	**Kokofine** *n* (%)	**Matukar** *n* (%)	**Megiar** *n* (%)	**Mirap** *n* (%)	**Wasab** *n* (%)
Single	Human	16 (47.1)	2 (50.0)	1 (33.3)	-	6 (54.6)	202 (42.1)	342 (62.4)
	Pig	4 (11.8)	1 (25.0)	1 (33.3)	-	2 (18.2)	66 (13.8)	70 (12.8)
	Dog	8 (23.5)	1 (25.0)	0 (0.0)	-	1 (9.1)	151 (31.5)	53 (9.7)
Mixed	Human/Pig	2 (5.9)	0 (0.0)	0 (0.0)	-	1 (9.1)	19 (4.0)	22 (4.0)
	Human/Dog	2 (5.9)	0 (0.0)	1 (33.3)	-	1 (9.1)	27 (5.6)	47 (8.6)
	Pig/Dog	2 (5.9)	0 (0.0)	0 (0.0)	-	0 (0.0)	12 (2.5)	9 (1.6)
	Human/Pig/Dog	0 (0.00)	0 (0.0)	0 (0.0)	-	0 (0.0)	3 (0.6)	5 (0.9)

Table S6.5. *An. longirostris* blood-meal sources. Numbers outside parenthesis are mosquito counts (*n*) and inside parenthesis are percent of column-wise totals. Dashes ("-") along columns indicate villages with zero blood-meal samples for this mosquito species.

		Villages						
Blood-meal type	**Host**	**Bulal** *n* (%)	**Dimer** *n* (%)	**Kokofine** *n* (%)	**Matukar** *n* (%)	**Megiar** *n* (%)	**Mirap** *n* (%)	**Wasab** *n* (%)
Single	Human	-	3 (75.0)	-	0 (0.0)	-	3 (15.8)	57 (67.9)
	Pig	-	0 (0.0)	-	1 (50.0)	-	10 (52.6)	15 (17.9)
	Dog	-	1 (25.0)	-	0 (0.0)	-	3 (15.8)	3 (3.6)
Mixed	Human/Pig	-	0 (0.0)	-	0 (0.0)	-	1 (5.3)	9 (10.7)
	Human/Dog	-	0 (0.0)	-	0 (0.0)	-	2 (10.5)	0 (0.0)
	Pig/Dog	-	0 (0.0)	-	0 (0.0)	-	0 (0.0)	0 (0.0)
	Human/Pig/Dog	-	0 (0.0)	-	1 (50.0)	-	0 (0.0)	0 (0.0)

Table S6.6. *An. punctulatus* (*s.s.*) blood-meal sources. Numbers outside parenthesis are mosquito counts (*n*) and inside parenthesis are percent of column-wise totals. Dashes ("-") along columns indicate villages with zero blood-meal samples for this mosquito species.

		Villages						
Blood-meal type	**Host**	**Bulal** *n* (%)	**Dimer** *n* (%)	**Kokofine** *n* (%)	**Matukar** *n* (%)	**Megiar** *n* (%)	**Mirap** *n* (%)	**Wasab** *n* (%)
Single	Human	4 (50.0)	20 (58.8)	-	2 (50.0)	-	8 (32.0)	118 (72)
	Pig	1 (12.5)	2 (5.9)	-	2 (50.0)	-	13 (52.0)	17 (10.4)
	Dog	3 (37.5)	9 (26.5)	-	0 (0.0)	-	2 (8.0)	22 (13.4)
Mixed	Human/Pig	0 (0.0)	1 (2.9)	-	0 (0.0)	-	1 (4.0)	1 (0.6)
	Human/Dog	0 (0.0)	1 (2.9)	-	0 (0.0)	-	0 (0.0)	4 (2.4)
	Pig/Dog	0 (0.0)	1 (2.9)	-	0 (0.0)	-	1 (4.0)	1 (0.6)
	Human/Pig/Dog	0 (0.0)	0 (0.0)	-	0 (0.0)	-	0 (0.0)	1 (0.6)

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CHAPTER 7: NON-RANDOM HOST SELECTION AND MIXED HUMAN BLOOD MEALS BY *ANOPHELES* VECTORS OF MALARIA IN MADANG, PAPUA NEW GUINEA: IMPLICATIONS FOR MALARIA TRANSMISSION

7.1. Abstract

Non-random distribution of blood meals and multiple blood meals taken from humans by *Anopheles* vectors of malaria can multiply the force of transmission of malaria and allow the disease to persist even when interventions are in place. This study investigated these phenomena in Madang villages, using barrier screen sampling of blood fed mosquitoes, and human genotyping of blood meals and villagers using microsatellite loci. Blood meals with three or more alleles in multiple microsatellite loci signaled cryptic meals from more than one person. DNA profiles of mosquito collectors and village residents were analyzed and checked for matches with the blood-meal profiles. The distribution of blood meals from individual humans (collector-fed blood meals were excluded) fitted the negative binomial better than Poisson model, indicating non-random utilization of individual human hosts. Males and individuals 15–30 years old were statistically overrepresented in blood meals relative to females and younger or older age groups. However, the risk of malaria infection was generally similar across sexes and age groups. The proportion of cryptic mixed meals ranged from 5.5–15%. The probability of an interrupted feeding on a human leading to a second feeding on a different human ranged from 0.03–0.18 for six *Anopheles* populations across four villages. Because non-random human selection and cryptic mixed blood-feeding behaviors can multiply the basic reproduction number of malaria and vectorial capacity of vectors, malaria is likely to persist despite ongoing efforts to

suppress it by insecticidal bed net intervention programs. The biased feeding on young males emphasizes the importance of targeted rather than population-wide disease intervention.

7.2. Introduction

Analysis of mosquito blood meals using molecular methods to identify vertebrate host species fed on by mosquitoes is widely used and has enabled (i) evaluation of important aspects of mosquito-host interactions such as host selection that affect vector-borne disease ecology and (ii) assessment of effectiveness of vector control measures such as insecticide-treated bed nets (Fritz, et al. 2013; Gunathilaka, et al. 2016; Hamer, et al. 2011; Hamer, et al. 2009; Hurk, et al. 2007; Kent and Norris 2005; Keven, et al. 2017; Logue, et al. 2016; Meece, et al. 2005; Molaei, et al. 2006; Molaei, et al. 2010). In recent years, analysis of blood meals has been extended to include multilocus genotyping of microsatellite (aka short tandem repeat) markers that serve as unique DNA profiles (aka DNA fingerprints) to identify or differentiate individuals of a species, particularly humans in blood-meal samples (Benedictis, et al. 2003; Chow-Shaffer, et al. 2000; Harrington, et al. 2014; Keven, et al. 2019b; Michael, et al. 2001; Scott, et al. 2006; Soremekun, et al. 2004).

Identification of human individuals in mosquito blood meals has three important applications in studies of mosquito-borne disease ecology. First, if mosquito biting rates are heterogeneous so that some individuals in a community are bitten more frequently than others, these individuals are more likely to be infected and serve as reservoirs or even sinks of mosquito-borne pathogens. Thus, identification of such individuals or their characteristics can inform public health officials, particularly those in resource-limited countries, to conduct targeted rather than population-wide disease intervention. For example, distributing interventions (antimalarial

prophylactic drugs, insecticide treated bed nets) to those segments of the human population who are bitten in excess could reduce prevalence of infection in parasite reservoirs and thereby diminish rates of transmission, simultaneously increasing effectiveness of the intervention and reducing costs (Bousema, et al. 2010; Bousema, et al. 2012; Greenwood 2010; Nofal, et al. 2019; Noor, et al. 2010).

Second, assessing patterns of mosquito biting frequencies among individuals is important to prognose the course of disease epidemiology, particularly in the context of a disease control program. Malaria prognosis is usually based on two epidemiological quantities: (i) basic reproduction number (R_0), which is the number of new human infection cases in a susceptible population that arises as a result of transmission from a single primary case throughout its infective life and (ii) the vectorial capacity (V), which is the expected number of infective bites that arises from all the *Anopheles* that bite a single infectious person in a single day (Smith, et al. 2012). Indeed, V is a submodel of R_0. Some studies have shown that the values of both R_0 and V under non-random conditions of vector-human contact (whether socially or spatially) are higher than under random conditions (Dye and Hasibeder 1986; Hasibeder and Dye 1988). As malaria transmission increases with R_0 and V, such non-random biting patterns may permit transmission to persist with potential for resurgence, even when a vector control program is underway (Dye and Hasibeder 1986; Hasibeder and Dye 1988).

