Etude du risque de transmission du paludisme le long de la frontière birmano-thaïlandaise par l'utilisation de biomarqueurs spécifiques d'exposition humaine aux piqures d'Anopheles et au Plasmodium

Phubeth Ya-Umphan

THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

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École doctorale CBS2 n°168 - Sciences Chimiques et Biologiques pour la Santé

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Titre de la thèse

ETUDE DU RISQUE DE TRANSMISSION DU PALUDISME LE LONG DE LA FRONTIERE BIRMANO-THAÏLANDAISE PAR L'UTILISATION DE BIOMARQUEURS SPECIFIQUES D'EXPOSITION HUMAINE AUX PIQURES D'*ANOPHELES* ET AU *PLASMODIUM*

Présentée par Phubeth YA-UMPHAN

Le 24 novembre 2017

Sous la direction de Vincent CORBEL et Theeraphap CHAREONVIRIYAPAHP

Devant le jury composé de

Mme Catherine Bourgouin, Directeur de recherche à L'Institut Pasteur de Paris	Rapporteur
M. Lionel Almeras, Maître de conférences à l'Université Aix-Marseille	Rapporteur
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CONTENT

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INTRODUCTION

1. MALARIA

Malaria is a mosquito-borne infectious disease caused by protozoan parasites from the *Plasmodium* family that is transmitted by the bite of the *Anopheles* mosquito. Malaria has been noted for more than 4,000 years (beginning in 2700 BC in China) and comes from the Italian for "bad air", mal'aria. Malaria is considered as the most devastating vector borne disease worldwide and World Health Organization (WHO) estimates that about 1.4 billion people are leaving in area at risk of the disease (WHO 2016a).

1.1 Global distribution of malaria

The geographic distribution of malaria is in tropical and subtropical regions of the Americas, Asia and Africa (Figure 1). The environment of these regions are consistent with high temperatures, humidity and rainfall that are suitable for the development of both the parasites and the *Anopheles* mosquitoes vector.

An estimated of 212 million (range : 148-304 million) peoples of the world were infected with malaria in 2015 and an estimated of 429,000 people (range : 235,000-639,000) died (WHO 2016a), most of them children in Africa. Malaria mortality and morbidity have been reduced by 60 and 41% since the 2000 thank to the implementation of preventive and curative strategies, including early diagnosis and prompt access to treatment, Artemisinin-based Combination Therapy (ACT), and high coverage of long-lasting insecticidal treated mosquito nets (LLIN) (WHO 2016a). Most of malaria cases in 2015 were estimated to have occurred in the African (92%), followed by Southeast Asia (6%) and the Eastern Mediterranean Region (2%). Despite significant progress in reducing the overall disease burden, malaria remains a major killer of children, particularly in sub-Saharan Africa, taking the life of a child every 2 minutes (WHO 2016a).

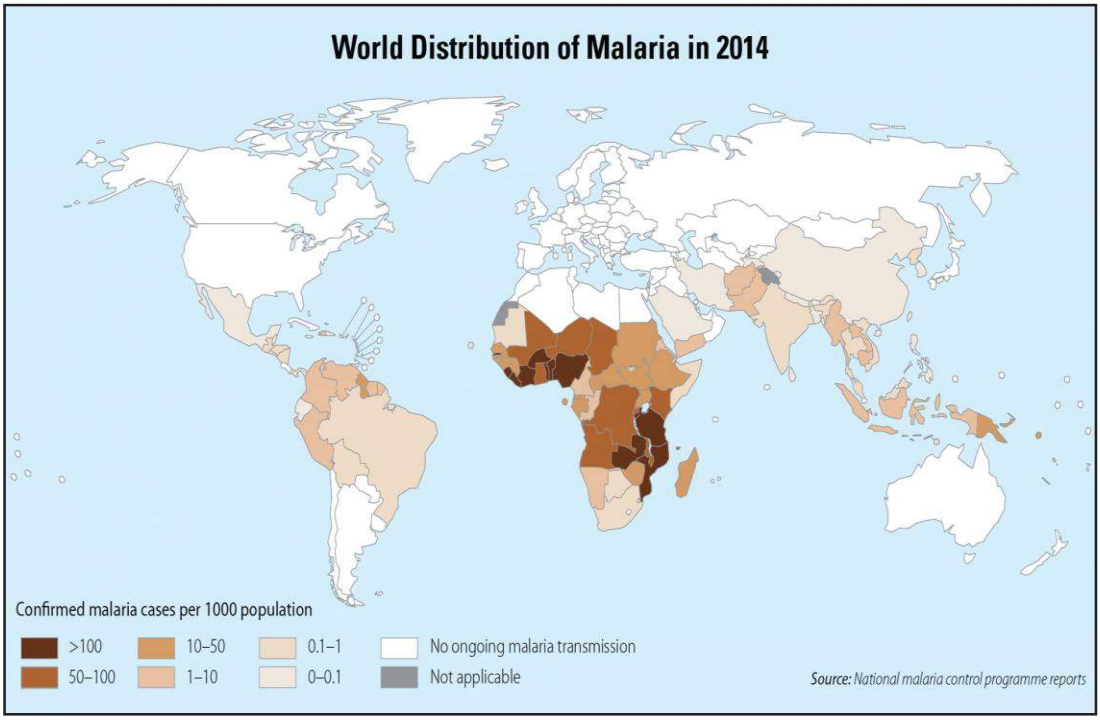

Figure 1. Countries with ongoing transmission of malaria. (Source: WHO 2014b)

1.2 Transmission

Malaria is a protozoan disease transmitted by *Anopheles* mosquitoes (White et al. 2014). In the human body, the parasites multiply in the liver, and then infect red blood cells. Usually, people get malaria by being bitten by an infective female *Anopheles* mosquito. However, in rare cases, transmission can occur without passage through a mosquito from one person to another one by blood transfusion (transfusion malaria) (Kitchen and Chiodini 2006) and from mother to child (congenital malaria) (Fischer 2003).

1.3 The *Plasmodium* parasite

Malaria is caused by seven species of the parasite belonging to the genus *Plasmodium* (White et al. 2014). Four species i.e. *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale* are human malaria species, which are spread from one person to another by female mosquitoes of the genus *Anopheles*. *Plasmodium falciparum* and *P. vivax* malaria pose the greatest public health challenge (WHO 2014b). The other malaria parasites (*P. knowlesi, P. cynomolgi and P.simimum*) are a species that causes malaria among monkeys but can be

transmitted occasionally from monkeys to humans (zoonotic transmission) (White 2008) (Figure 2).

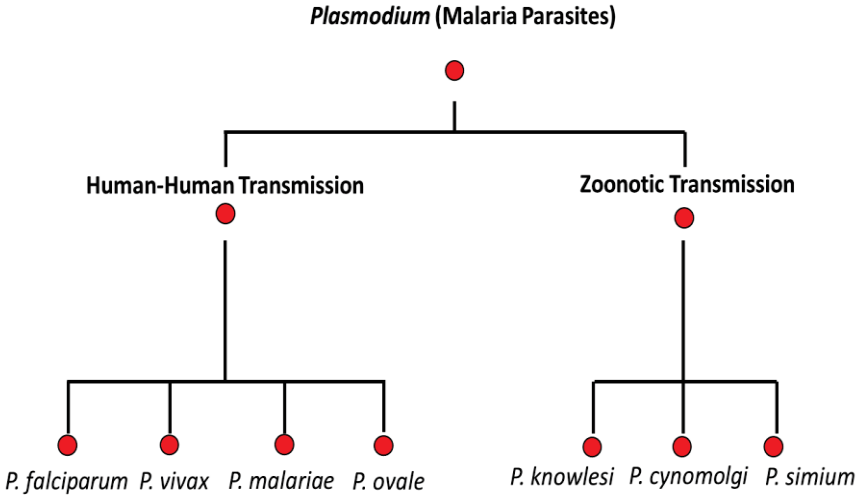

Figure 2. Malaria is caused by seven species of parasites belonging to the genus *Plasmodium*.

The different species are briefly described below;

Plasmodium falciparum, is found worldwide in tropical and subtropical areas. The vast majority of deaths (99%) are due to *P. falciparum* malaria and 90% of the deaths occured in Sub-sahara Africa (WHO 2016a). The most frequent and serious complications of malaria are severe neurologic complications (cerebral malaria) and severe anemia (Crutcher and Hoffman 1996 , Trampuz et al. 2003).

Plasmodium vivax, is found mostly in Asia, Central and South America, and in some parts of Africa (Howes et al. 2015). This species (as well as *P. ovale*) has dormant stages ("hypnozoites") that persist in the liver and cause relapses by invading the blood stream weeks, or even years later after the infected mosquito bite (White and Imwong 2012). *Plasmodium vivax* is less virulent than *P. falciparum*, however *P. vivax* malaria can lead to severe complications and death due to splenomegaly. The virtual absence of *P. vivax* infections in many areas of Africa is explained by the fact that most Africans do not have Duffy blood-group antigens, which apparently function as erythrocyte surface receptors for *P. vivax* merozoites; without the Duffy antigen, the parasites cannot invade (Crutcher and Hoffman 1996).

Plasmodium ovale, is found mostly in West Africa and the islands of the Western Pacific. In more recent years, there have been reports of *P. ovale* on the Asian mainland (Collins and Jeffery 2007). *Plasmodium ovale* is similar to *P.vivax* in both of biology and morphology. It's also has latent liver stages and is thus classified as one of the relapsing malaria parasites (Collins and Jeffery 2005). However, differently from *P. vivax*, it can infect individuals who are negative for the Duffy blood group. This explains the greater prevalence of *P. ovale* (over *P. vivax*) in African countries (https://www.cdc.gov/ malaria/about/biology/ parasites.html).

Plasmodium malariae, is widespread throughout sub-Saharan Africa, much of Southeast Asia, into Indonesia, and on many of the islands of the Western Pacific (Collins and Jeffery 2005). *Plasmodium malariae* has low prevalence and milder clinical manifestations compared to the other species. However, chronic infection with *P. malariae* is associated with the production of immune complexes in the kidneys, and can cause the nephrotic syndrome (Collins and Jeffery 2007).

Plasmodium knowlesi, has been found only in Southeast Asia (White 2008). This species causes malaria in long-tailed macaques (*Macaca fascicularis*), but it may also infect humans occasionally. It has recently been shown to be a significant cause of zoonotic malaria in Southeast Asia especially in forested areas of Malaysia (Kantele and Jokiranta 2011). The fever cause by *P. knowlesi* is quotidian. Parasite replicates and completes its blood stage cycle in about 24 hours hence resulting in high loads of parasite densities in a very short period of time. Consequently, infections caused by *P. knowlesi* can rapidly shift from uncomplicated to a severe malaria.

Plasmodium cynomolgi, is a malaria parasite that typically infects Asian macaque monkeys, and humans on rare occasions. This species is used as a model for human *P. vivax* infections. It is phylogenetically close to *P. vivax* and shares important biological features including the dormant liver form (hypnozoite) with this parasite (Sutton et al. 2016). The first and only known human case was found in a 39 years old Malay woman, from the east coast of peninsular Malaysia. The patient experienced 24-hour cycles of fever with chills and rigor, cough and cold. The symptoms had become worse after two weeks. A different molecular diagnostic methods confirmed that the patient was infected with *P. cynomolgi* and not with *P. vivax* (Ta et al. 2014).

Plasmodium simium, is a parasite from New World monkeys that is most closely related to the human malaria parasite *P. vivax* (Camargos et al. 2015). This species appears restricted to the Atlantic forest region in southeastern Brazil. Recently, a malaria outbreak in this area has been traced to zoonotic transmission from monkeys. *Plasmodium simium* does not produce particularly severe forms of human malaria, but the existence of a monkey reservoir for a malaria parasite capable of infecting humans will likely complicate efforts to eliminate malaria in Brazil (Brasil et al. 2017).

1.4 Life cycle of malaria parasites

The life cycle of malaria parasites involves a sequence of different stages in two types of hosts : humans (intermediate host) and female *Anopheles* mosquitoes (definitive host) (Figure 3).

1.4.1 Exo-erythrocytic cycle (Human liver stage)

After the bite of infected female *Anopheles* mosquito to human during a blood meal, the sporozoites are injected into the bloodstream via skin and are transported to the liver. The parasites grow and multiply in the liver cells and then mature into schizonts which rupture and release merozoites. For *P. vivax* and *P. ovale,* hypnozoites, the latent or dormant stage of the *Plasmodium* parasite in the liver can be relapsed and invade the blood several months or years later.

1.4.2 Erythrocytic cycle (Human blood stage)

After this initial replication in the liver, then the merozoites infect the red blood cells where they develop into ringshaped form, mature trophozoite form and schizonts stage that parasite divides several times to produce further merozoites and continue the cycle by invading other red cell within bloodstream. However, some merozoites differentiate into male or female gametocytes (sexual erythrocytic stages) which are taken up by the female *Anopheles* mosquito during a blood meal and they start different cycle of growth and multiplication in the mosquito.

1.4.3 Sporogonic cycle (Mosquito stage)

In the mosquito gut, the microgamete nucleus divides three times producing eight nuclei; each nucleus fertilizes a macrogamete forming a zygote. The zygote, after the fusion of

nuclei and the fertilization, becomes the so- called ookinete. The ookinete, then, penetrates the midgut wall of the mosquito, where it encysts into a formation called oocyst. Inside the oocyst, the ookinete nucleus divides to produce thousands of sporozoites (sporogony). The oocyst ruptures and the sporozoites are released inside the mosquito cavity and find their way to the salivary glands. Thus, when the infected mosquito takes a blood meal, it injects its saliva containing the sporozoites into the next human host marking the beginning of a new cycle.

1.5 Malaria symptoms

The symptoms of malaria typically occurring 7 days or more (usually 10-15 days) after an infective mosquito's bite. The initial symtoms are flu-like accompanied by headache, fever, chill and vomiting, may be mild and difficult to recognize as malaria (WHO 2016a). It's can be classified in two categories: uncomplicated and severe malaria (Bartoloni and Zammarchi 2012).

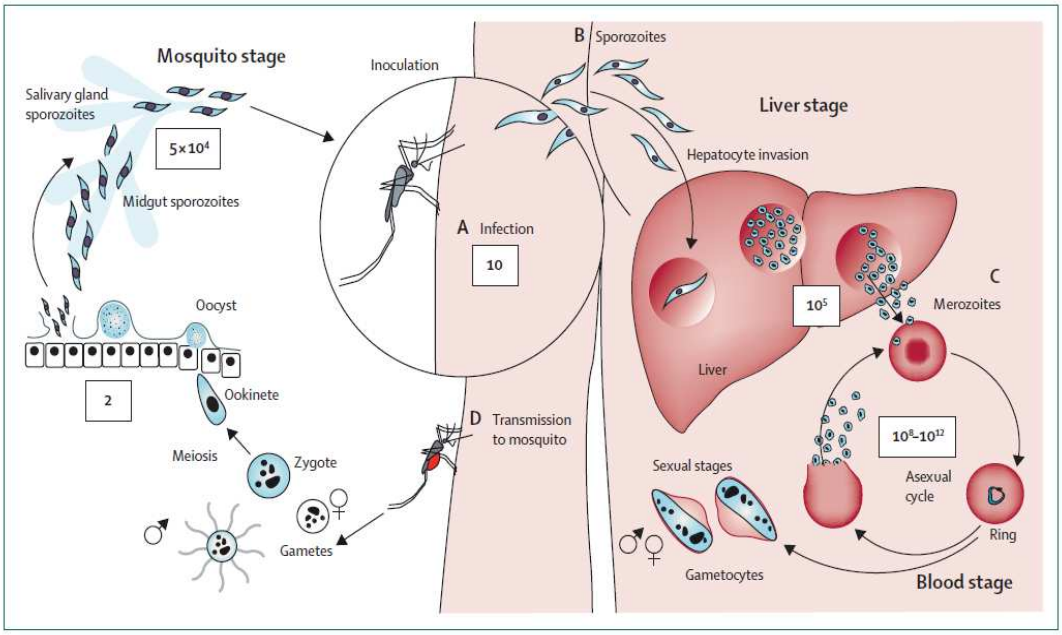

Figure 3. Lifecycle of *Plasmodium falciparum* in the human body and the *Anopheline* mosquito. The cycle begins with inoculation of motile sporozoites into the dermis (A; magnified), which then travel to the liver (B); each sporozoite invades a hepatocyte and then multiplies. After about a week, the liver schizonts burst, releasing into the bloodstream thousands of merozoites that invade red blood cells and begin the asexual cycle (C). Illness starts when total asexual parasite numbers in the circulation reach roughly 100 million. Some parasites develop into sexual forms (gametocytes). Gametocytes are taken up by a feeding *Anopheline* mosquito (D) and reproduce sexually, forming an ookinete and then an oocyst in the mosquito gut. The oocyst bursts and liberates sporozoites, which migrate to the salivary glands to await inoculation at the next blood feed. The entire cycle can take roughly

1 month. Estimated numbers of parasites are shown in boxes-a total body parasite burden of 10^{12} corresponds to roughly 2% parasitaemia in an adult (White et al. 2014).

Uncomplicated malaria (can be caused by all species of *Plasmodium*) is diagnosed when symptoms are present, but there are no clinical or laboratory signs to indicate a severe infection or the dysfunction of vital organs (WHO 2015b). The symptoms of uncomplicated malaria are non-specific and include fever. The same cycling pattern of symptoms, coldness followed by shivering and then fever and sweating, may repeat at intervals of every two days for *P. vivax* and *P. ovale* infections and every three days for for *P. malariae*. Whereas, *P. falciparum* infection can cause recurrent fever every 36-48 hours, or a less and almost continuous fever. If treatment is delayed, individuals suffering from this form can eventually develop severe complications of malaria.

Severe malaria (usually caused by *P. falciparum*) defined by clinical or laboratory evidence of vital organ dysfunction. Most of the severe malaria complications occur in non-immune subjects with falciparum malaria and involve central nervous system (cerebral malaria), pulmonary system (respiratory failure), renal system (acute renal failure) and/or hematopietic system (severe anaemia) (Bartoloni and Zammarchi 2012). Cerebral malaria is the most common complication and cause of death in severe *P. falciparum* infection.

1.6 Malaria diagnosis

Accurate diagnosis and prompt treatment of malaria is part of effective disease management and control (WHO 2015b). Diagnosis of malaria requires the identification of the parasite or its antigens/ products in the patient's blood to establish an appropriated treatment. The requirements of a diagnostic test are specificity, sensitivity, ease of performance and a reasonable cost.

1.6.1 Clinical diagnosis

Clinical diagnosis is based on the patient's symptoms and on physical findings at examination. This method is traditional among medical doctors, which is still challenging due to the non-specific initial symtoms of malaria (flu-like accompanied by headache, fever, and chill) most often overlapping with other diseases (common viral or bacterial infections). For these reasons,clinical examination should always be confirmed by a laboratory test for malaria (Tangpukdee et al. 2009).

1.6.2 Laboratory diagnosis

Laboratory diagnosis of malaria use different techniques, e.g. microscopic diagnosis by staining thin and thick peripheral blood smears and quantitative buffy coat (QBC) test, Immunological techniques e.g. rapid diagnostic test (RDT), Indirect fluorescent antibody test (IFA) and Enzyme linked immunosorbent assay (ELISA), and molecular biology techniques, such as polymerase chain reaction (PCR) (Tangpukdee et al. 2009).

1.6.2.1 Microscopic diagnosis

I. Microscopic diagnosis using stained thin and thick peripheral blood smears

Microscopic examination is the most commonly used method to detect malaria parasite because it is inexpensive to perform, able to quantify and distinguishing four major parasite species characteristics (WHO 2010a). However, this method is labor-intensive, required well-trained staff, the diagnostic accuracy depends on quality of blood smear and equipment. The procedure includes collecting peripheral blood, staining of smear with Giemsa stain and examination of red blood cells for malaria parasites under the microscope. Two sorts of blood film are traditionally used. Thick blood film are mainly used for screening the presence of malaria parasite and thin blood films are used for species identification and recognition of parasite forms like schizonts and gametocytes. Microscopy is the gold standard method, and serve as a reference in the evaluation of new tools for malaria diagnosis (Wongsrichanalai et al. 2007).

II. Quantative buffy coat (QBC) test

QBC test, which is based on principle of centrifugal stratification of blood components, is a well-known and a very sensitive technique which can be used for the detection of malarial parasites in peripheral blood (Ahmed and Samantaray 2014). This method involves staining parasite deoxyribonucleic acid (DNA) in a glass hematocrit tube with acridine orange stain (Pinto et al. 2001). Fluorescing parasites are then observed, with an ultra violet microscope. This technique is easier and faster than classic peripheral blood smear microscopy. However, QBC tests are expensive and require specialized equipment and cannot be used to determine species and accurate numbers of parasites (Vaidya and Sukesh 2012).

1.6.2.2 Immunological techniques

I. Serology tests

Serology detects antibodies against malaria parasites, using either indirect immunofluorescence (IFA) or enzyme-linked immunosorbent assay (ELISA). Serology does not detect current infection because of the time required for development of antibody. It is not practical for routine diagnosis of acute malaria. However, antibody detection may be useful to measure past exposure such as screening blood donors, testing a patient who has had repeated or chronic malaria infections (usually from an endemic area) and a patient who has been recently treated for malaria, whom the diagnosis is questioned.

Indirect fluorescent antibody test (IFA)

IFA method is based on the detection of antibodies against blood stage malaria parasite which can be used to confirm malaria infection or a history of infection, (Chotivanich et al. 2007). Specific antigen consists of infected blood or crude antigen bound to microscope slide. The homologous antibody in patient's serum react with antigen of parasites, forming an antigen-antibody (Ag-Ab) complex. Fluorescent-labeled anti-human antibody is then added, which attaches to the patient's malaria-specific antibodies. When the slide is examined with a fluorescence microscope, if parasites fluoresce an apple green color, a positive reaction has occurred (https://www.cdc.gov/malaria/diagnosis_treatment/serology .html). IFA is simple and sensitive, but time-consuming, requires fluorescence microscopy and well-trained technicians (Rosemary et al. 2007).

Enzyme linked immunosorbent assay (ELISA)

ELISA is a quantitative analytical methods that show Ag-Ab reactions through the color change obtained by using an enzyme-linked conjugate and enzyme substrate and that serve to identify the presence and concentration of molecules such as antigen (proteins, peptides, hormones, etc.) or antibody in biological fluids (Hornbeck 2001). ELISA can be performed with a number of modifications to the basic procedure: direct, indirect, sandwich or competitive. The key step, immobilization of the antigen of interest, can be accomplished by direct adsorption to the assay plate or indirectly via a capture antibody that has been attached to the plate. The antigen is then detected either directly (enzyme-labeled primary antibody) or

indirectly (enzyme-labeled secondary antibody). The detection antibodies are usually labeled with alkaline phosphatase (AP) or horseradish peroxidase (HRP). A large selection of substrates is available for performing the ELISA with an HRP or AP conjugate. The choice of substrate depends upon the required assay sensitivity and the instrumentation available for signal-detection (spectrophotometer, fluorometer or luminometer) (https://www.bosterbio.com/protocol-and-troubleshooting/elisa-principle) (Figure 4 and 5).

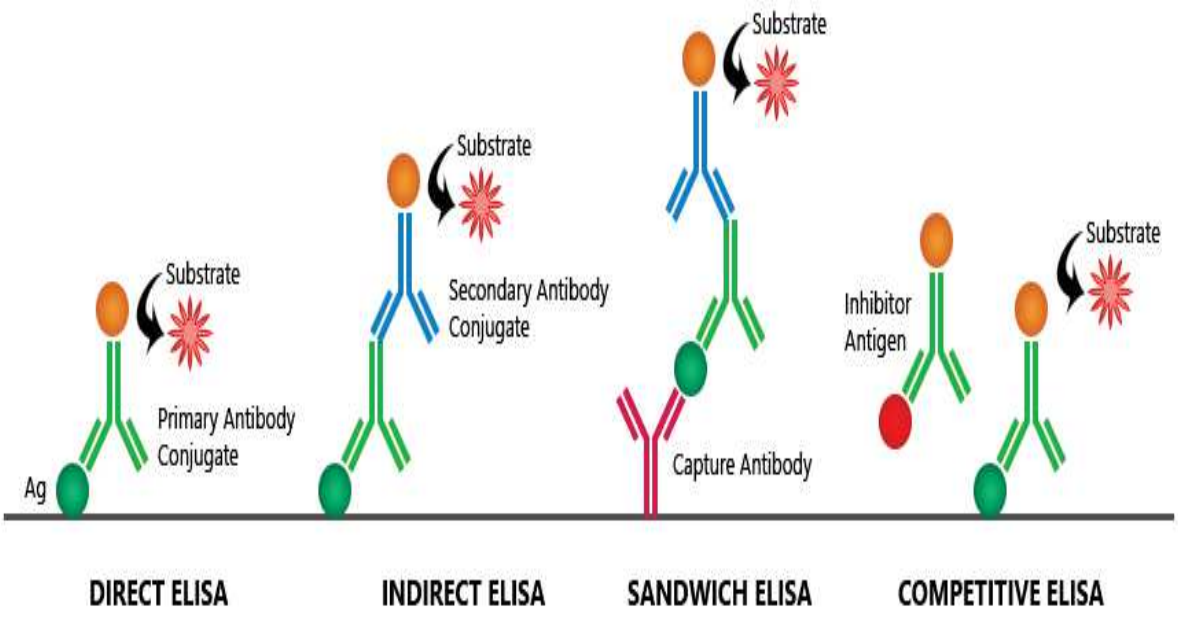

Figure 4. Four most common ELISA types. (https://www.bosterbio.com/protocol-and-troubleshooting/elisa-principle)

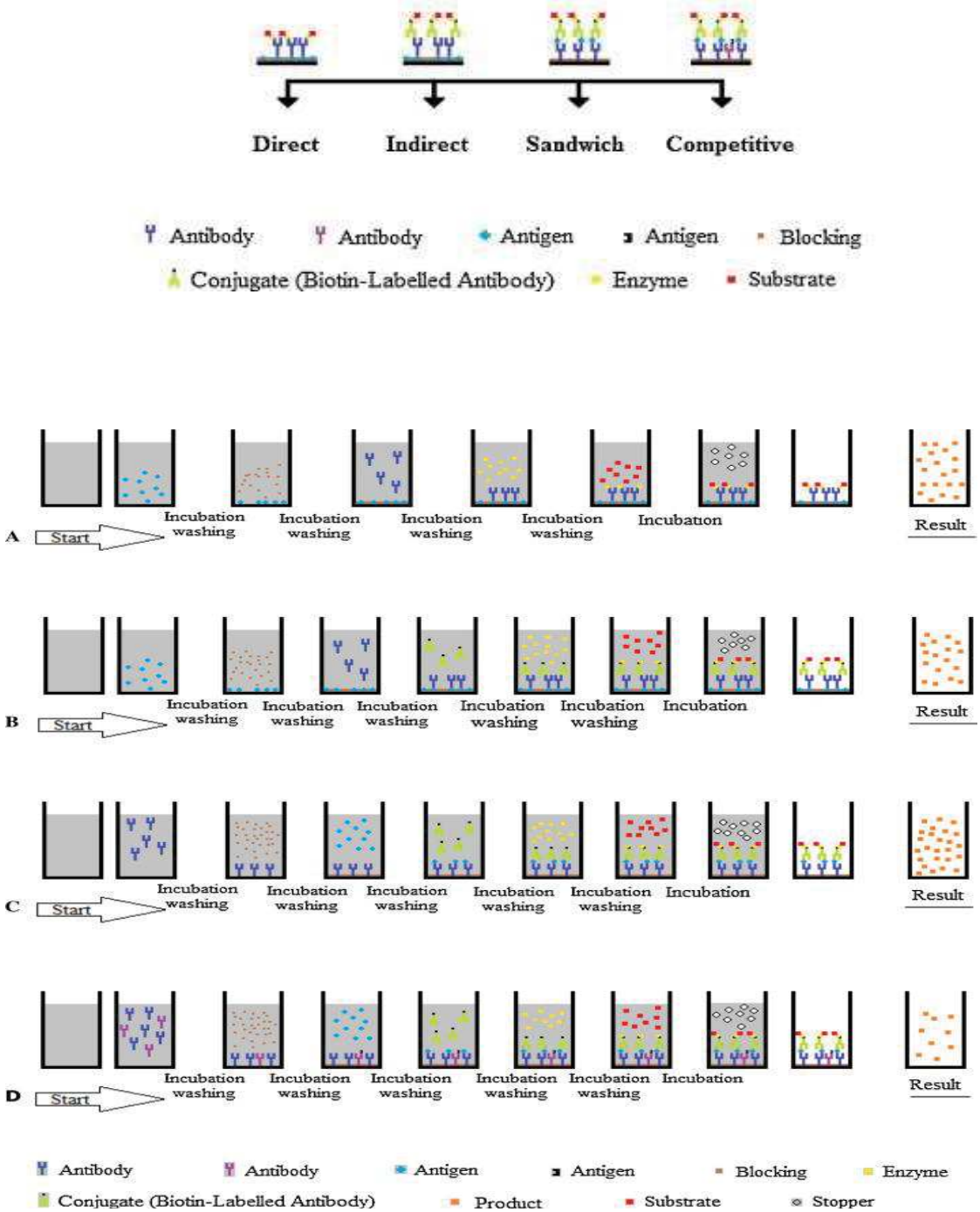

Figure 5. Schematic description of the direct (A), indirect (B), sandwich (C) and competitive (D) ELISA methods. (Aydin 2015)

ELISA is a sensitive and specific test that rapidly produces results. It has a wide area of application due to its ease of use and speed. Besides, it's more practical and does not require special equipment. The advantages and differences of ELISA tests are presented in Table 1.

Table 1. Strength and weakness of different ELISA assays. (Aydin 2015)

Types	Screening	Disadvantage	Advantages	Comment
Direct	Antibody	False-positive	Low sensitive	Detect the presence of small quantities of a substrate, either antigen or antibody. Reproducible.
Indirect	Antigen/Antibody	Immobilization non-specific	High sensitive	
Sandwich	Antigen		Very high sensitive	
Competitive	Antibody		High sensitive	

ELISA allows for testing large numbers of samples within a short time frame. High sensitivity and specificity of a histidine-rich protein 2 (HRP2)-based for the detection *P. falciparum* in patients of malaria clinics along the Thailand-Myanmar border were observed. Microscopy combined with ELISA reaches a sensitivity and specificity similar to PCR-adjusted microscopy for the diagnosis of *P. falciparum* while being considerably less expensive. Serology tools showed relevant to measure *Plasmodium* exposure risk at individual and community level (Helb et al. 2015) and to detect change in malaria transmission over time (Drakeley et al. 2005) (see section 3.2.2 for details).

II. Rapid diagnostic test (RDT)

WHO recommends prompt diagnosis of malaria either by microscopy or RDT in all patients with suspected malaria before treatment is administered (http://www.who.int/malaria /areas/diagnosis/overview/en/). Malaria RDT detect specific antigens (proteins) produced by malaria parasites in small amount of blood (5–15 μl) of infected individuals. Some RDT can detect only one species (*Plasmodium falciparum*) while others detect multiple species (*P. vivax*, *P. malariae* and *P. ovale*). Blood for the test is commonly obtained from a finger-prick. RDT are lateral flow immuno-chromatographic antigen-detection tests, which rely on the capture of dye-labeled antibodies to produce a visible band on a strip of nitro-cellulose, often encased in plastic housing, referred to as cassettes. With malaria RDT, the dye-labeled antibody first binds to a parasite antigen, and the resultant complex is captured on the strip by a band of bound antibody, forming a visible line (T - test line) in the results window. A control line (C-

control line) gives information on the integrity of the antibody-dye conjugate, but does not confirm the ability to detect parasite antigen. RDT is simple to perform, easy to interpret and doesn't require electricity so have the potential to detect malaria infections, especially in remote areas with limited access to good quality microscopy services. Recently, a new RDT method has been developed for detecting *P. knowlesi* (McCutchan et al. 2008).

1.6.2.3 Molecular biology techniques

I. Polymerase chain reaction (PCR)

PCR-based techniques were recently and are considered as the most specific and sensitive diagnostic methods, particularly for malaria cases with low parasitemia or mixed infection (Morassin et al. 2002). The first diagnosis of *Plasmodium* infection by means of PCR used rRNA sequence-specific oligonucleotides to detect *P. falciparum* DNA and *P. vivax* DNA from cultures and blood (Jaureguiberry et al. 1990). PCR assays have been developed for the laboratory diagnosis of malaria, including conventional and real-time PCR techniques (Myjak et al. 2002, Speers et al. 2003, Rougemont et al. 2004, Perandin et al. 2004 , Mangold et al. 2005). PCR can detect as few as 1-5 parasites/µl of blood (≤ 0.0001% of infected red blood cells) compared with around 50-100 parasites/µl of blood by microscopy or RDT. Moreover, a very sensitive and specific high-throughput high-volume qPCR method based on *Plasmodium spp* 18S RNA was developed for the detection of low-density parasitemias (>20 parasites/ml) (Imwong et al. 2014). The utility of this new method will allows assessment of the asymptomatic reservoir of parasitemic individuals with chronic very low-level *Plasmodium* infections, thereby providing an accurate assessment of the malaria epidemiology. PCR can also help detect drug-resistant parasites, mixed infections, and may be automated to process large numbers of samples (Swan et al. 2005, Hawkes and Kain 2007).

Molecular methods offer excellent specificity and sensitivity, and could be considered as a reference standard for malaria infection diagnosis. For routine use, the qRT-PCR method presents advantage of rapidity, less contamination and better standardization. Different methods are available for *Plasmodium* detection and quantification, each method having different sensitivity and specificity (review in Bourgeois et al. 2010).

The molecular detection of *Plasmodium* infection in vectors provides essential information on malaria epidemiology that is not accessible by conventional methods (either in

clinical or in entomological samples). Quantitative real-time polymerase chain reaction (qrtPCR) has significantly improved the sensitivity and the specificity of the detection and allowed for an accurate identification of the Plasmodial species in the *Anopheline* vectors. A wide variety of primers has been used in different assays, mostly adapted from molecular diagnosis of malaria in human. Many utilize primers designed against species specific regions in different PCR DNA targets of the nuclear (18S ssuRNA genes) or mitochondrial (COX I, COX III, other non-coding sequence) genome. Dilution series of standard DNA extracts (*Pf* and *Pv* sporozoites, oocysts and blood stages) were used to assess detection limits of the different methods and to produce standard curves for the absolute quantification of sporozoites in malaria vectors (Chaumeau et al. 2016).

Although PCR-based assays were found to be more sensitive than all conventional methods, it is of limited utility for the diagnosis of acutely ill patients in the standard healthcare setting. PCR results are not often available quickly enough to be of value in establishing the diagnosis of malaria infection (https://www.cdc.gov/malaria/diagnosis_ treatment/ diagnosis.html.) This method requires specific material and is more expensive than microscopy (Berry et al. 2005). In most areas with malaria transmission, factors such as limited financial and human resources, persistence of subclinical parasitaemia, inadequate laboratory infrastructures, and remote rural areas preclude PCR as a routine diagnostic method (Hanscheid and Grobusch 2002).

The choice of malaria-diagnostic method should take into account the level of malaria endemicity, the urgency of diagnosis, the experience of the physician, the effectiveness of healthcare workers and the budget.

1.7 Malaria treatment

Treatment of malaria depends on many factors such as type of infection, severity of infection, status of the host and associated conditions or diseases. Patients with *P. falciparum* malaria should be evaluated in potential seriousness of the disease and possibility of resistance to anti-malarial drugs. Antimalarial drugs are used against malaria and to prevent infections in individuals visiting a malaria endemic region who have no immunity (malaria prophylaxis). Current practice in treating cases of malaria is based on the concept of combination therapy. This offers several advantages, including reduced risk of treatment failure, risk of developing

resistance and reduced side effects. The combination therapy can be defined as, the simultaneous use of two or more blood schizonticidal drugs with independent modes of action and different biochemical targets in the parasite. Artemisinin based combination therapies (ACT) are recommended by WHO as the first-line treatment for uncomplicated malaria. ACT includes artemether + lumefantrine, artesunate + amodiaquine, artesunate + mefloquine, artesunate + sulfadoxin + pyrimethamine and dihydroartemisinin + piperaquine (WHO 2015b). These derivatives are highly effective against malaria and aims to reduce the biomass of asexual parasites rapidly while also exerting strong gametocytocidal (Price et al. 1996).

For *P.vivax* malaria, chloroquine and primaquine are recommended as first-line treatments (WHO 2015b). ACT and chloroquine are recommended to treat uncomplicated malaria caused by *P. vivax*, *P.ovale*, *P. malariae* or *P.knowlesi* in adults and children (WHO 2015b). Preventing relapse from *P.vivax* or *P.ovale* malaria, 14 day course (0.25-0.5 mg/kg body weight-daily) of primaquine is highly recommended (WHO 2015b). However, the use of primaquine is associated with hemolysis risk in people with glucose-6-phosphate dehydrogenase (G6PD) deficiency (Beutler 1959). RDT are now available for rapid detection of G6PD deficiency at community level (Satyagraha et al. 2016).

For severe malaria, WHO recommend to treat adults and children (including infants, pregnant women in all trimesters and lactating women) with intravenous or intramuscular artesunate for at least 24 h and until they can tolerate oral medication (WHO 2015b). Once a patient has received at least 24 h of parenteral therapy and can tolerate oral therapy, a complete treatment with 3 days of an ACT is advocated.

The choice of ACT differ between countries. In Thailand, the first-line treatment of uncomplicated malaria caused by *P. falciparum* are Dihydroartemisinin-Piperaquine (DP) (3 days) + Primaquine (PQ) (given on day 1 of DP). Treatment of uncomplicated malaria caused by *P. vivax* or *P. ovale* are Chloroquine (CQ) (3 days) + Primaquine (14 days). Treatment of uncomplicated of malaria mixed infection caused by *P. falciparum* and *P. vivax* or *P. ovale* are DP (3 days) + PQ (14 days). Treatment of uncomplicated of malaria caused by *P. knowlesi* are CQ (3 days).

ACT and other antimalarial drugs are widely used for the treatment and prevention of malaria infection. Unfortunately, the emergence of multi drug resistance could seriously

threaten progress achieved to date, and could lead to a rise in the disease burden worlwide (WHO 2013a).

1.7.1 Drug resistance

One of the major threats to malaria control and elimination efforts is the ongoing spread and emergence of resistance towards commonly used antimalarial drugs to treat *P. falciparum* and *P. vivax* infections. During 1950s-1960s, Chloroquine was the main drug of choice in the WHO Global Malaria Eradication Programmes (WHO 2008). This antimalarial drugs proved to be one of the most successful and important drugs ever deployed against an infectious disease. The tremendous success of chloroquine and its heavy use through the decades led to chloroquine resistance in *Plasmodium spp* (Wellems and Plowe 2001). Chloroquine resistance was first detected in the early sixties in Cambodia (Eyles et al. 1963). Resistant strains from these areas then spread steadily in the 1960s and 1970s through South America, Southeast Asia, and India. Until the late 1970s, chloroquine resistance was absent in Africa; the sweep of resistant *P. falciparum* across that continent occured within a decade (Peter 1987). Chloroquine resistance has represented a severe problem for both prophylaxis and treatment of malaria (Payne 1987). Due to the absence of new drug for Chloroquine replacement morbidity and mortality resurged in Africa, notably among children (Greenberg et al. 1989, Trape et al. 1998).

Today, WHO recommends artemisinin-based combination therapies (ACT) for the treatment of uncomplicated malaria caused by *P. falciparum* (section 1.7). ACT have been integral to the remarkable recent successes in global malaria control, and there is broad consensus that protecting the efficacy of these medicine combinations is an urgent priority. However, *P. falciparum* artemisinin resistance emerged in Cambodia (Dondorp et al. 2009) and has spread to four countries of the Greater Mekong Subregion: the Lao People's Democratic Republic, Myanmar, Thailand and Vietnam. Artemisinin Resistance containment plan has been set up in all countries as part of a multi-stakeholder effort (http://www.who.int /malaria/areas/drug_resistance/overview/en/). In low transmission settings such as Southeast Asia, elimination of *P. falciparum* malaria is the only strategy that can prevent the spread of artemisinin resistance (WHO 2015a). The race has started to try to eliminate *P. falciparum* malaria before this happens, as the consequence of letting resistance reach the African continent will be that millions will die (White et al. 1999).

Till date, drug resistance has been documented in *P. falciparum*, *P. vivax*, and *P. malariae* (WHO 2010b). *Plasmodium falciparum* has developed resistance to nearly all antimalarial drugs currently in use; *P. vivax* has been found to be resistant to chloroquine and primaquine; and *P. malariae* has been reported to be resistant to chloroquine and pyrimethamine in some areas (WHO 2010b, Maguire et al. 2002, Young 1957, http:// www.malariasite.com/drug-resistance/).

1.8 Global strategy for malaria prevention, control and elimination

Although effective tools have been developed to combat malaria since 2000, malaria still continue to strongly impact on the health and socio-economic development of low and middle income countries. Considering the highly adaptable nature of the mosquito vector and parasites, combination of cost effective tools are needed. Research will play also a key role in development of norms and policies to achieve millennium development goals for malaria.

1.8.1 WHO recommended strategies for malaria prevention, control and elimination

The main recommended interventions for malaria control are based on effective vector control, chemoprevention and case management (WHO 2015d) (Figure 6).

Vector control, which focuses on blocking the transmission of parasites by the mosquito vector from humans to mosquitoes and then back to humans, thereby reducing the disease burden. The main interventions are insecticide-treated mosquito nets (ITN), indoor residual spraying (IRS) and, in some settings, mosquito larval control (see section 2.3 for details).

Chemoprevention aims to suppress the blood stage infection in humans and hence prevent malaria disease. Preventive chemotherapy is the use of complete treatment courses of antimalarial medicines as a prophylactic in populations that are particularly at risk of malaria, with the goal of reducing malaria-related morbidity and mortality. The three preventive therapies presently recommended by WHO are intermittent preventive treatment in pregnancy, intermittent preventive treatment in infants, and seasonal malaria chemoprevention for children aged 3–59 months.

Case management, which involves prompt diagnosis and treatment of malaria infections with appropriate antimalarial medicines aims to reduce the likelihood of progression to severe disease and death from malaria.

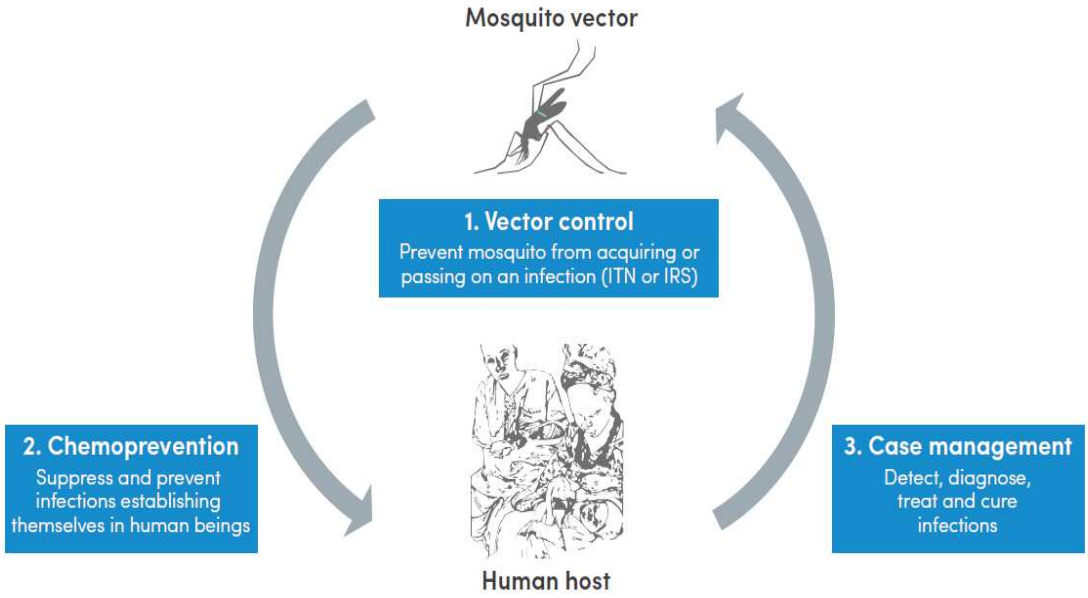

Figure 6. Main strategies to prevent and treat malaria. (WHO 2015d)

WHO is also accelerating efforts towards elimination of malaria and attainment of malaria-free status. Up to date, 17 countries eliminated malaria (i.e. attained zero indigenous cases for 3 years or more); six of these countries have been certified as malaria free by WHO (WHO 2016a). WHO has established a Strategy for Malaria Elimination in the Greater Mekong subregion (2015–2030) considering recent improvements in malaria control in four countries (Cambodia, Lao People's Democratic Republic, Myanmar and Vietnam). The ultimate goal is to eliminate *P. falciparum* malaria by 2025, and all malaria by 2030, in all countries in the Greater Mekong subregion including areas with multidrug resistance. The principle is based on scaling up a combinations core interventions, including improved malaria case and entomological surveillance, monitoring and evaluation, and stratification by malaria disease burden (WHO 2015d).

1.8.2 Progress in vaccine development

The development of a safe and effective malaria vaccines is an area of intensive research. Current approaches are focusing on recombinant protein and attenuated whole organism vaccines (Strugnell et al. 2011). However, the complexity of the *Plasmodium* parasite and the lack of understanding of critical processes, such as host immune protection and disease pathogenesis, have hampered vaccine development efforts. There is no licensed vaccine against malaria so far (https://www.cdc.gov/malaria/malaria_worldwide/reduction /vaccine.html) despite intensive research and development in the last 50 years. Several vaccine candidates have been however developed and tested in clinical trials, with various levels of efficacy (Figure 7). The most clinically advanced candidate, RTS,S, is presently undergoing Phase 3 clinical trials. " Final study results, published in The Lancet in April 2015, includes analysis of vaccine efficacy, immunogenicity, safety, and impact of RTS,S/AS01 over a median of 48 months of follow-up after the first dose and the effect of a fourth dose of vaccine. The final results demonstrated that efficacy against clinical malaria was 39 percent over four years of follow-up in children receiving four doses. Vaccination with the 3-dose primary series reduced clinical malaria cases by 26 percent in young children to the end of the study. The addition of a fourth dose of RTS,S, administered 18 months after the primary series, resulted in a reduction of clinical cases by 39 percent over an average of four years of follow-up. Administration of the fourth dose prolonged protection against clinical malaria, with 1,774 cases of malaria averted per 1,000 children vaccinated, on average, across all sites (site-specific cases averted ranged from 205 to 6,565 per 1,000 children vaccinated). Vaccine efficacy waned over time following the fourth dose, and further studies are ongoing to assess longer-term efficacy and the need for additional doses." (RTS,S Clinical Trials Partnership 2015, RTS,S malaria vaccine candidate (http://www.malariavacci ne.org/resources/rtss -malaria-vaccine-candidate).

1.8.3 Mass drug administration for malaria elimination

Mass drug administration (MDA) is the practice of treating a whole population within a given geographical area, irrespective of the presence of symptoms and without diagnostic testing (WHO 2011a). MDA was a component of many malaria elimination programs during the eradication era (Newby et al. 2015). Since then, it has been viewed with skepticism due to concerns regarding its efficacy, logistical feasibility, sustainability as a malaria control tool, and fear of accelerating drug resistance. But in light of the availability of transmission-reducing

antimalarials, e.g. artemisinin-based combination therapies and primaquine, and the limitations of current diagnostic tools to detect sub-patent infections, there has been renewed interest in the role of MDA as an elimination tool (Feachem et al. 2009, Shanks 2012).

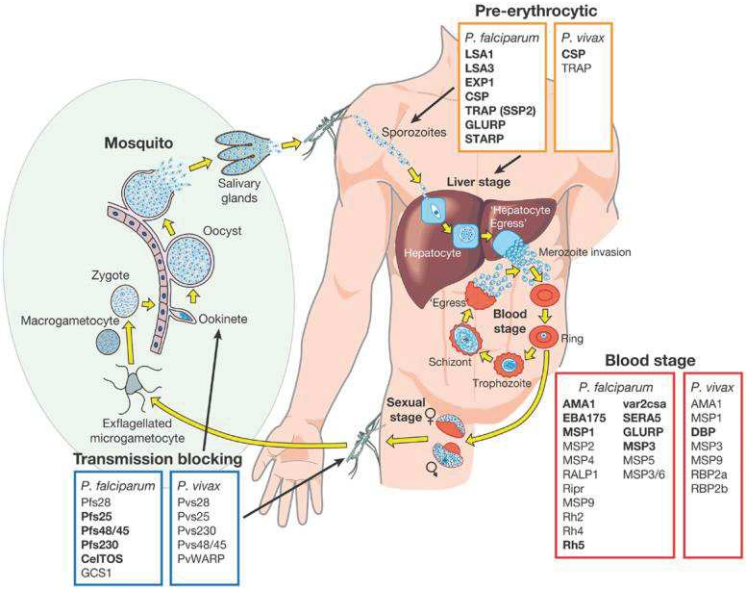

Figure 7. Malaria vaccine candidate antigens. All candidate antigens for *Plasmodium falciparum* and *Plasmodium vivax* are superimposed on the *Plasmodium* lifecycle, to indicate the category of malaria vaccine being developed and the lifecycle stage targeted. Antigens indicated in bold are those that are currently being evaluated in pre-clinical trials or have entered at least Phase1 clinical trials according to the WHO malaria vaccine rainbow tables (WHO 2013d). The *P. vivax* latent stages known as "hyponozoites" are not shown but these occur in the liver stage (Barry et al. 2014).

Numerous field studies have implemented MDA both to eliminate malaria and as an epidemic response, and while most failed, there are several examples where MDA, in combination with other malaria control measures, had success. A systematic review of the studies of MDA for malaria from 1913-2011 was recently published; Twelve studies met a definition of success applicable to malaria elimination settings with zero indigenous malaria cases in the target population maintained for at least 6 months after the end of all drug administration (Newby et al. 2015). The majority of the published studies (63%) had a follow-up period of less than 6 months, preventing an assessment of the interventions long-term effects on transmission. Many studies were able to reduce parasite prevalence in the target population

temporarily, but either were not able to reach zero prevalence or were followed by an increase in prevalence shortly after drug administration ceased (Poirot et al. 2013, Newby et al. 2015). The primary factors determining the success of MDA were: achieving at least 80% or even 90% coverage of the target population with drug administration, directly observed treatment (DOT), strong community engagement, high coverage with concomitant vector control interventions, and the use of 8-aminoquinolines, particularly in *P. vivax* transmission settings (Newby et al. 2015).

Strong community participation is also crucial for the MDA success for malaria elimination (Kaneko et al. 2000, Song et al. 2010). Local health workers or volunteers should be used for drug distribution, since they understand the environment and local customs. However, even well-staffed with local workers, and involved exhaustive community outreach to ensure participation, yet coverage was only 67% for the MDA campaign in the Solomon Islands due to refusal of the targeted population to take drugs when they were not ill. The 3-day drug regimen further contributed to lack of participation. Another factor leading to effective MDA is vector control. Co-interventions such as used of IRS, ITN, chemical or biological larval control and environmental management is essential and should be used prior to commencement of, or concurrently with, MDA to bring transmission down to low levels. Even vector control is less of a priority in highly seasonal *P. vivax* settings where there are regular periods of zero transmission with no vectors present, but in other transmission settings, every effort should be made to minimize vector-human contact either prior to or concurrent with MDA implementation. Vector control should be included as a central part of an MDA strategy, particularly for *P. falciparum* elimination (Newby et al. 2015).

In summary, MDA can be used to reduce and, in some circumstances, interrupt transmission of both *P. falciparum* and *P. vivax* malaria in specific settings, and should be considered for operational implementation as one component of a comprehensive elimination strategy. In areas with low transmission or seasonal *P. vivax* transmission, any MDA strategies used should include close monitoring for coverage, safety, and population impact on transmission rates.

2. THE *ANOPHELES* MOSQUITO

2.1 *Anopheline* life cycle

There are four development stages of *Anopheles* mosquito life cycle: egg, larva, pupa and adult mosquito (Figure 8). *Anopheles* mosquito can develop from egg to adult mosquito usually in about 5-14 days, depending on the species and the ambient temperature. *Anopheles* mosquito acts as malaria vector. The adult females can live up to a month (or more in captivity) but most probably do not live more than 1-2 weeks in nature (https://www.cdc.gov /malaria/about/biology/mosquitoes/index.html).

Eggs

To develop eggs a female mosquito needs to have a blood meal and after few days it is ready to lay eggs. Adult females lay 50-200 eggs per oviposition. Eggs are laid singly directly on water and are unique in having floats on either side. The length of time the eggs take to hatch into larvae largely depends on temperature such at about 30°C, eggs hatch into larvae in about 2-3 days where as in temperate zones (16°C) take time about 7-14 days (Williams and Pinto 2012).

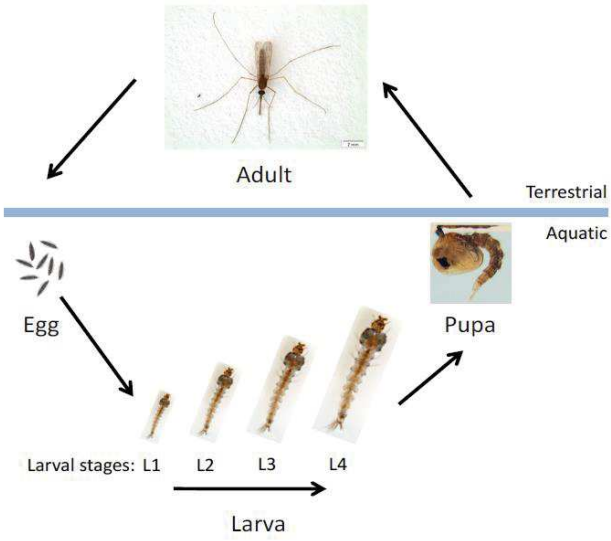

Figure 8. Stages of the *Anopheles* mosquito life-cycle (Williams and Pinto 2012)

Larvae

The larva has a well-developed head with “mouth brushes” used for feeding (filter feeders). The larva feeds on micro-organisms (e.g. algae, bacteria) and organic matter in the water where they breed. The *Anopheles* larva has no respiratory siphon. It lies parallel to surface of water in order to breath. *Anopheles* mosquitoes develop though four larval sizes or instars before pupating (Foster and Walker 2009).

Pupae

The pupa is comma-shaped and stays at the surface of the water to breathe through a pair of respiratory trumpets on the cephalothorax. Unlike the pupae of many other insects, mosquito pupae are very mobile; they use the paddle at the end of their abdomen to quickly move through their aquatic habitat. The pupal stage does not feed (Foster and Walker 2009).

Adults

After emerging from the pupa, the adult mosquito rests for a short time in order to harden its body. Adult mosquitoes usually mate within a few days after emerging from the pupal stage, and the females search for blood meals that are necessary for egg development. After encountering and biting a host, the female mosquito finds a resting place where it digests the blood and evaporates water. The resting time is temperature-dependent (shorter at higher temperatures) and is usually 2–3 days in tropical areas. After digesting the blood, the mosquito flies in search of a water body to lay the eggs before seeking a host again to repeat the feeding cycle (Figure 9). Usually, mosquitoes begin host-seeking at the same time every night. If they are unsuccessful in biting, they rest through the day and try again the next night. The probability that a mosquito is successful in completing a feeding cycle depends on a variety of factors, including whether the available human hosts are protected by ITN or IRS (Chitnis et al. 2010).

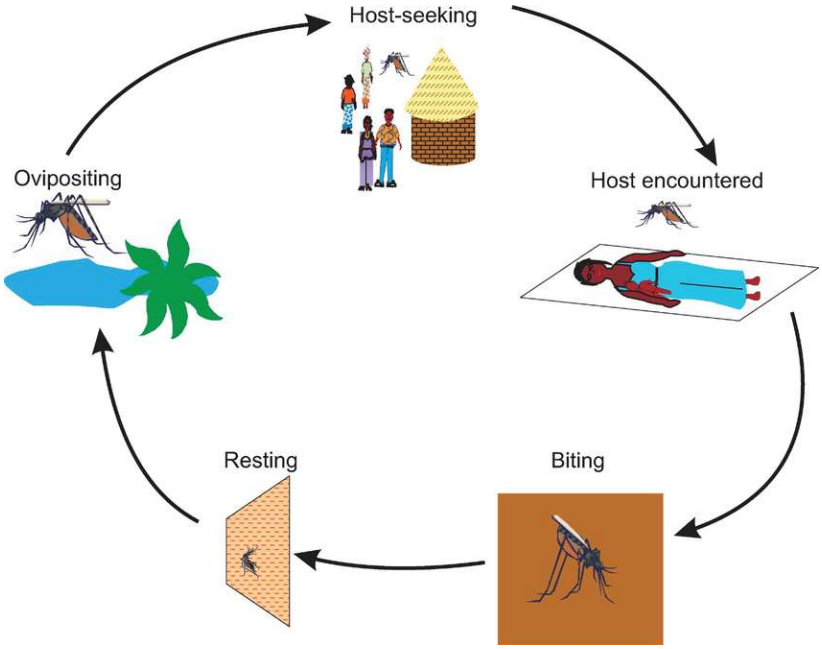

Figure 9. The feeding (or gonotrophic) cycle of the female mosquito. After emergence, mosquitoes seek and bite hosts, rest and lay eggs, before seeking hosts again. The mosquito experiences varying levels of risk in each state (Chitnis et al. 2008).

Mosquito vectors require blood meals for egg development, and the rate of digestion of these blood meals is normally directly proportional to increase in temperature. Increased frequency in egg-laying would require increased rates of feeding on human hosts resulting in enhanced vectorial capacity (vectorial capacity refers to all of the environmental, behavioral, cellular, and biochemical factors that may have an influence on the association between a vector, the pathogen transmitted by the vector, and the vertebrate host to which the pathogen is transmitted (Beerntsen et al. 2000). Moreover, seasonal increases in ambient temperature may accelerate *Plasmodium* parasites maturation rates and consequently enhance the vectorial capacity (Mala et al. 2014).

2.2 *Anopheles* malaria vectors

Plasmodium parasite are transmitted to humans by female mosquitoes of the genus *Anopheles*. Only around 25 of the more than 400 *Anopheline* species are good vectors (Sinka et al. 2012). The factor characteristic of mosquito species to be an effctive at transmitting malaria to people are including abundance, behavior, longevity, capacity and contact with human. There are malaria vectors in all continents of the world, exception of the

Antarctica. Several vector species can occur in sympatry, i.e. in the same area and at the same time. A global map of dominant vector species / species complex is shown in figure 10. Depending on the geographic region, the *Anopheles* species composition varies, and hence the vectors responsible for the transmission of malaria also vary from one region to another. Of the 25 described-*Anopheles* complexes worldwide, 11 occur in Asia, 10 of which are recognized in Thailand (Harbach et al. 2013, Manguin et al. 2013). Because individual species within a complex may differ significantly in biological, ecological, and behavioral characteristics, which can greatly influence their potential as disease-transmitting agents, incorrect species identification of individual complex members may result in failure to distinguish between a vector and non-vector species, and thus lead to misdirected and ineffective vector control programs (Van Bortel et al. 2001, Singh et al. 2010). *Anopheles gambiae* is one of the best known malaria vector, because of its predominant role in the transmission of the most dangerous malaria parasite species to humans (*P. falciparum*) in Africa (Manguin et al. 2013). *Anopheles arabiansis* and *An.funestus* are also co-dominant across much the Africa continent. Whereas in the Asia and Pacific region show a highly complex situation with coexistence of a numerous species complexes.

In Southeast Asia mainland (includes Cambodia, Lao PDR, Myanmar, Thailand, Vietnam and Peninsular Malaysia), three main malaria vectors are recognized: *An. dirus sensu lato (s.l.)* (Dirus complex), *An. minimus s.l.* (Minimus complex) and *An. maculatus s.l.* (Maculatus complex). Among the Dirus complex, two main malaria vectors, *An. dirus* and *An. baimaii* are considered as forest and forest-fringe malaria vectors exhibiting anthropophilic and exophagic preferences (Suwonkerd et al. 2013). The Minimus complex, comprises of three sibling species : *An. minimus* (formerly species A) and *An. harrisoni* (species C) which are broadly distribution in Southeast Asia, and *An. yaeyamaensis* (species E) which is found only in Japan. This species is mainly found in or near hilly and mountainous areas, where it uses a variety of larval habitats, including seepage waters, ditches, rice fields, ponds, stream margins, swamps and lakes (Williams and Pinto 2012). *Anopheles minimus* appears to more frequently anthropophilic in areas where cattle are not present. Whereas *An. harrisoni* is generally zoophilic, but more studies are needed to better understand his seeking and biting behaviour. In Thailand, *An. minimus s.s*. is considered endophagic while *An. harrisoni* is typically exophagic (Parker et al. 2015a). In the forested areas, the presence of both *An. dirus s.l.* during rainy season and *An. minimus s.l.* during the dry season can contribute to ensure perenial

malaria transmission. Species of the *An. maculatus* is typically found in or near hilly, forested regions. It is also known to inhabit forest camps (logging camps) and mountain areas above 1200 m. It breeds in shallow, rocky and sandy pools near clean rivers or streams, with direct sunlight. It is known to opportunistically feed on both animals and humans (review in Parker et al.2015a).

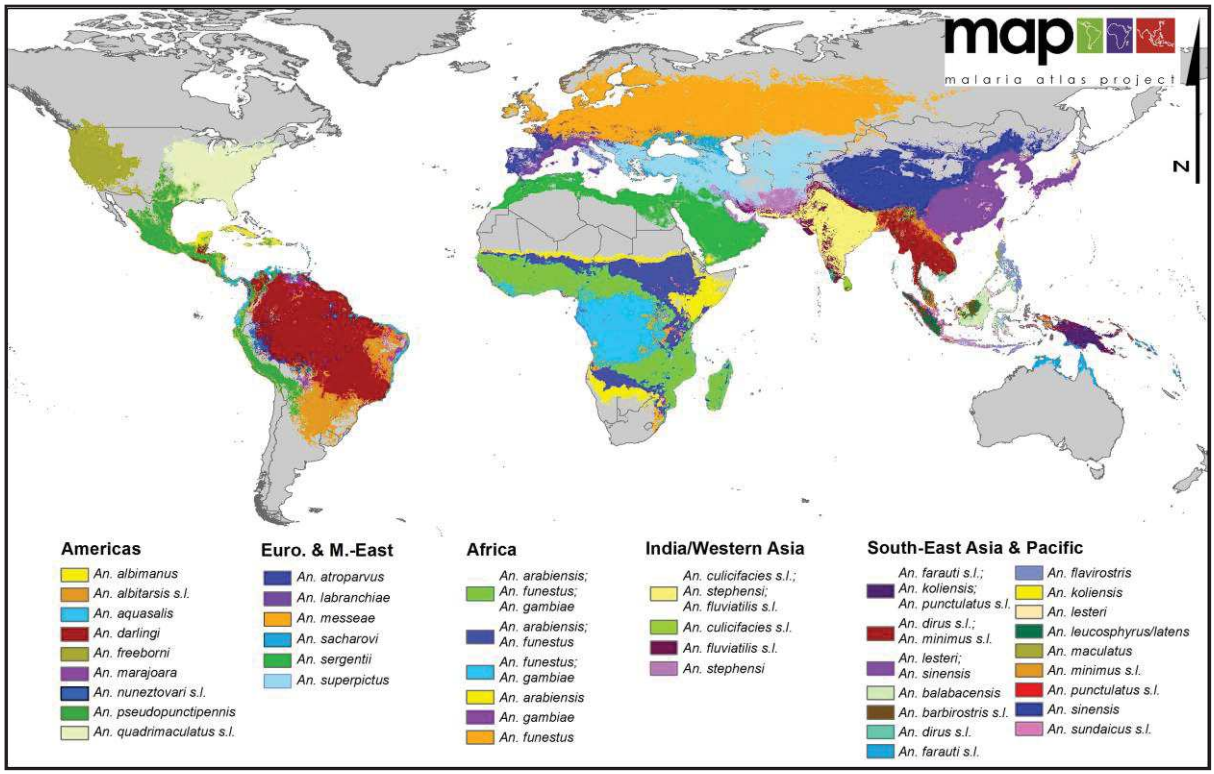

Figure 10. A global map of dominant malaria vector species. (Sinka et al. 2012)

2.3 Vector control strategies

Controlling mosquito vectors is fundamental to reduce mosquito-borne diseases by targeting vectorial capacity and hence the transmission. Effective vector-control strategies have been pivotal in reducing worldwide malaria mortality and morbidity (WHO 2015c). Recent estimates indicates that vector control will constitute 50–60% of all investments required to eliminate and control malaria and vector borne diseases from now until 2030. Over the past decade, the main vector-control tools used for malaria control were pesticides and

pesticide based products (WHO 2015c). The two core, broadly applicable measures for malaria vector control are long-lasting insecticidal nets (LLIN) and indoor residual spraying (IRS). In specific settings and under special circumstances, the core vector-control interventions of LLIN and IRS can be supplemented by other methods, such as larval source management (LSM). A brief description of each method is providing in the following sections.

