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Adapt or perish: defining malaria vector behaviours in a changing world

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Contributions

Chapter	**Contributions**
1. Introduction to the thesis	Bram van de Straat wrote the chapter; Tom Burkot and Tanya Russell provided input and edits to the content of the chapter.
2. Zoonotic malaria and land-use change in Southeast Asia: what is known about the vectors	Bram van de Straat and Tom Burkot conceived the review and analysis of existing data. Bram van de Straat performed the initial literature search and wrote the original draft of the manuscript. Boni Sebayang, Tanya Russell, Triwibowo Ambar Garjito and Indra Vythilingam provided significant input and edits to the manuscript as well as additional literature and invaluable insights on the vectors; Kyran Staunton, Tanya Russell and Matt Grigg provided input and literature on macaque biology and human land-use change. All authors read and approved the final manuscript.
3. A global assessment of surveillance methods for dominant malaria vectors	Bram van de Straat, Tanya Russell and Tom Burkot conceived the research. Bram van de Straat analysed the data and wrote the original draft of the manuscript. Tanya Russell, Kyran Staunton and Marianne Sinka provided input on the data analyses. All authors reviewed and approved the final manuscript.
4. Evaluation of different methods to collect human and simian malaria vectors in North Sumatra, Indonesia	Bram van de Straat, Tanya Russell and Tom Burkot conceived the study; Tom Burkot, Tanya Russell, Triwibowo Ambar Garjito and Inke Lubis advised on the fieldwork and laboratory analyses. Bram van de Straat and Ahadi Kurniawan conducted the fieldwork, and Bram van de Straat and Boni Sebayang performed the molecular analyses. Bram van de Straat analysed the data and wrote the initial draft of the manuscript. All authors read and approved the final manuscript.
5. Characterisation of *Anopheles* bionomics across different land-use types in North Sumatra, Indonesia	Bram van de Straat, Tanya Russell and Tom Burkot conceived the study; Tom Burkot, Tanya Russell, and Inke Lubis advised on the fieldwork and laboratory analyses. Bram van de Straat and Ahadi Kurniawan conducted the fieldwork, and Bram van de Straat and Boni Sebayang performed the molecular analyses. Bram van de Straat analysed the data and wrote the initial draft of the manuscript. All authors read and approved the final manuscript.
6. Emergence of outdoor biting and reduced anthropophagy in African malaria vector species before the scale-up in insecticide-based control	Bram van de Straat, Tanya Russell and Tom Burkot conceived the research. Marianne Sinka led the compilation of the dataset. Bram van de Straat analysed the data and wrote the original draft of the manuscript. Tanya Russell provided input on the data analyses. All authors read and approved the final manuscript.
7. Key findings, implications, and conclusions	Bram van de Straat wrote the chapter; Tom Burkot and Tanya Russell provided input and edits to the content of the chapter.

Personal note

The scope of this thesis has changed a fair bit since its initial conception. The original idea was to create a PhD thesis which was fully based on experimental data gathered in the field, specifically in North Sumatra and Kalimantan, Indonesia. This idea was conceptualised between September and December 2019, and my application to James Cook University was formally approved in February 2020. Consequently, travel was arranged, and it was decided that I would commence my PhD journey in Australia on 24 March 2020. Never have any of my plans changed so dramatically.

Two days before I was supposed to board my flight to Australia, the world went in full lockdown and all travel was suspended until further notice. Not only did this heavily affect my living situation (I had to move in with my parents, after ten years of independence), it also meant that the scope of my PhD project had to change. Instead of focussing on field research in Indonesia, I had to be flexible and resilient if I was to complete a PhD within the expected time. I made the most of my time while waiting for the pandemic to subside by conducting analyses on an extensive global dataset on vector surveillance and behaviours based on all the published literature. These subjects were highly relevant for my original thesis topic. Therefore, I believe that these analyses are valuable and integral additions to the thesis, resulting in two research chapters at a global perspective that situate the local data chapters. One of the global data analysis chapters has been published and the other submitted for publication.

In March 2022, two years after commencing my PhD journey, the situation in the world was finally allowing international travel again. This marked the start of the preparations for the fieldwork in Indonesia, which would begin in July 2022, after at least a year of delays. The results of this fieldwork are presented on chapters 4 and 5, both of which will be submitted as manuscript for publication as soon as possible.

Thesis abstract

Simian malaria infections in humans are an increasing public health concern across Southeast Asia, notably in rural areas of Malaysia and Indonesia. While reported case numbers of human malarias in the region are decreasing and several countries are approaching elimination of human malaria, the number of case reports of simian malaria in humans is increasing. Human infections with simian malaria were historically associated with the deep forest, but land development from deforestation and changing agricultural practices are changing this paradigm. The aim of this thesis was to define the bionomics of the simian malaria vectors, and to study the effects of different land-use types on vector distributions and behaviours in a rural area in North Sumatra, Indonesia. This work was put into context by an analysis of surveillance methods for dominant malaria vectors deployed globally and an analysis of dominant malaria vector behavioural changes over a 26-year period.

This thesis is composed of two broad areas of work: analyses of published data and original field research. The existing literature on vectors of simian malarias in Southeast Asia was first compiled in a literature review and analysed for strength of evidence of what is known about the vectors. A global data analysis of collection methods was then conducted to understand representative sampling methods for malaria vectors to inform the original field studies.

The field studies consisted of two stages. The objective of the first stage was to investigate the sensitivity and specificity of multiple sampling methods for potential simian malaria vectors. Human landing collections, animal-baited tent traps and three mechanical traps baited with CO_2 were tested in a Latin square design. The second stage consisted of a year-long surveillance study of potential simian malaria vectors in three common land-use types in rural North Sumatra using human landing collections, which was the most efficient sampling method according to the Latin square trials. Finally, a comprehensive global dataset of dominant vector behaviours was analysed to investigate whether changes in vector behaviours might explain the global reductions in malaria mortality and morbidity while programmatic vector control was largely absent.

Knowledge on the vectors that transmit simian malaria to humans is based on a small number of studies. Additionally, adhering to current dogmas which are based on limited knowledge to design vector sampling strategies might reinforce biases in surveillance data. Representative sampling by tailor-made surveillance could detect behavioural shifts of vectors more rapidly and could thus help prevent diminishing effectiveness of deployed vector control strategies. Comparing multiple sampling methods to find a representative sampling method for potential simian malaria vectors in North Sumatra showed that *Anopheles leucosphyrus* Group females could be collected

effectively by human landing collections or cow-baited tent traps. Due to logistical challenges with the cow-baited tent traps, human landing collections were used to define *Anopheles* bionomics for one year in a village in North Sumatra, encompassing residential areas, a mixed agricultural area, and an oil palm plantation. Clear differences in *Anopheles* occurrence and density were observed between the three land-use types. *Anopheles leucosphyrus* Group females, the suspected vectors of simian malaria to humans, showed peak biting well before most people went inside their houses, with the highest biting rates in the mixed agricultural area and the lowest in the oil palm plantation. *Anopheles maculatus* Group females, a suspected vector of human malaria in Indonesia, showed the highest biting rates in the oil palm plantation.

Shifts in behaviours of dominant malaria vectors in Africa were found with human blood feeding and indoor biting diminishing from 1985 to 2011. It appears that selective pressure inside houses, possibly house improvements and the use of untreated bed nets, among other unknown anthropogenic drivers, may have been created on malaria vector populations in sub-Sahara Africa. In Southeast Asia, habitat fragmentation because of agricultural development and human encroachment on existing habitats can increase human interactions with both macaques and simian malaria vectors, especially in forest edges. Predicting the risk of simian malaria infections in specific land-use types remains a challenge, requiring knowledge on the bionomics and infection rates of vectors, and behaviours of humans and macaques. To effectively control emerging simian malaria cases in humans, epidemiologically relevant and unbiased surveillance of malaria vectors remains vital to understand where the risk of transmission is greatest and what behaviours of the vectors make them most vulnerable to interventions.

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Chapter 1

A general overview of the current state of human malaria is provided, and some brief information on the transmission cycles of human and simian malaria parasites is given. Finally, the aims and scope of this thesis are introduced.

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3. Sampling methods used to define malaria vector behaviours across geographic regions
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6. Trends in malaria vector behaviours across geographic regions
7. General discussion, future research, conclusions

Introduction to the thesis

Human malarias

Human malaria is a disease caused by parasites in the *Plasmodium* genus, that are transmitted between persons by infected *Anopheles* mosquitoes. Historically, there are five *Plasmodium* species that are transmitted from human to human, causing symptomatic disease. The most prevalent and most lethal species is *Plasmodium falciparum* (>240 million cases in 2021), followed by *Plasmodium vivax* (4.9 million cases in 2021). *Plasmodium malariae*, *Plasmodium ovale curtisi*, and *Plasmodium ovale wallikeri* remain largely neglected tropical diseases because of their lower severity of symptomatic infections, even though their pooled prevalence is possibly higher than *P. vivax* (1). Symptoms of malaria include recurring periods of high fever, headaches, and chills, with severe malaria potentially leading to coma or death (2,3). The World Health Organization (WHO) estimated that there were 247 million malaria cases in 2021, spanning 84 countries (4). Ninety-five per cent (234 million) of malaria cases occurred in the WHO African Region, while the WHO Southeast Asian region, the second-most malarious region, accounted for approximately 2% or 5 million cases in 2021. Although the reductions in malaria mortality and morbidity have received global attention since the 1950s (5,6), the largest reductions in malaria were made since the year 2000. These advances were facilitated by large, multi-country programmes like Roll Back Malaria (starting in 1998), the Global Fund to Fight AIDS, Tuberculosis and Malaria (starting in 2002), and the President's Malaria Initiative (starting in 2005). Globally, malaria case numbers decreased from an estimated 262 million in 2000 to 214 million in 2015, while the mortality rate of malaria (the number of malaria deaths per 100,000 population at risk) reduced from 30 to 15 per 100,000 in the period 2000-2015 (7). These reductions are attributed mainly to three malaria intervention strategies: mosquito vector control, rapid diagnostic testing and treatment for malaria cases, and chemoprevention of malaria for pregnant women and children. Malaria vector control methods are especially effective, with insecticide-treated bed nets (ITNs) and indoor-residual spraying of insecticides (IRS) accounting for 68% and 13%, respectively, of the total reduction in malaria cases between 2000-2015 in sub-Sahara Africa, thereby averting hundreds of millions of malaria cases in the region (8). However, global malaria morbidity and mortality have been increasing since 2016, reflecting the vulnerability of present intervention strategies to many challenges (4).

Malaria transmission and development cycle

Plasmodium parasites have a complicated transmission cycle as they depend on both a vertebrate and a mosquito host. The cycle can roughly be divided in three parts: the host liver stages (or exo-erythrocytic schizogeny), the host blood stages (erythrocytic schizogeny), and the mosquito stages (sporogonic cycle) (Fig. 1.1). When a *Plasmodium*-infected mosquito feeds on a vertebrate host, the mosquito injects *Plasmodium* sporozoites into the host via its saliva. The inoculated sporozoites enter liver cells, where they replicate and mature as merozoites inside schizonts. Finally, the schizonts in the infected liver cells rupture simultaneously and release merozoites into the blood stream, which causes the symptomatic manifestations of malaria. The merozoites infect red blood cells, develop into rings, then into trophozoites and eventually into schizonts, inside of which blood stage merozoites develop. These schizonts rupture again simultaneously and release another wave of parasites into the blood stream. Most of these parasites are asexually reproducing merozoites that will infect additional erythrocytes of the host. A small proportion of parasites will differentiate into male and female gametocytes. To sexually reproduce, male and female gametocytes need to be ingested by a mosquito when it takes a blood meal, because sexual reproduction can only take place in the mosquito. The midgut environment in the mosquito triggers the male gametocytes to exflagellate into male gametes, which can fertilise the female gametes, and in so doing create a zygote. After meiosis, the zygote develops into a mobile ookinete that penetrates the midgut of the mosquito and attaches to the outside wall, where it forms an oocyst. Within an oocyst, many sporozoites will develop, which will be released in the haemolymph of the mosquito. From here, the mobile sporozoites will move through the entire

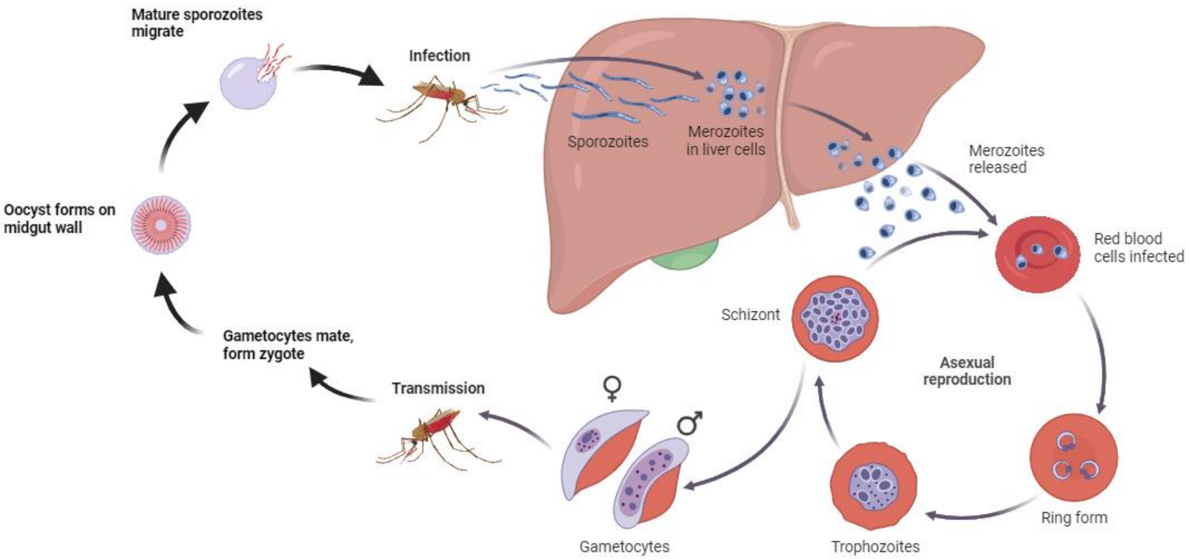


Figure 1.1 – Overview of the Plasmodium falciparum *transmission cycle. Figure created by Bram van de Straat in BioRender.*

body of the mosquito where some will invade the salivary glands, ready to infect a new host when the mosquito takes a blood meal.

The cycle described above is typical of *P. falciparum* and *P. malariae*. The developmental cycle of *P. vivax* and *P. ovale* (both species) is further complicated due to inclusion of a dormant hypnozoite stage in the liver. Some sporozoites do not develop into merozoites but form hypnozoites, which can lay dormant for weeks or months before they are released into the blood stream and initiate relapses after the initial blood stage infection. While the developmental cycle of *Plasmodium knowlesi*, the commonest and best-studied zoonotic malaria species in humans, is similar to that of *P. falciparum*, there are several notable differences. The asexual reproduction cycle of *P. knowlesi* takes only 24 hours, typically causing daily fever spikes in patients, as well as rapidly developing high parasitaemia in some patients (9). In addition, the long-tailed and pig-tailed macaques are the natural hosts of *P. knowlesi*. It is not known whether humans are true dead-end hosts or if long-chain human-mosquito-human transmission occurs to some extent to maintain *P. knowlesi* transmission in the absence of macaques (10). Hence, the transmission cycle of *P. knowlesi* is more complex than that of human *Plasmodium* species.

A key aspect of the *Plasmodium* transmission cycle is the life cycle of its vectors. All mosquitoes use plant sugars as nutritional resource, but to produce their eggs, female mosquitoes require a blood meal. Host preference of mosquitoes is determined by many factors, including genetic selection and the availability and density of host species (11). While some mosquito species are generalists and have no preference for blood from a certain host species (opportunistic feeding behaviour), others can express highly preferential behaviour for a vertebrate species. Some of the specialist feeders in the *Anopheles* genus (i.e., *Anopheles gambiae* in Africa, *Anopheles dirus* in Asia) have evolved a preference for human blood (anthropophily), and hence belong to the most important vectors of malaria to humans.

Malaria elimination (the reduction to zero parasite transmission in a specific area) can only be achieved by interrupting the transmission cycle (12). Malaria vector control methods like the indoor spraying of residual insecticides or the use of insecticide-treated bed nets are very effective because they physically disrupt the cycle of malaria transmission. Indoor residual spraying of insecticides kills vectors after they blood feed, while bed nets prevent blood feeding. When properly used and maintained, bed nets provide a physical barrier between humans and *Anopheles* females infected with *Plasmodium* that are looking for a bloodmeal (13). Insecticide-treated bed nets (ITNs) take this protection a step further as the insecticide on the net creates a lethal barrier for host-seeking mosquitoes that land on it, thus providing an additional protective effect for the community (14). In this way, non-infected *Anopheles* females cannot ingest blood which may contain *Plasmodium* gametocytes, and mosquito vectors already infected with *Plasmodium* are not able to transmit sporozoites to humans. Unlike ITNs, indoor residual spraying of insecticides (IRS) usually kills vectors after taking a blood meal, thus preventing further parasite transmission in subsequent feeding cycles.

Behaviours of mosquitoes for targeted interventions

Malaria vector control methods, or interventions, are most effective when they target specific vector behaviours. Presently, the primary vector control strategies recommended by the WHO are only indoor residual spraying (IRS) and insecticide-treated nets (ITNs) across all malaria transmission-endemic areas. As supplemental malaria vector control methods, the WHO recommends larval source management (LSM) in specific situations, and house improvements like screening.

Larval source management (LSM) by applying larvicides to breeding sites can be an effective control method and forms an important aspect of vector control in many regions (15). However, the effectiveness of LSM decreases when the target vector species breed in large water bodies (i.e., marshes or streams), since these are difficult to treat effectively with larvicides. Hence, the WHO recommends LSM as a supplemental control method in situations where larval habitats are few, fixed in location or in number and easily accessible, and such as where transmission is seasonal or in (peri-)urban areas.

House screening has a conditional WHO recommendation due to logistic concerns regarding how the intervention will be implemented and managed in houses suitable for screening, and if the resources for implementation are sufficient to undertake on a programmatic scale.

Spraying of residual insecticides on the inside walls of houses, which was the earliest intervention that was adopted on a global scale (5,16), is effective when malaria vectors are endophilic: they usually rest inside houses for some time, usually after taking a blood meal. Endophilic mosquitoes, like *Anopheles gambiae* s.s. in Africa and *Anopheles minimus* in Southeast Asia, are likely to rest indoors on a sprayed surface and thus to pick up a lethal dose of insecticide. However, some malaria vectors that are exophilic (i.e., that primarily rest outside) are less sensitive to IRS (17–19). Still, the transmission cycle can be disrupted using bed nets if vector species are endophagic (they regularly feed on humans inside houses). As mentioned earlier, (insecticide-treated) bed nets provide a potentially lethal protective barrier against infectious mosquito bites. The protective efficacy of ITNs and IRS is highly dependent on mosquito behaviours and having high access and use of these interventions (20). When coverage of ITNs and IRS is high, they confer community protection by depressing transmission, thereby even protecting individuals sleeping in houses without either of these interventions. When the peak biting time of the vectors in an area coincides with the times that most people are asleep, bed nets will provide maximum protection from potentially infectious mosquito bites. Many examples exist, however, of mosquito species that bite early in the evening (i.e., *An. gambiae* s.l. (21) and *Anopheles stephensi* (22) in some regions in Africa and *Anopheles dirus* in Southeast Asia (23)).

Besides mosquito populations that are naturally exophilic or that are early biters, malaria vectors can also actively avoid interventions. Behavioural plasticity of vectors was observed in the earliest trials with insecticide-treated bed nets, when most vectors seeking a blood host shifted from late-night biting to earlier in the evening, when most humans were not asleep yet (24). Another example of behavioural plasticity was observed in the Solomon Islands, where late night, indoor biting *Anopheles farauti* shifted towards early outdoor biting after DDT insecticide indoor spraying campaigns (25). The persistence of the behavioural change after withdrawal of IRS persisted in the absence of IRS, such as is seen in *An. farauti* populations in the Solomon Islands is termed behavioural resistance, implying a genetic change has occurred. Hence, behavioural resistance can be defined as behaviours that are heritable, evolved in response to the selective pressures exerted by a toxicant, that enhance the ability of a population to avoid the lethal effects of that toxicant and are persistent even after the selective pressure is removed (26,27). Although behavioural shifts in response to bed nets or indoor residual spraying were observed in several mosquito populations (28), outdoor-biting mosquitoes will occasionally enter houses to feed and hence ITNs can remain effective (14). Many other vector populations continued their late-night, indoor-biting behaviours (29), resulting in the large reductions in human malaria cases as well as the development of physiological resistance to insecticides.

Primate malarias

There are still many obstacles to overcome to eliminate human malaria. Besides the resistance of *P. falciparum* and *P. vivax* to anti-malarial drugs and the physiological and behavioural resistance of mosquito vectors to insecticides, zoonotic malaria species originating from monkeys are an emerging public health concern, especially in Southeast Asia. Zoonotic *Plasmodium knowlesi* was identified as a public health threat in 2004 and has since been reported throughout Southeast Asia where both its monkey hosts and mosquito vectors are found (30–32). Preliminary studies and several case studies in Sumatra and Kalimantan have confirmed the presence of zoonotic simian malaria cases in humans in Indonesia (33–35). Recently, the number of reported zoonotic malaria infections in humans has been increasing, notably in countries that have eliminated or are close to eliminating human-only *Plasmodium* transmission, like Malaysia (36). The growing numbers of zoonotic malaria infections will complicate malaria control in the Southeast Asia region and could prevent countries from meeting their malaria elimination targets. Control of *P. knowlesi* and other zoonotic malaria species is particularly challenging due to the ecology of its vectors. Historically, these were perceived to be forest mosquitoes, mainly preferring macaques and other primates as blood hosts, and thereby sustaining the enzootic transmission cycle of *P. knowlesi* and other non-human primate malaria species (i.e., *Plasmodium cynomolgi* and *Plasmodium inui*) (10). However, deforestation for agricultural development is hypothesised to divert mosquito biting from monkeys in the canopy, to humans on the ground (37). This scenario would increase spill over risk of zoonotic malaria infections to humans when combined with an intact enzootic transmission cycle within monkey populations (Fig. 1.2). Since many of the perceived vector species are biting and resting outdoors, current vector control methods like ITNs and IRS would have a limited effect on the transmission of zoonotic malarias to humans (38,39).

To understand the risk of infections in humans with simian malaria, it is essential to study the ecology and behaviour (bionomics) of the vectors. Vector bionomics consist of multiple elements, including biting times and locations, seasonality, human biting rates and entomological inoculation rates. When the bionomics of a vector species are understood, control measures can be selected to target behavioural vulnerabilities to maximise effectiveness.

Thesis scope, objectives, and structure

Simian malaria cases in humans are an increasing challenge in areas of rural Indonesia. Human infections were historically associated with areas of deep forest, but land development, deforestation, and changing agricultural practices are changing this paradigm. Preliminary studies and several case studies in Sumatra (Aceh) and Kalimantan have confirmed the presence of zoonotic simian malaria cases in humans in Indonesia, predominantly in Sumatra and Kalimantan (33–35).

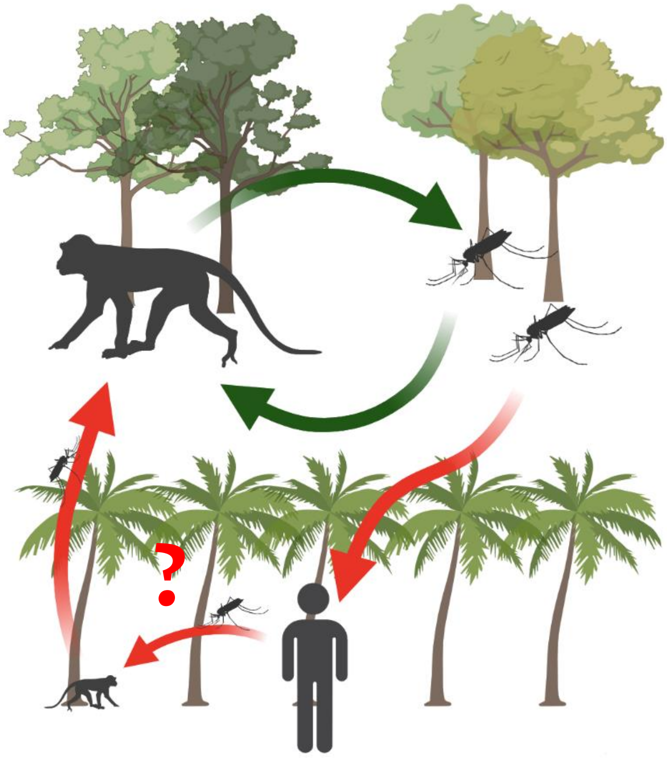


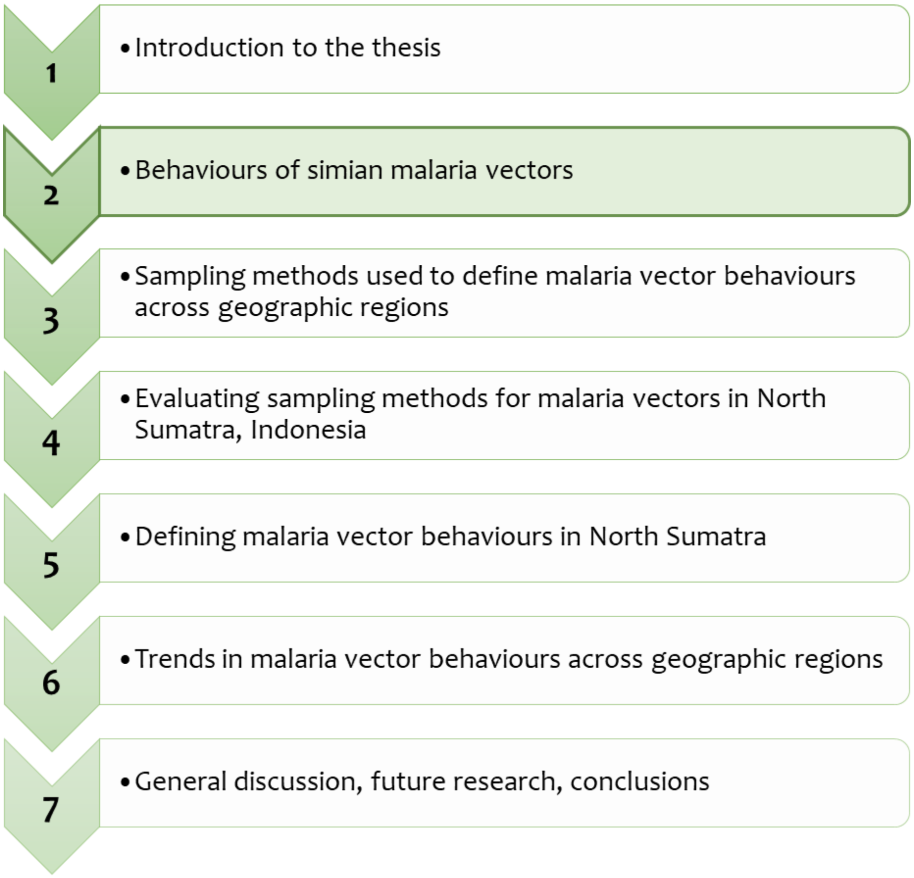
Figure 1.2 - The full transmission cycle of simian malarias, with the enzootic cycle above and the spill-over infections to humans below. Human-to-human transmission of simian malarias cannot be excluded but more evidence is needed.

The aim of this thesis was to define the bionomics of the simian malaria vectors in North Sumatra, Indonesia, and to study the effects of different land-use types on vector distributions and behaviours. The hypothesis is that that changing land uses impact the distribution and densities of the vectors, the macaque reservoir, and humans, thus changing the exposure of humans to bites by the vectors. Investigating this hypothesis requires understanding the present knowledge on simian malaria vector behaviours in Indonesia, as well as the efficacy and limitations of sampling methods used to incriminate vectors and to define their behaviours. This work was put into context by an analysis of surveillance methods for dominant malaria vectors deployed globally and an analysis of dominant malaria vector behavioural changes over a 26-year period.

Chapter 2 is a review of the existing literature covering what is known, and the strength of the evidence, about simian malaria vectors in Southeast Asia. In chapter 3, a precompiled dataset of all published papers on the dominant malaria vectors was analysed to investigate sampling methods used, not just in Indonesia or Southeast Asia but globally, to collect malaria vectors. This chapter provided key information on representative sampling methods for malaria vectors and directly informed the study design of the next chapter, a focussed investigation of sampling method sensitivity and specificity in North Sumatra, Indonesia. Chapter 4 thus describes field research conducted to evaluate different collection methods for *Anopheles* females including potential simian malaria vectors in North Sumatra. The objective of this research chapter was to find epidemiologically relevant and efficient collection methods by evaluating potential alternatives to methods currently used in Sumatra. In chapter 5, bionomics of *Anopheles* mosquitoes in North Sumatra were documented using the most efficient and relevant method of the sampling methods evaluated in the preceding chapter. Anopheline bionomics were studied across three dominant land-use types to quantify the potential simian malaria infection risk in humans. In chapter 6, the final research chapter, global behavioural patterns of malaria vectors were studied by geographic region (Africa, Asia, and the Americas). The objective of this database-driven study was to characterise long-term trends in dominant vector behaviours and what possible drivers were selecting for any changes in behaviours found. The results from the four research chapters are integrated in chapter 7. This final chapter also provides an outlook on the future of malaria control, with a focus on zoonotic malaria control, and the conclusions of the thesis.

Chapter 2

Here, an overview of the current knowledge and paradigms on zoonotic simian malarias in Southeast Asia is provided. Several knowledge gaps will be highlighted to lay a foundation for the forthcoming research chapters. This chapter was published in Malaria Journal (2022) 21:109.