Third, molecular methods that can distinguish between different individuals of a host species can be used to estimate the proportion of blood-fed mosquitoes that had obtained a blood meal from two or more individuals of that species, hereafter referred to as “cryptic mixed meals”. The term was used to distinguish this blood-meal type from those that obtained a blood meal from two or more individuals of different host species or “patent mixed meals” (Boreham

and Garrett-Jones 1973; Burkot, et al. 1988). Importantly, cryptic mixed meals occur within a single gonotrophic cycle, meaning there were multiple contacts between an individual female mosquito and humans rather than assuming (as is the case with transmission models) that there is only one contact per gonotrophic cycle. Cryptic mixed meals have been shown to increase both R_0 and V; an effect that has been termed a "force multiplier" (Tedrow, et al. 2019).

In this study, DNA profiles of humans in mosquito blood meals were analyzed and the data were used to quantify the proportion of cryptic mixed blood meals, estimate the probability of a human feed being interrupted, and test for non-random vector-human contacts in *Anopheles* vectors of malaria in PNG.

7.3. Methods

7.3.1. Mosquito and human blood samples

This study involved analysis of blood-fed female *Anopheles* mosquitoes from Bulal, Megiar, Mirap and Wasab villages. Geographic location (see Figure 2.1) and ecogeographic description of these villages are described in chapter 2. Mosquitoes were collected for six nights in Bulal (16th–21st March 2017), Megiar (4th–9th February 2017) and Mirap (11th–16th January 2017) and 12 nights in Wasab (5th–10th September and 4th–9th November 2016) using the barrier screen method and sampling procedure described in chapter 2. The mosquito species and their blood meal hosts were identified by PCR methods described in chapter 6. Census counts of male and female humans, and blood sample and age of consented individuals (including mosquito collectors) in Mirap and Wasab were obtained as described in chapter 3. Finger-pricked human blood samples (250 μl) were obtained from consented residents (ages 0.5–85 years; consent for minors obtained from parents or guardians) and the mosquito collectors working in the two

villages. The human blood samples were tested for *P. falciparum* and *P. vivax* infection using the qPCR method described in chapter 3.

7.3.2. Human genotyping

Human genotypes ("DNA profiles") of the village residents (including the mosquito collectors) of Mirap and Wasab and the *Anopheles* blood meals of all four villages were analyzed using a microsatellite genotyping assay used in forensic identification (Oostdik, et al. 2014). The assay utilized a multiplex PCR kit (PowerPlex Fusion System; catalog number, DC2402; Promega, Madison, WI, USA) which contained fluorophore-labelled primer pairs that co-amplified 22 autosomal microsatellite loci (D3S1358, D1S1656, D2S441, D10S1248, D13S317, Penta E, D16S539, D18S51, D2S1338, CSF1PO, Penta D, TH01, vWA, D21S11, D7S820, D5S818, TPOX, D8S1179, D12S391, D19S433, FGA, D22S1045), one Y-linked microsatellite locus (DYS391), and one locus serving as a sex-specific marker (Amel) (Oostdik, et al. 2014). The PCR reaction mixture (10 µl reaction volume) consisted of 6 µl of water, 1 µl of 5x Fusion Master Mix, 1 µl of 5x Fusion Primer Mix, 0.5 µl of 10x PCR Buffer (product number, N8080010; Thermo Fisher Scientific, Waltham, MA, USA), 0.5 µl of 25 mM $MgCl_2$ (product number, N8080010; Thermo Fisher Scientific) and 1 µl of template DNA. Thermal cycling consisted of 1 cycle of 96 °C for 1 minute followed by 30 cycles of 94 °C for 10 seconds, 59 °C for 1 minute, 72 °C for 30 seconds, and 1 cycle of 60 °C for 10 minutes.

For detection of amplicon size, solution mixtures consisting of 1 µl of the PCR products or Allelic Ladder (product number, DG381B; Promega) combined with 0.3 µl of WEN Internal Lane Standard 500 (product number, DG5001; Promega) and 9 µl of Hi-Di Formamide (catalog number, 4311320; Thermo Fisher Scientific) were analyzed by capillary electrophoresis (3500

Genetic Analyzer; Applied Biosystems). Results were analyzed using GeneMapper software version 4.1 (Applied Biosystems).

Microsatellite alleles were represented by the number of sequence repeats, which can be heterozygous or homozygous. The sex-specific marker indicated a female individual if it was homozygous (X, X) or a male if heterozygous (X, Y). For each sample, the genotype of each locus was listed in a Microsoft Excel spreadsheet. The alleles of each marker were separated by a comma; for heterozygous genotypes, the alleles were listed in order from small to large. Blood-meal samples with more than two alleles at one or more loci were considered cryptic mixed meals from different human hosts.

7.3.3. Data analyses

Procedure for identifying unique DNA profiles. A program was written in R that engaged loop functions and conditional statements to compare the genotype of each locus in a query sequence (i.e., a DNA profile) to its corresponding locus in a subject sequence (i.e., another DNA profile). The similarity of two DNA profiles was quantified based on their percent profile match, which is the proportion of matched loci times 100 (e.g., 18 matched loci/24 total loci x 100 = 75% profile match). In principle, DNA profiles from two blood-meal specimens that originated from a same human source will have 100% profile match, thus, match values < 100% indicate blood meals from different individuals. However, as some blood-meal specimens yield suboptimum human DNA (due to a tiny amount of engorged blood, for example), false mismatches due to allele dropouts can arise. To account for this, a value less than 100% was used as the criterion for determining a match. To determine this value, pairwise percent profile match analysis was performed on the DNA profiles of village residents. This generated $n(n\text{-}1)/2$ profile match results

or values, where *n* is the number of village residents. The highest value in the pairwise match output (excluding match results of monozygotic twins, which was 100%) was the criterion below which two DNA profiles were considered different.

Derivation of formulae for estimating probability of a human feed being interrupted. A blood-feeding mosquito that is interrupted by its human host before obtaining a full blood meal can complete it by feeding on another human (cryptic mixed meal) or a different host species (patent mixed meal) (Burkot, et al. 1988). The probability (*I*) of an interrupted feed resulting in a second meal, either cryptic or patent, can be estimated from the equation $I = \frac{(H_p * B) + H_c}{(H_p * B) + H_c + H_s}$, where H_p is the number of patent mixed human meals, *B* is the human blood index (proportion of all single blood meals that are humans), H_c is the number of cryptic mixed human meals and H_s is the number of single human meals. H_p consists of two groups of mosquitoes: those that feed on non-humans after being interrupted during feeding on a human (i.e., human to non-human), and those that feed on a human after being interrupted during feeding on a non-human (non-human to human). Because the interest is in the probability of a human feed (and not a non-human feed) being interrupted, the latter group (non-human to human) overestimates *I*. This is corrected by multiplying H_p with *B* in the equation, which eliminates the portion of H_p that consists of non-human to human patent mixed meals. The quantity *I* represents the probability of a human feed being interrupted that led to a second meal on another host of any species; not just humans. To estimate the probability (I_H) of a second meal on humans only, the patent mixed meal component in the numerator is eliminated to derive the equation $I_H = \frac{H_c}{(H_p * B) + H_c + H_s}$.