Larval source management (LSM)

Mosquito larval source management (LSM) is the management of water bodies (aquatic habitats) that are potential breeding sites for mosquitoes in order to prevent the completion of immature development. LSM can be further classified into habitat modification, habitat manipulation, biological control and larviciding (http://www.lcmcd. org/). Habitat modification is a permanent change of land and water, including landscaping, drainage of surface water, land reclamation and filling but also coverage of large water storage containers, wells and other potential breeding sites. Habitat manipulation is a recurrent activity, such as water-level manipulation, which includes measures like flushing, drain clearance, shading or exposing habitats to the sun depending on the ecology of the local vector. Biological control of mosquitoes refers to the introduction of natural enemies into aquatic habitats; these are predatory fish (such as *Gambusia affinis, Poecilia reticulata)* or invertebrates, parasites or disease organisms. Larviciding is the regular application of biological or chemical insecticides to water bodies for control of mosquitoes (Fillinger et al. 2011).

Insecticides available for larval control have different modes of action and include chemical insecticides (e.g. temephos, insect-growth regulators such as pyriproxyfen, methoprene and diflubenzuron), biological agents (e.g. bacteria *Bacillus thuringiensis israelensis* - *Bti, Bacillus sphaericus* - *Bs*) or toxins that kill larvae and pupae. The list of WHO-recommended compounds and formulations for the control of mosquito larvae is available on the WHO Pesticide Evaluation Scheme (WHOPES) (http://www.who.int/ whopes/en/).

The most common interventions for mosquito larval control are the application of *Bti* and *Bs*, temephos, filling and draining, and the introduction of fishes. LSM provides the dual benefits of not only reducing numbers of house-entering mosquitoes, but importantly, also those that bite outdoors. LSM represents a good complimentary vector control intervention particularly in locations where larval habitat are relatively few and easily identified (Fillinger

et al. 2011). Field evaluations under various eco-epidemiological conditions in Africa showed that hand-application of larvicides can reduce transmission by 70-90% in settings where the majority of aquatic mosquito larval habitats were defined and aquatic surface areas not too extensive (Fillinger et al. 2006, Shililu et al. 2007, Fillinger et al. 2009, Fillinger et al. 2011). In areas with very extensive water bodies, aerial application of larvicides would be best suited for the treatment of extensive flood plains and irrigation systems (Becker 2010). However, a frequent critique is that larviciding is too labor intensive and not sustainable. It needs intensive management systems for application, surveillance and evaluation, which are expensive and prone to failure (Vanek et al. 2006, Chaki et al. 2009). Accessibility of habitats in urban settings presents a major challenge because the majority of compounds are fenced for security reasons (Vanek et al. 2006). LSM is not a strategy for application in all habitats and is not a stand-alone intervention. However, LSM could be integrated into malaria control or general mosquito abatement programmes once transmission has been reduced to low or moderate levels by LLIN or IRS, or once these interventions have reached their maximum practical effect (WHO 2013f). Recent evidence showed that LSM can be particularly effective when combined with LLIN (Fillinger et al. 2009). In Kenya, ITN use was associated with a 31% reduction in the risk of new malaria infections, while residence in an area with additional larviciding reduced the risk of new infections by 56% (Fillinger et al. 2009). In Tanzania, microbial larviciding (*Bti*) was applied weekly through community-based program, reduced malaria infection risk among children 5 years of age provided protection at least as good as personal use of an insecticide treated net (Geissbuhler et al. 2009).

Indoor residual spraying (IRS)

IRS is the application of long-acting chemical insecticides on the walls and ceilings of household using insecticides with residual action, in order to kill the adult vector mosquitoes that land and rest on these surfaces (WHO 2006). The effectiveness of IRS depends to a large extent on the insecticide classes and formulation applied and vector's sensitivity to the insecticide used and how much they like to rest indoors. IRS are intended to reduce vector population and kill potentially infected *Anopheles* mosquitoes before the parasite they carry develops into an infective stage. Some of the insecticides used for IRS have also a repellent effect that reduce the number of mosquito vectors entering into the sprayed rooms, and thus reducing human-vector contact. One of the main advantages of IRS (compared to LLIN) is the ability to use a wide range of insecticide classes having different mode of actions. The list of

WHO-recommended insecticides for IRS is available on the WHO Pesticide Evaluation Scheme (WHOPES) (http://www.who.int/whopes/en/). The residual life of the insecticide on sprayed surfaces varies between different chemicals, but it is usually between three and six months. Frequency of IRS rounds will depend on the length of the transmission season, the outbreaks and the residual efficacy of the insecticide used.

The recent success of IRS in reducing malaria cases in South Africa by more than 80% has revived interest in this malaria prevention tool (https://www.cdc.gov/malaria /malaria_worldwide/reduction/irs.html). IRS reduced malaria transmission in young children by half compared to no IRS in an area of high transmission in Tanzania (Curtis 1999), and protected all age groups in an area of low transmission India and Pakistan (Rowland et al. 2000, Pluess et al. 2010). The meta-regression analysis shows that IRS is more effective in areas with high initial prevalence, multiple rounds of spraying and in regions where *P. falciparum* co exists with *P. vivax* (Kim et al. 2012).

Insecticide treated nets (ITN)

Another successful method of mosquito control relies on the use of bednets or curtains that can be sprayed with insecticides (insecticide treated nets; ITN). Since the *Anopheles* mosquito bites between dusk and dawn, sleeping under mosquito nets treated with pyrethroids provides critical protection against malaria transmission (http://www.malaria consortium.org/userfiles/file/Malaria%20resources/Insecticides%202.pdf). The net provides an effective barrier between the person who is sleeping under it and the mosquito vector, which can reduces the opportunity for biting and infection. The impregnated insecticide also acts to kill and repel any susceptible vector that rests on the net. ITN kill some of mosquitoes that come to bite, however, a protective effect at both the individual and community level (Birget et al. 2015). This “mass effect” does not always occur, but when it does, it provides benefits to everyone in the village (WHO 2006). Previous studies showed that ITN contributed to prevent around 50 percent of malaria cases in Africa and can reduce child deaths by an average 18 percent (Alonso et al. 1993).

Long-lasting insecticidal nets (LLIN) have been developed and increasingly distributed in place of conventionally treated nets (ITN) which need retreating with insecticide regularly. LLIN is a factory-treated mosquito net with insecticide incorporated into or bound around the

fibres, or a mosquito net treated with a long-lasting insecticidal treatment kit, that retains its biological activity for at least 20 WHO standard washes under laboratory conditions and 3 years of recommended use under field conditions without re-treatment (WHO 2013b). LLIN are considered by the WHO as the most cost effective and sustainable method for protection against malaria. About 1.2 billion LLIN have been distributed to world's population since 2004. A recent publication showed that about 70% of malaria cases were averted since 2000 due to the deployment of LLIN hence underlying the need to achieving high coverage in all transmission settings (Bhatt et al. 2015). In areas of high transmission, LLIN can reduce malaria incidence by 50% and in areas of low transmission, by 62% (Lengeler 2004). The list of WHO-recommended LLIN is available on the WHO Pesticide Evaluation Scheme (WHOPES) (http://www.who.int/whopes/en/).

The effectiveness of each vector control intervention (LLIN, IRS, LSM) will depends on a number of variables that includes bio-ecological traits of the mosquito vectors, habitat characteristics of the area and socio-economic/cultural aspects of the human population. The development of insecticide resistance in malaria vectors is also challenging the efficacy of LLIN that contains one insecticide class i.e the pyrethroids.

2.4 Vector resistance to insecticides

The extensive use of insecticides since the 1950s has led to the development of strong resistance in *Anopheles* mosquitoes hence representing a major threat to malaria control worldwide (Corbel and N'Guessan 2013). According to the WHO, resistance is defined as the ability of an insect to withstand the effects of an insecticide by becoming resistant to its toxic effects by means of natural selection and mutations (Davidson 1957). Resistance can involve several physiological and/or behavioural changes. Insecticides used for malaria control include organochlorine, organophosphorous, carbamate, and pyrethroid, with the latter being widely used for both IRS and LLIN (WHO 2009a). Resistance has naturally tended to follow the use and switches of these insecticides (Hemingway and Ranson 2000). Insecticide resistance has been reported in the main malaria vectors worldwide. Unfortunately, the highest levels of insecticide resistance were reported in Africa where malaria burden is still the highest in the world (WHO 2011b). Pyrethroid resistance, as measured by conventional bioassays, is clearly widespread in malaria vectors across Africa (Ranson et al. 2011). The immense challenge in Africa will be not to manage and control pyrethroid resistant mosquitoes only but to deal with

the development of “multiple resistant” populations that could resist to different class of public health insecticides. Few studies have evaluated the impact of resistance on malaria transmission in areas with known resistance. A meta-analysis found that even in areas with high pyrethroid resistance, LLIN continued to reduce blood feeding compared to untreated nets (Strode et al. 2014). Measurements on malaria transmission show that LLIN with a single pyrethroid maintain efficacy against malaria transmission to under-five year old children in an area of kdr resistance in West Africa (Henry et al. 2005) and in a cohort study in East Africa with enzyme based resistance (Lindblade et al. 2015). There is to date no evidence for malaria control failure using LLIN due to pyrethroid resistance (https://malariaworld.org/blog/llin-new-products-and-impact-insecticide-resistance) but new tools are need to prevent further spread. Recently, "combination nets" i.e. that combine a pyrethroid with piperonyl butoxide (PBO), have been developed to provide better control pyrethroid reistant mosquitoes (https://malariaworld.org /blog/llin-new-products-and-impact-insecticide-resistance). Five such products have a WHO prequalification listing as LLIN (WHOPES) (http://www.who.int/whopes/en/). Epidemiological data from a randomized controlled trial in Tanzania have demonstrated that one type of pyrethroid LLIN containing PBO has public health value in an area of confirmed metabolic pyrethroid resistance (Corbel, pers comm). Further studies are however needed to confirm this trend in different settings of malaria transmission and resistance pattern.

2.5 New paradigm for vector control

The later part of the 20th century has seen an expansion of areas with insecticide resistance in malaria vectors, coupled with low investment in development of public health pesticides. Hence, there is currently a dearth of effective vector-control products for malaria prevention and control. New and innovative tools (e.g. Spatial repellents, Attractive toxic sugar bait, Eave tubes, etc) are currently under evaluation by the vector community and by the WHO. Those new tools have been classified as new paradigms for tackling malaria transmission in area where mosquitoes developed high resistance to pyrethroids (http://www. who.int/neglected_diseases/vector_ecology/Summary_VCAG_paradigm_reviews.pdf?ua=1). A brief overview of these new tools/strategies are providing below.

Spatial repellents

The general concept of spatial repellency is to prevent an arthropod from entering a space occupied by a potential human host to reduce encounters between humans and vectors thereby eliminating or reducing the risk of pathogen transmission to either insect or human (Achee et al. 2012). Spatial repellents do not require physical contact of the mosquito with treated surfaces like insecticides used in IRS and LLIN, but act in the vapour state at a distance. The airborne pyrethroids minimize human-vector contact through reduced and delayed blood feeding. Mosquito coils, candles and emanators impregnated with volatile pyrethroids and other compounds such as plant terpines are collectively known as spatial repellents (Ogoma et al. 2014). The best strategy for application of spatial repellents depends on the vector. Growing evidence supports the use of spatial repellents to protect against arthropod-borne diseases in settings where existing products have reached their limits of efficacy (Hill et al. 2014, Syafruddin et al. 2014).

Attractive toxic sugar baits

Attractive toxic sugar baits (ATSB) are a new strategy for controlling mosquitoes and other biting flies. Female and male mosquitoes need plant derived sugars and carbohydrates to maintain energy for survival. This almost daily need for sugar presents an opportunity to leverage the sugar-feeding process with a bait containing a toxicant. The basic approach of ATSBs is to lure mosquitoes to a toxic bait and kill them (http://apps.who.int /iris/bitstream/10665/162861/1/9789241508674_eng.pdf?ua=1). Numerous field trials in Israel and Mali suggest that ATSB can reduce *Anopheles* mosquito densities, and thus vectorial capacity (Muller et al. 2008, Muller et al. 2010, Beier et al. 2012).

Eave tubes

The eave tube exploits the natural host-seeking behaviour of African malaria mosquitoes, which preferentially enter houses through the open space between the roof and walls (the so-called eave). When these eaves are closed and eave tubes are installed every 1-1.5 m the natural airflow and ventilation inside the house is maintained. Host seeking mosquitoes respond to odours from house occupants that emanate from the tubes. When they enter the tubes they encounter insecticide-treated electrostatic netting that has resistance-breaking potential (http://cordis.europa.eu/result/rcn/189746_en.html.). Eave tube technology

does not only consist of eave tubes but also window screening (with untreated netting) as well as sealing of cracks and gaps in walls and (whenever possible) improvement of the door (Knols et al. 2016). Pyrethroid-treated eave curtains have been shown to reduce malaria morbidity and mortality (Lengeler 2004, Bradley et al. 2013) which clearly demonstrates that the eave is an effective place to target host-seeking mosquitoes when they try to enter (Spitzen et al. 2016). The eave tubes could be a viable, cost-effective, and acceptable control tool for endophilic and endophagic *Anophelines*, and possibly other (nuisance) mosquitoes. The approach could be applicable in a wide variety of housing in sub-Saharan Africa, and possibly beyond, for vectors that use the eave as their primary house entry point (Knols et al. 2016).

In addition, biocontrol strategies (e.g. *Wolbachia*, sterile insect, entomopathogen fungi, etc) aim to be sustainable and target different stages of the mosquito lifecycle to reduce the current reliance on insecticide-based mosquito control.

Wolbachia Endosymbiotic Bacteria

Wolbachia are maternally transmitted intracellular bacteria that invade insect populations by manipulating their reproduction and immunity and thus limiting the spread of numerous human pathogens. The use of *Wolbachia* for malaria control will require a stable infection that is transmitted vertically to offspring. However, the challenging nature of mosquito microinjection has hampered the progress in transferring *Wolbachia* between mosquito species. The transfer of *Wolbachia* into mosquito embryos is intrinsically more difficult, since mosquito embryos are less amenable to inoculation, especially *Anopheles* embryos (Walker et al. 2011). A recent study identified stable *Wolbachia* infections in natural populations of two important malaria vectors, *Anopheles gambiae* and *Anopheles coluzzii*, in Burkina Faso, a country with a high malaria burden. The evidence of *Wolbachia* infections in natural *Anopheles* populations promotes further investigations on the possible use of natural *Wolbachia-Anopheles* associations to limit malaria transmission (Baldini et al. 2014).

Entomopathogenic fungi

Entomopathogenic fungi produce infective spores (conidia) that attach to and penetrate the cuticle of mosquitoes, releasing toxins that result in mosquito death (Scholte et al. 2004). Laboratory and field studies demonstrate that entomopathogenic fungi can efficiently kill adult *Anopheline* mosquitoes (Kanzok et al.2006, Scholte et al. 2008). These fungi have a distinct

advantage over the biological control agents, in that they do not need to be ingested to infect and kill the insects (Scholte et al. 2008). To infect mosquitoes in the field requires physical contact with fungal spores sprayed on surfaces such as walls, nets, or other resting targets in and around domestic dwellings (George et al. 2013). However, evidence from a number of systems suggests that some insects can detect and actively avoid fungal spores to reduce infection risk (Roy et al. 2006).

The Sterile Insect Technique

The Sterile Insect Technique is a genetic suppression strategy that involves rearing large numbers of males of the target species and either irradiating or treating them with chemosterilizing agents to generate chromosomal aberrations and dominant lethal mutations in sperm. These sterilized male insects are released and when they mate with wild females produce no progeny (Benelli et al. 2016). In Africa, the study on the feasibility of the sterile insect technique as a malaria vector control were demonstrated (Helinski et al. 2008, Munhenga et al. 2011). However, the use of this technique has been limited due to the reduced performance of sterilized males caused by sterilization and the difficulty of the initial need to reduce the wild population densities, prior to the release of sterile males (Benelli et al. 2016).

Clearly, more investigations are needed to demonstrate the add value of these new tools for malaria control.

3. ASSESSING THE RISK OF MALARIA TRANSMISSION

The risk of malaria is highly dependent on interactions between the human host, the *Plasmodium* parasite, the mosquito vector, and the environment. Changes in any one of these elements can profoundly impact on the malaria transmission (Figure 11).

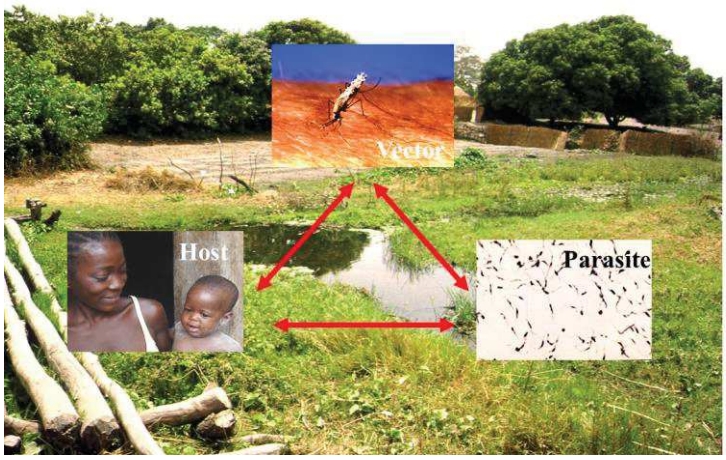

Figure 11. Malaria transmission cycle.

The risk of malaria transmission can be estimated by studying the mosquito vector (entomology investigation) and/or the human host (parasitology and serology investigation).

3.1 Vector : Entomological indicator of transmission

The risk of malaria transmission can be assessed by entomological investigations that provide information on vector species, density and their distribution (WHO 2013c). *Anopheles* population sampling is an essential step in a malaria survey program, to identify the vector population, to control and evaluate the efficacy of vector-control strategies. Different methods of mosquito catching are used to measure the intensity of human exposure to the vector, thus assessing human-vector contact (Silver et al. 2008). Human landing catch (HLC) is the gold standard method based on human bait for sampling host-seeking mosquitoes and estimation of the human biting rates (HBR), i.e. the number of bites received per man per unit of time. Although HBR does not precisely reflect the intensity of malaria transmission (as measured by the Entomological Inoculation Rates-EIR), it can be used as a proxy to measure vector abundance in given place at a given time and evaluate the spatiotemporal risk of transmission (Cottrell et al. 2012).

3.1.1 Human biting rates (HBR)

The HBR, an important parameter for measure of human-vector contact, is commonly estimated by HLC. HLC are typically conducted by volunteers trained to collect host-seeking mosquitoes that land on exposed body parts during the evening and night hours when

Anopheline vectors are most active. The advantage of this method is that it directly measures the biting rate of anthropophagic mosquitoes considered to be representative of the vector population responsible for malaria transmission (Davis et al. 1995). However, this method has some major disadvantages and is facing opposition. HLC are extremely labor-intensive, which limits the number of data points that may be simultaneously collected (Fornadel et al. 2010). An all-night man-landing catch requires well-motivated staff and close supervision if the results are to be reliable (WHO 1995). HLC are also logistically difficult to sustain on long term and expensive to carry out because volunteers need to be recruited and trained and, sorting and identification of collected non-target species is time-consuming. The differential attractiveness of individual collectors to mosquitoes, fatigue and ineffectiveness and/or misconduct of collectors, may also affected the outcomes (Overgaard et al. 2012). Furthermore, this technique poses safety issues in areas endemic for disease because the occupational exposure to host-seeking *Anophelines* may place the collectors at an increased risk of being bitten by infected mosquitoes (Service 1977). The HBR seems to be insensitive within small geographical areas (Mbogo et al. 2003, Orlandi-Pradines et al. 2009), is used at the community level measure (Poinsignon et al. 2008a) and is not suitable to evaluate the short-term impact of vector control programs (Doucoure et al. 2015b).

WHO recommends avoiding HLC unless absolutely essential, especially if safer techniques are available to provide proxy estimates of HBR (WHO 2003). Therefore, it is currently emphasized the need to develop new tools assessing reliably human malaria risk and control interventions, and monitoring changes over time at both population and individual levels (WHO 2009b, The malERA consultative group on monitoring, evaluation, and surveillance 2011).

3.1.2. Entomological inoculation rates (EIR)

The EIR, is the number of infectious bites per person per unit time, usually measured or expressed per year. It is the product of the HBR and the sporozoite rate:

$$\text{EIR} = MaS$$

The human biting rate (Ma) is the number of vectors biting an individual over a fixed period of time. *M* equals the number of *Anopheles* per person and *a* equals the average number of persons bitten by one *Anopheles* in one day. The sporozoite rate (*S*) is the proportion of infected

vector mosquitoes infectious, i.e. *Anopheles* with sporozoites in their salivary glands (Warrell and Gilles 2002, Snow and Marsh 2002).

The EIR is the most direct approach to estimate the human exposure to infectious bites and mosquito population monitoring (Badu et al. 2015). The proportion of infected mosquitoes in a locality reflects the capacity of the vectors to transmit malaria. The EIR determines to a large extent the epidemiology of malaria and the pattern of clinical disease in an area. The upper end of the EIR range is found in a few parts of tropical Africa, where rates of 500-1000 infected bites per person per year can be reached (Hay et al. 2000). At the lower end of the range, EIR of $\leqslant 0.01$ are found in the temperate climates of the Caucasus and Central Asia, where malaria transmission is only barely sustained. Between these extremes are situations of unstable seasonal malaria, such as in much of Asia and Latin America, where the EIR are < 10 and often 1-2, and situations of stable but seasonal malaria, as in much of West Africa, where the EIR is 10-100. (WHO 2015b).

The EIR is a flexible and accurate metric of malaria transmission. The relationships between the EIR and other epidemiological metrics (such as malaria incidence, prevalence rates, annual incidence rates of severe disease and a *P. falciparum* parasitemia), show that the EIR is the most useful measure for focusing vector interventions on the areas of highest transmission and, is the only metric sensitive enough to detect the differences in transmission in the final steps towards malaria elimination (Ulrich et al. 2013). This relationship is, however, affected by vectorial capacity, the pattern of acquisition and loss of immunity to malaria and access to effective drug treatment in the area. (WHO 2015b). Unfortunately, in the areas of low transmission, the EIR may lack sensitivity due to the low prevalence of malaria infections in the human population or infected mosquitoes (Cook et al. 2010, Tusting et al. 2014). Furthermore, both entomological and parasitological measures may be affected by seasonality (Kelly-Hope et al. 2009), so hotspots of transmission could be missed. Finally, the impact of increased malaria control interventions as well as the effects of inter-annual climate variability make understanding trends in malaria transmission in the areas particularly difficult (Lynch et al. 2016).

3.2 Human : Parasitological and serological indicator of transmission

3.2.1 Parasitological markers

Malaria transmission can be evaluated in human host by measuring parasite density and prevalence. Parasite density is defined as the number of asexual forms of parasite relative to a blood volume (e.g. microliter), which provides information on the severity of infection. Parasite prevalence (PR) is defined by the number of individuals who carrying *Plasmodium* (both symptomatic and asymptomatic) using thick blood smear (Beier et al. 1999). However, parasite prevalence may be underestimated due to asymptomatic individuals, and is depending on the sensitivity of parasite detection. As a result, more sensitive and standardized metrics are needed to assess transmission intensity in real time, to assess interventions, to acquire data necessary for planning appropriate control programs especially in areas of low transmission (Beier et al. 1999, Alonso et al.2011).

3.2.2 Serological markers

The risk of malaria transmission can be assessed also by the use of serological markers (Wong et al. 2014). The serological method used in the field are the rapid diagnostic tests (RDT), which are based on the detection of histidine-rich protein II (HRP-II) and parasite lactate dehydrogenase (pLDH). Antibodies to malaria antigens are sensitive biomarkers of population-level malaria exposure and can be used to identify hotspots of malaria transmission, estimate transmission levels, monitor changes over time or the impact of interventions on transmission, confirm malaria elimination, and monitor re-emergence of malaria (Elliott et al. 2014). Serological markers of transmission show greater sensitivity in low transmission areas, as seroprevalence reflects cumulative exposure and thus is less affected by seasonality due to the longer duration of specific antibody responses (Cook et al. 2010). However, the potential disadvantage of the serological approach is that if antibody responses saturate at low transmission intensity or are very long-lived, then serology may not detect significant recent deviations from the historic pattern of transmission (Drakeley et al. 2005).

The dynamics of antibody acquisition and maintenance vary based on exposure intensity and age (Helb et al. 2015). By analyzing 856 *Pf* antigens among Ugandan children by protein microarray, these authors revealed that *Pf*-Specific antibody profiles showed increased antibody reactivity in participants who were more recently infected. However, only few *Pf*

antigens showed accurate predictions of an individual's exposure history of *Pf* infection (Helb et al. 2015). Different *Pf* antigens elicit antibody responses with different magnitudes and kinetics, providing a large and diverse set of potential biomarkers of malaria exposure (Meraldi et al. 2004, Gray et al. 2007, Osier et al. 2008, Akpogheneta et al. 2008, Elliott et al. 2014, Stanisic et al. 2015).

The measurement of human antibodies to various synthetic peptides or recombinant proteins, as source of antigen, was also explored as a measure of malaria exposure. Many antigens are conserved across *P. falciparum* and *P. vivax*, including circumsporozoite protein (CSP), merozoite surface protein-1 (MSP-1), and apical merozoite antigen-1 (AMA-1), that have been most studied in serosurveillance (Elliott et al. 2014). Antibody response to several other *P. falciparum* antigens have been explored as alternative means to estimate malaria transmission intensity, including blood stage proteins, such as *Pf*MSP-2 (Drakeley et al. 2005), *Pf*MSP-1_{42} (Hsiang et al.2012) and *Pf* glutamate-rich protein (GLURP) (Cook et al. 2012), and liver-stage proteins, such as liver-stage antigen-1 (LSA-1) and thrombospondin- related adhesive protein (TRAP) (Webster et al. 1992, John et al. 2003, Noland et al. 2008, Campo et al. 2011) (review in Elliott et al. 2014).

The C-terminal 19-kDa fragment of MSP-1 is well conserved among *P. falciparum* isolates (Mazumdar et al. 2010) and was described as the most suitable immunological marker for assessing malaria transmission intensity (Elliott et al. 2014). Sero-surveillance based on *Pf*MSP-1_{19} seroconversion rates have been strongly correlated with other indicators of transmission intensity (EIR, parasite rate, malaria incidence, and altitude) (Drakeley et al. 2005, Satoguina et al. 2009, Stewart et al. 2009, Bousema et al. 2010b, Badu et al. 2012) and has facilitated the identification of transmission "hotspots" (Bousema et al. 2010a) and changes in transmission intensity over time (Stewart et al. 2009). Mathematical model showed that the village-specific annual rate of seroconversion is very closely correlated with independent estimates of the EIR. The analysis of age-specific seroprevalence rates may enabled differentiation of recent (seasonal) changes in transmission intensity from longer-term transmission trends (Kerkoff et al. 2016). The data suggest that, once acquired, antibody responses to *Pf*MSP-1_{19} seem to persist for many years and may indeed be lifelong. In the absence of effective anti-malarial treatment, frequent or persistent subpatent malaria infection is sufficient to maintain seropositivity (Drakeley et al. 2005).

Circumsporozoite protein are important sporozoite antigens that are relatively more conserved compared to merozoite surface antigens (Doolan et al. 2003, Kaiser et al. 2004, Kariu et al. 2006). The sporozoite stages of *P. falciparum* are exposed to the immune system for only short periods after inoculation. Anti-sporozoite antibodies would most commonly be detected in individuals with frequent or recent exposure and may be ideal candidates for estimating malaria transmission intensity (review in Kusil et al. 2014). The detection of significant antibody titers to CSP indicates previous inoculation with sporozoites, but does not necessarily lead to the development of the disease (Nothdurft et al. 1999). The use of CSP reflect a direct exposure to sporozoites, as opposed to models based on blood stage antigens, which may not reflect recent exposure to sporozoites. In persons living in areas of endemicity, prevalence and levels of CSP antibodies have shown to correlate well with the EIR and can serve as a proxy of malaria transmission (Druilhe et al. 1986, Del et al. 1987, Esposito et al. 1988, Webster et al. 1992). Circumsporozoite antibodies have been shown to be reliable indicators of the malaria transmission risk for short-term travelers to East Africa (Nothdurft et al. 1999). Kusil et al. showed that anti-CSP antibodies can be exploited for the development of models for predicting seasonal, short-term changes in transmission intensity in malaria-endemic areas, especially in areas of low transmission where malaria elimination becomes an achievable goal (Kusil et al. 2014). Anti-CSP antibody prevalence has been shown to be associated with malaria transmission in Africa and Asia (Druilhe et al. 1986, Ramasamy et al. 1994, Campo et al. 2011) and has also been shown to correlate with seasonal transmission patterns determined by parasitological and entomological measurements in Southeast Asia (Webster et al. 1992) (review in Kusil et al. 2014). Circumsporozoite protein antibody appeared to reflect more recent population exposure to mosquito inoculation of *P. falciparum* sporozoites and may then provide a relevant measure of malaria transmission dynamics (Webster et al. 1992). Risk mapping in low transmission areas can support improved targeting of remaining clusters of malaria and help programs reduce malaria burden and eliminate the disease. Suitability of different indicators of malaria transmission for risk mapping in low transmission settings (Sturrock et al. 2016) is shown in table 2.

Table 2. Suitability of different indicators of malaria transmission for risk mapping in low transmission settings. (Sturrock et al. 2016)

Indicator	Advantages	Disadvantages	Potential suitability for low transmission risk mapping
Entomological inoculation rate	Considered by some to be gold standard transmission metric	-Operationally challenging, labour intensive, and expensive - Only provides picture at one point in time - ethical issues	Low
Parasite prevalence	-Well-established and widely used method with established statistical methods and comparisons over time -Straightforward to obtain via cross-sectional surveys	-Sample size requirement in low transmission often prohibitively large, especially to obtain spatial resolution required for risk mapping -Only provides picture at one point in time - Cross-sectional surveys can be expensive, done infrequently	Low
Serology	-Ability to integrate information on exposure over time increases sensitivity and may allow higher precision with smaller sample sizes than PR -Ability to estimate exposure over multiple timescales may provide information on recent risk as well as suitability of an area to historically sustain transmission (vectorial capacity) - Incremental cost to augment active surveillance data is very low	- Methods to distinguish very recent from more distant exposure in low transmission settings are not well established -Like PR, requires the expense of dedicated collection of samples and data, e.g., from cross-sectional surveys, done infrequently -Existing markers may not be good indicators of current and/or future transmission	Medium (with currently available tools) - High (if precise markers of very recent exposure are developed)
Clinical incidence	-Cheap - data routinely collected at health facility level -Provides data over continuous time	-Quality of data highly dependent on quality of diagnoses, completeness of reporting, and variations in treatment seeking behavior -Could lead to spurious results without information on whether cases are local or imported -Location data often restricted to health facility limiting spatial resolution of risk maps -Only useful if spatial distribution of symptomatic cases is reflective of overall transmission, which may be driven by asymptomatic reservoirs	High (if data are of sufficient quality)

4. SALIVARY BIOMARKER

As described above, entomological and parasitological data are routinely used to estimate the level of exposure of human populations to *Anopheles* vector bites and malaria parasites. However, these methods are labor intensive, and lack of sensitivity especially in low exposure/transmission areas. In the recent years, new serological biomarkers relying on the human antibody response against arthropod salivary proteins have been developed (Doucoure et al. 2015a). These new biomarkers are promising because they may provide direct and accurate evaluation of the human exposure to vector bites, at both community and individual levels and can contribute to evaluate disease transmission risk and new vector control interventions (Doucoure et al. 2015a).

4.1 Salivary biomarker of human exposure to Arthropod bites

Vector-borne diseases are infections caused by pathogens and parasites, which are transmitted by the bite of infected arthropod species. Most of the vectors are blood sucking insects, which ingest and transmit microbes in the human host during a blood meal. “During the contact with the human host, the arthropod proceeds to an exploratory phase called the “probing.” This mechanism can vary according to insects. The success of the blood meal intake and the transmission of pathogens depend on a complex equilibrium of interactions between the arthropod and the vertebrate host during the bite. These interactions are sustained by the physiological properties of arthropod saliva that represent key elements in the interface of the arthropods and the vertebrate hosts during the bite. Some salivary proteins help to keep the bleeding by inhibiting essential hemostatic functions and, on the other hand, annihilate the human defense mechanisms” (review in Doucoure et al. 2015a).

The exploration of the close interactions between the human host and the vector through the antigenic salivary proteins of hematophagous arthropods has led to the development of new salivary biomarkers. “The concept is based on the fact that the arthropod saliva injected to the human host during the vector bite is antigenic and can induce an adaptive humoral host response. Therefore, a logical positive correlation between the human exposure level to vector bites and human anti-vector saliva Ab level can be expected. In this way, anti-saliva Ab response can be a pertinent epidemiological biomarker of human exposure to vector bites” (review in Drame et al. 2013a). The physiological interaction between the human and the vector was showed in Figure 12. “The interaction between the vector and the human involves several

steps: (1) the vector injects its salivary proteins in the human skin during the probing. Once in the skin, the salivary proteins (2) take the control of the human hemostatic system by inhibiting the platelet activation, clotting mechanism, and inflammatory system. (3) The salivary proteins modulate the human immune response and promote the production of anti-saliva antibodies. (4) If ever the vector carries a pathogen, the salivary proteins contribute to its transmission into the human" (review in Doucoure et al. 2015a).

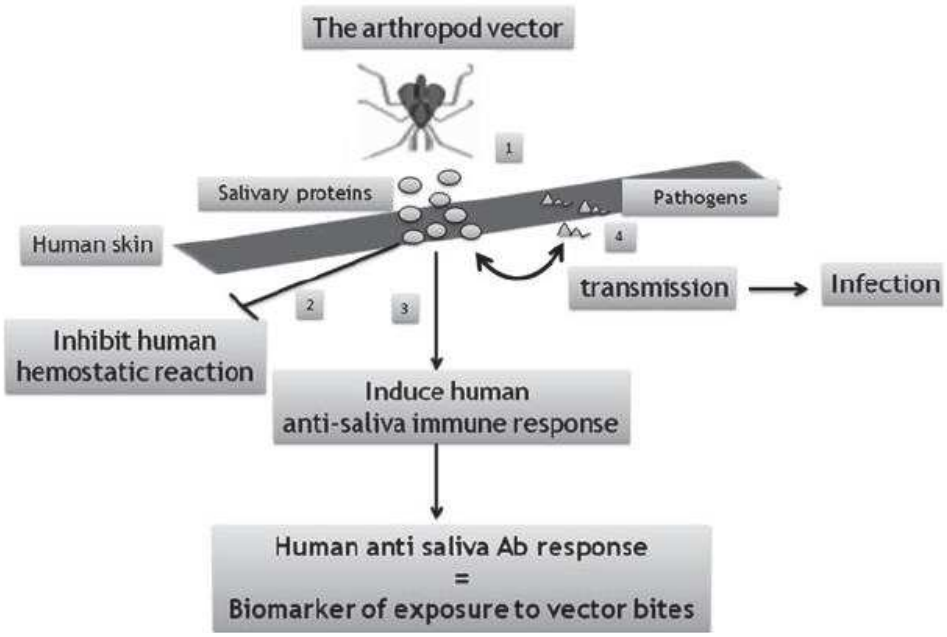

Figure 12. The physiological interaction between the human and the vector. (Source: Souleymane Doucoure, Institut de Recherche pour le De'veloppement)

Antibody responses to the saliva of a number of vectors (Table 3), including *Triatoma* (Chagas disease) (Schwarz et al. 2009a,b), *Phlebotomus* (Leishmaniasis) (Rohousova et al. 2005), *Glossina* (Human African Trypanosomiasis) (Poinsignon et al. 2008b) and *Ixodes* (Tick-borne disease) (Vu Hai et al. 2013a,b) have been identified as promising biomarkers for vector exposure. Antibody response to the saliva has been used also as potential biomarker of human exposure to mosquitoes, including *Culex tarsalis* (Trevejo and Reeves 2005), *Aedes aegypti* (Doucoure et al. 2012b), *Aedes albopictus* (Doucoure et al. 2014), *Anopheles gambiae* (Remoue et al. 2006), *Anopheles dirus* (Waitayakul et al. 2006) and *Anopheles darlingi* (Andrade et al. 2009) (review in Doucoure et al. 2012a). These salivary biomarkers showed

great potential to assess the risk of disease transmission and were used to evaluate the efficacy of vector control tools (Drame et al. 2010a, Doucoure et al. 2015a).

The use of vector-derived factors, such as arthropod saliva are also attractive targets for the development of novel vaccines against vector-borne diseases, since protection cannot be bypassed through mutations in pathogen-associated proteins and since they may prevent infection rather than inducing an immune response against an establishing or established infection. “The objective of these vaccines is simple and elegant : Targeting vector saliva molecules that assist pathogens during infection may either “unmask” the infectious inoculum and allow the host’s immune system to eliminate it, or induce an immune response that interferes with the establishment of an infection by the vector-borne pathogen” (Leitner et al. 2015). The next generation of vaccines against vector-borne diseases may need to comprise components of the pathogen as well as vector saliva factors to induce robust protection (Leitner et al. 2011).

Table 3. Antibody response to vector salivary proteins used as biomarkers of exposure. The table lists all salivary biomarker that have been used so far to measure the contact between the vertebrate hosts and the vectors. (Source: Souleymane Doucoure, Institut de Recherche pour le Developpement)

Vector	Salivary antigens	Vertebrate host	Biomarker application	Reference
Aedes aegypti	SGE	Children	Infection outcome	Machain-Williams et al. 2011
Aedes aegypti	SGE	Adult human	Urban exposure	Doucoure et al. 2012b
Aedes aegypti	Nterm-34 kDa peptide	Adult human	First validation of a *Aedes* peptide	Elanga Ndille et al. 2012
Aedes aegypti	Nterm-34kDa peptide	Adult human	Evaluate the efficacy of vector Control	Elanga Ndille et al. 2016
Aedes aegypti	SGE	Adult human	Risk of disease	Londono-Renteria et al. 2015c
Aedes albopictus	SGE	Adult human	Evaluate the efficacy of vector Control	Doucoure et al. 2014
Aedes albopictus	SGE	Adult human	Specific antigenic response	Doucoure et al. 2012a
Anopheles	Peptides: gSG6-P1 to gSG6-P5	Children	First validation of a gSG6 peptide	Poinsignon et al. 2008a
Anopheles	gSG6-P1 peptide	Adult human	Evaluation ITN	Drame et al. 2010a
Anopheles	SGE	Horses	Transmission of human malaria	Boulanger et al. 2011

Vector	Salivary antigens	Vertebrate host	Biomarker application	Reference
Anopheles	gSG6-P1 peptide	Adult human	Evaluation of VCP	Drame et al. 2013b
Anopheles darlingi	SGE	Adult human	Infection status	Andrade et al. 2009
Anopheles dirus	SGE	Adult human	Humoral immune response	Waitayakul et al. 2006
Anopheles funestus	gSG6-P1 peptide	Children	Biomarker relevance	Poinsignon et al. 2010
Anopheles gambiae	SGE	Children	Infection outcome	Remoue et al. 2006
Anopheles gambiae	SGE	Children	Biomarker relevance	Cornelie et al. 2007
Anopheles gambiae	SGE	Adult human	Evaluation ITN	Drame et al. 2010b
Anopheles gambiae, Aedes aegypti	SGE	Adult human	Antibody response in travelers	Orlandi-Pradines et al. 2007
Anopheles gambiae, Anopheles funestus	Recombinant proteins: gSG6, g-50nuc, fSG6, f-50 nuc	Adult human	Species-specific antigenic biomarkers	Ali et al. 2012
Culex tarsalis	SGE	Chicken	Arboviral surveillance	Trevejo and Reeves 2005
Glossina morsitans	Recombinant protein : rTsal1	Mice and Pigs	Biomarker relevance	Caljon et al. 2014
Glossina morsitans submorsitan	$Tsal1_{52–75}$	Cattle	Biomarker relevance	Somda et al. 2016
Glossina palpalis	Saliva	Cattle	Biomarker relevance	Somda et al. 2013
Glossina palpalis gambiensis	Saliva	Adult human	Biomarker relevance	Dama et al. 2013
Ixodes ricinus, Rhipicephalus sanguineus, Dermacentor reticulatus	SGE	Rabbit	Biomarker relevance	Vu Hai et al. 2013a,b
Ixodes scapularis	SGE	Experimentally bitten mice	Specific antigenic response	Alarcon-Chaidez et al. 2006
Leishmania infantum	SGE	Dog	Transmission intensity	Quinnell et al. 2018
Lutzomyia intermedia	Recombinant protein: rLinB-13	Adult human	Biomarker relevance	Carvalho et al.2017
Lutzomyia longipalpis	SGE, Recombinant protein	Children	Biomarker relevance	Barral et al. 2000
Lutzomyia longipalpis	Recombinant proteins: LJM11, LJM17	Adult human	Epidemiological biomarker	Souza et al. 2010
Lutzomyia longipalpis	SGE Recombinant proteins : rLJM17, rLJM11	Chicken	Biomarker relevance	Soares et al.2013
Phlebotomus argentipes	SGE	Adult human	Biomarker relevance	Clements et al. 2010
Phlebotomus papatasi	Recombinant protein: PpSP32	Children and Adult human	Biomarker relevance	Marzouki et al. 2015

Vector	Salivary antigens	Vertebrate host	Biomarker application	Reference
Phlebotomus papatasi	Recombinant protein: PpSP32	Adult human	Epidemiological marker	Mondragon-Shem et al.2015
Phlebotomus sergenti, P. papatasi, Lutzomyia longipalpis	SGE	Adult human, experimentally bitten mice	Risk of disease	Rohousova et al. 2005
Phlebotomus sergenti, P. papatasi, P. arabicus	SGE	Experimentally bitten mice	Specific antigenic response	Drahota et al. 2009
Phlebotomus, Lutzomyia	Salivary proteins	Humans and animal	Biomarker relevance, Risk of disease	Review in Andrade et al.2012
Triatoma infestans	SGE	Peridomestic animals	Epidemiological marker, disease surveillance	Schwarz et al. 2009b
Triatoma infestans	SGE	Sentinel guinea pigs	Evaluation ITN	Schwarz et al. 2011
Triatoma, Rhodnius	Recombinant protein: rTiSP14.6	Experimentally exposed animals	Epidemiological marker	Schwarz et al. 2009a

4.2 Salivary biomarker of human exposure to *Anopheles* bites

Recently, in order to assess the risk of malaria transmission and evaluate the efficacy of vector control program for malaria elimination, new salivary biomarkers of human exposure to *Anopheles* bites have been developed (Remoue et al. 2006). The development of such biomarkers (or indicators) can represent an alternative to current referent entomological and parasitological methods which present several limitations especially in low exposure/transmission contexts as previously described. The figure below shows the effects of *Anopheles* saliva on hemostatic, inflammatory and immune reactions of the human to the vector bites (Figure 13).

First experiments used salivary grand extracts (SGE) from mosquitoes as the source of antigens. Observational studies in Thailand demonstrated that anti-*Anopheles* salivary protein antibody occurred predominantly in patients with *P. falciparum* or *P. vivax* malaria compared to people from non-malarious area, which had no such antibody response (Waitayakul et al. 2006). The high level of Anti-SGE IgG were also observed in children in Senegal who developed clinical *P. falciparum* malaria within 3 months compared to children who did not. Moreover, the level of Anti-SGE IgG increased significantly with the level of *Anopheles* exposure, as evaluated by conventional entomological measurements. These findings suggest

that the evaluation of anti-salivary Ab response could be useful approach to identify biomarkers of malaria transmission (Remoue et al. 2006). However, the use of whole SGE as a salivary biomaker was not accurate and reliable enough due to the presence of a cocktail of various components that are not specific to *Anopheles* genus. Consequently, cross-reactivity between vector species within families or genera may occur. The production of SGE was time consuming, and required dissecting a large number of mosquitoes to have optimal protein concentration for immunological test. Finally, SGE lack of reproducibility between saliva batch and can be affected by several parameters such as age, physiological status or infectivity of the *Anopheles* mosquitoes (Ribeiro and Francischtti 2003).

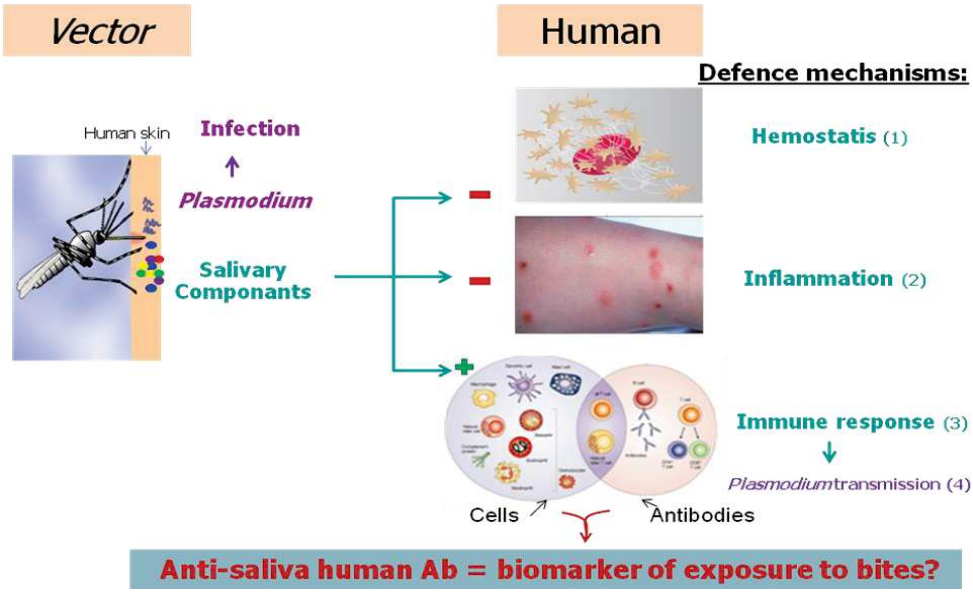

Figure 13. Effects of *Anopheles* saliva on hemostatic, inflammatory and immune reactions of the human to the vector bites. (Drame et al. 2013a)

Recent progress in sialotranscriptomic studies have allowed the identification of more specific antigens of *Anopheles* mosquitoes that have enhanced the specificity of the salivary biomarker (Ribeiro and Francischtti 2003). Among them, the gSG6 protein : protein which was first described in *An. gambiae* (Lanfrancotti et al. 2002), has been reported to be specific to *Anopheles spp* bites and has been further selected for validating its potential as an immunological biomarker to human exposure to malaria vector bites. Specific IgG antibodies to the gSG6 recombinant protein were observed in children living in area exposed to malaria in Senegal (Poinsignon et al. 2008a), and have been reported to be potentially antigenic in

travelers exposed to *Anopheles* bites for short period of time (Orlandi-Pradines et al. 2007). However, the gSG6 protein may contain more than one epitope and batch to batch variations in the production of recombinant proteins was reported. With the objective to optimize *Anopheles* specificity and reproducibility of the immunological assay, five gSG6-based peptide sequences (gSG6-P1 - gSG6-P5) were identified by bioinformatics. The IgG antibody response to these peptide was observed in children living in a Senegalese area exposed to malaria. The five gSG6 peptides showed different antigenic properties, with gSG6-P1 and gSG6-P2 exhibiting the highest antigenicity. However, a significant increase in the specific IgG response during the rainy season and a positive association between the IgG level and the level of exposure to *An. gambiae* bites was significant only for gSG6-P1 (Poinsignon et al. 2008a). The gSG6-P1 peptide demonstrated high reproducibility and specificity due to the presence of only one epitope. This peptide was then successfully used to identify malaria transmission risk in Africa (Poinsignon et al. 2009, Drame et al. 2010b) and America (Londono-Renteria et al. 2015a).

In addition to gSG6, the anti-cE5 IgG response is shown to be a sensitive indicator of human exposure to *Anopheline* vectors (Rizzo et al. 2014). The cE5 protein was initially identified during an *Anopheles gambiae* salivary gland transcriptome study (Arcà et al. 1999). The cE5 protein was then found to be a member of the *Anopheline* family of anti-thrombin peptides (Ronca et al. 2012). This protein was highly immunogenic and triggered in exposed individuals at relatively long-lasting antibody response, as shown by its unchanged persistence after a few months with no or very low exposure. The anti-cE5 IgG response may be especially useful in conditions of low vector density to monitor transiently exposed individuals (i.e. travellers/workers/soldiers spending a few months in tropical Africa). The gSG6 and cE5 salivary proteins were shown to trigger in exposed individuals a strikingly different immune response with (i) gSG6 evoking a short-lived IgG response, characterized by high IgG4 levels and most likely induction of immune tolerance, and (ii) cE5 eliciting a longer-living IgG response, dominated by anti-cE5 IgG1 antibodies and not inducing tolerance mechanisms (Rizzo et al. 2014).

CONTEXT OF THE THESIS

1. BACKGROUND

1.1 Malaria situation along the Thailand-Myanmar border

As in other Southeast Asian countries, malaria in Thailand exhibits the "border malaria" type (Corbel et al. 2013) with most of the malaria cases concentrated in foci located in forested areas along the borders with Myanmar (Carrara et al. 2013) and Cambodia (Khamsiriwatchara et al. 2011) (Figure 14). In these areas, malaria control remains difficults to implement due to complexities in scaling up malaria control measures and effective interventions in all villages where malaria is endemic, as well as building the capacity of local health personnel in malaria prevention (Wangdi et al. 2015). The predominant parasite species along the Thailand-Myanmar border (TMB) are *P. falciparum* and *P. vivax*. These have had nearly equal prevalence since 1997, although *P. vivax* has become more prevalent (60%) than *P. falciparum* (40%) since the turn of the century (Sattabongkot et al. 2004). Widespread use of artemisinin combination therapies (ACT) is a plausible explanation for the shift in parasite species incidence in the country (Carrara et al. 2013). These area present also very efficient vectors particularly selected members of the Dirus, Maculatus, and Minimus (Manguin et al. 2013) as well as secondary vectors such as *An. barbirostris* and *An. annularis* that may play increasing role in malaria tranmission (Sriwichai et al. 2016). The complexity of malaria epidemiology along the TMB represents a formidable challenge for malaria elimination goals planned in the region by 2030 (WHO 2015a).

1.1.1 Human populations

Cross-border malaria encompasses malaria transmission along international borders as a result of interconnections between human settlements and population movement (Wangdi et al. 2015). The border between Thailand and Myanmar is 2,107 km long and is mostly forested and mountainous and is inhabited by a several different ethnic minority groups who are collectively referred to as 'Hill Tribes' (with the largest group being the Karen) (Carrara et al. 2013). They all vary in socio-cultures, languages, and lifestyles. Along TMB, major population centres occur near rivers which have historically been the most reliable sources of year-round movement and transportation. However, most people along this border region live

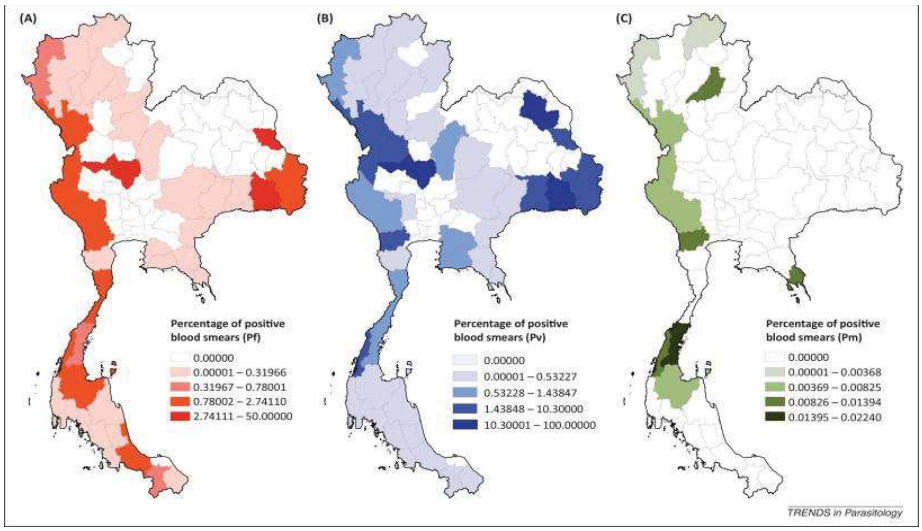

Figure 14. Malaria incidence in Thailand. Maps showing the distribution of malaria infections by (A) *Plasmodium falciparum*, (B) *Plasmodium vivax*, and (C) *Plasmodium malariae* in Thai and migrant populations in 2012. (Source: Bureau of Epidemiology, Ministry of Public Health, Thailand, www.boe.moph.go.th). The percentage of positive blood smears with *P. vivax* (*Pv*), *P. falciparum* (*Pf*), and *P. malariae* (*Pm*) were 1% (n = 16,471 positive blood smears), 0.7% (n =11,553), and 0.003% (n = 57), respectively (total number of blood smears inspected was 1,716, 811). Mixed infections (*Pf/Pv*) represent barely 0.5% of the total malaria cases. (Corbel et al. 2013)

in rural areas, with some inhabiting extremely remote, difficult-to-access locales along both sides of the international border (Parker et al. 2015a). Several of these minority groups have also been at war with the Myanmar government, meaning that they have been isolated along the Myanmar border and many have lived as refugees or displaced persons on the Thai side of the border (Parker et al. 2015b). Decades of internal conflicts in Myanmar have resulted in massive population displacements, and over 150,000 refugees now live in camps in Thailand (Carrara et al. 2013). In 1985, malaria was the main cause of consultations and mortality in the Karen refugee camps (Decludt et al. 1991). Between 1995 and 2000 the burden of malaria has fallen dramatically in the refugee camps as a result of the strategy designed at Shoklo Malaria Research Unit (SMRU) in Mae sot, Tak province, Thailand and followed by all medical NGOs. Since 2013, malaria has become a less public health problem in the camps and the mortality is limited to patients presenting late, usually from Myanmar. However, this progress is being challenged by the emergence of *P. falciparum* infections that are resistant to the artemisinine derivatives (Phyo et al. 2012) and by difficuties to implement malaria control campaign in the remoted villages .

Due to geographical uplands, hills, hillside slope areas, and valleys, border crossings at immigration checkpoints are easier for migration of cross-border people. This group of people are at the greater risk of malaria infections because they frequently revisit forest at multiple locations on or surrounding the border. Cross-border persons who carrying malaria infection during an incubation period are exposed to multiple bites of *Anopheles* vectors at multiple locations and this can spread malaria during a prodromal period until seeking treatment (Bhumiratana et al. 2013a).

Malaria control in border areas is particularly challenging, especially if effective control measures are not deployed on both sides of the border (Carrara et al. 2013). Moreover, the undergoing economic development to growing urban settlements in this region may lead to changes in economy, demography, rural-urban inhabitation and movement pattern (Parker et al. 2015a). Adult workers migrating according to work availability (e.g. mining, agriculture, the construction of dams, roads, and irrigation projects) and newly arrived people either displaced or in search of employment may result in increased human-vector contact and malaria transmission in the area. This population of migrant workers, especially those living in the border areas, constitutes a major challenge to the elimination of malaria in the region and is now probably the major factor contributing to the spread of resistant strains of malaria (SMRU : http://www.shoklo-unit.com).

1.1.2 Malaria parasites

Table 4 shows percent of all confirmed malaria cases in Thailand and Myanmar during 2012-2015 (WHO 2012, WHO 2013g, WHO 2014b, WHO 2015b). In Thailand and Myanmar, the predominant parasite species are *P.falciparum* and *P.vivax. Plasmodium ovale*, *P. malariae* and *P. knowlesi* infections are all found along the border, but are rare (Jongwutiwes et al. 2004, WHO 2008).

Malaria surveys conducted along the TMB during 1999-2011 showed that the incidence of *P. falciparum* infections in population living on the TMB has declined. During 2000-2002, *P. falciparum* infections were predominant over *P. vivax* infections. However, the use of rapid diagnostic test (RDT) for early detection and antimalarial treatment with highly effective artemisinin-based antimalarial therapies, since 2003 reduced the relative prevalence of *P. falciparum* over *P. vivax* (Carrara et al. 2013) (Figure 15). The effectiveness of ACT is

explained by the artemisinin derivatives that rapidly reduce the biomass of asexual parasites while also exerting strong gametocytocidal activity against early-stage sexual forms (Price et al. 1996). When combined with a slowly eliminated partner drug, the artemisinin derivatives minimize the risk of recrudescence and reduce *P. falciparum* transmissibility (Price et al. 1999).

Table 4. Percent of all confirmed malaria cases by P. *falciparum* and *P. vivax* in Thailand and Myanmar during 2012-2015.

Year	Thailand		Myanmar	
	***P.falciparum* (%)**	***P. vivax* (%)**	***P.falciparum* (%)**	***P. vivax* (%)**
2012	40	60	68	32
2013	40	60	65	35
2014	44	47	74	26
2015	38	54	75	25

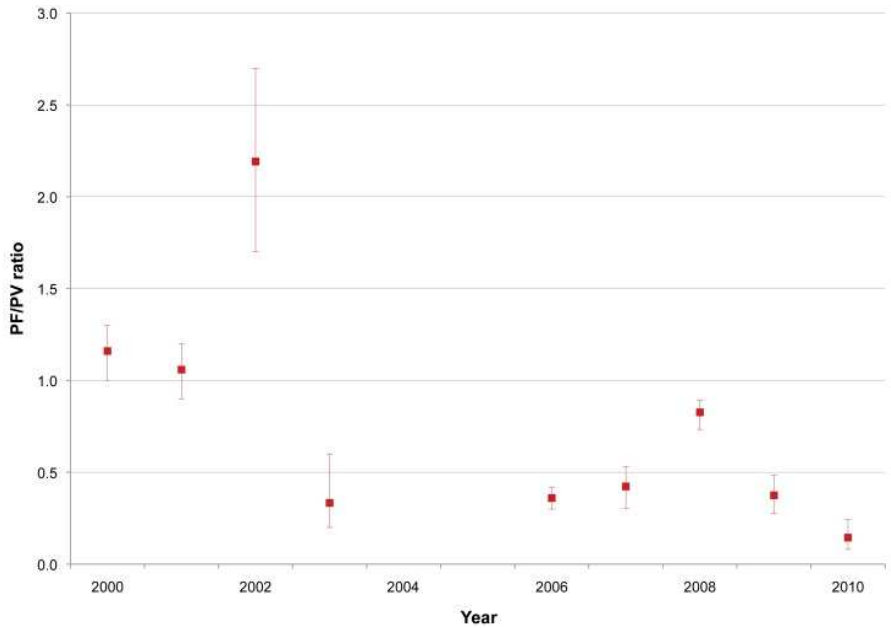

Figure 15. *Plasmodium falciparum* infection / *P. vivax* infection ratio from village surveys in Myanmar within SMRU clinic/health post catchment area. Red squares indicate the *P. falciparum* infection / *P. vivax* infection (*Pf/Pv*) ratio; bars indicate 95% CIs. A ratio above 1 indicates the predominance of *P. falciparum* over *P. vivax* ; a ratio below 1 indicates the predominance of *P. vivax* (Carrara et al. 2013).

1.1.3 Malaria vectors

In Thailand, at least 74 species of *Anopheles* mosquitoes have been documented, and seven *Anopheles* species have been incriminated as important malaria vectors including *An. baimaii*, *An. dirus*, *An. minimus*, *An. maculatus*, *An. pseudowillmori*, *An. sawadwongporni* and *An. aconitus* (Tainchum et al. 2015). In addition, *An. epiroticus* and *An. campestris* have been incriminated as secondary/incidental vectors in the country (Tainchum et al. 2015).

In Myanmar, a neigbouring country of Thailand, 36 *Anopheles* species were reported among which ten species have been found infected with malarial parasites. These included *An. aconitus*, *An. annularis*, *An. barbirostris*, *An. culicifacies*, *An. dirus*, *An. maculatus* , *An.minimus*, *An. sinensis* , *An. stephensi* (replaced by *An. sundaicus* complex in some coastal sites) and *An. subpictus*. The primary malaria vectors responsible for the majority of malaria infections are *An. dirus s.l.* and *An. minimus s.l.*. These two species are the major malaria vectors in the hilly regions such TMB. For *An. maculatus*, it has been reported as a primary vector, especially in Tanintharyi Division : the southern TMB, and elsewhere as a secondary vector depending on the location. Secondary vectors are include *An. aconitus*, *An.annularis s.l.*, *An. culicifacies s.l.*, *An. sinensis*, *An. jeyporiensis*, *An. philippinensis*, and *An. sundaicus s.l.* (Suwonkerd et al. 2013).

Malaria transmission along TMB is difficult to control due to the present of very efficient vectors. From the survey of natural human *plasmodium* infections in major *Anopheles* mosquitoes in western Thailand, *An. minimus s.l.* and *An. maculatus s.l*. were confirmed to be the major vector along TMB (Manguin et al. 2013). To understand malaria risk, the knowledge of mosquito behavior and their ecology is essential.

Anopheles dirus sensu lato

The Dirus complex belongs to the the Leucosphyrus group and is represented by 10 species in Thailand, including *Anopheles baimaii*, *An. dirus*, *An. cracens*, *An. hackeri*, *An.introlatus*, *An. latens*, *An. macarthuri*, *An. nemophilous*, *An. pujutensis* and *An. scanloni* (Tainchum et al. 2015). Within this complex, *An. baimaii* and *An. dirus* are considered to be malaria vectors in Thailand (Rattanarithikul et al. 2006). The sympatry of *An. dirus* with *An. baimaii* in Kanchanaburi province (Western Thailand) and along the TMB were reported (Rattanarithikul et al. 1995, Tananchai et al. 2012). *Anopheles dirus s.l.* is deep forest, forest-

fringe and foothills inhabiting mosquitoes. The immature stages were found in rock pools along the banks of thickly shaded streams, in cut bamboo stumps and in temporary standing or slow moving water under shade. *Anopheles dirus s.l.* are relatively long-lived and tend to be highly anthropophilic, making them a highly effective vector. While they are typically considered exophillic, biting can occur as frequency indoors as it does outdoors in open houses in forest. Feeding patterns (early versus late) are sometimes contradictory, even in the same site and species across different years or locations (Suwonkerd et al. 2013, Parker et al. 2015a). However, strong zoophilic behavior of *An. dirus s.l.* has been reported in a malaria endemic area of western Thailand. A recent study showed a significantly greater number of *An. dirus* and *An. baimaii* collected from cattle-baited traps as compared to human-landing collections (Tananchai et al. 2012).