Zoonotic malaria and land-use change in Southeast Asia: what is known about the vectors

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Zoonotic malaria, *Plasmodium knowlesi*, Leucosphyrus Group, Mosquito vectors, Vector behaviour, Human land-use

Abstract

Zoonotic *Plasmodium* infections in humans in many Southeast Asian countries have been increasing, including in countries approaching elimination of human-only malaria transmission. Most simian malarias in humans are caused by *Plasmodium knowlesi*, but recent research shows that humans are at risk of many different simian *Plasmodium* species. In Southeast Asia, simian *Plasmodium* species are mainly transmitted by mosquitoes in the *Anopheles leucosphyrus* and *Anopheles dirus* complexes. Although there is some evidence of species outside the Leucosphyrus Group transmitting simian *Plasmodium* species, these await confirmation of transmission to humans. The vectors of monkey malarias are mostly found in forests and forest fringes, where they readily bite long-tailed and pig-tailed macaques (the natural reservoir hosts) and humans. How changing land-uses influence zoonotic malaria vectors is still poorly understood. Fragmentation of forests from logging, agriculture and other human activities is associated with increased zoonotic *Plasmodium* vector exposure. This is thought to occur through altered macaque and mosquito distributions and behaviours, and importantly, increased proximity of humans, macaques, and mosquito vectors. Underlying the increase in vector densities is the issue that the land-use change and human activities create more oviposition sites and, in correlation, increases availably of human blood hosts.

The current understanding of zoonotic malaria vector species is largely based on a small number of studies in geographically restricted areas. What is known about the vectors is limited: the data is strongest for distribution and density with only weak evidence for a limited number of species in the Leucosphyrus Group for resting habits, insecticide resistance, blood feeding habits and larval habitats. More data are needed on vector diversity and bionomics in additional geographic areas to understand both the impacts on transmission of anthropogenic land-use change and how this significant disease in humans might be controlled.

Background

Since the turn of the millennium, substantial progress has been made to reduce the global incidence of human malaria caused by *Plasmodium falciparum* and *Plasmodium vivax*. Indeed, elimination of *P. falciparum* and *P. vivax* has been achieved in a number of countries (8). Largely responsible for this success has been the wide-scale use of insecticide-treated nets (ITNs) and indoor residual spraying (IRS), coupled with improved point-of-care diagnostics and treatment with artemisinin-based combination therapy. Recently, the number of reported zoonotic *Plasmodium* species infections in humans have been increasing, including in countries that have eliminated human malaria (Singapore, Brunei) as well as in countries such as Malaysia, which is close to eliminating human-only malaria transmission (36,40,41).

The most common cause of zoonotic malaria in Southeast Asia is due to the natural macaque host parasite *Plasmodium knowlesi*. *The Primate Malarias* (1971) (42) provides an excellent review of the discovery, in 1932, and subsequent transmission experiments of *P. knowlesi* to humans (43). Due to the discovery of a naturally transmitted *P. knowlesi* case (44), *The Primate Malarias* presciently warned that *P. knowlesi* could form a potential threat to humans as a zoonosis. However, it was thought at the time that zoonotic cases of *P. knowlesi* (and other simian malarias) were rare due to the strongly sylvan nature of both its primate hosts and mosquito vectors. Now, fifty years after the publication of *The Primate Malarias*, the understanding of how human activities are affecting, and potentially facilitating, the natural transmission of ‘monkey malaria’ to man is only just beginning.

Natural transmission of *P. knowlesi* to humans on a large scale was first described in 2004 in Sarawak, East Malaysia (30). Cases have since been discovered throughout the Southeast Asian region, including Indonesia (34,45), Lao PDR (46), Malaysia (31,47), Myanmar (48), the Philippines (49), Singapore (50,51), Brunei (52), Cambodia (53), Thailand (54) and Vietnam (55). Indeed, while human malaria transmission is waning, zoonotic *Plasmodium* infections in humans are rising and *P. knowlesi* malaria is now the dominant malaria in humans in Malaysia (36,56). *Plasmodium knowlesi* is primarily a parasite of non-human primates, especially of long-tailed macaques, and northern and southern pig-tailed macaques (*Macaca fascicularis*, *Macaca leonina*, and *Macaca nemestrina,* respectively). Human-to-human transmission via a mosquito vector, demonstrated experimentally, cannot be excluded as occurring in nature (44,57). Additionally, both the detection of *Plasmodium inui*, *Plasmodium inui*-like, *Plasmodium cynomolgi*, *Plasmodium knowlesi*, and *Plasmodium coatneyi* parasites in blood samples from Malaysia (58) and recent reports from both Peninsular Malaysia (59) and Malaysian Borneo (60,61) show naturally acquired human infections of several of the most common *Plasmodium* species in mosquitoes and macaques in Southeast Asia. The emergence of novel zoonotic malarias will complicate malaria control in the region.

This review will focus on transmission of *P. knowlesi* to humans in Southeast Asia, as such infections account for the highest incidence of zoonotic malaria and is the species for which relatively more is known about the transmission and vectors. The influence of anthropogenic land-use changes on the distributions and behaviours of the vectors of *P. knowlesi* malaria in Southeast Asia, with consequent spill-over transmission to humans, will be highlighted.

Vectors of zoonotic malaria in Southeast Asia

Tables 2.1 and 2.2 summarize what is known and the strength of the evidence for vector behaviour and transmission indicators (62) for human biting species known or strongly suspected to vector *P. knowlesi* to humans. Here, vector status is defined as regards *P. knowlesi* transmission to humans as confirmed, incriminated, or suspected. Confirmed vectors are species in which *P. knowlesi* sporozoites were found in the salivary glands, incriminated vectors are species in which *P. knowlesi* DNA was identified by PCR and suspected vectors refer to confirmed vectors of other simian malarias that occur in areas of *P. knowlesi* transmission. Although *Anopheles hackeri* was found to be a potential vector of simian malaria species including *P. knowlesi* in Peninsular Malaysia (63), it was later found to be mainly (if not entirely) zoophagic and not attracted to humans (64,65). Also, it has been suggested that *Anopheles kochi* might act as a vector of simian malaria species in Singapore (50). However, *An. kochi* is strongly zoophagic and bites humans only very sporadically (65–67). Hence, both *An. hackeri* and *An. kochi* are not considered to be important vectors of *P. knowlesi* and other simian malaria species to humans and will not be discussed further in this manuscript.

Although some indicators (occurrence, abundance, biting location) are relatively well-studied for most species, little to no evidence exists for many important indicators (resting location, larval habitats, sporozoite rates and EIR) (Table 2.1). What is known about the vectors' behaviours, especially the *Anopheles leucosphyrus* complex, is based on limited knowledge from a few point sources within the geographical distribution of the Leucosphyrus Group (Fig. 2.1). Additionally, even for *Anopheles balabacensis* and *Anopheles latens*, the best-studied and, as thus far known, most competent vectors of *P. knowlesi*, strong evidence is lacking for more than half of the entomological surveillance indicators (Table 2.1). Therefore, quantification of vector control target behaviours like biting location and peak biting times, as well as risk assessment of zoonotic malaria transmission to humans, remains challenging and should be a key focal point of future research.

Table 2.1 - Vector species of Plasmodium knowlesi: evidence for WHO indicators.

	WHO Indicators											
***P. knowlesi* vector species**	**Occurrence**	***Density***	***Biting time***	***Biting location***	***HBR***	***Resting location***	***Resistance***	***Larval habitat***	***HBI***	***Sporozoite rate***	***EIR***	**Literature**
An. latens	***	**	**	**	**	°		*	*	**	*	(10,68–74)
An. leucosphyrus	**			°								(75–77)
An. balabacensis	***	***	***	***	**		°			**	*	(57,69,71,74,78–85)
An. introlatus	**	**	*	**	*							(70,86–88)
An. dirus	***	***	**	**	**		**	**		**	°	(55,73,89–94)
An. cracens	**	**	**	**	*			*	°	**	*	(47,75,84,95)
An. donaldi	**	**	*	*	*					*		(74,78–80)
An. sundaicus	*	*										(96)
An. letifer	*	*		°						*		(69,87)

*This table includes published evidence on biological indicators of species that are known or strongly suspected to transmit zoonotic malaria, based on the WHO indicators for vector control. Only the publications that studies a species in its role as vector for zoonotic malaria are included; i.e., An. dirus is also a vector for human malarias but only research on its role as vector for zoonotic malaria is included. Evidence of direct findings are indicated by asterisks: * weak evidence (information from a single publication, or only mentioned as a sidenote in other publications); ** medium evidence (information from less than 5 publications, of which only a minority was mentioned as sidenote); *** strong evidence (information from 5 or more publications, none of which mentioned the evidence as a sidenote). Circumstantial evidence indicated by a. HBR human biting rate, HBI human blood index, EIR entomological inoculation rate.*

Table 2.2 - Information on vector behaviours

	WHO Indicators											
***P. knowlesi* vector species**	***Occurrence***	***Density***	***HBR***	***HBI***	***Biting time***	***Biting location***	***Resting location***	***Resistance***	***Larval habitat***	***Sporozoite rate***	***EIR***	**Literature**
An. latens	Borneo: Sarawak, South/Central/North Kalimantan	1 | 1073	Highest in forest fringe and forest	Human - monkey = 1:1.12[6]; 1.3:1[7]	Starts early, peak varies (20.00-01.00)	Outdoors	°		Temporary freshwater pools, puddles; still, shaded water; little vegetation	0.70-1.40%	*	(10,68–74)
An. leucosphyrus	Sumatra					°						(75–77)
An. balabacensis	Malaysia: Sabah, North Sarawak; Indonesia: North/South Kalimantan, Sumatra	1 | 1791	Highest in forest edge and plantations		Early, peaks between 18.00-21.00	Mainly outdoor biting but some indoor biting present in villages		°		1.03-3.42% (100%)	*	(57,69,71,74,78–85)
An. introlatus	Peninsular Malaysia	4 | 135	Highest in forest		Early, peaks between 19.00-21.00	Outdoors						(70,86–88)
An. dirus	Thailand, Lao PDR, Cambodia, Vietnam, Peninsular Malaysia	8 | 5686	Highest in forest 0.3-17.4		Early peak, biting continues through the night	Outdoors, occasional indoor biting		**	Freshwater pools, puddles; still, shaded water, sometimes along slow streams	(0%), 0.54-2.0%	°	(55,73,89–94)

Table 2.2 (continued) - Information on vector behaviours

	WHO Indicators											
***P. knowlesi* vector species**	***Occurrence***	***Density***	***Biting time***	***HBI***	***Biting time***	***Biting location***	***Resting location***	***Resistance***	***Larval habitat***	***Sporozoite rate***	***EIR***	**Literature**
An. cracens	Peninsular Malaysia	40 | 648	High in forest and fruit farm	°	Early, peaks between 19.00-21.00	Outdoors			Temporary freshwater pools, puddles; still, shaded water; little vegetation	0.60-2.90%	*	(47,75,84,95)
An. donaldi	Malaysia: Sarawak, Sabah,	3 | 251	High in forest		Early, peaks between 18.00-19.00	Outdoors				*		(74,78–80)
An. sundaicus	As possible *P. knowlesi* vector: Nicobar Islands, India	350										(96)
An. letifer	Malaysian Borneo	172				°				3.48%		(69,87)

This table includes information based on published data on biological indicators of species that are known to transmit P. knowlesi malaria to humans, based on the WHO indicators for vector control. The table only includes publications that studies a species in its role as vector for zoonotic malaria; i.e., An. dirus is also a vector for human malarias but only research on its role as vector for zoonotic malaria is included; An. donaldi, An. letifer and An. sundaicus await confirmation. HBR human biting rate, HBI human blood index, EIR entomological inoculation rate

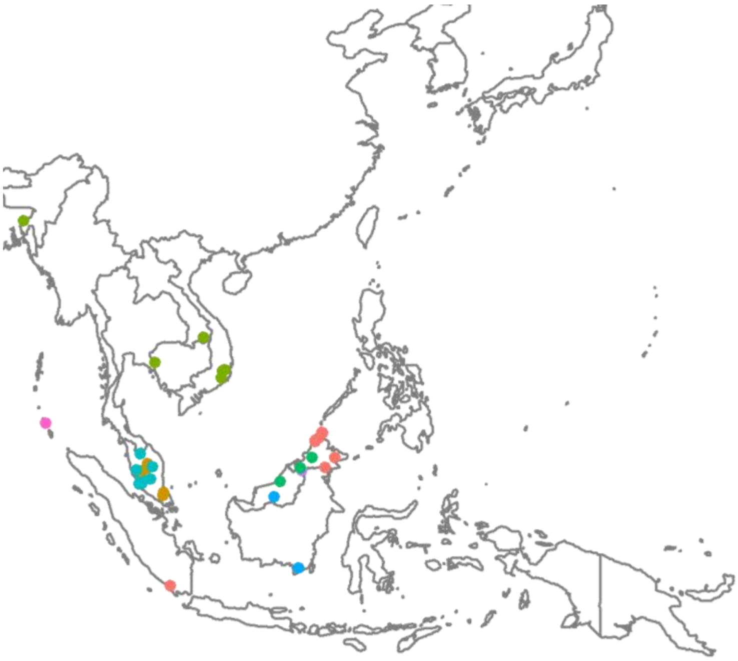

Figure 2.1 - The geographical distribution of research focussing on P. knowlesi *vectors and vector behaviours. Papers were included only when the research concerned (suspected)* P. knowlesi *or other simian malaria species transmission. Maps were made with R statistical software (R version 4.0.2), packages 'tidyverse' and 'maps'.*

Distribution

Zoonotic malaria is transmitted by *Anopheles* mosquitoes. In Asia, the main species transmitting *P. knowlesi* and other zoonotic malaria parasites, as well as human-only malaria species in some areas, belong predominantly in the *Anopheles* Leucosphyrus Group (68,97,98) (see Table 2.1, Fig. 2.2). These species are found across a wide geographic range, stretching from Northeast India and Myanmar eastward to Indonesia and the Philippines (99). The Leucosphyrus Group contains 21 species in three subgroups (Leucosphyrus, Hackeri and Riparis) (68). The Leucosphyrus subgroup is of most interest from a public health perspective as many species are incriminated as *P. knowlesi* vectors. The Leucosphyrus subgroup contains thirteen species, of which twelve belong in two cryptic species complexes: *An. leucosphyrus* and *Anopheles dirus* (68,97). The *An. dirus* complex is the most biodiverse and contains eight known species, all of which occur in continental Southeast Asia (89), with *Anopheles cracens* also found on Sumatra as well as in peninsular Thailand and Malaysia (97). The four known member species of the *An. leucosphyrus* complex are found in southern Thailand, Malaysia, Indonesia, and the Philippines (100).

Interestingly, the distribution of the Leucosphyrus Group mosquitoes roughly matches the distribution of the long-tailed macaque (*M. fascicularis*). Moreover, the distribution of the *An. leucosphyrus* complex overlaps that of the Southern pig-tailed macaque (*M. nemestrina*) (100). In

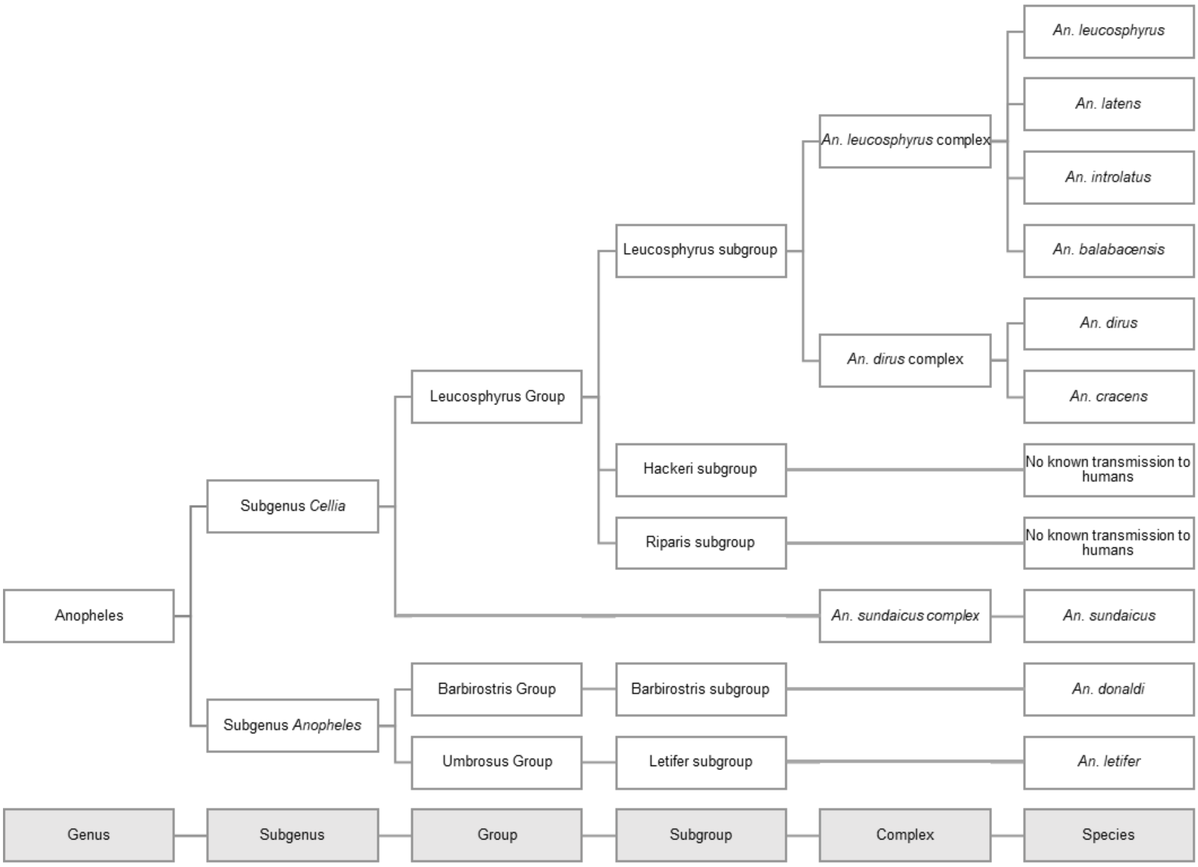

Figure 2.2 - Overview showing the relatedness of all mosquito species that are known or suspected vectors of Plasmodium knowlesi *to humans.*

addition, the distribution of the *An. dirus* complex closely matches the distribution of the Northern pig-tailed macaque (*M. leonina*). The *An. dirus* complex is bound to continental Southeast Asia (except *An. cracens*, which is found in North Sumatra as well (97)), while the *An. leucosphyrus* complex has a smaller geographical distribution (99).

Recently, studies in Sarawak and Sabah, East Malaysia, incriminated two species not in the Leucosphyrus Group, *Anopheles letifer* (69) and *Anopheles donaldi* (79,101), as *P. knowlesi* vectors in these regions. The mosquitoes were collected biting humans in both Sarawak and Sabah, and *P. knowlesi* was detected by PCR in the salivary glands and the whole mosquito, respectively. However, sporozoite or oocyst presence was not confirmed by microscopy of the salivary glands or midgut, and salivary glands were only examined separately by PCR in one study. Therefore, vector status of *An. donaldi* and *An. letifer* awaits official confirmation. Additionally, *P. knowlesi* DNA was found in a small pool of *Anopheles sundaicus* in the Nicobar district, India (96). However, no sporozoites were found and the study was too small to confirm the vector status of *An. sundaicus* for *P. knowlesi*. Further research is required to determine whether *An. sundaicus* may be a vector for *P. knowlesi* to humans. Still, the detection of parasites represents a remarkable exception to the widely accepted paradigm that *P. knowlesi* and *P. cynomolgi* zoonotic malaria are only transmitted by Leucosphyrus Group mosquitoes. It raises the possibility that other anopheline species may be capable of transmitting simian *Plasmodium* species to humans. Hence, public health officials and researchers of zoonotic *Plasmodium* species need to consider more comprehensive studies of anopheline species.

Vector behaviour

Species within the *An. dirus* and *An. leucosphyrus* complexes are historically considered to be forest and forest fringe dwelling species (68,75,97,99). The species for which moderate or strong evidence exists for their *P. knowlesi* vector status and behaviours (Table 2.1) feed predominantly outdoors on both humans and other animals, including macaques. Indoor human biting has been observed; however, these observations have indicated low densities for *An. balabacensis* in Sabah (80) and *An. latens* in Sarawak (70), Malaysia, and higher densities for *An. dirus* in Vietnam (90) and Lao PDR (91). In these studies, *An. balabacensis* and *An. latens* were collected in houses and a longhouse, respectively. However, *An. dirus* was only collected inside open-walled farm huts or houses devoid of proper walls, leaving the question unresolved of whether *An. dirus* will enter more permanently enclosed houses to feed. Biting occurs generally early in the evening, between 18.00 and 21.00, with sustained low biting rates throughout the night (70–72,80,81,92), although with some recently observed exceptions (Table 2.2).

Field studies investigating host blood meal choice in Leucosphyrus Group mosquitoes showed highly opportunistic biting behaviour. *Anopheles latens* feeds on humans in the forest fringe and both humans and macaques in the forest in Sarawak, East Malaysia (70). Additionally, biting behaviour may also depend on height above the ground in the forest canopy. Height-dependent biting behaviour in *An. leucosphyrus* complex mosquitoes was shown by Harbach et al. in the 1980s in South Kalimantan (71), with a higher human biting rate in the forest canopy than on the ground. Another field study in Sarawak, comparing the human landing catch with a monkey-baited trap, showed that *An. latens* fed more on macaques than humans in the canopy but fed more on humans than macaques near the ground (72). A similar pattern was noted in Peninsular Malaysia, for *An. leucosphyrus* (102) and for *Anopheles introlatus* (formerly *An. balabacencis introlatus*) (86). In Sabah, Malaysian Borneo, *An. balabacensis* bites humans more at ground level than in the canopy during paired human landing catches (82). However, no direct comparisons to macaques were made, so it remains unclear if potential vectors were diverted to macaque hosts in the canopy (82). Still, these observations are consistent with the overall pattern observed for the *An. leucosphyrus* complex. Interestingly, *An. dirus* in Cambodia was shown to preferentially bite humans on the ground and macaques in the canopy (103). This regularly observed propensity to feed on both humans and macaques in forests, forest fringes and fragmented forest habitats means that these mosquitoes can act as bridge vectors to transmit simian malaria species to humans.

Several suspected *P. knowlesi* vector species remain severely understudied. *Anopheles introlatus*, a potential vector in Peninsular Malaysia, bites both humans and macaques (86). Although *P. knowlesi* oocytes have been detected in 2014 (87), evidence that *An. introlatus* can develop a sporozoite stage infection of *P. knowlesi* was found very recently(10) (unpublished data, Vythilingam, UM). This is contrary to *An. donaldi* and *An. letifer*, for which there exists some molecular evidence (69,79). Additionally, *P. inui* and *Plasmodium fieldi* sporozoites, two other simian *Plasmodium* species which might be transmitted to humans, were detected in *An. introlatus* and *An. cracens* (104). It is known from Vietnam that, when *An. dirus* complex mosquitoes can develop sporozoites for one simian malaria species, they have the ability to develop sporozoites for all other species (92,105). This implies that *An. leucosphyrus* Group mosquitoes are able to develop all five species of simian malaria (*P. knowlesi, P. cynomolgi, P. inui, P. coatneyi, P. fieldi*). Additionally, almost nothing is known about the host preference, feeding habits or infection rates of *An. leucosphyrus*. This species is suspected to be confined to Sumatra (76) and further research is required to ascertain whether this species may transmit *P. knowlesi* to humans to guide appropriate vector control.

All species from the *An. leucosphyrus* complex, except for *An. leucosphyrus*, have been found carrying *P. knowlesi* sporozoites and hence have the potential of transmitting *P. knowlesi* malaria to humans (72,81,87). However, only two species from the *An. dirus* complex were found positive for *P. knowlesi* sporozoites. These are *An. dirus* in Vietnam (55,73), and *An. cracens* (106) in Peninsular Malaysia. Of these, *An. dirus* is of most concern due to its wide distribution across multiple Southeast Asian countries and the high numbers in which it often occurs (107,108). Although infected *An. dirus* were only found in South-Central Vietnam (55,109), the similarity of its distribution with that of long-tailed and pig-tailed macaques and its opportunistic blood feeding behaviour could make it a highly probable vector (100). Especially people who stay overnight in forest or forest fringe areas where *An. dirus* is present are at risk of infection with *P. knowlesi* (92). The range of *An. cracens* comprises areas of Peninsular Malaysia and Sumatra (97), where it has been incriminated as an important vector of human *P. knowlesi* infections in the former area (38,47).

Larval habitats

All members of the Leucosphyrus Group are essentially forest mosquitoes, and their larval habitats reflect this. However, recent research found associations between *An. balabacensis* larval habitats, distance from (rubber) plantations and forest fragmentation, supporting the hypothesis that the vector has adapted to changing land-use patterns (95). Although larval habitat documentation is sparse (see Table 2.1), there is much overlap among the larval habitat preferences of the studied species (*An. dirus sensu lato* (*s.l.*), *An. leucosphyrus s.l.*, *An. balabacensis*) (68,97). Larvae are mostly found in freshwater pools and puddles that are often temporary (Table 2.2). These water bodies can originate from almost any source after sufficient rainfall, including elephant footprints and wheel tracks to larger puddles on the ground (93). Common characteristics of these habitats are that they are, at least partially, shaded and that the water is still (68,99), with little to no vegetation present. Larvae can occur in large densities (MJ Bangs, pers. comm.), which leads to the assumption that these species are most abundant during the rainy season or after a prolonged period of precipitation during the dry season. *Anopheles leucosphyrus* complex mosquitoes seem to prefer temporary water bodies rather than more permanent water bodies like streams, while *An. dirus* can also be found along streams when the current is slow (89,110).

Drivers for transmission

Zoonotic malaria was traditionally considered a ‘forest malaria’ with infections mainly in people who enter the forest for work, like loggers or hunters (55,105). However, expansion of human activities and the resulting fragmentation of forests in large parts of Southeast Asia has been associated with increasing numbers of zoonotic *Plasmodium* species infections, not only in forest workers, but notably also in agricultural workers who remain relatively close to their resident village (111–114). Zoonotic infection spill-over is notoriously hard to predict, as it often crosses various phylogenetic and spatiotemporal scales (115). The behaviour of the monkeys, mosquitoes and people influences their interactions with each other, all of which are heterogeneous in space and time. One of the most important drivers of zoonotic malaria spill-over to humans is the ecology of mosquito vectors and reservoir hosts (116). To become infected with zoonotic *Plasmodium* species, infectious mosquitoes must bite humans. This requires proximity to infectious vectors, which is often associated with changes in land-use, occupation, and house construction (117). Additionally, wildlife harbouring the parasites (the reservoir hosts) needs to be close to both humans and vectors that readily blood-feed on both humans and the reservoir species. *P. knowlesi* (as well as *P. cynomolgi*) usually results in benign, chronic infections in natural macaque hosts (42,118). Hence, infected monkeys form an ideal reservoir for parasite spill over to humans as the monkeys are not restricted in their normal behaviours by disease (42). As the interaction between monkeys, mosquitoes and humans influences their respective behaviours, the factors discussed in the following subsections are in fact closely connected and interacting.

Vector diversity

The large number of sympatric species in Southeast Asia that can transmit malaria, including zoonotic malarias, makes it hard to determine the dominant vector in a geographic region. Variation in behaviours and distributions of individual vectors associated with a high diversity of vector species are likely to affect the transmission dynamics of zoonotic malaria, especially when vector abundance increases (119). Although this depends on the competence of the present vector species, a higher number of sympatric vector species in an area will generally facilitate increased zoonotic malaria transmission (120). Hawkes et al. (101) observed increased *Anopheles* species richness and abundance, as well as a higher infection rate, in forest edges compared to human settlements and plantations. Higher species richness can also extend the duration of seasonality in pathogen transmission, thus enabling a longer period of mosquito biting activity (121). In addition, the high degree of behavioural plasticity observed in many species plays an important role in the large variation in dominance of different vector species (122). This variation makes targeting zoonotic malaria vectors challenging, because species can display different behaviours by geographic area (89).

Human activities can drastically change the community composition of both vector and reservoir species. Anthropogenic exploitation of natural resources, like logging or hunting, in addition to the expansion of human settlements and, to a lesser extent, plantations, can cause a general loss of biodiversity (123,124). As a result of this development, the vector community composition can change, as has been observed in Kinabatangan, Malaysian Borneo, where *An. donaldi* may have replaced *An. balabacensis* as the primary malaria vector in certain areas (125). In Sarawak, Malaysian Borneo, *An. donaldi* and other suspected malaria vector abundance decreased while *Aedes albopictus* numbers increased after anthropogenic disturbance, thereby contributing to a shift in the relative disease risk from malaria to arboviruses (126). Additionally, if biodiversity loss is more severe in vertebrates than in invertebrates (127), an amplification effect for pathogen transmission can occur. Hence, high vector species richness concentrates blood feeding on the limited vertebrate species, resulting in higher biting on reservoir species. However, research is required to clarify the exact implications of this theoretical mechanism for zoonotic malaria and, specifically, *P. knowlesi* transmission.

Host diversity and distribution

As the primary hosts and vectors of *P. knowlesi* and *P. cynomolgi* are originally forest-dwelling species, contacts between humans, macaques and mosquitoes were few and transmission was thought to be very rare (64,86). However, human activities can lead to provision (unintentional or intentional feeding) of macaque troops (128), thus eventually drawing potential carriers of zoonotic malaria species towards areas of human settlement. The macaques are highly invasive and readily adapt their behaviour to thrive in fragmented landscapes by raiding crops, exhibiting aggressive behaviour and becoming an urban nuisance (129). Macaque behaviour is disturbed by deforestation, and changes have been observed in the macaque troop home range size, movement speeds and use of different habitat types (128,129). Long-tailed and pig-tailed macaques are frugivores but will switch readily to other, more abundant food sources in the absence of fruits (130). Pig-tailed macaques in Peninsular Malaysia extended their home range significantly to forage in oil palm plantations, which provided them with abundant, year-round food sources (131). Food provision in the vicinity of human settlements likely causes macaque troops to remain around these settlements. Ruslin et al. (132) showed that long-tailed macaques will readily feed on anthropogenic food and food waste. Additionally, Stark et al. (129) suggested that long-tailed macaques in Sabah, East Malaysia actively avoid human logging activities, spending more time in other habitats including farmland and thus bringing the *P. knowlesi* reservoir closer to humans.