Test of non-random human selection. To eliminate collector-feeding bias in the analysis of non-random human selection by mosquitoes, blood-meal samples whose DNA profile

matched that of the people who collected them were removed from the data. Cryptic mixed meals were not useful for this analysis and were also removed from the data. The number of different human individuals in the blood-meal sample was determined from the number of unique DNA profiles in the sample. The number of occurrences of each unique profile in the blood-meal sample represented the number of mosquitoes that fed on each human individual. These data were used to construct frequency distribution histograms which relate the number of different human individuals (y-axis) to the number of blood-meal occurrences (x-axis). The observed frequency distributions were fitted to zero-truncated Poisson and zero-truncated negative binomial frequency distribution models using the functions *zerotrunc* and *rootogram* of the package *countreg* (Kleiber and Zeileis 2016) in R software. Statistically supported fit to the Poisson model indicates random selection of human individuals by the mosquitoes, presumably reflecting no bias, whereas fit to the negative binomial indicates non-random selection, presumably reflecting bias. The observed frequency of blood meals taken on human individuals of a specific sex or age group was compared with their expected frequency (obtained from village census data) by the two-tailed exact binomial test. The likelihood of being infected with *P. falciparum* and *P. vivax* in human individuals of different age groups and sexes was tested by logistic regression of binary data. The two tests were performed using the functions *binom.test* and *glm* of the R package *stats*. Significance level (all tests) was based on type I error rate of 5%.

7.4. Results

7.4.1. Percent match values of human profiles

DNA profiles of 427 villagers of Mirap (205 males, 222 females) and 164 villagers of Wasab (90 males, 74 females), all consented residents, were successfully analyzed. Pairwise percent match

analysis of these DNA profiles resulted in 90,951 and 13,366 pairwise percent match values for Mirap and Wasab, respectively. For both villages, these pairwise results consisted of 18 unique percent match values, each with a varying number of occurrences. The distribution of the probability (i.e., proportion) of occurrence of the unique values is shown in Figure S7.1 (Appendix 7.1). The lowest percent match value was 0% with 0.05 probability of occurrence, and the highest value was 71% (i.e., 17/24 matched loci) with 10^{-4} probability of occurrence. Although 71% was the maximum percent match value, 79% (which allows for five false mismatched loci) whose probability of occurrence was zero was chosen as the criterion below which two DNA profiles were considered as belonging to different people.

7.4.2. Cryptic mixed meals and probability of interrupted feeds

Of 881 human-fed *Anopheles* blood meals that were genotyped, 85% (n = 753) yielded a DNA profile; the other 15% (n = 128) either did not PCR-amplify or had grossly incomplete profiles. Of those blood-meal samples that yielded a DNA profile, 18 were from Bulal, 124 from Megiar, 233 from Mirap and 378 from Wasab. The distribution of these profiles according to mosquito species, namely *An. bancroftii*, *An. farauti* (*s.s.*), *An. koliensis*, *An. longirostris* and *An. punctulatus* (*s.s.*), is presented in Table 7.1. The proportion of cryptic mixed meals for *Anopheles* spp. in each village and for three populations with sample size > 100 is shown in Figure 7.1. The numbers of cryptic mixed (H_c), patent mixed (H_p) and single (H_s) human meals for six *Anopheles* populations across the four villages are shown in Table 7.2; other populations (see Table 7.1) were excluded due to small sample size (n < 10). These three quantities were then used together with B (human blood index) to quantify I (probability of an interrupted blood meal on a human leading to a blood meal on another host of any species) and I_H (probability of an interrupted

blood meal on a human leading to a second blood meal on another human) for each of the six populations (Table 7.2). The values of *B* in Table 7.2 were computed from blood-meal data in Table S7.1 (Appendix 7.2). The estimated values of *I* ranged from 0.09–0.30, and of I_H ranged from 0.03-0.18 (Table 7.2). As expected, the mean of *I* (0.2 ± 0.08) was statistically greater than that of I_H (0.1 ± 0.06) (t-test: $t_{(9)} = 2.28$, $P = 0.047$).

Table 7.1. Distribution of successfully genotyped blood meals according to *Anopheles* species.

***Anopheles* species**	**Bulal**	**Megiar**	**Mirap**	**Wasab**
An. bancroftii	0	0	1	0
An. farauti (*s.s.*)	0	116	31	3
An. koliensis	15	8	200	339
An. longirostris	0	0	1	0
An. punctulatus (*s.s.*)	3	0	0	36

Table 7.2. Estimates of blood host choice parameters for six *Anopheles* populations.

Village	***Anopheles* species**	**Year**	***N***	H_s	H_p	H_c	***B***	***I***	I_H
Bulal	*An. koliensis*	2017	15	10	3	2	0.57	0.27	0.15
Megiar	*An. farauti* (*s.s.*)	2017	116	93	16	7	0.54	0.14	0.06
Mirap	*An. farauti* (*s.s.*)	2017	31	21	5	5	0.29	0.23	0.18
Mirap	*An. koliensis*	2017	200	145	44	11	0.47	0.18	0.06
Wasab	*An. koliensis*	2016	339	227	60	52	0.72	0.30	0.16
Wasab	*An. punctulatus* (*s.s.*)	2016	36	32	3	1	0.78	0.09	0.03

Abbreviations: *N*, number of successfully genotyped human blood meals; H_s, number of single human meals; H_p, number of patent mixed human meals; H_c, number of cryptic mixed human meals; *B,* human blood index; *I*, probability of an interrupted blood meal on a human leading to blood meal on another host of any species; I_H, probability of an interrupted blood meal on a human leading to blood meal on another human.

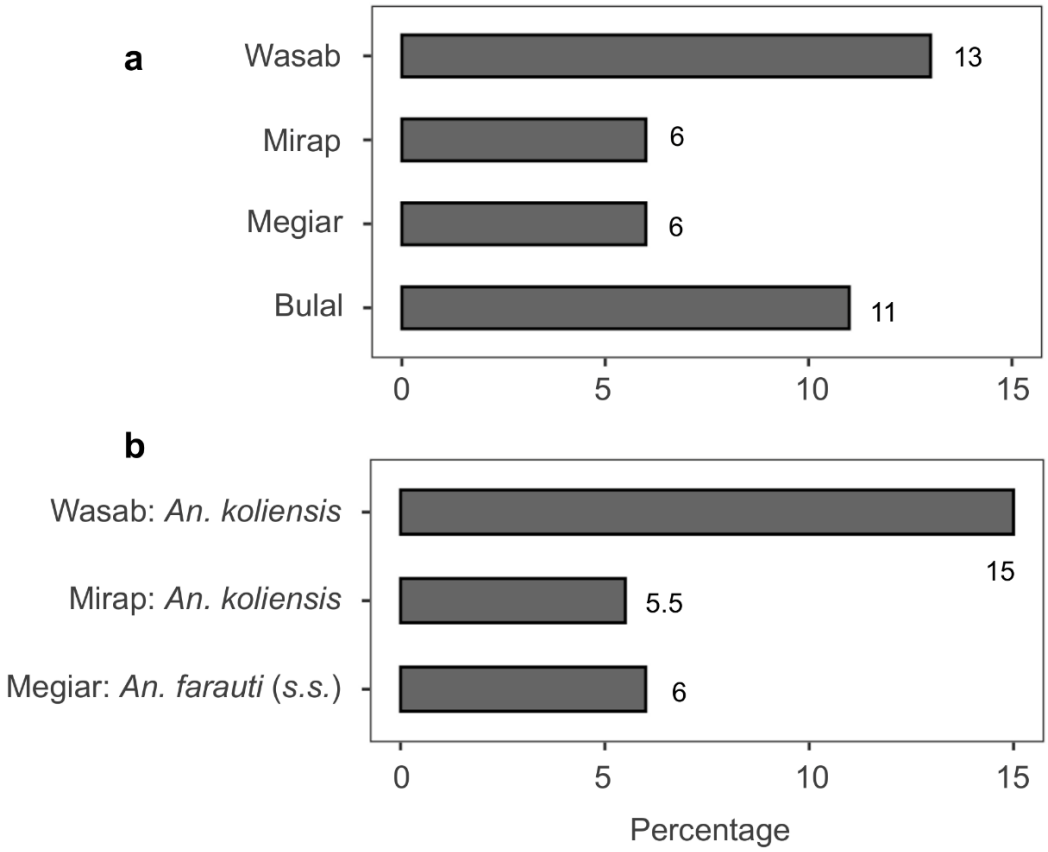

Figure 7.1. Percentage of cryptic mixed human meals. (a) For *Anopheles* spp. in Bulal (*n* = 18), Megiar (*n* = 124), Mirap (*n* = 233) and Wasab (*n* = 378). (b) For three populations: *An. farauti* (*s.s.*) in Megiar (*n* = 116), *An. koliensis* in Mirap (*n* = 200) and *An. koliensis* in Wasab (*n* = 339). Numbers at the tip of the bar are the estimated percentage.