Anopheles minimus sensu lato

The Minimus complex belongs to the the Funestus group and is represented by 2 species in Thailand, including *Anopheles minimus s.s.* and *An. harrisoni* (Tainchum et al. 2015). *Anopheles minimus s.s*. is the most common and widespread vector throughout Thailand, whereas *An. harrisoni* appears to be restricted in western and northern Thailand and occurs sometimes in sympathy with *An. minimus s.s*. (Rattanarithikul et al. 2006, Sungvornyothin et al. 2006). *Anopheles minimus s.l.* is primarily a mosquito of hilly regions, low rolling foothills to narrow river valleys in more mountainous areas (Suwonkerd et al. 2013). *Anopheles minimus s.s.* occupies a greater variety of habitats, including small streams or canals with slow running, clear and cool water, at dense forest and open agricultural fields. Conversely, *An. harrisoni* has a narrower habitat preference, being more closely linked to recently altered landscapes (deforested agricultural fields, etc) (Parker et al. 2015a). The adult behaviour of *An. minimus s.l.* is reported as highly diverse with the degree of anthropophily/zoophily, depends on the availability of alternative hosts such the presence of cattle can influence host choice behaviour in both *An. minimus s.s*. and *An. harrisoni* (Van Bortel et al. 2004, Trung et al. 2005). *Anopheles minimus s.s.* appears to feed more frequently on humans in areas where cattle are not present (Parker et al. 2015a). *Anopheles minimus* in Mae Sot shows both zoophilic and anthropophilic behaviors with no stronger preference for one host over the other (Sungvornyothin et al. 2006, Tananchai et al. 2012, Tisgratog et al. 2012 Tainchum et al. 2014). *Anopheles harrisoni* is generally zoophilic, but more studies are needed to better understand the host seeking preferences of this species (Parker et al. 2015a). In Thailand, *An.*

minimus s.s. is considered as endophagic while *An. harrisoni* is typically exophagic (Sungvornyothin et al. 2006, Trung et al. 2005).

Anopheles maculatus sensu lato

The Maculatus complex belongs to the Maculatus group and is represented by 7 species in Thailand, including *An. maculatus*, *An. pseudowillmori*, *An. sawadwongporni*, *An. dravidicus* , *An. notanandai* , *An. willmori* and *An. rampae* (Tainchum et al. 2015). Within this complex, *An. maculatus s.s*, *An. pseudowillmori*, *An. sawadwongporni*, are considered to be malaria vectors in Thailand (Rattanarithikul et al. 2006). *Anopheles maculatus s.l.* is typically found in forested foothills, around deep forest camps and in mountainous areas. The immature stages are found in diverse habitats such as pools, rocky, ponds, lakes, swamps, rice fields, occasionally tree holes and bamboo stumps, waterfall and rivers or streams of clear water that are often exposed to direct sunlight. *An. maculatus s.l.* is known to feed opportunistically on both animals and humans and mainly during the first and second quarters of the night (18:00-24:00) and exhibits typically exophagic behaviour (Tainchum et al. 2014, Parker et al. 2015a).

Other species contributing to malaria transmission

Many other species are consider to play secondary role in malaria transmission in Thailand. A secondary vector is thought to play a lesser role in transmission than the principal vector but is capable of maintaining malaria transmission at a reduced level or at particular period of the year (WHO 2016b). Recently, *An. annularis s.l.* and *An. barbirostris s.l.* were identified as secondary vectors along the TMB with potential importance for outdoor malaria transmission after the wet season (Sriwichai et al. 2016). In Myanmar, *An. aconitus* is a secondary vector in certain localities which commonly found in hilly tracts, foothills and also in the rice fields in the plains of central and southern Myanmar. *Anopheles annularis s.l.* has been found in stagnant water with thick grassy edges in permanent ponds, ground pits, tanks, swamps, stagnant drains and rice fields. Both *An. aconitus* and *An. annularis*, tend to play only a focal role in malaria transmission within their respective ranges and are often considered as secondary or incidental (Sinka et al. 2012). *Anopheles barbirostris/campestris* assemblage (these species could not be reliably distinguished) is probable vector of malaria in Sa Kaeo province (Limrat et al. 2001, Apiwathnasorn et al. 2002), Kanchanaburi province (Green, et al. 1991) and Chantaburi province, (Sriwichai et al. 2014) Thailand.

1.2 Limitation of the current malaria control strategies along the Thailand-Myanmar border

Malaria control remains difficult to implement along the TMB due to complexities in scaling up WHO malaria control measures and effective interventions in all villages where malaria is endemic. Malaria control and elimination in the region is also challenging by the difficulty to build the capacity of local health personnel in malaria detection. Moreover the emergence of artemisinin resistance in *P. falciparum* and the limited efficacy of conventional vector control tools (IRS and LLIN) against outdoor/early feeding mosquitoes may favor the re-emergence of malaria in these areas (Corbel et al. 2013).

1.2.1 Limitations in malaria diagnosis and treatment

Accurate diagnosis and prompt treatment of malaria is part of effective disease management and control. Light microscopy using Giemsa-stained thick and thin blood smears and antigen-detecting rapid diagnostic tests (RDT) are the primary diagnostic tools for the confirmation and management of suspected clinical malaria in all epidemiological situations, including areas of low transmission such TMB. However, microscopy and/or RDT, have a limitation because it's underestimate the prevalence of low density parasite infections (<100 parasites/μl) (WHO 2013f). The prevalence of *P. falciparum* infection by microscopy was, on average, around half of that measured by polymerase chain reaction (PCR)-based techniques (Okell et al. 2009). WHO recommend that the use of more sensitive diagnostic tools should be considered where there is already widespread implementation of malaria diagnostic tests and treatment and low parasite prevalence rates (e.g. < 10%) (WHO 2013f).

From recent surveys carried by the SMRU along the TMB, the prevalence of malaria infection, as measured by ultra-sensitive PCR detection (HVUsqPCR), showed to be much higher than expected due to the presence of a high proportion of submicroscopic carriers of *Plasmodium spp.* (Imwong et al. 2015). Along the TMB the *Plasmodium* prevalence shown approximately four times higher than estimates based on microscopy or RDT. Submicroscopic *P. falciparum* and *P. vivax* infections are common in low as well as high transmission settings (WHO 2013f) and relatively more common in adults than in children and in low rather than in high endemic settings. These carrier can be the source of 20-50% of all human to mosquito transmission when transmission reaches a very low level (Okell et al. 2012). Individuals with

asymptomatic malaria seemly a healthy people, but represent important reservoir of "parasites" between seasons (Figure 16). This suggest that conventional screening and treatment activities can only target a minority of parasitaemic individuals, which will be not enough to eliminate malaria and contain artemisinin and multidrug resistance.

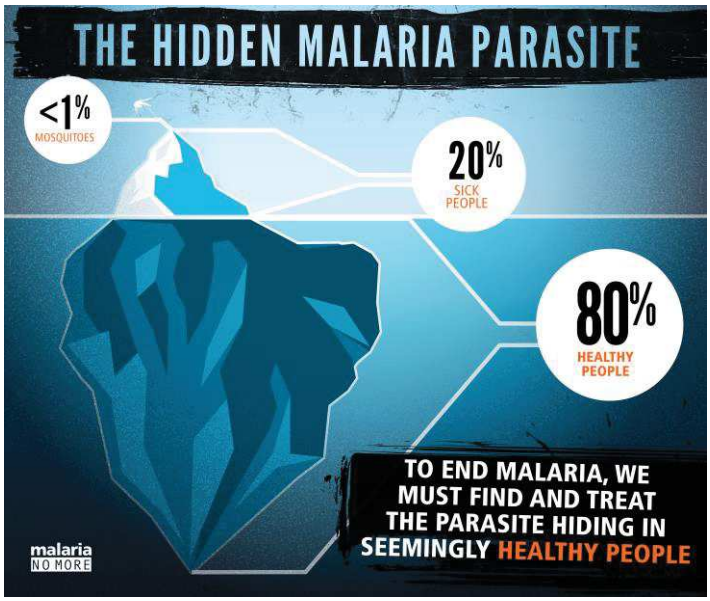

Figure 16. The hidden malaria parasite. The biggest proportion of malaria is asymptomatic and submicroscopic along the TMB. Source : https://www.malarianomore.org/news/blog/5-challenges-to-end-malaria-find-the-parasite

1.2.2 Antimalarial drug resistance and glucose-6-phosphate dehydrogenase deficiency

Recent studies conducted in the western border of Thailand showed evidence for the development of artemisinin-resistant in *P. falciparum* parasites in the region (Phyo et al. 2012, Phyo et al. 2016). This phenotype is characterized by a lower parasite clearance from the circulation (assessed by microscopy) following treatment with an artemisinin derivative. From >3,000 malaria patients in the northwestern border of Thailand, routinely admitted to malaria clinics run by the SMRU unit during 2001-2010, the mean of parasite clearance half-life increased from 2.6 hour to 3.7 hours. The proportion of slow-clearing infections (half-life ≥6.2 h) increased from 0.6% in 2001, to 20% in 2010. The results suggest that, at this parasite

clearance rate, artemisinin-resistant status will reach 5.5 hours in 2–6 years (Phyo et al. 2012). Between 2003 and 2013, the efficacy of mefloquine–artesunate (MAS3) in 1,005 patients on the TMB with uncomplicated *P. falciparum* malaria in relation to molecular markers of resistance were studied. Polymerase chain reaction (PCR)-adjusted cure rates declined from 100% in 2003 to 81.1% in 2013 as the proportions of isolates with the multidrug-resistance gene 1 (*Pf*mdr1) copies doubled from 32.4% to 64.7% and those with K13 mutations (a marker of artemisinin resistance) increased from 6.7% to 83.4% (Figure 17). The results suggest that, the increasing prevalence of K13 mutations was the decisive factor for the recent and rapid decline in efficacy of artemisinin-based combination (MAS3) on the TMB (Phyo et al. 2016).

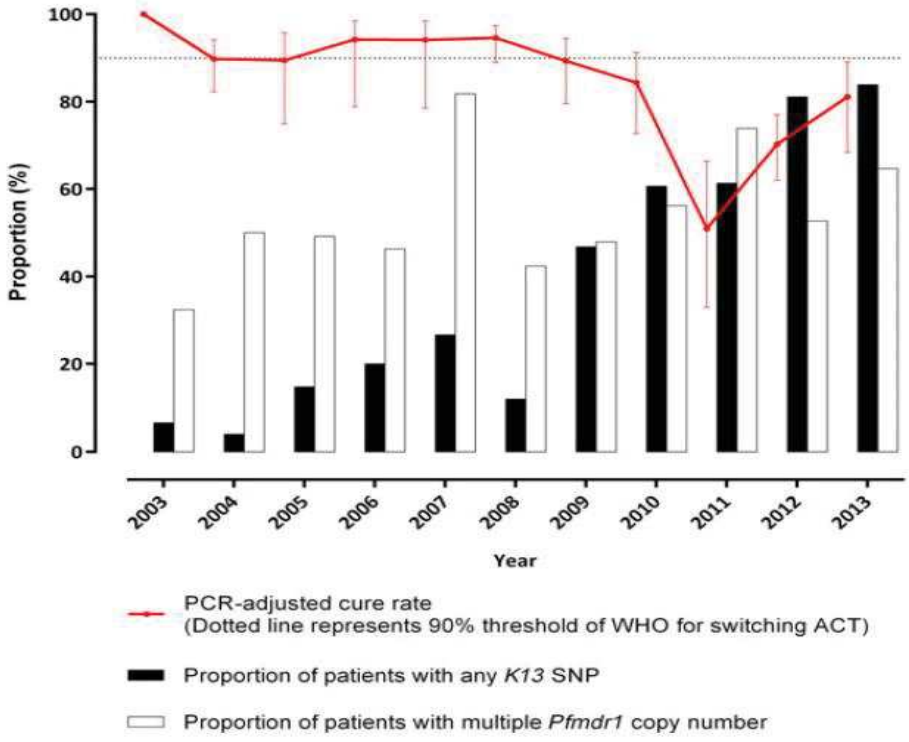

Figure 17. Annual proportions of day 42 polymerase chain reaction (PCR)-adjusted cure rates, summed K13 mutations, and amplified *Pf*mdr1. Abbreviations: ACT, artemisinin-based combination treatment; SNP, single-nucleotide polymorphism; WHO, World Health Organization (Phyo et al. 2016).

For *P.vivax* treatments, chloroquine and primaquine are recommended as first-line treatments in this region. However, the efficacy of chloroquine in the treatment of *P. vivax* infections is declining on the TMB (Phyo et al. 2011). Furthermore, to eliminate of *P. vivax*, primaquine has remained the only licensed drug capable of clearing the intra-hepatic schizonts and hypnozoites of this species, but its efficacy is highly dependent on the concurrent

administration of chloroquine, a blood schizontocidal agent and the recommended dose against vivax malaria is 0.5 mg/kg/d for 14 days for radical cure, so some patient may not completed the treatment dose (Fernando et al. 2011). Moreover, primaquine may cause clinically hemolysis in glucose-6-phosphate dehydrogenase deficiency (G6PD), a genetically inherited disorder found in 4–15% of the population in this region (Corbel et al. 2013). Currently, G6PD deficiency rapid diagnostic test are not yet integrate in routine into different health settings despite the fact that some products showed to be highly performant and sensitive in various epidemiological settings (Adu-Gyasi et al. 2015, Ley et al. 2015). The studies in Myanmar indicate that, a single dose of primaquine (0.25 mg base/kg of body weight) weekly for 8 weeks is however adequate for the treatment of *P. falciparum* gametocytes and/or *P. vivax* malaria patients with genotypes of red cell G6PD deficiency status, as no case of acute hemolysis was observed (Landier et al. 2017).

1.2.3 Residual malaria transmission

Vector behaviour is recognized as important parameter influencing the performances of vector control tools (Moiroux et al. 2012, Moiroux et al. 2014). Indoor residual spraying (IRS) and conventional insecticide treated mosquito nets (ITN) are expected to provide protection against highly anthropophilic and endophagic/endophilic mosquitoes. In Thailand, IRS and ITN, are the main vector control methods used in high and moderate/ low malaria endemic areas respectively (http://www.thaivbd.org/). Deltamethrin has been widely used in the malaria control program for IRS once or twice a year according to the endemic malaria zoning category as determined by the Bureau of Vector Borne Disease (BVBD) (http://www.thaivbd.org). According to annual malaria report from BVBD 2015, bifenthrin and alpha-cypermethrin have been used also for IRS whereas permethrin, and deltamethrin are the two main pyrethroids used for ITN (http://www.thaivbd.org).

Unfortunately, most of the malaria vectors in this area are forested mosquitoes feeding early in the evening and preferentially outdoors, such that the impact of IRS and ITN is limited (Trung et al. 2005, Tainchum et al. 2014). An outdoor biting mosquito might be responsible for maintaining malaria transmission despite high coverage of ITN and IRS, and this is defined as "residual transmission" (Durnez and Coosemans 2013). A combination of human and vector behaviours are responsible for this residual transmission, for example when people reside in or visit forest areas or do not sleep in protected houses (Bhumiratana et al.2013b) or when local

mosquito vector species exhibit one or more behaviours that allow them to avoid the core interventions (WHO 2014a).

In order to address vector bionomics and malaria transmission along TMB, IRD and SMRU conducted baseline entomological surveys in 4 villages before the implementation of a Targeted Chemo-Elimination (TCE) project for malaria elimination (TCE, previously termed mass drug administration (MDA)). The scope was to assess the feasibility, safety and acceptability of TCE on malaria transmission and its impact on the prevalence of artemisinin-resistance molecular markers. The second objective was to investigate the vector behaviour, insecticide resistance, *Plasmodium* infection rates in the *Anopheles* vectors and to quantify the the human-vector contact in communities along Thailand-Myanmar border where high prevalence of sub-microscopic parasitaemia has been reported. Twelve *Anopheles* species were identified including *An. minimus s.l.*, *An. maculatus s.l.*, *An. aconitus s.l.*, *An. dirus s.l.*, *An. annularis s.l.*, *An. barbirostris s.l.*, *An. hyrcanus s.l.*, *An. jamesi s.l.*, *An. kochi s.l.*, *An. subpictus s.l.*, *An. culicifacies B s.l.* and *An. tessellatus s.l.*. The dominant species are *An. minimus s.l.* (43.5%), *An. subpictus s.l.* (18.6%) and *An. maculatus s.l.* (17.5%). *Anopheles minimus s.l.* was by far the most abundant collected on humans (33.9%). *Anopheles minimus* can be considered as the most important malaria vector in the villages due to its wide distribution high abundance and high sporozoite rate (1.4%) (Kwansomboon et al. 2017). Molecular identification of sibling species by allele specific-PCR showed that *An. minimus* (formerly A) represented >98% of the Minimus Complex members collected with <0.1% identified as *An. harrisoni*. Regarding Maculatus group, four species i.e. *An. sawadwongporni*, *An. maculatus*, *An. pseudowillmori* and *An. dravidicus* were found in sympatry. *Anopheles sawadwongporni* was the predominant species followed by *An. maculatus*. *Anopheles dirus* complex was found exclusively on humans and was made of *An. baimaii* only (Kwansomboon et al. 2017).

The host seeking behavior of malaria vectors was investigated by estimating the zoophilic and exophagic preferences for *An. minimus*, *An. maculatus*, *An. sawadwongporni* and *An. pseudowillmori*. All *Anopheles spp.* showed a preference to feed outdoor (Exophagic Indexes (EI) range from 0.57 - 0.76) (Figure 18). All vector species showed greater predilection to feed on the animal bait but *An. minimus* showed the lowest zoophilic index (0.70) comparatively to *An. sawadwongporni* (0.96), *An. maculatus* (0.90) and *An. pseudowillormi* (0.93) (Figure 18). *Anopheles baimaii* showed high antropophagic rates but the low sample size preclude robust interpretation on its host biting preference. *Anopheles sawadwongporni*, *An.*

maculatus and *An. pseudowillmori* collected on humans showed earlier biting activity (22.00-00.00) than *An. minimus* (01.00-02.00) (Figure 19) (Kwansomboon et al 2017). The host seeking and biting preferences of *An. minimus* support his role in transmission. Indeed *An. minimus* showed higher anthropophilic and endophagic indexes compared to other malaria vector species and was able to bite humans all night long. Interestingly, this study showed that *An. sawadwongporni* and, to a lesser extent *An. maculatus* and *An. pseudowillmori* exhibited different night biting pattern according to the trapping method (median catching time of these species were one hour later when they were collected on the cow compared to humans.), hence suggesting some phenotypic plasticity in response to the availability of the vertebrate host (Kwansomboon et al 2017).

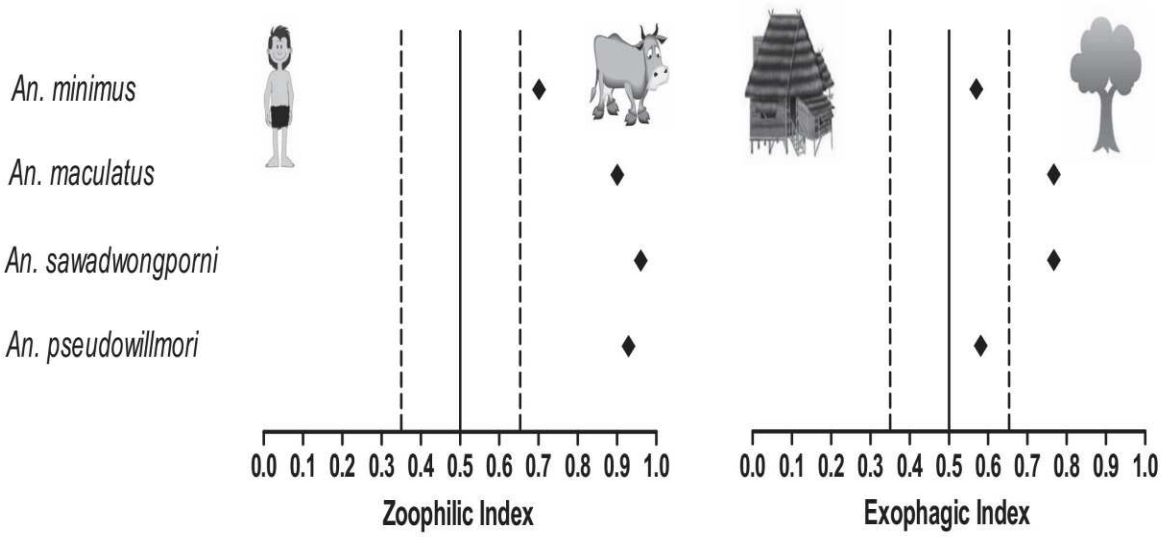

Figure 18. Host-biting preferences of primary malaria vectors according to the host and location. Calculating the zoophilic index (ZI) as the total number of bites received per cow for a given species compared to the total number of bites (cow + human). Calculating the exophagic index (EI) as the total number of bites received per human outdoors for a given species compared to the total number of bites received per human (indoors+outdoors). Results are presented for species having appropriate sample size to allow data analysis and interpretation (Kwansomboon et al. 2017).

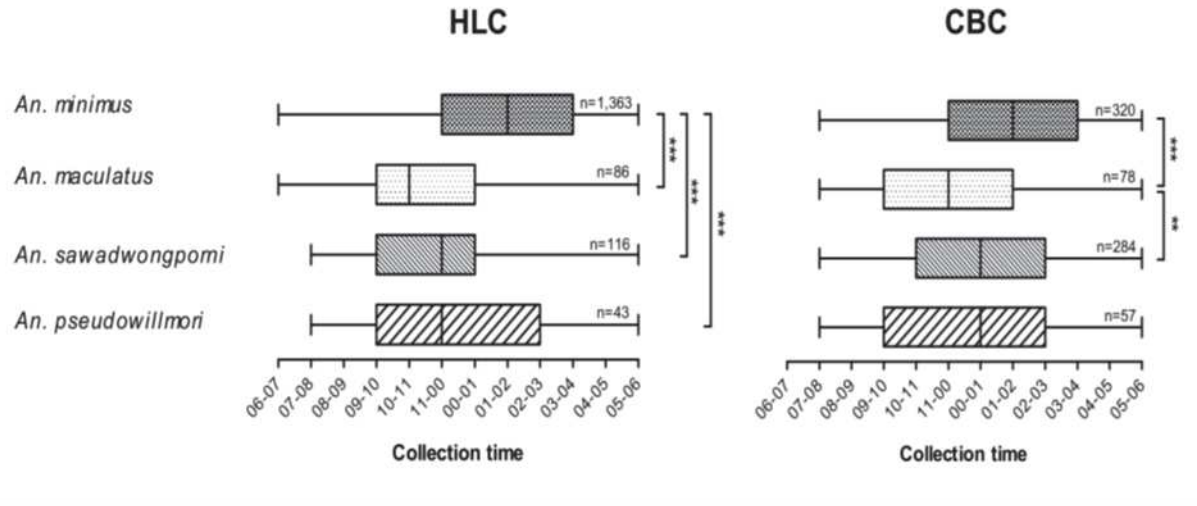

Figure 19. Median Catching Time (MCT) of malaria vectors. MCT was compared for *An. minimus*, *An. maculatus*, *An. Sawadwongporni*, and *An. pseudowillmori* collected by HLC and CBC and compared using Kruskal-Wallis test and Dunn's multiple comparison test (P-value <0.01 are represented as ** and P-value <0.001 are represented as ***) (Kwansomboon et al. 2017).

Complexity and diversity of malaria vectors and the heterogeneity of transmission together with low *Pf* sporozoite rates along the TMB pose increasing challenges to the successful implementation of malaria control and elimination. In low transmission settings, transmission "hot spots" remains and contribute to maintain a reservoir of parasites that are very difficult to detect and treat. With regards to the vector, control strategies will have to be adapted to the local ecology in order to ensure effective personal protection for populations at risk (mobile people, forest workers, etc). In addition, pyrethroid resistance in malaria vectors is on the rise along the TMB, and may represents an increasing threat to malaria vector control (Chaumeau et al. 2017).

To conclude, the complex interactions between, humans, malaria parasites, mosquito vectors and the environment render difficult the control and elimination of malaria along TMB (Table 5). Treating symptomatic patients and preventing transmission with conventional vector control tools cannot be succesuful to achieve malaria elimination. The low parasite clearance and the apparently healthy individuals who have asymptomatic malaria parasitaemia can sustain malaria over the dry season and maintain foci of transmission. Better understanding of the malaria epidemiology in low-endemic settings is needed to guide malaria control and elimination in the Great Mekong region.

Table 5. Malaria situation along Thailand-Myanmar border.

Interaction	Humans	Parasites	Vectors
Factor of transmission	Refugee community Cross-border population Migrant/forest workers	*Plasmodium falciparum* *Plasmodium vivax* Mixed *Pf*/*Pv* cases	*Anopheles minimus* *Anopheles maculatus* *Anopheles dirus* *Secondary vectors*
Malaria control strategies	Light microscopy Rapid diagnostic test	Antimalarial drug Artemisinin based combination therapies	LLIN / IRS
Limitations	Sub-microscopic reservoirs of parasites (asymptomatic carriers)	Multidrug resistance G6PD	Residual/outdoor transmission Complex biology/behavior of malaria vectors.

1.3 Mass drug administration (MDA) for malaria elimination and artemisinin resistance containment

Treated infections are an important source of transmission especially in areas of low, unstable malaria transmission. The effects of an unstable malarial outbreak are typically more severe than those associated with stable malaria. The reason for this is that those living in an area of stable malaria deal with it routinely and are therefore more experienced in the management and treatment of its associated problems than those who encounter malaria cases occasionally (http://www.smcm.edu/gambia/wp-content/uploads/sites/31/2014/11/98-chapter 3.pdf). In an area of stable malaria, individuals living in areas of stable malaria gradually acquire immunity against the major local strains of malaria parasites. Whereas unstable malaria, epidemics can be due to changes in human behavior, environmental and climate factors. For example, human migration and resettlement can introduce malaria into an area that did not have it previously, and then expose a naive population (i.e. that didn't acquired any immunity) to the disease (http://www.open.ed u/openlearncreate/mod/oucontent/view.ph p?id=88&printable=1).

Currently artemisinin-based combination therapy (ACT) is recommended for the treatment of *P. falciparum* malaria. Artemesinin-combination therapies reduce gametocyte carriage, and therefore reduce transmission, although ACT does not eliminate mature transmissible *P. falciparum* gametocytes. Preventing these patients' infections from transmitting falciparum malaria requires treatment with a specific gametocytocide, and the only

generally available drug is the 8-aminoquinoline, primaquine. Primaquine acts on mature gametocytes which are present usually in the circulation at the time when the patient presents for treatment. Following primaquine administration *P. falciparum* gametocytes are sterilized within hours, whereas clearance from blood takes days. Gametocytaemia (the rate of gametocyte clearance) and clearance times are determined predominantly by the more numerous female gametocytes, which are generally less drug sensitive than the minority of male gametocytes, whereas transmission-blocking activity and thus infectivity is determined by the more sensitive male forms (review in White et al. 2014).

In efforts to contain and eliminate artemisinin-resistant falciparum malaria, it is important to ensure that all effective measures to reduce malaria transmission are implemented. According to the WHO, a single dose of primaquine at 0.25 mg base/kg is effective in blocking transmission and is unlikely to cause serious toxicity in individuals with any of the G6PD-deficiency variants. Therefore 0.25 mg base/kg primaquine should be given to all patients (except for pregnant women and infants <1 year of age) with parasitologically-confirmed *P. falciparum* malaria on the first day of treatment in addition to an ACT (WHO 2015e).

The majority of populations along the TMB show high prevalence of sub-microscopic parasitemia (Imwong et al. 2015), with no recent history of fever. The presence of large foci of asymptomatic carriage of *P. falciparum* parasites is the main obstacle to the rapid elimination of falciparum malaria in this region of low and unstable transmission. Consequently, malaria control and elimination activities need a radical rethink. In this context, the SMRU lunched a pilot open-label cluster-randomized controlled trial evaluate the feasibility, safety and the acceptability of mass-drug administration (MDA) to reduce the asymptomatic parasite reservoir and accelerate elimination of falciparum malaria. Four villages (HPN, HKT, KNH and TOT) along the Thailand-Myanmar border with ≥30% malaria prevalence were selected after engagement of the community as described elsewhere (Landier et al. 2017). A malaria post was set up in each village. Two villages were randomly assigned to MDA immediately and two were followed for 9 months before receiving MDA (cross over design, Figure 20). A 3-day course of dihydroartemisinin-piperaquine (DP) and single low dose primaquine was given under supervision monthly for 3 months to all participants. As part of this study, the prevalence of malaria in quarterly surveys using ultrasensitive qPCR and the incidence of clinical malaria were measured over 24 months and indicators of transmission were measured using monthly entomology collections.

Along the TMB, local malaria transmission is not uniformly distributed and the risk of malaria may be confined to geographically small high risk areas. Identifying and targeting these malaria-transmission hot spots is therefore essential to remove remaining sources of transmission and to achieve malaria elimination. However, measuring local malaria transmission poses considerable challenges because of the lack of sensitivity of the classical entomological indicator (Human biting rate - HBR) for estimate the risk of malaria transmission. Therefore, it is currently emphasized the need to develop new epidemiological tool to assess the risk of malaria transmission on the TMB.

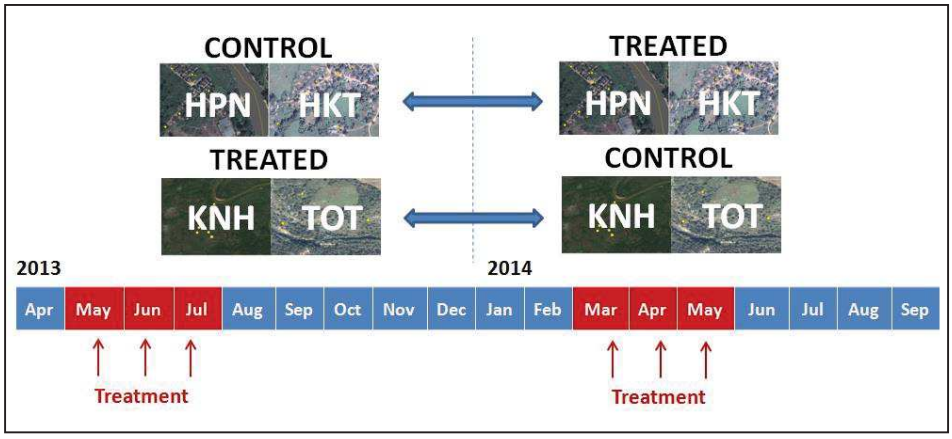

Figure 20. Round of MDA treatments.

My thesis was conducted in the framework of the MDA pilot project implemented along the TMB with the support of the Bill & Melinda Gates foundation and the Global Fund. The scope was to better understand the pattern of malaria transmission in a context of mass drug administration by the use of specific biomarkers of human exposure to malaria vector bites and *Plasmodium falciparum* infections. The specific outcomes were to understand the factors associated with the risk of human exposure to both the vectors and the parasite and to identify whether malaria transmission hotspots remained after MDA. The results were expected to guide decision making for deploying more effective vector control tools to durably interupt the transmission.

2. OBJECTIVE OF THE THESIS

2.1 To estimate the spatial and temporal changes in human-vector contact by the use of specific salivary biomarker of *Anopheles* bites.

The gSG6-P1 peptide, based on *An. gambiae* SG6 protein sequence, has been validated as a specific biomarker of *Anopheles* exposure in various settings including Africa and the Americas (Poinsignon et al. 2009, Drame et al. 2010b, Londono-Renteria et al. 2015a). Several studies in Africa showed that human antibody response to gSG6-P1 salivary peptide is a quantitative and specific biomarker to measure recent exposure of individuals to *Anopheles* bites , even in a context of low level of exposure to malaria vector bites, as well as to evaluate the human risk of malaria transmission. Moreover, the gSG6 protein and especially gSG6-P1 peptide showed to be well conserved among major *Anopheles* species. The applicability of gSG6-P1 to measure exposure to different *Anopheles* species in South east Asia has however not been evaluated. The first part of this thesis was therefore to i) validate the use of gSG6-P1 to estimate spatial and temporal changes in malaria transmision risk along the TMB and ii) to identify the determinants modulating the human-vector contact.

2.2 To address the relevance of using *Anopheles* salivary biomarker as a proxy for estimating *P. falciparum* malaria exposure risk along the Thailand-Myanmar border.

In the TMB, malaria cases were reduced by more than 50% in the last 15 years due to the improvements in malaria control. Consequently, a shift towards more heterogeneous malaria transmission has been observed. Malaria clusters along the TMB is associated with forests and forest edges with the cases often occur in hotspots, with close spatial associations with vector-breeding habitats, and in certain ‘high-risk’ sub-sets of the population (those with higher exposure to vector-breeding habitats). The malaria problem is often considered a spillover effect, with population movement and the rise and development of urban centres seems to have disrupted malaria cycles in the region. The risk of malaria transmission along TMB strongly vary in space and time and is influenced by the environment and human practices. Nevertheless, the relationship between human exposure to *Anopheles* bites and *Plasmodium falciparum* malaria remains unknown. The second part of the thesis aimed to i) determine the factors modulating the malaria transmission on the TMB and ii) to address

relevance of using *Anopheles* salivary biomarker to measure micro-epidemiological variations in exposure to *Pf* malaria in a context of malaria elimination campaign.

3. THESIS FRAMEWORK

Epidemiological, immunological and entomological surveys were conducted in four villages (HPN, HKT, KNH and TOT) during 18 months between April 2013 to December 2014. A community based malaria clinic ("malaria post") was set up in each village. A census was performed prior to the surveys and demographic information was collected at that time. Malaria prevalence was measured in each village during surveys of the entire village population by ultrasensitive PCR (μPCR) according to methods described previously (Imwong et al. 2015). Mosquito collections using human landing catch technique were conducted to determine the composition and abundance of malaria vectors in the study area. Dried blood spots were collected in filter papers among all inhabitants during epidemiological surveys at baseline (M0) and then every three months up to 18 months (M3, M6, M9, M12, M15 and M18). The real contact between *Anopheles* vector and human individuals were estimated by the use of a new biomarker based on the evaluation of human antibody response to specific *Anopheles* salivary proteins (gSG6-P1). In addition, exposure of human populations to *Plasmodium spp* were investigated through the measurement of human IgG against a panel of *P. falciparum* antigens (*Pf*CSP and *Pf*MSP-1_{19}). Appropriate statistical models were used to assess the human-vector contact and human exposure to *Plasmodium spp* of people in 4 village. The data collected during the TCE project (entomological, demographical, immunological, epidemiological and environmental data) were allow to set up a GIS (spatial clustering analysis) to better understand the pattern of malaria transmission in the study area (Figure 21).

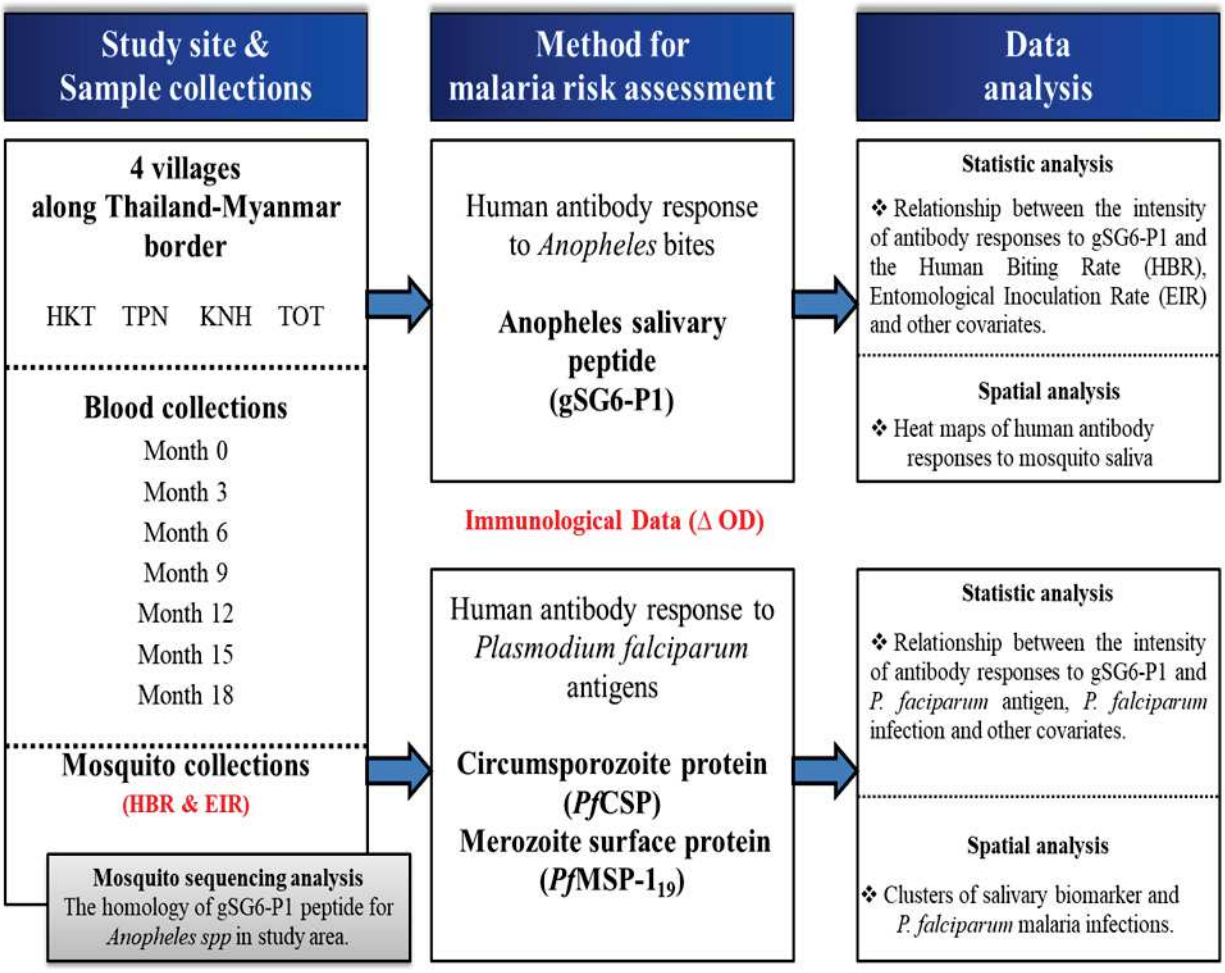

Figure 21. Thesis framework

4. STUDY DESIGN AND METHOD

4.1 Study site

The study was conducted in four sentinel sites located within 10 km of the Thailand border considered representative of the area in terms of environment, ecology, population, and behavior. Villages were Htoo Pyin Nyar or TPN (17° 14' N, 98 ° 29' E), Tar Au Ta or TOT (16° 36' N, 98° 57' E), Ka Nu Hta or KNH (17° 18 'N, 98° 24' E) and Htee Kaw Taw or HKT (16° 85 'N, 98° 47' E) (Figure 22). The villages were selected according to inclusion criteria were to dry season malaria prevalence exceeding 30% with *P. falciparum* comprising at least 30% of infections (data obtained by qPCR on a blood volume > 1 ml). The EIR at the beginning of the rainy season at HKT there was a mean of EIR*Pf* and EIR*Pv* were 3.0 and 11.4 infective bites per human per month, respectively and at KNH there was a mean of only EIRP*v* was 1.2 infective bites per human per month. EIR was nil at TPN and TOT but the low proportion of malaria vectors collected at TOT (n=326) could partially explain the outcomes. When considering the entire area (4 villages) the mean EIR*Pf* and EIRP*v* were 0.8 and 3.2 infected bites per man per month, respectively (Kwansomboon et al. 2017).

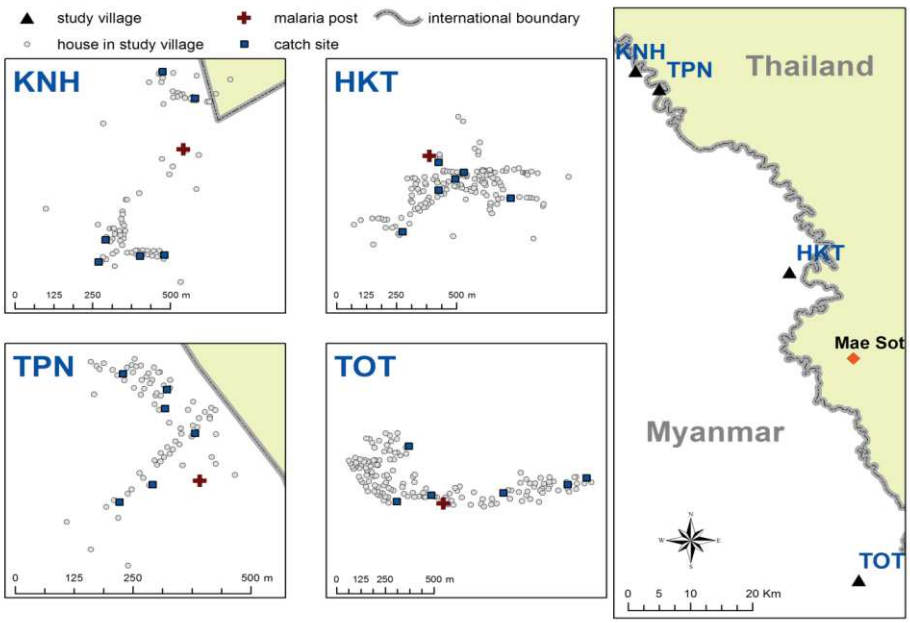

Figure 22. Map of the study area. The map shows the mosquito collection sites carried in four villages (TOT, TPN, KNH, and HKT) along the Thailand-Myanmar border. The map shows the malaria post (red cross), the catch sites (blue square), and households (grey circles).

4.2 Sample collections

4.2.1 Mosquito collections

Mosquito collections were conducted using human landing catch (HLC) between April 2013 and October 2014 (Table 6), to address the abundance of vectors and the dynamic of malaria transmission (as described in Kwansouboon et al. 2017). In each site, mosquito collections were carried out in 5 points from 06.00 pm to 06.00 am and for 5 consecutive nights (Figure 23). Five sites were used for HLC (outdoor and indoor) with one supervisor being present at each point to ensure no discrepancies in mosquito collection. Each night the team of collectors were rotated according to a Latin square design. Every morning the supervisors collected the cups containing the mosquitoes and brought them to SMRU for morphological identification. Mosquito identification at genus level (*Anopheles*, *Culex*, and *Aedes*) was carried out by entomologists present at SMRU. All A*nopheles* females were labelled, put in 1.5 ml tubes containing silica gel and kept at -20°C. Mosquitoes were then sent to the Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok and identified to the species level using morphological keys for Southeast Asian *Anophelines* (Rattanarithikul et al. 2006).

No	Village	Site 1 (HLC)			Site 2 (HLC)			Site 3 (HLC)			Site 4 (HLC)			Site 5 (HLC)		
		IN	OUT	Superv.	IN	OUT	Superv.	IN	OUT	Superv.	IN	OUT	Superv.	IN	OUT	Superv.
1	TPN	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	TOT	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
3	KNH	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4	HKT	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Figure 23. Design of the mosquito collection. The table shows the number of collectors and supervisor present at each site and each collection point at the same time.

Table 6. Mosquito collection date.

Survey	HPN	TOT	KNH	HKT
1	23-27 April 2013	19-23 May 2013	11-15 June 2013	23-27 June 2013
2	26-30 May 2013	14-18 July 2013	7-11 July 2013	28-29 July 2013 (2days)
3	21-25 July 2013	11-15 August 2013	4-8 August 2013	25-29 August 2013
4	18-22 August 2013	8-12 September 2013	1-5 September 2013	22-26 September 2013
5	15-19 September 2013	6-10 October 2013	29 September - 3 October 2013	20-24 October 2013
6	13-17 October 2013	3-7 November 2013	27-31 October 2013	17-21 November 2013
7	10-14 November 2013	1-5 December 2013	24-28 November 2013	15-19 December 2013
8	8-12 December 2013	26-30 January	22-26 December 2013	12-16 January 2014
9	5-9 January 2014	23-27 February 2014	19-23 January 2014	9-13 February 2014
10	2-6 February 2014	23-27 March 2014	16-20 February2014	9-13 March 2014
11	2-6 March 2014	20-24 April 2014	16-20 March 2014	6-10 April 2014
12	30 March - 3 April 2014	18-22 May 2014	13-17 April 2014	4-8 May 2014
13	27 April - 1 May 2014	15-19 June 2014	11-15 May 2014	1-5 June 2014
14	25-29 May 2014	13-17 July 2014	8-12 June 2014	29 June - 3 July 2014
15	22-26 June 2014	10-14 August 2014	6-10 July 2014	27-31 July 2014
16	20-24 July 2014	7-11 September 2014	3-7 August 2014	24-27 August 2014
17	17-21 August 2014	5-9 October 2014	31 August - 4 September 2014	21-25 September 2014
18	14-18 September 2014	2-6 November 2014	28 September - 2 October 2014	19-23 October 2014

4.2.2 Blood collections

Blood dried spots were collected from all inhabitants every 3 months from May 2013 to December 2014 (seven epidemiological surveys, Table 7). In each village a committee was

formed composed of village leaders, village malaria workers, and volunteers to assist the SMRU staff in organizing the surveys and in engaging and mobilizing the community (Imwong et al. 2015). A census was performed prior to the surveys and demographic information was collected at that time. All individuals aged 6 months or above were invited to participate, including temporary residents and migrant workers. Individual informed consent was obtained from adults, and parental consent for the participation of children under 16 years. Brief history of travels, professional activity, and impregnated bednet usage were also obtained. At each survey dried blood spots on filter Whatman papers were collected among inhabitants and properly labeled for further ELISA assays.

Table 7. Blood collection date.

Village	HPN	TOT	KNH	HKT
M0	20 May 2013	14 June 2013	14 June 2013	4 July 2013
M3	23 August 2013	12 September 2013	12 September 2013	07 October 2013
M6	7 November 2013	26 November 2013	11 December 2013	8 January 2014
M9	28 January 2014	18 February 2014	5 March 2014	1 May 2014
M12	23 April 2014	15 May 2014	26 May 2014	24 June 2014
M15	14 July 2014	06 August 2014	21 August 2014	16 September 2014
M18	08 October 2014	30 October 2014	13 November 2014	8 December 2014

4.3 Measurement of human IgG against *Anopheles* salivary and *P. falciparum* antigens

4.3.1 Elution of blood from dried blood spots

The standardized dried blood spots (1 cm diameter) were eluted by incubation in 400 µl of phosphate buffered saline (PBS-Tween 0.1%) at 4 °C for 24 hours. Thereafter, remove the filter paper and keep a samples at -20°C.

4.3.2 Antigens production

The gSG6-P1-specific *Anopheles* peptide was designed using bioinformatics (Poinsignon et al. 2008a). It was synthesized and purified (95%) by Genepep SA (St-Cle´ment

de Riviere, France). The *Pf*MSP-1_{19} and *Pf*CSP peptides (Ambrosino et al. 2010, Drakeley et al. 2005) were synthesized and purified (95%) by Vaximax, France. All peptide batches were shipped in lyophilized form and then suspended in ultra-filtered water and frozen in aliquots at -20 °C until use for immunological tests (ELISA).

4.3.3 ELISA assays

Maxisorp plates (Nunc, Rosklide, Denmark) were coated with the specific peptide. For gSG6-P1: the gSG6-P1-specific *Anopheles* peptide (20 µg/ml) in phosphate buffered saline for 2 hours and 30 min at 37°C. After washing (with a solution of PBS-Tween 0.1%), the plates were blocked for 1 hour at 37 °C with 300 µl of blocking buffer (Pierce, Thermo Scientific USA). Thereafter, each eluated was incubated in duplicate at 4 °C overnight at 1/20 dilution (in PBS-Tween 1%). Mouse biotinylated Ab to human IgG (BD Pharmingen, USA) was incubated at a 1/1,000 dilution at 37 °C for 1 hour and 30 min and peroxidase-conjugated streptavidine (GE Healthcare, UK) was added following the same conditions for 1 hour. Colorimetric development was carried out using ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline 6-sulfonic acid) diammonium salt) (Pierce, Thermo Scientific USA) in 0.05 M citrate buffer (pH 4) containing 0.003% H_2O_2, and absorbance was measured at 405 nm. In parallel, each test sample was assessed in a blank well containing no gSG6-P1-specific *Anopheles* peptide (ODn) to measure non-specific reactions.

For the *Pf*MSP-1_{19} / *Pf*CSP peptide: the *Pf*MSP-1_{19} / *Pf*CSP peptide (1 µg/ml) in coating buffer (PBS + Phenol red 1%) at 4 °C overnight. After washing (with a solution of PBS-Tween 0.1% + NaCl), the plates were blocked for 1 hour under stirring at room temperature with 150 µl of saturation buffer (PBS-Tween 0.1%+ milk powder 3%). Thereafter, each eluted was incubated in duplicate at 2 hours under stirring at room temperature at 1/20 dilution in sample dilution buffer (PBS-Tween 0.1%+ milk powder 1%+ sodium azide 0.02%) Anti-human IgG coupled to the peroxidase (Invitrogen, USA) was incubated at a 1/3,000 dilution at 1 hour under stirring at room temperature and substrate TMB one (3,3′,5,5′-tetramethylbenzidine) (Promega, USA) was added for 30 mininutes at room temperature protected from light then stopping the reaction with 0.2 M H_2SO_4 , and absorbance was measured at 450 nm. In parallel, each test sample was assessed in a blank well containing no *Pf*MSP-1_{19} / *Pf*CSP peptide (ODn) to measure non-specific reactions.

Individual results were expressed as the ΔOD value: ΔOD= ODx-ODn. ODx and ODn represent the mean of individual optical density (OD) in 2 antigen wells and 1 blank well containing no antigen, respectively. Specific IgG response were also assayed in non-*Anopheles* exposed / non-malaria exposed individuals (Negative samples from France: n = 14) in order to quantify the non-specific background Ab level and to calculate the cut-off value of the immune response (Cut off = mean (ΔDOneg) +3SDs). Based on our findings, a participant was classified as an immune responder to gSG6-P1, *Pf*CSP and *Pf*MSP-1_{19} if their ΔOD was >0.450, > 0.115 and > 0.162, respectively.

4.3.4 Sequence alignment of gSG6-P1 for *Anopheles* species

Molecular analysis were used to determination of the protein sequence of *Anopheles* collected in the study area. Sequence alignments were done with the Tblastn program in Vectorbase database (https://www.vectorbase.org/) which enabled comparing a sequence of gSG6 peptides with that of African vectors (*An. gambiae*). The nucleotide sequence were translated to the amino acid sequence with bioinformatics software (http://web.expasy.org/ translate/). Immunogenicity prediction of these epitopes were performed by computerized predictions of antigenicity based on physico-chemical properties of the amino acid sequences by different programs such as, BCEPred (http://www.imtech.res.in/raghava/bcepred/) , ABCPred (http:// www.imtech.res.in/raghava/abcpred/) and BepiPred (http://www. cbs.dtu.dk /services/BepiPred/).

4.4. Data analysis

4.4.1 Covariates

Individual level covariates included age group (sorted in 4 classes; <5 years old; 5-15 years old, 16-59 years old and 60 up years old) and sex. Household-level covariates included LLIN use, based on questionnaire conducted at the baseline (whether the participants had and used a LLIN “every night”, “some nights” and “never”). At the village level, the population size at each survey, the temperature and relative humidity (2 time-dependent variables defined as the estimated mean and max humidity during 2 weeks preceding the mosquito collections) were recorded. The mean Human Biting Rates (HBR) and Entomological Inoculation Rates (EIR) were estimated at each catching site 1 month prior the blood sample collection. Seasons were grouped as “Hot season” (mid February - mid May), “Rainy season” (mid May to mid

October) and “Cool season” (mid October-mid February) according to the Thai Meteorological Department (http://www.tmd.go.th/en/archive/thailand_ climate.pdf). All covariates used in this study acted as proxies for social, demographic or environmental status. The use of artemisinin combination treatments (ACT), consisting of DP accompanied by single dose PQ, as a part of MDA for the elimination of *P. falciparum* malaria were recorded. Participation to MDA was defined as a binary variable equal to 0 when an individual did not take MDA and equal to 1 when an individual took 1, 2 or 3 doses of treatment over the course of the study. Human exposure to *Anopheles* bites was defined as the intensity of Ab response to the gSG6-P1 (ΔOD) at each survey and was sorted in 4 categories (low, medium, high and very high) based on quartiles as described in Table 8.

4.4.2 Statistical approach

The relationship between the intensity of the human antibody response (ΔOD) and entomological indicators of transmission (HBR and EIR) was studied using a multivariate 3-level (house, individual, and measurement) mixed model analysis. We considered (1) the HBR (or EIR) of total *Anopheles* mosquitoes, (2) the HBR (or EIR) of the primary vectors, and (3) the HBR (or EIR) of the secondary vectors, in 6 separated analyses. The potential adjustment factors were all of the covariates described above (Table 8). In each analysis, the HBR variable was categorized in 4 classes (according to the quartiles) to avoid the assumption of a linear relationship between the HBR and the human antibody response. For the EIR model, data were categorized as a binary variable (0 and >0) because of the high number of data collected from uninfected mosquitoes. In all models, a univariate analysis was first performed, where we estimated the relationship between each adjustment factor and the antibody response through a univariate mixed model. In a second step, we entered in a multivariate mixed model all of the adjustment factors with a P value of <.2 from the univariate analysis and then removed sequentially all the adjustment factors with a P value of >.05 (backward selection).

To investigate the association between Ab responses specific for P. falciparum CSP and MSP-1_{19} (binary variable: positive, negative) and the intensity of Ab response to *Anopheles* salivary antigen (gSG6-P1), we used a multivariate logistic mixed regression model with adjustment for relevant covariates. Individual level covariates included age group, sex, Ab response to *Anopheles* bites (categorical variable: low, medium, high, very high; based on quartiles) and MDA treatment (a time-dependent individual binary variable, 0 as long the

individual as not taken any drug, 1 when individuals has received 1, 2 or 3 doses). Household-level covariates included LLIN use (never, sometimes, every night). At the village level, the population size at each survey, the temperature and relative humidity were taking into account in the model (i.e. second order polynomial). Season was defined according to the Thai Meteorological Department (http://www.tmd.go.th/en/archive/thailand_ climate.pdf.) All covariates acted as proxies for social, demographic or environmental status. First, a univariate analysis was conducted by entering each independent variable in a univariate logistic regression model. In a second step, variables were retained for the multivariate analysis if their p-value <0.2 in the univariate model. A backward stepwise selection procedure was applied to retain only the significant covariates ($p<0.05$) in the final model. In a second analysis, we investigated the crude relationship between the intensity of Ab response to *Anopheles* salivary antigens (4 categories) and P. falciparum malaria infection (binary variable: positive, negative by uPCR) using a multivariate mixed logistic regression. Statistical analyses were done with Stata version 13.0 (StataCorp LP, College Station, Texas). Graphs were constructed using GraphPad Prism 5 software (San Diego, CA, USA).

Table 8 : Classification of variable.

Blood survey	M0,M3,M6,M9,M12,M15,M18
gSG6-P1	Delta OD value
	Responder, Non-responder (catagorized by cut off = 0.450)
	High (2.505-1.034), Medium (1.033-0.610), Low (0.609-0.451), Non responder (<0.450) (catagorized by percentile <25%,25-75%,>75%)
***Pf*CSP**	Delta OD value
	Reactive, Non-reactive (catagorized by cut off = 0.115)
***Pf*MSP-1_{19}**	Delta OD value
	Positive, Negative (catagorized by cut off = 0.162)
***Pf* malaria infection**	Positive, Negative by uPCR
HBR	At each catching site 1 month prior the blood sample collection
	The nearest distance from house to collection sites 1-5 were calculated
	All *Anopheles* (Class 1:<96, Class 2 : 96-204, Class 3: 204-531, Class 4 : ≥531)
	Primary vector (*An.maculatus*, *An.minimus*, *An.dirus*) (Class 1:< 46.5, Class 2 : 46.5-159, Class 3: 159-468, Class 4 : ≥468
	Secondary vector (*An.barbirostris*, *An. Annularis*, *An aconitus*)
EIR	At each catching site 1 month prior the blood sample collection
	0 = no infected mosquito, >0 = presence of infected mosquitoes
Village	4 groups (HKT, TPN, KNH and TOT)
Age	4 groups
	1 = 0-4 years old
	2 = 5-15 years old
	3 = 16-59 years old
	4 = 60 up years old
Sex	2 groups (1= Male, 2= Female)
Season	3 groups : Related on mosquito collection date :
	Hot (16 February -15 May)
	Rainy (16 May -15 October)
	Cool (16 October- 15 February)
Temperature	Two weeks before mosquito collection date
	Average / Minimum / Maximum of temperature
RH	Two weeks before mosquito collection date
	Average / Minimum / Maximum of relative humidity
Bednet	No. of bednet distribution
	Sleep in bednet (Never / Some night / Everynight)
MDA	The use of artemisinin combination treatments for the elimination of *P. falciparum* malaria (0 = an individual did not take MDA, 1 = an individual took 1, 2 or 3 doses of treatment over the course of the study)

4.4.3 Spatial analysis

Geographic references (latitude and longitude) were recorded for all households in the study villages. Each house was given an identification code and all study participants could be linked back to their respective houses using the identification code.

Heat map raster layers were created for IgG responses among individuals within each village and survey time point, using QGIS 2.4 (http://www.qgis.org/). The raster layers give a smoothed representation of IgG intensity within study villages. Mean HBRs for malaria vectors and EIR positives (meaning >0) were also plotted in the maps to indicate catch site location, vector abundance, and malaria transmission foci. Spatial autocorrelation (clustering) of IgG values was calculated for each village and time point, using 2 approaches: the Moran I statistic (a global clustering method) and local indicators of spatial autocorrelation (LISAs) (Anselin 1995). The Moran I tests give a single test statistic and associated P value for each village/ month combination, while the LISAs give a test statistic and P value for each individual/month combination. We used a Benjamini-Hochberg correction to account for multiple testing. All results were mapped using ArcMap 10.2 (http:// www.esri.com/).

Scan statistics were used to test for statistically significant clusters of salivary biomarker (gSG6-P1) and *P. falciparum* malaria infections as measured by uPCR. A discrete space-time Poisson model was used to test for statistically significant clusters for each of these outcomes and for each study village across all screenings (M0 – M18) (Kulldorff 1997, Kulldorff 2009). The scan statistic uses a moving window (a spherical kernel) that centers on each point (house) in the village and calculates the *P. falciparum* malaria incidence within and outside of the window. The window increases in size until half of the village population is contained and then moves to the next point. Likelihood ratios are calculated for each window location, size and time point and p-values are calculated using Monte Carlo simulations for the largest ranking clusters. Scan statistics were calculated using SaTScan software (https://www.satscan.org/).

RESULTS

CHAPTER 1: USE OF SALIVARY BIOMARKER (gSG6-P1 PEPTIDE) FOR ASSESSING SPATIAL AND TEMPORAL CHANGES IN HUMAN EXPOSURE TO MALARIA VECTOR BITES

As described in the section 1.2.1, malaria along the TMB displays geographical heterogeneity and is characterized by high prevalence of submicroscopic carriage and the emergence of artemisinin-resistant strains of *P. falciparum*. In order to reach elimination in this region, a better understanding of the spatial and temporal dynamic of malaria transmission is a priority. Recently, the exploration of the close interactions between the human host and the vector through the antigenic salivary proteins of hematophagous arthropods has lead to the development of new salivary biomarkers. The gSG6-P1 peptide has been validated as a specific biomarker for *Anopheles* exposure for malaria epidemiological studies in various settings in Africa and the Americas (Poinsignon et al. 2009, Drame et al. 2010, Londono-Renteria et al. 2015a), but has never been explored in South East Asia. This study hence represents the first attempt to validate the gSG6-P1 peptide as an epidemiological tool for evaluating the direct exposure of human populations to *Anopheles spp*. in malaria hotspots in South East Asia. Seven serologic surveys were conducted from May 2013 to December 2014 in 4 sentinel villages. More than 9,400 blood specimens were collected in filter papers from all inhabitants at baseline and then every 3 months thereafter, up to 18 months, for analysis by enzyme-linked immunosorbent assay. The relationship between the intensity of the human antibody response and entomological indicators of transmission (human biting rates [HBR] and entomological inoculation rates [EIRs]) was studied using a multivariate 3-level mixed model analysis. Heat maps for human immunoglobulin G (IgG) responses for each village and survey time point were created using QGIS 2.4.

The results of this study was published in *The Journal of Infectious Disease (February 2017) 215 (3): 396-404: Use of an Anopheles Salivary Biomarker to Assess Malaria Transmission Risk Along the Thailand-Myanmar Border.*

Summary of results

This study demonstrated for the first time the relevance of using a specific *Anopheles* salivary biomarker to measure the risk of human exposure to *Anopheles* bites in malaria hotspots along the TMB. First, a sequencing study showed high identities of *An. gambiae* gSG6-P1 sequences with the dominant malaria vector species *An. minimus s.l.* , *An. aconitus* (87%), and *An. maculatus s.l.* (83%), hence confirming that the gSG6-P1 antigen is highly conserved among malaria vectors worldwide (Calvo et al. 2009). We couldn't demonstrate however whether secondary vectors and non-vectors can efficiently induce an antibody response, considering the unsuccessful alignment of SG6-P1 peptide sequences for those *Anopheles* species.

In a second time, we demonstrated a high gSG6-P1 seroprevalence (approximately 70%) among the populations along the TMB, which is consistent with previous findings in West Africa (Rizzo et al. 2014) and the Americas (Londono-Renteria et al. 2015a). IgG antibody level against gSG6-P1 varied according to villages and surveys. Multivariate analyses showed a highly significant and positive "dose-response" relationship between the intensity of antibody responses to gSG6-P1 and the degree of exposure to *Anopheles* bites (both using the HBR of total *Anopheles* and primary malaria vectors, $P<0.001$). This findings are consitent with the fact that *An. minimus* and *An. maculatus* were the two dominant species in the study villages. Our results showed that a significant relationship between age ($P <0.001$), season ($P <0.001$), and village ($P <0.001$) and the antibody response to *Anopheles* salivary peptide. Human behaviors and agricultural practices are suspected to modulate the human-vector contact in the area. Moreover, our findings highlighted a strong association between the gSG6-P1 antibody response and the entomological innocalution rates (EIR), indicating that heterogeneity in malaria transmission was directly associated with heterogenity in biting behavior among villages.

Finally, spatial clusters of individuals with high immune responses to vector bites were identified in all villages that correlated well with vector abundance and transmission risk. Our results showed that locations of hotspots varied according to season and tended to be more dispersed during the rainy season and tightly clustered in small pockets during the dry season. This was consistent with malaria epidemiology along the TMB, where spatial clustering in malaria infections were also observed during the dry season (Parker et al.

2015b). This finding indicated that the salivary biomarker is promising to measure small-scale variation in malaria vector bites in an area of low transmission intensity.

In conclusion, the gSG6-P1 serologic biomarker is capable of providing good estimates of malaria transmission risk along the TMB and has great potential for malaria epidemiology studies. The possible applications of this biomarker in evaluating the effectiveness of protection measures against mosquito bites (i.e. LLIN, repellent) in this region were discussed.

The Journal of Infectious Diseases

MAJOR ARTICLE

Use of an *Anopheles* Salivary Biomarker to Assess Malaria Transmission Risk Along the Thailand-Myanmar Border

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Background. The modalities of malaria transmission along the Thailand-Myanmar border are poorly understood. Here we address the relevance of using a specific *Anopheles* salivary biomarker to measure the risk among humans of exposure to *Anopheles* bites.

Methods. Serologic surveys were conducted from May 2013 to December 2014 in 4 sentinel villages. More than 9400 blood specimens were collected in filter papers from all inhabitants at baseline and then every 3 months thereafter, for up to 18 months, for analysis by enzyme-linked immunosorbent assay. The relationship between the intensity of the human antibody response and entomological indicators of transmission (human biting rates and entomological inoculation rates [EIRs]) was studied using a multivariate 3-level mixed model analysis. Heat maps for human immunoglobulin G (IgG) responses for each village and survey time point were created using QGIS 2.4.

Results. The levels of IgG response among participants varied significantly according to village, season, and age ($P<.001$) and were positively associated with the abundance of total *Anopheles* species and primary malaria vectors and the EIR ($P<.001$). Spatial clusters of high-IgG responders were identified across space and time within study villages.