Changes in biodiversity have the potential to affect the risk of infectious disease emergence (133). If vectors can select bloodmeals from a variety of host species that differ in their reservoir competence, the probability of a parasite being transmitted from host to vector will be diminished. The presence of low-capacity hosts (incompetent reservoirs) has been hypothesized to dilute the effect of the highly competent reservoir hosts, thus reducing disease risk, and is termed the dilution effect (133).

Environmental change

The emergence of zoonotic malaria in Southeast Asia is thought to be strongly driven by environmental changes caused by humans. When the first large focus of *P. knowlesi* malaria was discovered in 2004 (30), it was hypothesized that *P. knowlesi* infections were contracted away from human settlements in the forest. However, later research in the same region revealed that infective *An. latens*, the dominant vector species in the region, preferred to bite humans in farm areas and forest fringes (70). A similar pattern was observed in Sabah, where the predominant *P. knowlesi* vector, *An. balabacensis*, had the highest abundance in villages but the highest vectorial capacity in farms and forest fringes, based on parous rate and life expectancy (81). In mainland Southeast Asia, the dominant vector species *An. dirus* occurred in high densities in the forest rather than forest fringes or villages, but human invasion and sustained activities in the forests exposed people to infectious bites (80,89,100). Indeed, increased human activities in the forest may increase vector density in the forest and forest edge, relative to the village, by both creating more oviposition sites through human activities (e.g., puddles in muddy roads) (95) and by providing more human blood hosts (93).

The strongest environmental driver of *P. knowlesi* infections is the fragmentation of forests resulting from anthropogenic land-use (134). Fornace et al. (135) found that the decline of forest cover, both recent and historical, in the vicinity of human settlements was associated with a greater *P. knowlesi* incidence in Sabah, Malaysia. In addition, when human land-use and movements during peak biting times were considered, the highest risk of exposure to infectious mosquito bites was found in forest fringes, rather than the forest where higher vector abundance was observed (136). More specifically, factors that increase *P. knowlesi* infection risk were all associated with increased human activities in forest fringes or disturbed, fragmented forests (111). Occupation has been a consistent major risk factor, with oil palm plantation work and subsistence farmers having a higher exposure risk (111). The aforementioned anthropogenic land-use changes can affect the transmission of zoonotic malaria to individuals as well as populations. Besides when humans enter the forest (i.e., for work) and thus the zoonotic cycle, the removal of intact forest corridors can force the macaque reservoir hosts of zoonotic malaria into human territory, after which the mosquito vectors will most likely follow (with transmission to the human population) (137). Additionally, the replacement of primary forest with farms or plantations, notably large-scale oil palm, causes significant biodiversity loss in both vertebrates and, to a slightly lesser extent, invertebrates (138,139). The adaptation of *P. knowlesi* reservoir hosts and vectors to human habituation, combined with the loss of any dilution effect, can further increase the exposure risk of humans (140). It is important to keep in mind that the knowledge base is too limited to inform on possible mechanisms that regulate zoonotic malaria vector behaviours and how anthropogenic land-use changes might influence these behaviours.

Conclusion

Current information on zoonotic malaria vector species is largely based on a limited number of studies in geographically restricted areas (predominantly in Malaysia). The dearth of information on key vector behaviours stands in the way of effective vector control, especially considering the strong increase in zoonotic malaria infections in the past decade. Additional data is particularly needed from currently understudied regions where previously incriminated or suspected zoonotic malaria vectors occur. The way that humans change the environment results in increased exposure to simian malaria species and could facilitate vector adaptation to humans. However, how changing human land-use influences zoonotic malaria vectors is still poorly understood. Hence, more data are needed on vector diversity and bionomics in relation to anthropogenic land-use change. Understanding the individual vectors involved in zoonotic malaria transmission and the variation in their behaviour is imperative to deploy effective mosquito control methods, which remain key to reducing the malaria burden.

Declarations

Ethics approval and consent

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Competing interests

The authors declare that they have no competing interest.

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Author's contributions

B.v.d.S. and T.R.B. conceived the review and analysis of existing data. B.v.d.S. performed the initial literature search and wrote the original draft of the manuscript. B.S., T.L.R., T.a.G and I.V. provided significant input and edits to the manuscript as well as additional literature and invaluable insights on the vectors; K.S., T.L.R. and M.J.G. provided input and literature on macaque biology and human land-use change. All authors read and approved the final manuscript.

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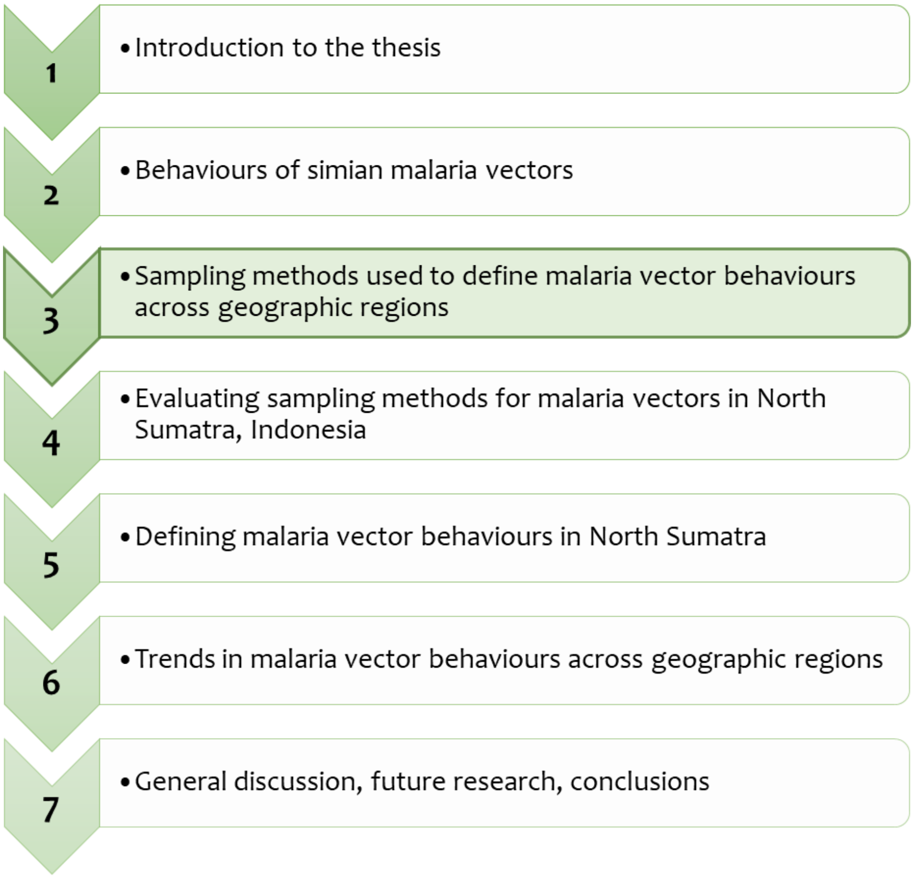
Chapter 3

The previous chapter highlighted the limited knowledge on the distributions and behaviours of the vectors of simian malaria. Therefore, an in-depth, global analysis of collection methods for dominant malaria vectors was performed to evaluate the most-used methods across the three largest malarious regions in the world, to inform which methods might be applicable for collecting simian malaria vectors in Indonesia. This chapter was published in Scientific Reports (2021) 11:15337.



A global assessment of surveillance methods for dominant malaria vectors

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Abstract

The epidemiology of human malaria differs considerably between and within geographic regions due, in part, to variability in mosquito species behaviours. Recently, the WHO emphasised stratifying interventions using local surveillance data to reduce malaria. The usefulness of vector surveillance is entirely dependent on the biases inherent in the sampling methods deployed to monitor mosquito populations. To understand and interpret mosquito surveillance data, the frequency of use of malaria vector collection methods was analysed from a georeferenced vector dataset (>10,000 data records), extracted from 875 manuscripts across Africa, the Americas, and the Asia-Pacific region. Commonly deployed mosquito collection methods tend to target anticipated vector behaviours in a region to maximise sample size (and by default, ignoring other behaviours). Mosquito collection methods targeting both host-seeking and resting behaviours were seldomly deployed concurrently at the same site. A balanced sampling design using multiple methods would improve the understanding of the range of vector behaviours, leading to improved surveillance and more effective vector control.

Abbreviations

ABT: animal-baited trap

Data record: a unique site-collection period-species combination which corresponds with a single row in the original database

DVS: dominant vector species

HLC: human landing catch

HBT: human-baited double net trap

IRS: indoor residual spraying

LLIN: long-lasting insecticidal bed net

NMCP: National Malaria Control Program

PSC: pyrethrum spray collection

WHO: World Health Organisation

Introduction

Substantial progress has been made to reduce the global incidence of human malarias. As malaria transmission diminishes, malaria cases become more spatially heterogenous (141,142). The latest World Health Organisation (WHO) guidance to national malaria programs encourages the use of local evidence to select interventions by transmission stratum, rather than utilising a one-size-fits-all approach (143). Vector surveillance data is thus increasingly critical to support this informed decision-making process, with surveillance, including vector surveillance, now considered a core intervention (144). The WHO recommends monitoring a set of vector surveillance indicators based on transmission intensity and vector control measures deployed (62,145,146). These indicators are species identification, abundance, peak biting times, resting and biting locations, insecticide susceptibility and infection rates (62). However, national programme capacity limitations (e.g., inadequate strategic frameworks, logistics shortfalls, limitations in human resources or financial constraints) often prohibit monitoring the complete set of indicators (147). Guidance on which surveillance tools should be used to measure the recommended indicators are provided by the WHO. Each of these surveillance tools has associated biases and limitations (148).

Globally, there are 41 dominant malaria vector species (DVS) responsible for most human malaria transmission. Each DVS has unique combinations of behaviours. In Africa, seven DVS are

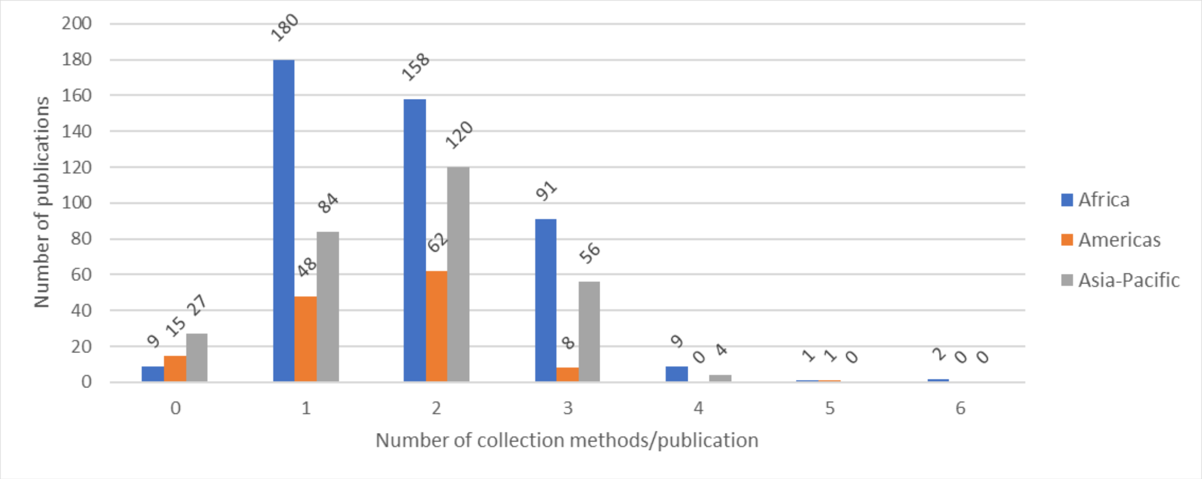

Figure 3.1 - Number of publications per continent. For each continent, the number of publications is shown (y-axis), as well as the number of collection methods that were reported in each publication (x-axix). Zero collection methods used means that there was no specific information available about the type of collection method(s) used in a publication.

recognised (149). Nine DVS are found in the Americas (North, Central and South America) (150) and a staggering nineteen DVS are recognised in the Asia-Pacific region (151). Additionally, species complexes containing cryptic species are found across all regions. One consequence of the regional variability in mosquito fauna is that the epidemiology of human malaria transmission and the effectiveness of vector control measures differs considerably between Africa, the Americas, and the Asia-Pacific region (152,153).

Representative vector surveillance requires unbiased sampling of mosquito populations. The high variability in biting and resting behaviours between regions and by vector species may impact the efficacy of sampling methods and potentially delay detecting changes in vector behaviours that reduce the efficacy of malaria control methods and thereby delay changing strategies to better control the vectors. Due to these differences in vector bionomics, certain patterns of behaviours are commonly associated with geographic areas. Many DVS in Africa are described as being distributed rurally, anthropophagic (preferring to bite humans) and predominantly biting late at night and indoors. Contrastingly, while also predominantly rural in distribution, many American and Asian DVS are considered to predominantly bite and rest outdoors and are opportunistic (more zoophagic) in their blood feeding preferences (149–151,154,155). Exceptions abound: for example, *An. culicifacies* is an urban, indoor biting and resting mosquito found in Asia (156) and *An. arabiensis* is an African vector that can exhibit opportunistic, outdoor blood feeding habits (157). We hypothesise that these behavioural differences will impact collection method efficacy (i.e., the number of mosquitoes captured). Hence, collection methods might be selected on the basis of efficacy to maximise numbers captured. Thus, the use of traps might vary in the frequency with which they are deployed by geographic area. Consequently, the predominant vector behaviours reported in an area may also reflect the biases associated with the collection method used.

Here, the frequency of use of the most commonly deployed malaria vector collection methods in Africa, the Americas, and the Asia-Pacific region between 1981 to 2015 was analysed from an extensive database of spatially defined information on anopheline vector bionomics (108). Geographical patterns of collection method use from the three regions were compared with requirements for accurately monitoring malaria vectors.

Results

The database contained 5,678 data records from 450 publications from Africa, 1,346 data records from 134 publications from the Americas and 3,898 data records from 291 publications from the Asia-Pacific region. Of these 875 publications, 51 (9 from Africa, 15 from the Americas, 27 from the Asia-Pacific region) did not contain specific information on collection methods. The majority of publications reported one (n = 312) or two (n = 340) collection methods used in the study, while three different collection methods were used in 155 publications (see figure 3.1). More than three collection methods were reported in only a few publications.

Individual data records (rows in the database) showed unique site-collection period-species combinations. The count of collection methods used in Africa was 9,824 with a mean number of 1.74 collection methods per data record. The count of collection methods used in the Americas was 1,925 with a mean number of 1.47 collection methods per data record. The count of collection methods used in Asia-Pacific was 5,819 with a mean number of 1.75 collection methods per data record. The different collection methods recorded in the database targeting host-seeking or resting mosquitoes were categorised as by Farlow et al.(148), which showed that the same seven collection method groups were used in Africa, the Americas and the Asia-Pacific region (Table 3.1, 3.2).

Sampling host-seeking anophelines

Global analysis of the data showed that indoor and outdoor human landing catches (HLCs) were by far the most frequently used methods to collect anophelines. The proportional use of HLCs in the Asia-Pacific region was 0.597 ($n_{ASIA\text{-}PACIFIC} = 3{,}475$) which was not significantly different to Africa at 0.604 ($n_{AFRICA} = 5{,}934$) (Table 3). Contrastingly, the proportion of HLCs in the Americas was much higher, at 0.890 ($n_{AMERICAS} = 1{,}713$). Comparing within continents, statistically there was no discernible preference for indoor or outdoor use of HLCs in Africa ($HLC_{OUT} = 25.82\%$, $HLC_{IN} = 31.03\%$; Mann-Whitney U, p = 0.28, n.s.), the Americas ($HLC_{OUT} = 47.48\%$, $HLC_{IN} = 33.82\%$; Mann-Whitney U, p

Table 3.1 - Sampling methods for host-seeking mosquitoes. The different sampling methods recorded in Massey et al. (2016) targeting host-seeking mosquitoes were grouped into categories of techniques/tools used as proposed by Farlow et al. (2020).

Sampling method group	Definition/tool used	Sampling method (indoor)	Sampling method (outdoor)
Human landing catch	Mosquitoes attracted to collectors are captured by aspiration on exposed lower legs.	HLC (Human Landing Catch) MB (Man biting, location unspecified) MBI (Man biting indoors)	MBO (Man biting outdoors)
Light trap	CO_2-baited CDC trap with light bulb	ILT (Indoor light trap)	OLT (Outdoor light trap)
Human-baited double net trap	A collection method composed of two mesh tents with one inside the other. The inner tent contains and protects a human that acts as bait for host-seeking mosquitoes. The outer tent has a gap between the mesh wall bottom and the ground or open tent doors to allow mosquitoes to enter. Mosquitoes are trapped and retained between the tent walls and are periodically collected by aspiration.		HBT (Human-baited tent) HBN (Human-baited net)
Animal-baited trap	As human-baited trap, but containing an animal (cow/goat/pig/monkey) instead of human.	ABI (Animal-baited inside, in animal shelter) AB (Animal-baited, location unspecified)	CBT (Cow-baited trap) ABT (Animal-baited trap) ABO (Animal-baited outside) ABN (Animal-baited net trap)
Odour trap	A mechanical trap releasing CO_2 and/or host odours while capturing attracted mosquitoes		Odour-bait

Table 3.2 - Sampling methods for resting and other behaviours. The different sampling methods recorded in Massey et al. (2016) targeting resting mosquitoes were grouped into categories of techniques/tools used as proposed by Farlow et al. (2020). Artificial resting sites are specifically constructed to lure and catch resting mosquitoes, while Natural resting sites are not, i.e., a house is not specifically constructed to lure and catch resting mosquitoes, so it is considered a Natural resting site.

Sampling method group	Definition/tool used	Sampling method (indoor)	Sampling method (outdoor)
Natural resting site collections	Capture of mosquitoes by oral, battery-powered and backpack aspirators; includes knockdown spray catches targeting resting mosquitoes in which an insecticide fog immobilises mosquitoes that fall to the floor and are collected	HRI (House resting indoors) RO (shelter)/RO (ani-shelter): Resting in natural or animal shelters	RO (Resting outdoors)
Artificial resting site collections	Includes capture of mosquitoes in constructed shelters and Window Exit traps	WinExit: wire mesh (glue optional) covering the windows, which traps mosquitoes trying to exit houses	RO (pit): Resting outdoors in pit traps and other man-made shelters like clay pots, resting boxes, barrier traps and Malaise traps

= 0.85, n.s.) and the Asia-Pacific region (HLC_{OUT} = 29.20%, HLC_{IN} = 25.90%; Mann-Whitney U, p = 0.71, n.s.). However, when comparing between continents, outdoor HLCs tended to be more common in the Americas as well as the Asia-Pacific region when compared to Africa where there was a tendency to perform indoor HLCs, reflecting perceived vector biting characteristics (Table 3). Sampling location was not recorded for a small number of the HLCs (Africa: 3.55%, Americas: 7.69%, Asia-Pacific: 4.62%).

The data frame was manipulated to summarise the combination of collection methods used per data record, ensuring that the location (indoors or outdoors) for each sampling effort was noted. When individual data records were examined in further detail, HLC collections were used in three sampling strategies: indoor-only, outdoor only and simultaneously indoor and outdoor. Simultaneous indoor and outdoor HLCs were common practice in all regions. In the analysed data, 77.4% of HLCs in Africa, 50.1% of HLCs in the Americas and 67.5% of HLCs in the Asia-Pacific region were deployed indoors and outdoors simultaneously. Interestingly, the proportion of outdoor-only HLC collections is much larger in the Americas and the Asia-Pacific region than in Africa (Americas: 21.1%, Asia-Pacific: 21.1%, Africa: 3.5%). The opposite is true for indoor-only HLC collections, which occurred more often in Africa (Africa: 19.1%, Americas: 0.6%, Asia-Pacific: 11.4%).

Table 3.3 – The number of data records for each collection method used in Africa, the Americas and the Asia-Pacific region, categorised by 5-year period.

		HLC			Human-baited trap	Animal-baited trap	Light trap		Odour trap	Natural resting site collection		Resting (other)	
		Indoor	*Outdoor*	*Unknown*			*Indoor*	*Outdoor*		*Indoor*	*Outdoor*		*Total*
Africa	1981 - 1985	71	22	1	0	0	2	1	0	12	0	2	111
	1986 - 1990	512	351	61	19	16	124	6	0	332	35	84	1540
	1991 - 1995	535	385	81	11	0	144	4	2	428	41	59	1690
	1996 - 2000	545	541	58	15	0	85	1	9	762	17	24	2057
	2001 - 2005	796	762	77	8	2	127	19	4	497	71	72	2435
	2006 - 2010	537	448	38	4	5	156	69	1	344	7	95	1704
	2011 - 2015	13	14	0	0	0	14	8	0	17	0	3	69
	NA	0	0	0	0	1	0	0	0	0	0	0	1
	Total	*3048*	*2537*	*349*	*57*	*24*	*670*	*108*	*20*	*2489*	*173*	*349*	***9824***
	Prop.	31.03%	25.82%	3.55%	0.58%	0.24%	6.82%	1.10%	0.20%	25.34%	1.76%	3.55%	100%
Americas	1981 - 1985	20	20	0	0	9	0	0	0	2	0	4	55
	1986 - 1990	143	146	3	0	8	2	1	0	19	27	0	349
	1991 - 1995	134	140	16	0	18	1	53	0	1	10	0	373
	1996 - 2000	44	160	23	0	0	11	0	0	2	3	0	243
	2001 - 2005	99	185	99	8	0	0	1	0	1	12	0	405
	2006 - 2010	209	222	7	0	0	6	0	8	0	3	0	455
	2011 - 2015	2	2	0	1	0	0	0	0	0	0	1	6
	NA	0	39	0	0	0	0	0	0	0	0	0	39
	Total	651	914	148	9	35	20	55	8	25	55	5	***1925***
	Prop.	33.82%	47.48%	7.69%	0.46%	1.82%	1.04%	2.86%	0.42%	1.29%	2.86%	0.26%	100%
Asia-Pacific region	1981 - 1985	204	233	0	4	91	0	8	0	98	22	0	660
	1986 - 1990	450	366	152	23	10	43	44	0	308	72	5	1473
	1991 - 1995	187	286	28	18	41	16	51	7	535	2	4	1175
	1996 - 2000	418	494	8	0	27	31	19	1	195	47	1	1241
	2001 - 2005	114	162	10	8	8	14	11	0	186	54	8	575
	2006 - 2010	131	147	57	29	3	51	8	0	169	11	10	616
	2011 - 2015	0	2	0	0	0	3	1	0	2	3	0	11
	NA	3	9	14	15	6	4	0	0	14	0	3	68
	Total	*1507*	*1699*	*269*	*97*	*186*	*162*	*142*	*8*	*1507*	*211*	*31*	***5819***
	Prop.	25.90%	29.20%	4.62%	1.67%	3.20%	2.78%	2.44%	0.14%	25.90%	3.63%	0.53%	100%

Indoor and outdoor light trap collections in Africa showed large differences (Table 3.3). Indoor light trap collections were deployed 6-times more frequently than outdoor light trap collections (light trap_{IN} = 6.82%, light trap_{OUT} = 1.10%, Mann-Whitney U, $p = 0.03$). Indoor and outdoor light trap collections in the Asia-Pacific region accounted for only 2.78% and 2.44% of total sampling effort, respectively, and did not significantly differ (Mann-Whitney U, $p = 0.42$, n.s.) (Table 3.3). In the Americas, indoor and outdoor light trap collections were deployed infrequently as well (light trap_{IN} = 1.04%, light trap_{OUT} = 2.86%, Mann-Whitney U, $p = 0.28$, n.s.). Indoor light trap collections were more often deployed in Africa than in the Asia-Pacific region, although the difference was not statistically significant ($n_{\text{AFRICA}} = 670$, $n_{\text{ASIA}} = 162$; Mann-Whitney U, $p = 0.086$).

Alternative sampling methods to HLCs designed to collect host-seeking mosquitoes – human-baited tent traps (HBTs) and animal-baited tent traps (ABTs) – were more commonly used in the Asia-Pacific region than in Africa and the Americas, albeit infrequently (Table 3). Animal-baited trap use accounted for 3.2% (n = 186), while HBT use accounted for only 2.0% (n = 97) of the total sampling effort in the Asia-Pacific region. The use of ABTs in the Asia-Pacific region decreased strongly after the year 2000, while relative HBT use varied strongly between the five-year periods. In Africa, HBTs were rarely used (n = 57). Their use peaked at 0.6% in 1986-1990 and decreased thereafter with no data records of HBT use in the period 2011-2015. ABT deployment in Africa was even less common, accounting for only 0.24% of data records (n = 24), of which 16 occurred between 1986-1990. ABT and HBT use was also uncommon in the Americas ($n_{\text{ABT}} = 35$, $n_{\text{HBT}} = 9$), where the deployment of ABTs was not recorded in the database after 1995.

Sampling resting anophelines

The collection of anophelines from natural indoor resting sites (defined as structures, including houses and animal shelters, not constructed specifically to lure resting mosquitoes (158)) was the second most commonly used method in both the Asia-Pacific region (29.5%, n = 1,718) and Africa (27.1%, n = 2,662; Table 3.3). While the collection of anophelines from natural resting sites was also the second most frequently used method in the Americas, it was infrequently used (4.15%, n = 80). The ratio between indoor and outdoor collections was highly skewed in Africa and the Asia-Pacific region, with indoor resting collections exceeding outdoor resting collections from vegetation 7-fold in the Asia-Pacific region (nat. $\text{resting}_{\text{OUT}}$ = 3.63%, nat. $\text{resting}_{\text{IN}}$ = 25.90%; Mann-Whitney U, $p = 0.01$) to almost 15-fold in Africa (nat. $\text{resting}_{\text{OUT}}$ = 1.76%, nat. $\text{resting}_{\text{IN}}$ = 25.34%; Mann-Whitney U, p =

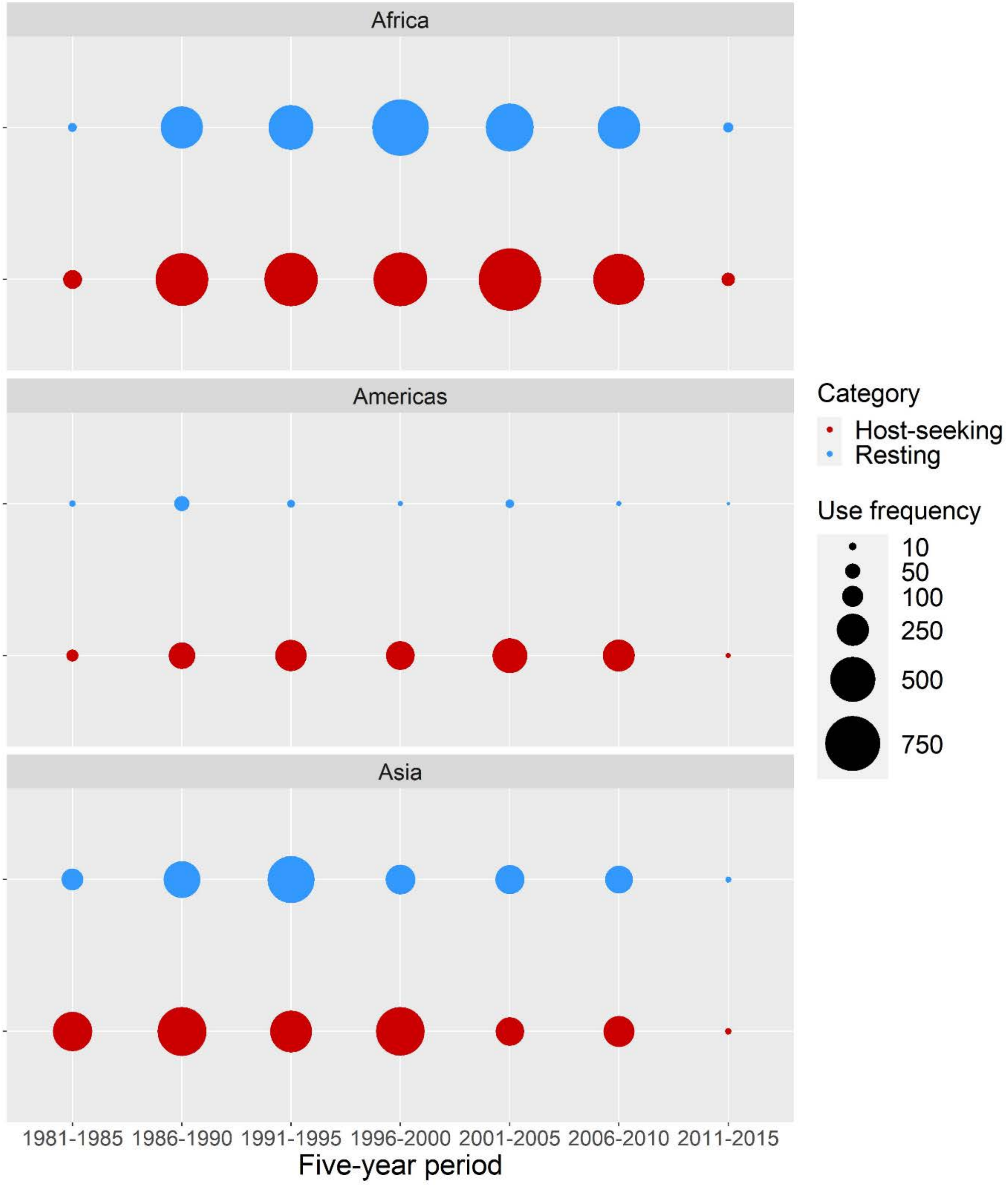

Figure 3.2 - The number of data records for the two categories of collection methods, host-seeking and resting, presented per 5-year time period.