7.4.3. Non-random human host selection

Statistical tests of non-random human selection were performed on mosquitoes from Mirap and Wasab only as it was possible to identify and eliminate the collector-fed blood meals in these samples. Also, the test was performed on *Anopheles* in general; analysis according to species entailed working with low sample size. Of 233 successfully genotyped Mirap blood-meal samples, 94% (*n* = 218) were single-human meals (i.e., not cryptic mixed meals). Of 378 successfully genotyped Wasab blood-meal samples, 87% (*n* = 328) were single-human meals. Of the 218 Mirap single-human blood-meal profiles, 29% (*n* = 63) matched the collector's profile.

Of the 328 Wasab single-human blood-meal profiles, 46% (n = 152) matched the collector's profile. After excluding the collector-fed blood-meals from the data, 88 different human individuals were identified in the Mirap blood meals (n = 155) and 79 individuals were found in the Wasab blood meals (n = 176). For each village, the number of occurrence of each human individual in the blood-meal sample was used to construct a plot which showed the frequency of occurrence (i.e., number of blood meals taken on an individual) on the x-axis and the number of individuals having a particular frequency of occurrence on the y-axis (Figure S7.2, Appendix 7.3). Fits of the frequency distribution data of both Mirap and Wasab to zero-truncated negative binomial and zero-truncated Poisson distribution models are shown in Figure 7.2. Data fit well to the negative binomial, with nonsignificant differences between observed and expected frequency distributions, and poorly to the Poisson model with significant differences between the observed and expected frequency distributions. Of the 88 different individuals identified in Mirap blood meals, most (70%) had one blood meal in the sample, 24% of people had 2–4 blood meals, and 6% had more than 4 blood meals. There were two individuals with 10 blood meals in the sample from Mirap. Of the 79 different individuals in Wasab blood meals, 68% had one blood meal, 24% of people had 2–4 blood meals, and 8% had more than 4 blood meals. There were 23 blood meals from the same person in the sample from Wasab.

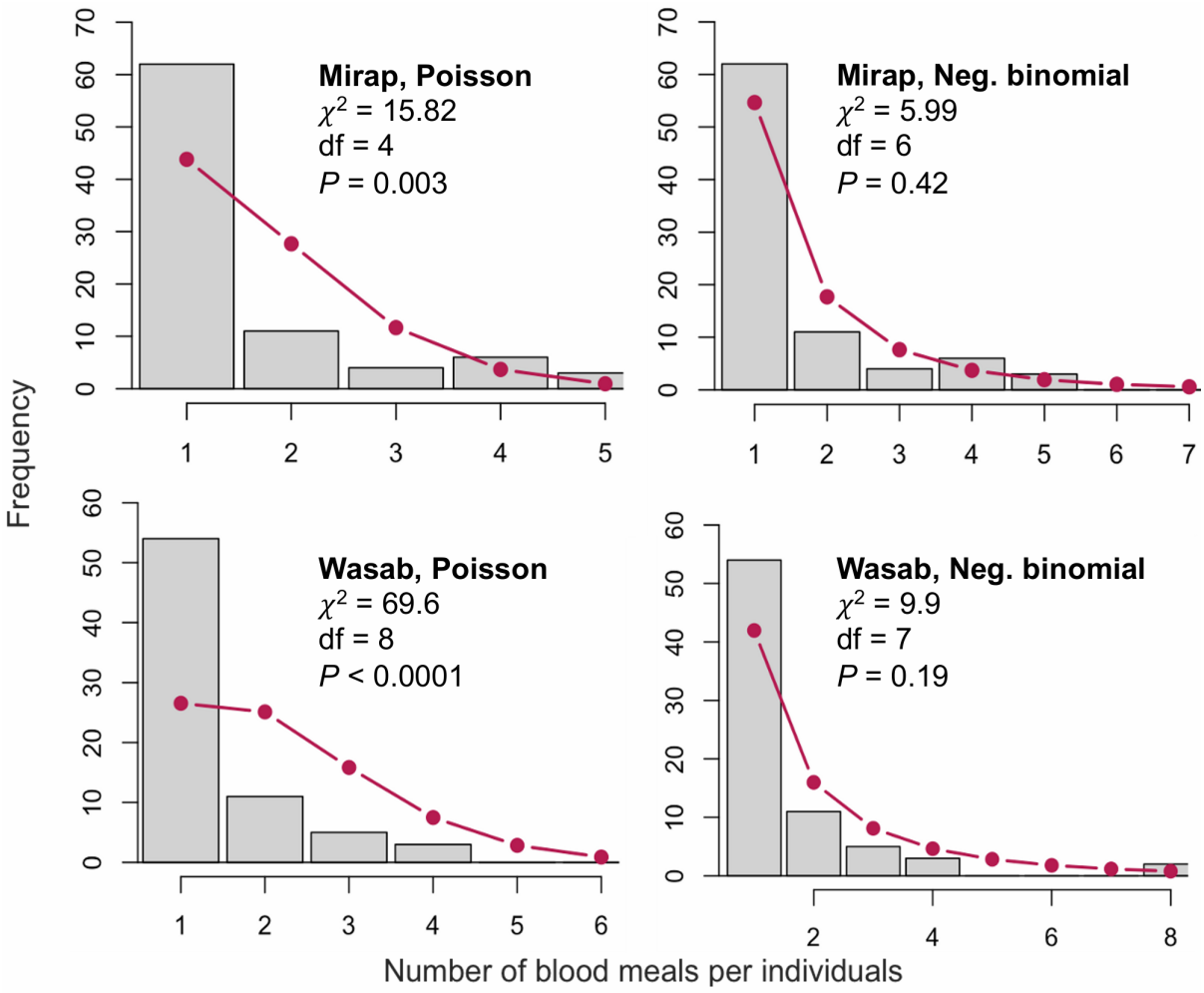

Figure 7.2. Chi-square test of fit of zero-truncated Poisson and zero-truncated negative binomial expected distribution (red curves) to observed distribution (vertical bars) of the frequency of occurrence of unique blood-meal profiles in mosquito samples of Mirap (top row) and Wasab (bottom row) villages.

The number of *Anopheles* spp. that fed on a female or male individual and the result of statistical analysis (exact binomial test) comparing the proportion of female-fed and male-fed blood meals to their expected proportion based on census data are shown in Table 7.3. Despite near equal proportion of both sexes in both Mirap (51% females, 49% males) and Wasab (49% females, 51% males), there were significantly more male-fed (Wasab, 69%; Mirap, 72%) and less female-fed (Wasab, 31%; Mirap, 28%) mosquitoes than expected. Twelve percent (19/155)

of Mirap and 24% (43/176) of Wasab *Anopheles* spp. blood-meal profiles were successfully matched to a village resident of known age (in years). The number of the blood-meal profiles that matched an individual from each of five age groups and the results of statistical analysis (exact binomial test) comparing the proportion of blood meals in each age group to their expected proportion are shown in Table 7.4. In both villages, the proportion of blood meals in the three older age groups did not deviate from the expected proportion whereas significant deviation was observed in the two younger age groups. Blood meals obtained from individuals < 15 years old were underrepresented whereas those obtained from 15–30 years old were overrepresented in both villages (Table 7.4).

Prevalence of infection for *P. falciparum* and *P. vivax* in villagers residing in Mirap and Wasab is shown in Figure 7.3. Result of logistic regression tests which compared the likelihood of being infected with *P. falciparum* or *P. vivax* in male individuals relative to females (reference group), and for individuals in four age groups relative to those in the < 15 years group (reference group) are shown for Mirap (n = 440 study participants) in Table 7.5 and for Wasab (n = 285 participants) in Table 7.6. In Mirap, the likelihood of infection with *P. falciparum* was statistically higher in the age groups 15–30 years and 31–45 years compared to the < 15 years age group; no difference was observed for the two older age groups or between the sexes (Table 7.5). For *P. vivax* in Mirap (Table 7.5) and both malaria species in Wasab (Table 7.6), no statistical variation was observed among the age groups or between sexes. Only the main effects were evaluated, as the interaction between age and sex was not significant in either case.