Conclusions. The gSG6-P1 biomarker has great potential to address the risk of transmission along the Thailand-Myanmar border and represents a promising tool to guide malaria interventions.

Keywords. Thailand-Myanmar border; malaria vectors; transmission; human antibody response; Salivary Biomarker; gSG6-P1.

In Thailand, malaria displays geographical heterogeneity and is exemplified by the so-called border malaria type, with most of the malaria cases concentrated along the borders with Myanmar [1]. Malaria transmission along the Thailand-Myanmar border is high because of extensive population movement across the border, especially mobile and forest workers, who make a substantial contribution to the regional malaria burden [2]. The forest area along the border presents very efficient vectors species, including *Anopheles minimus sensu lato, Anopheles maculatus sensu lato*, and *Anopheles dirus sensu lato* [3, 4]. The vectorial capacity and relative importance of these vector species in malaria transmission are, however, poorly understood, hence representing a threat to the success of malaria control and elimination in the region [2].

The emergence of artemisinin-resistant *Plasmodium falciparum* is a threat to malaria control. Given the paucity of new antimalarials, the only viable option is elimination of the parasite. Eliminating malaria requires accurate tools for monitoring local malaria transmission intensity [5]. The gold standard for estimating malaria transmission is the entomological inoculation rate (EIR), which is defined by the number of infected bites received per human per unit of time [6]. The EIR is estimated by human-landing collection events that are strongly dependent on the density of human-biting mosquitoes in a given time [5]. However, the density of vectors has been shown to greatly vary according to collection site and season and seems to be insensitive within small geographical areas [7–9]. Moreover, mosquito collections are time-consuming, costly, difficult to sustain for the long term, and pose ethical challenges in areas of endemicity for vector-borne diseases [10]. In settings of low malaria transmission, where people received generally <1 infected bite per person per year [11], the EIR may lack sensitivity because the number of *Plasmodium*-positive samples is inadequate to estimate of the sporozoite index [12–14]. Effectively using limited resources for malaria elimination and evaluating interventions require new measurements of the risk of being infected with *Plasmodium* at both population and individual levels [15, 16].

Recently, alternative serological methods for monitoring human-vector contact by measuring the intensity of antibody response to mosquito bites have been developed [17]. Positive

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correlation between the human exposure level to *Anopheles* bites and human anti–mosquito saliva antibody level has been extensively reviewed [18, 19]. The gSG6-P1 peptide, based on the *Anopheles gambiae* SG6 protein sequence, has been validated as a specific biomarker of *Anopheles* exposure in various settings, including Africa and the Americas [20–22]. Several studies in Africa showed that human antibody response to gSG6-P1 salivary peptide is a quantitative and specific biomarker to measure recent exposure of individuals to *Anopheles* bites [23–26], even in a context of a low level of exposure to malaria vector bites [20, 27], as well as to evaluate the human risk of malaria transmission [28–31]. The gSG6 protein and especially gSG6-P1 peptide showed to be well conserved among major *Anopheles* species [32] hence representing a promising tool for estimating the risk of malaria transmission in Southeast Asia.

This study represents the first attempt to validate the gSG6-P1 peptide as an epidemiological tool for evaluating the direct exposure of human populations to *Anopheles* species in malaria hot spots along the Thailand-Myanmar border. Here we investigated the relationships between the anti–gSG6-P1 antibody response and entomological indicators of transmission—the human biting rate (HBR) and the EIR—through a cohort of approximately 2600 participants followed up every 3 months for 18 months. This study demonstrates that the *Anopheles* gSG6-P1 salivary biomarker has great potential to quantify human exposure to malaria vectors and to estimate the risk of malaria transmission along the Thailand-Myanmar border.

METHODS

Study Site

The study was conducted in 4 sentinel Myanmar villages located within 10 km of the Thailand border that are considered representative of the area in terms of environment, ecology, population, and behavior. Villages were Htoo Pyin Nyar (TPN; 17°14′N, 98°29′E), Tar Au Ta (TOT; 16°36′N, 98°57′E), Ka Nu Hta (KNH; 17°18′N, 98°24′E), and Htee Kaw Taw (HKT; 16°85′N, 98°47′E). These villages were selected because they showed the highest prevalence of *P. falciparum* (2%–12%) and *Plasmodium vivax* (7%–24%) submicroscopic infections in the area [33].

Study Design, Populations, and Sampling Methods

Seven serologic surveys were performed every 3 months from May 2013 to December 2014. In each village, a committee composed of village leaders, village malaria workers, and volunteers was formed to assist the Shoklo Malaria Research Unit (SMRU) staff in organizing the surveys and in engaging and mobilizing the community [33]. Informed consent was obtained directly from participating adults, and parental consent was obtained on behalf of participating children aged <16 years. Brief history of travels, professional activity, and insecticide-impregnated bed net use was also obtained. At each survey, blood specimens from inhabitants were collected on Whatman filter papers, using the dried blood spot technique, and properly labeled for analysis by enzyme-linked immunosorbent assay (ELISA).

In each village, mosquitoes were collected monthly, using the human-landing collection technique, to determine the vector abundance and composition [14]. Briefly, mosquitoes were collected in the same 5 catching sites (indoor and outdoor) from 6:00 PM to 6:00 AM for 5 consecutive nights per month. Mosquitoes landing on humans, at the time of collection, were caught individually by glass tubes and brought back to the laboratory for morphological identification [34] and assessment of sporozoite rates, using a real-time polymerase chain reaction assay [35]. *Anopheles minimus s.l.*, *An. maculatus s.l.*, and *An. dirus s.l.* were considered primary vectors [4], whereas secondary vectors were *Anopheles aconitus sensu lato*, *Anopheles barbirostris sensu lato*, and *Anopheles annularis sensu lato* [36]. All houses and mosquito collection sites were georeferenced using Garmin etrex 20 global positioning system units. Temperature and relative hygrometry were recorded daily, using captors located in a central house of the village.

Sequence Alignment of gSG6-P1 for Southeast Asian *Anopheles* Species

Ten samples of each *Anopheles* species collected were sequenced for clustal alignment of SG6-P1 salivary peptides. Alignments were done with ClustalW, which enabled comparison of the sequence of gSG6 peptide from local *Anopheles* species to that of the reference African (*An. gambiae*) vector [23]. The gSG6-P1–specific *Anopheles* peptide was synthesized and purified (95%) by Genepep (St-Clément de Riviere, France).

Measurement of Human Antibody Levels to *Anopheles* Saliva Antigens

Serologic testing of human exposure to gSG6-P1 saliva peptide was carried out by ELISA as described in [25] but with some modifications (Supplementary Materials). The intensity of the immunoglobulin G (IgG) response was measured at the individual level and was expressed as the ΔOD, calculated as OD*x* – OD*n*, where OD*x* and OD*n* represent the mean of the individual ODs in 2 antigen wells and the OD in 1 blank well containing no gSG6-P1 antigen, respectively. As a negative control, the specific anti–gSG6-P1 IgG response was also assayed in 16 non–*Anopheles*-exposed individuals from France and a Thai citizen who were living in Bangkok for >2 months, to quantify the nonspecific background antibody level and to calculate the cutoff (calculated as the mean ΔOD + 3 SDs). Based on our findings, a participant was classified as an immune responder if their ΔOD was >0.450.

Statistical Analysis

Covariates

Individual-level covariates included age group (sorted in 4 classes: <5 years, 5–15 years, 16–59 years, and ≥60 years) and sex. Household-level covariates included long-lasting insecticide-treated bed net (LLIN) use, based on a questionnaire conducted at the baseline visit (whether the participants had and

used a LLIN "every night," "some nights," or "never"). At the village level, the population size at each survey, temperature, and relative humidity (2 time-dependent variables defined as the estimated mean and maximum humidity during the 2 weeks preceding mosquito collection events) were recorded. The mean HBR and EIR were estimated at each catching site 1 month before blood sample collection. Seasons were grouped as the hot season (mid February–mid May), the rainy season (mid May–mid October), and the cool season (mid October–mid February) according to the Thai Meteorological Department [37].

Statistical Approach

The relationship between the intensity of the human antibody response (ΔOD) and entomological indicators of transmission (HBR and EIR) was studied using a multivariate 3-level (house, individual, and measurement) mixed model analysis. We considered (1) the HBR (or EIR) of total *Anopheles* mosquitoes, (2) the HBR (or EIR) of the primary vectors, and (3) the HBR (or EIR) of the secondary vectors, in 6 separated analyses. The potential adjustment factors were all of the covariates described above. In each analysis, the HBR variable was categorized in 4 classes (according to the quartiles) to avoid the assumption of a linear relationship between the HBR and the human antibody response. For the EIR model, data were categorized as a binary variable (0 and >0) because of the high number of data collected from uninfected mosquitoes. In all models, a univariate analysis was first performed, where we estimated the relationship between each adjustment factor and the antibody response through a univariate mixed model. In a second step, we entered in a multivariate mixed model all of the adjustment factors with a *P* value of <.2 from the univariate analysis and then removed sequentially all the adjustment factors with a *P* value of >.05 (backward selection). Statistical analyses were done with Stata, version 13.0 (StataCorp, College Station, TX). Graphs were constructed using GraphPad Prism 5 software (San Diego, CA).

Spatial Analysis

Heat map raster layers were created for IgG responses among individuals within each village and survey time point, using QGIS 2.4 (available at: http://www.qgis.org/). The raster layers give a smoothed representation of IgG intensity within study villages (Supplementary Materials). Mean HBRs for malaria vectors and EIR positives (meaning >0) were also plotted in the maps to indicate catch site location, vector abundance, and malaria transmission foci.

Spatial autocorrelation (clustering) of IgG values was calculated for each village and time point, using 2 approaches: the Moran *I* statistic (a global clustering method) and local indicators of spatial autocorrelation (LISAs) [38]. The Moran *I* tests give a single test statistic and associated *P* value for each village/month combination, while the LISAs give a test statistic and *P* value for each individual/month combination. We used a Benjamini-Hochberg correction to account for multiple testing. All results were mapped using ArcMap 10.2 (available at: http://www.esri.com/).

Ethical Statement

The Ethics Review Committee for Research Involving Human Research Subjects Health Science Group, Chulalongkorn University, Thailand, approved the study (096.1/56; 16 October 2014). The protocols for blood sample collection and the dried spot technique have been approved by the Oxford Tropical Research Ethics Committee (1015-13; 29 April 2013).

RESULTS

Characteristic of the Study Populations and Immunological Outcomes

Table 1 describes the population characteristics during the period of the study. Participants consisted in 2602 people followed up every 3 months over 18 months. Participants from the 4 study villages were comparable in age, and the sex ratio varied from 0.46 (in KNH) to 0.52 (in TOT). A total of 1906, 1970, 2046, and 3503 blood specimens were collected using the dried spot technique and analyzed at TPN, TOT, KNH, and HKT, respectively. The proportion of participants with an immune response to *Anopheles* salivary antigen ranged from 59% at TPN to 86% at HKT (Supplementary Materials).

Entomology Outcomes

A total of 58 833 *Anopheles* mosquitoes were collected on human volunteers over 18 months. The overall abundance of *Anopheles*

Table 1. Descriptive Statistics of Participants, by Study Sites

Characteristics	Htoo Pyin Nyar (n = 452)	Tar Au Ta (n = 659)	Ka Nu Hta (n = 459)	Htee Kaw Taw (n = 1032)
Age, y, median (range)	21 (0–66)	19 (0–80)	22 (0–73)	19 (0–94)
Female sex, %	47	52	46	50
Antibody prevalence, visits, % (proportion)				
All ages	59.3 (1131/1906)	68.8 (1356/1970)	61.4 (1256/2046)	86.3 (3024/3503)
Ages 0–4 y	57.4 (139/242)	57.6 (175/304)	52.5 (117/223)	82.4 (375/455)
Ages 5–15 y	59.9 (332/554)	68.2 (433/635)	56.8 (303/533)	86.6 (1101/1272)
Ages 16–59 y	59.2 (629/1062)	72.6 (682/939)	64.5 (786/1218)	87.2 (1467/1683)
Ages >60 y	64.6 (31/48)	71.7 (66/92)	69.4 (50/72)	87.1 (81/93)

species was higher in TOT (n = 27 903) as compared to other villages, where it ranged from 7228 in TPN to 10 786 in HKT (Figure 1A). Twelve *Anopheles* species were identified, including *An. minimus s.l., An. maculatus s.l., An. aconitus s.l., An. dirus s.l. An. annularis s.l.*, *An. barbirostris s.l.*, *Anopheles hyrcanus sensu lato*, *Anopheles jamesi*, *Anopheles kochi*, *Anopheles subpictus*, *Anopheles culicifacies* species B, and *Anopheles tessellatus* (Figure 1B). The malaria vectors *An. minimus s.l.* and *An. maculatus s.l.* were by far the 2 dominant species, representing >70% of the total *Anopheles* collected. A total of 123 *Plasmodium*-positive *Anopheles* mosquitoes (sporozoite index, 0.23%; n = 47 914) were identified through 18 surveys, including 104 *An. minimus s.l.* (n = 35 177), 12 *An. maculatus s.l.* (n = 7251), 5 *An. dirus s.l.* (n = 1071), and 2 *An. barbirostris s.l.* (n = 4415).

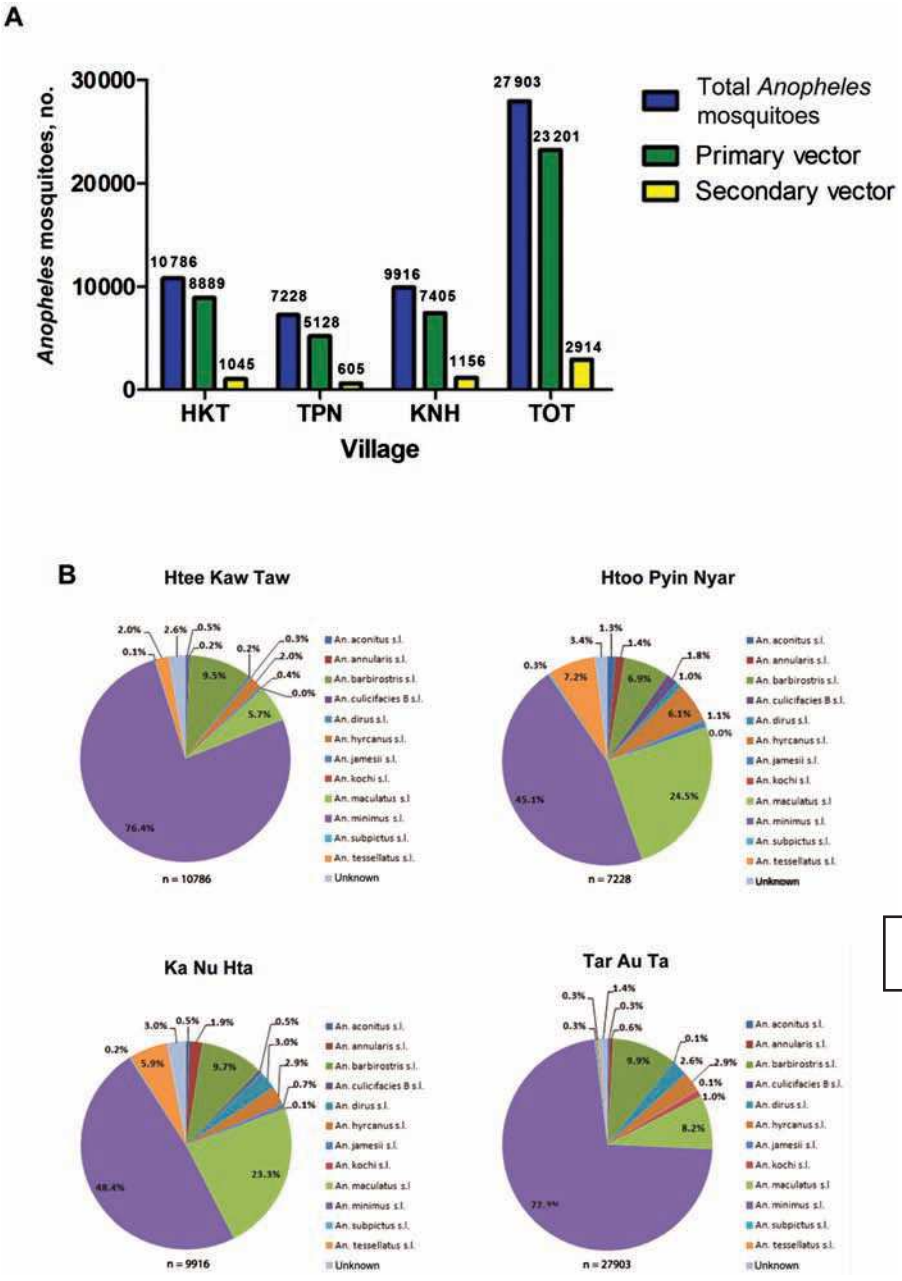

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Figure 1. Abundance and diversity of *Anopheles* mosquitoes, according to village. *A*, Total *Anopheles* represents the numbers of *Anopheles* mosquitoes collected in each village, based on monthly collections over 18 months. *B*, *Anopheles* composition in the study area. *Anopheles minimus sensu lato*, *Anopheles maculatus sensu lato*, and *Anopheles dirus sensu lato* were considered as primary vectors [4], whereas secondary vectors were *Anopheles aconitus sensu lato*, *Anopheles barbirostris sensu lato*, and *Anopheles annularis sensu lato* [36]. Five sites were used for human landing collections (HLCs) to cover all subareas of the village. Human catch sites were separated by a minimum 50 m from each other to avoid potential bias in attracting mosquitoes. Five teams of 2 volunteers were rotated between catching sites for 5 successive nights (equivalent to 50 human-nights of collection) to mitigate potential collector bias. HLCs lasted for 45 minutes per hour, followed by a 15-minute break for collectors.

Sequence Alignment of gSG6-P1 for Local *Anopheles* Species

The homology of gSG6-P1 peptide sequence with that of *An. gambiae* was high for *An. minimus, An. aconitus*, and *An. maculatus* (Figure 2). A lower score was found for *An. dirus*. The peptide sequence for these malaria vector species were antigenic as determined by computerized predictions of antigenicity based on physicochemical properties of the amino acid sequences by different programs (BCEPred, ABCPred, and BepiPred). Sequencing of gSG6-P1 peptide for all other *Anopheles* species was unsuccessful.

Human Antibody Response to the gSG6-P1 to Quantify *Anopheles* Exposure and Estimate Malaria Transmission Risk

Multivariate analyses were performed on 2602 participants and the mean number of visits per individual was 3.8 (range, 1–7 visits). Multivariate analyses showed a highly significant and positive dose-response relationship between the intensity of antibody responses to gSG6-P1 and the HBR of both the total *Anopheles* population recovered (P <.001) and the primary malaria vectors (P<.001; Table 2). Post hoc analyses showed that adjusted mean IgG response intensities were significantly different between all HBR classes (Supplementary Materials). Interestingly, we also found a significant and positive relationship between the intensity of antibody responses and the EIR for total *Anopheles* (P <.001) and primary vectors (P <.001; Table 3). The HBR and EIR models were, however, not significant for secondary vectors, probably because of the limited sample size (P >.05; data not shown).

Demographic, Social, and Environmental Factors Associated with Human Vector Contact

For all models, after the univariate analysis, all covariates (except the sex) were selected for the multivariate analyses. Spatial (villages) and temporal (surveys) heterogeneity in IgG intensity was apparent across and within the study villages (Figure 3). The multivariate analysis showed that the IgG response to malaria vector bites differed according to village (P<.001); the mean antibody response was higher at KNH than at other villages when adjusted for HBR (both for malaria vectors and total *Anopheles*) and other covariates (Table 2). A higher intensity of the antibody response was recorded during the rainy season, compared with the cool season (P <.001) and the hot season (P<.001). A positive monotonic relationship between the age and the intensity of antibody response was noted (P <.001). A positive relationship was found between the population size and the intensity of the antibody response (P <.001). Conversely, bed net use was not significant in any multivariate models.

Spatial Clustering of Human Antibody Response To Malaria Vector Bites Within Villages

Heat maps of the IgG antibody response to gSG6-P1 indicated variation in the spatial distribution of the IgG antibody across space and time within villages and surveys. In all villages, areas of mid-to-high IgG intensity were detected in almost every survey in the same place. For example, in TPN the highest IgG intensity during each month occurred in a patch in the northern part of the village and was evident in both rainy and dry season (Figure 4A and 4B). Conversely, HKT had high-intensity patches in each survey month. Spatial clustering of high-IgG responders occurred within all 4 villages but varied over time (Supplementary Materials). Furthermore, LISAs indicated statistically significant clusters of individuals and houses with high antibody values near other high antibody values (green squares) during most surveys. High IgG intensity appeared more dispersed in the villages during the rainy season (Figure 4B) and patchier during the dry season (Figure 4A). This was well illustrated in TOT, where houses with residents in whom a high IgG intensity was detected occurred in a single location in the eastern portion of the village during the dry and hot seasons but were dispersed throughout much of the village during the rainy season.

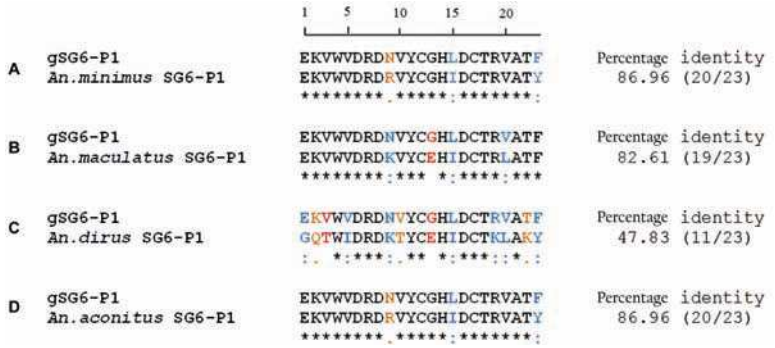

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Figure 2. Clustal alignment and sequence identity of the gSG6-P1 salivary peptide for *Anopheles minimus, Anopheles maculatus, Anopheles dirus*, and *Anopheles aconitus*. The amino acid sequence of the gSG6-P1 peptide of *Anopheles gambiae* (gi:13537666) is presented as reference. Sequence identities are marked with an asterisk, strong amino acid conservations are marked with a colon, and weak amino acid conservations are marked with a period. Sequence alignment showed 87% identity (20 of 23 amino acids) for *An. minimus* and *An. aconitus*, 83% identity (19 of 23 amino acids) for *An. maculates*, and 48% identity (11 of 23 amino acids) for *An. dirus*.

Table 2. Multivariate Linear Mixed Model Showing the Relationship Between the Intensity of Antibody Responses to gSG6-P1 and the Human Biting Rate (HBR) and Other Covariates

Characteristic	Intensity for All *Anopheles*		Intensity for Primary Vectors	
	Mean Difference[a]	*P*	Mean Difference[a]	*P*
HBR class		<.001[c]		<.001[c]
Low	Reference		Reference	
Medium	0.06	<.001	0.06	<.001
High	0.10	<.001	0.09	<.001
Very high	0.19	<.001	0.18	<.001
Age, y		<.001[c]		<.001[c]
<5	Reference		Reference	
5–15	0.09	<.001	0.09	<.001
15–59	0.13	<.001	0.13	<.001
≥59	0.16	<.001	0.16	<.001
Village		<.001[c]		<.001[c]
Htee Kaw Taw	Reference		Reference	
Htoo Pyin Nyar	0.05		0.04	
Ka Nu Hta	0.15	<.001	0.15	<.001
Tar Au Ta	−0.08	<.001	−0.09	<.001
Season		<.001[c]		<.001[c]
Cool	Reference		Reference	
Hot	0.06	<.001	0.08	<.001
Rainy	0.08	<.001	0.05	<.001

Analyses were adjusted for temperature and humidity variables, in addition to the specified variables.

[a]Defined as the difference between each class and the reference class.

[b]HBR classes for total *Anopheles* were <96 for low HBR, 96–204 for medium HBR, 204–531 for high HBR, and ≥531 for very high HBR. HBR classes for primary malaria vectors were <46.5 for low HBR, 46.5–159 for medium HBR, 159–468 for high HBR, and ≥468 for very high HBR.

[c]By the likelihood ratio test, for analysis of the global effect of the variable.

Table 3. Multivariate Linear Mixed Model Showing the Relationship Between the Intensity of Antibody Responses to gSG6-P1 and Entomological Inoculation Rates (EIRs)

Characteristics	Intensity for All *Anopheles*		Intensity for Primary Vectors	
	Mean Difference	*P*	Mean Difference	*P*
EIR[a]		<.001[b]		<.001[b]
0	Reference		Reference	
>0	0.14	<.001	0.15	<.001

Analyses were adjusted for temperature, humidity, age, season, and village.

[a]By the likelihood ratio test, for analysis of the global effect of the variable.

[b]A value of 0 indicates no transmission, and a value of >0 indicates transmission.

DISCUSSION

In this study, we demonstrated the usefulness of an innovative serological marker for quantifying human-vector contact and estimating malaria transmission risk in areas exhibiting a high prevalence of subclinical malaria infections [33]. The serological evaluation of the antibody response to mosquito saliva and its association with the exposure to malaria vectors has received increasing attention because of the limitations of current techniques in estimating malaria transmission [39]. The relevance of the gSG6-P1 biomarker for malaria epidemiologic studies has been validated in various settings worldwide [19, 40], with the exception of Southeast Asia. Here, we first demonstrated high identities of *An. gambiae* gSG6-P1 sequences with the dominant malaria vector species *An. minimus s.l.*, *An. aconitus* (87%), and *An. maculatus s.l.* (83%), hence confirming that the gSG6-P1 antigen is highly conserved among malaria vectors worldwide [32]. The lower match observed with *An. dirus* (48%) does not indicates an absence of antibody response to this species, because *An. dirus* salivary proteins were detected in patient with malaria in Thailand, where *An. dirus s.l.* was the main vector [41]. We were unable, however, to demonstrate whether secondary vectors and nonvectors can efficiently induce an antibody response, considering the unsuccessful alignment of SG6-P1 peptide sequences for those *Anopheles* species.

Our study also revealed a high gSG6-P1 seroprevalence (approximately 70%) among the populations, which is consistent with previous findings in West Africa [17] and the Americas [21]. Our study first demonstrated a dose-response relationship between the intensity of antibody responses to gSG6-P1 and the degree of exposure to *Anopheles* bites. The fact that the 2 HBR models (ie, primary vector versus total *Anopheles*) showed a similar trend is consistent with the fact that *An. minimus* and *An. maculatus* are the 2 dominant species in the study villages. Strikingly, our findings highlighted a strong association between the gSG6-P1 antibody response and the EIR, indicating that heterogeneity in malaria transmission is associated with heterogeneous biting behavior. The salivary biomarker looks promising for identifying malaria hot spots and measuring small-scale variation in malaria exposure rates in an area of low transmission intensity.

Our findings showed that the antibody response to *Anopheles* salivary peptide varies according to age, season, and village. The intensity of the response was higher during the rainy season than during the cool and hot seasons, when adjusted for other covariates. This indicates that seasonal changes in biting patterns can reflect similar changes in antibody responses. Similarly, age was positively correlated with the intensity of antibody responses in all villages. The increase in the IgG response with age is generally consistent with the gradual acquisition of immunity against *Anopheles* mosquito saliva [30] following the development of individual factors and behaviors that increase the probability of human-vector contact. Human behaviors and agricultural practices are expected to modulate the human-vector contact in the study area. The population is essentially made up of local and temporary farmers working in rice paddies and cornfields around the villages during the rainy season, when vector density is the highest. During harvest time, men and women will, quite frequently, spend nights in the

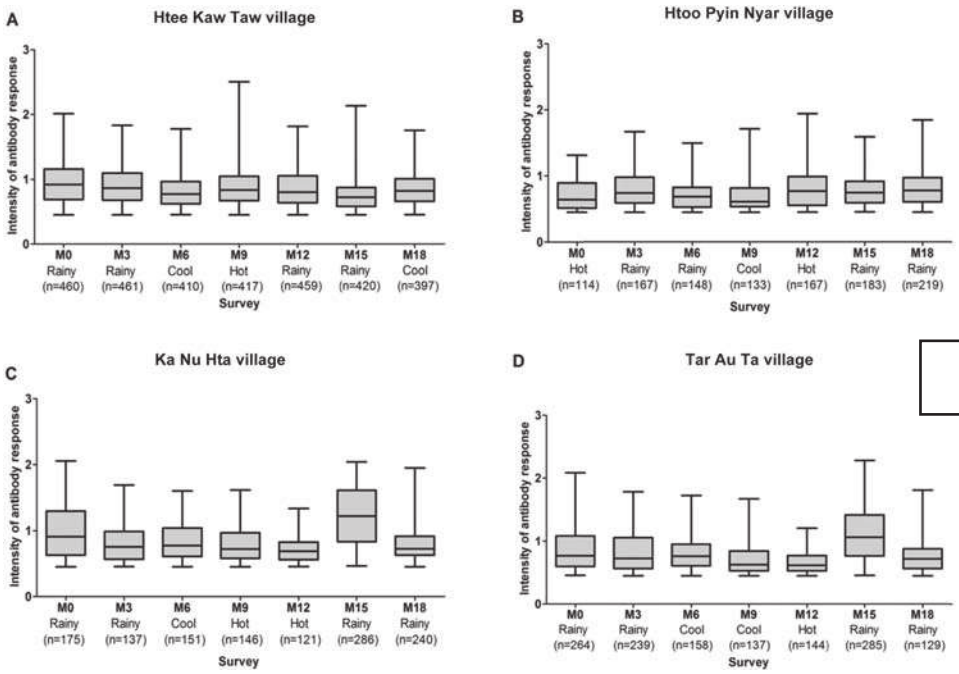

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Figure 3. Changes in immunoglobulin (IgG) response intensity to gSG6-P1 peptide, according to surveys and village. Boxes display the median ΔOD for IgG responders (ΔOD >0.450) at each survey (at month 0 [M0], M3, M6, M9, M12, M15, and M18) with 25th and 75th percentiles. The whiskers show the 5th/95th percentiles.

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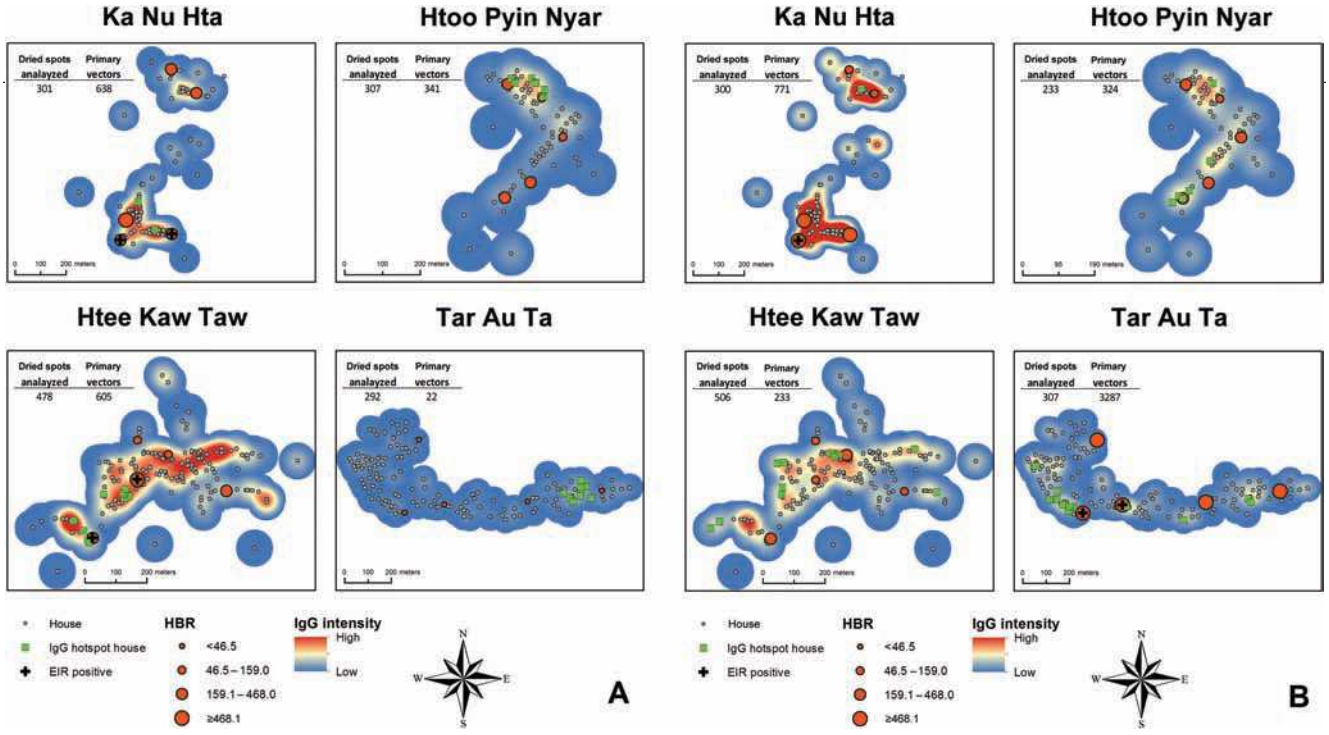

Figure 4. Heat maps of human immunoglobulin G (IgG) responses to mosquito saliva for each village in dry (*A*) and rainy (*B*) seasons (month 15 for the rainy season and month 9 for the dry season). The smoothed maps indicate relative intensities of IgG values, with dark blue denoting low intensity, yellow denoting medium intensity, and dark red denoting high. Houses are represented by gray circles, and clusters of neighbors with higher than expected IgG values (from local indicators of spatial autocorrelation statistics) are indicated by bright green squares. The human biting rates (HBRs) for each survey time are indicated by dark orange graduated cylinders, whereas foci of malaria transmission (positive entomological inoculation rates [EIRs]) are indicated by a black cross.

farms and may be particularly exposed to malaria vector bites. This behavior probably explains the absence of a sex effect on the intensity of human antibody responses to *Anopheles* bites. Regarding village, participants from KNH exhibited a higher specific IgG response than those from other sites, when analysis adjusted for HBR and other covariates. The reason for a higher vector exposure in this population is unknown, but we assume that this may reflect different human behavior, agricultural and vector control practices, population movement, and/or immunogenicity characteristics. More information on vector ecology, demographic characteristics, and socioeconomic structure in the study villages are needed to better understand the factors associated with human-vector contact and malaria transmission [42].

Interestingly, bed net use was not significant in univariate analysis, despite the fact that 79% of people declared sleeping under bed nets every night. Although this result has to be taken with caution, considering potential biases in measuring LLIN use, we suspect that insecticide-treated bed nets might offer limited personal protection against mosquito bites. In the study area, malaria vectors exhibit strong behavioral plasticity [43] and are known to feed preferentially outdoors and in the early evening, when people are not protected by bed nets [44]. The salivary biomarker may then be particularly relevant for national malaria control programs willing to evaluate the efficacy of new malaria vector control tools, such as insecticide-treated materials and repellents Finally, we identified spatial clusters of individuals with high immune responses to vector bites in all villages that correlated well with vector abundance and transmission risk. Our results showed that locations of hot spots varied according to season and tended to be more dispersed during the rainy season and tightly clustered in small pockets during the dry season. This is consistent with malaria epidemiology along the Thailand-Myanmar border, where spatial clustering of *P. vivax* infections was also observed during the dry season [45]. Clustering of anti-gSG6 IgG responders is less obvious in the rainy season, most probably because vectors tend to be dispersed throughout the village, owing to multiplication of larval breeding habitats. Interestingly, TOT seems to differ from other villages (especially in the dry season), because several hot spots of immune responders occurred without clear indication of high IgG responders and vector abundance. In this case, we suspect that those people may have been extensively exposed to *Anopheles* bites outside the village.

In conclusion, our results showed that the gSG6-P1 serologic biomarker is capable of providing accurate estimates of the malaria transmission risk along the Thailand-Myanmar border and has great potential for malaria epidemiologic studies. Timely identification of population subsets at high risk of exposure to malaria vectors could help national malaria control programs implement hot spot–targeted interventions with the aim to eliminate potential transmission sources and achieve malaria elimination.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases online*. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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V. C., T. C., and F. N. designed the study. P. Y. and D. C. performed all ELISAs and jointly conduct data analysis. F. R., A. P., and C. B. conducted sequence alignment and provided useful comments on the manuscript. G. C. and P. Y. worked on the statistical analysis. D. P. performed spatial mapping and the GIS study. P. Y., D. C., and V. C. managed the data. All authors contributed to the writing of versions of the manuscript. All authors read and approved the final manuscript.

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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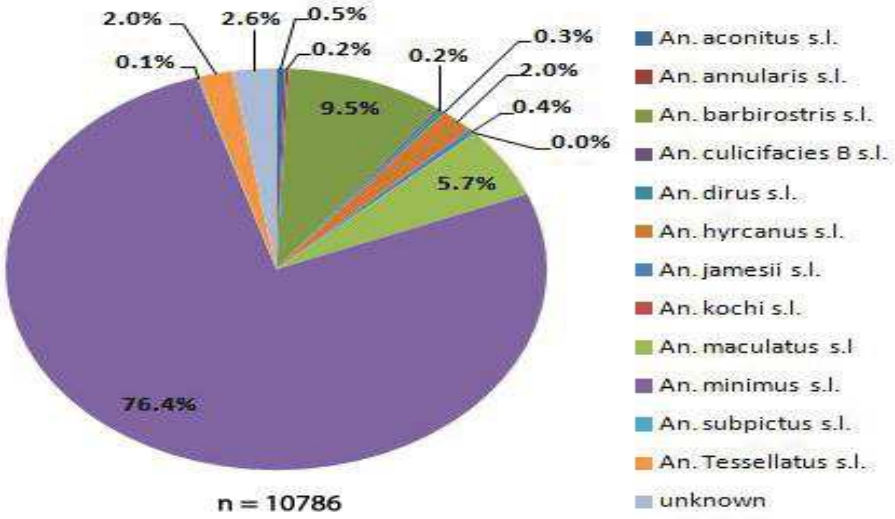
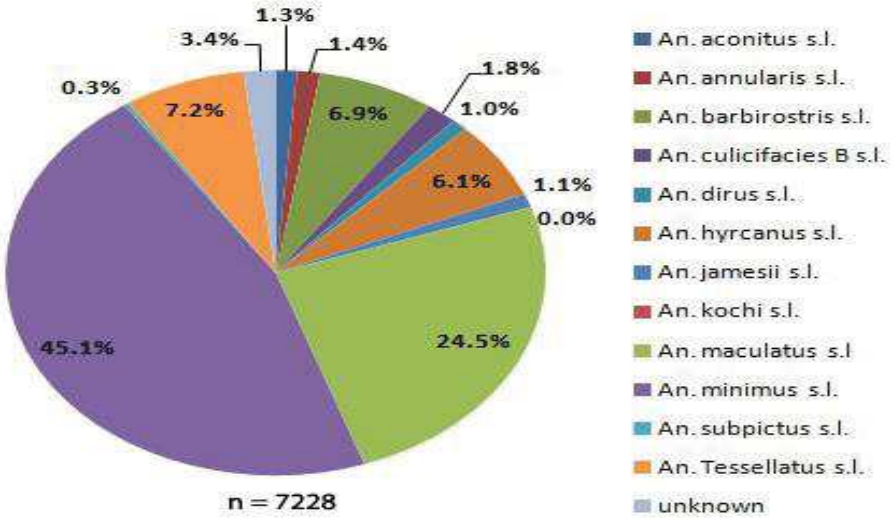
Figure 1B

B

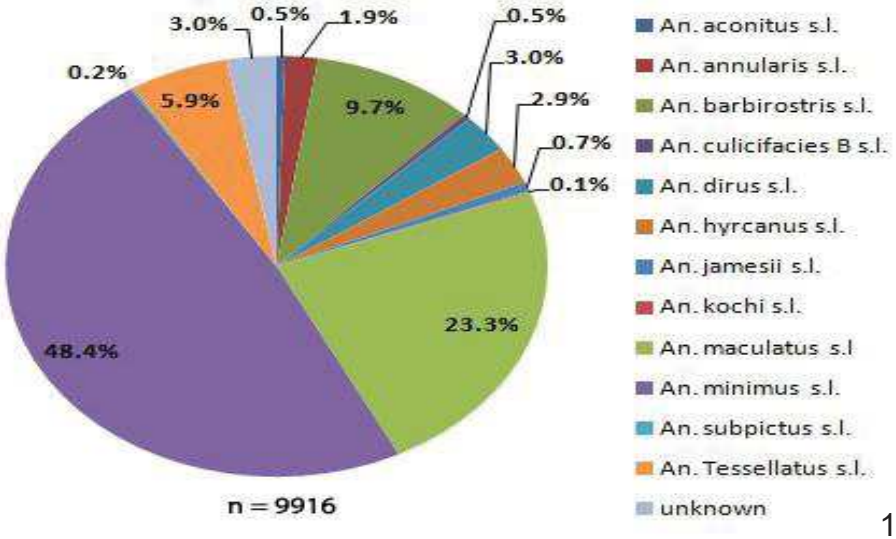
Htee Kaw Taw



Htoo Pyin Nyar



Ka Nu Hta



Tar Au ta

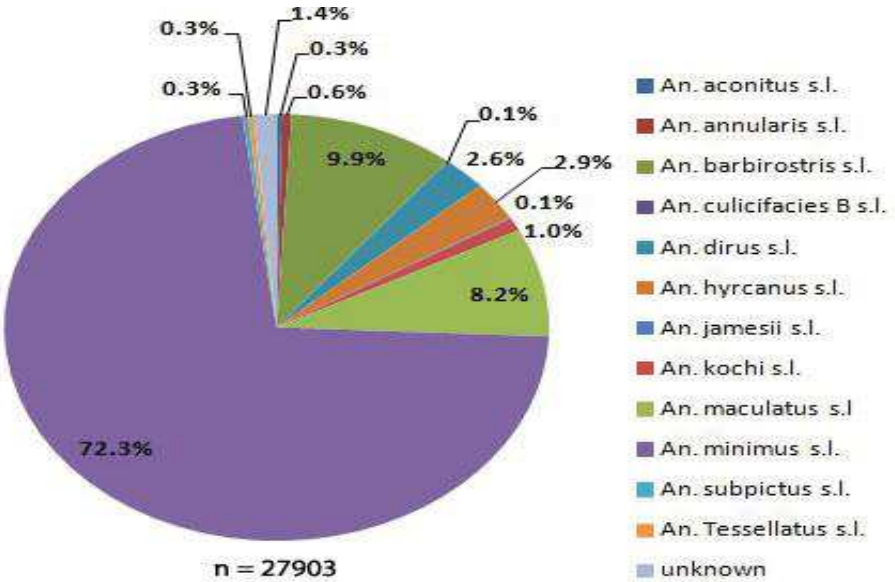


Figure 2

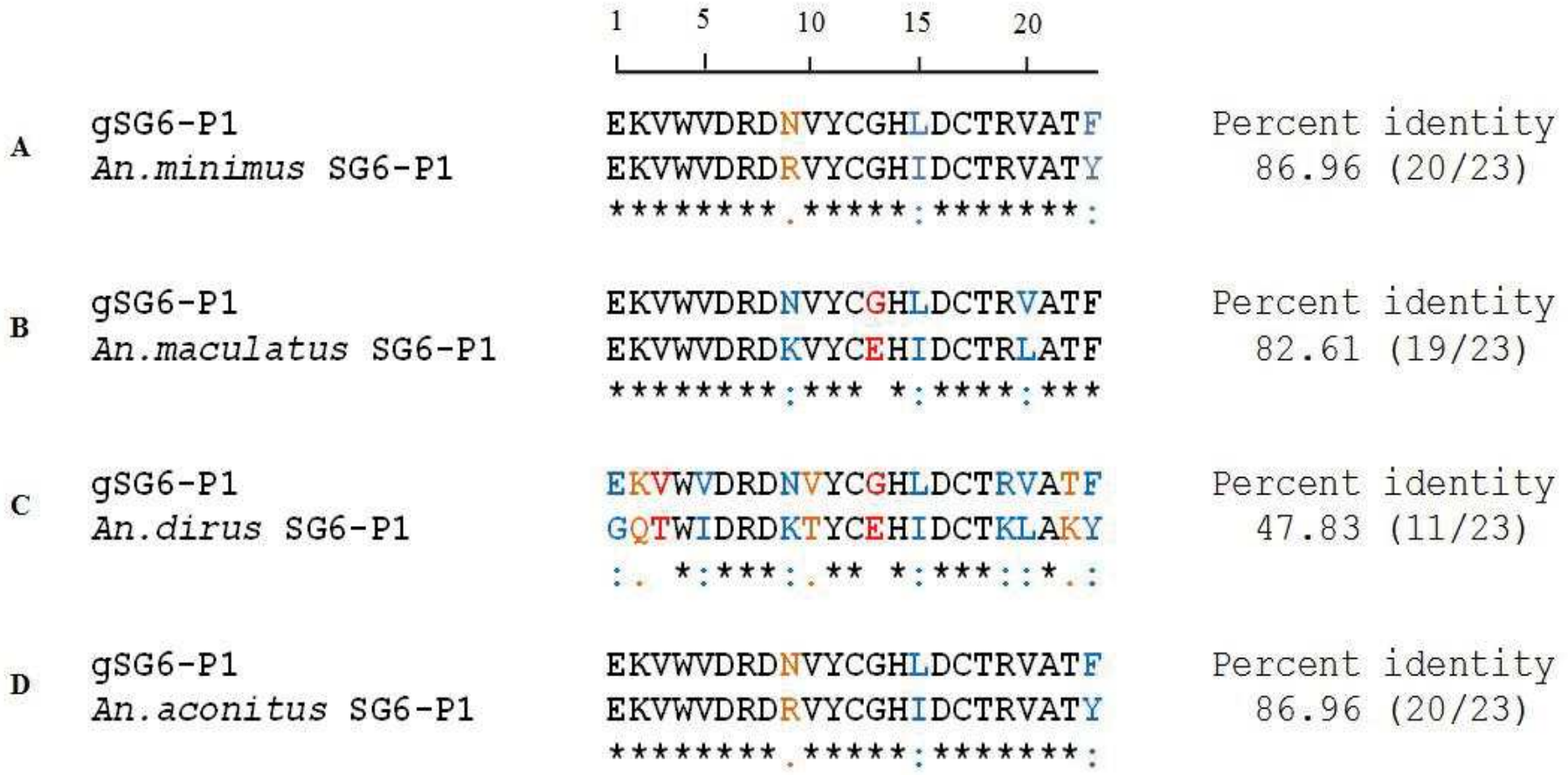

1 5 10 15 20
A
gSG6-P1
An.minimus SG6-P1
EKVWVDRDNVYCGHLDCTRVATF
EKVWVDRDRVYCGHIDCTRVATY
Percent identity
86.96 (20/23)
B
gSG6-P1
An.maculatus SG6-P1
EKVWVDRDNVYCGHLDCTRVATF
EKVWVDRDKVYCEHIDCTRLATF
Percent identity
82.61 (19/23)
C
gSG6-P1
An.dirus SG6-P1
EKVWVDRDNVYCGHLDCTRVATF
GQTWIDRDKTYCEHIDCTKLAKY
Percent identity
47.83 (11/23)
D
gSG6-P1
An.aconitus SG6-P1
EKVWVDRDNVYCGHLDCTRVATF
EKVWVDRDRVYCGHIDCTRVATY
Percent identity
86.96 (20/23)

Figure 3

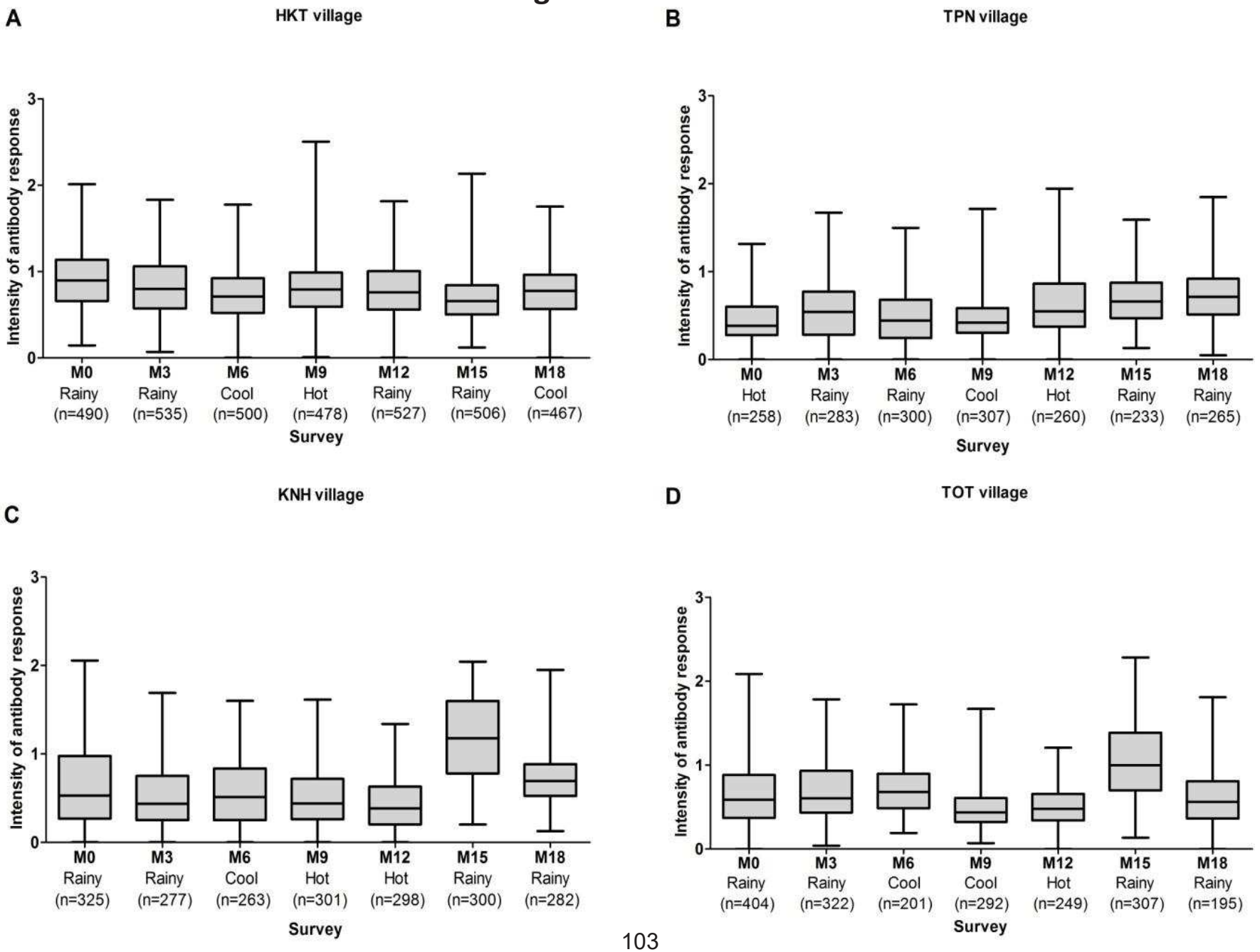

A
HKT village
Intensity of antibody response
0
1
2
3
M0 Rainy (n=490)
M3 Rainy (n=535)
M6 Cool (n=500)
M9 Hot (n=478)
M12 Rainy (n=527)
M15 Rainy (n=506)
M18 Cool (n=467)
Survey
B
TPN village
Intensity of antibody response
M0 Hot (n=258)
M3 Rainy (n=283)
M6 Rainy (n=300)
M9 Cool (n=307)
M12 Hot (n=260)
M15 Rainy (n=233)
M18 Rainy (n=265)
Survey
C
KNH village
Intensity of antibody response
M0 Rainy (n=325)
M3 Rainy (n=277)
M6 Cool (n=263)
M9 Hot (n=301)
M12 Hot (n=298)
M15 Rainy (n=300)
M18 Rainy (n=282)
Survey
D
TOT village
Intensity of antibody response
M0 Rainy (n=404)
M3 Rainy (n=322)
M6 Cool (n=201)
M9 Cool (n=292)
M12 Hot (n=249)
M15 Rainy (n=307)
M18 Rainy (n=195)
Survey

Figure 4A

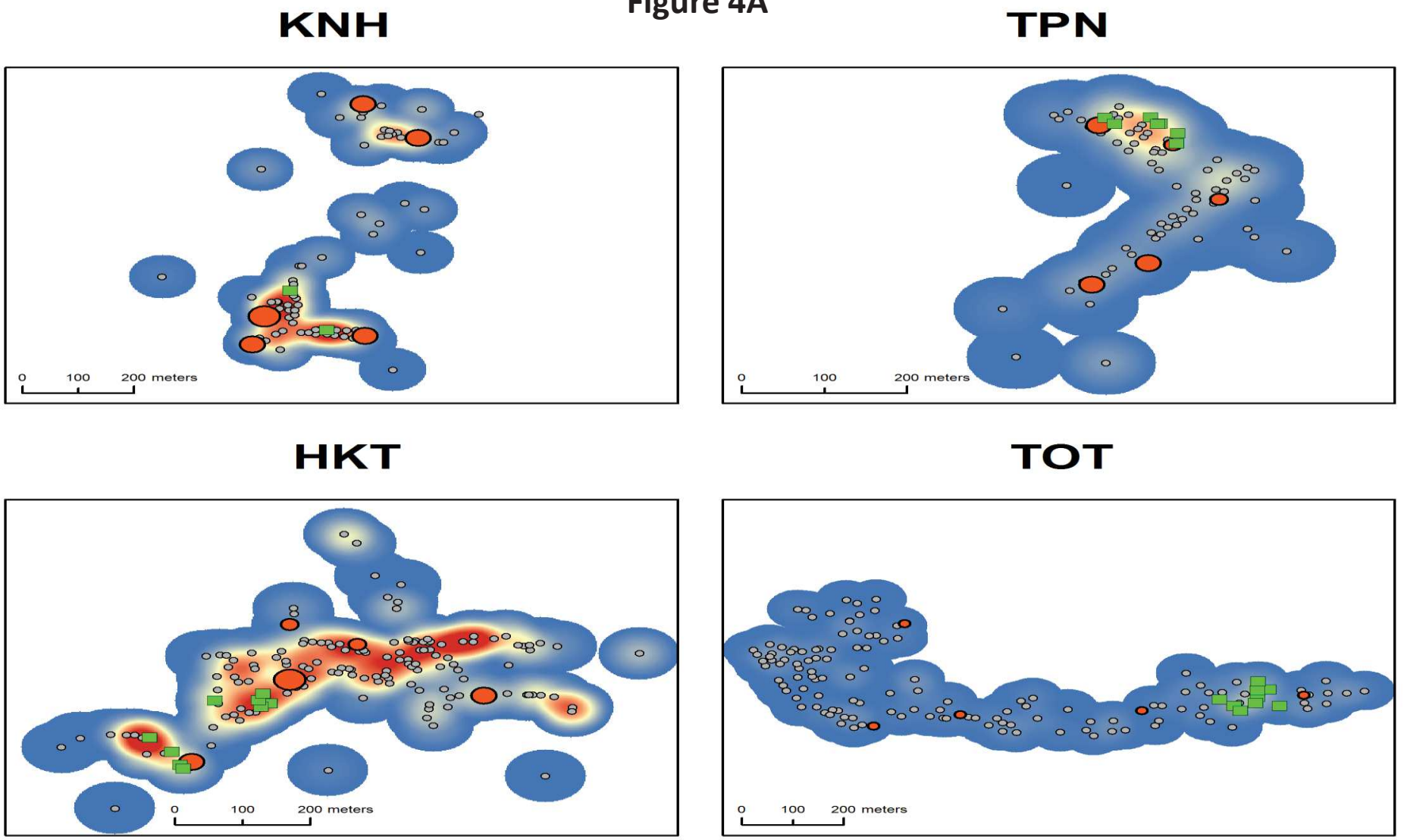
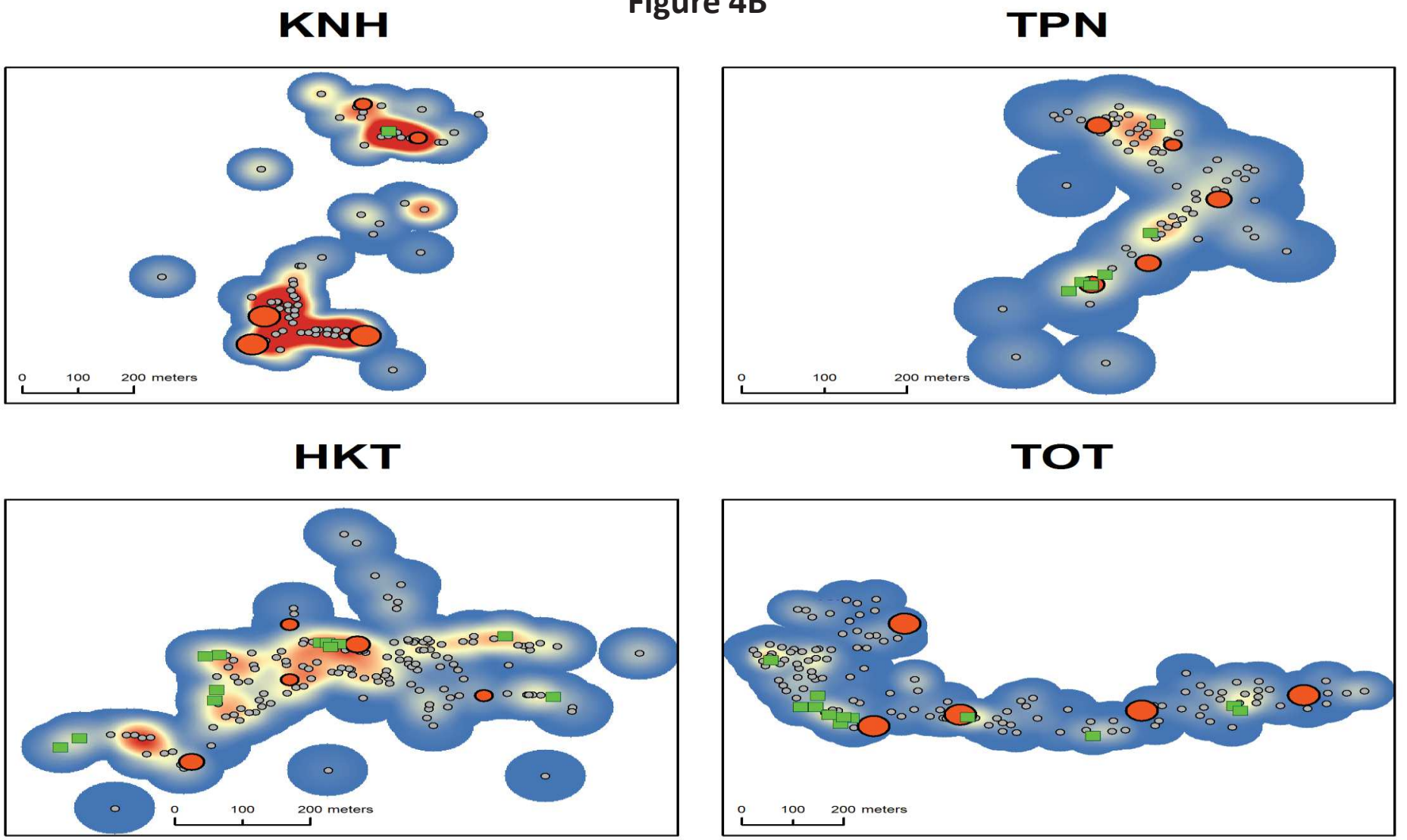

KNH
TPN
HKT
TOT
0 100 200 meters
house
IgG hotspot house
HBR
< 46.5
46.5 - 159.0
159.1 - 468.0
468.1 +
IgG intensity
High
Low
N
W
E
S
A

Figure 4B


KNH
TPN
HKT
TOT
0 100 200 meters
house
IgG hotspot house
HBR
< 46.5
46.5 - 159.0
159.1 - 468.0
468.1 +
IgG intensity
High
Low
N
W
E
S

B

CHAPTER 2 : THE RELEVANCE OF USING *ANOPHELES* SALIVARY BIOMARKER (gSG6-P1 PEPTIDE) TO ESTIMATE *PLASMODIUM FALCIPARUM* MALARIA ALONG THE THAILAND-MYANMAR BORDER

Monitoring malaria transmission is crucial to estimate the long term impact of mass drug administration (MDA) for malaria elimination. Transmission is however difficult to measure using classical entomological methods and endpoints (see section 3.1). In the previous chapter, we demonstrated the pertinence of using salivary biomarker for quantifying human-vector contact and estimating malaria transmission risk along the TMB. We showed that the risk of transmission strongly varied in space and time and was influenced by environmental factors and human practices. Here, the aim was to address whether *Anopheles* salivary biomarker could be relevant to detect small scale variations in human exposure to *P. falciparum* malaria along the TMB in a context of MDA for malaria elimination. This information is pivotal in public health programs, especially to identify remaining sources of transmission and guide malaria vector control.

The same cohort of ≈2600 participants (see Chapter 1) was followed up to address the relationship between *Anopheles spp*. exposure (as measured by Ab specific response to gSG6-P1 salivary antigen) and the Ab responses to *P. falciparum* malaria antigens (*Pf*CSP, *Pf*MSP-1_{19}) and the prevalence of *P. falciparum* malaria infections (as measured by ultra-sensitive PCR). Scan statistics was used to map potential clusters of *Pf* malaria infections and vector exposure before and after introduction of MDA. Multivariate logistic mixed regression models were used to address significant association between variables.

This work is currently under evaluation in the journal "*American Journal of Tropical medicine and Hygiene"* (http://www.ajtmh.org/).

Summary of results

Overall we demonstrated a significant relationship between *Anopheles* exposure and *P. falciparum* malaria exposure in areas of low endemicity along the TMB. Multivariate logistic mixed analyses showed that there was a positive "dose-response" relationship between *Pf*CSP or *Pf*MSP-1_{19} seroprevalence and the intensity of Ab response to gSG6-P1 ($p<.005$). Interestingly, we found that very high responders to gSG6-P1 (top 25%) were also at higher risk o*f P. falciparum* malaria infections (OR 2.30, p=0.004). This confirms previous results in the same area showing a significant correlation between entomological inoculation rate (EIR) and Ab responses to gSG6-P1 salivary antigen (Ya-Umphan et al. 2017). Altogether, these findings suggest that anti-gSG6-P1 reactivity correlate well with local exposure to infected mosquito bites and could be used as a proxy to identify malaria "hotspots" as part as malaria surveillance and elimination.

Spatial scan statistics showed that *P. falciparum* infections at baseline appear to cluster in specific parts of the study villages which partially correlate geographically to vector mosquito exposure. This suggest that the heterogeneity in *P. falciparum* malaria is positively associated with heterogeneous mosquito biting behavior ("focal" transmission risk). This finding suggest that the submicroscopic carriers probably play a role in malaria transmission in the study villages. Cluster of the spatial autocorrelation between vector exposure and *P. falciparum* exposure was less obvious over time most probably because of the successive introduction of MDA in the study villages that contributed to eliminate >95% *P. falciparum* reservoir (Landier et al. 2017). The reasons for higher malaria and vector risk in particular locations across villages are not known but we assume that this may reflect different human behavior and socio-economic conditions, environment (presence of mosquito breeding habitats), vector control practices, and/or immunogenicity characteristics as demonstrated by other (Parker et al. 2015b).

Another risk factor associated to sero-reactivity to *P. falciparum* infections was MDA, but the strength and direction of the association surprisingly differed according to the antigen (*Pf*CSP or *Pf*MSP-1_{19}). The reason for such discordance is not known but we suspect that difference in immunogenic properties (e.g. longevity of antibody responses) and exposure to

the immune system (immunogenicity of antigens) may explain the outcomes (Biggs et al. 2017, Mosha et al. 2014). Potential cross-reactivity between *P. falciprum* and *P. vivax* cannot be totally discard as previously observed along the TMB (Freya communications) and this might contribute to maintain specific Ab responses to *Pf*MSP-1_{19} in an area where *P. vivax* persists after MDA (Landier et al. 2017).