0.03). In the Americas, outdoor resting collections amongst vegetation exceeded indoor resting collections 2-fold but this was not significant (nat. resting$_{OUT}$ = 2.86%, nat. resting$_{IN}$ = 1.29%; Mann-Whitney U, p = 0.82). It is important to note that the small proportion of outdoor resting collections generally reflected the difficulty of collecting mosquitoes outdoors and not the absence of outdoor resting mosquitoes.

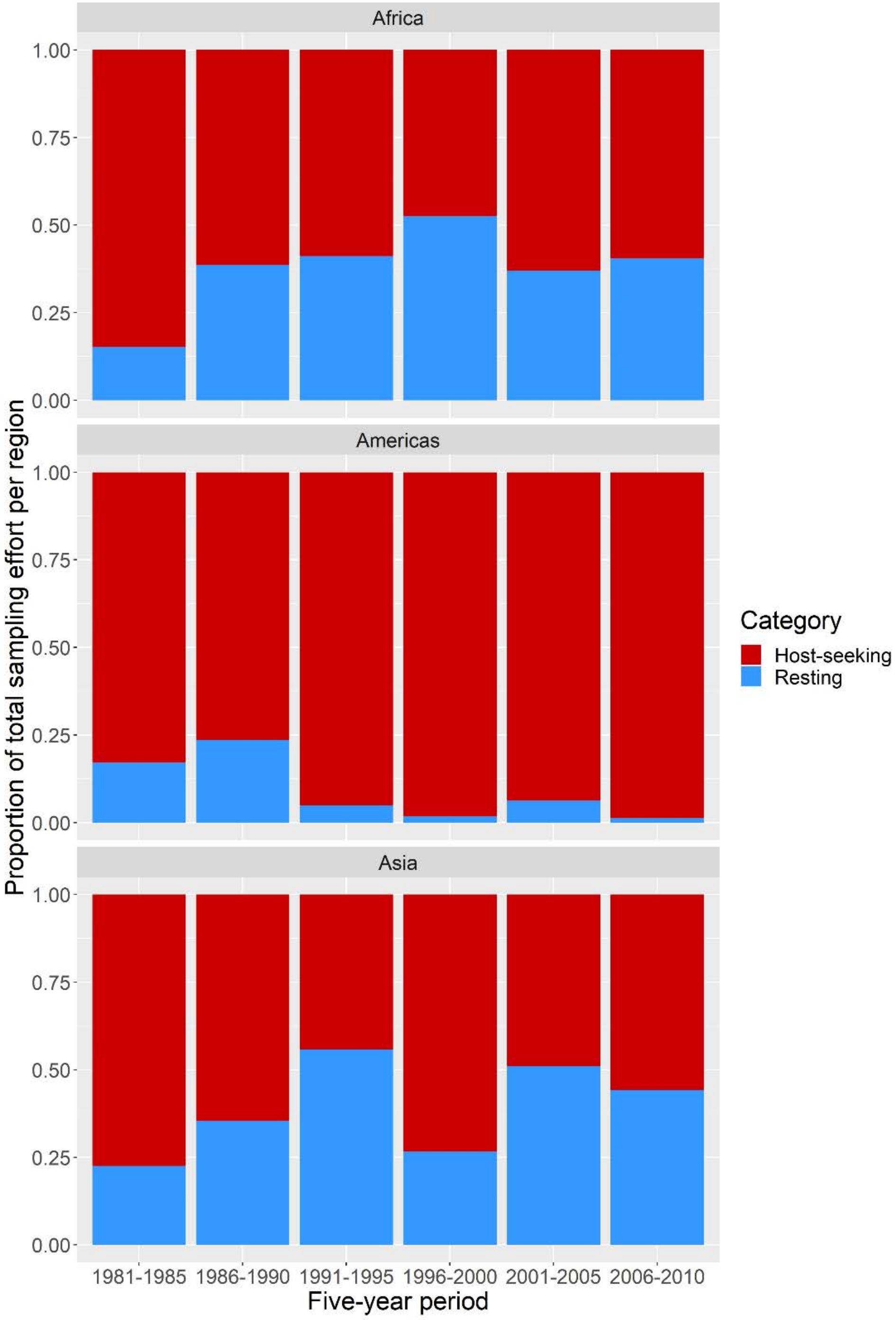

Figure 3.3 - Proportion of host-seeking and resting collections. The proportion of host-seeking (red) and resting (blue) collections presented per 5-year time period for each region analysed.

When sampling location per data record was taken into account, natural resting collections showed a sharp contrast in both Africa and the Asia-Pacific region, with indoor-only resting collections (Africa: n = 2248, 92.9%; Asia-Pacific region: n = 1325, 86.3%) far outnumbering simultaneous indoor and outdoor resting collections (Africa: n = 144, 5.9%; Asia-Pacific region: n = 168, 10.9%) as well as outdoor-only resting collections (Africa: n = 27, 1.1%; Asia-Pacific region: n = 43, 2.8%). The Americas contrasted with the other two geographic regions analysed, because outdoor-

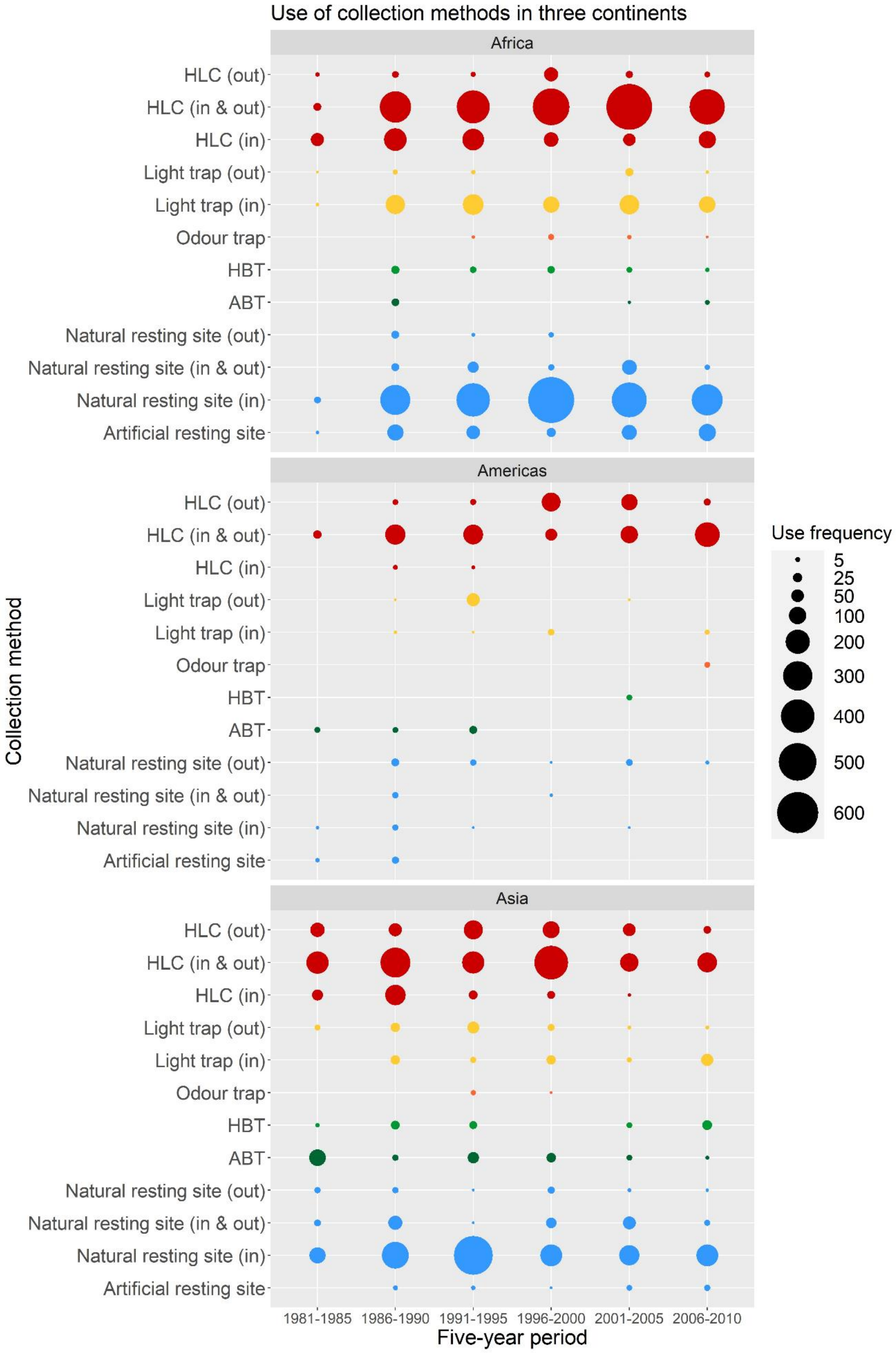

Figure 3.4 - Number of data records per collection method. The number of data records for each collection method used in (a) Africa, (b) the Americas, and (c) the Asia-Pacific region, presented per 5-year time period. ABT = animal-baited trap, HLC = human landing catch, HBT = human-baited double net trap.

only resting collections from vegetation (n = 43, 63.2%) outnumbered indoor-only resting collections (n = 13, 19.1%) and simultaneous indoor and outdoor resting collections (n = 12, 17.6%).

Pit traps and other artificial resting sites (e.g., clay pots, box traps etc.) can be used as alternatives to vegetation aspiration to increase mosquito numbers in outdoor resting collections (159). Only information on the use of pit traps was found in the dataset for artificial resting site collections. Pit traps were rarely used in the Americas (n = 4) and the Asia-Pacific region (n = 15), where aspiration of natural vegetation was more common. In Africa however, pit trap use was more frequent (n = 156) and comparable to the aspiration of vegetation (n = 173). Pit traps were used in 27 African studies included in the database and, in most of these studies, used in lieu of the aspiration of vegetation.

Temporal patterns in sampling methods

In Africa, malaria vector sampling increased in the late 1980s for both host-seeking as well as resting collections (Fig. 3.2a). Total sampling effort remained quite stable in the following years to 2010. In the Americas (Fig. 3.2b), the abundance of host-seeking collections followed a similar pattern to Africa: between 1986-2010 the abundance of host-seeking collections remained quite stable. Resting collection sampling frequency, on the other hand, decreased in the 1990s and remained a very infrequently deployed sampling method until 2011-2015 (the final recorded period of the database). In the Asia-Pacific region, the abundance of host-seeking and resting collections varied more than in the other continents (Fig. 3.2c). Remarkably, the total sampling effort in the Asia-Pacific region was much lower in the 21st century than it was in the 20th century, while the total sampling effort in Africa and the Americas did not show such a decrease. Between 2011-2015, sampling effort declined sharply in the three analysed regions, which is most likely due to the small number of published records.

Although a slight upward trend can be seen in the proportion of resting collections, relative to host-seeking collections in Africa, this was by no means significant (Fig. 3.3a). The ratio between host-seeking and resting collections in Africa was the most stable of the three geographic areas analysed. The Americas was the only region in this analysis which showed a highly skewed ratio between host-seeking and resting collections (Fig. 3.3b). Here, resting collections were being used regularly in the 1980s, but the sampling effort in the Americas consisted almost entirely of host-seeking collections since the 1990s. In the Asia-Pacific region, the proportions of host-seeking and resting collections showed a slight upward trend but was not as stable as in Africa (Fig. 3.3c). The more varying ratios in the Asia-Pacific were not significantly different from the ratios observed in Africa (GLM, Tukey's Post-hoc comparison, $p > 0.99$). However, the clear decrease in the proportion of resting collections since the 1990s in the Americas was significantly different from Africa (GLM, Tukey's Post-hoc comparison, $p = 0.0014$) and the Asia-Pacific region (GLM, Tukey's Post-hoc comparison, $p = 0.0022$). The variation in resting collections among the analysed periods was not significant (GLM, Tukey's Post-hoc comparison, $p > 0.65$).

The total sampling effort for each region is displayed in Figure 3.4. In Africa, HLC (simultaneously indoors & outdoors) and indoor resting collections dominated the entire database time frame. Light traps were consistently deployed indoors but were almost never deployed outdoors. Other alternative methods to HLCs, specifically HBTs, ABTs and odour traps, were used infrequently in Africa. In the Americas, HLCs made up the bulk of host-seeking mosquito collections. Sampling of natural resting sites occurred mainly outdoors since the 1990s, which contrasts with the indoor natural resting site collections in Africa and the Asia-Pacific region. Alternatives to HLC were rarely deployed. Together with the infrequent use of resting collections, this creates a highly singular dependence on surveillance data from HLCs. The Asia-Pacific region, like Africa, showed that surveillance data depended largely on HLCs and indoor resting collections. However, the deployment location of light traps was more evenly divided between indoors and outdoors than in Africa. Additionally, HBTs and ABTs were more often used as an alternative or complementary method to HLCs in the Asia-Pacific region than either in Africa or the Americas. Animal-baited traps were consistently used since 1980, whereas HBTs were used less frequently, and not at all in 1996-2000. What is also remarkable is that the overall mosquito sampling effort decreased in the periods 2001-2005 and 2006-2010 in the Asia-Pacific region, while sampling effort in Africa and the Americas remained stable.

Geospatial patterns in sampling method use

Visual inspection of the plotted locations of collection methods in the three analysed regions suggested that there was a clustering of collection method use in specific areas (Fig. 3.5). In Africa, the locations of indoor/outdoor HLCs and indoor natural resting collections were confined to West and East Africa, with very limited sampling in Central Africa (Fig. 3.5a). However, the data did not immediately show a geospatial pattern in the spread of both methods. 30 and 48 density-based clusters were detected of HLC (indoor/outdoor) and indoor natural resting collections, respectively. The detected clusters for both methods were spread across the continent, not being confined to one country and were mostly of small size, indicating that the sampling effort in these few areas was high. It is possible that these clusters roughly reflect the locations of research facilities. Computing Moran's I for all the locations in Africa where surveys occurred supported the absence of continent-wide clustering of any sampling method (Moran's I = 0.09, $p > 0.95$, n.s.).

In the Americas, the locations of indoor/outdoor HLCs were widespread across the region, with only a few locations of natural resting collections (Fig. 3.5b). The density-based cluster calculation detected six clusters of HLC (indoor/outdoor) locations, but these were not country-specific and additionally, five of the six clusters overlapped each other. Density-based cluster analyses were unable to detect any significant spatial clustering in other sampling methods due to the limited data. However, computing Moran's I for all locations where sampling occurred indicated that there was spatial autocorrelation of sampling methods (Moran's I = 0.19, $p < 0.001$).

In the Asia-Pacific region, a distinction could be seen in the spread of the sampling methods most often used (Fig. 3.5c). Indoor/outdoor HLCs were deployed across the entire Asia-Pacific region with the exception of India, where indoor natural resting collections were mainly deployed while sparsely used in the rest of the region. This was also shown by the density-based cluster detection, which detected 13 clusters of HLC (indoor/outdoor), of which the largest were not country-specific and were all found in the Greater Mekong Subregion (Myanmar, Thailand, Vietnam, Lao PDR, and Cambodia). Smaller, country-specific clusters of indoor/outdoor HLCs were detected in the Philippines, Papua New Guinea and the Solomon Islands. Sixteen clusters of indoor natural resting collections were detected by density-based clustering, fourteen of which were (partially) in India. One large cluster was found in Myanmar and the final cluster was in Sri-Lanka. Outside these countries, indoor natural resting collections were too few in number and too widespread to fall into a cluster. Clustering of methods was supported by Moran's I (Moran's I = 0.27, $p < 0.001$), which showed that similar methods were more often deployed near each other than randomly spread.

Discussion

This investigation analysed data from 875 distinct studies to examine collection methods use in Africa, the Americas, and the Asia-Pacific region. Differences among the methods used to collect anophelines were observed among the regions and the potential causes for these differences explored. All analyses performed in this research were based on a precompiled bionomics dataset, which collated data from published studies. While the dataset was very rich, it did not contain data from national malaria control programmes (NMCPs) and other routine surveillance programs. Such documents are rarely publicly available and are often not published in English, which made finding these publications hard and data extraction unreliable. Therefore, we assumed that the dataset

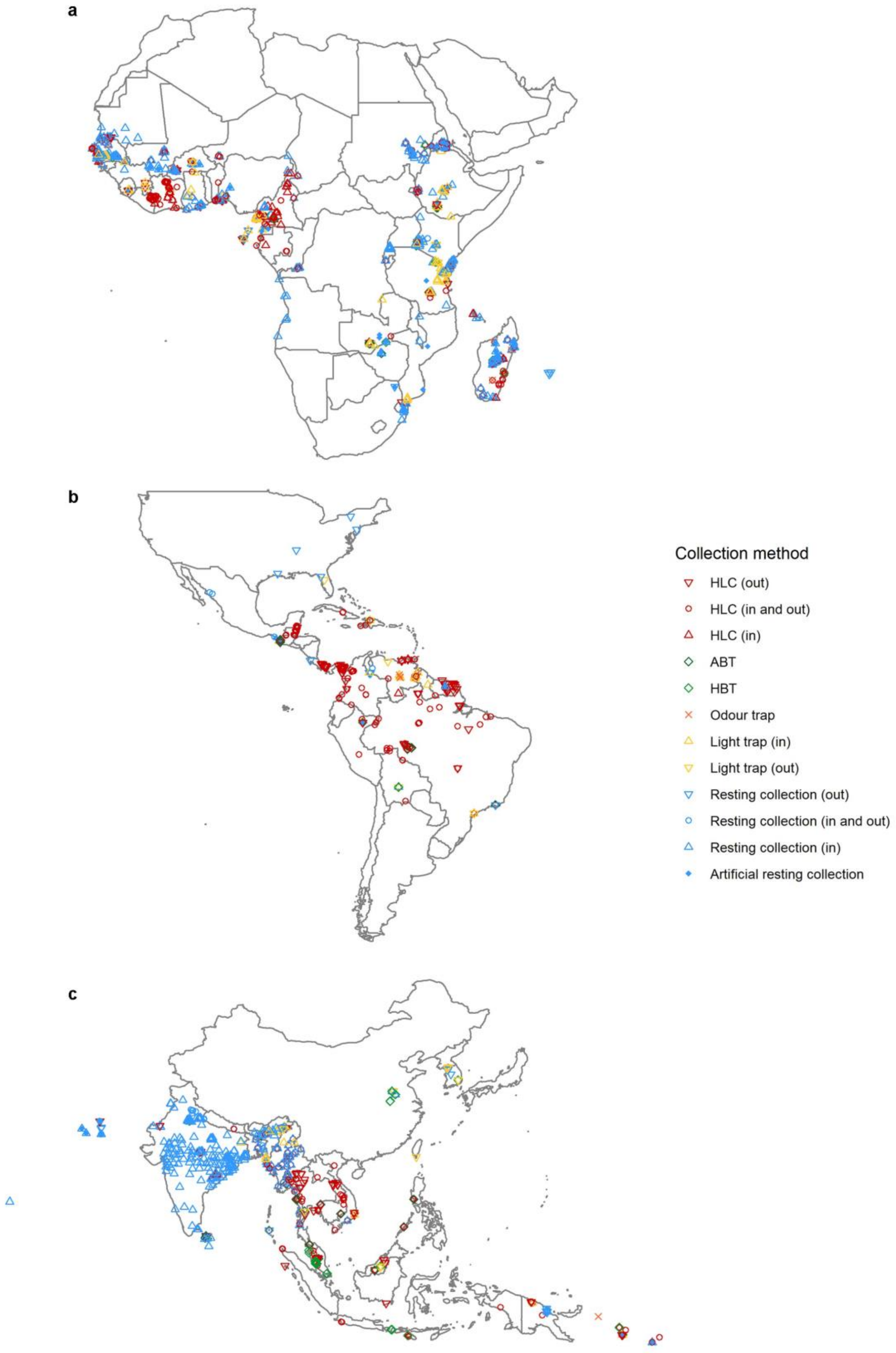

Figure 3.5 - Geographical distribution of the different collection methods used in (a) Africa, (b) the Americas, (c) the Asia-Pacific region. ABT = animal-baited trap, HLC = human landing catch, HBT = human-baited double net trap. Maps were made with R statistical software (R version 4.0.2), packages 'tidyverse' and 'maps'.

used for our analyses was a representative sample of the existing data on malaria vectors in Africa, the Americas, and the Asia-Pacific region.

The HLC was the most commonly used method to sample mosquitoes in the three regions analysed. The HLC is considered the 'gold standard' in mosquito collections, because it gives the most accurate estimate of the human exposure to mosquito bites (148). However, biting rates from HLCs are crude and likely overestimate true human biting rates since collectors, in contrast to the general public, do not make any attempts to ward off mosquitoes (159,160). While concerns are often raised that collectors may be bitten by a mosquito before they can collect it, human landing collectors actually have a reduced risk of acquiring malaria due to prophylaxis (161). Despite the reduced malaria risk, collectors are still vulnerable to arbovirus exposure and thus many attempts have been made to find an alternative to HLCs (65,162–167). Despite its drawbacks, HLC still remains the most frequently used method for collecting malaria vectors.

The use of CDC light traps did not differ significantly between Africa, the Americas, and the Asia-Pacific region. Outdoor light traps were not commonly deployed in any region, but indoor light traps were more often deployed in Africa than in the Americas or the Asia-Pacific region. Although this difference is not statistically significant, sampling more frequently indoors in Africa is consistent with the current dogma that most of the African DVS are endophagic (149,154). The fact that the African DVS are perceived as endophagic and anthropophilic is consistent with the very limited use of animal-baited traps (24 data records from 6 studies) in this region. Additionally, bionomics data on African anophelines shows that their indoor/outdoor biting ratio is around 50/50 (108). By focussing on sampling indoors on human hosts, researchers might miss potentially important shifts in mosquito behaviours toward increased rates of opportunistic feeding or an increase in outdoor biting (168). This might overestimate the perceived vulnerability of vectors to indoor interventions and thus overestimate the effectiveness of the currently recommended control methods, long-lasting insecticidal nets (LLINs) and indoor residual spraying of insecticides (IRS), that protect people from mosquito bites when they are indoors (122,169,170).

Animal-baited traps were more commonly used in Asia-Pacific than in Africa or the Americas. However, this collection method was not as popular as expected when considering the perceived more opportunistic biting behaviour of many Asian vectors (151), being only used in 25 out of 292 studies. The limited use of ABTs may reflect their significant operational constraints of being labour intensive and requiring substantial training for efficient deployment (146,148). Hence, animal-baited traps were mainly used as a complementary method to HLC, instead of an alternative, thus covering a wider range of mosquito biting behaviours. Increasing the amount of entomological data from a wider array of surveillance methods for different vector behaviours is recommended for both researchers and NMCPs (62). Animal-baited collection methods can play an important role in understanding vector behaviours more completely.

Natural resting site collection of anophelines was the second-most commonly used mosquito sampling method. This method encompasses indoor and outdoor handheld aspirators, backpack aspirator collections and pyrethrum spray collections (PSC). While indoor collections of resting mosquitoes are easier to conduct and yield reasonable sample numbers, outdoor collections of resting mosquitoes are notoriously difficult due to mosquitoes' wide and heterogeneously dispersed resting sites (171,172). This is consistent with observations in this study that showed that a large majority of resting collections (>85% in Africa and the Asia-Pacific region) were conducted indoors. Although some inference on outdoor resting can be made by examining abdominal status of mosquitoes collected indoors (173–175), the tendency to preferably collect resting mosquitoes by indoor sampling may have generated a bias towards indoor resting in our understanding of resting behaviours. Additionally, the tendency towards indoor resting collections may have delayed detection of changes in resting behaviours (e.g., behavioural resistance) following scale-up of indoor residual spraying. The current assumption, especially in Asia, is that the majority of malaria vectors rest outdoors (151). Hence, surveillance strategies that only sample resting mosquitoes indoors will maintain the present knowledge gap on (outdoor) resting behaviours of malaria vectors.

Artificial resting sites (pit traps) were used in Africa as an alternative collection method to sample outdoor-resting anophelines. Other ways to create artificial resting sites for outdoor mosquito collections (clay pots, box shelters, etc. (159)) were not documented in the dataset analysed, so direct comparisons between respective sampling efforts of pit traps and other methods could not be made. However, Odiere et al. (2007) (171) reported that clay pots were more successful and practical than pit traps in collecting both the more endophilic *An. gambiae* s.s. and the more exophilic *An. arabiensis*. In contrast, more recent research showed that clay pots were somewhat less productive than pit traps in collecting *An. gambiae* s.l. (176,177). However, clay pots yielded a comparable relative abundance of anopheline species and were more practical in many situations, thereby offsetting their lower productivity. In areas with traditionally high or increased (e.g., resulting from high IRS coverage) levels of exophily, outdoor resting surveillance is crucial in understanding the ecology of malaria vectors and the effectivity of applied interventions. In such areas, not only in Africa but also in the Americas and the Asia-Pacific region, artificial resting site collections could be a valuable asset in vector surveillance.

The geospatial pattern of published mosquito collections in the Asia-Pacific region shows that HLCs, both indoor and outdoor, are the main surveillance method in the entire region except India where indoor resting collections are common practice. The astonishing number of data records from India (almost 50% of the data from the Asia-Pacific region comes from India) were almost exclusively acquired by indoor resting collections and may reflect an emphasis on one DVS, *Anopheles culicifacies*, which rests and bites traditionally indoors. However, expert opinions and bionomics data show that *An. culicifacies* can also be found resting outdoors (108,151).

The use of a single method or insufficient sampling sites used to define a vector's resting behaviours may limit the capacity to detect potential changes in its resting behaviour in response to selective pressure from indoor residual spraying or LLINs (e.g., resting indoors or outdoors, duration of resting at one location, peak biting time, endophagy/exophagy or choice of blood host) (151,178,179). Most resting collections (mechanical aspirator collections, PSCs) are conducted in the early morning (159) and cannot detect shifts in temporal or spatial resting patterns. Furthermore, data on host-seeking or biting behaviours cannot be derived from resting collections. A comparable argument can be made when HLCs alone are used to define biting behaviours. HLCs can detect spatio-temporal shifts in host-seeking and biting behaviours when contemporaneous indoor and outdoor collections are made, but cannot be analysed for changes in blood host choice (179).

The use of one sampling method, specifically the method which results in the highest vector numbers, is a cost-effective way of acquiring large numbers of mosquitoes to define a limited number of vector indicators. In contrast, collecting mosquitoes by using multiple sampling methods for biting and resting, indoors and outdoors (and by representative sampling), yields more epidemiologically relevant data for researchers and policy makers. Therefore, entomological surveillance should utilise multiple complementary collection methods across different micro-habitats to sample different behaviours. Concurrent use of complementary collection methods will enable a more comprehensive characterisation of vector behaviours, will better define vector species richness and community composition, as well as enable the early detection of behavioural shifts that may threaten the effectiveness of malaria vector control (122).

Conclusions

We observed a tendency towards using collection methods to potentially maximise the number of mosquitoes captured based on anticipated vector behaviours in a geographic region by targeting specific vector behaviours (and by default, ignoring other behaviours). Although similar malaria vector collection methods were used in the three regions, their frequency of use varied between Africa, the Americas, and the Asia-Pacific region. Their frequency of use may have resulted from biases in the perceived behaviours of the DVS in each region in order to maximise the numbers captured. Adherence to current dogmas and expert opinions to design vector sampling strategies may reinforce biases in surveillance data and can delay the detection of behavioural shifts of vectors which could lead to reduced vector control. A more varied, tailor-made surveillance effort integrating multiple collection methods for specific regions can provide better insights in vector behaviour and changes in vector behaviours.

Methods

All analyses in this study were based on the bionomics data extracted and collated from literature published between 1981 and 2015 on the global DVS (108). A detailed structure of the database as well as original references are in the original publication. The database contained 10,922 data records from 875 publications. Information on *Anopheles* species included the (georeferenced) study location, starting month and year of the study, mosquito collection methods used, biting and resting locations, preferred blood hosts and peak biting times. The methods used to collect malaria vectors in the WHO regions 'Africa', 'Americas' and 'Asia-Pacific' were analysed because 96.4% of the global malaria burden is found in these regions (145), where malaria vector behaviours differ.

Individual data records (rows in the database) show unique site-collection period-species combinations. A single study or publication could therefore comprise multiple data records (rows), depending on: 1) the number of sites studied, 2) the number of sampling events included in the study, 3) the commencement date and 4) the species collected. Collection sites were recorded with geographical latitude and longitude, without differentiating between the area size, which included "point" (≤ 10 km^2), "wide area" (>10 and ≤ 25 km^2), "small polygon" (>25 and ≤ 100 km^2) and "large polygon" (>100 km^2). While month and year defined the start as well as the end of an experiment (two data records could have similar start dates but different end dates), only the study starting year was used to categorise the time of the study. Accurate estimates of the number of collection nights for each individual study (the sampling effort) could not be extracted because only start and end month were recorded and the number of surveys, traps and workhours were not recorded in the database.

Collection methods were only analysed if they captured species of interest. In the construction of the dataset, mosquito collection methods were categorised as: 'vector biology sampling', 'infection sampling', 'human biting rate collection' and 'resting collection' depending on the bionomic metric the data were informing. These categories were not mutually exclusive, so for each data record a collection method could be recorded twice. The data frame was manipulated to summarise the combination of collection methods used per data record, ensuring that the location (indoors or outdoors) for each sampling effort was noted. The different collection methods recorded in the database targeting host-seeking or resting mosquitoes were categorised as by Farlow et al. (148) (Table 3.1, 3.2).