Table 7.3. Results of exact binomial test comparing the observed proportion of each sex in the blood meals with their expected proportion based on village census count.

Village	Sex	Blood-meal count	Expected Proportion	Blood-meal proportion (95% CI)	*P* value
Mirap	Females	44	0.51	0.28 (0.21–0.36)	< 0.0001*
	Males	111	0.49	0.72 (0.64–0.79)	< 0.0001*
Wasab	Females	54	0.49	0.31 (0.24–0.38)	< 0.0001*
	Males	122	0.51	0.69 (0.62–0.76)	< 0.0001*

Note: Total number of people in census data were 671 in Mirap and 292 in Wasab. Asterisks (*) indicate significant *P* value.

Table 7.4. Results of exact binomial test comparing the observed proportion of each age group in the blood meals with their expected proportion based on village census count.

Village	Age group	Blood-meal count	Expected proportion	Blood-meal proportion (95% CI)	*P* value
Mirap	< 15 years	3	0.48	0.16 (0.03–0.40)	0.005*
	15–30 years	11	0.23	0.58 (0.33–0.80)	0.001*
	31–45 years	2	0.19	0.11 (0.01–0.33)	0.56
	46–60 years	2	0.07	0.11 (0.01–0.33)	0.39
	> 60 years	1	0.03	0.05 (0.001–0.26)	0.44
Wasab	< 15 years	11	0.48	0.26 (0.14–0.41)	0.003*
	15–30 years	19	0.25	0.44 (0.29–0.60)	0.007*
	31–45 years	9	0.16	0.21 (0.10–0.36)	0.40
	46–60 years	3	0.07	0.07 (0.01–0.19)	1.0
	> 60 years	1	0.02	0.02 (0.001–0.12)	1.0

Note: Total number of people in census data were 671 in Mirap and 292 in Wasab. Asterisks (*) indicate significant *P* value.

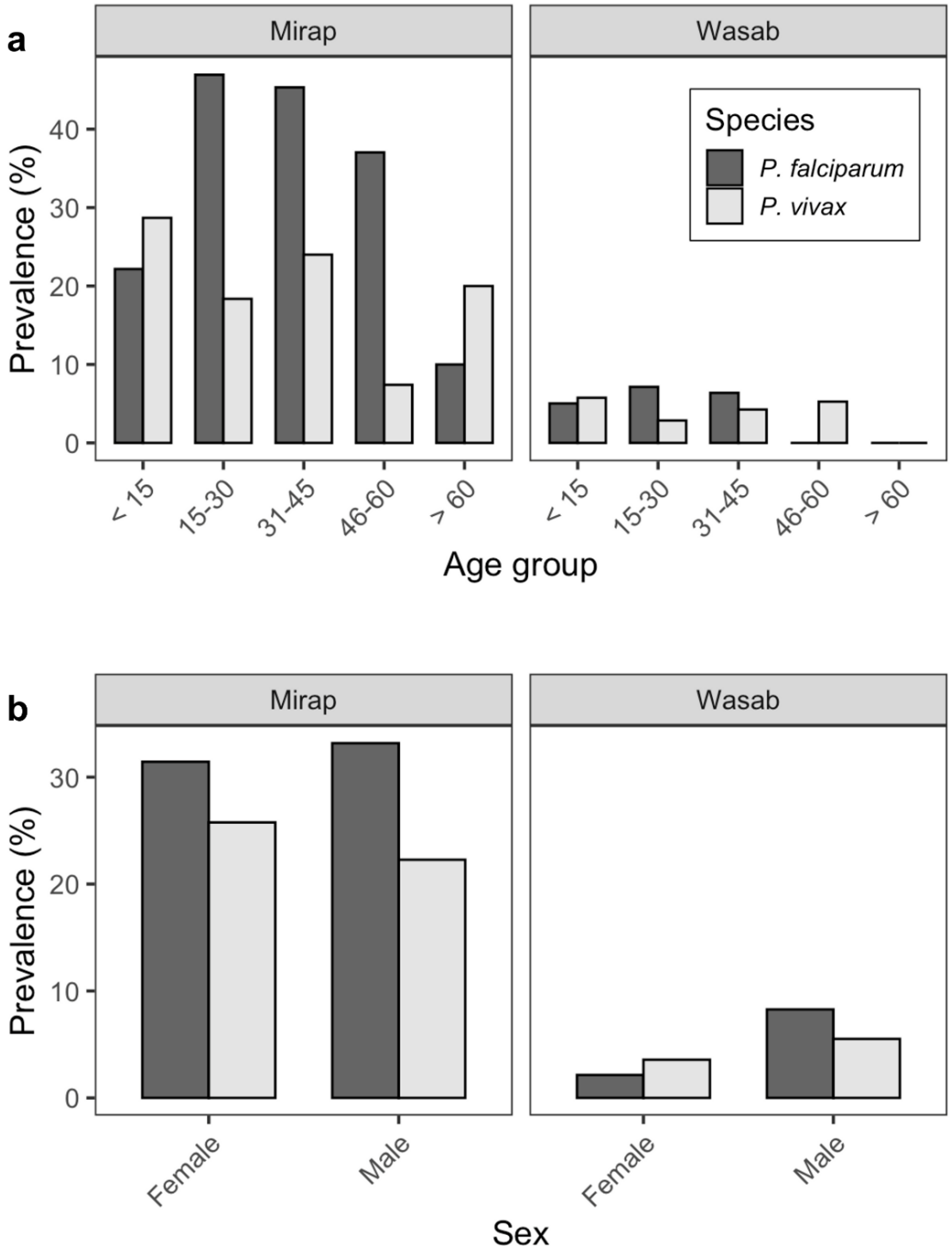

Figure 7.3. Prevalence of infection for *P. falciparum* and *P. vivax* in residents of Mirap and Wasab villages. (a) Prevalence of infection by age group. (b) Prevalence of infection by sex.

Table 7.5. Logistic regression results for test of variation in likelihood of malaria infection among age and sex groups in Mirap.

Reference variable	Variable	β	Std. Error	*P* value
P. falciparum	15–30 years	1.32	0.37	0.0003*
	31–45 years	1.15	0.39	0.0033*
	46–60 years	1.04	0.55	0.0563
	> 60 years	-14.17	650.87	0.9826
	Male	0.28	0.32	0.3883
P. vivax	15–30 years	-0.53	0.41	0.179
	31–45 years	-0.16	0.41	0.686
	46–60 years	-1.18	0.78	0.131
	> 60 years	-0.55	1.14	0.63
	Male	-0.15	0.29	0.618

Note: Each of the age group was compared with < 15 years as the reference group and male was compared with female as the reference group. Asterisks (*) indicate significant *P* value.

Table 7.6. Logistic regression results for test of variation in likelihood of malaria infection among age and sex groups in Wasab.

Reference variable	Variable	β	Std. Error	*P* value
P. falciparum	15–30 years	1.67×10^{1}	1.36×10^{3}	0.99
	31–45 years	1.64×10^{1}	1.36×10^{3}	0.99
	46–60 years	-5.58×10^{-11}	3.51×10^{3}	1.00
	> 60 years	-5.62×10^{-11}	6.36×10^{3}	1.00
	Male	1.73×10^{1}	1.36×10^{3}	0.99
P. vivax	15–30 years	-0.59	1.17	0.617
	31–45 years	-0.22	1.18	0.85
	46–60 years	-15.57	2.0×10^{3}	0.994
	> 60 years	-15.57	3.8×10^{3}	0.997
	Male	0.34	0.76	0.648

Note: Each of the age group was compared with < 15 years as the reference group and male was compared with female as the reference group.