Finally, we showed that bednet use was not associated with a reduction of *P. falciparum* malaria exposure, which is consistent with previous findings showing an absence of correlation between net use and the intensity of Ab response to *Anopheles* bites (Ya-umphan et al. 2017). This finding suggests that more appropriate personal protection tools (e.g. repellents, insecticide treated clothes) should be delivered to people at risk of malaria to strengthen malaria control and elimination efforts.

In conclusion, these results indicated that the gSG6-P1 salivary biomarker shows promising for assessing *P. falciparum* malaria exposure risk along the TMB and could serve to implement hotspot targeted vector control interventions with the aim of achieving malaria elimination.

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Anopheles salivary biomarker as a proxy for estimating Plasmodium falciparum malaria exposure on the Thailand-Myanmar border

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Key Words:	Plasmodium falciparum malaria, salivary biomarker, Thailand-Myanmar border, ELISA

Anopheles salivary biomarker as a proxy for estimating *Plasmodium falciparum* malaria exposure on the Thailand-Myanmar border

Running title: *P. falciparum* malaria risk on the TMB

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ABSTRACT

Timely identification and treatment of malaria-transmission "hot spots" is essential to achieve malaria elimination. Here we investigate the relevance of using an Anopheles salivary biomarker to estimate *P. falciparum* malaria exposure risk along the Thailand-Myanmar border to guide malaria control. Between May 2013 and December 2014, >9,000 blood samples collected in a cluster randomized control trial were screened with serological assays to measure the Ab responses to anopheles salivary antigen (gSG6-P1) and *P. falciparum* malaria antigens (CSP, MSP-1_{19}). *Plasmodium falciparum* infections were monitored through passive and active case detection. Seroprevalence to gSG6-P1, MSP-1_{19} and CSP were 71.8% (95% Confidence interval, [CI], 70.9, 72.7), 68.6% (95%CI, 67.7, 69.5) and 8.6% (95%CI 8.0, 9.2), respectively. Multivariate analysis showed that individuals with the highest Ab response to gSG6-P1 had 6 times the odds of being positive to CSP antigens (P<.001) and 2 times the odds of *P. falciparum* infection compared to low gSG6-P1 responders (p=0.004). Spatial scan statistics revealed the presence of clusters of gSG6-P1 that partially overlapped *P. falciparum* infections. The gSG6-P1 salivary biomarker represent a good proxy for estimating *P. falciparum* malaria risk and could serve to implement hot spot–targeted vector control interventions to achieve malaria elimination.

Key words; Plasmodium falciparum malaria, salivary biomarker, ELISA, Thailand-Myanmar border

64

65 **INTRODUCTION**

66 Malaria along the Thailand-Myanmar border (TMB) is characterized by foci of high prevalences
67 of asymptomatic and submicroscopic *Plasmodium* spp. carriage [1]. Over the past several decades
68 malaria caused by *P. falciparum* infection has continuously declined in populations living along
69 the TMB which has mainly be attributed to the use of rapid diagnostic tests for early malaria
70 detection and treatment of malaria with highly effective artemisinin-based combination
71 antimalarial therapies and, to a lesser extend to prevention of mosquito bites [2, 3]. The recent
72 emergence of *P. falciparum* parasites that are resistant to artemsisin and artemisinin partner
73 drugs along the TMB raised concern about the potential spread of artemisinin resistance to India
74 and Africa and threatens recent gains in the reduction in the burden of malaria in the region and
75 globally [4, 5]. To contain *P. falciparum* artemisinin resistance, the Shoklo Malaria Research Unit
76 and partners conducted a Mass Drug Adminsitration (MDA) pilot-trial with the aim to eliminate
77 sub-microscopic reservoirs of *Plasmodium* spp. parasites and interupting malaria transmission [6,
78 7]. Along the TMB, local malaria transmission is heterogeneously distributed and the risk of
79 malaria may be confined to geographically small high risk areas and subsets of the population [3].
80 Identifying and targeting these malaria-transmission "hot spots" and "hot pops" is therefore
81 essential to remove remaining sources of transmission and to achieve malaria elimination [8].

82 Measuring local malaria transmission poses considerable challenges because of the lack of
83 sensitivity of commonly used entomology methods [9, 10]. Assessing malaria transmission intensity
84 by determining the entomological inoculation rate (EIR) is challenging considering the low
85 frequency of mosquitoes positive for infection and spatial and temporal variations in mosquito
86 densities and composition necessitate long-term intensive sampling [11]. Serological biomarkers

using antibodies specific for *Plasmodium* spp. are increasingly used to estimate changes in malaria transmission in areas of low endemicity [12, 13, 14, 15, 16]. Measuring anti-malarial antibodies to detect malaria transmission offer several advantages (compared to entomological and parasite outcomes) because of the longer duration of specific antibody responses, they are indicative of recent malaria exposure rather than point prevalence [12, 17]. Recently, new serological biomarkers that measure the intensity of human exposure to mosquito salivary antigens have been identified (reviewed in: [18]). Compared to other serological tools, salivary markers offer great potential for measuring small-scale variation in the exposure to malaria vectors as they provide shorter-lived antibody responses [18]. In a previous study, we demonstrated that antibodies specific for the salivary biomarker gSG6-P1 are relevant to quantify human-vector contact and estimate malaria transmission risk at the TMB [19]. We showed that the risk of malaria transmission strongly varied in space and time and was influenced by the environment and human behavior. Nevertheless, the relationship between human exposure to Anopheles bites and the risk of being infected by *P. falciparum* malaria was not elucidated.

The aim of the present study was to address whether Anopheles salivary biomarker can detect small scale variations in human exposure to *P. falciparum* malaria in a context of malaria elimination. This information is pivotal in public health programs to improve malaria surveillance and guide vector control programs.

MATERIAL AND METHODS

Study site and survey procedure

Four villages, TPN, TOT, KNH and HKT with >10% *P. falciparum* prevalence by high volume ultra-sensitive qPCR (uPCR) were selected after engagement of the community [1]. A community based malaria clinic ("malaria post") was set up in each village to monitor malaria infections with SD Bioline Pf/Pv rapid diagnostic test (RDT) [20]. A census was performed prior to the surveys and demographic information was collected [19].Two villages (TOT and KNH) were randomly assigned to MDA intervention immediately and two (HKT, TPN) were followed for 9 months (control period) before receiving MDA as described in Landier et al [20]. Briefly a 3–day treatment course of dihydroartemisinin-piperaquine (DP) and a single 0·25mg base/kg dose of primaquine was administered orally under supervision every month for 3 months to all participants. Long lasting impregnated nets (LLIN) were distributed to all households at the start of the study (M0). Seven household surveys were carried in each village every three months (month 0, 3, 6, 9, 12, 15, 18) from May 2013 to December 2014. At each survey, dried blood spots on Whatman filter papers (3MM) were collected during surveys of the entire village population for Enzyme-Linked Immunosorbent Assay (ELISA). *Plasmodium* spp. infections were also recorded by uPCR according to methods described previously [1, 21].

Antigen selection

The gambiae salivary peptide gSG6-P1 was selected because it is highly antigenic and highly specific to the Anopheles genus, with no relevant cross-reactivity with epitopes from other proteins or vectors of parasites [22, 23] The synthetic nature largely ensures high reproducibility of the assay and it induces short-term (up to 2 months) and specific host humoral response [18]. The *P. falciparum* antigens MSP-1_{19} and CSP were selected for their capacity to detect different life cycle stages of the plasmodium (sporozoite *versus* merozoite) and for their difference in immunogenicity and persistence [15, 24].

ELISA: Human Antibody response to *Plasmodium falciparum* antigens

Serologic testing of human exposure to MSP-1_{19} and CSP antigens was carried out by ELISA as described in [15] but with some modifications (Supplementary Material S1). Individual responses were expressed as the ΔOD value: ΔOD= ODx-ODn. ODx and ODn represent the mean of individual optical density (OD) in 2 antigen wells and 1 blank well containing no PfMSP1-19 / CSP peptide, respectively. Specific anti- MSP-1_{19} / CSP IgG response were also determined by ELISA in non-malaria exposed individuals (negative samples from France: n=18) in order to quantify the non-specific background Ab level and to calculate the cut-off value for seropositivity (mean (ΔDOneg) +3SD). Procedures for testing human exposure to gSG6-P1 salivary biomarker have previously been described [19].

Statistical analysis

To investigate the association between the intensity of Ab response to anopheles salivary antigen (gSG6-P1; categorical variable: low, medium, high, very high; based on quartiles) and Ab responses specific for *P. falciparum* CSP and MSP-1_{19} (binary variable: positive, negative), we used a multivariate multilevel logistic mixed regression model with adjustment for relevant covariates which represented proxies for social, demographic or environmental status. At the individual level, covariates included Ab response to gSG6-P1, age group, sex and MDA treatment (a time-dependent individual binary variable, 0 as long the individual as not taken any drug, 1 when individuals has received 1, 2 or 3 doses). Household-level covariates included LLIN use (never, sometimes, every night). At the village level, the population size at each survey, the temperature and relative humidity were taken into account in the model (i.e. second order polynomial) and season was defined according to the Thai Meteorological Department. In a second analysis, we investigated the relationship between the intensity of Ab response to

anopheles salivary antigens (4 categories) and *P. falciparum* malaria infections (binary variable: positive, negative) using a multivariate mixed logistic regression adjusted for covariates described above. Statistical analyses were done with Stata version 13.0 (StataCorp LP, College Station, Texas). Graphs were constructed using GraphPad Prism 5 software (San Diego, CA, USA).

Spatial analysis

Geographic references (latitude and longitude) were recorded for all households in the study villages. Each house was given an identification code and all study participants could be linked back to their respective houses using the identification code. Scan statistics were used to test for statistically significant clusters of salivary biomarker (gSG6-P1) and *P. falciparum* malaria infections as measured by uPCR (surveys) and RDT (malaria post). A discrete space-time Poisson model was used to test for statistically significant clusters for each of these outcomes and for each study village across all screenings (M0 – M18) [25, 26]. The scan statistic uses a moving window (a spherical kernel) that centers on each point (house) in the village and calculates the number of cases (*P. falciparum* infections or biomarker positives) within the window and the number of expected cases within the window, given the population within the window and the distribution of cases and population across the entire village. The window increases in size until half of the village population is contained and then moves to the next point. Likelihood ratios are calculated for each window location, size and time point and p-values are calculated using Monte Carlo simulations for the largest ranking clusters. Scan statistics were calculated using SaTScan software (https://www.satscan.org/).

Ethical approval

This study was part of a multicentre cluster-randomized control trial conducted in several sites in the Greater Mekong sub-region and registered on ClinicalTrials.gov: NCT01872702. Study protocol was reviewed and approved by OxTREC (reference no. 1017–13 and 1015–13).

RESULTS

Study populations and outcomes

Participants consisted of 2,602 people followed up every 3 months over 18 months and are described in Table 1. The compositions of the four study villages were comparable in age and sex. The overall prevalence of *P. falciparum* infections over the surveys ranged from 0.88% (17/1,934) in TPN to 3.14% (64/2,036) in KNH. The incidence of *P. falciparum* recorded at MP ranged from 1 per 1000 people per year at HKT to 45.5 at TOT. A total of 9,373 and 9,401 dried spots were analyzed for CSP and MSP-1_{19}, respectively. Overall, Ab seroprevalence was higher for MSP-1_{19} (range: 23.9% - 100%) than for CSP (range: 0.4% - 42%) and increased with age in each village (Table 1). The seroprevalence to gSG6-P1 ranged from 59% at TPN to 86% at HKT.

Seroprevalence in antibody response to Anopheles spp. and *P. falciparum* antigens

The Ab response to the MSP-1_{19} and CSP antigens varied according to village and survey (Figure 1). For MSP-1_{19}, seroprevalence greatly increased from M3 to M9 in all sites, except at HKT where it remained constant all along the study (≈60%) (Supplementary Table1). In contrast, seroprevalence to CSP increased by ≈2 fold from M0 to M3 in all sites and then continuously declined until M18 (Supplementary Table 2). The seroprevalence to gSG6-P1 was high (71.8%, 95% CI 70.9, 72.7) and strongly varied across villages and surveys (Supplementary Table 3).

Relationship between *P. falciparum* malaria and vector exposure

198 The mean number of measurements per individual was 3.4 (range, 1–6). Multivariate logistic
199 mixed analyses showed a highly significant and positive dose-response relationship between Ab
200 response to gSG6-P1 and the odds of a positive Ab response against *P. falciparum* CSP and
201 MSP-1_{19} (Table 2). Individuals with the 25% highest Ab response to gSG6-P1 (i.e. "very high"
202 responders) had 6 times the odds of being positive to CSP antigens (OR 5.94, 95%CI 3.72-9.48,
203 P<.001) compared to low gSG6-P1 responders. Increased odds of positive MSP-1_{19} responses
204 was also demonstrated but the magnitude of effect was smaller (OR 2.91, 95%CI 1.86-3.67,
205 P<.005). *Ad hoc* multivariate analysis showed that the very high responders to gSG6-P1 were
206 also associated with increased odds of *P. falciparum* infection after adjustment for other
207 covariates (OR, 2.27, 1.35-3.80, P=0.002, Table 3).

208 Multivariate logistic analysis also showed that the odds of *P. falciparum* Ab prevalence strongly
209 increased with age and was slightly lower in females compared to males (Table 2). The
210 seroprevalence of *P. falciparum* Ab varied according to village (P<.001) and season (P<.001) but
211 the strength and direction of the association differed according to antigen. The highest
212 seroprevalence to CSP was in HKT whereas KNH showed the highest seroprevalence to MSP-
213 1_{19} (Table 2). The rainy and cool seasons were associated with higher odds of positive CSP
214 responses compared to hot season (OR 9.47, 95% CI, 2.69, 33.35, P<.001 and 8.34, 95% CI
215 2.47, 28.11, P=0.001). MDA was associated with decreased odds of CSP seroprevalence (OR
216 0.49, 95% CI, 0.33, 0.74, p=.001) but increased odds of MSP-1_{19} Ab seroprevalence (OR 4.18,
217 95% CI, 3.15, 5.54, p<.001). Finally, there was no strong evidence of an association between bed
218 net use and the seroprevalence of *P. falciparum* Ab (Table 2).

219 **Spatial patterns in *P. falciparum* malaria infections**

We mapped together the very high responders to anopheles salivary bites with *P. falciparum* infections monitored either at surveys or malaria posts during the rainy season, at baseline (M0, no MDA) and then 15 months after implementation of MDA (M15). At baseline, three villages (KNH, TOT, HKT) had statistically significant clusters of *P. falciparum* malaria that partially overlapped with clusters of gSG6-P1 at KNH and HKT (Figure 2). TPN showed a detectable hotspot of *P. falciparum* infections in the middle of the village, with no apparent hotspot of vector exposure (the number of *P. falciparum* infections were however low). AT TOT, *P. falcip*arum infections were dispersed within the village (no clustering) and a wide cluster of vector exposure was seen ($p < 0.0001$; RR = 16.15). *P. falciparum* infections were drastically reduced by M15 in all villages receiving MDA (Figure 2), except at TOT were remaining pockets of *P. falciparum* malaria infections and vector exposure persisted in the western part of the village.

DISCUSSION

In the present study, we used antibody responses to demonstrate a strong and significant relationship between Anopheles exposure and *P. falciparum* malaria in areas of low endemicity along the TMB. This association was shown for antibody responses to two different *P. falciparum* antigens (MSP-1_{19} and CSP) and confirmed by the observation that very high responders to gSG6-P1 were also at higher risk of *P. falciparum* infections. This was further supported by spatial statistics showing that *P. falciparum* clusters partially overlap the gSG6-P1 clusters before and after the use of MDA for malaria elimination. This confirms previous results in the same villages showing a significant correlation between entomological inoculation rate (EIR) and Ab responses to gSG6-P1 salivary antigen [19]. Altogether, these findings suggest that

anti-gSG6-P1 could be used to estimate population vulnerability to malaria vector bites and hence be used as a proxy to estimate malaria transmission risk as part as malaria surveillance and elimination.

The spatial mapping data showed that *P. falciparum* infections clustered in specific parts of the study villages which were partially geographically correlated to vector mosquito exposure. While it is likely that some *P. falciparum* malaria are imported in the study villages by migration and population movement [27], our findings indicate that heterogeneity in *P. falciparum* malaria is positively associated with heterogeneous mosquito biting behavior, hence reflecting "focal" transmission risk. Spatial autocorrelation was less obvious over time probably because of the successive introduction of MDA that eliminated >95% *P. falciparum* reservoir [20]. Despite that, the salivary biomarker was successful to identify remaining sources of *P. falciparum* transmission at TOT where low coverage (75%) of MDA was reported [20]. Absence of spatial autocorrelation between vector exposure and *P. falciparum* exposure in some surveys suggest also that a non-negligible part of malaria transmission probably occurred outside the villages.

Our study showed that the risk of exposure to malaria and the vector vary in space (village) and time (season). The reasons for higher malaria risk in particular locations within villages are currently unknown but we assume that this may reflect different human behavior and socio economic conditions, environment (presence of mosquito breeding habitats), vector control practices, and/or immunogenicity characteristics as demonstrated by others [3]. As expected, a clear age dependent response in malaria specific immune responses was seen, which confirms exposure-driven acquisition of antibody responses [13, 28, 29]. This trend was more pronounced with $MSP\text{-}1_{19}$ than CSP, probably due to the stronger immunogenic property of this antigen. Interestingly, the higher odds of *P. falciparum* seropositivity to CSP and MSP in older age group

266 correlates well with the age-dependent response to anopheles exposure observed previously in
267 the area [19]. We showed that females were also at lower risk of *P. falciparum* malaria exposure
268 than males confirming the predominance of *P. falciparum* infections in adult males along the
269 TMB due to different behavior, movement and occupation [2].

270 Other risk factors associated with sero-reactivity to *P. falciparum* infections was MDA, but the
271 strength and direction of the association differed according to antigen (CSP or MSP-1_{19}). The
272 observed differences between antigens may be due to differences in immunogenic properties (eg
273 longevity of antibody responses) or differences in exposure of life-cycle specific antigens to the
274 immune system (immunogenicity of antigens); sporozoite antigens such as CSP is exposed to the
275 immune system for a shorter duration than blood-stage merozoite antigens such as MSP-1_{19} [30, 31].
276 Finally, potential cross-reactivity between *P. falciparum* and *P. vivax* antigens cannot be ruled
277 out. In areas co-endemic for *P. falciparum* and *P. vivax*, homologous antigens may elicit cross-
278 reactive antibodies [32] and MSP-1_{19} shares ~50% amino acid identity between the two species.
279 Cross-reactivity may also have contributed to the maintenance of specific Ab responses to MSP
280 in areas where *P. vivax* persists after MDA [20].

281 Finally our study revealed that frequent bednet use ("self-reporting") was not associated with a
282 reduction of *P. falciparum* malaria exposure, which is consistent with previous findings showing
283 an absence of correlation between net use and the Ab response to anopheles bites [19]. Along the
284 TMB, malaria vectors bite preferentially outdoors and in early evening when people are not
285 protected by insecticide treated nets [33, 34]. This finding suggests that more appropriate personal
286 protection tools (eg repellents, treated clothes) should be delivered to people at risk of malaria to
287 strengthen malaria control and elimination efforts. Our results also confirm the great potential of
288 using salivary markers to evaluate the efficacy of vector control interventions where malaria

prevalence and intensity of transmission become low [35]. The development of factory made rapid diagnostic device for detection of human exposure to anopheles bites would be useful to replace labour and intensive ELISA and be routinely incorporated into elimination programmes.

CONCLUSION

In communities living in areas of low seasonal malaria transmission, where reservoirs of sub-microscopic infections have been identified, the bio-marker of exposure to vector correlates well with the risk of *P. falciparum* infections. In this study, the relationship between vector exposure (biomarker) and risk of malaria carriage (Ab) is complex, partly because of MDA and because of the complexities of malaria ecology in the region. Bio-marker could be a useful tool to measure the impact of targeted-vector control measures deployed as part of an elimination effort that addresses the parasite reservoirs in the "hot-spots" villages. These vector control measures are important since LLIN may provide little protective efficacy against anopheles vectors in this region.

Authors' contributions

VC and FN designed the study. PYU and DC carried out all ELISA assays conduct data analysis. GC and PYU worked on the statistical analysis. DP carried out spatial analysis and mapping. PYU, DC and VC managed the data. FF interpreted data. All authors contributed to the writing of the paper. All authors have contributed to writing of the versions of the paper. All authors read and approved the final manuscript.

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Table 1: Descriptive statistics of participants, by Study sites

Characteristics	**Htoo Pyin Nyar**		**Tar Au Ta**		**Ka Nu Hta**		**Htee Kaw Taw**	
Age, y, median (range)	21 (0-66)		19 (0-80)		22 (0-73)		19 (0-94)	
Female sex, %	47		52		46		50	
Pf Prevalence by uPCR % (no. positive/no. tested)	0.88 (17/1934)		2.77 (51/1843)		3.14 (64/2036)		1.62 (59/3648)	
Pf incidence by RDT (cases per 1000 per year)	11.4		45.5		6.6		1.0	
Antibody prevalence to gSG6-P1	59.3 (1131/1906)		68.8 (1356/1970)		61.4 (1256/2046)		86.3 (3024/3503)	
Antibody prevalence to Pf Antigen, visits, (%)	CSP	$MSP\text{-}1_{19}$	CSP	$MSP\text{-}1_{19}$	CSP	$MSP\text{-}1_{19}$	CSP	$MSP\text{-}1_{19}$
All ages	6.0 (113/1892)	66.7 (1268/1902)	14.5 (284/1958)	73.7 (1452/1969)	8.7 (179/2050)	77.9 (1591/2043)	6.7 (231/3469)	61.3 (2136/3484)

Ages 0-4 y	0.4	51.4	3.2	28.0	1.3	44.0	1.5	23.9
	(1/241)	(125/243)	(10/309)	(87/311)	(3/225)	(99/225)	(7/453)	(109/456)
Ages 5-15 y	3.6	63.6	5.4	66.4	4.1	68.8	3.4	54.0
	(20/551)	(353/555)	(34/627)	(420/633)	(22/540)	(368/535)	(431/1262)	(683/1264)
Ages 16-59 y	8.7	70.9	21.7	91.8	11.9	86.9	9.9	76.2
	(91/1051)	(748/1055)	(202/932)	(857/934)	(145/1215)	(1055/1214)	(165/1661)	(1273/1671)
Ages > 60 y	2.0	85.7	42.2	96.7	12.9	100.0	17.2	76.3
	(1/49)	(42/49)	(38/90)	(88/91)	(9/70)	(69/69)	(16/93)	(71/93)

Table 2. Multivariate logistic regression mixed model showing the relationship between *Plasmodium falciparum* antibody responders (CSP, MSP-1_{19}) and the intensity of antibody responses to gSG6-P1 salivary antigen and other covariates.

	Ab response to CSP			Ab response to MSP-1_{19}		
Characteristics	OR	95% CI	*p-value*	OR	95% CI	*p-value*
Ab response to gSG6-P1						
Low	1			1		
Medium	2.08	1.31-3.32	0.002	1.81	1.35-2.44	<0.001
High	3.74	2.35-5.95	<0.001	1.71	1.25-2.34	0.001
Very high	5.94	3.72-9.48	<0.001	2.61	1.86-3.67	<0.001
Age, y						
<5	1			1		
5-15	5.05	1.89-13.47	0.001	28.83	15.67-52.97	<0.001
16-59	32.90	12.57-86.12	<0.001	285.60	147.36-553.51	<0.001
> 59	96.89	26.15-359.03	<0.001	1629.94	345.53-7688.80	<0.001
Sex						
Male	1			1		
Female	0.70	0.46-1.05	0.082	0.46	0.31-0.68	<0.001
Village						
Htee Kaw Taw	1			1		
Htoo Pyin Nyar	0.03	0.01-0.10	<0.001	10.16	3.96-26.11	<0.001

Ka Nu Hta	0.05	0.02-0.17	<0.001	22.60	8.21-62.15	<0.001
Tar Au Ta	0.78	0.34-1.81	0.561	3.21	1.46-7.02	0.004
Season						
Hot	1			1		
Cool	9.48	2.69-33.35	<0.001	0.59	0.23-1.48	0.268
Rainy	8.34	2.47-28.1	0.001	0.45	0.18-1.09	0.079
MDA						
No MDA	1			1		
MDA	0.49	0.33-0.74	0.001	4.18	3.15-5.54	<0.001
Sleep under Bednet						
Never	1			1		
Some night	0.72	0.16-3.27	0.675	2.01	0.45-9.02	0.364
Every night	0.97	0.55-1.72	0.912	1.80	1.02-3.17	0.043

The multilevel model included Ab response to gSG6-P1, age group, sex and MDA at the individual level, LLIN use at the household level and season, the population size at each survey, the temperature and relative humidity at the village level.

Table 3. Multivariate logistic regression mixed model showing the relationship between the *Plasmodium falciparum* infections and the intensity of antibody responses to gSG6-P1 salivary antigen.

	Plasmodium falciparum Infections		
Characteristics	OR	95% CI	*p-value*
Ab response to gSG6-P1*			
Low	1		
Medium	0.88	0.49-1.55	0.664
High	1.45	0.84-2.52	0.178
Very high	2.27	1.35-3.82	0.002

* gSG6-P1 classes were OD<0.4125 for low responders, 0.4125<x<0.6345 for medium responders, 0.6345<x<0.9045 for high HB responders and ≥0.9045 for very high responders. Analyses were adjusted for temperature, humidity, age, season, and village.

FIGURE LEGEND

Figure 1: Seroprevalence data for antibodies against *Plasmodium falciparum* merozoite surface protein 119 (MSP-1_{19}), B) and *P. falciparum* sporozoite (CSP), according to villages (KNH, TOT, HKT, TPN) and surveys (7 surveys over 18 months). Seropositivity for MSP-1_{19} and CSP was calculated based on cut off values (ΔOD) > 0.162 and > 0.115, respectively.

Figure 2: Micogeographical clusters of Pf malaria infections and vector exposure in the study villages at baseline (M0) and then after the implementation of the MDA pilot-trial (M15). The large red (*P. falciparum* infections as measured by either uPCR or RDT), and green (very high responder to gSG6-P1) are the statistically significant hotspots detected by spatial scan statistics ($p < 0.001$). Pf infections are show in red diamond. Before MDA (M0), three villages (TOT, KNH, HKT) had multiple clusters of gSG6-P1 responders (Relative Risk, RR= 16.15, 9.29, 8.83, and, $p<0.0001$, respectively) that partially overlapped with clusters of *P. falciparum* infections at KNH (RR= 30.20, $p<0.0001$) and HKT (RR= 19.46, $p<0.0001$). After MDA (M15 conducted at the end of the rainy season), *P. falciparum* infections almost disappeared in all villages except at TOT where a cluster of *P. falciparum* infections was detected in the western part of the village (RR= 77.23, $P<0.0001$) that mostly overlapped with the cluster of gSG6-P1 (RR= 5.86, $P<0.0001$).

Supplementary material:

See at Page 219

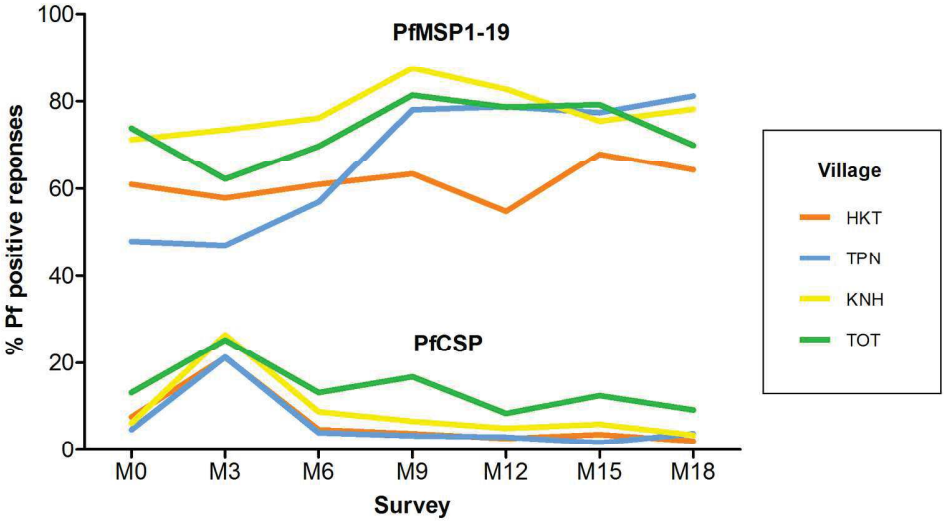

Figure 1

177x103mm (300 x 300 DPI)

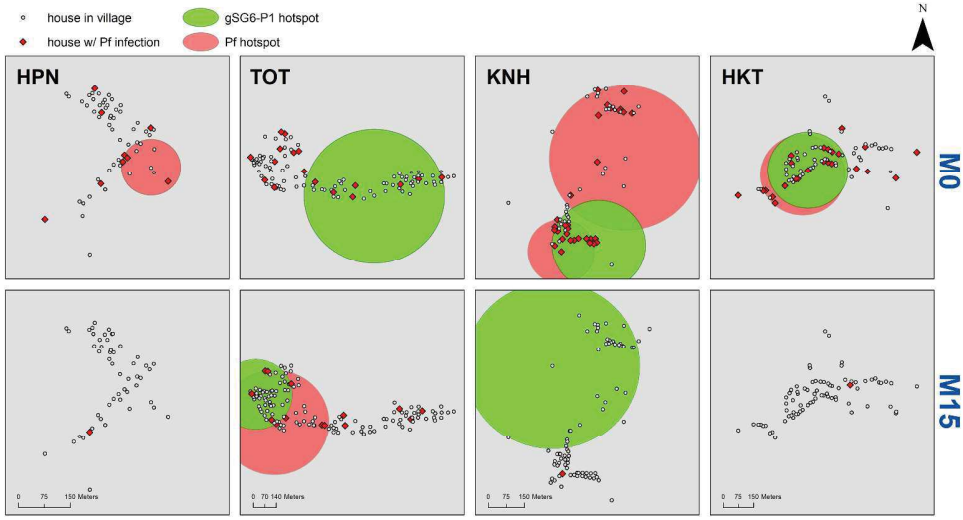

Figure 2

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DISCUSSION & PERSPECTIVES

Over the past 15 years the malaria situation in the Greater Mekong Subregion (GMS) has greatly improved and is reflected in the steady decline in annual malaria incidence and deaths (WHO 2015e). However, GMS nations still face daunting challenges as malaria epidemiology in this region exhibits enormous geographical heterogeneity (Cui et al. 2012). Within each country, malaria distribution is uneven, exemplified by high transmission occurring along international borders, and in forests and forest fringes. In Thailand, malaria incidence and deaths have declined continuously from 2000 to 2012 (i.e. morbidity reduced by 35% annually and mortality reduced by 30%) but it remains an important public health problem along the country borders with Myanmar, Lao PDR and Cambodia. The Tak province along the Thailand-Myanmar border (TMB) records each year the highest number of cases in the country (http://www.thaivbd.org/). Malaria transmission in this hilly forested area is intense due to the presence of highly efficient vectors and intensive population movement that render difficult the control and prevention of the disease. Moreover, the occurence of artemisinin-resistant strains of *P. falciparum* (Phyo et al. 2016) represent an increasing threat to the control and elimination of malaria, as *P. falciparum* malaria could become untreatable with currently available drugs within a few year. The consequence of letting such resistance reach the African continent will be that millions will die (White et al. 1999).

Along the TMB, malaria elimination has become a priority with the scope to contain the spread of artemisinin resistance in *P. falciparum* (Thu et al. 2017). Timely identification and treatment of all infected patients are then require to eliminate the remaining sources of transmission. The lack of sensitivity of current malaria diagnostic methods to detect low *P. falciparum* density infections pose a serious challenges considering that the majority of patients are asymptomatic and sub-microscopic (Imwong et al. 2015, Landier et al. 2017). The persistent "reservoir" represents the main obstacle to the rapid elimination of falciparum malaria in the GMS (Canier et al. 2013, Maude et al. 2014, Imwong et al. 2011). In such settings, 20% of individuals generally receive 80% of all infections (Smith et al. 2005), and these 20% of

individuals represent small groups of households or “transmission hotspots” that play an essential role in malaria transmission. Therefore, the use of new epidemiological tools to rapidly identify malaria “hotspots” is needed to enable countries to maximize progress toward malaria control and elimination.

This thesis was conducted in the framework of the MDA pilot-project implemented along the TMB for malaria elimination and resistance containment (Landier et al. 2017). The objectives were to investigate the relevance of using serological tools to measure micro geographical changes in human-vector exposure and malaria transmission risk in 4 pilot villages on the TMB. Overall we showed that the use of specific biomarker of human exposure to malaria vector bites (gSG6-P1) was successful to detect small-scale differences in *P. falciparum* malaria exposure in low transmission settings and could be used to guide the implementation of hot spot-targeted vector control interventions. With goals of malaria elimination in the Greater Mekong Subregion, the salivary biomarker represents a promising tool to evaluate the efficacy of vector control interventions in areas where conventional malariometric markers may fail (Figure 24).

The following sections will address strength and weakness of serological biomarkers for malaria epidemiology studies and provide guidance on how these tools could be incorporated into national programme for malaria control and elimination.

Serology biomarkers to overcome limitations of conventional entomology methods

In an era of malaria elimination, methods to accurately identify transmission hotspots are crucial. Classical entomological indicators (Human biting rate - HBR and Entomological Inoculation Rates-EIR) have major limitations in the measurement of malaria transmission intensity (e.g. high variability and dispersion, low sensitivity, high cost, etc) and are not adequate to address malaria exposure risk at individual level. This is particularly true in low transmission settings where the prevalence of infections (in both humans and vectors) remain low and most often below the threshold of detection. Then large sample size is needed to minimize the influence of outliers or extreme observations and to generate the best estimates of transmission

intensity (Coran et al. 2007). This is well illustrated in our MDA-pilot project where only 106 *Plasmodium* infected mosquitoes of 57,474 tested by qPCR were detected over the 2 years follow up (Chaumeau, in prep). Obtaining large sample size then requires large number of collection sites and surveys that pose financial, logistic and ethical constraints.

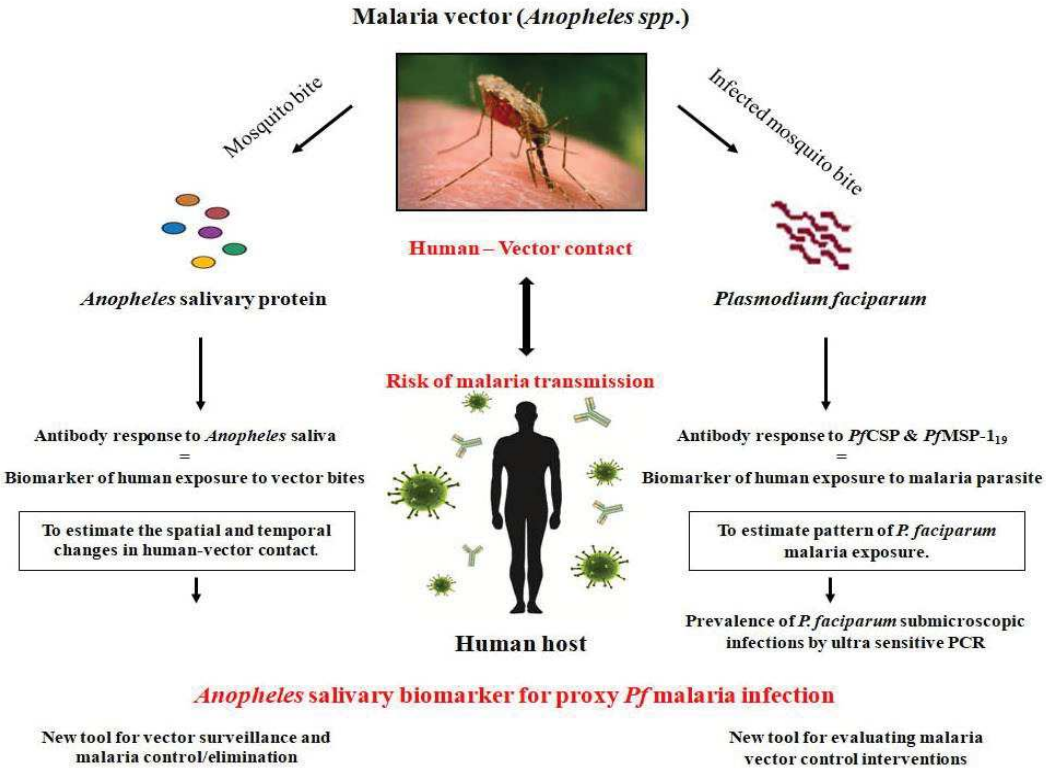

Figure 24. Outcomes of thesis

Consequently, serological assays have been increasingly used for the surveillance and monitoring of malaria transmission where existing metrics such as entomological indicators, parasite prevalence and/or incidence of clinical cases have become less sensitive (Yman et al. 2016). Seroconversion rates, based on antibody prevalence to *P. falciparum* asexual blood-stage antigens, provide estimates of transmission intensity that correlate well with entomological inoculation rates but lack precision in settings where seroprevalence is still high (Yman et al.

2016). The antibody acquisition models, based on cross-sectional data on individual antibody levels, were developed to improve serological estimates of malaria transmission intensity. This method enables sensitive detection of transmission changes and provide more precise estimates, due to a higher power to detect changes when seroprevalence is high, but may not be applicable if data are not log-normally distributed, e.g. if there are many zero measurements (Yman et al. 2016). Serology studies showed promising also to address malaria transmission risk in low transmission areas (Kerkhof et al. 2016a, Sturrock et al. 2016). In Cambodia, Kerkhof and colleagues (2016) could identified serological markers of human exposure to *P. falciparum* with a "relatively" short half-life (6-12 months) such Ags *Pf*GLURP.R2, LSA3.RE, GLURP, *Pf*MSP-1_{19} and *Pf*CSP. These serological markers were successfully used to identify malaria pockets with more precision and accuracy than conventional methods and were capable to distinguish recent from past malaria exposure (Kerkhof et al. 2016a,b). The use of antibody responses against parasite proteins has however some limitations. Actually, people exposed to malaria can be seropositive during several months or years (Druilhe et al. 1986, Greenhouse et al. 2011), even after transmission has stopped (Druilhe et al.1986) or in the context of low transmission (Clark et al. 2012).

More recently, serological methods for monitoring human-vector contact by the measure of the intensity of antibody response to mosquito bites have been developed (Drame et al. 2013). Compared to conventional serological tools of malaria exposure, salivary biomarkers offer great potential for measuring small-scale variation in the recent exposure to malaria vector bites. The gambiae salivary peptide gSG6-P1 is known to be highly antigenic and highly specific to the *Anopheles* genus (Arca et al. 2005), with no relevant cross-reactivity with epitopes from other proteins or vectors of parasites (Poinsignon et al. 2008 a,b). The gSG6-P1 declines much faster than *P. falciparum* antigens and it confers no protection to malaria, thus avoiding confounding factors associated with immunity and malaria incidence. Finally, high sequence homology of gSG6-P1 was shown for primary malaria vectors collected in the study area compared to the reference *An. gambiae* (Ya-Umphan et al 2017), hence confirming its great potential for malaria epidemiology in Southeast Asia.

***Anopheles* salivary biomarker to estimate changes in human-vector contact.**

The first part of the thesis aimed to validate the use of the *Anopheles* salivary biomarker to detect changes in human-vector exposure and malaria tranmssion risk in four karen villages along the TMB. Our study demonstrated a dose-response relationship between the intensity of antibody responses to gSG6-P1 and the degree of exposure to *Anopheles* bites. Spatial clusters of individuals with high immune responses to vector bites were identified in all villages hence reflecting high heterogeneity in vector abundance and biting pattern. Interestingly a strong association between the gSG6-P1 antibody response and the EIR was seen, hence indicating that this specific salivary biomarker was sensitive for measuring the heterogeneity of malaria risk, in areas with low-levels of malaria transmission.

Our results showed that significant risk factors for *Anopheles* bites were age, season and villages. The increase in the IgG response with age is consistent with the gradual acquisition of immunity against *Anopheles* mosquito saliva following the development of individual factors and behaviors that increase the probability of human-vector contact (Drame et al. 2010). The intensity of the response was higher during the rainy season than during the cool and hot seasons, but lower spatial clustering of anti-gSG6 IgG responders was noted during the rainy season. This may reflect a higher dispersion of mosquito throughout the village during the rainy season due to the multiplication of breeding habitats. The higher vector exposure risk in some areas of the villages may reflect also factors ultimately related to the ecology, demography and socio-economic structure of the village. For example, some individuals may share behavioral traits or norms, including a lack of bed net use or occupational and subsistence strategies, that could differentially put them at greater risk of infection (Parker et al. 2015b). Morover, agricultural practices are known to modulate the human-vector exposure in the study area (Parker et al. 2015b). Indeed, the population is essentially made up of local and temporary farmers working in rice paddies and cornfields around the villages during the rainy season, when vector density is the highest. Consequently, during harvest time, men and women will, quite frequently, spend nights in farms and may be particularly exposed to malaria vector bites outside the villages. This is particularly well captured at TOT where several clusters of high IgG responders occurred

without clear indication of vector abundance. In this case, we suspect that those people may have been extensively exposed to *Anopheles* bites when they moved to farms and/or forest plantations, logging, bamboo cutting, charcoaling and foraging (Bhumiratana et al. 2013a). Timely identification of people at high risk of malaria vector bites have practical implications for malaria control with the scope to provide them with more appropriate personal protection tools.

Finally, we showed that vector control was not necessarily the primary factor modulating human-vector contact in the study villages as no reduction in Ab response to *Anopheles* bites was associated with a frequent use of treated bednets. Although this result has to be taken with caution considering potential biases in measuring LLIN use (self-reporting), we suspect that insecticide-treated bed nets might offer limited personal protection against malaria in areas where vectors tend to bite preferentially outdoor and at early evening or morning (Somboon et al. 1993, Trung et al. 2005, Kwansomboon et al. 2017). This highlights the need to deploy new and locally adapted vector control tools to improve malaria elimination in the region.

***Anopheles* salivary biomarker as a tool to identify *Plasmodium falciparum* malaria hotspots.**

Since the initiation of the WHO's Mekong Malaria Program a decade ago, malaria incidence in the GMS has been reduced, hence resulting in very low malaria transmission clustered in “hotspots” and “hotpops” (Canier et al. 2013, Maude et al. 2014, Sluydts et al. 2014). In general, the nature of malaria transmission in hotspots is strongly associated with intense mosquito exposure and high levels of (asymptomatic) parasite carriage in the human population (Bousema et al. 2012). When aiming for malaria elimination, these focused areas of malaria transmission pose considerable challenges for entomological and epidemiological surveillance and implementation of malaria control measures (Cook et al. 2012). Timely identification and treatment of malaria pockets is essential to eliminate remaining sources of transmission and achieve elimination (Kerkhof et al. 2016b).

The second part of the thesis aimed to address whether the gSG6-P1 salivary biomarker could be relevant to detect small scale differences in *P. falciparum* malaria before and after the

deployment of MDA for malaria elimination. Overall we showed that individuals with the 25% highest Ab response to gSG6-P1 ("very high" responders) had 6 times the odds of being positive to *Pf*CSP antigens and 2 times the odds of *P. falciparum* (submicroscopic) infections compared to low gSG6-P1 responders. Furthermore, spatial scan statistics revealed the presence of clusters of gSG6-P1 that partially overlapped *P. falciparum* infections in absence of antimalarial treatment. Mathematical models using entomology data collected in the study area demonstrated a strong and positive association between the prevalence of submicroscopic carriers and the EIR, i.e. the EIR increased >200 times when the prevalence of submicroscopic infections passed from 0 to 100% (Chaumeau et al. unpublished). No such correlation was seen with the clinical *P. falciparum* infections hence indicating that the submicroscopic reservoir probably contribute to an important part of the malaria transmission in the study villages.

In our study, a clear age dependent response in malaria specific immune responses was seen, which confirms exposure-driven acquisition of antibody responses (Longley et al. 2017, Biggs et al. 2017, Kerkhof et al. 2106a). This trend was more pronounced with *Pf*MSP-1_{19} than *Pf*CSP, probably due to the stronger immunogenic property of this antigen. The odds of *P. falciparum* Ab prevalence was slightly lower in females compared to males but this was significant only for *Pf*MSP-1_{19}. This is consistent with the fact that the populations at high risk for malaria along TMB are adult males, especially migrants and other mobile people living and working in forested border areas (Parker et al. 2015a). Consequently, those individuals who are bitten most often are most likely to be infected and can amplify transmission by transmitting the malaria parasites to a large number of mosquitoes. In the recent past, ethnic minority groups practicing swidden agriculture were the largest and most important populations in the GMS in terms of malaria burden. Among them, whole families but especially adult males spend days or weeks away from their villages, tending forest plots, gathering forest products or hunting (Singhanetra et al. 1993). As a result, the cycle of transmission may continue in these communities, even if it is interrupted or decreased within the village (Dysoley et al. 2008). This is confirmed by our findings showing *P. falciparum* malaria hotspots in some locations with no indication of "mosquito hotspots".

In our study we also showed that MDA was associated with decreased odds of *Pf*CSP seroprevalence but increased odds of *Pf*MSP-1_{19} Ab seroprevalence. Although the explanation remains unclear, we suspect that differences in immunogenic properties (e.g. longevity of antibody responses) or differences in exposure of life-cycle specific antigens to the immune system may explain the outcomes. Indeed, the availability of *Pf*CSP to the immune system is considered as "short-term" response, as sporozoites from new infective bites are present in the blood in small numbers and for only a short duration (Wong et al. 2014, Singer et al. 2003). In contrast, the blood-stage antigen merozoite surface protein *Pf*MSP-1_{19} induced more prolonged stimulation of immune system (Fowkes et al. 2010, Fowkes et al. 2012, Badu et al. 2015). This could explained the persistence of *Pf*MSP-1_{19} clusters at M9 and M15 despite the elimination of *P. falciparum* submicroscopic infections by MDA (Landier et al. 2017). Finally, potential cross-reactivity between *P. falciparum* and *P. vivax* antigens cannot be ruled out. In areas co-endemic for *P. falciparum* and *P. vivax*, homologous antigens may elicit cross-reactive antibodies (Nagao et al. 2008) and *Pf*MSP-1_{19} shares ~50% amino acid identity between the two species. Cross-reactivity may also have contributed to the maintenance of high Ab responses to *Pf*MSP-1_{19} in areas where *P. vivax* persists after MDA (Landier et al. 2017).

To conclude, our findings showed that salivary biomarker has great potential to identify sub-sets of the population at high risk of malaria in areas where transmission occurs at levels too low to be detected by microscopy or RDT. However, there is a need to develop factory made rapid diagnostic device for detection of human exposure to *Anopheles* bites to replace labour and intensive ELISA assays and be routinely incorporated into elimination programmes.

Anopheles salivary biomarker as a tool for malaria surveillance and elimination

As a result of intensified global and regional efforts for elimination and improving socioeconomic conditions, many countries in the Asia Pacific Region have made great strides in moving toward elimination (WHO 2015e). However, elimination requires a different strategy than sustained control. New challenges have emerged that require collective and proactive actions, including the threats of artemisinin and insecticide resistance and residual transmission

(Corbel et al. 2013), alongside with other ongoing challenges in improving malaria case detection, entomological surveillance, monitoring and evaluation, and stratification of malaria risk to optimize implementation of malaria intervention (http://apmen.org/). The strategy for malaria elimination in the GMS (2015-2030) emphasizes the progression from burden reduction, which needs to be pursued in high transmission areas, and the elimination phase with rigorous norms for surveillance and management of active foci. The strategy has an objectives to interrupt transmission of *P. falciparum* in areas of multidrug resistance, including ACT resistance in all areas of the GMS, to reduce malaria in all high-transmission areas at risk and to maintain malaria free area and prevent reintroduction in areas where it has been interrupted. In Thailand, the authorities optimistically expect that 80 percent of Districts will be free from malaria transmission by the year 2020 and malaria will be eliminated by 2025 (WHO 2015e).

In the country, malaria risk is stratified by province according to 4 categories (Satimai et al. 2012); A1 areas with perennial transmission; A2 areas with periodic transmission; B1 areas with no transmission during the previous three years but still at risk of malaria epidemic due to the presence of vectors and a persistent suitable environment for malaria transmission and; B2 areas with non-transmission which, in principle, are not susceptible to transmission. This classification which has been defined for malaria control may be not adequate anymore when entering in a pre-elimination phase (i.e. what scale should be used to define malaria risk when incidence has drastically reduce?). In remote settings, it is difficult to cross-check the accuracy and reliability of parasite counting from all blood slides hence biasing the estimation of malaria endpoints. In addition, the presence/absence of transmission is difficult to predict as an “area” without any detectable infected vector doesn’t indicate an absence of transmission.

Consequently, the salivary biomarker could be beneficial in all steps of the elimination strategy and be used to establish an early warning system to monitor malaria risk factors in terms of population vulnerability and receptivity to malaria vector bites. The advantage is not only to identify micro geographical “areas” at high risk of vector abundance (“mosquito hotspots”) but to provide fine scale mapping of high risk communities due to different behavior, movement and occupation. The development of such novel tools may be more adequate to respond to existing

and new challenges and could be directly incorporated into Malaria Information System of the Bureau of Vector-borne Diseases, Ministry of Public Health, Thailand (together with demographic, parasitological, clinical data, etc) to boost the national surveillance system. In pre elimination era, this could ensure proactive identification and treatment of people at high malaria risk (Satimai et al. 2012). In the post-elimination era, the biomarker of exposure to bites could be used to target the population that is more likely to receive new infected bites if the parasite is reintroduced in the area. Regardless the settings, the inclusion of serology data would imply to train public health officers to facilitate rapid uptake of the tool and to follow up on immuno-epidemiological data. Further evidence is needed however to propose malaria risk stratification based on serological data.

Anopheles salivary biomarker as a tool to evaluate the effectiveness of vector control measures

In 2017, the WHO has developed the Global Vector Control Response (2017-2030), with the aim to reduce the burden and threat of vector-borne diseases through effective locally adapted sustainable vector control (http://www. who.int/malaria/areas/vector_control/Draft-WHO-GVCR-2017-2030.pdf). The goals are to reduce mortality, case incidence due to vector-borne diseases globally and prevent epidemics of vector-borne diseases by rapid detection of outbreaks and curtailment before spread beyond country. To achieve the global targets, enhancing vector surveillance and monitoring and evaluation of interventions is required as well as new indicators to evaluate the efficacy of vector-control strategies. The use of *Anopheles* salivary biomarker is particularly relevant within the scope of the new vector control agenda, and offer multiple applications to guide vector control planning and implementation, and to improve the evaluation of innovative vector control interventions.

The use of gSG6-P1 as a biomarker to evaluate the effectiveness of vector control is not new and has been reported in various settings in Africa and America (review in Drame & Doucouré 2015). In Angola, anti-gSG6-P1 IgG responses showed to reflect the success of ITN-based malaria vector control in a malaria-endemic area (Drame et al 2010a). Interestingly, the

specific IgG Ab level to gSG6-P1 was pertinent to evaluate ITN protective efficacy at individual level. In Dakar, the use of gSG6-P1 was considered as reliable tool to assess the effectiveness of ITN, mosquito coils, spray bombs and ventilation (electric fans / air conditioning) in low-exposure/low-transmission settings (Drame et al. 2013). In America, Londono-Renteria et al showed a 10-fold increase in anti- gSG6-P1 titer during the spring and summer when mosquito exposure was likely to be the highest. After introduction of long-lasting permethrin-impregnated clothing to populations, the Ab responses to salivary antigens in subjects wearing treated uniforms was 2- to 2.5-fold lower than that of control subjects (Londono-Renteria et al. 2015b). These studies highlighted the potential of the salivary biomarker to evaluate the efficacy of vector control interventions in different malaria transmission settings.

In Thailand, the national strategic plan for malaria elimination (2017-2026) recognized the deployment of effective vector control measures in malaria risk areas (http://www.thaivbd .org/n/projects/download/128). Currently the main vector control tools implemented in all active transmission areas rely on the distribution of Insecticide-treated nets (ITN), Long Lasting Insecticide-treated nets (LLIN), topical repellents and Insecticide Treated Hammocks (ITH) (Malaria Elimination Strategy 2017-2026 : http://www.thaivbd.org/n/ projects/down load/128). Unfortunately, there's few evidence demonstrating the impact of those tools for malaria prevention and control in the Mekong region (Luxemburger et al. 1994 , Sluydts et al. 2016). For example, only marginal impact of mosquito nets on clinical and epidemiological indicators of malaria was reported in the study area (Dolan et al. 1993, Carrara et al. 2013, Smithuis et al. 2013a,b). Despite such contrasting results, there has been massive funding to ensure universal coverage of ITN in the GMS and ITN coverage has now reached saturation in many areas. Questions remains about the added value of ITN for malaria elimination in areas where malaria vectors bite preferentially at early evening or morning and outdoor (when people are not protected by ITN (Sriwichai et al. 2016, Kwansomboon et al. 2017). For example, 60% of infected bites occur before bed time in Cambodia (Sluydts et al. 2016) and 65% of them occurred outside the "window" of bednet protection (i.e. 09PM-05AM indoor) in our study villages (Chaumeau et al. in prep). This is further supported by our data showing an absence of significant correlation between bednet use and the intensity of Ab response to both *Anopheles*

bites and *Plasmodium* infections. The use of salivary biomarker could be particularly relevant in the SEA region to address where and when ITN are more likely to provide the optimal impact in term of personal and community protection. The salivary biomarker may represent also a "cost-effective" approach to assess the performances of new tools such as topical and spatial repellents, impregnated clothes and combat uniforms and/or treated hammocks for which evaluation are urgently need (Dondorp et al. 2017).

Along the TMB, the Malaria Elimination Task Force (METF) is integrating MDA with other interventions in a demonstration project. The key interventions included the establishment of a dense network of community level, early malaria diagnosis and treatment clinics, targeted MDA and locally adapted vector control tools. As part of this project, Long Lasting Insecticide-Treated Clothes (LLITC) will be distributed to >2,000 Karen people to contribute to malaria elimination efforts and the salivary biomarker has been selected to evaluate the protective efficacy of this new tool. The results will be important to validate the utility of the gSG6-P1 salivary peptide to evaluate the efficacy of innovative vector control measures in Southeast Asia.

Limitations and practical problems for measuring IgG response to salivary antigens

In this study, we used dry blood spots to measure the Ab levels to gSG6-P1, *Pf*CSP and *Pf*MSP-1_{19} antigens by ELISA. Dry blood spots (DBS) collected into filter papers represent an alternative (and attractive) method to plasma or serum for serological studies (easy to collect, transport and store). In remote field settings, DBS have great potential to empower healthcare workers by making laboratory-based diagnostic tests more readily accessible. Estimates of malaria transmission intensity obtained from serum and from blood spots showed similar, and values obtained using blood spots agreed well with entomologically determined values (Corran et al. 2008). However, environmental conditions (temperature and humidity) are known to affect the quality of DBS. This can be impeded by keeping desiccated spots below 4°C for extended periods. A challenge to overcome is also the improved elution of antibodies from filter papers overtime which may bias age-seroconversion estimates (Fowkes, personal communication).

Regarding immunological assays, we used ELISA to address the human antibody response to specific salivary antigen. The method is relatively cheap and provide both qualitative (yes/no) and quantitative detection of Ab reponses to specific antigen. ELISA requires however skilled and trained staff and well equipped laboratory that are not easily implementable in rural areas with limited access to electricity and human resources. In addition, ELISA can exhibit cross-reactivity between *Plasmodium* antigens (Nagao et al. 2008) and suffers from variability from plate to plate. This impose to control room conditions during the assays, to add reference samples in each plate (to check for variations in OD values) and to proceed a large number of samples to get consistent/reliable results. Multiplex immunoassays are now in use in many lab and represent a alternative option to save time and efforts, with the potential to provide quantitative data via multiple analyses. These assays require substantially less sample and reagents than the traditional ELISA which is further limited by its ability to measure only a single antigen (Fu et al. 2010). For example, multiplex immunoassays based on the Luminex technology were successfully used in Cambodia to measure the antibody responses against >19 different *Plasmodium* specific antigens and 2 vector saliva antigens from >8,000 blood samples (Kerkhof et al. 2016) and showed to be sensitive enough to estimate half-lives of Ab response to different antigens and to estimate short and long-term malaria transmission trends.

New insights into the development of rapid diagnostic tools of human-vector exposure.

To promote the use of salivary biomarker as an epidemiological tool for estimating malaria transmission risk and for the evaluation of vector control, there is a need to develop sensitive and specific serological kit that could be easily used in the field by non-specialized workers. Recently, a rapid immuno-chromatographic lateral flow test (autoreactive dipstick) to measure the level of human exposure to *Anopheles* bites has been investigated through public-private partnership. This device that is still under development should enable the rapid and easy detection of human IgG antibody response to *Anopheles* gSG6-P1 salivary peptide. The lateral flow test detects the presence or absence of human IgG antibody response to *Anopheles* gSG6-P1 salivary peptide in a liquid sample. Color lines may appear after applying a finger prick of blood to the test well. The presence of only one line indicates a negative result whereas the presence of

two color lines indicates a positive result (i.e. above a certain threshold). Moreover, the intensity of the colored test line could be measured with a lateral flow portable reader for a rapid "quantitative" diagnostic of human exposure to *Anopheles* bites (e.g. low, medium and high) (Figure 25). This biomarker may have 2 applications: first this dipstick could be used by public health workers to identify malaria vector risk areas and to guide malaria targeted-control measures. The device could be used also to monitor short to long term efficacy of malaria vector interventions. The rapid test will have to be robust, sensitive, cheap and simple-to-use in order to be widely advocated. The development stage is currently under the form of "go / no go" with a calibration and development of POC ("point of care") dispstick in laboratory followed by its validation in the field using a human cohort of vector-exposed individuals living in malaria endemic area. The ultimate goal is to develop a device that will ensure stratification of malaria transmission risk based on different Ab response threshold.

Figure 25. POC prototype intended for the detection of human IgG Ab response against *Anopheles* gSG6-P1 salivary antigen, B-The intensity of the colored test line could be measured with a lateral flow portable reader for a rapid quantitative diagnostic.

Development of new salivary biomarker to evaluate the exposure to *Anopheles* infective bites

In the field, the majority of bites are not infective by *Plasmodium* parasites (Beier et al. 1999, Drakeley et al. 2003), and consequently the measurement of human-vector contact may not necessarily reflect the real situation of malaria transmission. As less than 1% of the bites are infective in the Mekong region (Imwong et al. 2011), the Ab response to salivary antigens could overestimate the risk of transmission if infective *versus* non-infective bites are not distinguished. It is known that proteins synthesized in the salivary glands of the *An. gambiae* mosquito play important role in the life cycle of the malaria parasite *Plasmodium (*Choumet et al. 2007*)*. These authors demonstrated that the expression levels of five secreted proteins were altered when the parasite was present (Choumet et al. 2007). Therefore, attempts were made to develop specific biomarkers of *Plasmodium* infected bites. The principle of a biomarker of infective bites is based on the use of immunogenic salivary protein marker of transmission. The expression of some salivary proteins could be induced or regulated when salivary glands are infected. Therefore, if one of such protein presents immunogenic properties, the specific immune response to this protein may be a marker of transmission (Drame et al. 2013). By using proteomic approach combining 2D-DIGE and mass spectrometry, Marie et al shown that five salivary proteins (gSG6, gSG1b, TRIO, SG5 and long form D7) were overexpressed (from 1.4 to 2 fold) in the infected salivary glands. Moreover, the presence of wild *P. falciparum* in salivary glands modulated the expression of several salivary proteins and appeared to induce post-translational modifications (Marie et al. 2014). Although promising, the authors failed to identify specific proteins exclusively expressed in presence of *P. falciparum* infections (Marie et al. 2014). The development of specific biomarker of *Anopheles* infective bites is promising to identify the real risk of malaria transmission but this will require further efforts and investigations. Consequently, the gSG6-P1 salivary peptide remain the best candidate developed so far to estimate *Anopheles* exposure and malaria transmission risk.

CONCLUSION

The present study highlights the great potential of using specific *Anopheles* salivary peptide (gSG6-P1) as an epidemiological tool to evaluate human exposure to *Anopheles* bites and to identify *P. falciparum* transmission risk areas along the TMB. The use of antibodies levels to specific *Anopheles* salivary proteins correlated well with malaria vector abundance and transmission and could be used to overcome current limitations of conventional entomology methods. This tool could be used in low transmission settings to guide vector surveillance and vector control policies for malaria elimination. The development of rapid diagnostic kits of human exposure to *Anopheles* bites is however needed to ensure the incorporation of salivary biomarker into national malaria control programmes.

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ANNEX

SUPPLEMENTARY DATA

Published

Phubeth Ya-umphan, Dominique Cerqueira, Daniel M. Parker, Gilles Cottrell, Anne Poinsignon, Franck Remoue, Cecile Brengues, Theeraphap Chareonviriyaphap, Francois Nosten and Vincent Corbel. Use of an *Anopheles* salivary biomarker to assess malaria transmission risk along the Thailand-Myanmar border. *The Journal of Infectious Diseases* 2017 ; 215 (3): 396-404. DOI: https://doi.org/10.1093/infdis/jiw543

Supplementary data S1- ELISA procedures

Serologic testing of human exposure to gSG6-P1 saliva peptide was carried out by Enzyme-Linked Immunosorbent Assay (ELISA) as described in [25] but with the following modifications. First standardized dried blood spots (1 cm diameter) were eluted by incubation in 400 µl of phosphate buffered saline (PBS-Tween 0.1%) at 4 °C for 24 hours. Maxisorp plates (Nunc, Rosklide, Denmark) were coated with the gSG6-P1 specific *Anopheles* peptide (20 µg/mL) in phosphate buffered saline for 2 hours and 30 min at 37°C. After washing (with a solution of PBS-Tween 0.1%), the plates were blocked for 1 hour at 37 °C with 300 µl of blocking buffer (Pierce, Thermo Scientific USA). Thereafter, each eluate was incubated in duplicate at 4°C overnight at 1/20 dilution (in PBS-Tween 1%). Mouse biotinylated Ab to human IgG (BD Pharmingen, USA) was incubated at a 1/1000 dilution at 37°C for 1 hour and 30 min and peroxidase-conjugated streptavidine (GE Healthcare, UK) was added following the same conditions for 1 hour. Colorimetric development was carried out using ABTS (2,29-azino-bis(3-ethylbenzthiazoline 6-sulfonic acid diammonium) (Pierce, Thermo Scientific USA) in 0.05 M citrate buffer (pH 4) containing 0.003% H2O2, and absorbance was measured at 405 nm. In parallel, each test sample was assessed in a blank well containing no gSG6-P1-specific *Anopheles* peptide (ODn) to measure non specific reactions. Intensity of IgG response was measured at individual level and was expressed as the ΔOD value:

ΔOD= ODx-ODn where;

ODx and ODn represent the mean of individual optical density (OD) in 2 antigen wells and 1 blank well containing no gSG6-P1 antigen, respectively.

Specific anti-gSG6-P1 IgG response was also assayed in 16 non-*Anopheles* exposed individuals from France and Thai citizen staying at Bangkok >2 months in order to quantify the non-specific background Ab level and to calculate the cut-off value (mean (ΔDOneg) +3SD). Based on our findings, a participant was classified as an immune responder if its ΔOD was > 0.450.