All geo-referenced data records were compiled, summarised by continent, stratified by year and method, and mapped in R Studio. The Mann-Whitney U test analysed within-continent differences between indoor and outdoor sampling methods. To explore trends in the proportional use of host-seeking and resting collections over the entire database time period, a generalised linear model was constructed with ‘proportion host-seeking collections’ as dependent variable and ‘start year of the research’ as independent variable. Running the model with a quasibinomial distribution accounted for overdispersion of the data. The data were summarised by 5-year periods for visualisation. Potential geospatial patterns of collection method use were first studied visually in each of the three regions analysed. Consequently, the two sampling methods used most often that showed at least some degree of clustering were analysed by density-based clustering following the OPTICS algorithm, described in detail by Hahsler et al. (2019) (180). OPTICS starts with a random data point and provides the order in which new points are explored and added to a cluster. Follow-up ξ-extraction is required to detect clusters of variable density and provide the cluster hierarchy. This means that the OPTICS method can detect clusters within clusters. Afterwards, Moran’s I for spatial autocorrelation tested whether the detected clustering of the analysed sampling methods was significant, or the locations where a sampling method was deployed were spread randomly across a continent/region. Data analyses were performed in R Studio with R statistical software (R version 4.0.2) using the packages ‘tidyverse’, ‘maps’, ‘gganimate’, ‘geosphere’, ‘ape’, ‘psych’, ‘dbscan’ and ‘sjmisc’.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files. Original databases can also be found in Massey NC, Garrod G, Wiebe A, Henry AJ, Huang Z et al. *Sci Data*. A global bionomic database for the dominant vectors of human malaria. 2016;3:1-13. doi: 10.1038/sdata.2016.14.

Competing interests

The authors declare that they have no competing interests.

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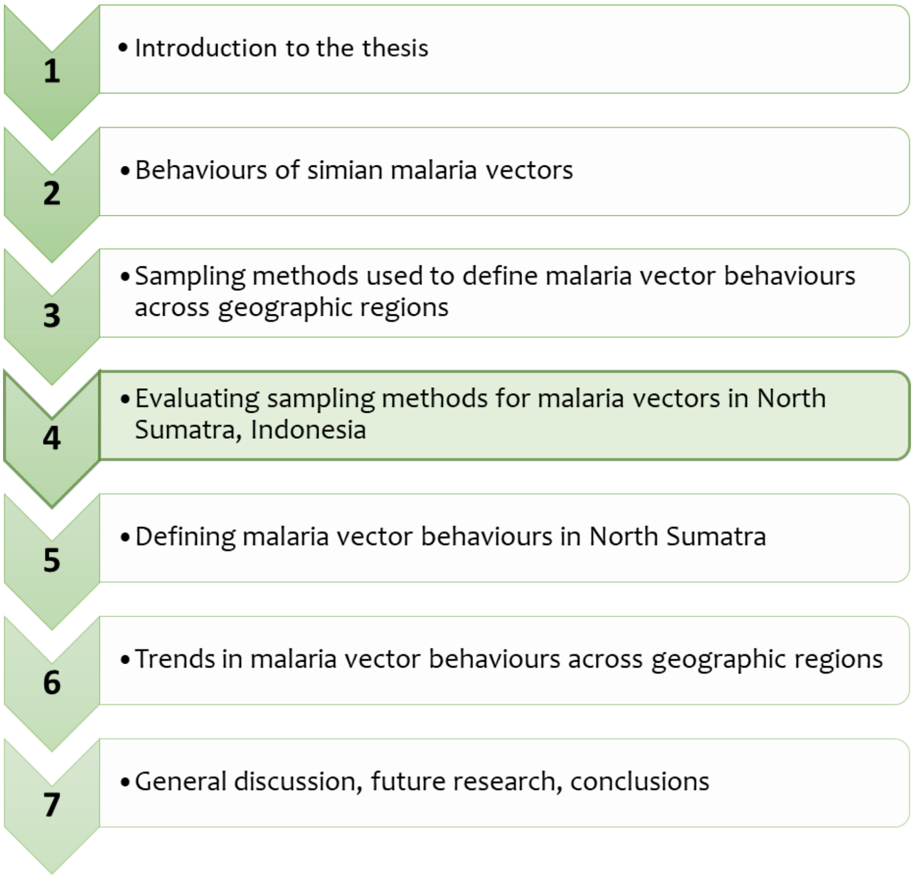
Authors' contributions

BvdS, TLR and TRB conceived the research. BvdS analysed the data and wrote the original draft of the manuscript. TLR, KS and MES provided input on the data analyses. All authors reviewed and approved the final manuscript.

Chapter 4

The previous chapter showed that the gold standard for collecting Anopheles mosquitoes in Asia is the human landing catch, with animal-baited tent traps being the second-most used method. In this chapter, the sensitivity and specificity of these two methods, along with several mechanical traps for sampling Anopheles *collection, and specifically* Anopheles leucosphyrus *complex mosquitoes, were evaluated in two sites in an area endemic for simian malaria.*



Evaluation of different methods to collect human and simian malaria vectors in North Sumatra, Indonesia

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Abstract

Background

Malaria transmission to humans is increasing in complexity in Indonesia with simian malaria cases in humans increasing as human malaria parasites diminish in transmission. Reliable, epidemiologically relevant, logistically feasible, and cost-effective collection methods are needed to define the transmission risk to humans and the efficacy of vector control for both simian and human malarias. Alternative and/or complementary representative sampling methods to the labour-intensive human landing collections (HLC) and animal-baited tent traps (ABT) currently used for mosquito surveillance in Indonesia are needed.

Methods

Three consecutive Latin square experiments were conducted to determine the sensitivity and specificity of different CO_2 lures and collection methods for sampling potential malaria vectors in North Sumatra. Carbon dioxide gas from a compressed cylinder and from yeast/sugar fermentation were used as lures in Passive box, CDC light and BG Sentinel 2 mosquito traps against human landing catches (HLCs) and animal baited traps (ABTs) with a cow as bait in Latin square comparisons across three land use types (oil palm plantation, mixed-crop agricultural area and village) common in North Sumatra.

Results

The ABT with cow as bait was the most sensitive sampling method for all anopheline species in a direct comparison to the BG-Sentinel 2 trap and HLC in North Sumatra, capturing 3 to 5 times more anophelines, respectively. The Passive Box and CDC light traps were not sensitive for anophelines regardless of the CO_2 source. The HLC showed the highest specificity for members of the *An. leucosphyrus* complex including *Anopheles dirus* (up to 35% of mosquitoes collected), and also captured *An. kochi*, *An. barbirostris*, and *An. maculatus*.

Abundance of anophelines varied between land-use types. *Anopheles leucosphyrus* complex numbers were greater in the small-scale, mixed-crop agricultural area, while the *An. maculatus* Group abundance was greater in the oil palm plantation. All *Anopheles* species were less abundant in the village sites.

Conclusions

Trapping methods incorporating humans or cows were more sensitive for anopheline species in North Sumatra than Passive Box or CDC light traps using CO_2 sources from tanks or yeast fermentation. However, the BG Sentinel trap showed some potential for anopheline sampling of *An. leucosphyrus* complex and *An. barbirostris*.

Keywords

Anopheles leucosphyrus, *Anopheles dirus*, *Anopheles maculatus*, *Anopheles barbirostris*, malaria, vector, human landing catch, animal-baited tent trap

Introduction

Human malaria cases caused by *Plasmodium falciparum* and *Plasmodium vivax* are diminishing in Southeast Asia including Indonesia (181), while simian malaria cases in humans are increasing in all countries in Southeast Asia except Timor-Leste (31,34,45–49,51–55). Improved vector surveillance is a key strategy to better understand transmission to humans of simian malaria species (59–61), as well as to define areas of transmission risk and to evaluate the efficacy of vector control strategies against both human and simian malarias.

Nine species of the *Anopheles leucosphyrus* and *Anopheles dirus* species complexes are implicated as vectors of simian and human malarias (55,72,92,98), but little is known about the behaviours of these vectors in many regions in Southeast Asia (182). Field studies of Leucosphyrus Group mosquitoes showed opportunistic blood host choice for many species. Direct comparisons between human landing catches and monkey-baited traps showed that *Anopheles leucosphyrus* complex mosquitoes fed on both macaques and humans in the forest in Malaysia (64,72,86) and Cambodia (103). This opportunistic biting behaviour means that these mosquitoes may transmit simian malaria species to humans.

Identifying the transmission risk to humans requires understanding what the vector species are, as well as where and when contact between the vectors and humans occurs, a function of vector density, indoor and outdoor human biting rates (HBR), biting time, seasonality of the vectors, and vector species richness (160). Quantifying these parameters requires reliable, epidemiologically relevant, and cost-effective collection methods that are operable in the often-remote areas where residual transmission of simian and human malarias to humans occurs (148).

Commonly used collection methods for anophelines, including the simian malaria vectors in the *An. leucosphyrus* complex, are human landing catches (HLCs) and animal-baited traps (ABTs) (70,78,81,82). Although both methods are effective in sampling anophelines, these methods are labour-intensive and expensive, oftentimes limiting their deployment. Additionally, ABTs require an animal as bait. While, in the case of simian malaria transmission, ideally a macaque or other monkey would provide an epidemiologically relevant bait to directly measure bites on monkeys, animal ethics in many countries including Indonesia prohibit the use of monkeys in animal-baited traps. Alternatives, including cows, can be used as bait. Previous studies in Southeast Asia, including in Indonesia, have shown that a cow-baited trap can collect high numbers of anophelines, including species that are known malaria vectors to humans (65,183). Also, cows are usually more docile and easier to keep in a tent than monkeys.

Mechanical mosquito traps could overcome the limitations of human- and animal-baited traps as many require less labour to set up and monitor than HLCs and ABTs, which allows wider deployment. The Mosquito Magnet trap was reported to be statistically comparable to HLCs for sampling *An. leucosphyrus* Group members in Malaysia. However, sample numbers captured by the Mosquito Magnet were lower than by HLC, and its setup and establishment costs remain logistically and financially challenging. Other mechanical traps, that require less labour to set up than the Mosquito Magnet trap, include the Centers for Disease Control (CDC) light trap (184), the BG-Sentinel 2 trap (a fan-powered counter current trap) (185) and the Passive Box trap (PBT) (186). The PBT has been successful in collecting host seeking anophelines in Australia, and is relatively cheap, requires no electricity, and was designed to provide a relatively undisturbed plume of CO_2 via natural air flow for mosquitoes to follow (187). Although the BG-Sentinel 2 trap is chiefly used to collect *Aedes* mosquitoes, there is some evidence that it can be attractive for anophelines when it is baited with CO_2 (187). Nevertheless, mechanical traps to collect malaria vectors including *An. leucosphyrus* Group species in Indonesia have not been tested extensively, and results from previous evaluations were inconsistent (74,88,104).

This study aimed to identify sensitive collection methods for anopheline malaria vectors that are logistically operable in remote areas of Sumatra where simian and human malarias are presumably transmitted (e.g., oil palm plantations and forest fringes) (99). To maximise numbers of anophelines and assess the effectivity of the tested collection methods, this study was conducted in three land-use types that are common throughout rural North Sumatra. Hence, this study examined both the effectiveness of each collection method as well as their ease and cost of deployment in representative field sites.

Methods

Study sites

The study was conducted in Ujung Bandar village, subdistrict Salapian, Langkat Regency, North Sumatra province, Indonesia, in July 2022 and October 2022. The climate is tropical, with over 3000 mm rain/year and mean daytime temperatures ranging between 29-34°C and 19-24°C at night in the study period. The hilly landscape (from 400-650 meters above sea level) is dominated by large-scale oil palm plantations interspersed with small-scale, mixed-crop agriculture areas, and small patches of disturbed forest. Ujung Bandar village consists of nine sub-villages or dusun, in two of which (Dusun 2 and Dusun 5) the study took place. Dusun 2 is surrounded by oil palm plantations, while Dusun 5 (located approximately 3km away) is a larger sub-village with small mixed-crop agricultural areas bordering on disturbed forest patches. Within Ujung Bandar village, 4 human infections with *Plasmodium knowlesi* were detected between March 2022 – March 2023, along with 6 *P. vivax*, 22 *P. malariae*, and 10 *Plasmodium* (genus only) infections (I. Lubis, personal communication, 2023). Long-tailed macaques (*Macaca fascicularis*) are common, with pig-tailed macaques (*Macaca nemestrina*) also inhabiting the area. Domestic animals (mainly cows, chickens, and dogs) roam free in and around both dusun.

Study design

This Latin square study consisted of three experiments conducted in Dusun 2 in July 2022, and repeated in Dusun 5 in October 2022. Mosquito collections were conducted at fixed sampling stations in two concurrent Latin squares per experiment from 18.30 to 0600. Each collection method was assigned to one sampling station for one night, and collection methods were rotated to a different station each night within a Latin square until each collection method was tested in each sampling station. Sampling stations were at least 50 meters apart and fixed in location over the study. Outputs were the *Anopheles* abundance per collection method (sensitivity) and the number of species of mosquitoes collected by each collection method (specificity).

The first Latin square experiment (3x3 design) compared the human landing catch (HLC) to two Passive Box traps (PBTs) (186) and was run in duplicate in each dusun (2x3 collection nights per dusun). Teams of two collectors concurrently conducted HLC by collecting all mosquitoes that landed on their lower legs with mouth aspirators. Passive box traps were hung with the entrance 1.5m above the ground, one baited with CO_2 from a gas cylinder (flow rate: 250 mL/minute) with the second PBT baited with CO_2 generated from yeast/sugar fermentation (variable flow rate). Carbon dioxide flow rates were measured daily during trap setup by a gas flow meter (Kytola Instruments, Muurame, Finland). The yeast/sugar fermentation mixture consisted of 500g sugar and 20g dry yeast in 2L water, in a 5L plastic container, made daily at 4PM, 2 hours before the start of the experiment. Carbon dioxide gas was delivered to the PBTs via 4mm diameter tubing.

The second Latin square experiment (4x4 design), run in duplicate in Dusun 2 and Dusun 5 (2x4 collection nights per dusun), compared the HLC (as described above) to three mechanical traps (the PBT, the BioGents Sentinel 2 trap [BioGents AG, Germany] and the CDC Miniature light trap [John W. Hock Company, Gainesville FL, USA]), each baited with CO_2. Because of the limited samples from Latin square round 1, this mechanical trap comparison was conducted concurrently in duplicates, varying only in the CO_2 source in each replicate (one Latin square had traps baited with CO_2 from a gas cylinder and the other simultaneously had traps baited with CO_2 from yeast/sugar fermentation). The CDC light trap and the PBT were suspended with trap entrances 1.5m above the ground; the CDC light trap was also baited with an incandescent light bulb. The BG-Sentinel 2 trap was placed on the ground under a large umbrella to protect against rain. After four collection nights, the best mechanical trap was selected based on maximum numbers and species of anophelines collected.

The third Latin square experiment (3x3 design), which was also run in duplicate in each dusun (2x3 collection nights per dusun), compared the best mechanical trap (from Latin square round 2) baited with tank CO_2 source to the HLC, as described in Latin square 1, and an animal-baited tent trap (ABT) using a cow as bait. The ABT placed a cow with food and water inside a rectangular mosquito net (L*W*H = 3m * 3m * 2m) lifted approximately 50cm from the ground to allow mosquitoes to enter. The inside of the net was searched hourly for 10 minutes by trained collectors with mouth aspirators. The cow owner accompanied the mosquito collectors to minimise stress to the cow and to intervene in the event of stress.

All mosquitoes collected during the three Latin square experiments were transferred to paper cups covered with mesh netting, sedated with chloroform, and identified by morphology to genus and to species for *Anopheles* and *Aedes*, when possible (188). Afterwards, all mosquitoes were stored dry, on cotton wool and silica gel beads, in 1.5mL Eppendorf tubes. All females identified morphologically as *An. leucosphyrus* complex, as well as a random subsample of other anophelines, were subjected individually to species confirmation (or, for *An. leucosphyrus* complex, species complex confirmation) by PCR amplification of the ITS2 gene. Mosquito DNA was extracted using the DNEasy Blood & Tissue kit (Qiagen, Germany), after which the ITS2 gene was amplified using the ITS2A and ITS2B primers (189). The cycling parameters were initial denaturation at 95°C for 120 sec, then 35 cycles of denaturation (60 sec, 95°C), annealing (30 sec, 51°C), and extension (60 sec, 72°C), followed by a final extension at 72°C for 10 min. The amplified product was run on a 1.5% agarose gel at 90 V/400 mA for 90 minutes and visualised under ultraviolet light. A random subset of the *An. leucosphyrus* complex samples (n = 25) were analysed by Sanger sequencing to identify species within the complex (Macrogen Indonesia).

Table 4.1 – the pooled number of Anopheles females collected by each collection method in every experiment across the two dusun. HLC: human landing catch with two collectors per sampling station; PBT: passive box trap; ABT: animal-baited tent trap.

		An. barbirostris	*An. kochi*	*An. leucosphyrus*	*An. maculatus*	*An. vagus*
Dusun 2	HLC	1	0	5	2	0
	PBT + tank	0	0	0	0	0
	PBT + yeast & sugar	0	0	1	0	0
Dusun 5	HLC	0	0	26	0	0
	PBT + tank	0	0	1	0	0
	PBT + yeast & sugar	0	0	0	0	0
	Experiment 1 - Total	*1*	*0*	*33*	*3*	*0*
Dusun 2	HLC	0	0	12	1	0
	BG-Sentinel 2 + yeast & sugar	0	0	0	0	0
	BG-Sentinel 2 + tank	0	0	0	0	0
	CDC light trap + yeast & sugar	0	0	1	0	0
	CDC light trap + tank	0	0	0	0	0
	PBT + yeast & sugar	0	0	1	0	0
	PBT + tank	0	0	0	0	0
Dusun 5	HLC	0	0	49	1	0
	BG-Sentinel 2 + yeast & sugar	0	0	2	0	0
	BG-Sentinel 2 + tank	1	0	0	1	0
	CDC light trap + yeast & sugar	0	0	0	0	0
	CDC light trap + tank	0	0	0	0	0
	PBT + yeast & sugar	0	0	1	0	0
	PBT + tank	0	0	1	0	0
	Experiment 2 - Total	*1*	*0*	*67*	*3*	*0*
Dusun 2	HLC	0	3	0	13	0
	ABT	0	12	17	163	4
	BG-Sentinel 2 + tank CO_2	0	0	1	0	0
Dusun 5	HLC	0	0	7	0	0
	ABT	3	2	13	39	0
	BG-Sentinel 2 + tank CO_2	5	0	17	9	0
	Experiment 3 - Total	*8*	*17*	*55*	*224*	*4*

Statistical analysis

Nightly catch numbers of *An. leucosphyrus* complex and *An. maculatus* Group females were compared between the different collection methods in a General Linear Mixed Model (GLMM) with Poisson distribution and log-link function. In this model, 'collection method' and 'dusun' were independent variables, while 'collection night' and 'sampling station' were random variables to account for variability between nights and stations. Overdispersion was addressed by fitting a second, negative binomial GLMM with quasipoisson distribution with the same explanatory variables to the data. Hereafter, the model that best fitted the data was determined by an ANOVA. Because the three Latin Square experiments were set up similarly, the statistical analysis described here was applied to all experiments. Following observations of varying *Anopheles* abundance across the three land-use types in which the experiments were conducted, a preliminary analysis was conducted. The association of different land-use types on the relative abundance of mosquitoes was tested by comparing the proportions of mosquitoes in a Chi-square test of independence. All data analyses were performed in R (R version 4.1.1; R Foundation for Statistical Computing, Vienna, Austria) with the most recent version of the 'tidyverse' and 'lme4' packages.

Results

25 of 155 *An. leucosphyrus* complex mosquitoes were identified by sequencing the ITS2 gene as *Anopheles dirus*. Because of the limited sample size of confirmed identifications, these mosquitoes will be referred to as *An. leucosphyrus* complex.

Mechanical trap + different CO_2 sources compared to HLC

Across both duplicates in the two Dusun comparing CO_2 sources to HLC, 33 *Anopheles leucosphyrus* complex, 3 *Anopheles maculatus* Group and 1 *Anopheles barbirostris* females were collected (Table 4.1). In addition, 13 *Aedes albopictus*, 1 *Aedes finlaya*, 33 *Culex* spp., and 7 *Armigeres* spp were collected. Differences between collection methods were highly significant, with HLC yielding the highest mean nightly catch of *An. leucosphyrus* complex females (GLMM, $X^2 = 22.155$, df = 2, $p < 0.001$). The PBT was less attractive for mosquitoes, regardless of the CO_2 source. Two *An. leucosphyrus* complex females were collected, one by each CO_2 source, and one *Culex* spp. was collected in a PBT baited with the yeast/sugar mixture while one *Armigeres* spp. was found in a PBT baited with CO_2 from a gas cylinder. The proportion of *An. leucosphyrus* complex mosquitoes in the HLC collections was 0.356, indicating a relatively high specificity (Fig. 4.1a).

Mechanical traps compared to HLC

In the mechanical trap comparison experiment, 67 *An. leucosphyrus* complex, 3 *An. maculatus* and 1 *An. barbirostris* were collected in the two Dusun combined (Table 4.1), as well as 50 *Ae. albopictus*, 5 *Aedes* spp., 80 *Culex* spp., 30 *Armigeres* spp., and 2 unidentified mosquitoes. The mean nightly catch of *An. leucosphyrus* complex by the HLC was significantly higher than the numbers collected by any of the mechanical traps (GLMM, , $X^2 = 53.702$, df = 3, $p < 0.001$). The number of *An. leucosphyrus* complex or other *Anopheles* collected did not differ significantly between the three mechanical traps, and the CO_2 source did not significantly affect the number of *Anopheles* mosquitoes trapped (GLMM, n.s.). The relative abundance of *An. leucosphyrus* complex mosquitoes in HLC collections was 0.311 (Fig. 4.1b).

HLC, ABT and BG-Sentinel 2 comparison

The BG-Sentinel 2 trap baited with CO_2 from a gas cylinder was compared to the HLC and ABT in the final experimental round. A total of 55 *An. leucosphyrus* complex, 224 *An. maculatus* Group, 17 *Anopheles kochi*, 8 *An. barbirostris*, and 4 *Anopheles vagus* were collected across the two dusun (Table 4.1). Furthermore, 27 *Ae. albopictus*, 23 other *Aedes* spp., 146 *Culex* spp., 329 *Armigeres* spp., and 4 *Toxorhynchitis* spp. were collected. Highly significant differences in the mean nightly catch numbers of *An. leucosphyrus* complex mosquitoes were found with the ABT collecting three and

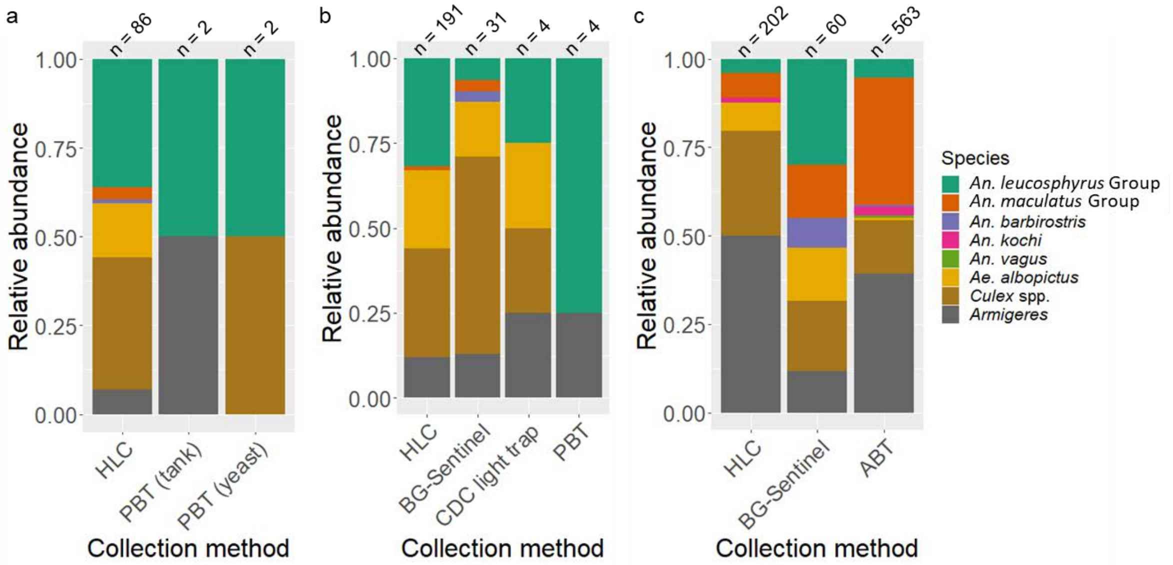


Figure 4.1 - Relative abundance of mosquito species collected during the three experiment rounds, pooled across the two Dusun. 'Other' species consists of Armigeres *spp. (>95%),* Aedes *spp., and* Toxorhynchites *spp.. a) Experiment round 1, which used Passive Box traps (PBT) to compare CO_2 from a gas tank to CO_2 from yeast/sugar fermentation and human landing catch (HLC); b) Experiment round 2, which compared the HLC to three mechanical traps; c) Experiment round 3, which compared the BG-Sentinel trap with CO_2 from a tank to the HLC and a cow-baited tent trap (ABT).*

five-fold greater catches than the BG-Sentinel 2 and HLC, respectively (GLMM, $X^2 = 28.167$, df = 2, $p < 0.001$). Collection numbers of *An. maculatus* Group mosquitoes were also significantly higher in the ABT than in the other two collection methods (GLMM, $X^2 = 144.858$, df = 2, $p < 0.0001$). Collection numbers of other anophelines were too low for statistical analysis. Additionally, the *Anopheles* species richness was highest in the ABT, which collected five *Anopheles* species and was more sensitive, capturing much higher numbers of anophelines than the other methods. In contrast, the BG-Sentinel 2 collected *An. leucosphyrus* complex (n = 18), *An. barbirostris* (n = 5), and *An. maculatus* (n = 9), while the HLC collected *An. leucosphyrus* complex (n = 7), *An. maculatus* (n = 13), and *An. kochi* (n = 3). The relative abundance of *An. leucosphyrus* complex mosquitoes was much lower than in the previous experiment rounds, with 0.038 in the HLC, 0.281 in the BG-Sentinel 2 trap, and 0.051 in the ABT (Fig 4.1c).

Land-use type effects

Across the different land-use types, large differences in mosquito catches were observed (Fig. 4.2). There was a significant effect of land-use type on the proportions of collected mosquito females by species across the full experiment ($X^2 = 840.92$, df = 14, $p < 0.001$). Collections in the small scale, mixed-crop agricultural area captured higher numbers of *An. leucosphyrus* complex and *An. barbirostris* than expected, while the relative abundances of *An. maculatus* Group, *An. kochi*, and *An. vagus* mosquitoes were higher than expected in the oil palm plantation. In the village, the proportion of other mosquito species (mainly *Armigeres* spp.) was much higher than expected, while the relative abundance of *Culex* spp. was lower than expected. Analysis of the residuals

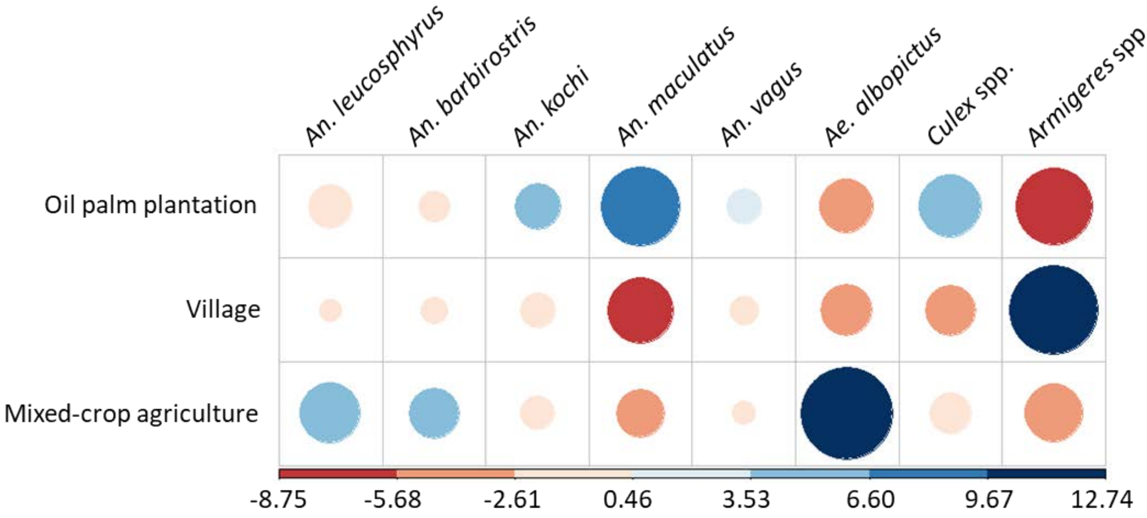


Figure 4.2 - Correlations of different mosquito species with the different land-use types in which the experiment was conducted. Blue dots indicate a positive association, red a negative association, as indicated by the standardised residuals; larger dots mean a stronger association.

showed that there was a significant positive correlation between *An. leucosphyrus* complex and the mixed crop agricultural area, as well as a strong positive correlation between *An. maculatus* Group and the oil palm plantation. In contrast, most mosquito species were negatively correlated with the village, except for *Armigeres* spp., which comprised >95% of the 'other mosquito species' group (Fig. 4.2). The remaining 5% of the 'other mosquito species' group consisted of *Aedes* spp. (n = 31) and *Toxorhynchites* spp. (n = 4).

Discussion

Mechanical collection methods successfully sampled anophelines including simian malaria vectors in this and other studies (37,47–49). However, the sensitivity of sampling methods for anopheline mosquitoes in this study was greatest when using humans or cattle as lures compared to mechanical mosquito collection methods, including the Passive Box trap, the CDC Miniature light trap, and the BG-Sentinel 2 trap, baited with CO_2.