7.5. Discussion

Sources of error in matching DNA profiles is an important consideration in a study of this kind. Here, the two primary sources of error are the degree of relatedness of villagers and the allele dropout rate per locus. If villagers are closely related to each other genetically, then the ability to discriminate among blood meals taken from different villagers is reduced. If too many alleles drop out of the analysis owing to insufficient DNA or inconsistency with priming during PCR, then false homozygosity would impede matching of DNA profiling. Of the two, the former is likely to be more important to error rate as the latter still yields a profile of some value. Villagers of New Ireland, New Britain and Bougainville provinces, PNG, (similar to most villages elsewhere in the country, such as Madang Province) have low genetic diversity and are likely

genetically related to many other members of the village through nuclear and extended family connections (Friedlaender, et al. 2008). This means that for most people, the percent profile match with many members of the village will be high and thus require a greater number of microsatellite markers to successfully differentiate them. In this study, the pairwise percent profile match based on 24 loci found that the highest percent match between any two non-monozygotic individuals was 71%, but the probability of observing this value was 10^{-4} or 1 in 10, 000 pairwise trials, which is extremely low. This means that a minimum of 17 markers (16 microsatellites and one sex-determining marker), which is similar to the number of markers used in other forensic identification protocols (Beleza, et al. 2004; Gorostiza, et al. 2007; Kim, et al. 2003), is sufficient to discriminate between two individuals from a typical PNG village. By choosing 79% (with zero probability of occurrence) instead of 71% as the criterion for deciding a match (match values ≥ 79%) or a mismatch (values < 79%), this decision rule allows for at most five erroneous mismatched loci of the 24 loci examined here, while maintaining the power to correctly discriminate between different individuals because of the remaining matched loci. Other studies (Benedictis, et al. 2003; Chow-Shaffer, et al. 2000; Harrington, et al. 2014; Michael, et al. 2001; Scott, et al. 2006; Soremekun, et al. 2004) that have conducted DNA profiling and matching of blood meals to human individuals have not considered this important element of the identification process and accordingly have not established this quite necessary rule of thumb, nor accurately incorporated these potential sources of error into their analysis.

The frequency of *Anopheles* blood meals taken on individual humans in the villages was not randomly distributed but rather was clumped or clustered. One likely explanation is that the *Anopheles* catches in households in these same villages was not randomly distributed but rather aggregated (see chapter 4), therefore, *Anopheles* females are most likely to encounter humans

where the mosquitoes themselves aggregate. In the malaria transmission system, those few human individuals who contribute most of the blood meals could be recognized as "super-spreaders", as they are likely to be infected and serve as reservoirs of the parasite to be spread to other members of the community (Bousema, et al. 2012; Woolhouse, et al. 1997). Non-random vector-human contact when measured by blood-meal genotyping has been observed in several other *Anopheles* and non-*Anopheles* populations and their associated mosquito-borne disease systems (Benedictis, et al. 2003; Harrington, et al. 2014; Michael, et al. 2001; Woolhouse, et al. 1997). In other villages near to the ones in the current study, people over the age of 20 who were infected with either *P. vivax* or *P. falciparum* malaria commonly harbored gametocytes (*P. vivax*, 40%; *P. falciparum*, 50%) but at lower densities of gametocytemia (*P. vivax* geometric mean, 2 gametocytes/ul; *P. falciparum* geometric mean, 0.5 gametocytes/ul) than younger age groups (Koepfli, et al. 2015). From these findings, it can be inferred that the older age groups found here to be fed upon at a greater than random encounter rate can be viewed as potential sources of malaria infection to mosquitoes rather than as sinks. Experimental feedings and other means of investigation could reveal this possibility.

Certain demographic groups were overrepresented or underrepresented in mosquito host choice. In both villages, the *Anopheles* obtained more blood meals from male individuals of the 15–30 years old age group compared to females or other age groups. A striking finding from the blood meals was under-representation of the youngest age group (< 15 years), which constituted a large proportion of the village censuses (52% of Mirap, 50% of Wasab). Human behavior and bed net usage could explain these patterns. From observation, in typical rural villages of PNG, males are shirtless most of the time (a response to high atmospheric temperature and humidity all year round) compared to their female counterparts. Thus, males have larger exposed body

surface for mosquitoes to land. Adult males (> 15 years) are less likely to sleep under a bed net than are females (Hetzel, et al. 2012). Also, from observation, youth greater than 15 years of age, particularly males, often remain active into the late night and retire to bed later in the morning hours compared to other age groups and to females. Thus, male individuals in the 15–30 years old age group would have greater exposure to *Anopheles* bites than those of the other age groups who retire under the bed nets earlier in the night. It is also worth noting that the guidelines for the bed net campaign prioritized distribution of nets to children and pregnant women (Hetzel, et al. 2014a; Hetzel, et al. 2014b), which could also have contributed to the observed demographic representations in the mosquito blood meals here. Similar findings to those here have been reported for populations of *Anopheles funestus* and *Anopheles gambiae* in villages of Tanzania. In that setting, males and individuals ≥ 20 years received more *Anopheles* bites than expected whereas females and those < 20 years old were bitten less frequently. In a village without bed nets, such variations were not observed (Soremekun, et al. 2004). In a Kenyan village, individuals < 20 years old received more bites than expected compared to those between 20–50 years old, but no variation was observed between sexes (Scott, et al. 2006). In five Zambian villages, the proportion of male-fed and female-fed blood meals were homogeneous with the expected proportions; data on age groups were not reported (Das, et al. 2017).

Given the heterogeneous utilization of humans by the vectors, it was predicted that the likelihood of malaria infection would be higher in male than female individuals and in individuals in the youth age (15–30 years) than the other age groups. However, this prediction held true only for the age group variable in Mirap *P. falciparum* infections; it was not the case for either parasite species in Wasab, nor for *P. vivax* in Mirap. Interestingly, malaria infection for both species was not different between sexes, which would not follow from observed biases in

Anopheles feeding. A plausible explanation for these inconsistencies in predictions is that the human biting rates (number of mosquito bites per person per unit time) in the underutilized human groups (i.e., females and non-youth age groups) were still high enough to support transmission across these demographic groups such that the probability of infection was homogeneous in all ages and sexes despite biased mosquito feeding patterns. This assertion is supported indirectly by a study which showed that the rate of malaria transmission, measured in terms of entomological inoculation rate (the number of malaria infective *Anopheles* bites received per person per year), must be reduced below 1.0 before appreciable reduction in prevalence of infection in the human population can be observed (Smith, et al. 2005).

The proportion of cryptic mixed human meals observed in *Anopheles* spp. in the four villages varied from 6–13% and in the three *Anopheles* populations with sufficient sample size varied from 5.5–15%. A similar study conducted in another Madang village in which the proportion of mosquitoes that fed on mother-child pair was quantified using ABO blood group markers showed that 13% of *Anopheles* spp., 11% of *An. punctulatus* (*s.s.*), 13% of *An. koliensis* and 18% of *An. farauti* (*s.s.*) had mixed meals (Burkot, et al. 1988). These results were in the same range as the ones produced in this current study. The proportion of cryptic mixed blood meals in PNG *Anopheles* observed in both the current and previous studies, were similar to those reported for *Anopheles funestus* (2–14%) and *Anopheles gambiae* (0–11%) in two Kenyan villages (Scott, et al. 2006), and *An. gambiae* (10%) in a Tanzanian village (Soremekun, et al. 2004). Similar values were also observed in non-anophelines (Benedictis, et al. 2003; Harrington, et al. 2014; Michael, et al. 2001), except for an *Aedes aegypti* population in Thailand where a much higher proportion (45%) was observed (Chow-Shaffer, et al. 2000).

The sensitivity of hosts to mosquito bites affects the outcome of a blood meal; the more intolerable a host is to mosquito bites, the more likely a blood-feeding attempt will be interrupted before a full meal is obtained (Anderson and Brust 1997; Boreham and Garrett-Jones 1973). An interrupted mosquito can either forgo further blood-feeding attempts (i.e., partial feed) or it can complete the blood meal by feeding on a different individual of the same host species or a different species. The former has important epidemiological consequences. In the case of malaria, both R_0 and V increase when a considerable proportion of the vectors obtain cryptic mixed human meals (Tedrow, et al. 2019). This is because those vectors can infect more than one humans if they are sporozoite-positive, and also have more than one chance of obtaining an infectious (i.e., gametocytemic) blood meal in a single gonotrophic cycle.