Supplementary data S2-Technical details for spatial analysis and maps

All houses and mosquito collection sites were geo-referenced using Garmin etrex 20 Global Positioning System units and extracted using DNRGPS (http://www.dnr.state.mn.us/mis/gis/DNRGPS/DNRGPS.html). Geographic references (latitude and longitude) were projected to Universal Transverse Mercator (UTM) projections, meaning that absolute distances between points are in meters. Both "global" (Moran's I) and "local" (LISA: Local indicators of spatial autocorrelation) spatial clustering approaches are based on connectivity matrices that specifies the underlying spatial relationship between individuals in the datasets. Spatial coordinates were collected at the house level, meaning that multiple individuals have the same spatial location if they share a house. Rather than creating summary measures for each house, which could result in the loss of information about inter-house heterogeneity, the data were analyzed at the individual level. Given that individuals cluster within houses, we used a nearest neighbor specification for the connectivity matrix. The average house size from in the study villages was 5.5, and we tested nearest neighbors matrices of 10, 20, and 40. Tests of spatial autocorrelation therefore investigated clustering among individuals and their 20 nearest neighbors (which would include both house members and house members of nearby houses). At 10 nearest neighbors most clusters occurred in individual houses. There were few differences between the clustering statistics when comparing 20 or 40 nearest neighbors. The analysis was primarily focused on levels of clustering above the house level therefore final clustering analyses were run using 20 nearest neighbors.

Supplementary data S3. Immune profile of the population per age group and surveys

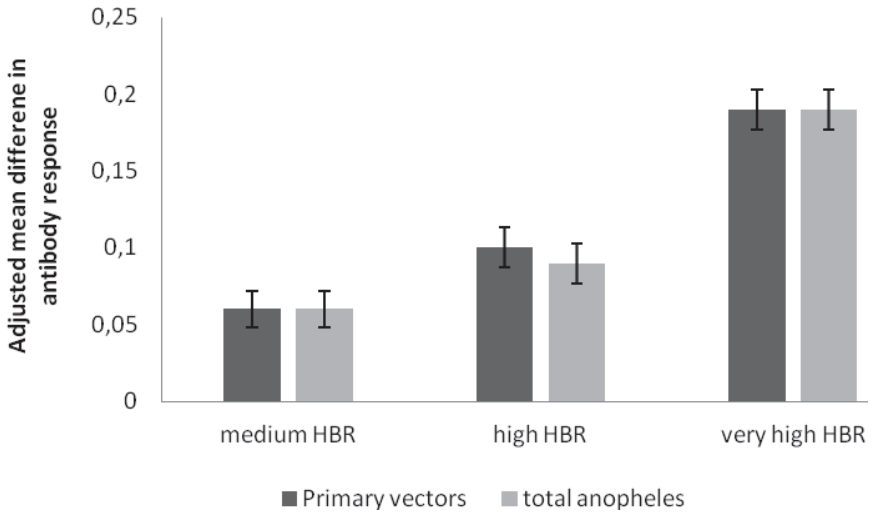
Village		TPN												
Survey of blood collection	Total population	Characteristic of blood spot						Antibody prevalence % , (n/N) , (95%CI)						
								Sex			Age			
		Blood Collection date	Number of dry blood spot	Male	Female	Median age (range) (y)		Male	Female	Total	0-4	5-15	15-59	60 up
M0	373	20-05-13	258	146	112	27.3 (1-66)	%	45.2	42.9	44.2	33.3	41.0	45.2	66.7
							(95% CI)	(37.0-53.6)	(33.7-52.6)	(38.1-50.0)	(6.0-75.9)	(30.2-52.8)	(37.6-53.1)	(24.1-94.0)
							(n/N)	(66/146)	(48/112)	(114/258)	(2/6)	(32/78)	(76/168)	(4/6)
M3	406	23-08-13	283	154	129	24.8 (1-66)	%	57.8	60.5	59.0	51.7	53.7	63.0	57.1
							(95% CI)	(49.6-65.6)	(51.5-68.9)	(53.0-64.8)	(32.9-70.1)	(42.4-64.6)	(55.1-70.3)	(20.2-88.2)
							(n/N)	(89/154)	(78/129)	(167/283)	(15/29)	(44/82)	(104-165)	(4/7)
M6	434	07-11-13	300	158	142	24.8 (0-66)	%	49.4	49.3	49.3	43.6	57.8	45.6	71.4
							(95% CI)	(41.4-57.4)	(40.9-57.8)	(43.6-55.1)	(28.2-60.2)	(46.5-68.4)	(38.0-53.4)	(30.3-94.9)
							(n/N)	(78/158)	(70/142)	(148/300)	(17/39)	(48/83)	(78/171)	(5/7)
M9	464	28-01-14	307	160	147	23.9 (0-66)	%	50.0	36.1	43.3	34.8	47.7	43.4	44.4
							(95% CI)	(42.0-58.0)	(28.4-44.4)	(37.7-49.1)	(21.8-50.3)	(36.9-58.7)	(35.8-51.3)	(15.3-77.3)
							(n/N)	(80/160)	(53/147)	(133/307)	(16/46)	(41/86)	(72/166)	(4/9)
M12	473	23-04-14	260	135	125	22.8 (0-66)	%	60.7	68.0	64.2	65.1	63.4	65.9	33.3
							(95% CI)	(51.9-68.9)	(59.0-75.9)	(58.0-70.0)	(49.0-78.6)	(52.0-73.6)	(57.0-73.9)	(6.0-75.9)
							(n/N)	(82/135)	(85/125)	(167/260)	(28/43)	(52/82)	(85/129)	(2/6)
M15	492	14-07-14	233	127	106	24.3 (0-66)	%	80.3	76.4	78.5	70.6	83.1	77.2	100.0
							(95% CI)	(72.1-86.6)	(67.0-83.9)	(72.6-83.5)	(52.3-84.3)	(71.3-90.9)	(68.7-84.0)	(56.1-100.0)
							(n/N)	(102/127)	(81/106)	(183/233)	(24/34)	(54/65)	(98/127)	(7/7)
M18	501	08-10-14	265	139	126	23.0 (0-66)	%	83.5	81.7	82.6	82.2	78.2	85.3	83.3
							(95% CI)	(76.0-89.0)	(73.7-87.9)	(77.4-86.9)	(67.4-91.5)	(67.2-86.5)	(78.0-90.6)	(36.5-99.1)
							(n/N)	(116/139)	(103-126)	(219/265)	(37/45)	(61/78)	(116/136)	(5/6)

Village		TOT												
Survey of blood collection	Total population	Characteristic of blood spot						Antibody prevalence % , (n/N) , (95%CI)						
								Sex			Age			
		Blood Collection date	Number of dry blood spot	Male	Female	Median age (range) (y)		Male	Female	Total	0-4	5-15	15-59	60 up
M0	740	14-06-13	404	192	212	26.8 (0-80)	%	68.2	62.7	65.3	42.9	58.1	72.4	50.0
							(95% CI)	(61.1-74.7)	(55.8-69.2)	(60.5-69.9)	(18.8-70.4)	(49.3-66.4)	(66.1-78.0)	(28.8-71.2)
							(n/N)	(131/192)	(133/212)	(264/404)	(6/14)	(79/136)	(168/232)	(11/22)
M3	785	12-09-13	322	149	173	22.2 (0-70)	%	77.2	71.7	74.2	63.6	70.3	78.8	92.9
							(95% CI)	(69.5-83.5)	(28.7-38.5)	(69.0-78.8)	(47.7-77.2)	(61.1-78.2)	(71.1-84.9)	(64.2-99.6)
							(n/N)	(115/149)	(124/173)	(239/322)	(28/44)	(83/118)	(115/146)	(13/14)
M6	789	26-11-13	201	98	103	24.1 (1-70)	%	75.5	81.6	78.6	72.0	73.2	82.6	92.3
							(95% CI)	(65.6-83.4)	(72.4-88.3)	(72.2-83.9)	(50.4-87.1)	(61.2-82.7)	(73.0-89.4)	(62.1-99.6)
							(n/N)	(74/98)	(84/103)	(158/201)	(18/25)	(52/71)	(76/92)	(12/13)
M9	814	18-02-14	292	144	148	21.7 (0-80)	%	46.5	47.3	46.9	40.0	51.1	45.7	63.6
							(95% CI)	(38.3-55.0)	(39.1-55.6)	(41.1-52.8)	(27.3-54.8)	40.3-61.9)	(37.2-54.3)	(31.6-87.6)
							(n/N)	(67/144)	(70/148)	(137/292)	(22/55)	(45/88)	(63/138)	(7/11)
M12	833	15-05-14	249	114	135	19.3 (0-70)	%	59.6	56.3	57.8	39.7	59.1	66.7	66.7
							(95% CI)	(50.0-68.6)	(47.5-64.7)	(51.4-64.0)	(27.8-52.8)	(46.3-70.8)	(57.0-75.2)	(30.9-91.0)
							(n/N)	(68/114)	(76/135)	(144/249)	(25/63)	(39/66)	(74/111)	(6/9)
M15	861	06-08-14	307	152	155	21.3 (0-80)	%	92.1	93.5	92.8	94.7	92.0	92.5	93.8
							(95% CI)	(86.3-95.7)	(88.1-96.7)	(89.2-95.4)	(84.5-98.6)	(84.4-96.2)	(86.4-96.2)	(67.7-99.7)
							(n/N)	(140/152)	(145/155)	(285/307)	(54/57)	(92/100)	(124/134)	(15/16)
M18	879	30-10-14	195	93	102	19.5 (0-70)	%	57.0	74.5	66.2	47.8	76.8	72.1	28.6
							(95% CI)	(46.3-67.1)	(64.8-82.4)	(59.0-72.7)	(33.1-62.9)	(63.3-86.6)	(61.2-81.0)	(5.1-69.7)
							(n/N)	(53/93)	(76/102)	(129/195)	(22/46)	(43/56)	(62/86)	(2/7)

Village		KNH												
Survey of blood collection	Total population	Characteristic of blood spot						Antibody prevalence % , (n/N) , (95%CI)						
								Sex			Age			
		Blood Collection date	Number of dry blood spot	Male	Female	Median age (range) (y)		Male	Female	Total	0-4	5-15	15-59	60 up
M0	348	14-06-13	325	180	145	29.1 (2-73)	%	55.6	51.7	53.8	33.3	40.2	59.3	69.2
							(95% CI)	(48.0-62.9)	(43.3-60.0)	(48.3-59.3)	(9.0-69.1)	(30.0-51.3)	(52.4-65.8)	(38.9-89.7)
							(n/N)	(100/180)	(75/145)	(175/325)	(3/9)	(35/87)	(128/216)	(9/13)
M3	417	12-09-13	277	147	130	28.1 (1-73)	%	47.6	51.5	49.5	30.4	38.4	56.7	50.0
							(95% CI)	(39.4-56.0)	(42.7-60.3)	(43.4-55.5)	(14.1-53.0)	(27.4-50.5)	(49.0-64.2)	(20.1-79.9)
							(n/N)	(70/147)	(67/130)	(137/277)	(7/23)	(28/73)	(97/171)	(5/10)
M6	435	11-12-13	263	141	122	28.1 (0-73)	%	61.7	52.5	57.4	59.1	55.7	56.5	80.0
							(95% CI)	(53.1-69.7)	(43.3-61.5)	(51.2-63.4)	(36.7-78.5)	(43.4-67.4)	(48.5-64.2)	(44.2-96.5)
							(n/N)	(87/141)	(64/122)	(151/263)	(13/22)	(39/70)	(91/161)	(8/10)
M9	449	05-03-14	301	155	146	25.9 (0-73)	%	47.7	49.3	48.5	43.2	45.2	52.1	36.4
							(95% CI)	(39.7-55.9)	(41.0-57.7)	(42.8-54.3)	(27.5-60.4)	(34.5-56.4)	(44.3-59.8)	(12.4-68.4)
							(n/N)	(74/155)	(72/146)	(146/301)	(16/37)	(38/84)	(88/169)	(4/11)
M12	469	26-05-14	298	160	138	25.1 (0-73)	%	41.9	39.1	40.6	31.7	47.6	38.4	55.6
							(95% CI)	(34.2-49.9)	(31.1-47.8)	(35.0-46.4)	(18.6-48.2)	(36.7-58.7)	(31.0-46.4)	(22.7-84.7)
							(n/N)	(67/160)	(54/138)	(121/298)	(13/41)	(40/84)	(63/164)	(5/9)
M15	485	21-08-14	300	162	138	26.0 (0-70)	%	94.4	96.4	95.3	81.0	97.1	97.8	100.0
							(95% CI)	(89.4-97.3)	(91.3-98.7)	(92.1-97.3)	(65.4-90.9)	(89.1-99.5)	(94.0-99.3)	(65.6-100.0)
							(n/N)	(153/162)	(133/138)	(286/300)	(34/42)	(68/70)	(174/178)	(10/10)
M18	495	13-11-14	282	154	128	24.6 (0-70)	%	86.4	83.6	85.1	63.3	84.6	91.2	100.0
							(95% CI)	(79.7-91.2)	(75.8-89.3)	(80.3-89.0)	(48.3-76.2)	(73.1-92.0)	(85.4-94.9)	(62.9-100.0)
							(n/N)	(133/154)	(107/128)	(240/282)	(31/49)	(55/65)	(145/159)	(9/9)

Village		HKT												
Survey of blood collection	Total population	Characteristic of blood spot						Antibody prevalence % , (n/N) , (95%CI)						
								Sex			Age			
		Blood Collection date	Number of dry blood spot	Male	Female	Median age (range) (y)		Male	Female	Total	0-4	5-15	15-59	60 up
M0	899	04-07-13	490	241	249	26.4 (0-94)	%	92.9	94.8	93.9	91.7	92.2	95.0	94.4
							(95% CI)	(88.8-95.7)	(91.0-97.1)	(91.3-95.8)	(71.5-98.6)	(86.8-95.6)	(91.6-97.2)	(70.6-99.7)
							(n/N)	(224/241)	(236/249)	(460/490)	(22/24)	(154/167)	(267/281)	(17/18)
M3	979	07-10-13	535	269	266	24.5 (0-89)	%	88.5	86.8	86.2	81.6	85.7	86.8	94.1
							(95% CI)	(80.6-89.4)	(82.0-90.5)	(83.9-89.0)	(67.5-90.8)	(79.6-90.3)	(82.2-90.4)	(69.2-99.7)
							(n/N)	(230/269)	(231/266)	(461/535)	(40/49)	(156/182)	(249/287)	(16/17)
M6	1029	08-01-14	500	243	257	22.1 (0-78)	%	78.2	85.6	82.0	75.8	81.9	84.8	63.6
							(95% CI)	(72.4-83.1)	(80.6-89.5)	(78.3-85.2)	(63.4-85.1)	(75.6-86.9)	(79.3-89.0)	(31.6-87.6)
							(n/N)	(190/243)	(220/257)	(410/500)	(50/66)	(158/193)	(195/230)	(7/11)
M9	1070	01-05-14	478	240	238	22.4 (0-78)	%	87.9	86.6	87.2	77.9	86.9	89.7	100.0
							(95% CI)	(83.0-91.6)	(81.4-90.5)	(83.8-90.0)	(65.9-86.7)	(80.6-91.4)	(84.9-93.2)	(62.9-100.0)
							(n/N)	(211/240)	(206/238)	(417/478)	(53/68)	(146/168)	(209/233)	(9/9)
M12	1192	24-06-14	527	269	258	21.7 (0-89)	%	85.1	89.1	87.1	87.6	88.8	85.7	84.6
							(95% CI)	(80.2-89.0)	(84.6-92.6)	(83.9-89.8)	(78.6-93.4)	(83.1-92.8)	(80.5-89.8)	(53.7-97.3)
							(n/N)	(229/269)	(230/258)	(459/527)	(78/89)	(166/187)	(204/238)	(11/13)
M15	1217	16-09-14	506	266	240	21.6 (0-78)	%	81.2	85.0	83.0	82.1	85.8	81.3	81.8
							(95% CI)	(75.9-85.6)	(79.7-89.1)	(79.4-86.1)	(71.9-89.3)	(79.6-90.4)	(75.6-85.9)	(47.8-96.8)
							(n/N)	(216/266)	(204/240)	(420/506)	(69/84)	(151/176)	(191/235)	(9/11)
M18	1236	08-12-14	467	233	234	20.0 (0-78)	%	82.8	87.2	85.0	84.0	85.4	84.9	85.7
							(95% CI)	(77.2-87.3)	(82.1-91.1)	(81.4-88.1)	(73.3-91.1)	(79.6-89.8)	(78.6-89.7)	(56.2-97.5)
							(n/N)	(193/233)	(204/234)	(397/467)	(63/75)	(170/199)	(152/179)	(12/14)

Supplementary data S4. Adjusted mean IgG response intensities are significantly different between all HBR classes

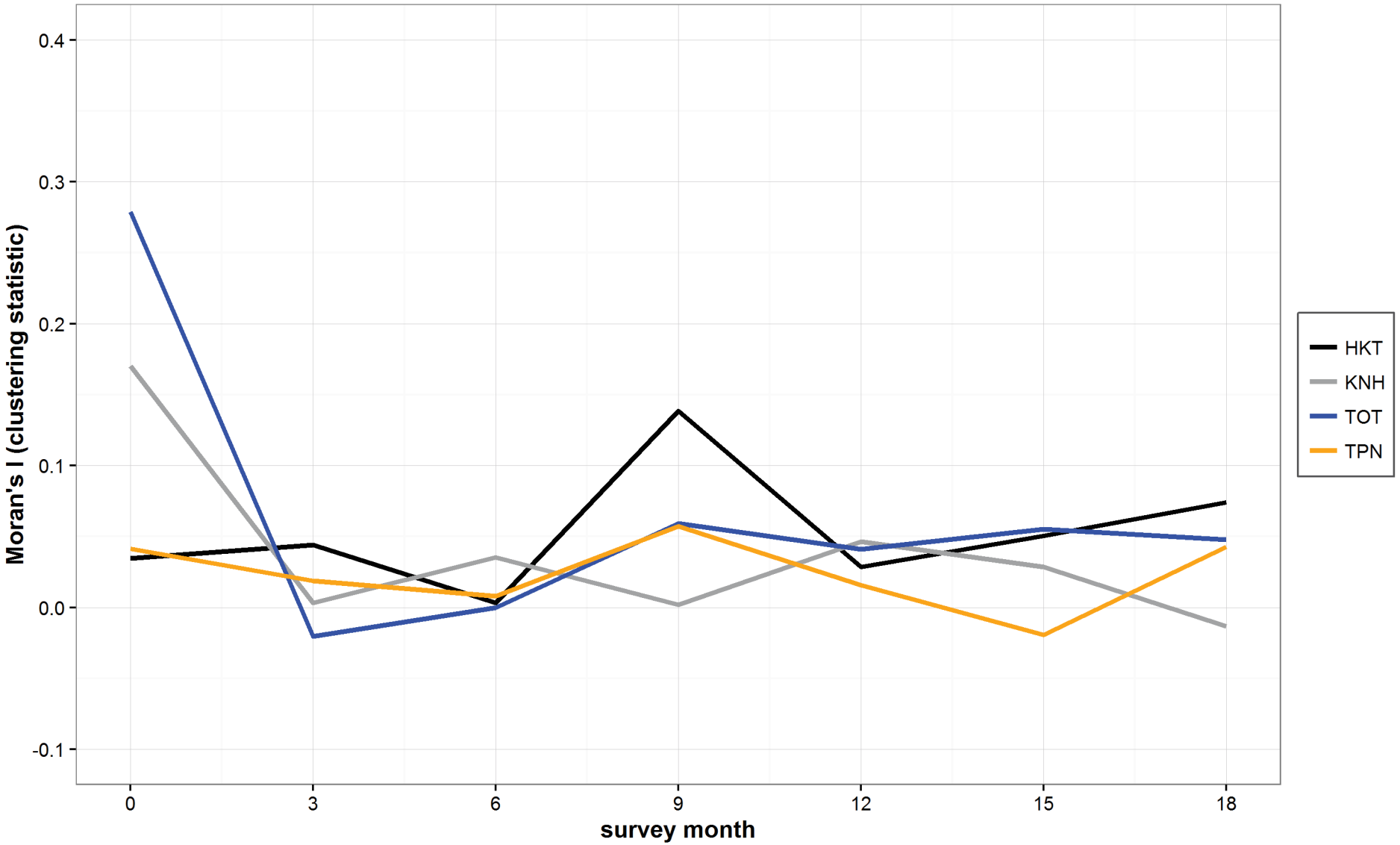


Dose-response relationship between the IgG antibody response against gSG6-P1 antigen and the abundance of Anopheles mosquitoes. The X-axis provides adjusted difference (with standard errors) estimated by the multivariate mixed model by taking the "low HBR" as reference class (data not shown). The mean Human biting rates of primary vectors (black) and total anopheles (grey) are categorized as medium, high and very high according to the quartile. HBR classes for total anopheles were <96 for low HBR (reference); [96 - 204[for medium HBR; [204 - 531[for high HBR and; ≥531 for very high HBR. HBR classes for primary malaria vectors were <46.5 for low HBR (reference); [46.5 - 159[for medium HBR; [159 - 468[(high HBR) and; >=468 very high HBR.

Supplementary data S5 - Moran's I statistics and associated p-values for villages by survey month (M0 – M18) and additional maps

*

Village	0		3		6		9		12		15		18	
	I	p-value	I	p-value	I	p-value	I	p-value	I	p-value	I	p-value	I	p-value
KNH	0.1702	0.0000	0.0031	0.7659	0.0354	0.0972	0.0019	0.7959	0.0465	0.0161	0.0284	0.1207	-0.0133	0.6583
TPN	0.0412	0.0867	0.0186	0.3084	0.0080	0.5798	0.0571	0.0021	0.0157	0.4169	-0.0193	0.5805	0.0427	0.0447
HKT	0.0347	0.0669	0.0440	0.0106	0.0034	0.7860	0.1384	0.0000	0.0284	0.0895	0.0503	0.0077	0.0741	0.0003
TOT	0.2789	0.0000	-0.0205	0.4534	-0.0001	0.8947	0.0592	0.0120	0.0411	0.1468	0.0552	0.0137	0.0477	0.1879


0.4
0.3
0.2
0.1
0.0
-0.1
Moran's I (clustering statistic)
0
3
6
9
12
15
18
survey month
HKT
KNH
TOT
TPN

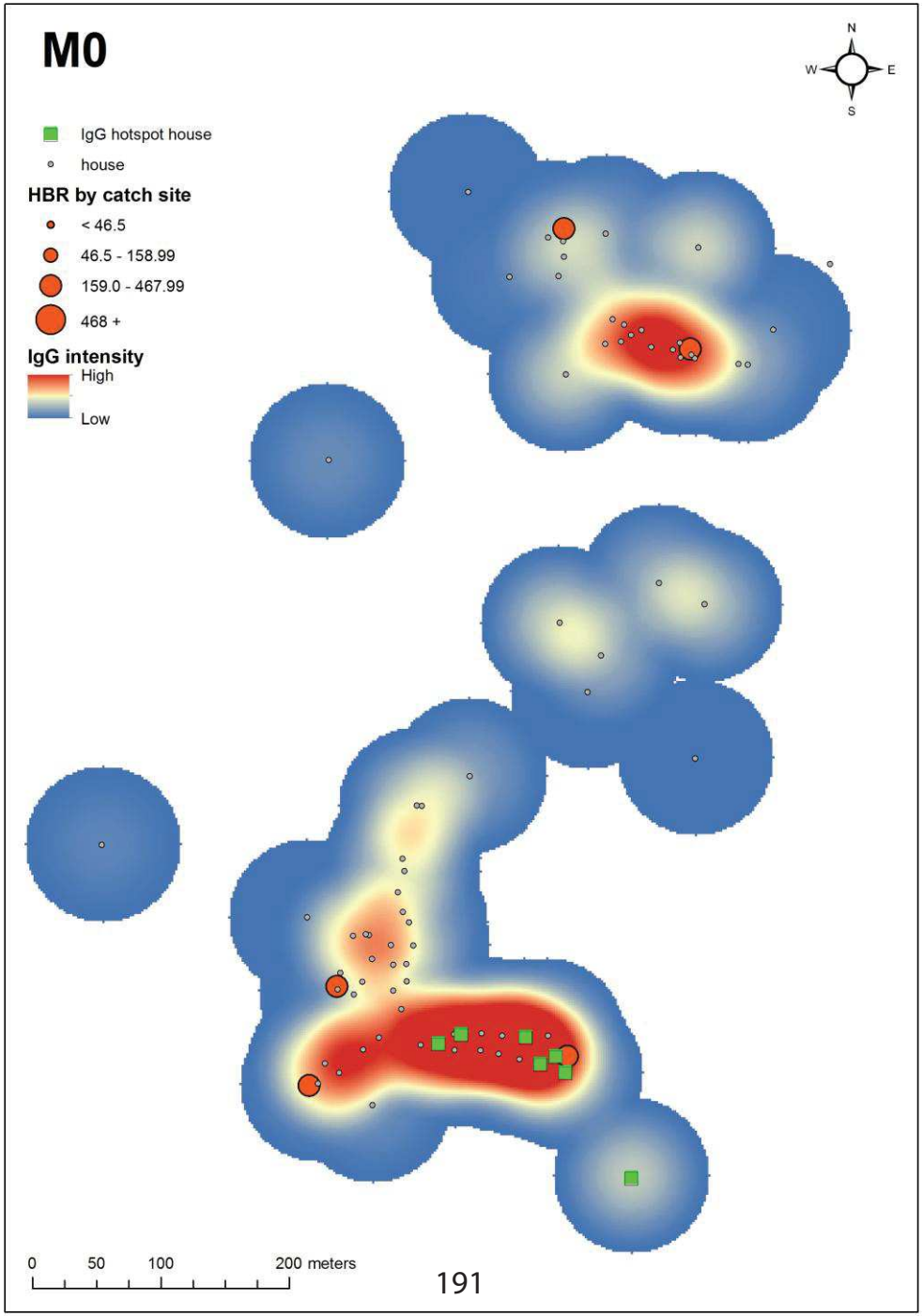
rainy season

cool season

hot season

* sig. Moran’s I

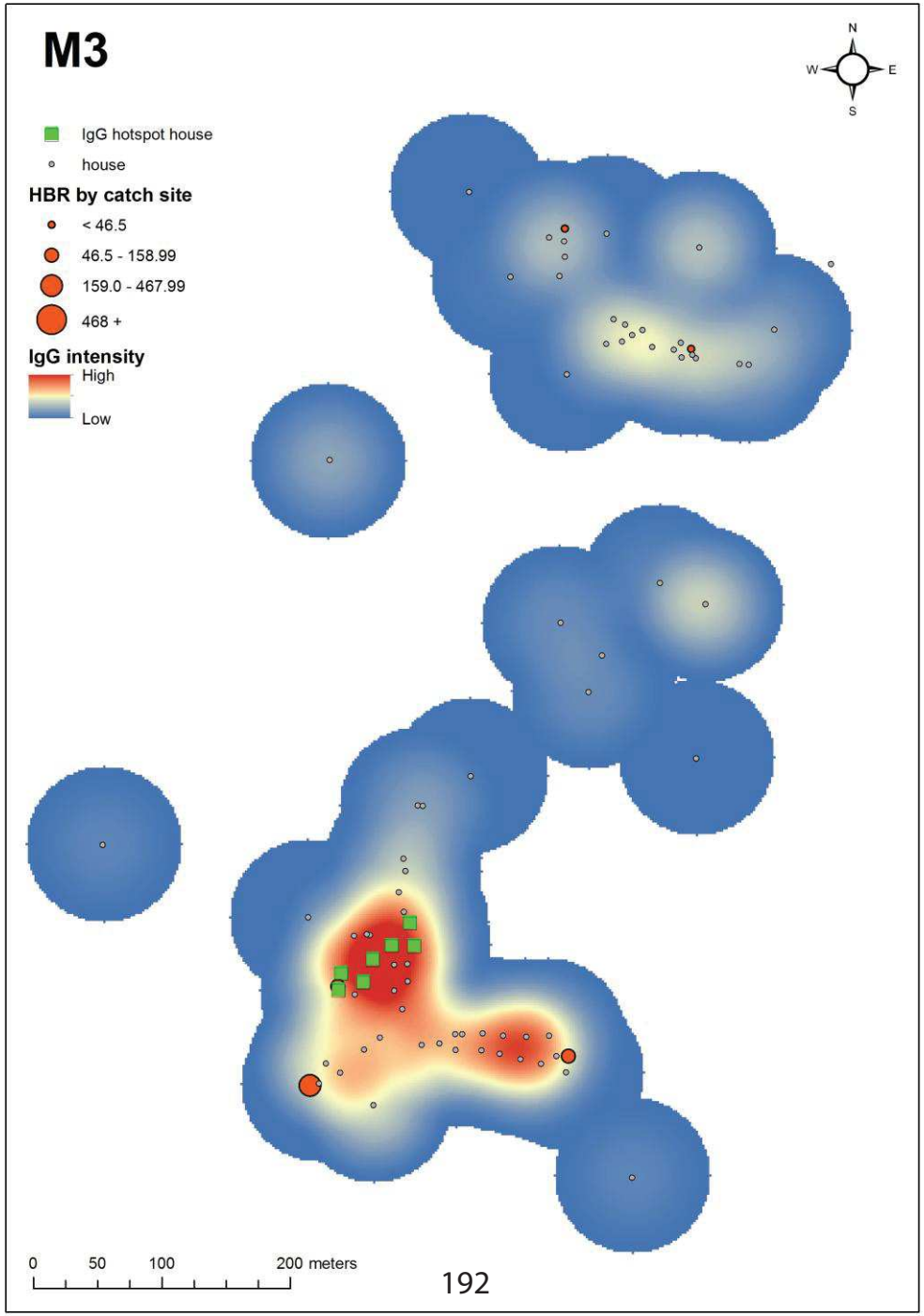
KNH


M0
N
W
E
S
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0
50
100
200 meters

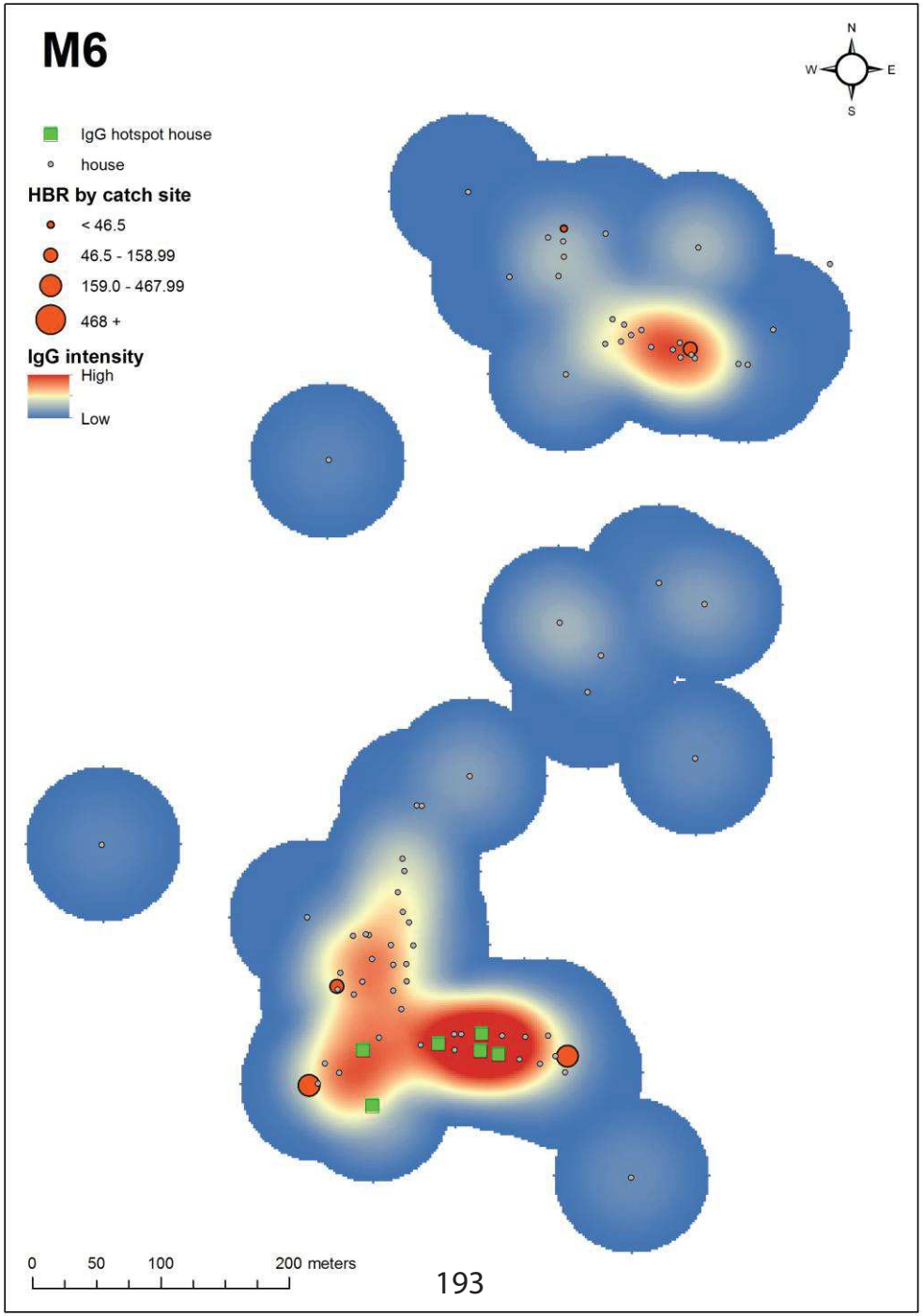
191

*

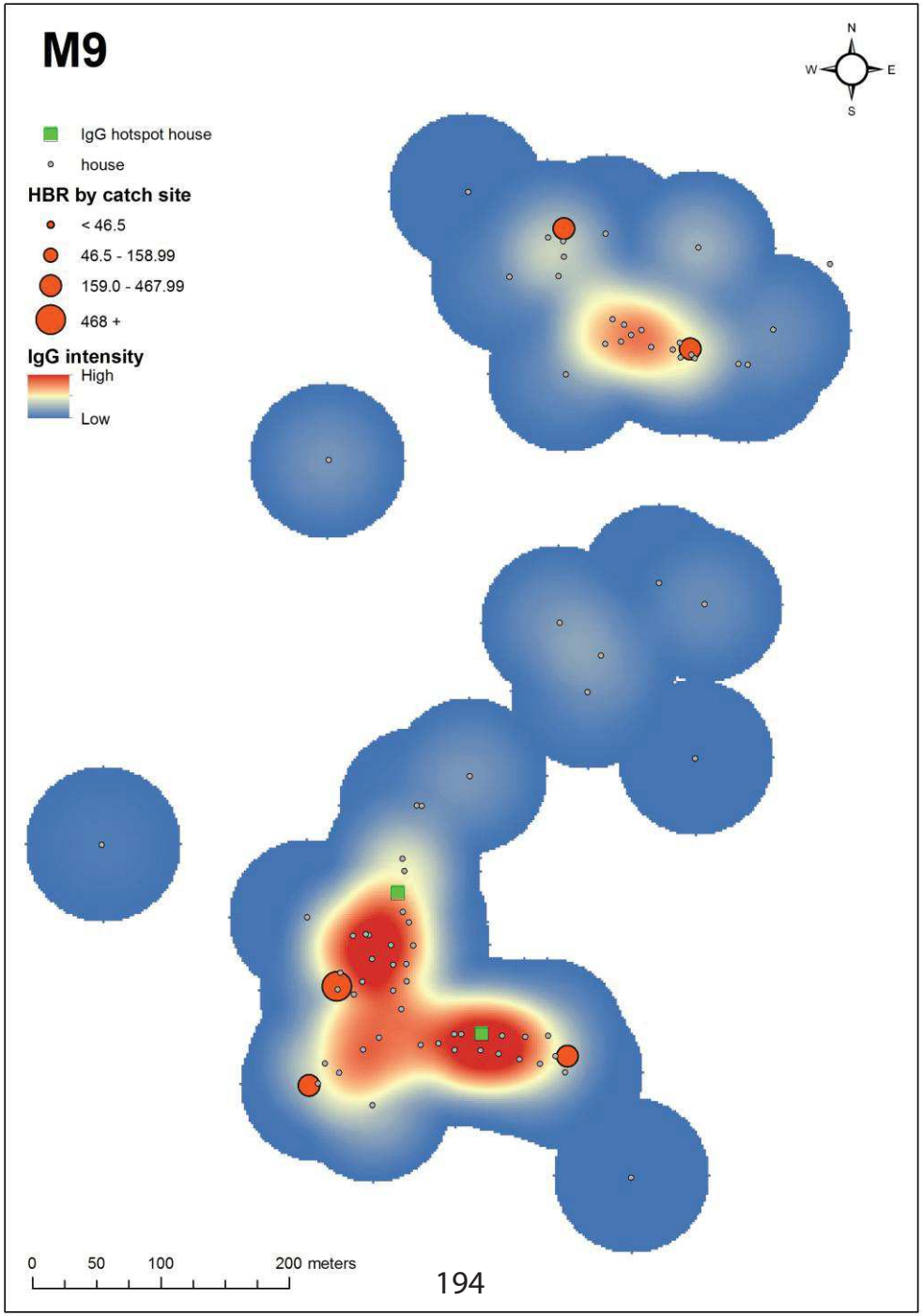
KNH


M3
N
W
E
S
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0
50
100
200 meters

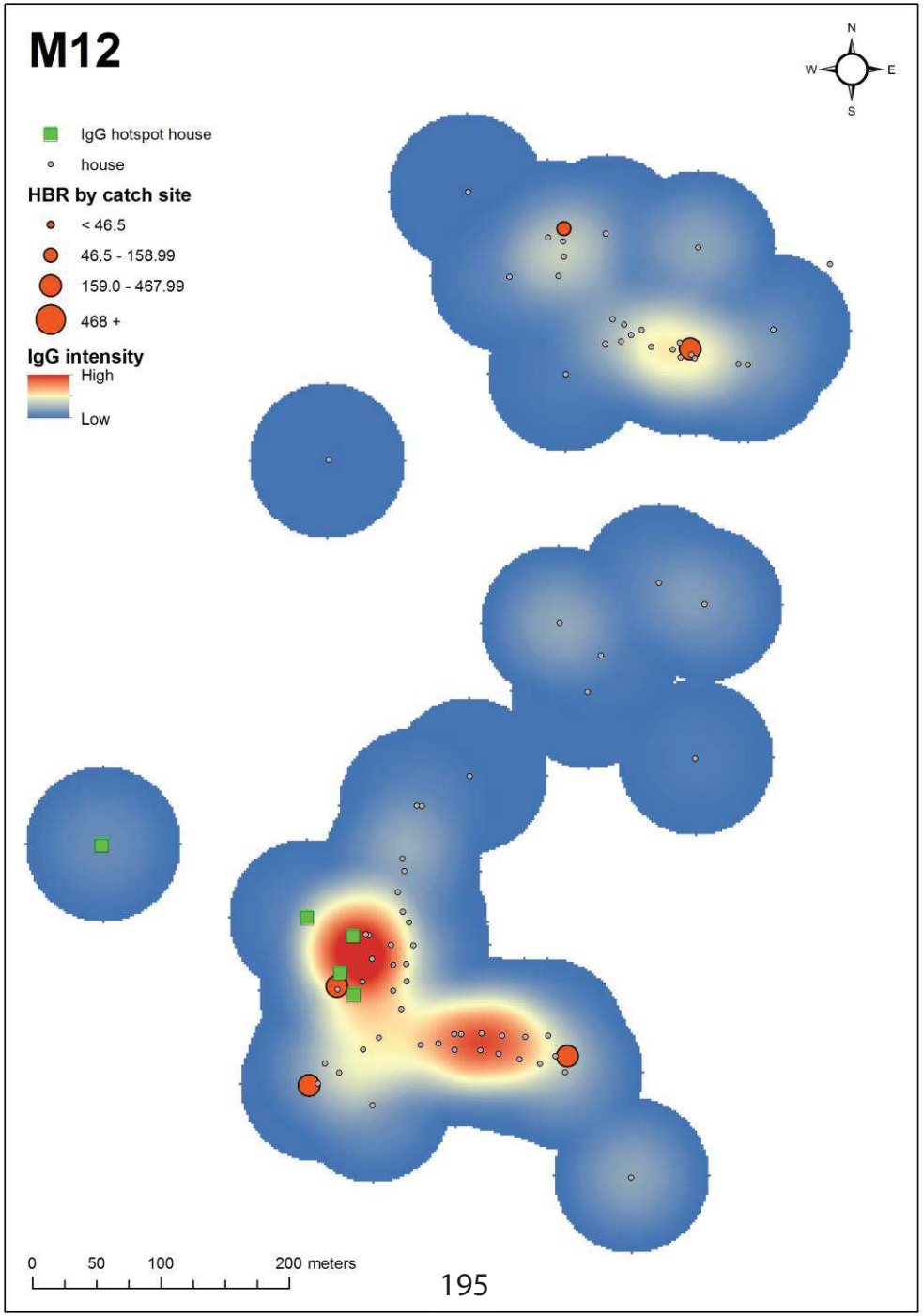
KNH


M6
N
W
E
S
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0
50
100
200 meters

KNH


M9
N
W
E
S
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0
50
100
200 meters

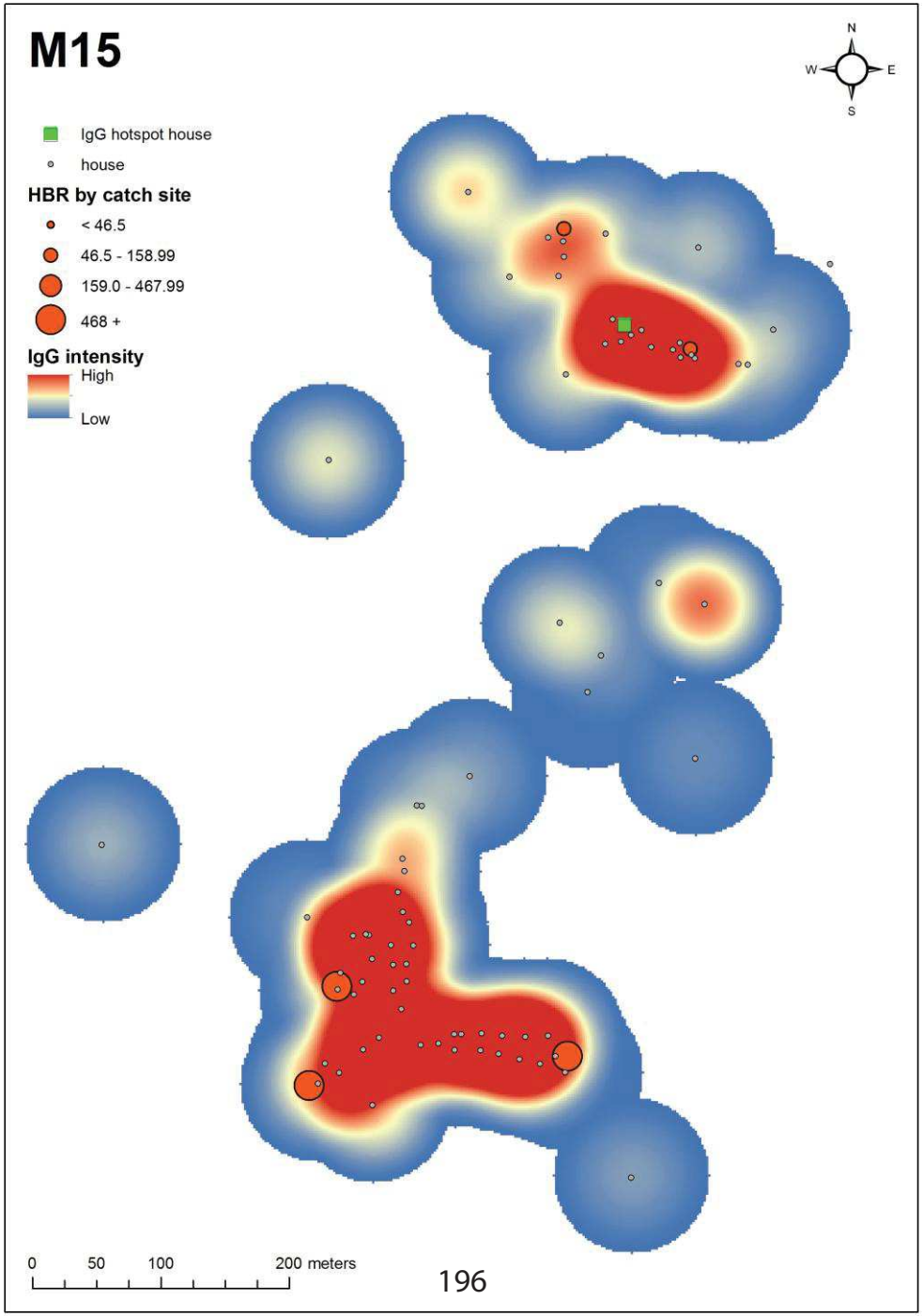
KNH


M12
N
W
E
S
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0
50
100
200 meters

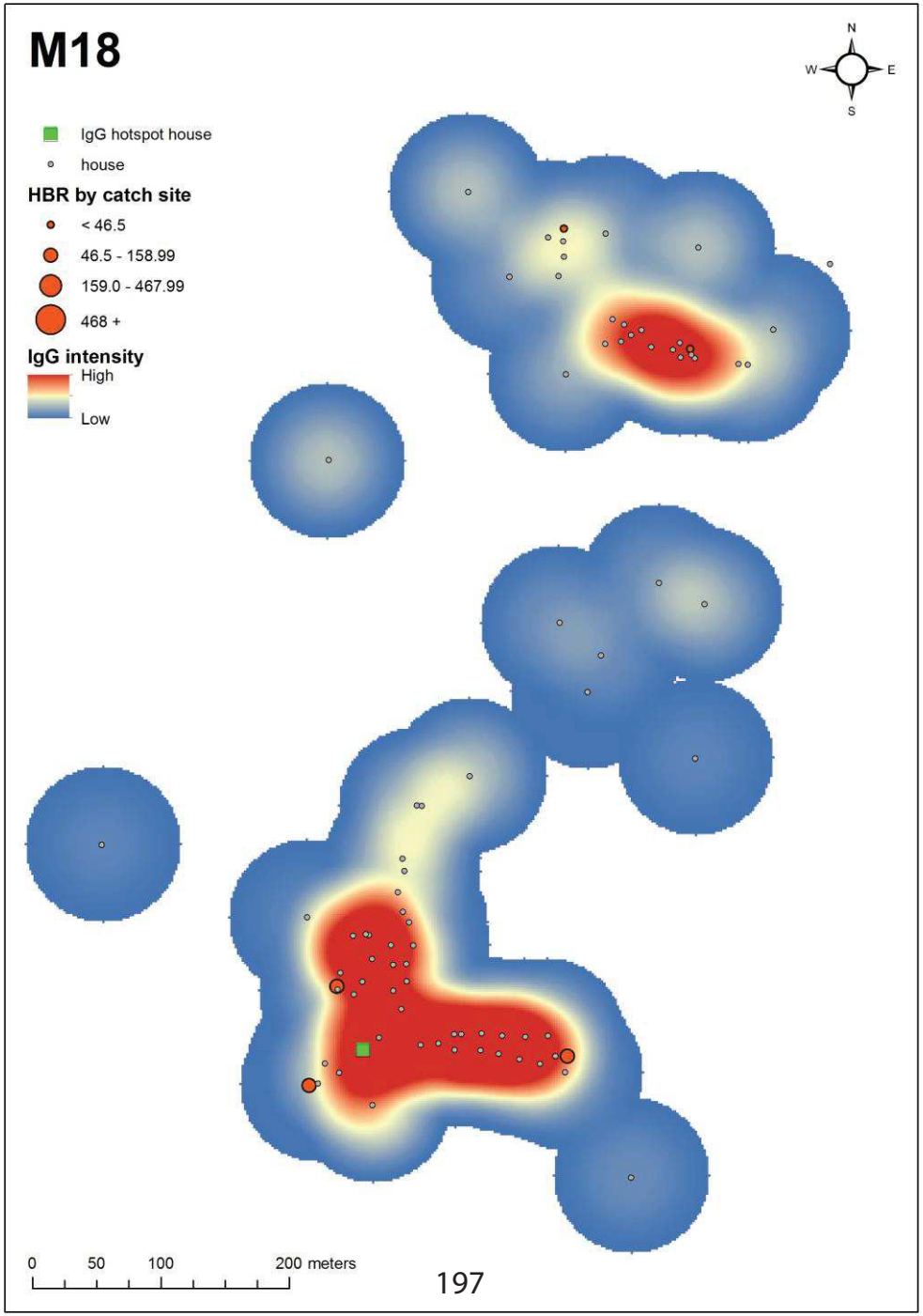
195

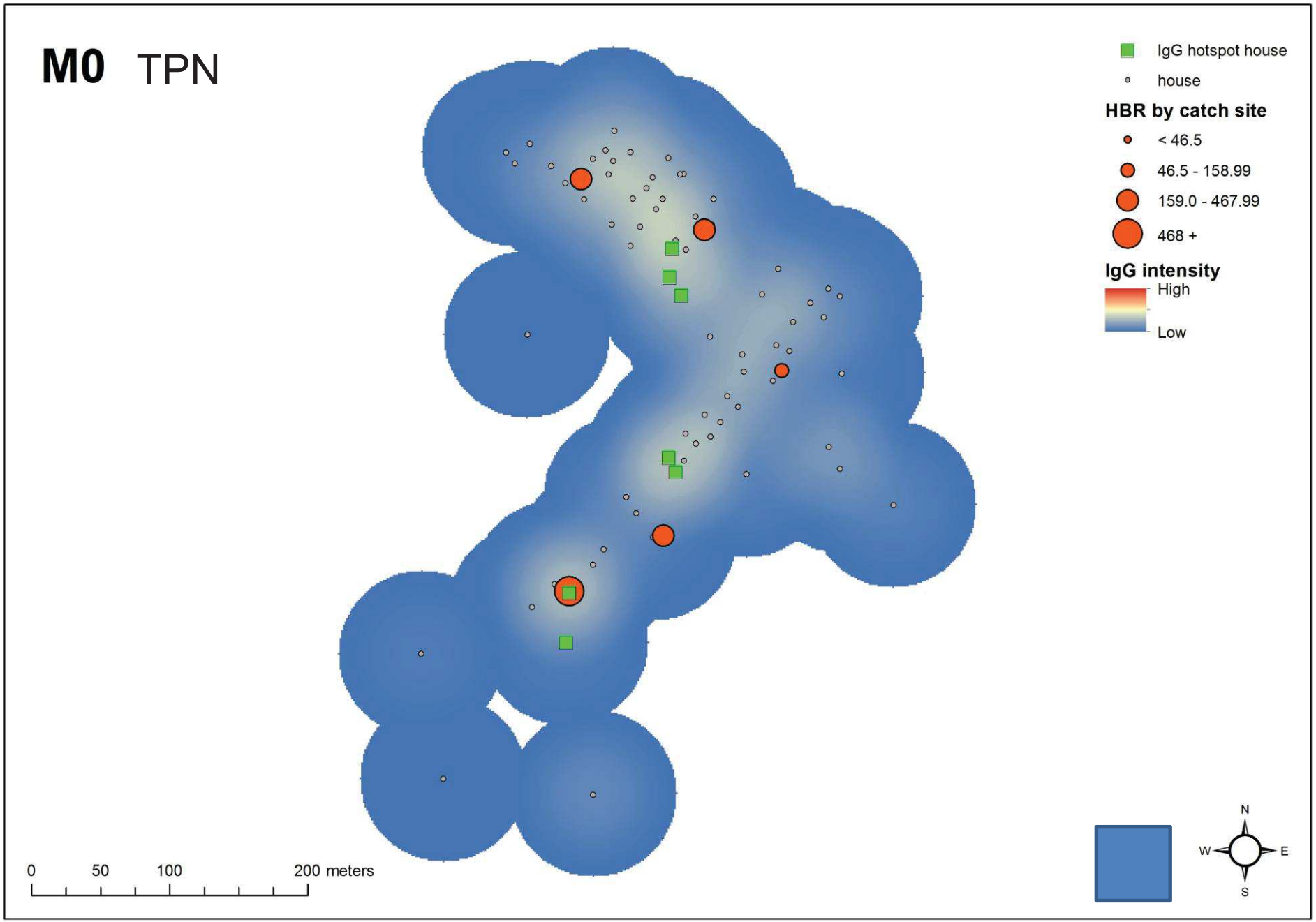
*

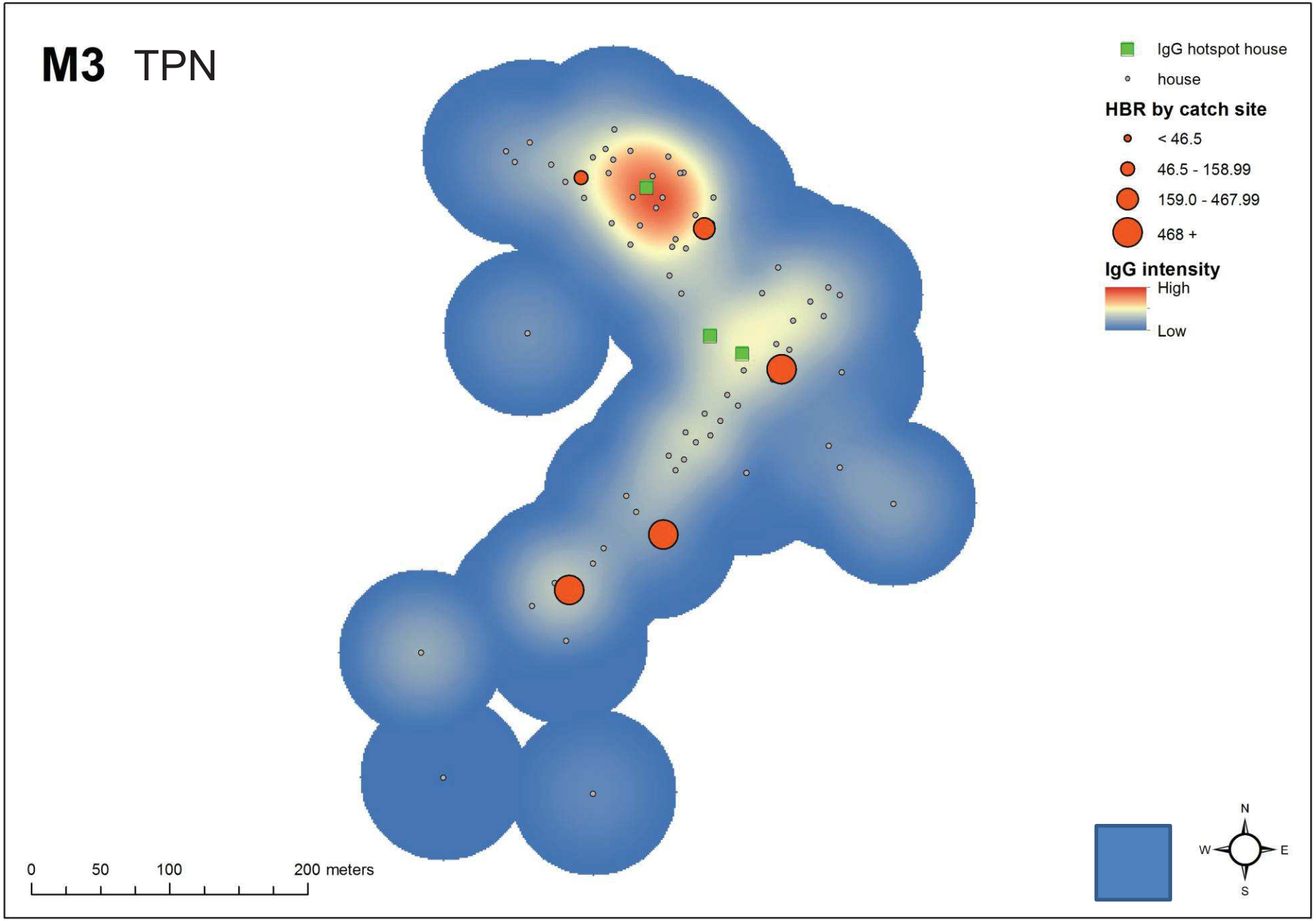
KNH

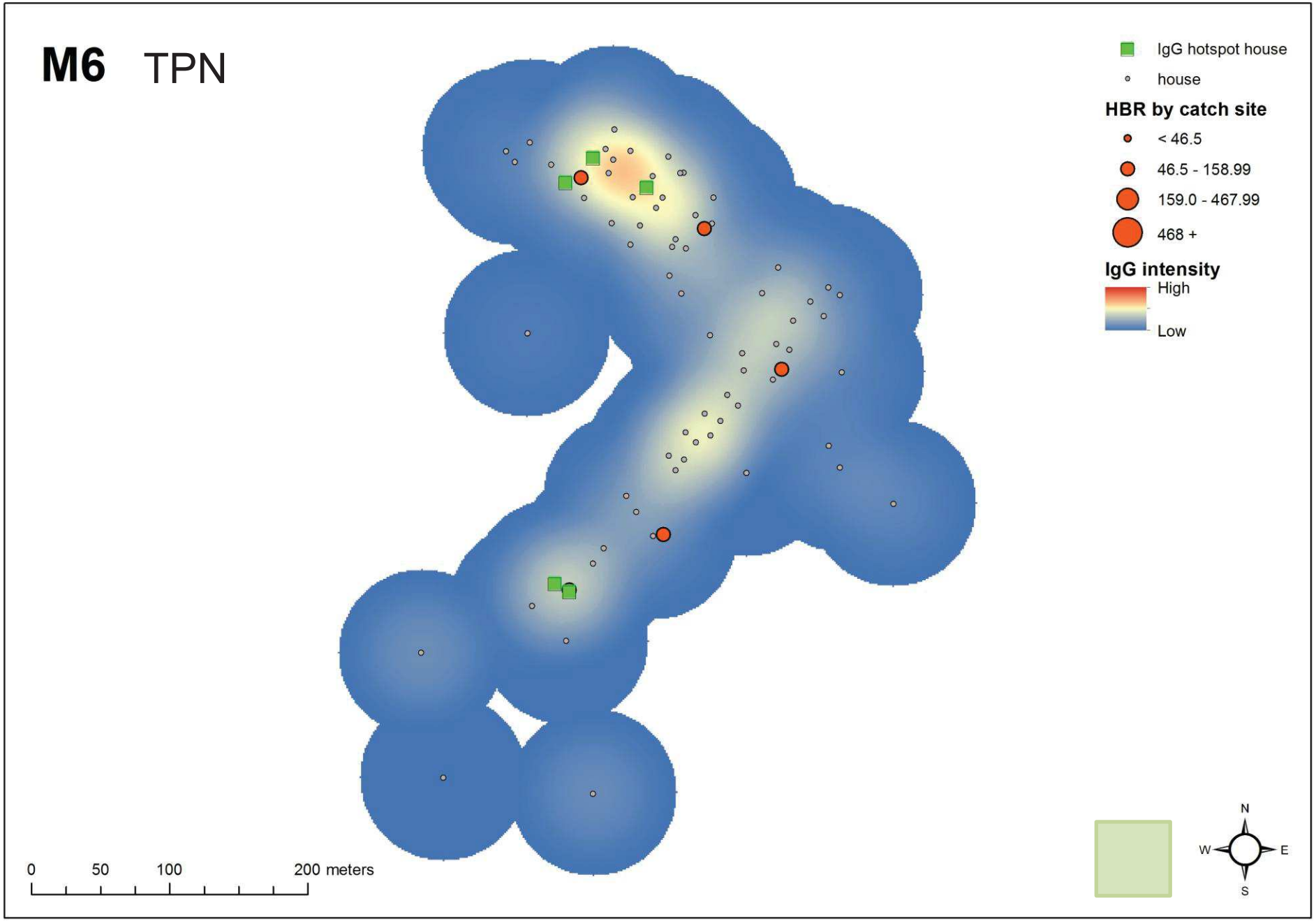

M15
N
W
E
S
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0
50
100
200 meters

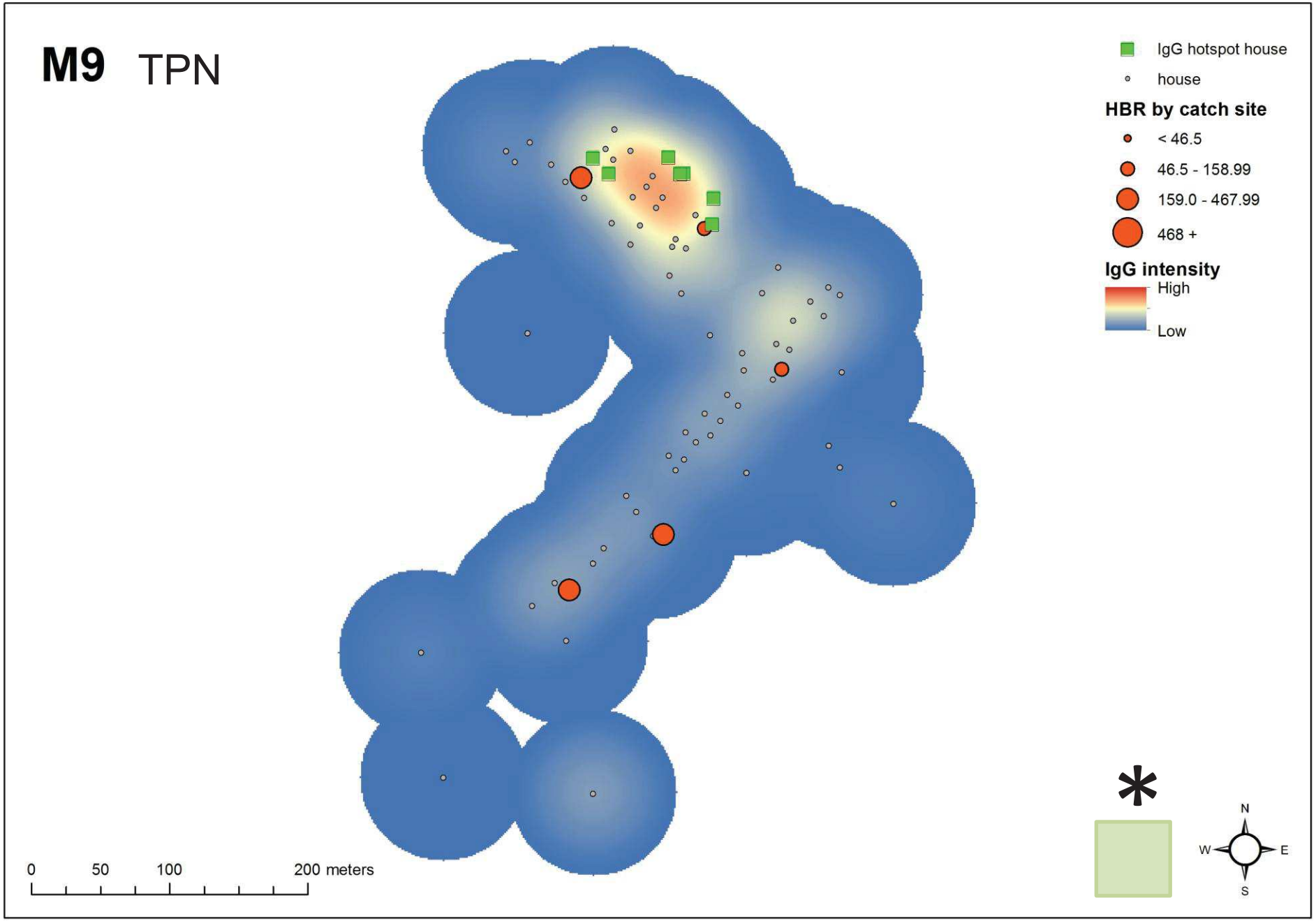
KNH

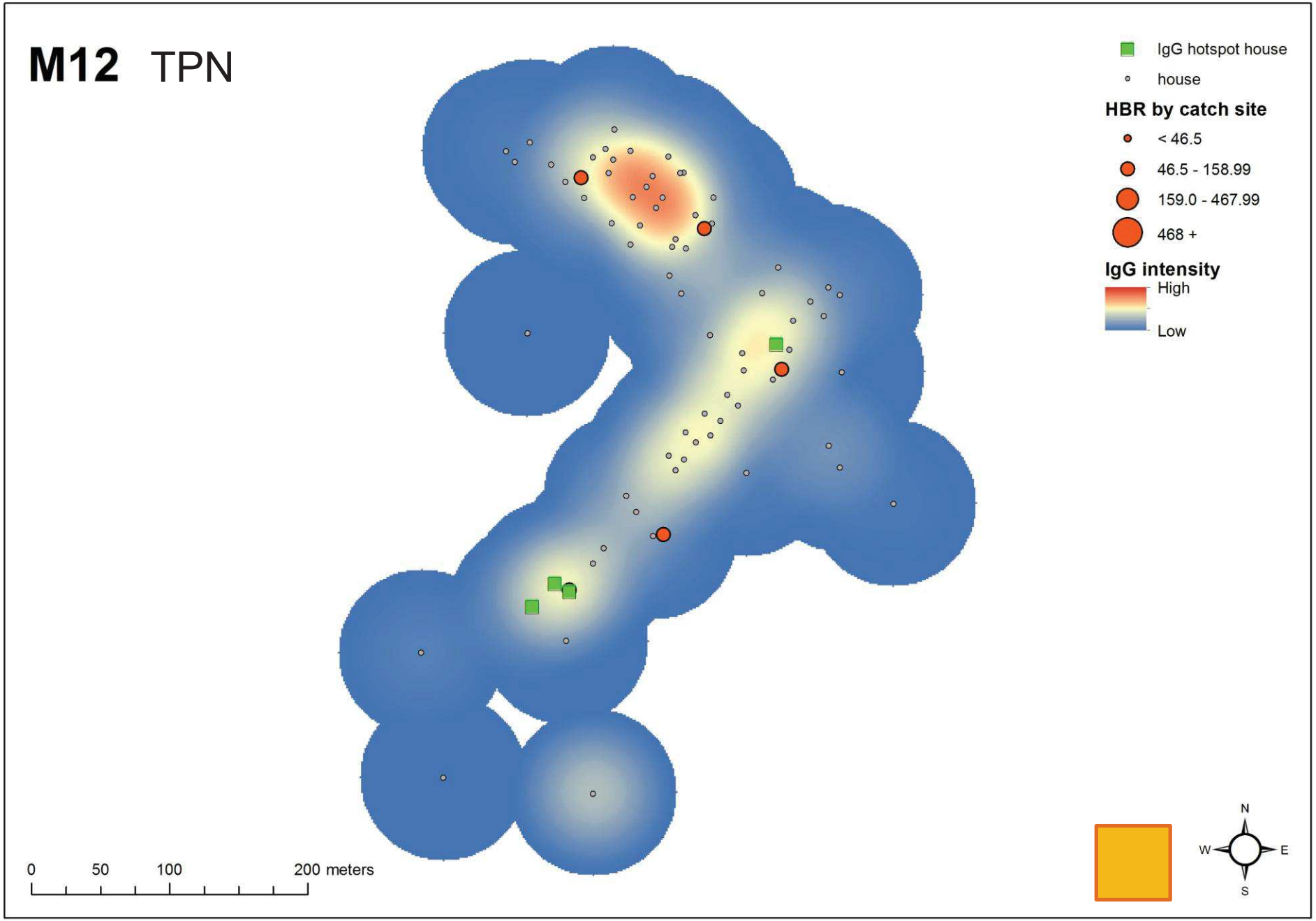

M18
N
W
E
S
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0
50
100
200 meters