While numbers of collected mosquitoes are often lower in traps baited with CO_2 from yeast/sugar fermentation compared to CO_2 from dry ice, other studies reported representative sampling of mosquitoes using yeast/sugar fermentation (190,191). Additionally, studies in Africa (192,193) and Australia (187,194) reported high numbers of mosquitoes including anophelines collected in traps with CO_2 produced by yeast/sugar fermentation, but no direct comparisons to HLC were made. The yeast fermentation may not have produced optimal amounts of CO_2 to attract large numbers of mosquitoes. However, CO_2 released from a tank at recommended rates (e.g., 250ml/min) was equally ineffective in attracting large numbers of mosquitoes. Another explanation for the low number of mosquitoes in the mechanical traps might be that the evaluated traps were either not efficient in capturing mosquitoes lured to them or were not attractive to mosquitoes as deployed. Adding an extra odour bait or heat source, or placement at a different elevation, might improve catch numbers (165,192,195).

Mechanical traps like the CDC light trap and the BG-Sentinel trap have collected mosquitoes for decades. In sub-Sahara Africa, CDC light traps and other mechanical traps supplemented or replaced HLCs for malaria vector collections to provide a rough estimate of human biting rates, despite their lower sensitivity (196). In Asia, however, mechanical traps have not been as promising. In Peninsular Malaysia, collection numbers of An. *leucosphyrus* Group mosquitoes in the Mosquito Magnet were lower, but not statistically different from HLCs, while CDC Miniature light traps yielded much lower numbers of these and other mosquitoes (104). Smaller mosquito surveys in Indonesia showed that *An. leucosphyrus* Group mosquitoes were collected by HLC but not CDC light traps in a direct comparison (197,198). In this study, a similar result was observed across the study sites, with CDC light traps and other mechanical traps collecting low numbers of mosquitoes compared to HLCs. The BG-Sentinel 2 trap had not, to our knowledge, been evaluated for *Anopheles* sampling in Indonesia before this study. However, a previous study in northern Queensland, Australia showed that a BG-Sentinel 2 trap baited with tank CO_2 collected *Anopheles* females (187). The third round of this study provides some evidence that the BG-Sentinel 2 trap, baited with CO_2 from a gas cylinder, could be at least comparable in sensitivity to the HLC to collect *Anopheles*. Especially when HLCs are logistically challenging (e.g., in deep forest locations) or when many locations need to be sampled simultaneously, the BG-Sentinel 2 trap could be a viable alternative. It would require further evaluation to understand why the BG-Sentinel 2 was more sensitive than the HLC in one of the two experimental rounds in which they were directly compared. Additionally, running a mechanical trap baited with a CO_2 tank will only be more cost-effective than a single team of HLC collectors when collecting for 16 nights or more, due to its relatively high establishment cost (Supplemental Material 4.1).

The cow-baited tent trap was sensitive, collecting high numbers of anophelines throughout the night. However, the ABT attracted much higher numbers of other mosquito species (e.g., *Armigeres* spp. and *Culex* spp.) than the HLC, as well as many zoophilic anophelines. Cow-baited tent traps are likely the best method to collect a wide range of species including vectors of malaria and arboviruses but are less accurate in estimating biting risk on humans (65). Cow-baited traps often yield much higher mosquito numbers than HLCs, which would require calibration against HLCs to estimate the biting rate on humans. Also, the number of non-target mosquitoes collected in ABTs baited with a cow is significantly higher compared to HLCs, which can hamper or delay mosquito identification in the field. Finally, the ABT is more challenging to deploy than other collection methods. During this study, challenges with the ABTs included finding suitably flat ground to set up the ABT, inadvertently causing stress to the cow despite the presence of the owner, escape attempts, and windy weather.

The primary hosts of *P. knowlesi* and other simian malarias that can infect humans are long-tailed macaques and pig-tailed macaques, which are originally forest-dwelling species (64,86) but will readily adapt to living and feeding in the vicinity of human settlements and oil palm plantations (129,131). Several studies conducted in Sarawak, Malaysia, have shown that *An. leucosphyrus* complex mosquitoes display opportunistic biting behaviours in forest and forest fringe areas, biting both humans and monkeys on the ground as well as in the canopy (64,70–72,82). Thus, the most accurate estimate of the transmission risk of simian malaria to humans and monkeys would be to simultaneously deploy a human-baited collection method (i.e., HLC) and a monkey-baited collection method (i.e., monkey-baited tent trap, monkey-baited electrocuting net trap) and compare biting rates of the suspected vectors (74). The use of a monkey-baited trap in the study was not possible due to ethical prohibition and while this can be considered a limitation of the study, the relatively high biting rates on both cows and humans show that *An. leucosphyrus* complex mosquitoes in the study area display opportunistic blood feeding behaviour. This information, combined with the confirmed presence of long-tailed macaques in the study area (personal observations), implies that these mosquitoes can act as bridge vectors for simian malaria transmission between macaques and humans.

A heterogeneity between the first two and the third Latin Square experiment comparison was seen in the relative sensitivity of the BG Sentinel 2 trap and the HLC in sampling *An. leucosphyrus* complex and *An. maculatus* Group mosquitoes. These results lack a clear reason, since Sanger sequencing of the ITS2 gene showed that all specimens in the random subsample were in the *Anopheles dirus* complex. RFLP analyses suggest that all members of the complex were one species (B. Sebayang, unpublished data). However, the sample size (n = 25) does not dismiss the possibility that more than one species was present (albeit the statistical likeness of this is small). Additionally, the sampling sites and individuals conducting the HLC were the same during all three rounds. These individuals were trained and supervised hourly. While the relative abundance of *An. leucosphyrus* complex mosquitoes captured by HLC decreased between rounds two and three, the relative abundance of other anophelines (both *An. maculatus* Group and *An. kochi*) increased for HLC. Since mosquito samples were collected hourly through the night, this ensured that collectors were performing as trained and were neither asleep nor wearing repellents.

The finding of *An. dirus* in this region of North Sumatra was unexpected, since this species was not previously reported here (89,99). However, a sibling species of *An. dirus* (*Anopheles cracens*) was reported in Sabang Island, Aceh (75), and more recently, *An. dirus* was collected in Central Kalimantan, indicating a wider geographical spread than previously known (199). Since *An. dirus* is a competent vector of malaria in many areas, further research across a wider geographical area is required to establish the true geographical boundaries of this species in Indonesia, and to estimate the potential risk of malaria transmission to humans in Indonesia.

Land-use type was associated with different mosquito species and abundance during the study period. Despite the small spatial scale of this study, *An. leucosphyrus* complex mosquitoes were predominantly collected in the mixed-crop agricultural area around Dusun 5. The presence of suitable larval habitats (B. F. Sebayang, personal communication) could partially explain the high abundance of *An. leucosphyrus* complex mosquitoes in the mixed-crop agricultural area. However, the distance between the village and the larval habitats was well within the flight range of *Anopheles* females, so it is likely that other, unknown factors are influencing *Anopheles* densities in this area of North Sumatra. Microclimatic data (e.g., temperature and rainfall) were not recorded for each sampling station, but these are unlikely to have affected mosquito numbers due to the proximity of sampling stations (50 – 100 meters between sampling stations within each Dusun). Some evidence for land-use type associations and *An. leucosphyrus* complex and *An. maculatus* Group distributions in the study area was found, but since these observations are based on a limited number of sampling nights, longer studies across multiple land-use types are needed to confirm if the land-use type effects observed here are valid across time and space.

Conclusion

Collection methods using humans or cows were the most sensitive for *Anopheles* species, including *An. leucosphyrus* complex, in North Sumatra. Mechanical traps baited with CO_2 were not very efficient, regardless of the CO_2 source or the trap type. The BG-Sentinel 2 trap baited with tank CO_2 showed some potential for sampling anophelines in Sumatra as it showed greater sensitivity for sampling *An. leucosphyrus* complex and *An. barbirostris* than the HLC in one of two direct Latin Square comparisons. Therefore, it could be useful in large-scale surveillance studies after additional studies to understand the variation in relative sensitivity reported here. Despite the higher absolute number of *An. leucosphyrus* complex in the ABT compared to the HLC in a direct comparison, HLCs produced high numbers and a high specificity for *An. leucosphyrus* complex mosquitoes in the other Latin square rounds. Therefore, HLC remains the best method to understand zoonotic malaria transmission to humans and to define high-risk areas of transmission in North Sumatra as it is relatively easy to establish at multiple concurrent sites and directly provides an epidemiologically relevant estimation of the biting risk to humans.

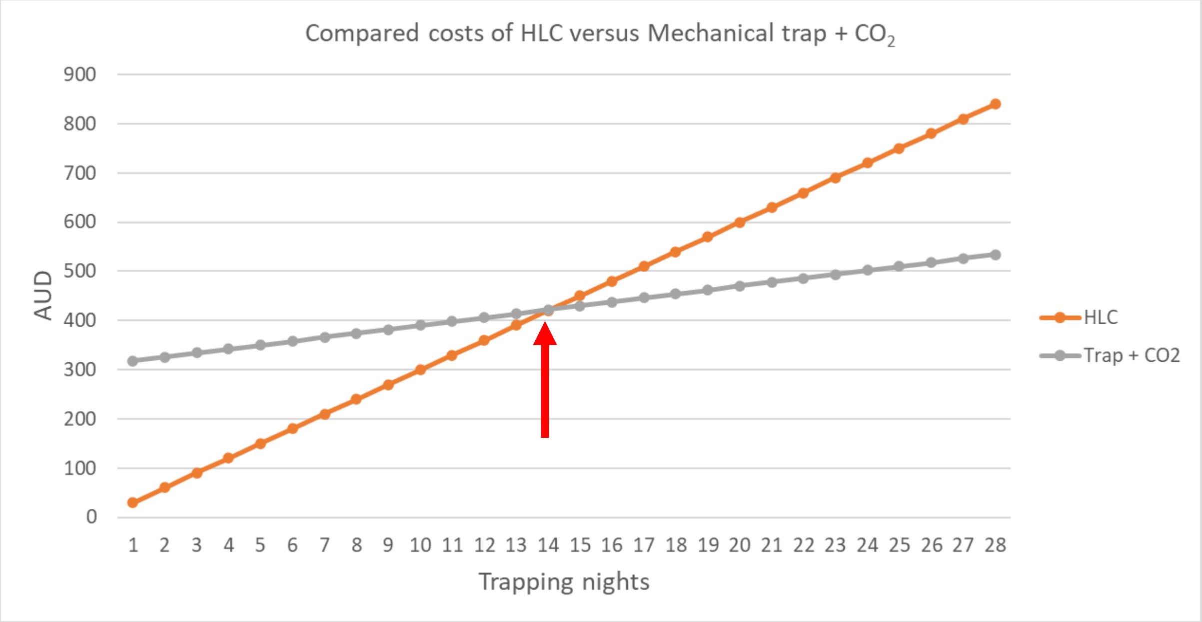
Supplementary Material 4.1

Cost of one BG-Sentinel 2 trap compared to one HLC team of two collectors.

One CO_2 tank can last reliably for 5 collection nights when set at a standard CO_2 release rate of 250 mL/min. The formula to calculate the cost of using a mechanical trap baited with CO_2 is

1) $y = 310 + 8x.$

Method	**Establishment costs**	**Nightly running costs**
BG-Sentinel 2 trap	AU$ 150	
CO_2 tank (incl. 1 gas fill)	IDR 2,000,000 (AU$ 200)	
Refill of CO_2 tank	IDR 400,000 (AU$ 40)	AU$ 8
HLC (per collector)		IDR 150,000 (AU$ 15)



Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Faculty of Medicine, Universitas Sumatera Utara (No. 723/KEP/USU/2021). Informed consent was given by collectors performing HLCs.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Competing interests

The authors declare that they have no competing interests.

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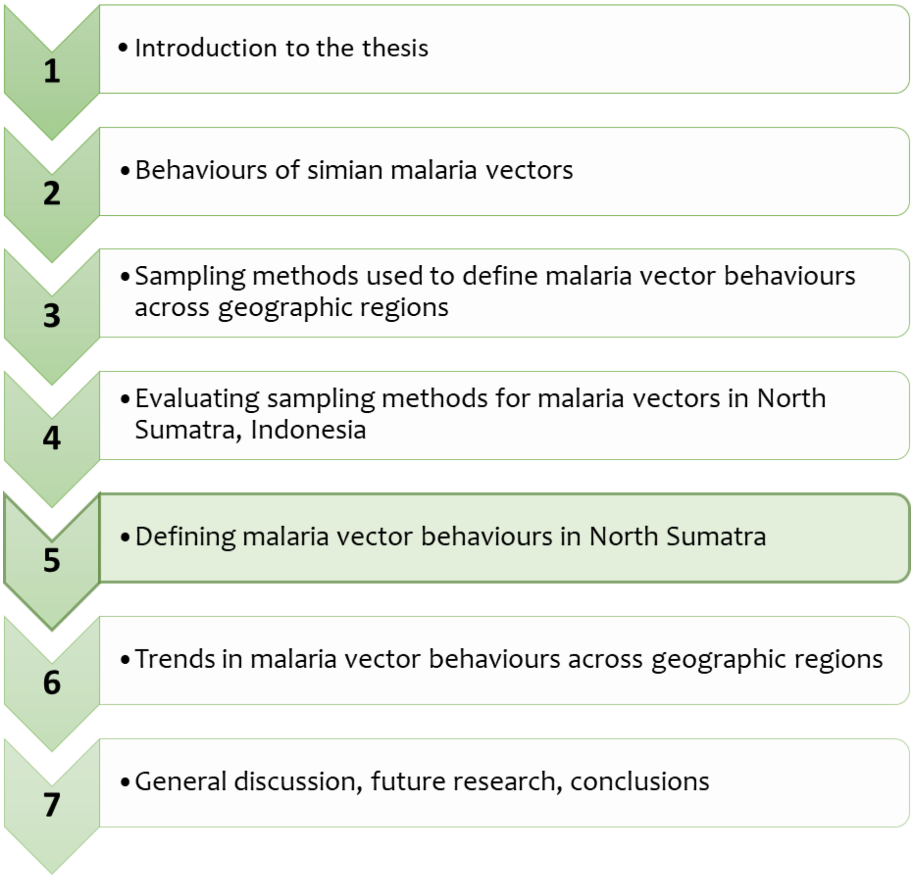
Authors' contributions

BvdS, TRB and TLR conceived the study; TRB, TLR, TAG and IL advised on the fieldwork and laboratory analyses. BvdS and AK conducted the fieldwork, and BvdS and BS performed the molecular analyses. BvdS analysed the data and wrote the initial draft of the manuscript. All authors read and approved the final manuscript.

Chapter 5

This chapter describes a year-long bionomics study of potential simian and human malaria vectors in North Sumatra. The previous chapter showed that mosquito distributions can differ strongly among land-use types. Therefore, the objectives of this chapter were to study the bionomics of host-seeking Anopheles *mosquitoes and to estimate the risk of malaria transmission to humans in different land-use types.*



Characterisation of *Anopheles* bionomics across different land-use types in North Sumatra, Indonesia

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Abstract

Background

Simian malaria cases in humans are increasing across Southeast Asia including major regions in Indonesia, but its transmission dynamics are still poorly understood. Incriminating the vector species and defining their behaviours is essential to understanding simian malaria transmission dynamics and to target vector behaviours vulnerable to interventions, to control human and simian malaria transmission to humans.

Methods

Adult mosquito collections were conducted from July 2022 to June 2023 in an oil palm plantation, a residential area, and a mixed-crop agriculture area, using all-night human landing collections in two sub villages (dusun) within Ujung Bandar village in Sumatra, Indonesia. A vegetation survey at each sampling station was conducted to study effects of vegetation density and composition on mosquito population composition.

Results

Biting rates of *Anopheles leucosphyrus* complex were highest in the mixed-crop agriculture area, located in Dusun 5 (mean bites/man-hour = 2.98 [95% CI +/- 1.23]), while *Anopheles maculatus* biting rates were highest in the oil palm plantation, located in Dusun 2 (mean bites/man-hour = 0.90 [95% CI +/- 0.43]). Additionally, *An. leucosphyrus* complex females showed higher biting rates in the village sites, located in Dusun 5, than *An. maculatus*. These opposing habitat preferences were reflected by the vegetation survey, which showed that *An. leucosphyrus* complex numbers were higher in the sampling stations with a high density of herbs. In contrast, *An. maculatus* numbers were higher in sampling stations with higher shrub densities.

Conclusions

Anopheles leucosphyrus complex and *An. maculatus* Group mosquitoes, potential vectors of human and simian malarias in North Sumatra, Indonesia, bite throughout the night in the study area, with peak biting rates before midnight and highest biting rates in the mixed-crop agricultural area and the oil palm plantation, respectively. The highest biting rates of *An. leucosphyrus* complex were in Dusun 5, which is surrounded by fragmented forest, suggesting that the risk to humans for potentially infectious bites is, at least in part, driven by high-level landscape structure in addition to habitat type.

Keywords

Anopheles leucosphyrus, Anopheles maculatus, zoonosis, simian malaria, vector

Introduction

Simian malaria cases of *Plasmodium knowlesi* in humans are increasing in Southeast Asia, while significant reductions in human malaria cases caused by *Plasmodium falciparum* and *Plasmodium vivax* across the region occur. Cases of simian malaria in humans have been reported in all countries in Southeast Asia except Timor-Leste (200). The most prevalent simian malaria species in humans is *Plasmodium knowlesi*, which can cause severe clinical disease and even death (9). However, the vectors transmitting simian malaria parasites to humans are poorly understood. To better understand transmission of simian malaria species to humans (59–61), surveillance of the vectors and defining their behaviours is crucial to selecting appropriate interventions for their control (148).

Plasmodium knowlesi infections in humans have been reported in association with changes in land-use from deforestation, including establishment of large-scale agricultural plantations including oil palm (134,135). Several studies in Malaysian Borneo have shown that *P. knowlesi* vectors have the highest vectorial capacity in forest fringes and prefer to bite humans in such areas (70,81). Agricultural development for large-scale oil palm plantations is hypothesised to divert mosquito biting from monkeys in the canopy to humans on the ground, thereby affecting the distribution and behaviour of the mosquito vectors (37,136). Consequentially, this increases the risk of infection to people primarily living or working in areas of large-scale agricultural development (111). Specifically, increased human activities in disturbed, fragmented forests and forest fringes are associated with increased risk of *P. knowlesi* infections in humans (111). Identifying the risk of *P. knowlesi* transmission to humans in different land-use types requires incrimination of the vector species in a specific region, as well as where and when contact between the vectors and humans occurs (160).

Nine species of the *Anopheles leucosphyrus* and *Anopheles dirus* species complexes are implicated as vectors of simian malarias to humans in Southeast Asia, and this large variation in vector species further complicates extrapolation of behaviours between species (55,72,92,98). *Anopheles leucosphyrus* complex vectors have been confirmed in Kalimantan and in Sumatra, Indonesia (71,201,202). Yet, little is known about the bionomics of these vectors in Indonesia, including vector densities, human biting rates and times, and seasonality (182). Additionally, how different land-use types and human behaviours might influence vector behaviours in North Sumatra is poorly understood (182). Better knowledge of the behaviours, distributions, and bionomics of simian and human malaria vectors can target vector control effectively. Therefore, this study aimed to define the bionomics and behaviours of *Anopheles* mosquitoes in different land-use types in North Sumatra, Indonesia.

Methods

Study sites

The study was conducted in Ujung Bandar village, subdistrict Salapian, Langkat Regency, North Sumatra province, Indonesia. The climate is tropical, with over 3000 mm rain/year and mean daytime and night temperatures ranging between 29-34°C and 19-24°C, respectively. The landscape surrounding Ujung Bandar village is hilly (350-600 meter above sea level), dominated by large-scale oil palm plantations interspersed with small-scale mixed agricultural areas and small patches of disturbed forest. Within Ujung Bandar village, 4 human infections with *Plasmodium knowlesi* were detected between March 2022 – March 2023, along with 6 *P. vivax*, 22 *P. malariae*, and 10 *Plasmodium* (only genus identified) infections (I. Lubis, personal communication, 2023). Ujung Bandar village consists of nine sub-villages ('dusun'), in two of which (Dusun 2 and 5) the study took place. Long-tailed macaques (*Macaca fascicularis*) are common, with pig-tailed macaques (*Macaca nemestrina*) also inhabiting the area. Domestic animals (mainly cows, chickens, and dogs) roam free in and around the dusun.

Study design

Outdoor mosquito collections were conducted monthly from July 2022 to June 2023 at six collection stations in Dusun 2 and six in Dusun 5. Dusun 2 consisted of scattered residences surrounded by large-scale oil palm plantations. All collection stations were in the oil palm plantation. Dusun 5 comprised two land-use types: a hamlet, and its adjacent subsistence farm area with mixed crops. Three collection stations were in each land-use type. The stations were fixed as to location and situated at least 50 meters apart. Mosquitoes were sampled with human landing collections (HLC) by teams of two collectors working concurrently from 18.30 (sunset) to 06.00 (sunrise), who aspirated all mosquitoes landing on their exposed lower legs into paper cups covered with mesh netting.

All collected mosquitoes were sedated with chloroform before identification by morphology at the field sites (188). Afterwards, samples were stored on cotton wool and silica gel beads in 1.5mL Eppendorf tubes to dry. The heads of all *Anopheles leucosphyrus* complex females were screened by qPCR for *Plasmodium* DNA (203). All *An. leucosphyrus* complex females, as well as a random subsample of other *Anopheles* species, were subjected to species confirmation (species complex confirmation for *An. leucosphyrus* complex mosquitoes) by PCR amplification of the ITS2 gene. Mosquito DNA was extracted from the thorax and legs using the DNEasy Blood & Tissue kit

(Qiagen, Germany) before amplifying the ITS2 gene (189). The cycling parameters were initial denaturation at 95°C for 120 sec, then 35 cycles of denaturation (60 sec, 95°C), annealing (30 sec, 51°C), and extension (60 sec, 72°C), followed by a final extension at 72°C for 10 min. The amplified product was run for 90 minutes on a 1.5% agarose gel at 90 V/400 mA and visualised with UV. A randomised subsample of *An. leucosphyrus* complex females were analysed by Sanger-sequencing to identify species (Macrogen, Indonesia).

Vegetation survey

Collection stations were characterised using a basic vegetation survey, conducted at the end of the experiment. All trees (single-stemmed woody plants), shrubs (multi-stemmed woody plants), herbs (non-woody plants), and grasses were counted along a 10 x 2-meter belt transect. The canopy cover was estimated by overlaying a 10 x 10 grid on photos of the canopy and counting the number of covered cells. The distance to the nearest plant ('Line of Sight') at a height of 10cm and 100cm was measured in four directions at five predetermined points along the transect to estimate the 'openness' of the landscape. Finally, the straight-line distance to the edge of the nearest forest patch was measured in QGIS (version 3.10, QGIS Association, www.qgis.org).

Statistical analyses

Nightly catch numbers of *An. leucosphyrus* complex and *An. maculatus* Group females were analysed in a Generalised Linear Mixed Model (GLMM) with Poisson distribution and log-link function. In this model, land-use type was an independent variable, while collection night and collection station were random variables to account for variability between nights and stations. Overdispersion was tackled by fitting a second, negative binomial GLMM with quasipoisson distribution with the same explanatory variables to the data. Hereafter, the model that best fitted the data was determined by an ANOVA. To study the effect of vegetation species composition and density on the mosquito communities in the three land-use types, the effects of the total number of trees, shrubs, herbs, ferns, and grasses, as well as the median Line of Sight at 10cm, distance to the nearest forest patch, and canopy cover, were evaluated in a multivariate GLM. Subsequently, the results were visualised using non-metric multidimensional distance scaling (nMDS). All data analyses were performed in R (R version 4.1.1; R Foundation for Statistical Computing, Vienna, Austria) with the most recent version of the 'tidyverse', 'lme4', 'mvabund', and 'vegan' packages (204).

Results

Mosquito occurrence

Six hundred twenty-three *An. leucosphyrus* Group mosquitoes were collected, together with 290 *Anopheles maculatus*, 38 *Anopheles kochi*, 6 *Anopheles vagus*, and 1 *Anopheles barbirostris*. Additionally, 731 *Culex* spp, 625 *Armigeres* spp, 372 *Aedes albopictus*, 23 other *Aedes* including 3 *Aedes aegypti*, and 3 *Mansonia annulifera* were collected. Of the *An. leucosphyrus* complex, 77 were identified as *Anopheles dirus* by sequencing the ITS2 gene. Because of the limited number of *An. leucosphyrus* complex whose identities were confirmed by sequencing, these mosquitoes will be referred to as *An. leucosphyrus* complex. There were large differences in the *Anopheles* species composition between the two areas. In Dusun 2, 81 *An. leucosphyrus* Group were collected, while collection numbers were more than 6.5 times higher in Dusun 5, totalling 542. In contrast, *An. maculatus* and *An. kochi* were much more abundant in Dusun 2 than in Dusun 5, with total collection numbers being 8 (*An. maculatus*) to 18 times (*An. kochi*) higher in the former. An additional spot survey in Dusun 5 with two HLC collectors at one indoor collection station for four nights showed that indoor biting of *An. leucosphyrus* complex was limited, with five specimens collected indoors against 33 outdoors in the village and 82 in the mixed-crop agricultural area for 4 nights. *Plasmodium*-positive mosquitoes were not collected during the study period (n_{TOTAL} = 266; 219 *An. leucosphyrus* complex and 42 *An. maculatus* tested).

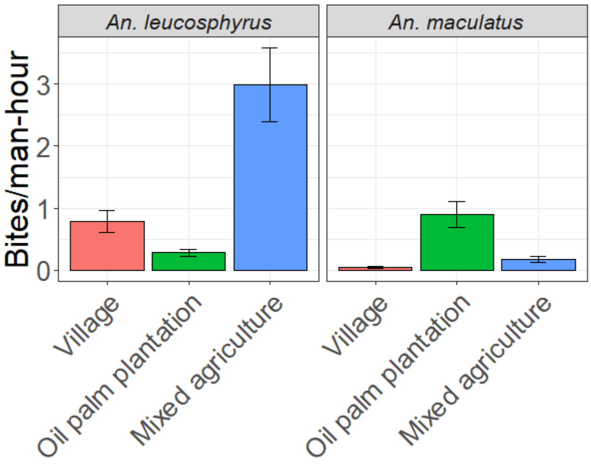

Figure 5.1 - Mean nightly collection numbers of An. leucosphyrus *complex (left) and* An. maculatus *Group (right) in three different land-use types. Note that the oil palm plantation sites were in Dusun 2, while the village and mixed agriculture sites were in Dusun 5. Error bars in both panels represent standard errors of the mean.*

Effects of land-use type

In addition to the significant difference in *An. leucosphyrus* complex numbers between Dusun 2 and 5 (GLMM, Est. = 1.9045, SE = 0.3038, $p < 0.0001$), there were also large differences in numbers of collected anophelines among collection stations within Dusun 5. The three collection stations in the village yielded a mean of 0.78 (95% CI +/- 0.36) *An. leucosphyrus* complex and 0.05 (95% CI +/-

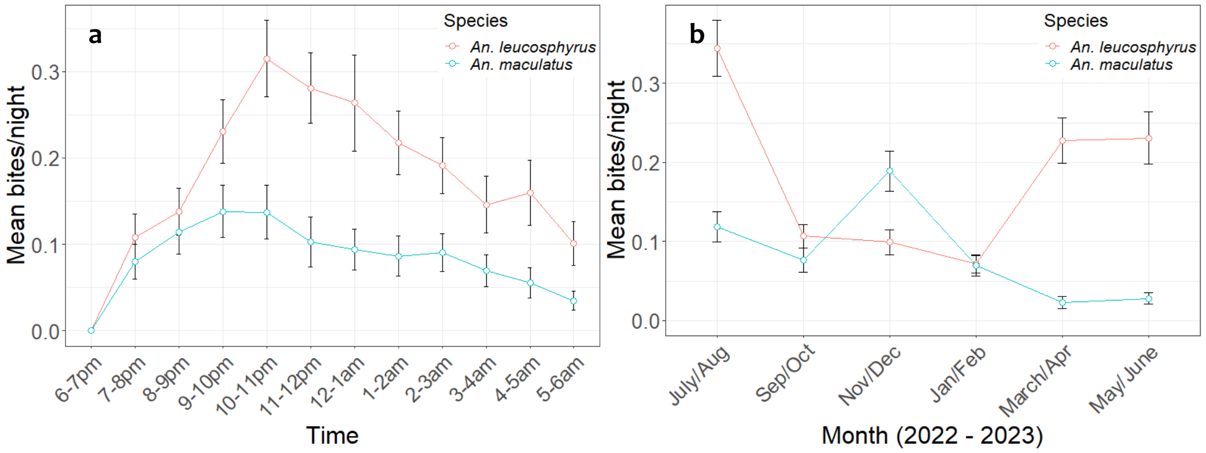

Figure 5.2 - (a) Mean biting rates of An. leucosphyrus *complex (red) and* An. maculatus *Group (blue) per night. (b) Seasonality of* An. leucosphyrus *complex and* An. maculatus *Group. Error bars represent standard errors of the mean.*

0.03) *An. maculatus* Group mosquitoes per man-hour of collection, while the three collection stations in the mixed agriculture area yielded a mean of 2.98 (95% CI +/- 1.23) *An. leucosphyrus* complex and 0.17 (95% CI +/- 0.10) *An. maculatus* Group mosquitoes per man-hour. The stations in Dusun 2, located within an oil palm plantation, yielded a mean of 0.28 (95% CI +/- 0.11) bites/man-hour for *An. leucosphyrus* complex females and 0.90 (95% CI +/- 0.43) bites/man-hour for *An. maculatus* Group females. The differences in mean number of *An. leucosphyrus* complex mosquitoes among the three land-use types were significant, with the mixed agricultural area in Dusun 5 having a positive effect (GLMM, Incidence Risk Ratio [IRR] = 4.3, $p < 0.001$) and the oil palm plantation in Dusun 2 having a negative effect on collection numbers (GLMM, IRR = 0.34, $p = 0.058$) compared to the village (Fig. 5.1). The difference in *An. maculatus* Group numbers between the village and the mixed agricultural area was not significant, but the oil palm plantation had a strong, significant effect on collection numbers (GLMM, IRR = 15.8, $p = 0.0005$) (Fig. 5.1).