Before DNA profiling was available, the proportion of human blood meals that were cryptic mixed (C) was estimated indirectly from mathematical models (Boreham and Garrett-Jones 1973; Burkot, et al. 1988). According to (Burkot, et al. 1988), $C = QI_H$, where Q is the probability of selecting a human as a host by a mosquito. Q can be calculated as $Q = P_H + P_M [I_H/(I_H + I_N)]$, where P_H is the proportion of all blood meals with only human blood, P_M is the proportion of all blood meals that are patent mixed human meals and I_N is the probability of a non-human feed being interrupted (Burkot, et al. 1988). Substituting the right hand side of this equation for Q in the previous one gives a new equation $C = (P_H + P_M [I_H/(I_H + I_N)])I_H$. Unlike P_H and P_M whose values were easily estimated directly from blood-meal analysis, it was difficult to estimate I_H and I_N empirically and researchers depended on assumed values to estimate C (Burkot, et al. 1988). With the availability of a DNA profiling method, C can be estimated directly, as demonstrated in the current study. However, as DNA profiling is expensive, many researchers may rely on the mathematical model. Thus, some empirical estimates of I_H, rather

than assumed values, are needed for such researchers to use in the theoretical model. In this current study a model for estimating I_H, which can also be used to estimate I_N, was derived (see method section) and used to estimate I_H for the six *Anopheles* populations in PNG. The results ranged from 0.03–0.18. Researchers working with malaria vector populations in PNG can now use these I_H values in the model to better estimate C. In addition to I_H, a related model for calculating the probability (I) of an interrupted human feed that undergoes a second feed on any host species (not just humans) was derived. The values of I for the six *Anopheles* populations ranged from 0.09-0.3. The values of I were statistically greater than I_H (t-test: $P = 0.047$), which was expected because of the high use of non-human hosts (in particular pigs and dogs) by these *Anopheles* species (Burkot, et al. 1989; Burkot, et al. 1988; Keven, et al. 2017; Russell, et al. 2016).

7.6. Conclusion

This study employed a microsatellite-based DNA profiling method to identify different human individuals in *Anopheles* blood meals of four PNG villages. Analysis of the data resulted in three epidemiologically important findings. First, 6–13% of all *Anopheles* that feed on humans were interrupted by their host and completed their blood meal on a different individual in a single gonotrophic cycle. By feeding on more than one human, the vectorial capacity of the vector population is increased. Second, the frequency of vector bites among individuals in the villages were not random but clustered. As heterogeneous exposure to vectors is known to increase malaria R_0, it is logical to postulate that non-random exposure to vector bites is a factor underlying the persistent and rebounding malaria transmission recently reported in the study villages (see chapter 3). Third, youth age male individuals were more frequently fed on by the

vectors compared to the females and individuals in other age groups. This is likely caused by differences in the behaviors and bed net usage among individuals in different sex and age groups. Despite overutilization of youth age males by mosquitoes, the likelihood of malaria infection was generally homogeneous among age groups and between sexes.

APPENDICES

Appendix 7.1. Percent profile match values

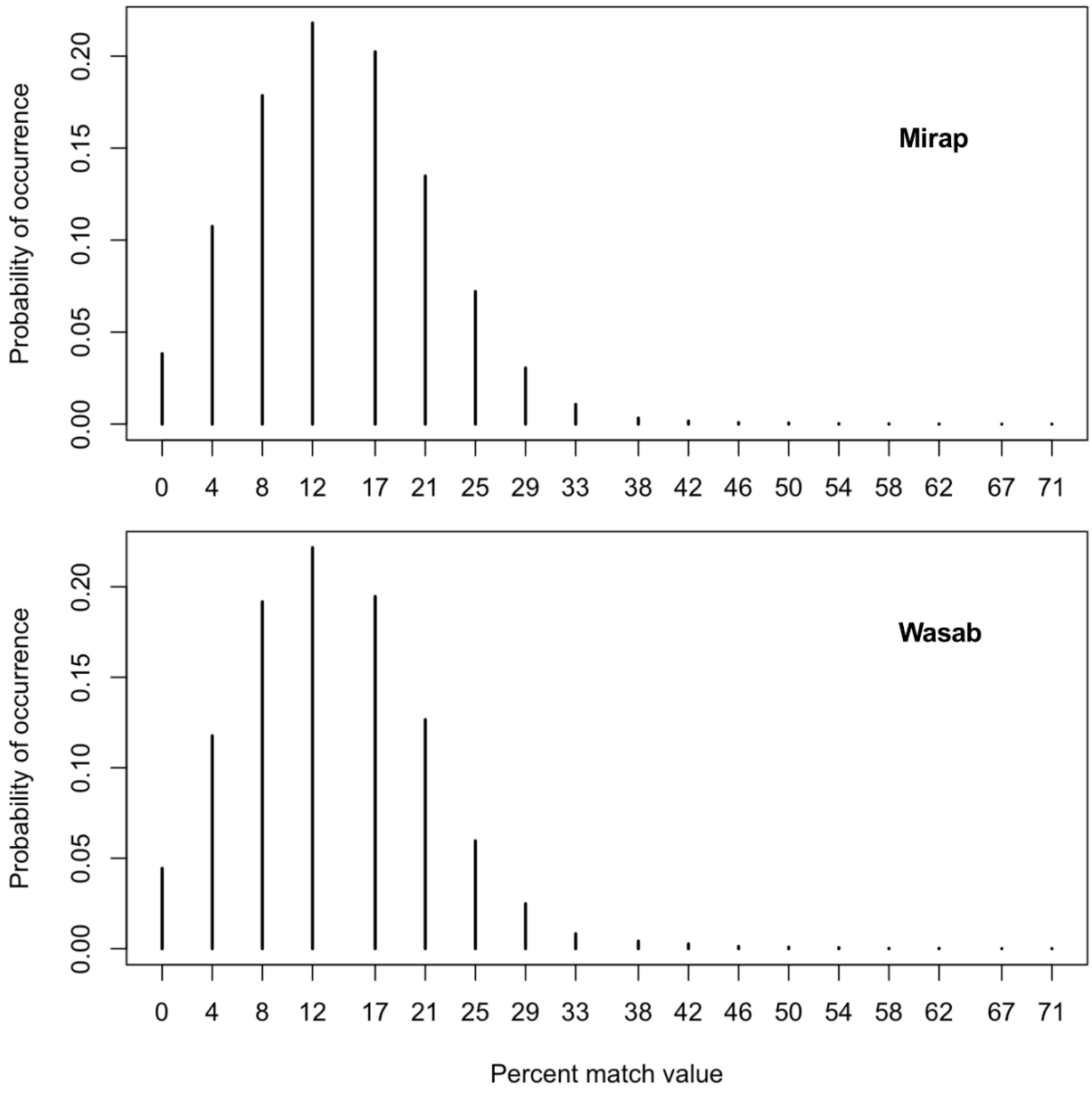

Figure S7.1. Probability of occurrence (y-axis) of different percent match values (x-axis) calculated from pairwise percent profile match analysis of 427 DNA profiles of Mirap and 164 DNA profiles of Wasab residents.

Appendix 7.2. Human blood index of *Anopheles* populations

Table S7.1. Human blood index (*B*) of six *Anopheles* populations.

Village	*Anopheles* species	Total tested	No. of non-human meals	No. of human meals	*B*
Bulal	*An. koliensis*	28	12	16	0.57
Megiar	*An. farauti* (*s.s.*)	191	88	103	0.54
Mirap	*An. farauti* (*s.s.*)	120	85	35	0.29
Mirap	*An. koliensis*	404	215	189	0.47
Wasab	*An. koliensis*	440	122	318	0.72
Wasab	*An. punctulatus* (*s.s.*)	54	12	42	0.78

Note: Mosquitoes were sampled, and the blood meal hosts were determined as described in the method section. The value of *B* was estimated by dividing the number of human blood meals by the total number of mosquitoes tested.