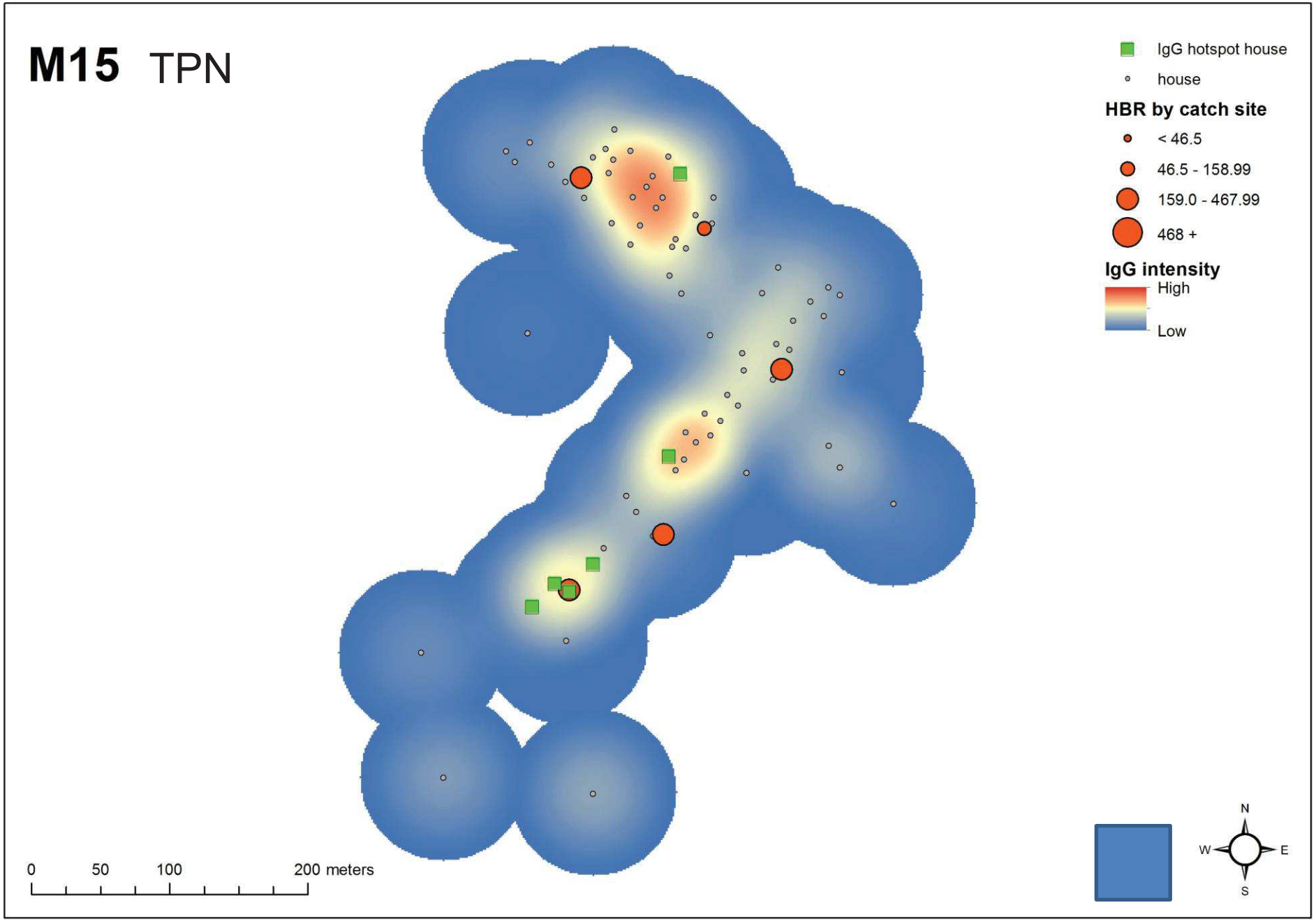

M0 TPN
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0
50
100
200 meters
N
W
E
S

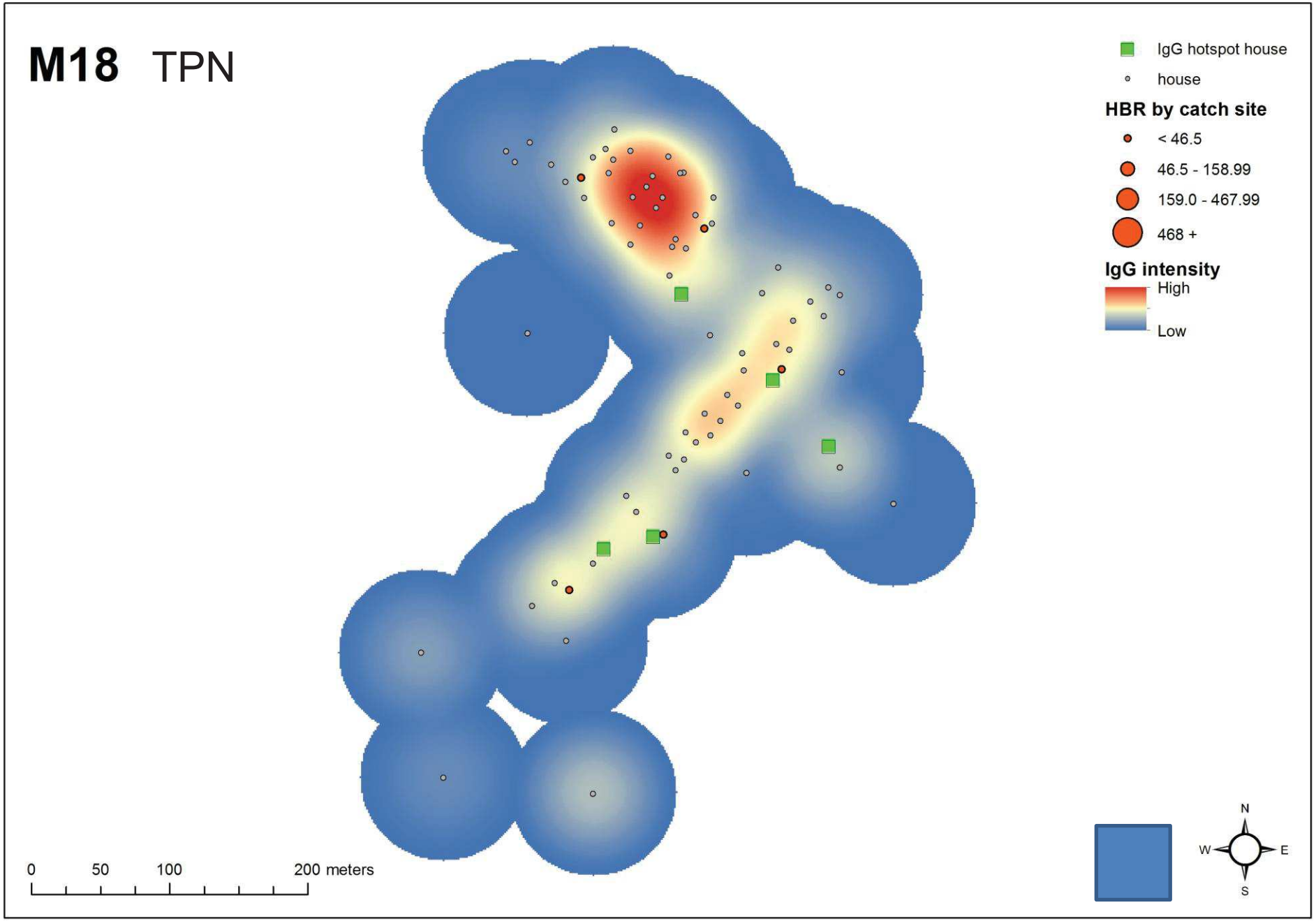

M3 TPN
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0
50
100
200 meters
N
W
E
S

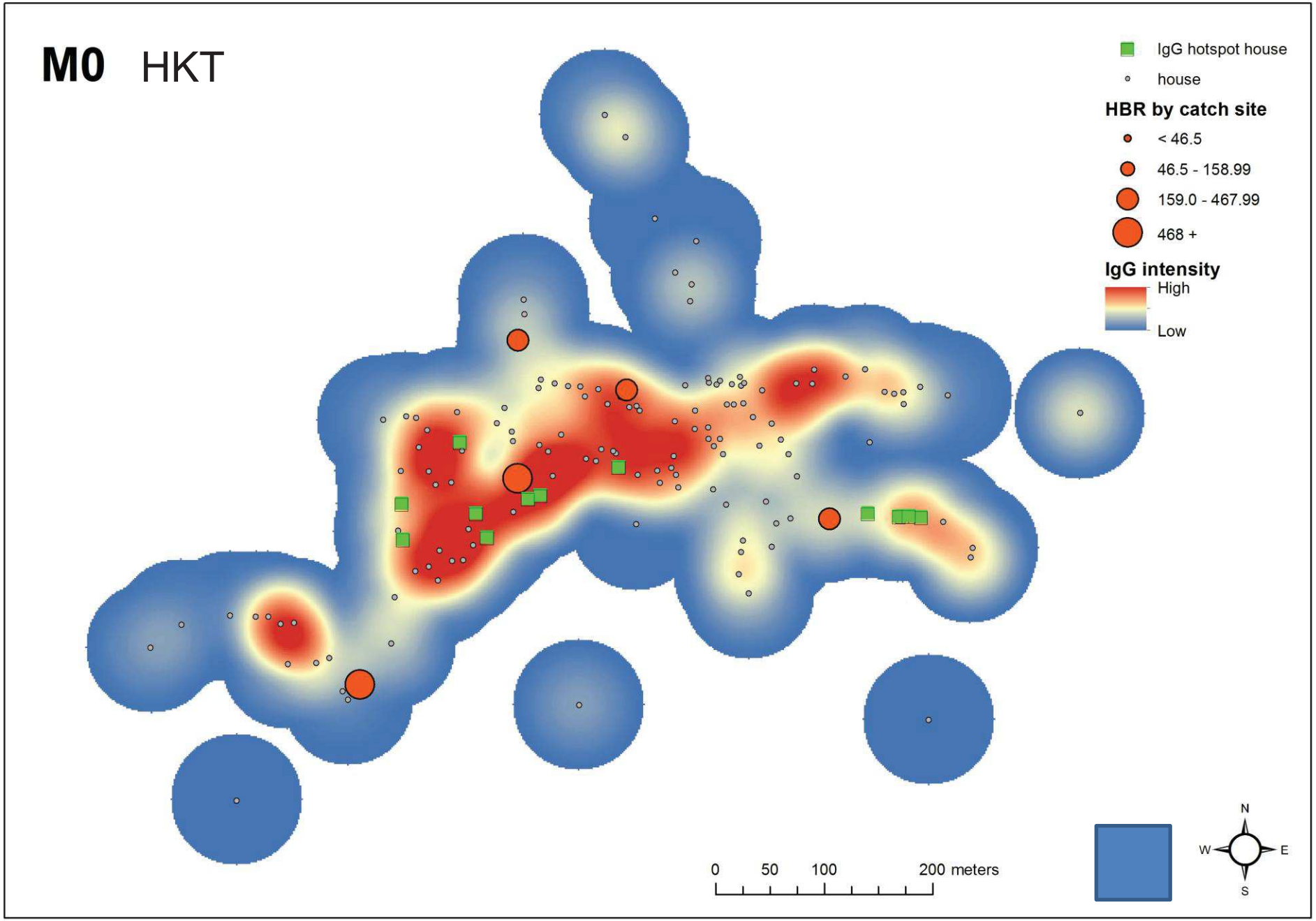

M6 TPN
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0
50
100
200 meters
N
W
E
S

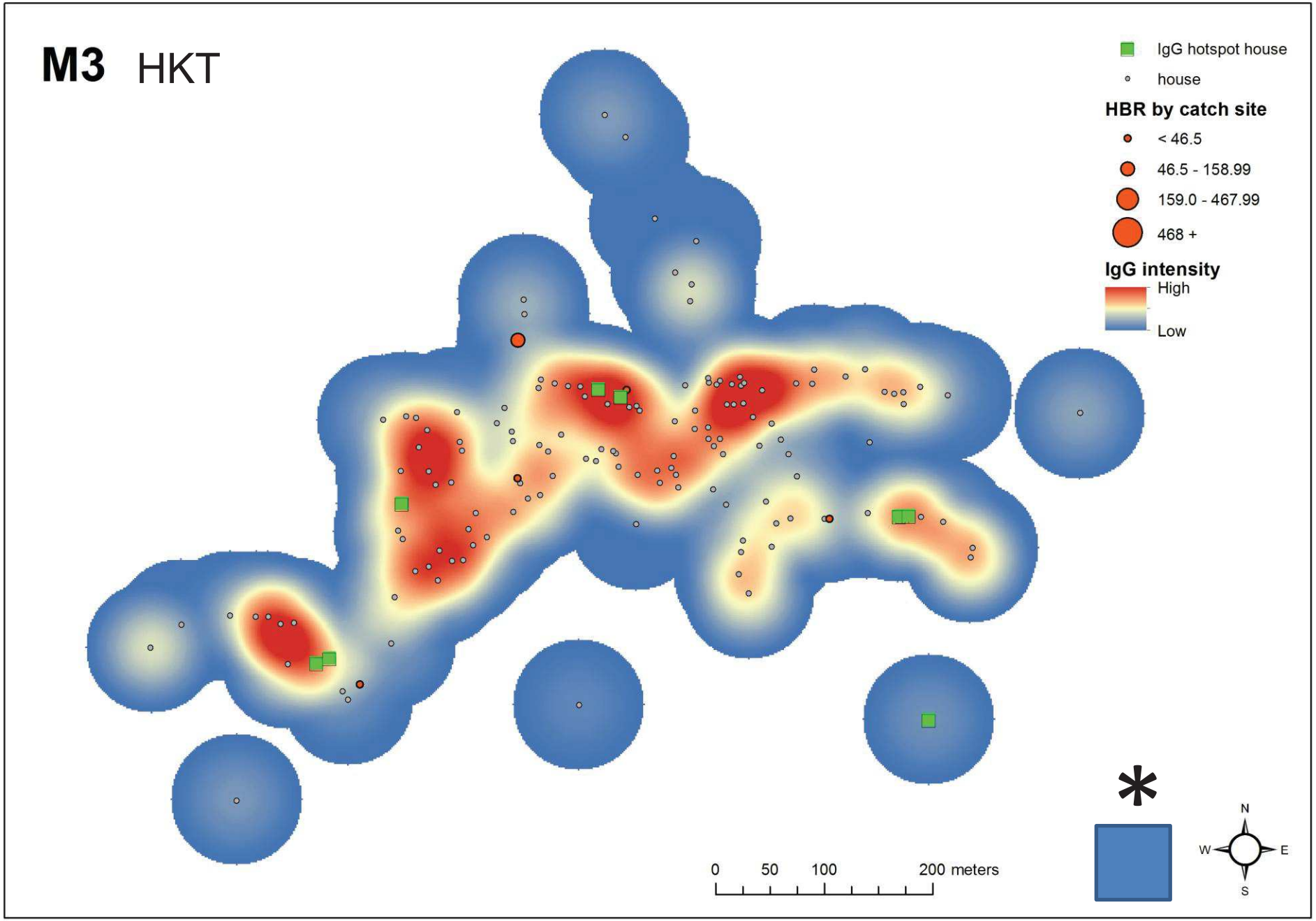

M9 TPN
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0
50
100
200 meters
N
W
E
S

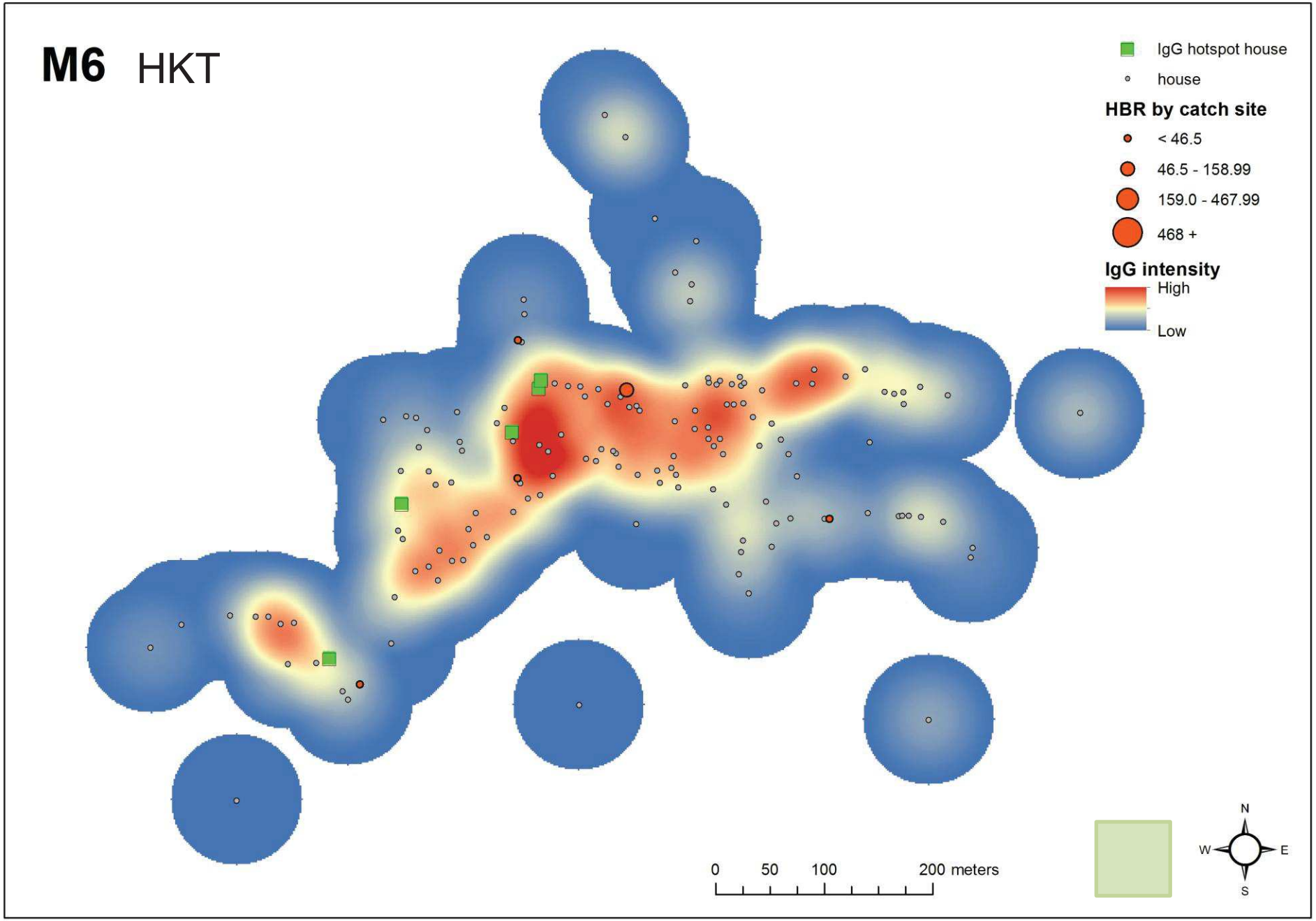

M12 TPN
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0
50
100
200 meters
N
W
E
S

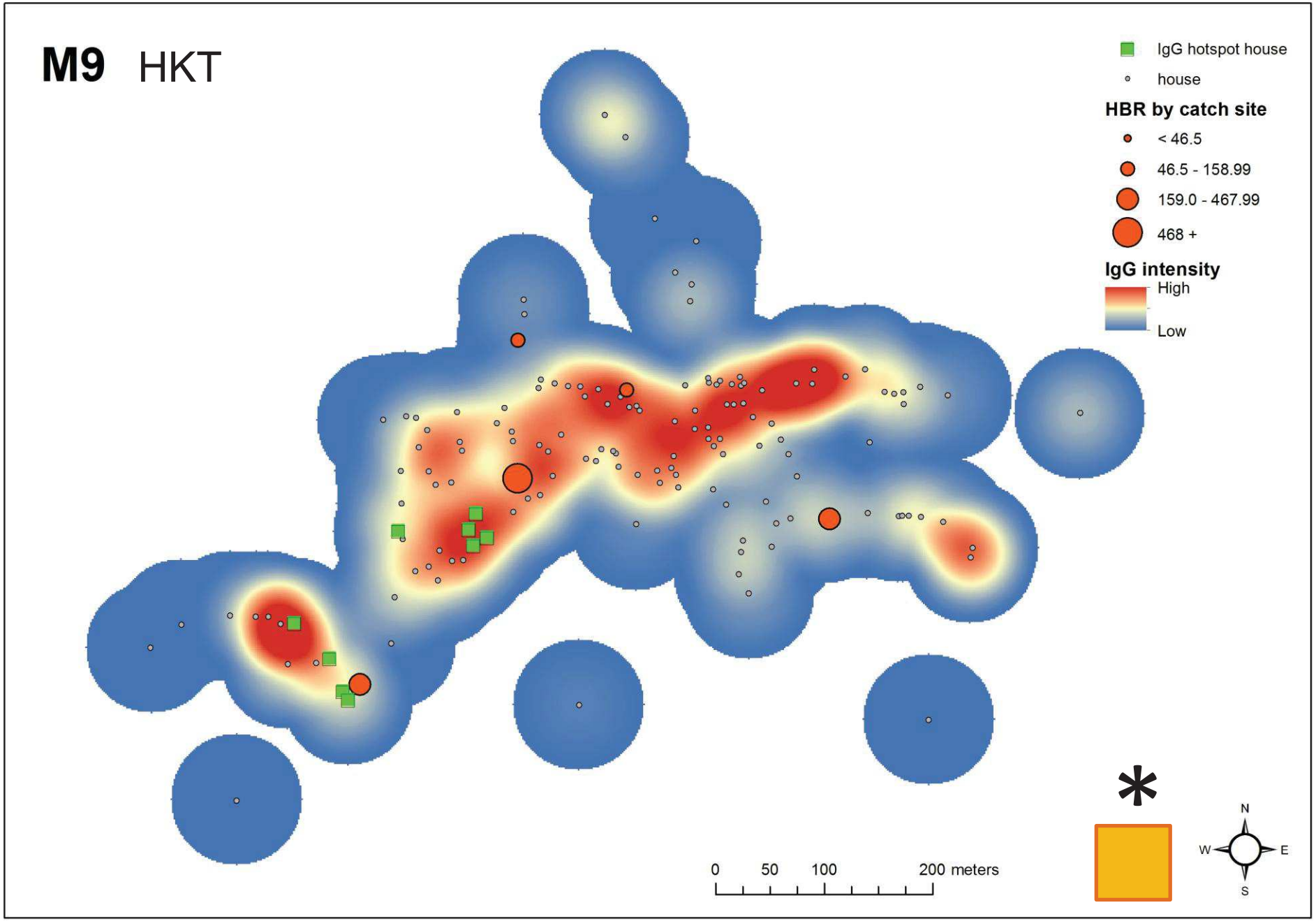

M15 TPN
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0
50
100
200 meters
N
W
E
S

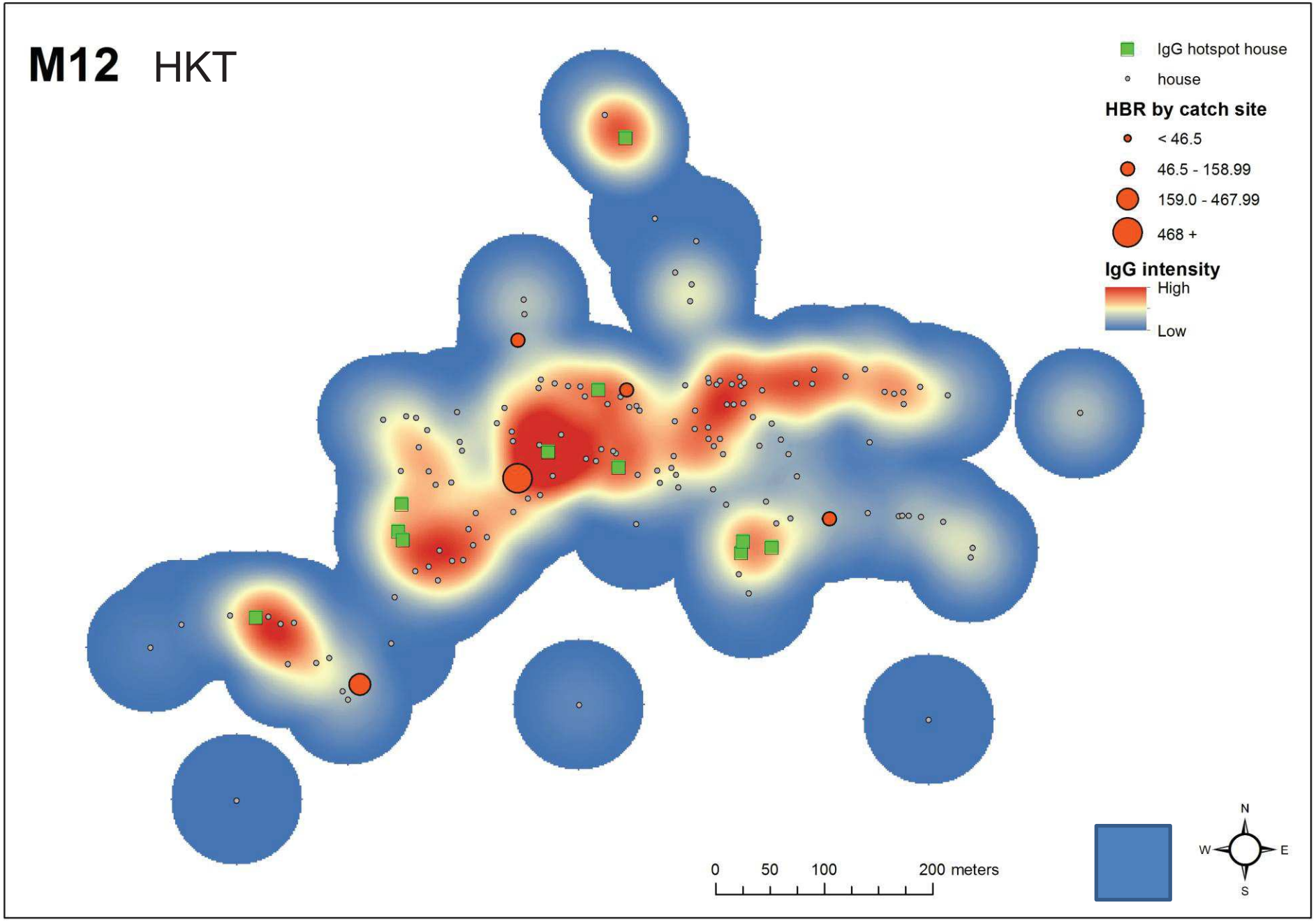

M18 TPN
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0
50
100
200 meters
N
W
E
S

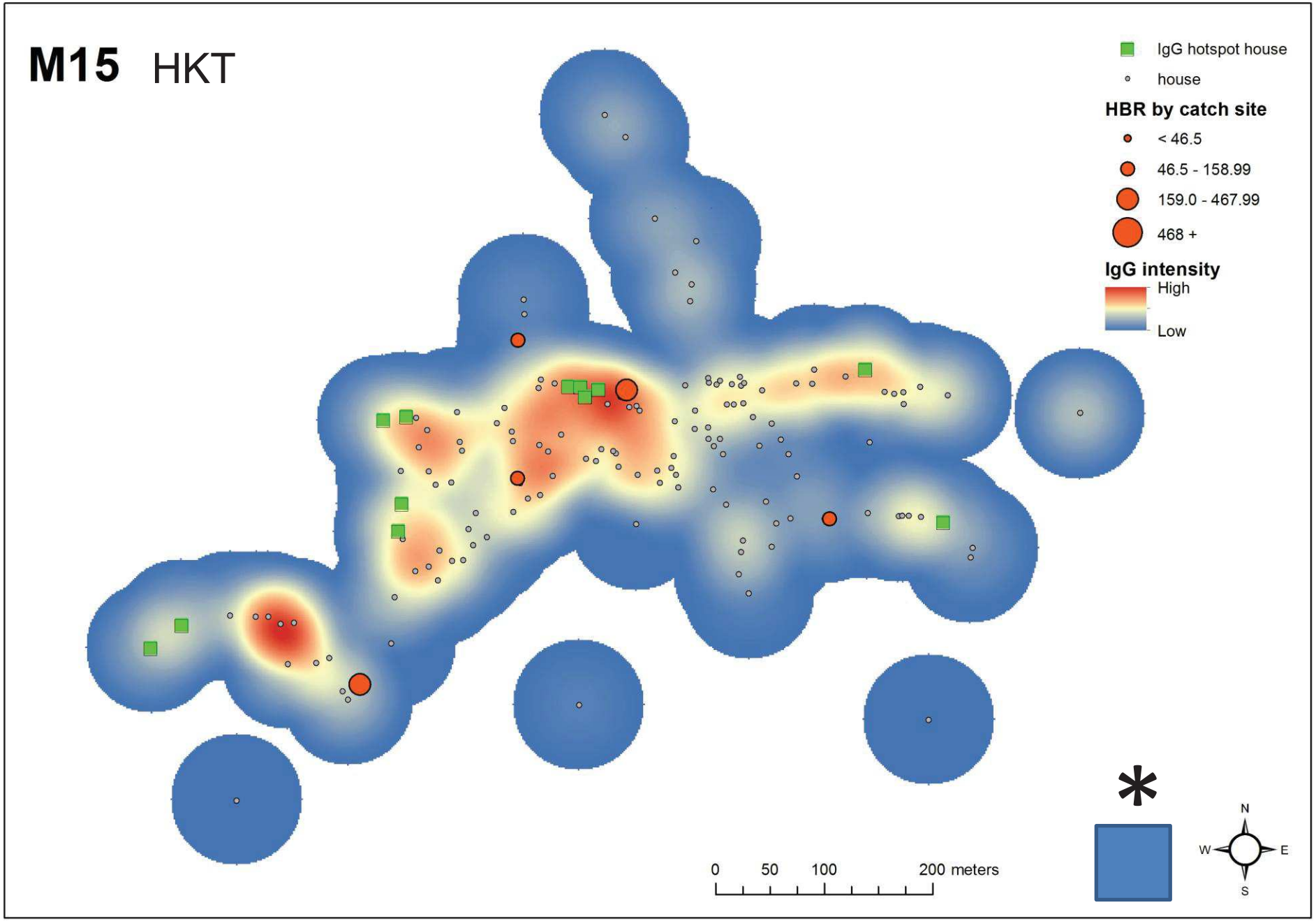

M0 HKT
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0 50 100 200 meters
N
W
E
S

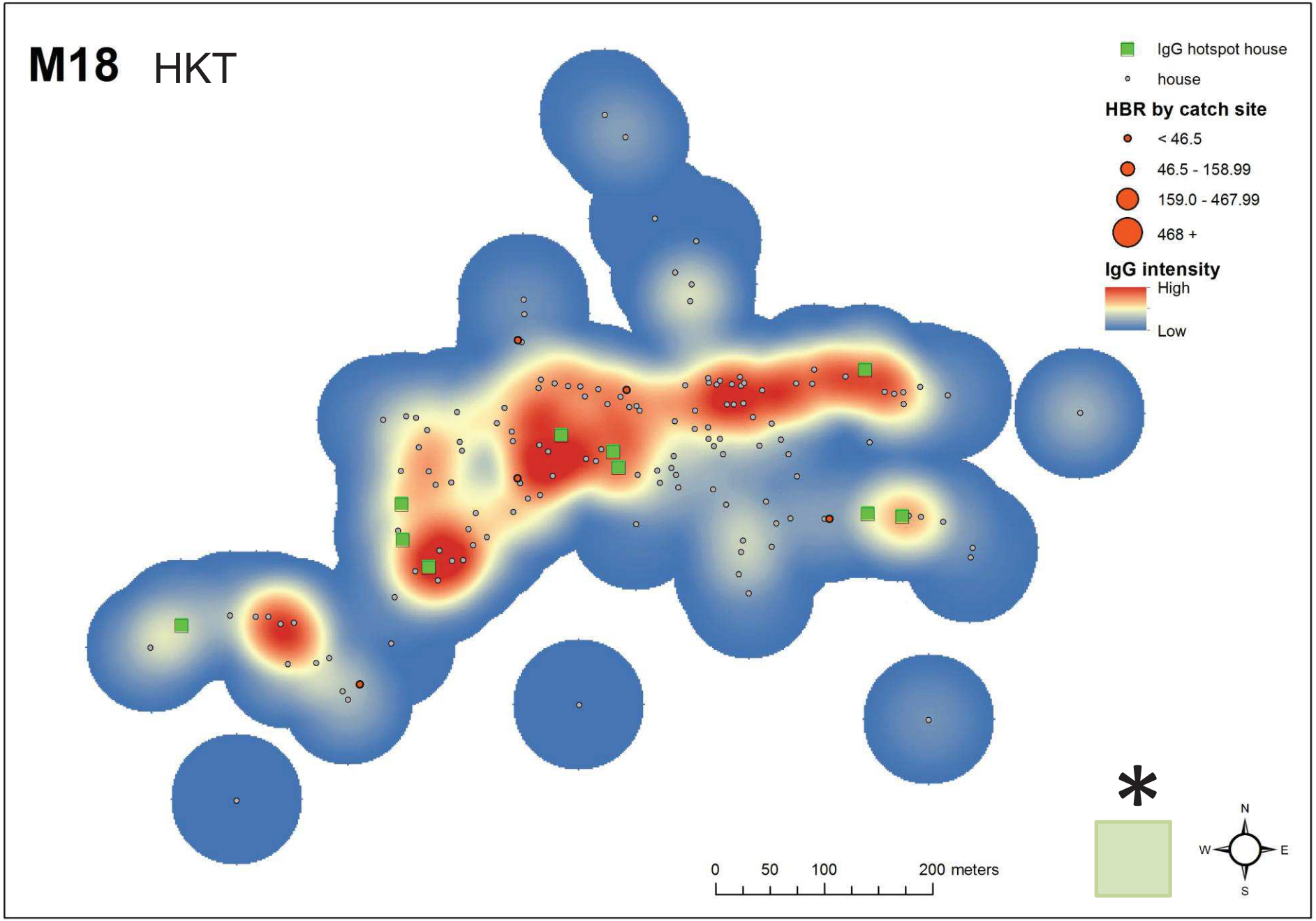

M3 HKT
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0 50 100 200 meters
N
W
E
S

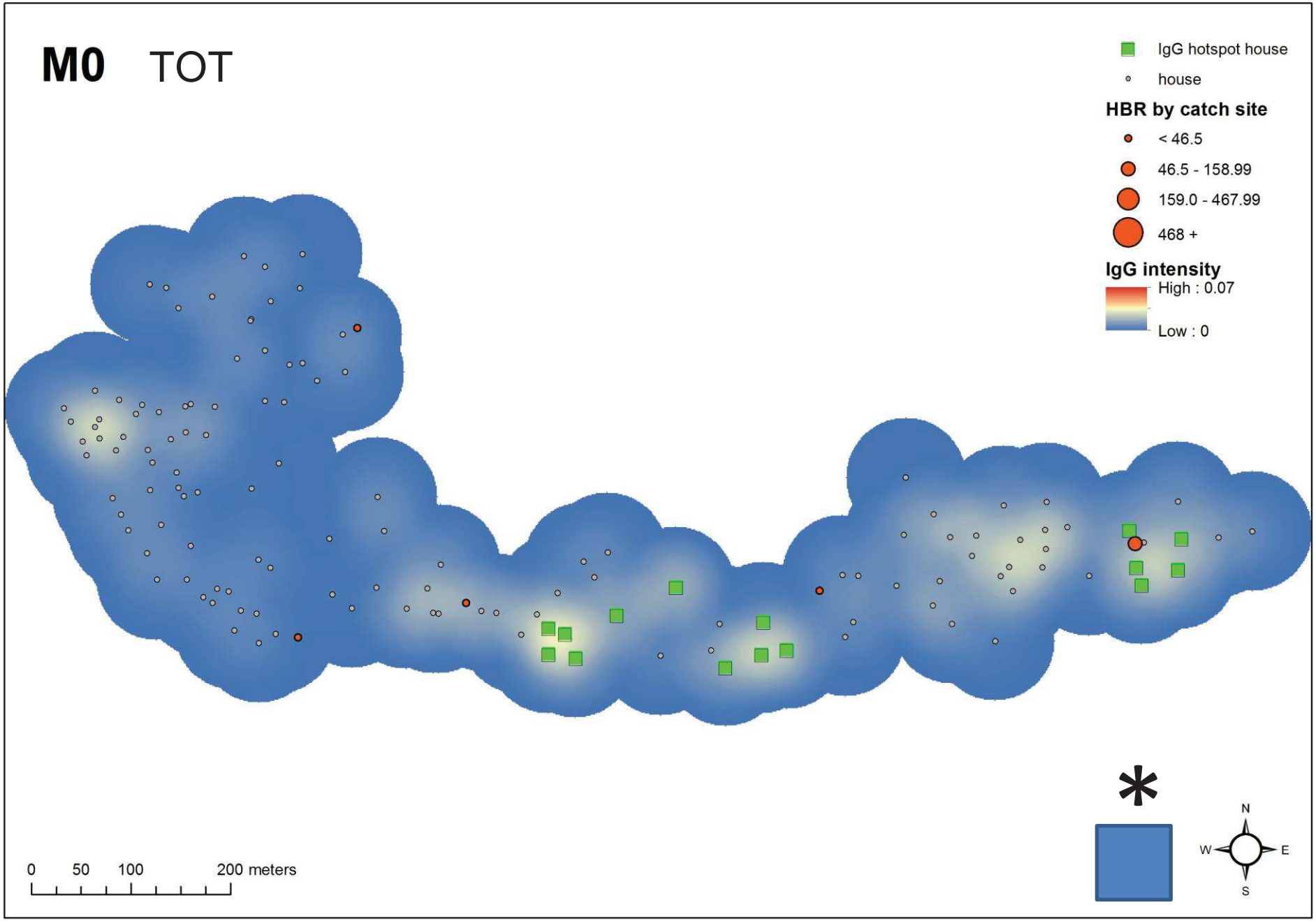

M6 HKT
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0 50 100 200 meters
N
W
E
S

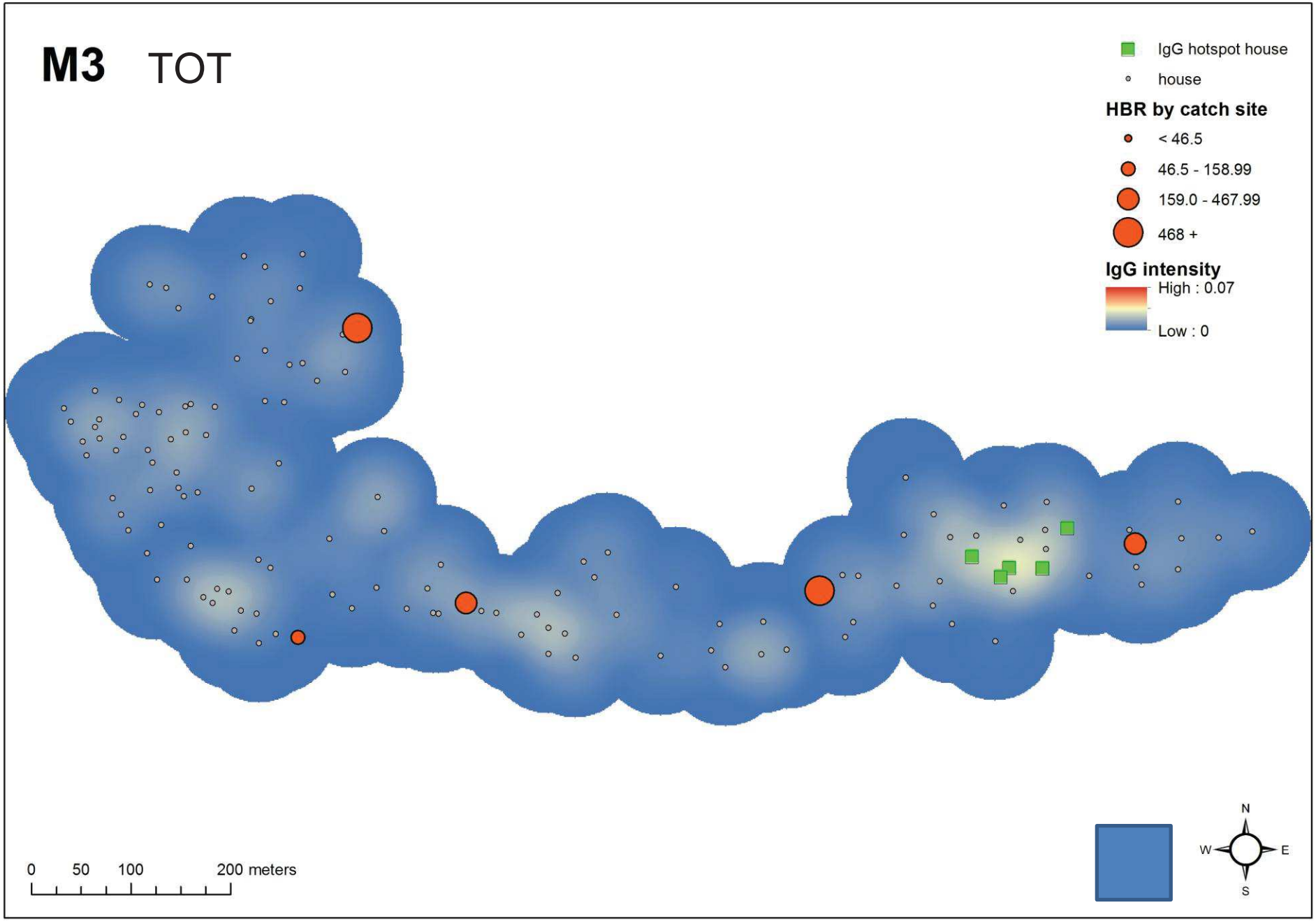

M9 HKT
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0 50 100 200 meters
N
W
E
S

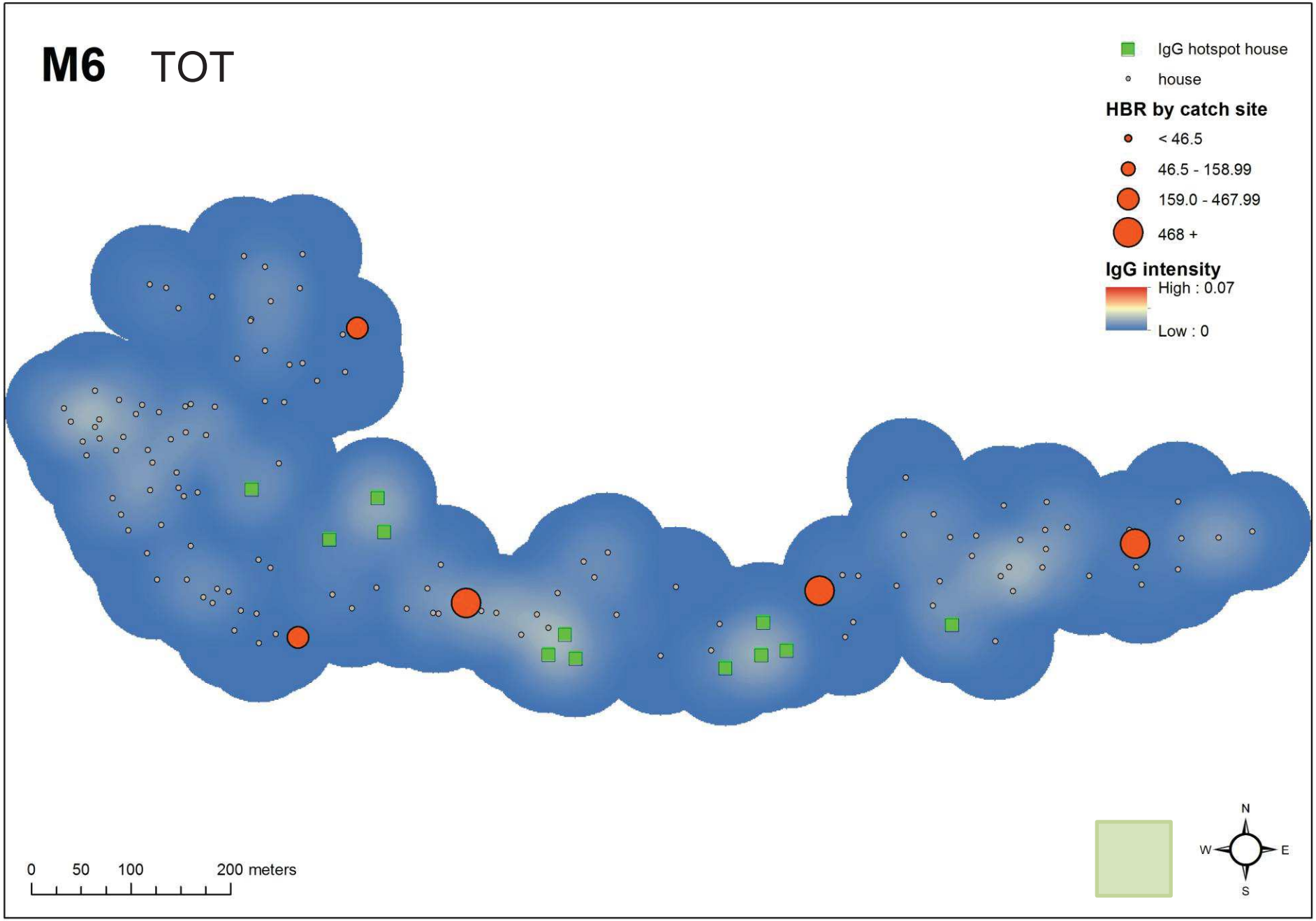

M12 HKT
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0 50 100 200 meters
N
W
E
S

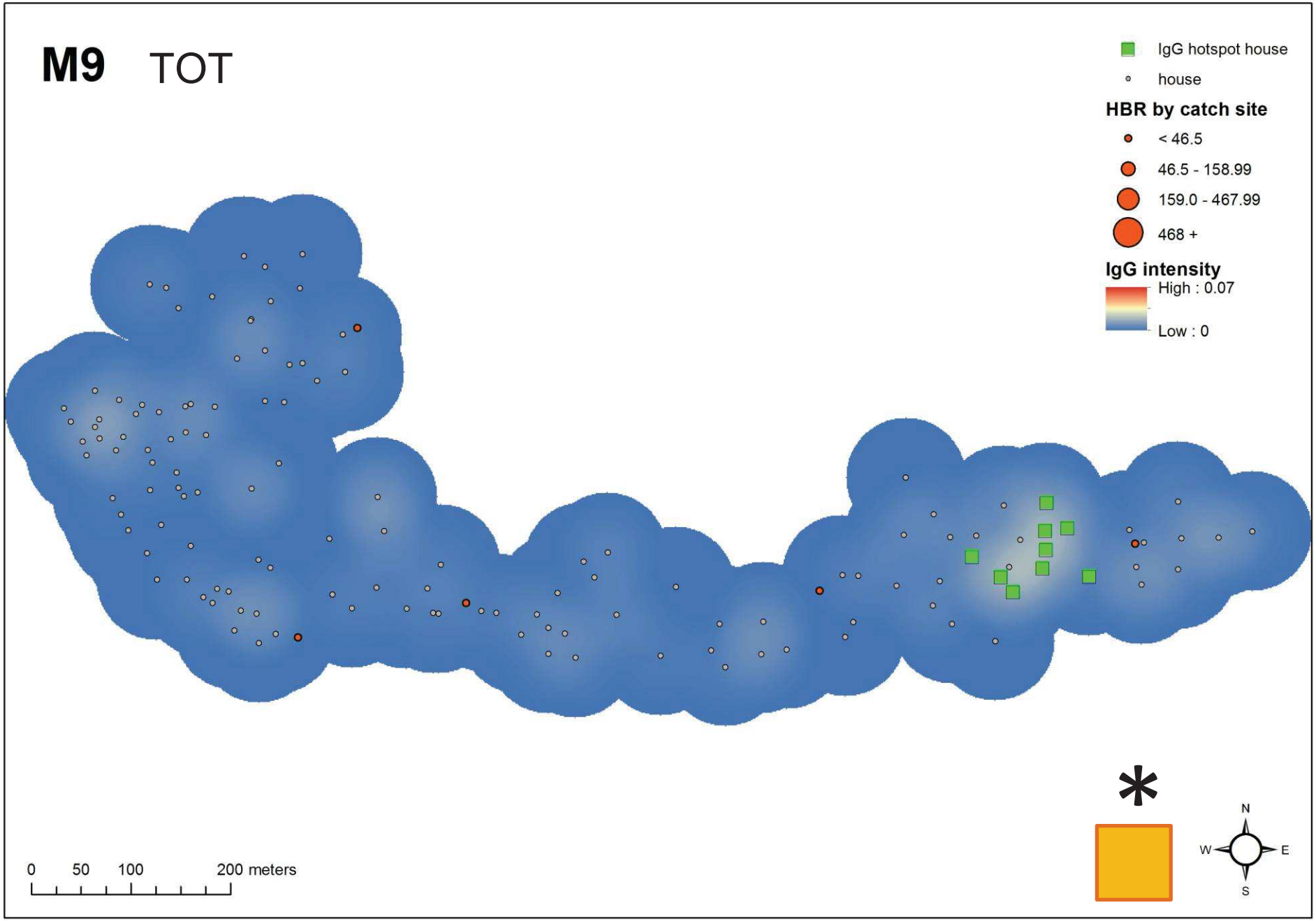

M15 HKT
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0 50 100 200 meters
N
W
E
S


M18 HKT
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High
Low
0 50 100 200 meters
N
W
E
S


M0 TOT
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High : 0.07
Low : 0
0 50 100 200 meters
N
W
E
S


M3 TOT
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High : 0.07
Low : 0
0 50 100 200 meters
N
W
E
S


M6 TOT
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High : 0.07
Low : 0
0 50 100 200 meters
N
W
E
S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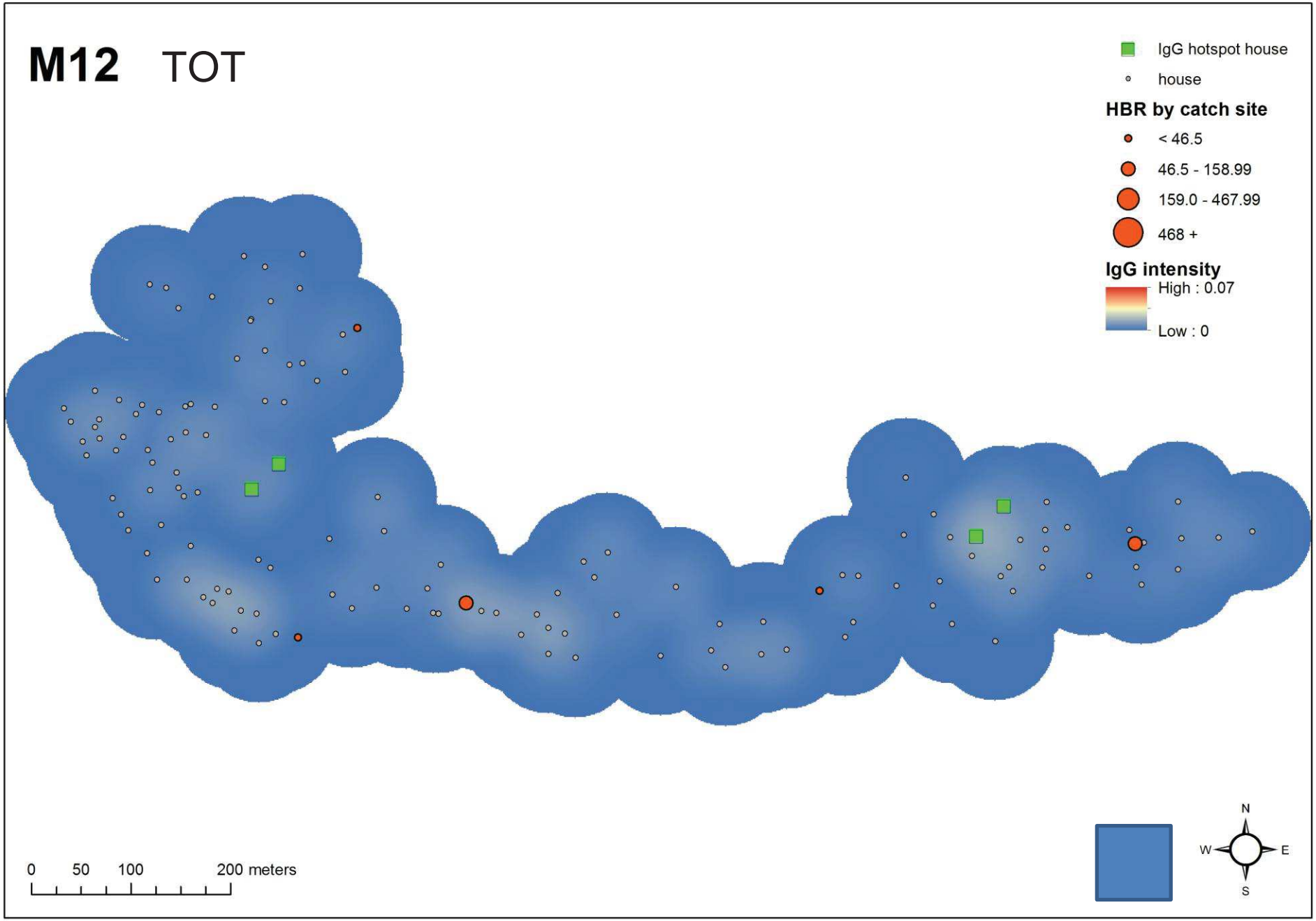
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High : 0.07
Low : 0
0 50 100 200 meters
N
W
E
S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IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
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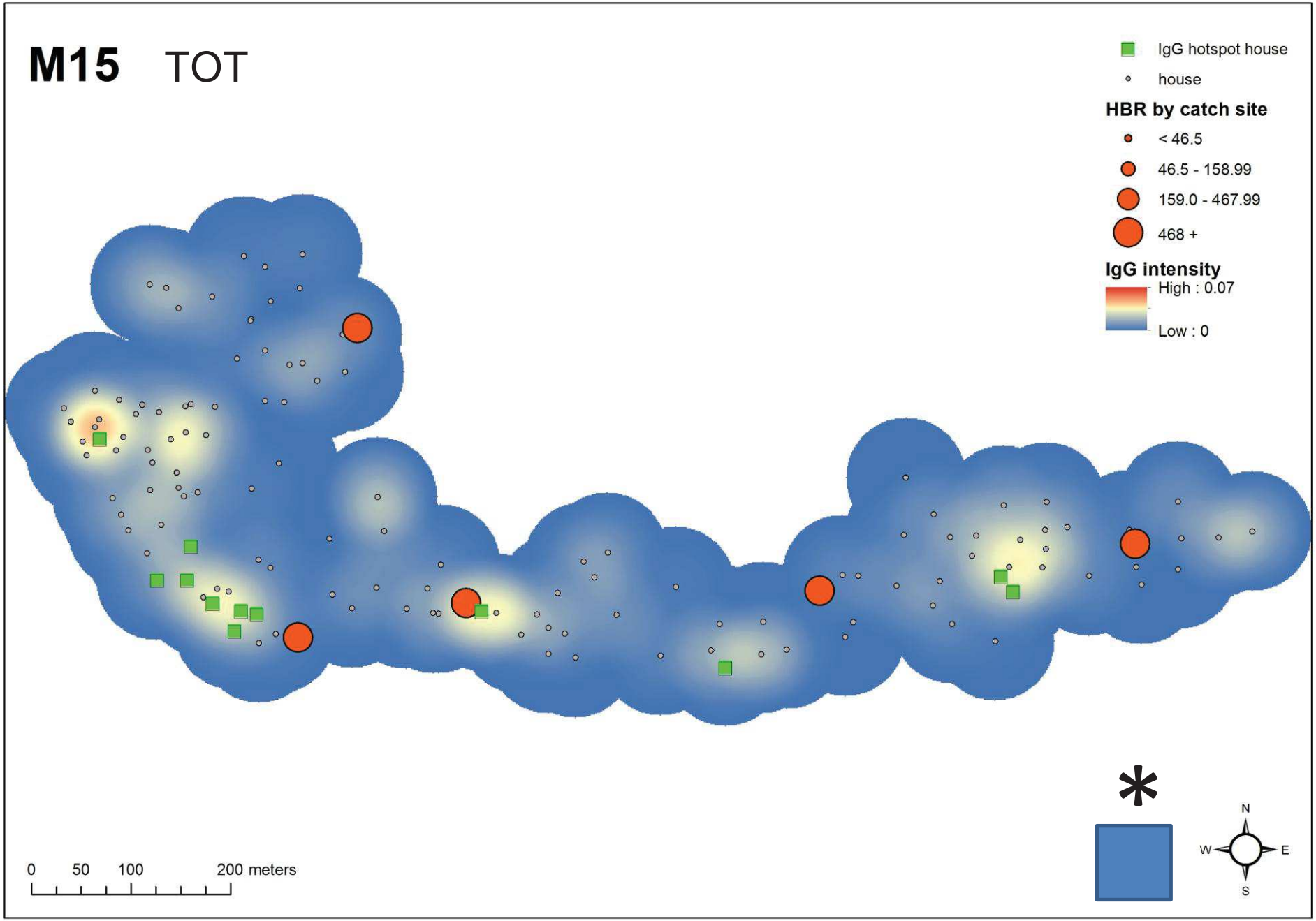
Low : 0
0 50 100 200 meters
N
W
E
S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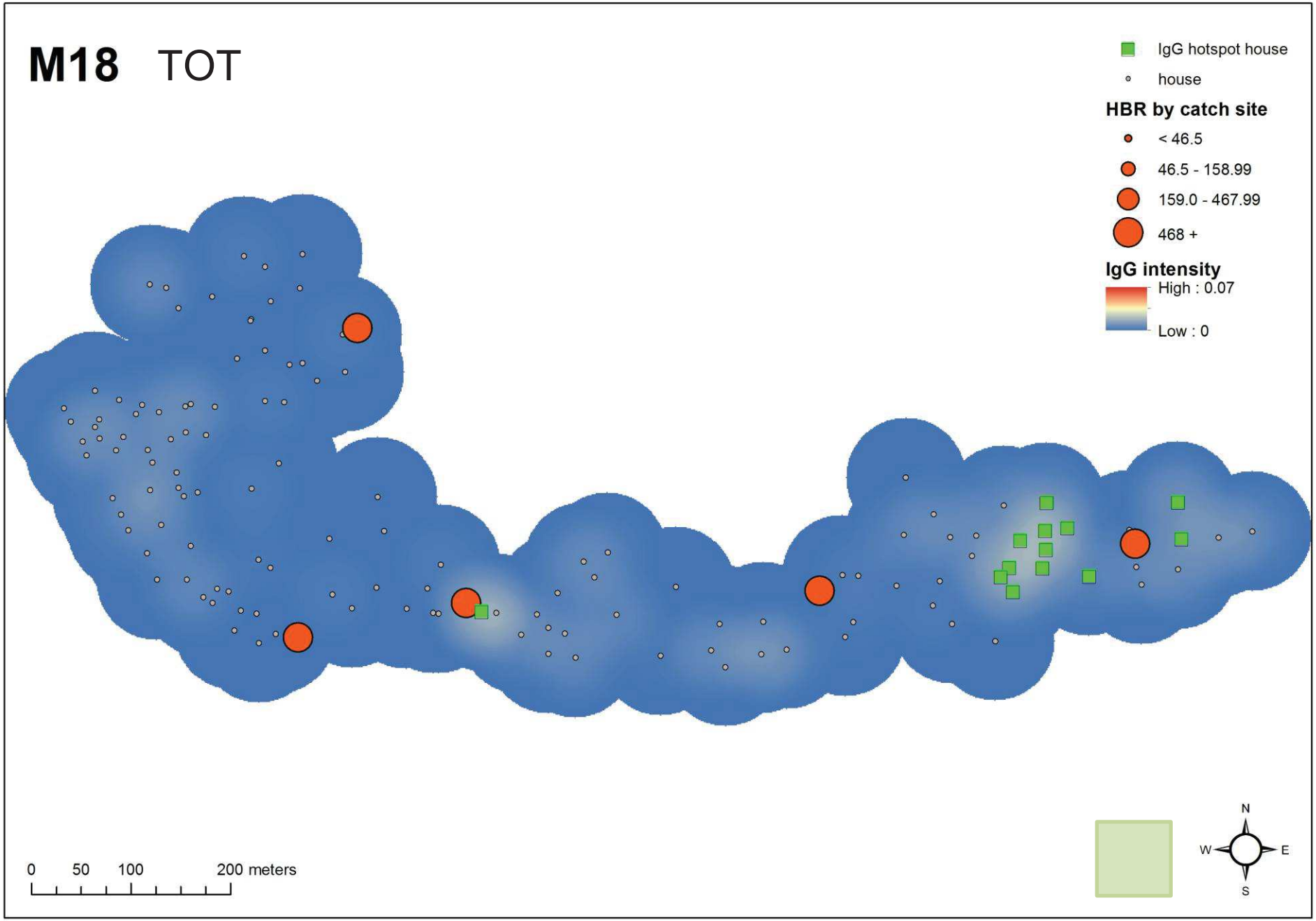
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High : 0.07
Low : 0
0 50 100 200 meters
N
W
E
S


M18 TOT
IgG hotspot house
house
HBR by catch site
< 46.5
46.5 - 158.99
159.0 - 467.99
468 +
IgG intensity
High : 0.07
Low : 0
0 50 100 200 meters
N
W
E
S

SUPPLEMENTARY DATA

Under evaluation

Phubeth Ya-umphan, Dominique Cerqueira, Gilles Cottrell, Daniel M. Parker, Freya J.I Fowkes, Francois Nosten and Vincent Corbel. "*Anopheles salivary biomarker as a proxy for estimating Plasmodium falciparum malaria exposure on the Thailand-Myanmar border"* This work is currently under evaluation in the journal "American Journal of Tropical medicine and Hygiene" (http://www.ajtmh.org/).