Biting times

Anopheles leucosphyrus complex and *An. maculatus* Group mosquitoes were collected by HLC throughout the night (Fig. 5.2a). For the *An. leucosphyrus* complex, the mean biting rate peaked between 22.00 – 23.00, with 61% of *An. leucosphyrus* complex females collected between 21.00 – 02.00. Peak biting time of *An. maculatus* Group was less pronounced, with the highest biting rates between 20.00 and 00.00 and lower biting rates sustained throughout the night.

Biting rates of *An. leucosphyrus* complex mosquitoes varied throughout the study period, with lower rates in September – February (Fig. 5.2b). *Anopheles maculatus* Group mosquitoes displayed a different biting pattern, with peak biting occurring in November and December. Similar collection patterns were observed in all three land-use types for both *An. leucosphyrus* complex and *An. maculatus* Group mosquitoes. Insufficient numbers of other *Anopheles* species were collected to permit statistical analysis.

Vegetation survey

The results of the vegetation survey at each station, together with the total number of *An. leucosphyrus* complex and *An. maculatus* Group mosquitoes collected, are summarised in Table 5.1. The landscape around all collection stations was open, with a median line of sight at 100 cm height of >40 meter in all directions. At 10 cm from the ground, the line of sight was more variable, but was not significantly associated with either *An. leucosphyrus* complex or *An. maculatus* Group numbers (GLMM, n.s.). Multivariate analysis showed that the assemblage of the mosquito community was significantly different between land-use types (manyglm, Likelihood Ratio Test = 880.6, p = 0.001), and the vegetation composition directly surrounding the sampling sites had a significant effect on the assemblage of the mosquito community (manyglm, Likelihood Ratio Test = 514.7, p = 0.001). However, the interaction between these variables was not significant. Follow-up univariate tests per mosquito species showed a significant effect of land-use type and vegetation composition on *An. leucosphyrus* complex, *An. maculatus* Group, and *An. kochi* densities. In addition, the density of non-target species (*Ae. albopictus*, *Culex* spp, *Armigeres* spp.) was also significantly affected by the vegetation composition around the sampling sites. Notwithstanding, visualising the differences between mosquito communities in the three land-use

Table 5.1 - Summarised results of the vegetation survey, with the total number of plants by plant type per belt transect and the total number of An. leucosphyrus *complex and* An. maculatus *Group caught per mosquito collection station. Station = mosquito collection station; L. o. S. = median Line of Sight from measurements in four directions.*

	Station	Trees	Shrubs	Herbs	L. o. S. (10 cm)	*An. leucosphyrus* complex	*An. maculatus* Group
Dusun 2	1	0	0	84	>40 m	4	54
	2	11	42	209	0.256 m	40	4
	3	1	2	25	0.475 m	7	19
	4	3	0	252	>40 m	9	51
	5	4	14	40	>40 m	13	29
	6	1	73	35	>40 m	8	101
Dusun 5	1	1	0	353	>40 m	38	4
	2	3	15	126	>40 m	10	2
	3	4	0	152	>40 m	65	1
	4	0	0	707	0.266 m	224	4
	5	0	0	531	0.168 m	122	18
	6	5	0	960	0.356 m	83	3

types by nMDS did not show significant separation, regardless of collection station or time of night of collection (Fig. 5.3).

Discussion

This study identifies host-seeking and biting behaviours of *An. leucosphyrus* complex and *An. maculatus* Group mosquitoes in North Sumatra, Indonesia. In 2020, Wibowo et al. (201) reported the collection of a *P. knowlesi* positive *An. balabacensis* specimen in Lampung Regency, Sumatra, but mosquito collections were limited to two nights. This research presents the first detailed behavioural data of *An. leucosphyrus* complex mosquitoes throughout an entire year in two Dusun of Ujung Bandar village in North Sumatra.

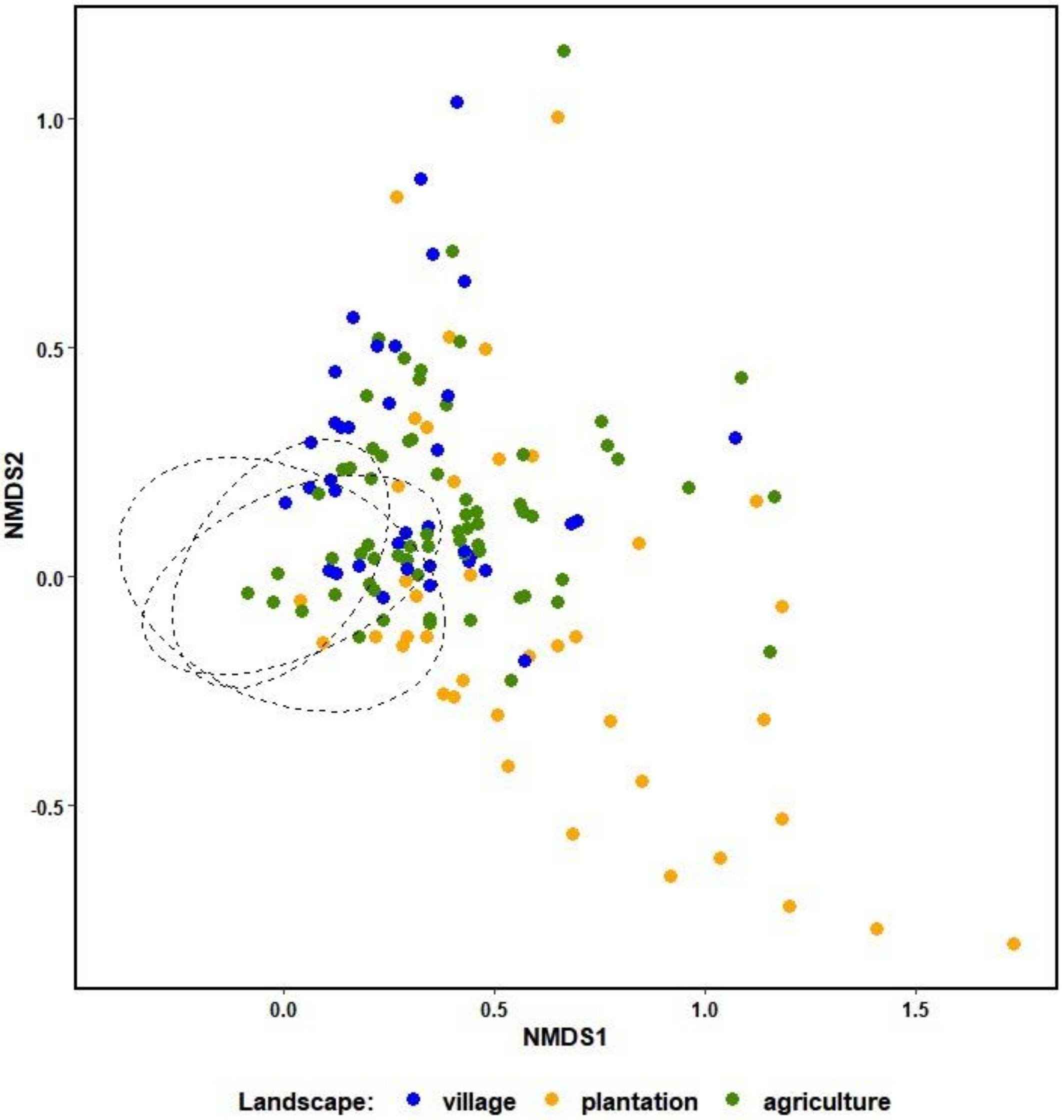

Figure 5.3 - nMDS plot of mosquito community composition comparing different anthropogenic land-use types. Despite a significant effect of land-use type on mosquito communities, there is substantial overlap of mosquito communities between land-use types.

During the study, the highest *An. leucosphyrus* complex biting rates were consistently found in the mixed agriculture area, but *An. leucosphyrus* complex mosquitoes were also found to bite throughout the night in the village, albeit in much lower numbers. A similar pattern was also observed in Sabah, Malaysia, where biting rates of *Anopheles balabacensis* (a member of the *An. leucosphyrus* complex) were much higher in the forest than in the area surrounding the study villages (39). However, people did not visit the forest at night but stayed in their village, and consequently the areas of the highest biting risk were in and around the study villages in Sabah, Malaysia (136). Another study in Sabah showed that *An. balabacensis* was biting humans around their houses in the early evening, thereby confirming that the peridomestic area can be a focus of simian malaria transmission to humans (80). If residents in Ujung Bandar have similar behaviours, most bites by *An. leucosphyrus* complex mosquitoes on humans would also occur near their homes. As peak biting was experienced between 21.00-01.00h, when people were often still outside socialising (personal observations), WHO-recommended mosquito control methods like insecticide-treated bed nets or indoor residual spraying, which are deployed indoors and mainly target indoor-biting mosquitoes, are likely less effective in preventing *An. leucosphyrus* complex bites.

There were large differences in *An. leucosphyrus* complex numbers between Dusun 2 and Dusun 5, with Dusun 5 having much higher numbers. The reasons for the different *Anopheles* abundances between the Dusun is uncertain. It may be that the landscape of Dusun 5 provides more potential larval habitats for *An. leucosphyrus* complex mosquitoes (B. Sebayang, unpublished data), more potential blood hosts like monkeys, or both. The vegetation survey shows that the number of herbaceous plants surrounding the collection stations was much higher in Dusun 5 than in Dusun 2, while the numbers of trees and shrubs were lower. Adult *An. leucosphyrus* complex females might use these herbaceous plants for shelter, although the flight range of most *Anopheles* is much larger than the small area covered by the vegetation survey and females could rest much further away from the collection stations at which they were captured. Aerial images of the two Dusun and their surrounding landscape (taken September 2022, unpublished data) show a varied landscape around Dusun 5, with relatively small patches of oil palm plantation, small mixed-crop agricultural areas, and many forested areas. Hence, the landscape around Dusun 5 provides more complex forest habitat (38,71) in which *An. leucosphyrus* complex mosquitoes would be found prior to deforestation and agricultural development with likely many resting places and larval habitats, as well as many potential human and primate blood hosts in the vicinity. The landscape around Dusun 2, while only three kilometres away from Dusun 5, is fundamentally different, consisting mainly of oil palm plantations, interspersed with small patches of remnant secondary forest. This near-monoculture landscape likely provides fewer larval habitats and resting spots for *An. leucosphyrus* complex mosquitoes, resulting in much lower numbers in the study area around Dusun 2. It is important to note that the habitat effects cannot be separated from the effects of Dusun, as each habitat type is not represented in each dusun. Notwithstanding, the landscape types of the two dusun in which the study was conducted were different from each other. Therefore, the dusun-level effects likely represent the broader effects of the landscape on *An. leucosphyrus* complex mosquitoes. The high-level landscape effects observed in the study area (i.e., higher total abundance of *An. leucosphyrus* complex in Dusun 5) are consistent with effects seen in studies in Malaysian Borneo, where *An. leucosphyrus* complex mosquitoes were biting more on humans in forest fringes (136,137). In Palawan in the Philippines, sampling across three habitat types also showed that *An. balabacensis* densities were highest in the forest edge, compared to agricultural or forest sites (205). This suggests that the habitat preferences and, consequentially, biting densities of *An. leucosphyrus* complex mosquitoes are at least partially driven by higher-level landscape structure (i.e., a highly varied, fragmented landscape) instead of the presence of individual habitat types.

Anopheles maculatus Group mosquitoes in this study showed contrasting distributions to *An. leucosphyrus* complex mosquitoes, with the highest biting rates for *An. maculatus* Group mosquitoes in the oil palm plantation. While this land-use type provided few larval habitats for *An. leucosphyrus* complex, many larval habitats of *An. maculatus* Group were found here (B. Sebayang, unpublished data). As land-use in many parts of Indonesia is transforming, with forests being supplanted with large-scale oil palm plantations, this shift could become associated with increases in *An. maculatus* Group mosquitoes which are considered important malaria vectors in several regions in Western Indonesia (206).

There was a large heterogeneity in mosquito numbers including *An. leucosphyrus* complex between the collection stations within each Dusun. Differences between stations were especially large in Dusun 5, where total *An. leucosphyrus* complex collections ranged between 10-65 for the stations in the village area and 83-224 for the stations in the mixed-crop agriculture area. Nevertheless, the distance to the nearest forest edge did not have a significant effect on *An. leucosphyrus* complex collections in both dusun. Even though microclimatic data (e.g., temperature and rainfall) were not recorded for each sampling station, these are unlikely to have affected mosquito numbers due to the proximity of sampling stations (50 – 100 meters between sampling stations within each Dusun). It is possible that the observed heterogeneity is an effect of sampling effort. The distribution of collection stations was limited to being relatively close to the villages due to collector concerns about potential hazards further away from the village.

Additionally, the non-independence between Dusun and habitat type might limit interpretation of the habitat effects. Currently, due to the nature of the landscape surrounding the two Dusun and their respective study sites, specific habitat type effects cannot be analysed independently from higher-level effects from the two Dusun. This complicates extrapolation of the results from this study to other areas with comparable habitat types in North Sumatra. Therefore, when possible, future mosquito surveys should aim to collect mosquitoes across areas that are comparable, at least on a higher landscape level, and in more remote sites, like forests, to better study occurrence and behaviours of potential simian malaria vectors.

Conclusion

Anopheles leucosphyrus complex and *An. maculatus* Group mosquitoes bite throughout the night in the three common land-use types within the study area. The highest biting rates for *An. leucosphyrus* complex were in the mixed-crop agricultural area, while the large-scale oil palm plantation had the highest biting rates for *An. maculatus* Group. Biting densities of *An. leucosphyrus* complex mosquitoes in specific habitat types in the study area are likely partially driven by the landscape structure surrounding these habitats.

Declarations

Ethics approval and consent to participate

This study was approved by the Universitas Sumatera Utara, Fakultas Kedokteran (application number 723/KEP/USU/2021) on 14 July 2021.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

BvdS, TRB and TLR conceived the study; TRB, TLR, and IL advised on the fieldwork and laboratory analyses. BvdS and AK conducted the fieldwork, and BvdS and BS performed the molecular analyses. BvdS analysed the data and wrote the initial draft of the manuscript. All authors read and approved the final manuscript.

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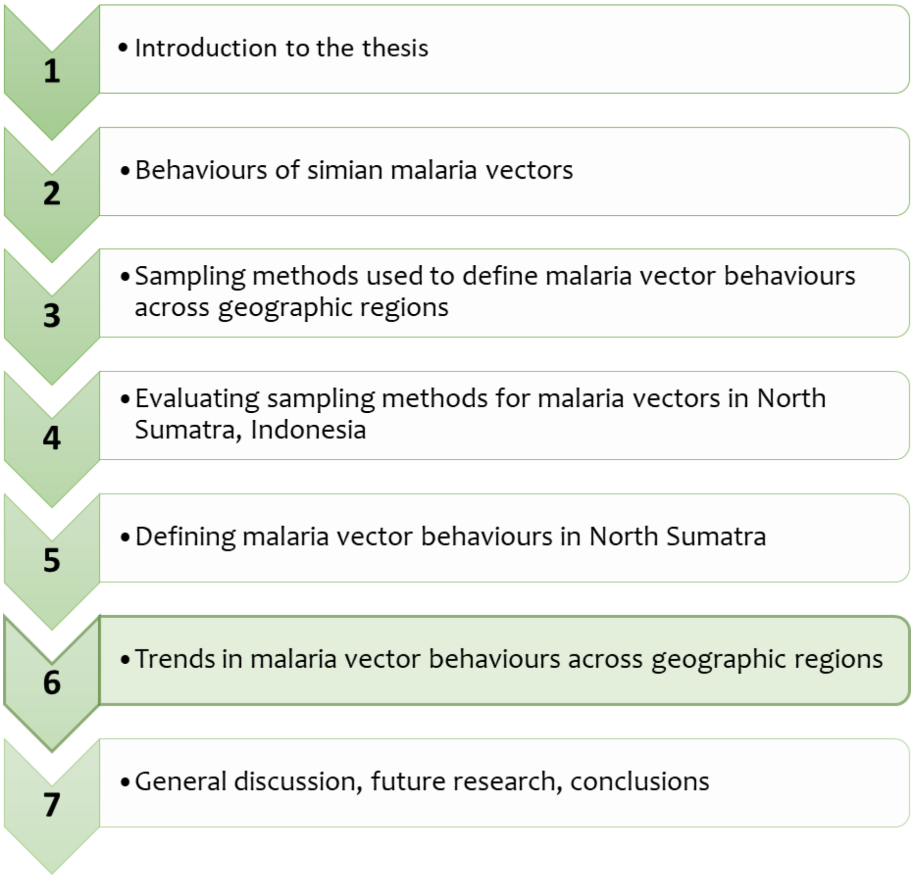
Chapter 6

Chapter 5 described bionomics of An. leucosphyrus *complex and* An. maculatus *Group mosquitoes in relation to different anthropogenic land-use types. In this chapter, historical global data were analysed for trends in dominant vector behaviours. This chapter has been submitted for publication to The Lancet Planetary Health.*



A meta-analysis for behavioural shifts of the dominant malaria vectors in Africa before the scale-up in insecticide-based vector control

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Abstract

Background

The decline in malaria prevalence in Africa from 2000 to 2015 was attributed primarily to the upscaling of access and use of insecticide-treated nets (ITNs) and indoor residual spraying (IRS). However, malaria cases had been declining for over 100 years, despite limited programmatic vector control during most of this time. The observed declines in malaria morbidity and mortality, which occurred prior to the widespread implementation of ITNs and IRS, were investigated for any associations with shifts in the behaviours of vector mosquitoes.

Methods

To investigate whether changes in vector behaviours might explain the reductions in malaria morbidity and mortality prior to the scale up in ITNs and IRS in the 2000s, a meta-analysis was conducted of global mosquito behaviours spanning 25 years (1985-2010). A comprehensive dataset of over 2,500 datapoints describing the behaviour and bionomics of the dominant malaria vectors in Africa, the Americas, and the Asia-Pacific regions was extracted from all publications from 1985-2013 included in the Malaria Atlas Project repository.

Findings

From 1985 to 2010 the proportion of blood meals on humans and the proportion of bites indoors by *Anopheles gambiae* s.s. and *Anopheles funestus* s.s. diminished, along with the proportion of bites indoors by *Anopheles arabiensis,* thus lowering the vectorial capacity of these three dominant malaria vectors in Africa.

Interpretation

Such behaviour shifts are characteristics of both behavioural resistance and plasticity in anophelines to insecticide-based interventions such as ITNs and IRS in which vectors avoid contact with the insecticides in ITNs and IRS inside houses. However, the behavioural changes across Africa reported here started before the onset of the scale-up in ITNs and IRS in the early 2000s, suggesting that other widespread drivers present during this time, such as house improvements and untreated bed nets, may have exerted pressure to prevent vector house entry and feeding on humans in a manner analogous to ITNs and IRS.

Funding

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Introduction

From 2000 until 2015, malaria mortality and morbidity in humans declined significantly in Africa, with 80% of the reduction in *Plasmodium falciparum* malaria cases attributed to the upscaling of insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS) (207,208). These interventions disrupt transmission by reducing contact rates between humans and mosquito vectors and by increasing vector mortality. However, the continued efficacy of IRS and ITNS is at risk due to the development of widespread physiological resistance to the insecticides in these tools (https://apps.who.int/malaria/maps/threats/#/) in which vectors exposed to the insecticides are not killed (209).

Because ITNs and IRS are deployed indoors, they disproportionally affect the more indoor biting (endophagic) and indoor resting (endophilic) vector species, such as *Anopheles gambiae* s.s. and *Anopheles funestus* s.s. in Africa. Thus, in response to these interventions applied indoors, the density of insecticide-susceptible endophilic and endophagic human-biting malaria vector populations can diminish more than exophagic and exophilic malaria vectors (168,210). For example, the more exophilic *Anopheles arabiensis* is now the dominant vector in some areas where prior to to the widespread use of ITNs and IRS the more endophilic *An. gambiae* and *An. funestus* were the dominant vectors (210).

The effectiveness of ITNs and IRS faces a second serious challenge: vector behavioural changes to avoid exposure to insecticides. Behavioural changes (either resistance or plasticity) to ITNs and IRS can develop over time being expressed as reductions in the human feeding (the human blood index (HBI)) and/or house entry (with lowered proportion of indoor biting) of mosquito populations, thereby minimising their exposure to insecticides inside houses. Changes in behaviours were first described as a consequence of exposure to DDT used in IRS by *Anopheles pseudopunctipennis* in Mexico (211). Similarly, the DDT-based IRS campaign in the Solomon Islands resulted in a rapid change from equal indoor and outdoor all-night biting to mainly outdoors and earlier biting in the dominant malaria vector, *Anopheles farauti* (25). Similar changes in behaviours in malaria vector species are now widespread in Africa following exposure to ITNs and IRS including in Benin (*An. funestus* s.s.), Kenya (*An. arabiensis*, *An. funestus* s.s.), Tanzania (*An. gambiae* s.s., *An arabiensis*, *An. funestus* s.s.) and Equatorial Guinea (*Anopheles coluzzii*, *Anopheles melas*) (26).

Spatiotemporal analyses of behaviour changes in vectors have been limited to recent studies on the consequences of exposure to IRS and ITNs in individual sites or meta-analyses in limited geographical areas (26). The establishment of the Global Fund in 2002 (www.theglobalfund.org) and the President's Malaria Initiative in 2005 (www.pmi.gov) saw significant increases in access and use of ITNs and IRS beginning in 2004. Here, a comprehensive dataset of behavioural data on malaria vectors spanning 25 years (1985-2010) was analysed to characterise behaviours and bionomic characteristics in the dominant malaria vectors in Africa, the Americas, and the Asia-Pacific regions (108). This research investigated whether the observed declines in malaria morbidity and mortality, prior to the widespread implementation of ITNs and IRS, was associated with any behaviour shifts of vectors. The capacity of a vector species to transmit malaria is affected by its intrinsic susceptibility to become infected, the density of biting mosquitoes on humans (the human biting rate (HBR)), its survivorship through the extrinsic incubation period, and the behaviours and bionomic characteristics that determine its propensity to blood feed on humans. This manuscript provides a descriptive analysis of a comprehensive global dataset of the behaviours and bionomics of the dominant malaria vectors in Africa, the Americas, and the Asia-Pacific regions. The dataset was extracted from all publications compiled by the Malaria Atlas Project, published from 1985-2013.

Methods

The analyses were carried out with bionomics data published between 1985 and 2013 and compiled by the Malaria Atlas Project on the dominant malaria vector species (see original publication for details of the dataset structure and original references) (108). The dataset contained 10,922 data records from 875 publications, spanning the three major malarial regions (Africa, the Asia-Pacific, and the Americas). The data on *Anopheles* species included the (georeferenced) study location, mosquito species or species complex, study period, indoor and outdoor human biting rate (HBR), indoor and outdoor human blood index (HBI), and biting location (indoor and outdoor). The data from the 875 publications in the dataset is not uniform: for example, HBR data was recorded as hourly, nightly, weekly, or occasionally monthly or per year in different publications. Consequently, all data in the dataset reflected the data as presented in the original source. Where possible all data reported in the source for a specific location time and species were combined on a single data line. When datasets had a temporal component, the data from each time period were recorded across multiple data lines. All data manipulations and analyses given here were performed in RStudio with R statistical software (v. 4.1.1, R Foundation of Statistical Computing, Vienna, Austria) using packages ‘tidyverse’, ‘stats’, ‘psych’, and ‘lme4’.

To analyse behaviours and bionomics, data subsets were created to include only records for each variable of interest (HBR, HBI, and proportion indoor biting). The methods used to collect malaria vectors in Africa were consistent from 1985 to 2010 (212). For the parameter of human biting rate

(HBR), only human landing collections (HLCs) conducted either indoors or outdoors were used. The proportion biting indoors was calculated only for sites where both indoor and outdoor human landing catches were performed concurrently. For determination of the HBI, indoor and outdoor collections for resting blood fed mosquitoes were conducted by pyrethroid spray catches indoors and manual searches outdoors. Further descriptions and analyses on the collection methods for dominant malaria vectors in the dataset are described in the Supplementary Information and elsewhere (108,212). Data were summarised by starting year of the study and species. Because the bionomics data were not consistently reported by taxonomic level, analyses of behaviours and bionomics were first conducted by species complex, after which a follow-up analysis to the species level was conducted. Species with the most robust data were further analysed and aggregated, where applicable, by species complexes which stratified the data by geographic regions.

Initially the data was analysed to clarify which species and or complexes had the highest HBI and HBR. Simple GLMs analysed differences between species complexes and between species for each bionomic indicator studied: HBI (for both indoor and outdoor-resting mosquitoes), proportion bites indoors (where concurrent data on indoor and outdoor biting was recorded), and HBR. The distributions of the models assessing HBI and proportion of bites indoors were binomial, while the distributions of the HBR models were gaussian with a log link function.

The overall models that analysed the temporal behaviours and bionomics for different vector species or species complexes were GLMMs separately constructed for each dependent factor: HBI, HBR and proportion of bites indoors. For the HBI and HBR models, independent factors were ‘starting year of the study’ and ‘biting location (indoor or outdoor)’ and the interaction between starting year and sampling location. For the proportion of bites indoors, the independent factor was ‘starting year of the study’. A random variable for sampling country was included in each model to account for natural geographic density variation. Separate models were run for each species and species complex.

Table 6.1 - The distribution of data records for all Anopheles species across the Africa, Asia-Pacific, and Americas WHO regions

Region	HBI	Biting location	HBR
Africa	*781 (74·4%)*	*340 (47·8%)*	*1527 (56·7%)*
An. gambiae *s.l.*	544	219	987
An. funestus *s.l.*	237	121	540
Americas	*84 (8·0%)*	*183 (25·7%)*	*97 (3·6%)*
Asia-Pacific	*185 (17·6%)*	*188 (26·5%)*	*1068 (39·7%)*
Total (100%)	1050	711	2692

To probe the dataset for changes in bionomic indicators prior to the scale-up of vector control, the data was truncated to the period 1985-2004, before the scale-up of mass insecticide-based vector control (ITNs and IRS) (213). Separate regression analyses were performed for each bionomic indicator, after which the slopes of the regressions over the truncated data were compared to the corresponding regression slopes over the full data (1985 to 2010).

Results

Individual bionomic indicators contained 2748 data records for HBR, 1060 data records for HBI, and 740 data records for proportional biting with, depending on the bionomic indicator, 50-75% of the data records from Africa (see table 6.1). Therefore, further analyses focussed on African malaria vector species. Most data records from Africa (n = 2648, >95%) concerned the two most important (primary) malaria vector complexes in this region: *Anopheles gambiae* s.l. (with *An. gambiae* s.s., *An. arabiensis*, *Anopheles quadriannulatus*, *An. melas*, *Anopheles merus*, *Anopheles amharicus*, *Anopheles bwanbae*, and *An. coluzzii*) and *An. funestus* s.l. (with *An. funestus* s.s., *Anopheles parensis*, *Anopheles rivulorum*, and *Anopheles leesoni*) (214,215). *Anopheles coluzzii* (a member of the *An. gambiae* complex) was only recognised as a separate species in 2013 (214). Hence, the analyses for *An. gambiae* s.s. reported here likely includes both *An. gambiae* s.s. and *An. coluzzii*.

The geographic origin of data did not change significantly in the reported period. Both Western Africa and Eastern/Southern Africa subregions showed a similar pattern in the number of

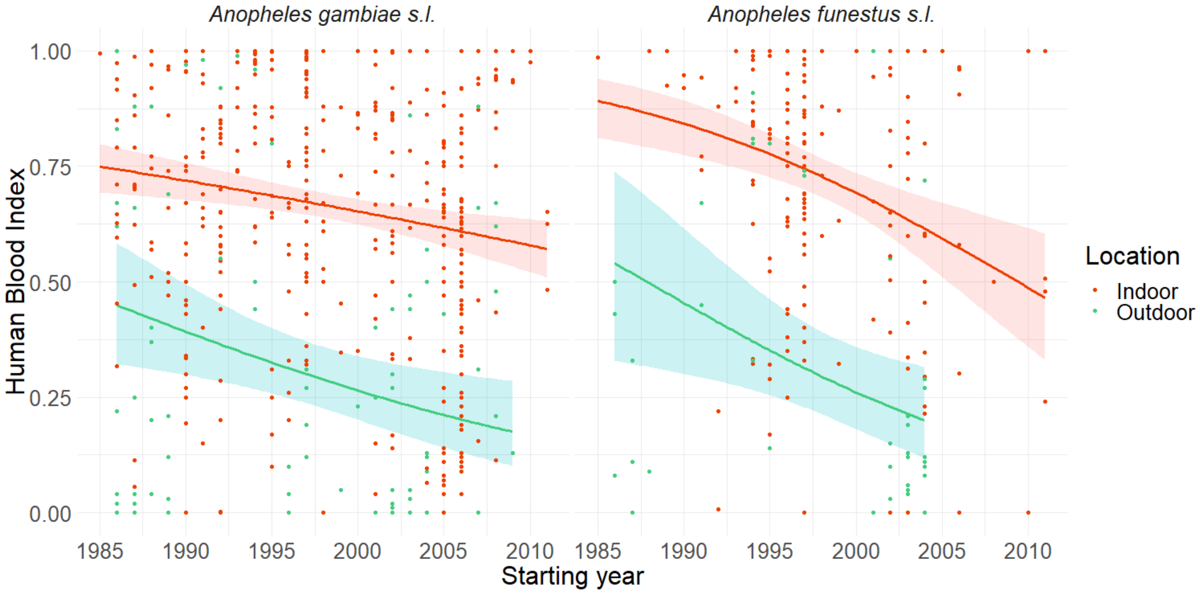


Figure 6.1 - Human Blood Index (HBI) for An. gambiae s.l. (left) and An. funestus s.l. (right) in Africa. In red, the HBI from indoor collections is displayed, while HBI from outdoor collections is shown in green. To show general trends in the data, GLM curves were fit (mean predicted values; solid lines). Shaded areas represent the 95% confidence interval of the fitted models.