Appendix 7.3. Frequency distribution of blood meals

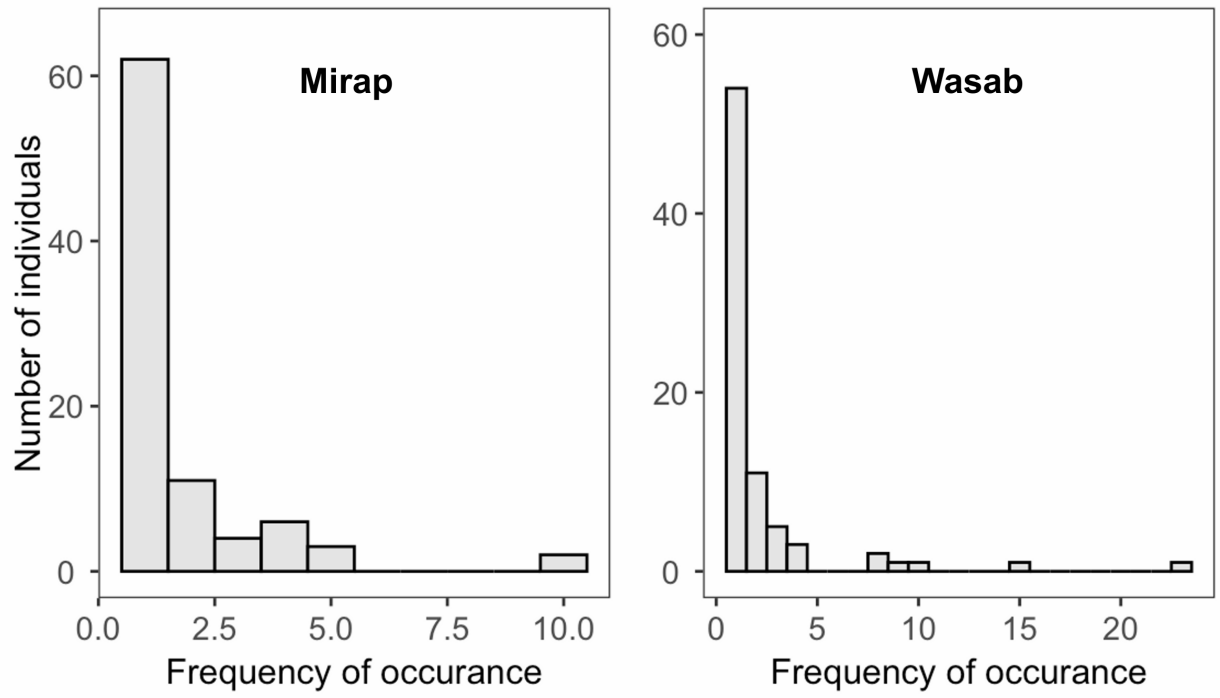

Figure S7.2. Distribution of frequency of blood meals taken on different human individuals by *Anopheles* mosquitoes in Mirap and Wasab village.

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CHAPTER 8: GENERAL CONCLUSION

In chapter 2–7, a number of primary and minor conclusions were arrived at as a result of inferences made from detailed evaluation of the data. This final chapter focuses on summarizing the main conclusions to cohesively address the overarching hypothesis of this dissertation. This hypothesis was that although malaria was reduced considerably by the nationwide bed net-based vector intervention program, further reduction in the rate of transmission has halted in some parts of the country owing to ecological conditions which allow local vectors to evade the bed nets. This allows malaria transmission to persist at relatively high rates and infection prevalence to rebound despite ongoing efforts to intensify the vector intervention program. Indeed, the study reported in chapter 3 shows conclusively that in the human communities in the north coast of Madang province, the annual *IBR* for *Plasmodium* spp. has risen above 15 infective *Anopheles* spp. bites per person-year since it was reduced below this level in 2011, and that the prevalence of infection has rebounded after it was reduced to its lowest levels in 2014 as a result of the vector intervention program.

Four vector-associated ecological factors were posited to be causing the persistent malaria transmission in this part of the country. The first was increased vector abundance and species diversity. As the purpose of the insecticide was to reduce vector abundance and consequently the *IBR* by killing the mosquitoes, high vector abundance signals infectiveness of the bed nets against the local vectors. The studies reported in chapter 2 and 3 show that in the north coast of Madang province, vector abundance and human biting rates have increased after plummeting to their lowest levels in 2011. Different *Anopheles* species or ecotypes vary in their biological attributes such as vector competence, duration of incubation period, and host

preference which affect their vectorial capacity. Thus, the presence of multiple vector species can increase malaria transmission potential. Vectors may also vary in host-seeking behaviors and biting cycles which can cause them to be differentially affected by the bed nets. Therefore, in communities with multiple vector species or ecotypes, those with vulnerable attributes (e.g., endophagy) can be affected by the bed nets but malaria will continue to be transmitted by the evasive ones. Thus, the presence of multiple vector species within communities and varying species composition among communities both indicate that the bed nets are not having the intended effect on the mosquitoes. Research reported in chapter 2 shows that diverse vector species live in sympatry in Madang villages and their relative composition vary even among villages that are < 1.0 km apart. Thus, it can be concluded that vector species abundance and diversity are helping to promote persistent malaria transmission in the study villages.

The second ecological factor is outdoor and evening biting habits of the mosquitoes. To minimize exposure to mosquito bites and hence the likelihood of getting infected, humans must retire to bed and be protected by the bed nets during the period of the night when mosquito host-seeking activity peaks. Also, as bed nets are deployed inside houses, mosquitoes that seek humans inside houses have higher risk of exposure to the insecticide than those the feed outdoors. In this study (chapter 4), it was observed that most (29–50% of total) of the vector bites taken on humans occurred before 22:00 hr when 75–90% of people were active (awake) and were unprotected by the bed nets, and 67–83% of the bites occurred before 2:00 hr before > 95% of the people retired to bed. Most (> 50%) of the bites taken on humans occurred outdoors where the risk of exposure to the bed nets was low. Thus, it can be concluded that outdoor and evening biting behaviors of the local vectors promote persistent transmission in the study villages.

The third factor is heterogeneity of human exposure to the intensity of vector bites. In any environment where malaria exists the R_0 of malaria has a specific value when the frequency of bites taken on individuals is homogeneous or randomly distributed. However, epidemiological models have shown that under the condition where the frequency of bites tend to cluster in a few spatial locations or on a small subset of individuals villagers, the R_0 tend to increase by some quantifiable multiplicative factors. By increasing the R_0, transmission tend to persist rather than dissipate. In this study, it was shown (chapter 4 and 7) that the distribution of bites by the local *Anopheles* spp. among spatial locations within villages and individual villagers were clustered rather than random and the R_0 in these villages were increased by multiplicative factors ≥ 1.6. Thus, it can be concluded that heterogeneity of human exposure to vector bites may play an important role in supporting persistent transmission in the study villages.

The final factor is the propensity of vectors to feed unbiasedly on blood host species. Mosquitoes are considered non-vectors if they are strictly zoophagous because they are unable to transmit malaria. If a population is strictly anthropophagous it is considered a good vector. Strictly anthropophagic vectors are faithful to humans despite prevailing environmental conditions that may limit access to human hosts. This makes them dangerous vectors, but only under ordinary condition. Under high bed net usage, the inflexibility of the anthropophagic vectors to utilize alternative hosts will cause them to pursue humans inside houses, rendering them susceptible to the insecticide. Thus, bed net-based malaria intervention can succeed when the mosquitoes are strictly anthropophagous. If, however, the mosquito population is opportunistic in its host selection, any host that is available and accessible will be unbiasedly utilized. In this respect, inaccessibility to human hosts due to bed nets does not affect their blood meal requirement as they can utilize alternative hosts instead of pursuing humans into the house

and being exposed to the insecticides. By feeding on alternative hosts, populations of opportunistic mosquitoes are able to maintain high vector abundance and perpetuate transmission by feeding on humans outdoors and during the times when humans are not protected by bed nets. Studies reported in chapter 5 and 6 show that the vector species in PNG are opportunistic blood feeders and can utilize pigs and dogs as blood meal hosts.

Although the main conclusions above were based on studies that were conducted in a few Madang villages, it can be extrapolated to other parts of PNG and the South West Pacific region. Thus, it can be concluded that in PNG and the South West Pacific region reliance on insecticidal bed nets alone as the primary malaria control method is insufficient to eliminate or reduce the disease to acceptably low levels. Alternative vector intervention methods, such as treatment of non-human hosts (e.g., pigs and dogs) with endectocides, need to be developed and implemented alongside the bed net program.