Supplementary data S1- ELISA procedures

The standardized dried blood spots (1 cm diameter) were eluted by incubation in 400 µl of phosphate buffered saline (PBS-Tween 0.1%) at 4 °C for 24 hours. Maxisorp plates (Nunc, Rosklide, Denmark) were coated with the *Pf*MSP-1_{19} / *Pf*CSP peptide (Vaximax, FRANCE) at a concentration of 1 µg/mL in coating buffer (PBS + Phenol Red 1%) at 4 °C overnight. After washing (with a solution of PBS-Tween 0.1% + NaCl), the plates were blocked for 1 hour under stirring at room temperature with 150 µl of saturation buffer (PBS-Tween 0.1%+ milk powder 3%). Thereafter, each eluted was incubated in duplicate at 2 hours under stirring at room temperature at 1/20 dilution in sample dilution buffer (PBS-Tween 0.1%+ milk powder 1%+ sodium azide 0.02%). Anti-human IgG coupled to the peroxidase (Invitrogen, USA) was incubated at a 1/3000 dilution at 1 hour under stirring at room temperature. Substrate TMB one (3,3′,5,5′-tetramethylbenzidine) (Promega, USA) was added for 30 minutes at room temperature protected from light then stopping the reaction with 0.2 M H_2SO_4 , and absorbance was measured at 450 nm. In parallel, each test sample was assessed in a blank well containing no *Pf*MSP-1_{19} / *Pf*CSP peptide (ODn) to measure non-specific reactions. Individual results were expressed as the ΔOD value: ΔOD= ODx-ODn. ODx and ODn represent the mean of individual optical density (OD) in 2 antigen wells and 1 blank well containing no *Pf*MSP-1_{19} / *Pf*CSP peptide, respectively. Specific anti-*Pf*MSP-1_{19} / *Pf*CSP IgG response were also assayed in non-malaria exposed individuals (negative samples from France : n = 18) in order to quantify the non-specific background Ab level and to calculate the cut-off value (mean (ΔDOneg) +3SD). Based on our findings, a participant was classified as an immune responder to *Pf*MSP-1_{19} and *Pf*CSP if ΔOD was> 0.162 and >0.115, respectively.

All peptide batches were shipped in lyophilized form and then suspended in ultra-filtered water and frozen in aliquots at -20 °C until use.

Supplementary data S2- Seroprevalence in antibody response to CSP per age group and surveys

Village		TPN												
Survey of blood collection	Total population	Characteristic of blood spot						Pf CSP Antibody prevalence % , (n/N) , (95%CI)						
								Sex			Age			
		Blood Collection date	Number of dry blood spot	Male	Female	Median age (range) (y)		Male	Female	Total	0-4	5-15	16-59	60 up
M0	373	20-05-13	257	145	112	27.4 (1-66)	%	5.5	3.6	4.7	16.7	3.9	4.8	0.0
							(95% CI)	(2.6-11.0)	(1.2-9.4)	(2.6-8.2)	(0.9-63.5)	(1.0-11.7)	(2.2-9.6)	(0.0-43.9)
							(n/N)	(8/145)	(4/112)	(12/257)	(1/6)	(3/77)	(8/167)	(0/7)
M3	406	23-08-13	267	143	124	25.1 (1-66)	%	21.7	21.0	21.4	0.0	16.0	28.7	0.0
							(95% CI)	(15.4-29.5)	(14.4-29.4)	(16.7-26.9)	(0.0-15.5)	(8.9-26.7)	(21.9-36.5)	(0.0-40.2)
							(n/N)	(31/143)	(26/124)	(57/267)	(0/27)	(12/75)	(45/157)	(0-8)
M6	434	07-11-13	300	159	141	24.6 (0-66)	%	3.1	5.0	4.0	0.0	0.0	7.1	0.0
							(95% CI)	(1.2-7.6)	(2.2-10.3)	(2.2-7.1)	(0.0-11.2)	(0.0-5.4)	(3.9-12.4)	(0.0-43.9)
							(n/N)	(5/159)	(7/141)	(12/300)	(0/39)	(0/85)	(12/169)	(0/7)
M9	464	28-01-14	307	160	147	24.0 (0-66)	%	3.1	3.4	3.3	0.0	1.2	5.4	0.0
							(95% CI)	(1.2-7.5)	(1.3-8.2)	(1.7-6.1)	(0.0-10.0)	(0.1-7.2)	(2.7-10.4)	(0.0-37.1)
							(n/N)	(5/160)	(5/147)	(10/307)	(0/46)	(1/86)	(9/166)	(0/9)
M12	473	23-04-14	264	138	126	22.2 (0-66)	%	3.6	2.4	3.0	0.0	1.2	5.4	0.0
							(95% CI)	(1.3-8.7)	(0.6-7.3)	(1.4-6.1)	(0.0-9.8)	(0.1-7.3)	(2.4-11.2)	(0.0-43.9)
							(n/N)	(5/138)	(3/126)	(8/264)	(0/45)	(1/85)	(7/130)	(0/7)
M15	492	14-07-14	234	127	107	24.4 (0-66)	%	2.4	0.9	1.7	0.0	1.5	2.4	57.1
							(95% CI)	(0.6-7.3)	(0.1-5.8)	(0.6-4.6)	(0.0-13.0)	(0.1-9.3)	(0.6-7.3)	(20.2-88.2)
							(n/N)	(3/127)	(1/107)	(4/234)	(0/33)	(1/66)	(3/127)	(4/7)
M18	501	08-10-14	266	139	127	22.8 (0-66)	%	5.0	2.4	3.8	0.0	2.5	5.2	16.7
							(95% CI)	(2.2-10.5)	(0.6-7.3)	(1.9-7.0)	(0.0-9.8)	(0.4-9.7)	(2.3-10.7)	(0.9-63.5)
							(n/N)	(7/139)	(3/127)	(10/266)	(0/45)	(2/79)	(7/136)	(1/6)

Village		TOT												
Survey of blood collection	Total population	Characteristic of blood spot						Pf CSP Antibody prevalence % , (n/N) , (95%CI)						
								Sex			Age			
		Blood Collection date	Number of dry blood spot	Male	Female	Median age (range) (y)		Male	Female	Total	0-4	5-15	16-59	60 up
M0	740	14-06-13	402	192	210	26.7 (0-80)	%	17.7	9.1	13.2	7.1	0.7	18.5	40.0
							(95% CI)	(2.7-24.0)	(5.7-14.0)	(10.1-17.0)	(3.7-35.8)	(0.0-4.7)	(13.8-24.2)	(20.0-63.6)
							(n/N)	(34/192)	(19/210)	(53/402)	(1/14)	(1/135)	(43/233)	(8/20)
M3	785	12-09-13	308	145	163	22.5 (0-70)	%	26.9	23.9	25.3	4.7	17.3	35.5	50.0
							(95% CI)	(20.0-35.0)	(17.8-31.4)	(20.6-30.6)	(0.8-17.1)	(11.0-25.9)	(27.7-44.0)	(24.0-76.0)
							(n/N)	(39/145)	(39/163)	(78/308)	(2/43)	(19/110)	(50/141)	(7/14)
M6	789	26-11-13	205	102	103	23.7 (1-70)	%	16.7	9.7	13.2	3.6	7.1	16.0	46.2
							(95% CI)	(10.3-25.6)	(5.0-17.5)	(9.0-18.8)	(0.2-20.2)	(2.7-16.6)	(9.5-25.3)	(20.4-73.9)
							(n/N)	(17/102)	(10/103)	(27/205)	(1/28)	(5/70)	(15/94)	(6/13)
M9	814	18-02-14	292	143	149	21.5 (0-80)	%	18.9	14.8	16.8	5.4	6.8	24.8	54.6
							(95% CI)	(13.0-26.5)	(9.7-21.7)	(12.8-21.7)	(1.4-15.8)	(2.8-14.8)	(18.0-33.1)	(24.6-81.9)
							(n/N)	(27/143)	(22/149)	(49/292)	(3/56)	(6/88)	(34/137)	(6/11)
M12	833	15-05-14	250	114	136	19.2 (0-70)	%	12.3	5.2	8.4	1.6	0.0	16.5	22.2
							(95% CI)	(7.1-20.1)	(2.3-10.7)	(5.4-12.7)	(0.1-9.7)	(0.0-6.6)	(10.3-25.1)	(4.0-59.8)
							(n/N)	(14/114)	(7/136)	(21/250)	(1/63)	(0/69)	(18/109)	(2/9)
M15	861	06-08-14	305	150	155	21.5 (0-80)	%	14.7	10.3	12.5	1.8	2.0	20.7	43.8
							(95% CI)	(9.6-21.6)	(6.2-16.5)	(9.1-16.8)	(0.1-10.8)	(0.4-7.9)	(14.4-28.7)	(20.8-69.5)
							(n/N)	(22/150)	(16/155)	(38/305)	(1/56)	(2/98)	(28/135)	(7/16)
M18	879	30-10-14	196	94	102	18.9 (0-70)	%	11.7	6.9	9.2	2.0	1.8	16.9	28.6
							(95% CI)	(6.3-20.4)	(3.0-14.1)	(5.7-14.3)	(0.1-12.2)	(0.1-10.6)	(9.9-27.0)	(5.1-69.7)
							(n/N)	(11/94)	(7/102)	(18/196)	(1/49)	(1/57)	(14/83)	(2/7)

Village		KNH												
Survey of blood collection	Total population	Characteristic of blood spot						Pf CSP Antibody prevalence % , (n/N) , (95%CI)						
								Sex			Age			
		Blood Collection date	Number of dry blood spot	Male	Female	Median age (range) (y)		Male	Female	Total	0-4	5-15	16-59	60 up
M0	348	14-06-13	323	178	145	29.0 (1-73)	%	7.9	4.1	6.2	0.0	1.2	8.0	15.4
							(95% CI)	(4.5-13.1)	(1.7-9.2)	(3.9-9.6)	(0.0-34.5)	(0.1-7.1)	(4.9-12.7)	(2.7-46.3)
							(n/N)	(14/178)	(6/145)	(20/323)	(0/10)	(1/87)	(17/213)	(2/13)
M3	417	12-09-13	275	148	127	27.9 (1-73)	%	26.4	26.8	26.5	13.0	17.6	32.7	20.0
							(95% CI)	(19.6-34.3)	(19.5-35.5)	(21.5-32.3)	(3.4-34.7)	(10.1-28.5)	(25.8-40.5)	(3.5-55.8)
							(n/N)	(39/148)	(34/127)	(73/275)	(3/23)	(13/74)	(55/168)	(2/10)
M6	435	11-12-13	261	140	121	28.0 (0-73)	%	12.9	4.1	8.8	0.0	2.8	12.0	2.0
							(95% CI)	(8.0-19.8)	(1.5-9.9)	(5.8-13.1)	(0-19.2)	(0.5-10.7)	(7.5-18.3)	(3.5-55.8)
							(n/N)	(18/140)	(5/121)	(23/261)	(0/21)	(2/71)	(19/159)	(2/10)
M9	449	05-03-14	303	155	148	25.9 (0-73)	%	2.6	4.1	6.6	0.0	2.4	9.5	18.2
							(95% CI)	(0.8-6.9)	(1.7-9.0)	(4.2-10.2)	(0.0-11.4)	(0.4-9.0)	(5.7-15.2)	(3.2-52.2)
							(n/N)	(14/155)	(6/148)	(20/303)	(0/38)	(2/85)	(16/169)	(2/11)
M12	469	26-05-14	301	160	141	24.9 (0-73)	%	7.5	2.1	5.0	0.0	1.2	8.3	0.0
							(95% CI)	(4.1-13.0)	(0.6-6.6)	(2.9-8.3)	(0.0-10.7)	(0.1-7.3)	(4.8-13.8)	(0.0-43.9)
							(n/N)	(12/160)	(3/141)	(15/301)	(0/41)	(1/85)	(14/169)	(0/7)
M15	485	21-08-14	303	164	139	25.8 (0-70)	%	8.5	2.9	5.9	0.0	4.2	7.9	10.0
							(95% CI)	(4.9-14.2)	(0.9-7.7)	(3.7-9.4)	(0.0-10.2)	(1.1-12.5)	(4.5-13.1)	(0.5-45.9)
							(n/N)	(14/164)	(4/139)	(18/303)	(0/43)	(3/72)	(14/178)	(1/10)
M18	495	13-11-14	285	155	130	24.6 (0.70)	%	5.2	1.5	3.5	0.0	0.0	6.3	0.0
							(95% CI)	(2.4-10.3)	(0.3-6.0)	(1.8-6.6)	(0.0-9.1)	(0.0-6.9)	(3.2-11.5)	(0-34.5)
							(n/N)	(8/155)	(2/130)	(10/285)	(0/49)	(0/66)	(10/160)	(0/10)

Village		HKT												
Survey of blood collection	Total population	Characteristic of blood spot						Pf CSP Antibody prevalence % , (n/N) , (95%CI)						
								Sex			Age			
		Blood Collection date	Number of dry blood spot	Male	Female	Median age (range) (y)		Male	Female	Total	0-4	5-15	16-59	60 up
M0	899	04-07-13	487	238	249	26.2 (0-94)	%	8.8	6.4	7.6	0.0	3.0	10.1	22.2
							(95% CI)	(5.7-13.4)	(3.8-10.4)	(5.5-10.4)	(0.0-16.7)	(1.1-7.2)	(6.9-14.4)	(7.4-48.1)
							(n/N)	(21/238)	(16/249)	(37/487)	(0/25)	(5/167)	(28/277)	(4/18)
M3	979	07-10-13	524	263	261	24.1 (0-89)	%	19.0	23.4	21.2	10.2	12.0	28.6	55.6
							(95% CI)	(4.6-24.4)	(18.5-29.1)	(17.8-25.0)	(3.8-23.0)	(7.9-17.8)	(23.4-34.4)	(12.1-58.5)
							(n/N)	(50/263)	(61/261)	(111/524)	(5/49)	(22/183)	(79/276)	(5/16)
M6	1029	08-01-14	493	241	252	21.9 (0-78)	%	5.0	4.4	4.7	0.0	2.1	7.2	27.3
							(95% CI)	(2.7-8.8)	(2.3-7.9)	(3.1-7.0)	(0.0-7.1)	(0.1-5.5)	(4.3-11.6)	(7.3-60.7)
							(n/N)	(12/241)	(11/252)	(23/493)	(0/64)	(4/195)	(16/223)	(3/11)
M9	1070	01-05-14	478	241	237	22.6 (0-78)	%	4.2	3.4	3.8	0.0	4.3	5.9	22.2
							(95% CI)	(2.1-7.7)	(1.6-6.8)	(2.3-6.0)	(0.0-6.8)	(0.2-4.7)	(3.4-10.0)	(4.0-59.8)
							(n/N)	(10/241)	(8/237)	(18/478)	(0/67)	(2/166)	(14/236)	(2/9)
M12	1192	24-06-14	512	257	255	21.8 (0-89)	%	3.9	1.6	2.7	0.0	1.1	4.7	7.7
							(95% CI)	(2.0-7.2)	(0.5-4.2)	(1.6-4.7)	(0.0-6.8)	(0.2-4.4)	(2.5-8.5)	(0.4-3.8)
							(n/N)	(10/257)	(4/255)	(14/512)	(0/88)	(2/178)	(11/233)	(1/13)
M15	1217	16-09-14	504	264	240	21.8 (0-78)	%	5.3	1.7	3.6	1.2	3.4	4.7	0.0
							(95% CI)	(3.0-8.9)	(0.5-4.5)	(2.2-5.7)	(0.1-7.6)	(1.4-7.7)	(2.5-8.5)	(0.0-30.1)
							(n/N)	(14/264)	(4/240)	(18/504)	(1/82)	(6/175)	(11/235)	(0/12)
M18	1236	08-12-14	471	233	238	19.9 (0-78)	%	2.6	1.7	2.2	1.3	1.0	3.3	7.1
							(95% CI)	(1.1-5.8)	(0.5-4.5)	(1.1-4.1)	(0.1-7.9)	(0.2-4.0)	(1.4-7.4)	(0.4-35.8)
							(n/N)	(6/233)	(4/238)	(10/471)	(1/78)	(2/198)	(6/181)	(1/14)

Supplementary data S3- Seroprevalence in antibody response to PfMSP per age group and surveys

Village		TPN												
Survey of blood collection	Total population	Characteristic of blood spot						Pf MSP1-19 Antibody prevalence % , (n/N) , (95%CI)						
								Sex			Age			
		Blood Collection date	Number of dry blood spot	Male	Female	Median age (range) (y)		Male	Female	Total	0-4	5-15	16-59	60 up
M0	373	20-05-13	256	145	111	27.2 (1-66)	%	49.7	46.0	48.1	16.7	30.8	56.6	66.7
							(95% CI)	(41.3-58.0)	(36.5-55.6)	(41.8-54.4)	(0.9-63.5)	(21.1-42.4)	(48.7-64.2)	(24.1-94.0)
							(n/N)	(72/145)	(51/111)	(123/256)	(1/6)	(24/78)	(94/166)	(4/6)
M3	406	23-08-13	278	148	130	25.3 (1-66)	%	49.3	44.6	47.1	21.4	40.5	54.0	62.5
							(95% CI)	(41.1-57.6)	(36.0-53.6)	(41.2-53.2)	(9.0-41.5)	(29.8-52.2)	(46.0-61.8)	(25.9-89.8)
							(n/N)	(73/148)	(58/130)	(131/278)	(6/28)	(32/79)	(88/163)	(5/8)
M6	434	07-11-13	299	158	141	24.6 (0-66)	%	58.2	55.3	56.9	33.3	56.0	61.5	85.7
							(95% CI)	(50.1-65.9)	(46.7-63.6)	(51.0-62.5)	(19.6-50.3)	(44.7-66.6)	(53.7-68.8)	(42.0-99.3)
							(n/N)	(92/158)	(78/141)	(170/299)	(13/39)	(47/84)	(104/169)	(6/7)
M9	464	28-01-14	306	160	146	23.9 (0-66)	%	80.0	76.0	78.1	58.7	82.6	80.0	100.0
							(95% CI)	(72.8-85.7)	(68.1-82.5)	(73.0-82.5)	(43.3-72.7)	(72.6-89.6)	(72.9-85.7)	(62.9-100.0)
							(n/N)	(128/160)	(111/146)	(239/306)	(27/46)	(71/86)	(132/165)	(9/9)
M12	473	23-04-14	264	138	125	22.2 (0.66)	%	82.6	75.2	78.8	60.0	79.5	83.9	100.0
							(95% CI)	(75.0-88.3)	(66.5-82.3)	(73.3-83.5)	(44.4-73.9)	(69.0-87.3)	(76.1-89.5)	(51.7-100.0)
							(n/N)	(114/138)	(94/125)	(208/264)	(27/45)	(66/83)	(109/130)	(6/6)
M15	492	14-07-14	235	137	108	24.3 (0-66)	%	72.3	75.9	77.0	58.8	72.7	82.8	100.0
							(95% CI)	(63.9-79.4)	(66.8-83.4)	(71.0-82.1)	(40.8-74.9)	(60.2-82.6)	(74.9-88.7)	(56.1-100.0)
							(n/N)	(99/137)	(82/108)	(181/235)	(20/34)	(48/66)	(106/128)	(7/7)
M18	501	08-10-14	266	139	127	22.8 (0-66)	%	82.7	79.5	81.2	68.9	82.3	84.6	83.3
							(95% CI)	(75.2-88.4)	(71.3-86.0)	(75.8-85.6)	(53.2-81.4)	(71.7-89.6)	(77.1-90.0)	(36.5-99.1)
							(n/N)	(115/139)	(101/127)	(216/266)	(31/45)	(65/79)	(115/136)	(5/6)

Village		TOT												
Survey of blood collection	Total population	Characteristic of blood spot						Pf MSP1-19 Antibody prevalence % , (n/N) , (95%CI)						
								Sex			Age			
		Blood Collection date	Number of dry blood spot	Male	Female	Median age (range) (y)		Male	Female	Total	0-4	5-15	16-59	60 up
M0	740	14-06-13	402	193	209	26.7 (0-80)	%	74.6	73.2	73.9	21.4	53.3	87.1	95.0
							(95% CI)	(67.8-80.5)	(66.6-79.0)	(69.2-78.1)	(5.7-51.2)	(44.6-61.9)	(82.0-91.0)	(73.1-99.7)
							(n/N)	(144/193)	(153/209)	(297/402)	(3/14)	(72/135)	(203/233)	(19/20)
M3	785	12-09-13	322	151	171	22.1 (0-70)	%	64.2	60.2	62.1	10.6	45.7	88.3	100.0
							(95% CI)	(56.0-71.8)	(52.5-67.5)	(56.5-67.4)	(4.0-23.9)	(36.5-55.2)	(81.6-92.8)	(73.2-100.0)
							(n/N)	(97/151)	(103/171)	(200/322)	(5/47)	(53/116)	(128/145)	(14/14)
M6	789	26-11-13	202	100	102	24.0 (1-70)	%	71.0	68.6	69.8	19.2	57.1	89.3	23.1
							(95% CI)	(60.9-79.4)	(58.6-77.3)	(62.9-75.9)	(7.3-40.0)	(44.8-68.7)	(80.7-94.4)	(6.2-54.0)
							(n/N)	(71/100)	(70/102)	(141/202)	(5/26)	(40/70)	(83/93)	(13/13)
M9	814	18-02-14	291	142	149	21.5 (0-80)	%	81.0	81.9	81.4	39.3	84.1	96.3	90.9
							(95% CI)	(73.4-86.9)	(74.6-87.5)	(76.4-85.6)	(26.8-53.3)	(74.4-90.7)	(91.2-98.6)	(57.1-99.5)
							(n/N)	(115/142)	(122/149)	(237/291)	(22/56)	(74/88)	(131/136)	(10/11)
M12	833	15-05-14	249	113	136	19.2 (0-70)	%	77.0	80.2	78.7	33.3	86.6	98.2	100.0
							(95% CI)	(67.9-84.2)	(72.3-86.3)	(73.0-83.5)	(22.3-46.4)	(75.5-93.3)	(92.9-99.7)	(62.9-100.0)
							(n/N)	(87/113)	(109/136)	(196/249)	(21/63)	(58/67)	(108/110)	(9/9)
M15	861	06-08-14	307	150	157	21.6 (0-80)	%	78.7	79.6	79.2	32.1	79.0	96.3	100.0
							(95% CI)	(71.1-84.8)	(72.3-85.5)	(74.1-83.5)	(20.7-46.1)	(69.5-86.3)	(91.1-98.6)	(77.1-100.0)
							(n/N)	(118/150)	(125/157)	(243/307)	(18/56)	(79/100)	(129/134)	(17/17)
M18	879	30-10-14	196	94	102	18.9 (0-70)	%	71.3	69.6	70.4	26.5	77.2	90.4	85.7
							(95% CI)	(60.9-79.9)	(59.6-78.1)	(63.4-76.6)	(1.5-41.3)	(63.9-86.8)	(81.4-95.5)	(42.0-99.3)
							(n/N)	(67/94)	(71/102)	(138/196)	(13/49)	(44/57)	(75/83)	(6/7)

Village		KNH												
Survey of blood collection	Total population	Characteristic of blood spot						Pf MSP1-19 Antibody prevalence % , (n/N) , (95%CI)						
								Sex			Age			
		Blood Collection date	Number of dry blood spot	Male	Female	Median age (range) (y)		Male	Female	Total	0-4	5-15	16-59	60 up
M0	348	14-06-13	321	178	143	29.0 (1-73)	%	75.9	65.7	71.3	20.0	53.5	79.3	100.0
							(95% CI)	(68.8-81.8)	(57.3-73.3)	(66.0-76.2)	(3.5-55.8)	(42.5-64.2)	(73.0-84.4)	(71.7-100.0)
							(n/N)	(135/178)	(94/143)	(229/321)	(2/10)	(46/86)	(168/212)	(13/13)
M3	417	12-09-13	272	146	126	28.1 (1-73)	%	76.7	69.8	73.5	18.2	55.6	86.9	100.0
							(95% CI)	(68.9-83.1)	(60.9-77.5)	(67.8-78.6)	(6.0-41.0)	(43.4-67.1)	(80.6-91.4)	(65.6-100.0)
							(n/N)	(112/146)	(88/126)	(200/272)	(4/22)	(40/72)	(146/168)	(10/10)
M6	435	11-12-13	260	139	121	28.3 (0-73)	%	81.3	70.3	76.2	45.5	60.9	85.5	100.0
							(95% CI)	(73.6-87.2)	(61.2-78.0)	(70.4-81.1)	(25.1-67.3)	(48.4-72.2)	(78.9-90.4)	(65.6-100.0)
							(n/N)	(113/139)	(85/121)	(198/260)	(10/22)	(42/69)	(136/159)	(10/10)
M9	449	05-03-14	304	156	148	25.9 (0-73)	%	90.4	84.5	87.5	68.4	82.4	93.5	100.0
							(95% CI)	(84.4-94.3)	(77.4-89.7)	(83.1-90.9)	(51.2-82.0)	(72.2-89.5)	(88.4-96.6)	(67.9-100.0)
							(n/N)	(141/156)	(125/148)	(266/304)	(26/38)	(70/85)	(159/170)	(11/11)
M12	469	26-05-14	300	159	141	24.7 (0-73)	%	84.3	80.9	82.7	46.3	82.4	90.5	100.0
							(95% CI)	(77.5-89.4)	(73.2-86.8)	(77.8-86.7)	(31.0-62.4)	(72.1-89.5)	(84.8-94.3)	(51.7-100.0)
							(n/N)	(134/159)	(114/141)	(248/300)	(19/41)	(70/85)	(153/169)	(6/6)
M15	485	21-08-14	302	163	139	25.7 (0-70)	%	76.1	74.8	75.5	39.5	68.1	85.9	100.0
							(95% CI)	(68.6-82.2)	(66.6-81.6)	(70.2-80.2)	(25.4-55.6)	(55.9-78.3)	(79.7-90.5)	(65.6-100.0)
							(n/N)	(124/163)	(104/139)	(228/302)	(17/43)	(49/72)	(152/177)	(10/10)
M18	495	13-11-14	285	155	130	24.6 (0-70)	%	79.4	76.2	77.9	42.9	77.3	87.6	100.0
							(95% CI)	(72.0-85.3)	(67.7-83.0)	(72.5-82.5)	(29.1-57.7)	(65.0-86.3)	(81.2-92.1)	(62.9-100.0)
							(n/N)	(123/155)	(99/130)	(222/285)	(21/49)	(51/66)	(141/161)	(9/9)

Village		HKT												
Survey of blood collection	Total population	Characteristic of blood spot						Pf MSP1-19 Antibody prevalence % , (n/N) , (95%CI)						
								Sex			Age			
		Blood Collection date	Number of dry blood spot	Male	Female	Median age (range) (y)		Male	Female	Total	0-4	5-15	16-59	60 up
M0	899	04-07-13	488	237	251	26.3 (0-94)	%	65.0	57.0	60.9	16.7	48.5	70.8	83.3
							(95% CI)	(58.5-71.0)	(50.6-63.1)	(56.4-65.2)	(5.5-38.2)	(40.8-56.3)	(65.0-76.0)	(57.7-95.6)
							(n/N)	(154/237)	(143/251)	(297/488)	(4/24)	(82/169)	(196/277)	(15/18)
M3	979	07-10-13	533	267	266	24.3 (0-89)	%	59.6	56.0	57.8	20.0	43.7	72.5	75.0
							(95% CI)	(53.4-65.4)	(49.8-62.0)	(53.5-62.0)	(10.5-34.1)	(36.5-51.2)	(66.9-77.6)	(47.4-91.7)
							(n/N)	(159/267)	(149/266)	(308/533)	(10/50)	(80/183)	(206/284)	(12/16)
M6	1029	08-01-14	496	240	256	22.0 (0-78)	%	64.6	57.4	60.9	20.0	53.9	77.5	81.8
							(95% CI)	(58.1-70.6)	(51.1-63.5)	(56.4-65.2)	(11.5-32.1)	(46.6-61.0)	(71.4-82.7)	(47.8-96.8)
							(n/N)	(155/240)	(147/256)	(302/496)	(13/65)	(104/193)	(176/227)	(9/11)
M9	1070	01-05-14	479	243	236	22.4 (0-78)	%	67.5	58.9	63.3	19.1	56.0	80.8	77.8
							(95% CI)	(61.2-73.3)	(52.3-65.2)	(58.8-67.6)	(11.0-30.8)	(48.1-63.5)	(75.0-85.5)	(40.2-96.1)
							(n/N)	(164/243)	(139/236)	(303/479)	(13/68)	(94/168)	(189/234)	(7/9)
M12	1192	24-06-14	513	258	255	21.8 (0-89)	%	58.5	51.0	54.8	20.5	49.7	71.2	61.5
							(95% CI)	(52.2-64.6)	(44.7-57.3)	(50.4-59.1)	(12.9-30.7)	(42.2-57.3)	(64.9-76.9)	(32.3-84.9)
							(n/N)	(151/258)	(130/255)	(281/513)	(18/88)	(89/179)	(166-233)	(8/13)
M15	1217	16-09-14	506	264	242	21.7 (0-78)	%	68.6	67.4	68.0	33.7	61.7	83.9	83.3
							(95% CI)	(62.5-74.0)	(61.0-73.1)	(63.7-72.0)	(24.0-45.0)	(54.0-68.9)	(78.4-88.2)	(50.9-97.1)
							(n/N)	(181/264)	(163/242)	(344/506)	(28/83)	(108/175)	(198/236)	(10/12)
M18	1236	08-12-14	469	231	238	19.9 (0-78)	%	67.5	60.9	64.2	29.5	64.0	78.9	71.4
							(95% CI)	(61.0-73.4)	(54.4-67.1)	(59.6-68.5)	(20.0-41.1)	(56.8-70.6)	(72.1-84.5)	(42.0-90.4)
							(n/N)	(156/231)	(145/238)	(301/469)	(23/78)	(126/197)	(142/180)	(10/14)

Supplementary data S4- Seroprevalence in antibody response to gSG6-P1 per age group and surveys.

Village		TPN												
Survey of blood collection	Total population	Characteristic of blood spot						Antibody prevalence % , (n/N) , (95%CI)						
								Sex			Age			
		Blood Collection date	Number of dry blood spot	Male	Female	Median age (range) (y)		Male	Female	Total	0-4	5-15	15-59	60 up
M0	373	20-05-13	258	146	112	27.3 (1-66)	%	45.2	42.9	44.2	33.3	41.0	45.2	66.7
							(95% CI)	(37.0-53.6)	(33.7-52.6)	(38.1-50.0)	(6.0-75.9)	(30.2-52.8)	(37.6-53.1)	(24.1-94.0)
							(n/N)	(66/146)	(48/112)	(114/258)	(2/6)	(32/78)	(76/168)	(4/6)
M3	406	23-08-13	283	154	129	24.8 (1-66)	%	57.8	60.5	59.0	51.7	53.7	63.0	57.1
							(95% CI)	(49.6-65.6)	(51.5-68.9)	(53.0-64.8)	(32.9-70.1)	(42.4-64.6)	(55.1-70.3)	(20.2-88.2)
							(n/N)	(89/154)	(78/129)	(167/283)	(15/29)	(44/82)	(104-165)	(4/7)
M6	434	07-11-13	300	158	142	24.8 (0-66)	%	49.4	49.3	49.3	43.6	57.8	45.6	71.4
							(95% CI)	(41.4-57.4)	(40.9-57.8)	(43.6-55.1)	(28.2-60.2)	(46.5-68.4)	(38.0-53.4)	(30.3-94.9)
							(n/N)	(78/158)	(70/142)	(148/300)	(17/39)	(48/83)	(78/171)	(5/7)
M9	464	28-01-14	307	160	147	23.9 (0-66)	%	50.0	36.1	43.3	34.8	47.7	43.4	44.4
							(95% CI)	(42.0-58.0)	(28.4-44.4)	(37.7-49.1)	(21.8-50.3)	(36.9-58.7)	(35.8-51.3)	(15.3-77.3)
							(n/N)	(80/160)	(53/147)	(133/307)	(16/46)	(41/86)	(72/166)	(4/9)
M12	473	23-04-14	260	135	125	22.8 (0-66)	%	60.7	68.0	64.2	65.1	63.4	65.9	33.3
							(95% CI)	(51.9-68.9)	(59.0-75.9)	(58.0-70.0)	(49.0-78.6)	(52.0-73.6)	(57.0-73.9)	(6.0-75.9)
							(n/N)	(82/135)	(85/125)	(167/260)	(28/43)	(52/82)	(85/129)	(2/6)
M15	492	14-07-14	233	127	106	24.3 (0-66)	%	80.3	76.4	78.5	70.6	83.1	77.2	100.0
							(95% CI)	(72.1-86.6)	(67.0-83.9)	(72.6-83.5)	(52.3-84.3)	(71.3-90.9)	(68.7-84.0)	(56.1-100.0)
							(n/N)	(102/127)	(81/106)	(183/233)	(24/34)	(54/65)	(98/127)	(7/7)
M18	501	08-10-14	265	139	126	23.0 (0-66)	%	83.5	81.7	82.6	82.2	78.2	85.3	83.3
							(95% CI)	(76.0-89.0)	(73.7-87.9)	(77.4-86.9)	(67.4-91.5)	(67.2-86.5)	(78.0-90.6)	(36.5-99.1)
							(n/N)	(116/139)	(103-126)	(219/265)	(37/45)	(61/78)	(116/136)	(5/6)

Village		TOT												
Survey of blood collection	Total population	Characteristic of blood spot						Antibody prevalence % , (n/N) , (95%CI)						
								Sex			Age			
		Blood Collection date	Number of dry blood spot	Male	Female	Median age (range) (y)		Male	Female	Total	0-4	5-15	15-59	60 up
M0	740	14-06-13	404	192	212	26.8 (0-80)	%	68.2	62.7	65.3	42.9	58.1	72.4	50.0
							(95% CI)	(61.1-74.7)	(55.8-69.2)	(60.5-69.9)	(18.8-70.4)	(49.3-66.4)	(66.1-78.0)	(28.8-71.2)
							(n/N)	(131/192)	(133/212)	(264/404)	(6/14)	(79/136)	(168/232)	(11/22)
M3	785	12-09-13	322	149	173	22.2 (0-70)	%	77.2	71.7	74.2	63.6	70.3	78.8	92.9
							(95% CI)	(69.5-83.5)	(28.7-38.5)	(69.0-78.8)	(47.7-77.2)	(61.1-78.2)	(71.1-84.9)	(64.2-99.6)
							(n/N)	(115/149)	(124/173)	(239/322)	(28/44)	(83/118)	(115/146)	(13/14)
M6	789	26-11-13	201	98	103	24.1 (1-70)	%	75.5	81.6	78.6	72.0	73.2	82.6	92.3
							(95% CI)	(65.6-83.4)	(72.4-88.3)	(72.2-83.9)	(50.4-87.1)	(61.2-82.7)	(73.0-89.4)	(62.1-99.6)
							(n/N)	(74/98)	(84/103)	(158/201)	(18/25)	(52/71)	(76/92)	(12/13)
M9	814	18-02-14	292	144	148	21.7 (0-80)	%	46.5	47.3	46.9	40.0	51.1	45.7	63.6
							(95% CI)	(38.3-55.0)	(39.1-55.6)	(41.1-52.8)	(27.3-54.8)	40.3-61.9)	(37.2-54.3)	(31.6-87.6)
							(n/N)	(67/144)	(70/148)	(137/292)	(22/55)	(45/88)	(63/138)	(7/11)
M12	833	15-05-14	249	114	135	19.3 (0-70)	%	59.6	56.3	57.8	39.7	59.1	66.7	66.7
							(95% CI)	(50.0-68.6)	(47.5-64.7)	(51.4-64.0)	(27.8-52.8)	(46.3-70.8)	(57.0-75.2)	(30.9-91.0)
							(n/N)	(68/114)	(76/135)	(144/249)	(25/63)	(39/66)	(74/111)	(6/9)
M15	861	06-08-14	307	152	155	21.3 (0-80)	%	92.1	93.5	92.8	94.7	92.0	92.5	93.8
							(95% CI)	(86.3-95.7)	(88.1-96.7)	(89.2-95.4)	(84.5-98.6)	(84.4-96.2)	(86.4-96.2)	(67.7-99.7)
							(n/N)	(140/152)	(145/155)	(285/307)	(54/57)	(92/100)	(124/134)	(15/16)
M18	879	30-10-14	195	93	102	19.5 (0-70)	%	57.0	74.5	66.2	47.8	76.8	72.1	28.6
							(95% CI)	(46.3-67.1)	(64.8-82.4)	(59.0-72.7)	(33.1-62.9)	(63.3-86.6)	(61.2-81.0)	(5.1-69.7)
							(n/N)	(53/93)	(76/102)	(129/195)	(22/46)	(43/56)	(62/86)	(2/7)

Village		KNH												
Survey of blood collection	Total population	Characteristic of blood spot						Antibody prevalence % , (n/N) , (95%CI)						
								Sex			Age			
		Blood Collection date	Number of dry blood spot	Male	Female	Median age (range) (y)		Male	Female	Total	0-4	5-15	15-59	60 up
M0	348	14-06-13	325	180	145	29.1 (2-73)	%	55.6	51.7	53.8	33.3	40.2	59.3	69.2
							(95% CI)	(48.0-62.9)	(43.3-60.0)	(48.3-59.3)	(9.0-69.1)	(30.0-51.3)	(52.4-65.8)	(38.9-89.7)
							(n/N)	(100/180)	(75/145)	(175/325)	(3/9)	(35/87)	(128/216)	(9/13)
M3	417	12-09-13	277	147	130	28.1 (1-73)	%	47.6	51.5	49.5	30.4	38.4	56.7	50.0
							(95% CI)	(39.4-56.0)	(42.7-60.3)	(43.4-55.5)	(14.1-53.0)	(27.4-50.5)	(49.0-64.2)	(20.1-79.9)
							(n/N)	(70/147)	(67/130)	(137/277)	(7/23)	(28/73)	(97/171)	(5/10)
M6	435	11-12-13	263	141	122	28.1 (0-73)	%	61.7	52.5	57.4	59.1	55.7	56.5	80.0
							(95% CI)	(53.1-69.7)	(43.3-61.5)	(51.2-63.4)	(36.7-78.5)	(43.4-67.4)	(48.5-64.2)	(44.2-96.5)
							(n/N)	(87/141)	(64/122)	(151/263)	(13/22)	(39/70)	(91/161)	(8/10)
M9	449	05-03-14	301	155	146	25.9 (0-73)	%	47.7	49.3	48.5	43.2	45.2	52.1	36.4
							(95% CI)	(39.7-55.9)	(41.0-57.7)	(42.8-54.3)	(27.5-60.4)	(34.5-56.4)	(44.3-59.8)	(12.4-68.4)
							(n/N)	(74/155)	(72/146)	(146/301)	(16/37)	(38/84)	(88/169)	(4/11)
M12	469	26-05-14	298	160	138	25.1 (0-73)	%	41.9	39.1	40.6	31.7	47.6	38.4	55.6
							(95% CI)	(34.2-49.9)	(31.1-47.8)	(35.0-46.4)	(18.6-48.2)	(36.7-58.7)	(31.0-46.4)	(22.7-84.7)
							(n/N)	(67/160)	(54/138)	(121/298)	(13/41)	(40/84)	(63/164)	(5/9)
M15	485	21-08-14	300	162	138	26.0 (0-70)	%	94.4	96.4	95.3	81.0	97.1	97.8	100.0
							(95% CI)	(89.4-97.3)	(91.3-98.7)	(92.1-97.3)	(65.4-90.9)	(89.1-99.5)	(94.0-99.3)	(65.6-100.0)
							(n/N)	(153/162)	(133/138)	(286/300)	(34/42)	(68/70)	(174/178)	(10/10)
M18	495	13-11-14	282	154	128	24.6 (0-70)	%	86.4	83.6	85.1	63.3	84.6	91.2	100.0
							(95% CI)	(79.7-91.2)	(75.8-89.3)	(80.3-89.0)	(48.3-76.2)	(73.1-92.0)	(85.4-94.9)	(62.9-100.0)
							(n/N)	(133/154)	(107/128)	(240/282)	(31/49)	(55/65)	(145/159)	(9/9)

Village		HKT												
Survey of blood collection	Total population	Characteristic of blood spot						Antibody prevalence % , (n/N) , (95%CI)						
								Sex			Age			
		Blood Collection date	Number of dry blood spot	Male	Female	Median age (range) (y)		Male	Female	Total	0-4	5-15	15-59	60 up
M0	899	04-07-13	490	241	249	26.4 (0-94)	%	92.9	94.8	93.9	91.7	92.2	95.0	94.4
							(95% CI)	(88.8-95.7)	(91.0-97.1)	(91.3-95.8)	(71.5-98.6)	(86.8-95.6)	(91.6-97.2)	(70.6-99.7)
							(n/N)	(224/241)	(236/249)	(460/490)	(22/24)	(154/167)	(267/281)	(17/18)
M3	979	07-10-13	535	269	266	24.5 (0-89)	%	88.5	86.8	86.2	81.6	85.7	86.8	94.1
							(95% CI)	(80.6-89.4)	(82.0-90.5)	(83.9-89.0)	(67.5-90.8)	(79.6-90.3)	(82.2-90.4)	(69.2-99.7)
							(n/N)	(230/269)	(231/266)	(461/535)	(40/49)	(156/182)	(249/287)	(16/17)
M6	1029	08-01-14	500	243	257	22.1 (0-78)	%	78.2	85.6	82.0	75.8	81.9	84.8	63.6
							(95% CI)	(72.4-83.1)	(80.6-89.5)	(78.3-85.2)	(63.4-85.1)	(75.6-86.9)	(79.3-89.0)	(31.6-87.6)
							(n/N)	(190/243)	(220/257)	(410/500)	(50/66)	(158/193)	(195/230)	(7/11)
M9	1070	01-05-14	478	240	238	22.4 (0-78)	%	87.9	86.6	87.2	77.9	86.9	89.7	100.0
							(95% CI)	(83.0-91.6)	(81.4-90.5)	(83.8-90.0)	(65.9-86.7)	(80.6-91.4)	(84.9-93.2)	(62.9-100.0)
							(n/N)	(211/240)	(206/238)	(417/478)	(53/68)	(146/168)	(209/233)	(9/9)
M12	1192	24-06-14	527	269	258	21.7 (0-89)	%	85.1	89.1	87.1	87.6	88.8	85.7	84.6
							(95% CI)	(80.2-89.0)	(84.6-92.6)	(83.9-89.8)	(78.6-93.4)	(83.1-92.8)	(80.5-89.8)	(53.7-97.3)
							(n/N)	(229/269)	(230/258)	(459/527)	(78/89)	(166/187)	(204/238)	(11/13)
M15	1217	16-09-14	506	266	240	21.6 (0-78)	%	81.2	85.0	83.0	82.1	85.8	81.3	81.8
							(95% CI)	(75.9-85.6)	(79.7-89.1)	(79.4-86.1)	(71.9-89.3)	(79.6-90.4)	(75.6-85.9)	(47.8-96.8)
							(n/N)	(216/266)	(204/240)	(420/506)	(69/84)	(151/176)	(191/235)	(9/11)
M18	1236	08-12-14	467	233	234	20.0 (0-78)	%	82.8	87.2	85.0	84.0	85.4	84.9	85.7
							(95% CI)	(77.2-87.3)	(82.1-91.1)	(81.4-88.1)	(73.3-91.1)	(79.6-89.8)	(78.6-89.7)	(56.2-97.5)
							(n/N)	(193/233)	(204/234)	(397/467)	(63/75)	(170/199)	(152/179)	(12/14)

S5- Spatial clusters of P. falcipraum malaria and vector exposure, as measured by relative risk calculation, in the study villages

	KNH			HPN			HKT			TOT		
	gsg6	csp	msp	gsg6	csp	msp	gsg6	csp	msp	gsg6	csp	msp
Month												
0	p < 0.0001; RR = 9.29						p < 0.0001; RR = 8.83			p < 0.0001; RR = 16.15		p < 0.0001; RR = 3.34
3		p < 0.0001; RR = 13.46		p < 0.0001; RR = 8.90	p < 0.0001; RR = 18.95			p < 0.0001; RR= 14.86			p < 0.0001; RR = 6.38	
6												
9			p < 0.0001; RR = 3.55			p < 0.0001; RR = 3.88						
12												
15	p < 0.0001; RR = 6.01								p < 0.0001; RR= 3.69	p < 0.0001; RR = 5.86		
18												
	statistically significant cluster											

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LIST OF ABBREVIATIONS

Ab : Antibody

ACT : Artemisinin-based Combination Therapy

Ag : Antigen

AMA : Apical Merozoite Antigen

AP : Alkaline Phosphatase

ATSB : Attractive Toxic Sugar Baits

Bs : *Bacillus sphaericus*

Bti : *Bacillus thuringiensis israelensis*

CBC : Cow Bait Collection

CQ : Chloroquine

CSP : CircumSporozoite Protein (CSP)

DNA : DeoxyriboNucleic Acid

DOT : Directly Observed Treatment

DP : Dihydroartemisinin-Piperaquine

EIR : Entomological Inoculation Rates

ELISA : EnzymeLinked ImmunoSorbent Assay

EPI : Expanded Program for Immunization

G6PD : Glucose-6-Phosphate Dehydrogenase

GIS : Geographic Information System

GLURP : GLUtamate-Rich Protein

HBR : Human Biting Rates

HLC : Human Landing Catch

HPR : Horseradish Peroxidase

HPR2 : Histidine-Rich Protein 2

IFA : Indirect fluorescent antibody test

Ig : ImmunoGlobulin

IRS : Indoor Residual Spraying

ITH : Insecticide Treated Hammocks

ITN : Insecticide-Treated mosquito Nets

kdr : knock-down resistance

LLIN : Long-Lasting Insecticidal Treated mosquito Nets

LLITC : Long Lasting Insecticide-Treated Clothes

LSA : Liver Stage Antigen

LSM : mosquito Larval Source Management

MCT : Median Catching Time

MDA : Mass Drug Administration

MPPT : Mass Primaquine Prophylactic Treatment

MSP : Merozoite Surface Protein

OD : Optical Density

PBO : Piperonyl Butoxide

PCR: Polymerase Chain Reaction

pLDH : parasite Lactate DeHydrogenase

PQ : Primaquine

PR : Parasite Prevalence

QBC : Quantitative Buffy Coat

qRT-PCR : quantitative Real-Time Polymerase Chain Reaction

RDT : Rapid Diagnostic Test

rRNA : ribosomal RiboNucleic Acid

s.l. : sensu lato

SGE : Salivary Grand Extracts

TCE : Targeted Chemo-Elimination

TMB : Thailand-Myanmar Border

TRAP : Thrombospondin-Related Adhesive Protein

UTM : Universal Transverse Mercator

WHO : Word Health Organisation

WHOPES : World Health Organization Pesticide Evaluation Scheme

Résumé de la thèse

Le long de la frontière entre la Thaïlande et le Myanmar (TMB), le paludisme montre une hétérogénéité spatiale et se caractérise par une prévalence élevée en portage submicroscopique et par l'émergence de souches de *Plasmodium falciparum* résistantes à l'artémisinine. Dans ce contexte, l'unité de recherche « Shoklo Malaria Research Unit » (SMRU) a évalué la faisabilité, l'efficacité et l'acceptabilité d'une stratégie de traitement médicamenteuse de masse (TdM) pour réduire le réservoir de parasites asymptomatiques et ainsi accélérer l'élimination du paludisme à *P. falciparum*. Cette thèse a été menée dans le cadre du projet TdM et portait sur l'étude du risque de transmission du paludisme par l'utilisation de biomarqueurs spécifiques d'exposition à la piqûre d'Anopheles (peptide gSG6-P1) et à *Plasmodium falciparum* (CSP & MSP-1_{19}).

La première partie de la thèse a consisté à estimer les changements spatio-temporels du risque vectoriel en mesurant la réponse immunitaire anti-salive d'anopheles dans 4 villages pilotes et à identifier les déterminants modulant le contact « homme-vecteur ». La seconde partie de la thèse a permis d'évaluer la pertinence d'utiliser ce biomarqueur salivaire pour estimer le risque d'exposition à *P. falciparum* et identifier les facteurs modulant le risque de transmission.

Des enquêtes épidémiologiques, immunologiques et entomologiques ont été menées dans quatre villages (HPN, HKT, KNH et TOT) pendant 18 mois entre avril 2013 et décembre 2014. Un centre communautaire de lutte contre le paludisme («poste palu») a été mise en place dans chaque village. Deux villages ont été assignés au hasard au TdM immédiatement et deux autres ont été suivis pendant 9 mois avant de recevoir le TdM. Un traitement de trois jours à la dihydroartémisinine-pipéraquine (DP) et à une dose faible et unique de primaquine à a été administré à tous les participants tous les mois pendant trois mois. Un recensement a été effectué avant les enquêtes et des informations démographiques ont été recueillies. La prévalence du paludisme a été mesurée dans chaque village au cours des enquêtes menées sur l'ensemble de la population du village par PCR ultrasensible (µPCR). Des collectes de moustiques utilisant la technique de capture sur sujet humain ont été menées pour déterminer la composition et l'abondance des vecteurs du paludisme dans la zone d'étude. Des papiers buvards contenant du sang de chaque participant ont été collectés au cours des enquêtes épidémiologiques en « baseline » (M0) puis tous les trois mois jusqu'à 18 mois (M3, M6, M9, M12, M15 et M18). Le

risque d'exposition de l'homme aux piqures de moustique Anopheles a été estimé par ELISA en mesurant la réponse d'anticorps humains à des protéines salivaires spécifiques d'Anopheles (gSG6-P1). En outre, l'exposition des populations humaines à *P. falciparum* a été étudiée par la mesure des IgG humains contre un panel d'antigènes spécifiques (*Pf*MSP-1_{19} et *Pf*CSP). De plus, des techniques de séquençage ont été effectuées pour mesurer l'homologie de séquence du peptide gSG6-P1 *d'An. gambaie* (référence) avec celui des espèces de moustique Anopheles présents dans la zone d'étude. Des modèles statistiques ont été utilisés pour analyser les données. Les données collectées durant le projet TdM (entomologiques, démographiques, immunologiques, épidémiologiques et environnementales) ont été utilisées pour cartographier les zones (« hotspots ») et groupes de personnes («hotpops ») à fort risque de transmission palustre.

Dans le premier chapitre de cette thèse, nous avons démontré pour la première fois la pertinence d'utiliser un biomarqueur salivaire spécifique d'Anopheles pour mesurer le risque d'exposition humaine aux piqûres d'Anopheles dans les « hotspots » de paludisme le long de la TMB. Premièrement, nous avons montré une forte homologie entre la séquence du peptide gSG6-P1 de An. gambiae et celles des espèces dominantes de vecteur, à savoir *An. minimus s.l., An. aconitus* (87%) et *An. maculatus s.l.* (83%). Ces résultats montrent que l'antigène gSG6-P1 est hautement conservé parmi les vecteurs du paludisme dans le monde (Calvo et al., 2009). En revanche, nous n'avons pas pu démontrer si les vecteurs secondaires et les espèces non vectrices pouvaient induire une réponse anticorps, compte tenu de l'alignement infructueux des séquences peptidiques de gSG6-P1.

Par la suite, nous avons démontré une forte séroprévalence dans la population étudiée au peptide salivaire gSG6-P1 (70%), ce qui était cohérent avec les résultats observés en Afrique de l'Ouest (Rizzo et al., 2014) et en Amérique (Londono-Renteria et al. 2015a). Le niveau d'anticorps IgG dirigé contre le peptide gSG6-P1 variait selon les villages et les enquêtes. Les analyses multivariées ont montré une relation positive et « dose-dépendante » entre l'intensité de la réponse anticorps à gSG6-P1 et le degré d'exposition aux piqûres d'Anopheles (mesuré par le nombre de piqures reçu par homme et par nuit). Ces résultats s'expliquent par le fait que *An. minimus* et *An. maculatus* étaient les deux espèces dominantes dans les villages étudiés. Nos résultats ont montré une relation significative entre l'âge, la saison et le village et la réponse anticorps anti-salive d'Anopheles. Les comportements humains et les pratiques agricoles sont

soupçonnés de moduler le contact « homme-vecteur » dans la région. De plus, nos résultats ont mis en évidence une forte association entre la réponse anticorps anti-gSG6-P1 et les taux d'inoculation entomologique (EIR), indiquant que l'hétérogénéité de la transmission du paludisme était directement associée à l'hétérogénéité du comportement de piqure des vecteurs dans la zone d'étude.

Enfin, des populations à risque ou « Hotpops », ayant des réponses immunitaires élevées aux piqûres de vecteurs, ont été identifiées dans tous les villages. Nos résultats ont montré que les emplacements de ces « hotpops » variaient selon la saison et avaient tendance à être plus dispersés pendant la saison des pluies et étroitement regroupés dans des « poches » pendant la saison sèche. Ces résultats traduisent bien l'épidémiologie du paludisme le long de la TMB, où des clusters d'infections palustres sont généralement observés pendant la saison sèche (Parker et al., 2015b). Ces premiers résultats suggèrent que le biomarqueur salivaire est prometteur pour estimer la variation de risque d'exposition aux piqures de vecteurs à l'échelle micro géographique dans des zones de faible intensité de transmission.

Dans le deuxième chapitre de cette thèse, nous avons pu démontrer une relation positive et «dose-dépendante» entre la séroprévalence à *Pf*CSP ou *Pf*MSP-1_{19} et l'intensité de la réponse anticorps anti-gSG6-P1. Une analyse multivariée a montré que les individus ayant la réponse anticorps anti-gSG6-P1 avaient 6 fois (IC 95% 3,7-9,5) plus de chance d'être positifs aux antigènes CSP et 2,3 fois (IC 95%, 1,4-3,8) plus de chance d'être infectés par *P. falciparum* par rapport aux faibles répondeurs. Ceci confirme nos premiers résultats montrant une corrélation significative entre le taux d'inoculation entomologique (EIR) et les réponses anticorps anti salive d'Anopheles (Ya-Umphan et al., 2017). Notre étude a ainsi permis de démontrer que la réponse anti-gSG6-P1 est fortement corrélée avec le risque de transmission et pourrait être utilisée comme « proxy » pour identifier les «hotpsots» de paludisme dans le cadre de la surveillance et de l'élimination du paludisme.

Les statistiques de balayage spatial ont montré que les zones d'infections sub-microscopiques *à P. falciparum* étaient géographiquement corrélées aux zones d'exposition des moustiques vecteurs avant introduction de la TdM. Cette découverte suggère que les porteurs submicroscopiques jouent probablement un rôle dans la transmission du paludisme dans les

villages étudiés. L'autocorrélation spatiale entre l'exposition du vecteur et l'exposition à *P. falciparum* était moins évidente au cours de l'étude en raison de l'introduction successive du TdM dans les villages qui ont contribué à éliminer> 95% du réservoir à *P. falciparum* (Landier et al., 2017).

Nos résultats ont aussi montré que le TdM était significativement corrélé à la séroprévalence de l'infection à *P. falciparum*, mais que la force et la direction de l'interaction variait selon l'antigène étudié (négativement corrélé pour *Pf*CSP et positivement corrélé pour *Pf*MSP-1_{19}). La raison d'une telle discordance n'est pas connue mais nous pensons que les différences immunogèniques (par exemple longévité des anticorps) et/ou la durée d'exposition du système immunitaire aux antigènes (immunogénicité des antigènes) peuvent expliquer les résultats (Biggs et al., 2017, Mosha et al. 2014). Une réaction croisée entre *P. falciparum* et *P. vivax* ne peut pas être totalement écartée car l'antigène MSP-1_{19} partage ~50% d'identité dans la séquence d'acide animique entre les deux espèces (Nagao et al 2008). Ceci pourrait donc expliquer le maintien d'une réponses anticorps élevé à *Pf*MSP-1_{19} dans une zone où *P. vivax* persiste après le TdM (Landier et al., 2017).

Enfin, nous avons montré que l'utilisation de moustiquaires n'était pas associée à une réduction de l'exposition de l'homme *à P. falciparum*, confirmant ainsi les résultats précédents montrant une absence de corrélation entre l'utilisation des moustiquaires et l'intensité de la réponse anti-salive d'anophèles (Ya-umphan et al. 2017). Cette découverte suggère que des outils de protection individuelle plus appropriés (par exemple des répulsifs, et/ou vêtements imprégnés d'insecticides) devraient être distribués aux personnes à risque pour renforcer les efforts de lutte et de prévention du paludisme dans la région.

Pour conclure, la présente étude a mis en évidence le fort potentiel d'utilisation du biomarqueur anti-salivaire d'Anopheles (gSG6-P1) comme outil épidémiologique pour évaluer l'exposition humaine aux piqûres de vecteurs et identifier les zones à risque de transmission de *P. falciparum* le long de la TMB. Ce biomarqueur pourrait être utilisé pour surmonter les limitations des méthodes entomologiques conventionnelles (par exemple le taux d'inoculation entomologique est peu sensible dans les zones de faible transmission car la prévalence d'infections des vecteurs collectés est quasi nulle). Avec les objectifs d'élimination du paludisme

dans la sous-région du Grand Mékong, le biomarqueur salivaire représente également une approche intéressante pour évaluer l'efficacité des interventions de lutte antivectorielle dans les zones où les marqueurs malariométriques conventionnels peuvent échouer. Cet outil pourrait être utilisé pour améliorer la surveillance des vecteurs et guider les politiques de santé publique pour l'élimination du paludisme. Le biomarqueur salivaire pourrait être bénéfique à toutes les étapes de la stratégie d'élimination et servir à établir un système d'alerte précoce pour surveiller la vulnérabilité des populations aux piqûres de vecteurs du paludisme. L'avantage est non seulement d'identifier des zones micro-géographiques à fort risque vectoriel («hotspots») mais également de fournir une cartographie fine des communautés à risque de transmission («hotpop»). Dans une région ou l'élimination du paludisme est une priorité, cela pourrait permettre d'effectuer des traitement préventifs et ciblés, principalement dans les zones forestières et transfrontalières difficiles d'accès (Satimai et al., 2012). En phase de post-élimination, le biomarqueur de l'exposition aux piqûres d'anopheles pourrait être utilisé pour cibler la part de population susceptible de recevoir de nouvelles piqûres infectantes si le parasite est réintroduit. Le développement de kits de diagnostic rapide de l'exposition humaine aux piqûres d'Anopheles est cependant nécessaire pour assurer l'incorporation de ce biomarqueur dans les programmes nationaux de lutte contre le paludisme.

Risk of malaria transmission along the Thailand-Myanmar border by the use of specific biomarker of human exposure to *Anopheles* bites and *Plasmodium spp.*

Malaria along the Thailand-Myanmar border (TMB) displays geographical heterogeneity and is characterized by high prevalence of submicroscopic carriage and the emergence artemisinin resistance in *P. falciparum*. Timely identification and elimination of remaining *P. falciparum* transmission "hotspots" is essential to contain artemisinin resistance. The aim of this study was to address the relevance of using serological biomarkers of human exposure to *Anopheles* bites (gSG6-P1) and *Plasmodium* antigens (CSP & $MSP1_{19}$) to identify remaining sources of transmission and to measure spatial and temporal changes in human vector contact along the TMB. Blood spots were collected in filter papers among a cohort of ≈2600 people followed every 3 months up to 18 months, and used for analysis by enzyme-linked immunosorbent assay (ELISA). Our findings showed that the levels of IgG responses to gSG6-P1 antigen varied according to village, season, and age and were positively associated with the abundance of total *Anopheles* species and primary malaria vectors. A significant and positive association was noted between the antibody response to gSG6-P1 and the entomological inoculation rate (EIR) hence demonstrating that heterogeneity in malaria transmission was directly associated with heterogeneous biting behavior. Further investigations showed that salivary biomarker was relevant to detect small scale variations in *P. falciparum* malaria. This was supported by scan statistics showing that *P. falciparum* clusters partially overlap the gSG6-P1 clusters. Altogether, these findings indicates *Anopheles* salivary biomarker as great potential for epidemiological studies and could be useful to guide the implementation of hotspot-targeted vector control interventions with the aim to achieve malaria elimination.

Key words: Thailand-Myanmar border, *Plasmodium falciparum* malaria, Serological biomarker, Salivary Biomarker, gSG6-P1, Human antibody response, Malaria vectors, Transmission

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Etude du risque de transmission du paludisme dans la zone thailando birmane par l'utilisation de biomarqueurs specifiques d'exposition humaine aux piqures d'*Anopheles* et au *Plasmodium*

Le long de la frontière entre la Thaïlande et le Myanmar (TMB), le paludisme se caractérise par une forte hétérogénéité de la transmission, une forte prévalence en porteurs sub-microscopiques et par l'émergence de la résistance à l'artémisinine chez *Plasmodium falciparum*. L'identification précoce des « foyers » infectieux et leurs éliminations sont nécessaires pour contenir la résistance à l'artémisinine. L'objectif de cette thèse était de démontrer l'intérêt d'utiliser des biomarqueurs sérologiques de l'exposition humaine aux piqûres *d'Anophèles* (gSG6-P1) et au *Plasmodium* (CSP & $MSP1_{19}$) pour quantifier le contact homme-vecteur et identifier les foyers résiduels de transmission. Des papiers filtres contenant du sang ont été prélevés sur une cohorte de 2600 personnes suivie tous les 3 mois jusqu'à 18 mois et analysés par dosage immuno-enzymatique (ELISA). Nos résultats ont montré que les niveaux de réponse IgG à l'antigène gSG6-P1 variaient selon le village, la saison et l'âge et étaient positivement corrélés à l'abondance des espèces *Anophèles* et des vecteurs primaires de paludisme. Une association significative et positive a été observée entre la réponse de l'anticorps au gSG6-P1 et le taux d'inoculation entomologique (EIR), démontrant ainsi que l'hétérogénéité de la transmission du paludisme était directement associée à un comportement de piqûre hétérogène. Des études complémentaires ont montré que le biomarqueur salivaire était pertinent pour détecter des variations micro géographiques dans la transmission à *P. falciparum*. Cela s'est traduit par des chevauchements significatifs entre les foyers infectieux à *P. falciparum* et ceux à forts répondeurs en anticorps anti-salive d'*Anopheles* (gSG6-P1). Dans l'ensemble, ces résultats indiquent que le biomarqueur salivaire d'*Anopheles* est prometteur pour les études épidémiologiques et pourrait guider la mise en œuvre d'interventions de lutte antivectorielle « ciblées » afin d'éliminer les foyers résiduels de paludisme.

Mots clés; Zone frontalière Thailando-Birmane, *Plasmodium falciparum*, marqueurs sérologiques, biomarqueur salivaire, gSG6-P1, Réponse anticorps, vecteurs de paludisme, Transmission