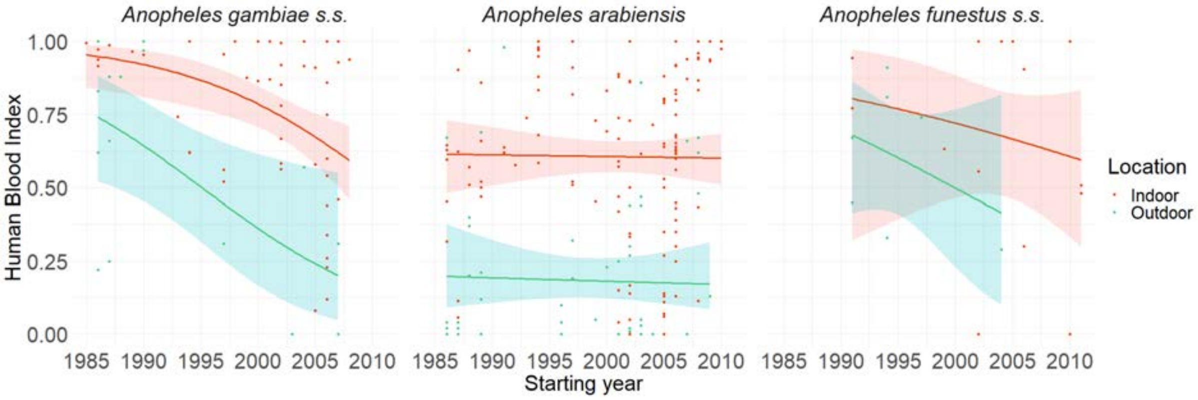


Figure 6.2 - Human Blood Index (HBI) for An. gambiae s.s. (left), An. arabiensis (middle), and An. funestus s.s. (right) in Africa. In red, the HBI from indoor collections is displayed, while HBI from outdoor collections is shown in green. To show general trends in the data, GLM curves were fit (mean predicted values; solid lines). Shaded areas represent the 95% confidence interval of the fitted models. These data form a subset from the species complex data, so the number of data points can be considerably smaller.

publications per year, with a clear peak in the 1990s, a dip around the year 2000, and a second peak from 2008-2011 (see Additional Fig. 6.3). The methods to collect malaria vectors in Africa were consistent from 1985 to 2010 (Supplementary Information and (212)). Human landing collections (HLCs) indoors, outdoors or conducted simultaneously were the dominant collection methods to study the HBR and the indoor biting proportion. Indoor and outdoor resting collections most often were used to determine the HBI. Further descriptions and analyses on the collection methods for dominant malaria vectors in the dataset are described elsewhere (212).

Human blood index of African vectors

The HBI is the proportion of human blood-fed mosquitoes collected in indoor and outdoor resting collections. There were 781 data points on the HBI for *An. gambiae* s.l. and *An. funestus* s.l., with *An. funestus* s.l. having significantly higher HBIs than *An. gambiae* s.l., indicating that *An. funestus* s.l. complex generally blood fed more on humans (GLM, OR = 1.18, 95% CI [1.15, 1.21], $p < 0·0001$). For both species complexes, there was a significant influence of the sampling year on the indoor HBI, decreasing by 17.8% (*An. gambiae* s.l.) and 42.6% (*An. funestus* s.l.), and outdoor HBI, decreasing by 27.3% (*An. gambiae* s.l.) and 34.0% (*An. funestus* s.l.) between 1985-2010 (Table 6.2; Fig. 6.1). Additionally, statistically significant differences in the HBI values between indoor and outdoor collections were found (Table 6.2), with outdoor collections consistently having lower HBI values for both species complexes.

The species with the most HBI data were *An. arabiensis* (n = 192), *An. gambiae* s.s. (n = 68), and *An. funestus* s.s. (n = 22). Differences in HBIs between species were significant, with *An. arabiensis* having lower indoor and outdoor HBIs than both *An. gambiae* s.s. and *An. funestus* s.s. (GLM, OR =

Table 6.2 – *Model outcomes for the human blood index (HBI).*

HBI - vector complex data			
	Estimate	**SE**	**p-value**
***An. gambiae* s.l.**			
Year	-0·074	0·0013	<0·0001***
Location (outdoors)	-80·440	0·764	<0·0001***
Year : Location (outdoors)	0·039	0·0004	<0·0001***
***An. funestus* s.l.**			
Year	-0·194	0·0046	<0·0001***
Location (outdoors)	-103·20	1·124	<0·0001***
Year : Location (outdoors)	0·051	0·0005	<0·0001***
HBI - vector species data			
	Estimate	**SE**	**p-value**
***An. gambiae* s.s.**			
Year	-0·067	0·006	<0·0001***
Location (outdoors)	-166·3	2·156	<0·0001***
Year : Location (outdoors)	0·082	0·0011	<0·0001***
An. arabiensis			
Year	-0·0007	0·0026	0·802
Location (outdoors)	-3·499	0·9302	<0·001***
Year : Location (outdoors)	0·001	0·0005	0·018*
***An. funestus* s.s.**			
Year	-0·355	0·0097	<0·0001***
Location (outdoors)	35·151	2·2526	<0·0001***
Year : Location (outdoors)	0·018	0·0011	<0·0001***

0.22, 95% CI [0.20, 0.24], p < 0·0001; Fig. 6.2). The overall indoor and outdoor HBIs for *An. gambiae* s.s. and *An. funestus* s.s. decreased significantly from 1985 to 2010, while indoor and outdoor HBI values for *An. arabiensis* remained constant (Table 6.2). For each of the three species, the outdoor HBIs were significantly lower than indoor HBIs, showing a lower anthropophagy by mosquitoes resting outdoors (Table 6.2).

Proportion of indoor biting African vectors

Although the overall proportion of indoor biting for both the *An. gambiae* s.l. and *An. funestus* s.l. complexes was higher than outdoor biting; with *An. funestus* s.l. (n = 219) having a higher proportion of indoor biting compared to *An. gambiae* s.l. (n = 121) (GLM, OR = 1.22, 95% CI [1.19, 1.24], p < 0·0001; Fig. 3). For *An. funestus* s.l., the proportion of indoor biting decreased significantly from 1985-2010, as did the proportion of indoor biting for *An. gambiae* s.l. (Table 6.3; Fig. 6.3).

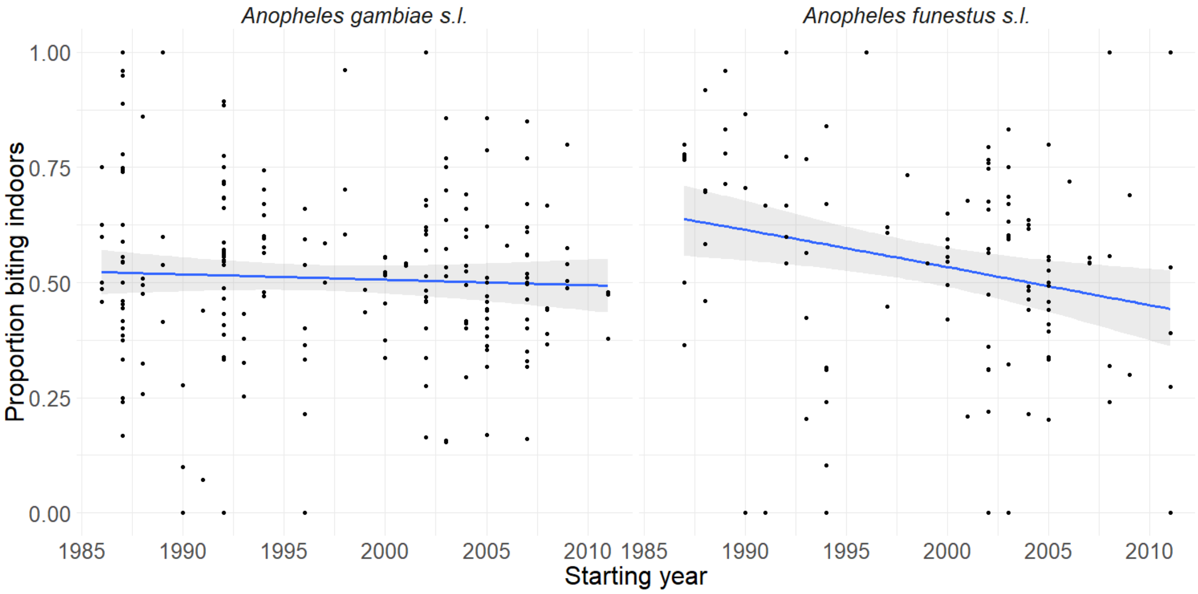


Figure 6.3 - The proportion of indoor biting for An. gambiae s.l. (left) and An. funestus s.l. (right) in Africa. To show the changing proportion of indoor biting, GLM curves were fit (mean predicted values; solid lines). Shaded areas represent the 95% confidence interval of the fitted models.

Paired indoor and outdoor biting data for *An. gambiae* s.s. (n = 55), *An. arabiensis* (n = 60), and *An. funestus* s.s. (n = 46) showed higher proportions of indoor biting in the three species (Table 6.3). However, all species showed significant decreases in indoor biting proportions over time, with *An. funestus* s.s. having the largest shift in its proportion indoor biting, going from 59.6% in 1985 to 40.8% in 2010 (Table 6.3, Fig. 6.4). For *An. gambiae* s.s., the mean indoor-biting proportion was 58·6%, significantly higher than *An. arabiensis* with an indoor biting proportion of 46·7% (GLM, OR = 0.90, 95% CI [0.87, 0.92], p < 0·0001).

Human biting rate of African vectors

Nightly human biting rates (HBR) of the principal African malaria vectors ranged from 0 – 500 for *An. gambiae* s.l. and from 0 – 120 for *An. funestus* s.l. (Fig. 6.5). Overall, the combined indoor and outdoor HBR for *An. funestus* s.l. was lower than for *An. gambiae* s.l. (GLM, Est. = -6·197, p-value = 0·0003). Nightly HBR values for both *An. gambiae* s.l. and *An. funestus* s.l. did not change significantly over the years, with no differences between indoor and outdoor HBR (Table 6.4).

Significant differences in the HBR (indoor or outdoor) were not found between *An. gambiae* s.s. (n = 61), *An. arabiensis* (GLM, n = 374, p-value = 0·263), and *An. funestus* s.s. (GLM, n = 29, p-value = 0·645) (Fig. 6.6). Despite a numerical decrease in HBR, the variation in overall HBR values for *An. arabiensis* was too large to show a statistically significant decrease. For *An. gambiae* s.s. and *An.*

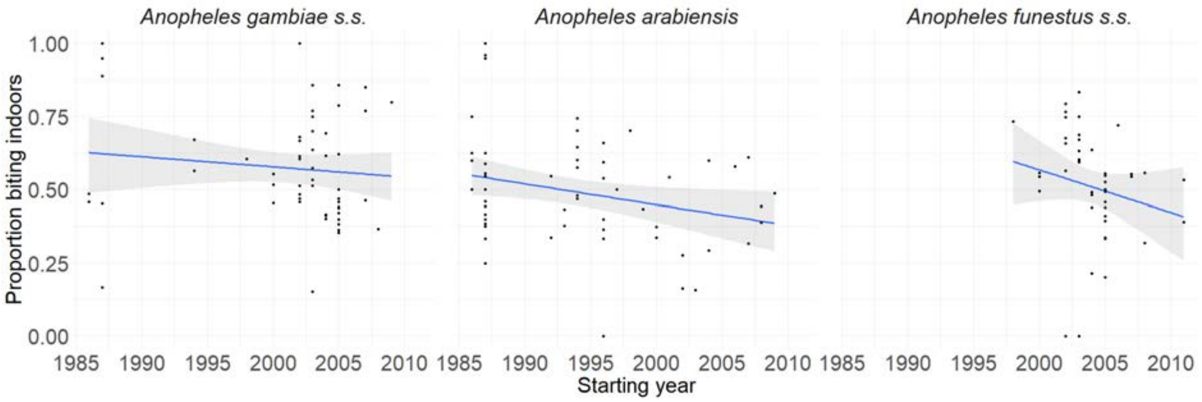


Figure 6.4 - The proportion of indoor biting for An. gambiae s.s. (left), An. arabiensis (middle), and An. funestus s.s. (right) in Africa. As these data form a subset from the species complex data, the number of data points are considerably smaller. Solid lines show mean predicted values, shaded areas represent the 95% confidence interval of the fitted models.

funestus s.s., the number of datapoints was small and the variation was large, which impeded trend analysis for both indoor and outdoor HBRs.

Effect of vector control on vector bionomics

Analysis of the 1985-2004 data showed a significant change in mosquito bionomics similar to the bionomic changes observed when analyses included the years with vector control (2004-2010) for two of three bionomic indicators. The HBI of both *An. gambiae* s.l. and *An. funestus* s.l. decreased significantly during 1985-2004. Similarly, the proportion indoor biting decreased from 1985-2004 for *An. gambiae* s.l. and *An. funestus* s.l.. However, there was no significant change in HBR in the period between 1985-2004. When the regression slopes of the fitted models for the 1985-2004 data subset and the full dataset were compared, the slopes were very similar, with significant overlap

Table 6.3 - Model outcomes for the proportion indoor biting.

Proportion indoor biting - vector complex data			
	Estimate	**SE**	**p-value**
***An. gambiae* s.l.**			
Year	-0·032	0·0013	<0·0001***
***An. funestus* s.l.**			
Year	-0·088	0·0025	<0·0001***
Proportion indoor biting - vector species data			
	Estimate	**SE**	**p-value**
***An. gambiae* s.s.**			
Year	-0·045	0·0063	<0·0001***
An. arabiensis			
Year	-0·048	0·0033	<0·0001***
***An. funestus* s.s.**			
Year	-0·139	0·0059	<0·0001***

between the standard error margins of the regression slopes for HBI, proportion indoor biting and HBR (Additional Fig. 6.2a, 6.2b and 6.2c).

Discussion

From 1985 to 2010 three dominant African species exhibited consistent changes in two key behaviours, which adversely impacted their capacity to transmit malaria. *Anopheles gambiae* s.s., *An. arabiensis* and *An. funestus* s.s. showed significant declines over time in the proportion of indoor biting. The HBI also decreased significantly for the two anthropophagic species, *An. gambiae* s.s. and *An. funestus* s.s., but did not change for the more zoophagic *An. arabiensis*. These behavioural changes (e.g., reductions in indoor biting and HBI) define both behavioural resistance and behavioural plasticity in response to the selective pressure exerted by widescale access and use of insecticide-based vector control with ITNs and IRS. However, the reductions in indoor biting

Table 6.4 - Model outcomes for the human biting rate (HBR).

HBR - vector complex data			
	Estimate	**SE**	**p-value**
***An. gambiae* s.l.**			
Year	0·113	0·2733	0·679
Location (outdoors)	-435·285	819·1919	0·595
Year : Location (outdoors)	0·2171	0·4092	0·596
***An. funestus* s.l.**			
Year	0·0007	0·1537	0·996
Location (outdoors)	170·3	556·4	0·760
Year : Location (outdoors)	-0·084	0·2778	0·761
HBR - vector species data			
	Estimate	**SE**	**p-value**
***An. gambiae* s.s.**			
Year	0·638	0·1865	0·0012**
Location (outdoors)	-5040·32	1949·62	0·0125*
Year : Location (outdoors)	2·515	0·9725	0·0124*
An. arabiensis			
Year	-1·275	0·7296	0·08
Location (outdoors)	-1853·5	2275·471	0·416
Year : Location (outdoors)	0·925	1·1361	0·416
***An. funestus* s.s.**			
Year	-0·415	0·2561	0·117
Location (outdoors)	2455·981	2720·034	0·375
Year : Location (outdoors)	-1·225	1·3556	0·375

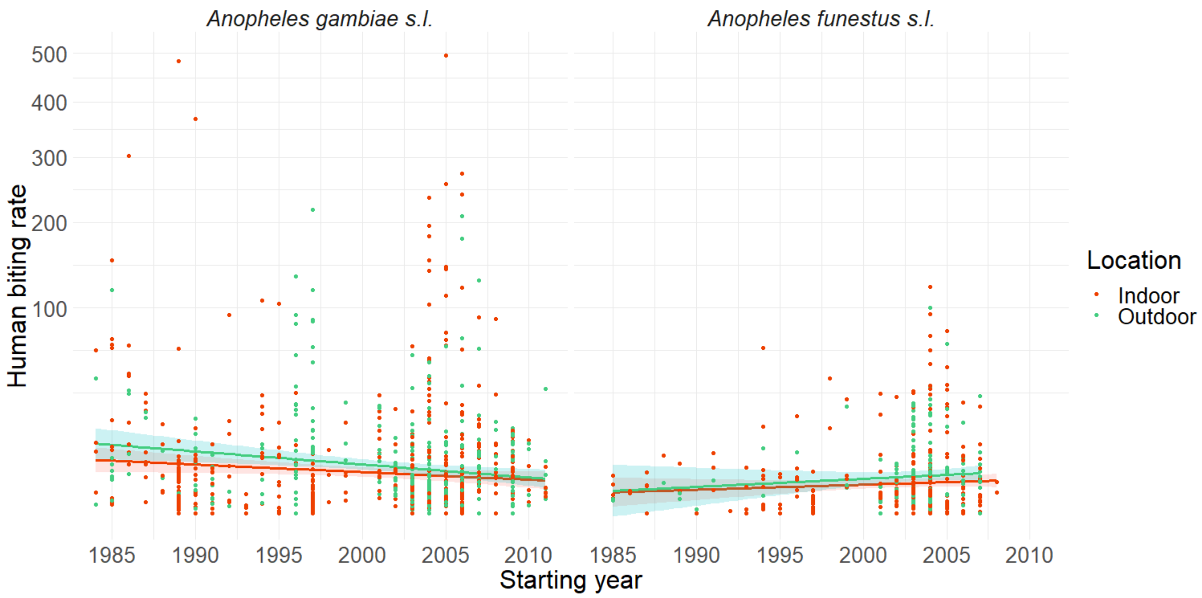


Figure 6.5 – Human biting rate for An. gambiae s.l. (left) and An. funestus s.l. (right). Solid lines represent mean predicted values and shaded areas represent the 95% confidence interval of the fitted models.

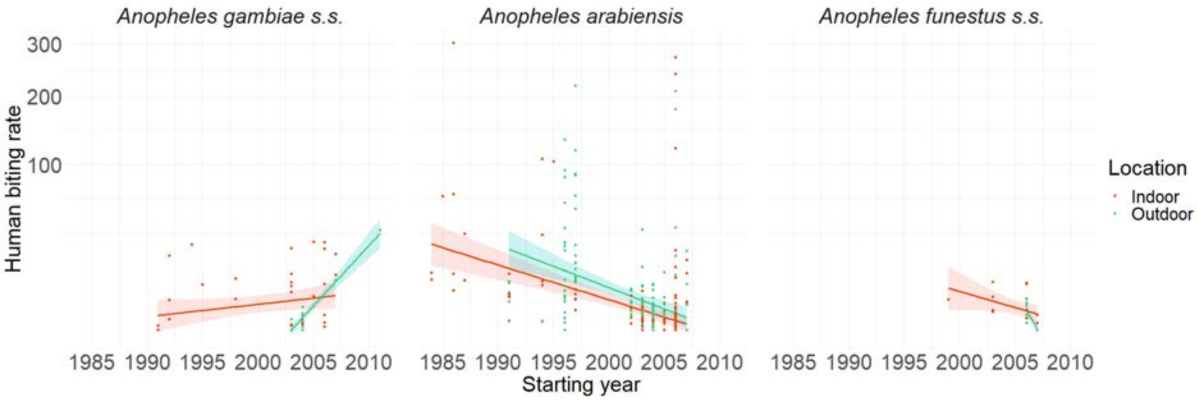


Figure 6.6 - Human biting rate for An. gambiae s.s. (left), An. arabiensis (middle), and An. funestus s.s. (right). Solid lines represent mean predicted values and shaded areas represent the 95% confidence interval of the fitted models.

and HBI documented here from 1985 started at least 19 years before significant mass deployment of ITNs and IRS began which was made possible, in large part, with the support of the Global Fund to Fight AIDs, TB, and Malaria (www.theglobalfund.org) and the President's Malaria Initiative (www.pmi.gov) (Fig. 6.7). By 2004, only 7 million of 737 million people at risk of malaria in Africa (< 1%) were living in houses protected by IRS, and only ~5% of people at malaria risk in Africa had access to ITNs (213,216).

The behavioural changes observed in this study suggest that one or more large scale drivers across Africa were exerting selection pressure on vector behaviours prior to but in a similar manner to the vector control programs that target mosquitoes indoors, where 80-100% of malaria transmission occurs in sub-Saharan Africa (217). The drivers selecting for reductions in human blood feeding and house entering from 1985 to 2004 are uncertain. However, as these are the same behaviours that describe behavioural changes in response to exposure to ITNs and IRS indoors, such drivers likely

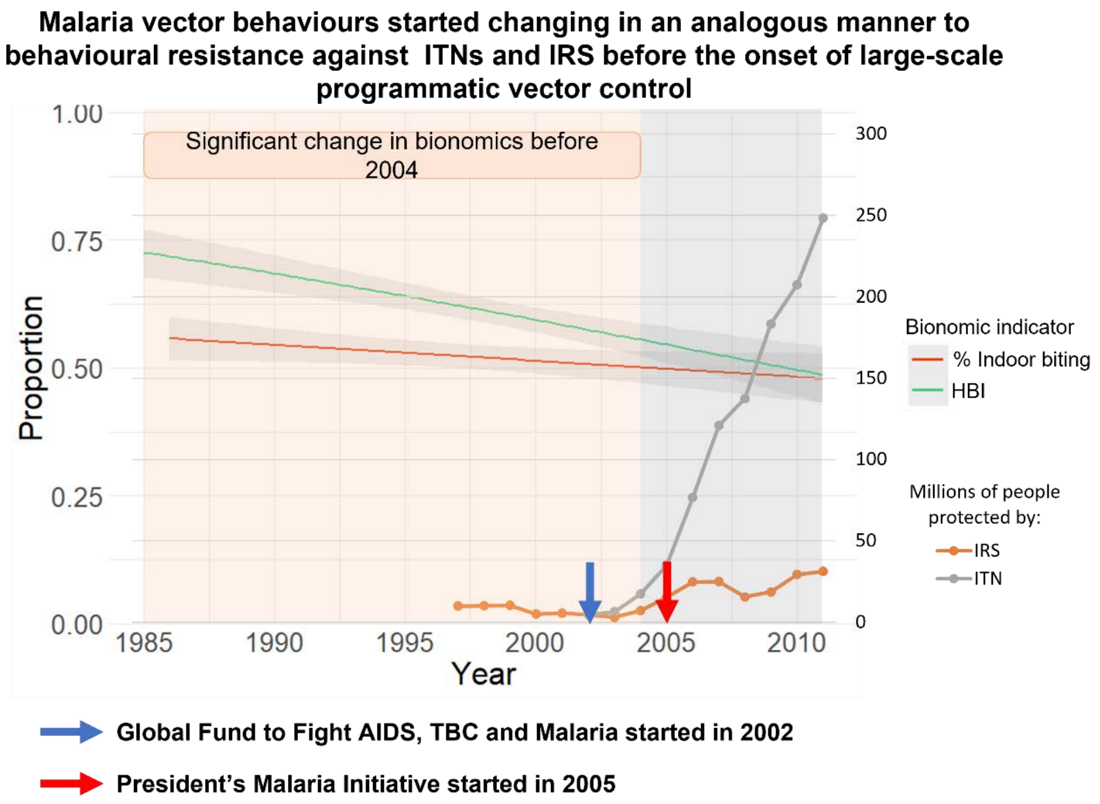


Figure 6.7 – The changes in behaviour in dominant malaria vectors in relation to the upscaling of programmatic vector control in Africa. The period in which programmatic vector control was absent is marked in orange.

acted on mosquitoes attempting to enter houses and feed on humans inside houses. These potential drivers might include improved housing and the use of non-insecticidal (untreated) bed nets.

Improved housing, defined here as houses made of durable materials such as concrete/brick walls and metal roofing, significantly prevent house entry by vectors and in so doing lower the proportion of blood meals on humans (218–220). Improving traditional mud-walled, thatched houses with more modern houses with cement floors, and metal roofs, with ceilings and closed eaves, restricts access of endophagic malaria vectors into houses (219,221). This would decrease the proportion of mosquitoes entering houses and thereby diminish access to humans as blood meal sources, lowering the HBIs, as found in this study, indicating protection of inhabitants from malaria vectors indoors.

Data on house improvements across sub-Sahara Africa before 2000 are limited, but some UNICEF Multiple Indicator Country Surveys from 1995-1996 recorded housing conditions (https://mics.unicef.org/surveys). Houses across sub-Sahara Africa improved with construction with durable materials along with increasingly prevalent access to water and sanitary facilities. In 1996, 65% of houses in Sierra Leone had concrete floors and 85% had zinc roofs, while in Angola 45% of the people were living in improved houses (e.g., with walls not made of mud or roofs not made of thatch). Housing improvements were less prevalent in the Democratic Republic of the Congo, where 22% of houses had cement floors and 32% had metal roofs (data from 1996).

A finished house was defined as having at least two of the following structures, roof, walls, or floors built with durable materials like cement or metal. In 2000, the prevalence of people living in finished houses was 25·6% in rural areas and 80·3% in urban areas across sub-Sahara Africa. By 2015, the prevalence of finished houses had increased to 44·7% and 91·5% in rural and urban areas, respectively (222).

Houses with improved walls and roofs affect mosquito numbers inside houses. In Tanzania, densities of both *An. gambiae* s.l. and *An. funestus* s.l. inside houses with brick walls or metal roofs were less compared to houses with mud walls or thatched roofs (223). In The Gambia, mosquito counts were lower inside houses with cement walls compared to houses constructed of mud walls (224). Another study in the Gambia showed reduced mosquito survival which would reduce malaria transmission in metal-roofed houses, which were hotter than thatched roof houses (225).

Untreated bed nets can significantly reduce the HBI of both indoor and outdoor resting mosquitoes and reduce the number of blood fed mosquitoes resting inside houses, as well as significantly reducing sporozoite rates and prevalence of malaria (13,226–228). Despite the absence of programmatic distributions of untreated bed nets, significant increases in untreated bed net ownership since the 1980s were reported (229,230). In 2003, 15% of children under five used a bed net in Africa, with more than 40% of children under five using untreated bed nets in Guinea-Bissau, Sao Tome & Principe, and The Gambia (230).

The analysis here provides a potential explanation for the lowering malaria incidence observed prior to the scale-up in programmatic insecticide-based malaria vector control: diminishing human blood feeding and house entry by malaria vectors would diminish vectorial capacity and might result in lower transmission (231). These behavioural changes may have been a response to the increasing use of untreated bed nets in the 1990s, combined with improvements in housing across Africa.

It is possible that other drivers may also have selected for reductions in the HBI of vectors and in the proportion of bites indoors. While climate change acts on vector populations through rainfall and temperature, thereby potentially altering the abundance and productivity of larval habitats (232), climate change has not been documented to impact the proportion of indoor biting or human blood feeding. It is possible that increased temperatures could diminish house entering. However, this might act through house improvements with iron roofing creating a less favourable indoor environment for mosquitoes. Additionally, if the numbers of large domestic animals increased relative to humans, this might decrease human blood feeding. From 1985-2004, overall large animal numbers in Africa increased by 55.3% from 567 million to 881 million (https://www.fao.org/ faostat/en/#data/QCL). During the same period, the human population increased by 62% (https://www.worldometers.info/world-population/africa-population/). Therefore, the relative abundance of large domestic animals to humans in Africa did not change and thus is not a likely explanation for the decrease in the HBI during the period of this study.

Bionomics data have been recorded by many researchers, often using varying methodologies, and as such, the data was extracted using a defined standardised process to create a comprehensive database of all data published from 1985 to 2013. While sampling effort was variable across sampling locations, the bionomic indicators were assessed using a limited number of sampling methods (indoor PSC and outdoor aspirations to collect resting mosquitoes for HBI determination, and HLC indoors and outdoors to estimate human biting rates and proportion of bites indoors). As in all cross-study meta-analyses, there is a risk of bias in compiling a large dataset. The size of the dataset and the use of standardised collection methods to study and report the bionomic indicators analysed minimised the impact of bias across the time frame and geographic scope of the data.

According to WHO guidelines, understanding the behaviours and bionomics of vector populations is critical to effective vector control as behaviours change over time and in response to vector control, and the findings here reinforce that vector behaviours are not static, even in the absence of insecticide-based vector control. Hence, continual vector surveillance to monitor vector behaviours is essential to ensure that any vector control deployed is targeting vulnerabilities in the vectors' behaviours to maximise effectiveness (233). It must be stressed that the results here, although they precede the deployment of ITNs, do not argue against the use of LLINs or IRS. These tools are effective against malaria even in areas where the vectors mostly bite and rest outdoors as a mosquito only needs to enter a house late at night during one feeding cycle over the course of the extrinsic incubation period to be exposed to the insecticides in ITNs or IRS to be killed (14).

Conclusion

Analyses of long-term data on a continental scale provides important insights on malaria vector behavioural trends not obvious from more spatially or temporally limited data. Shifts in dominant malaria vector behaviours were observed across Africa. These shifts were potentially driven by factors acting as unintentional de facto vector control, including possibly improved housing and the use of untreated bed nets. Untreated nets are substandard care today and thus not testable as a hypothesis. Improved housing is an active area of research, and the evidence supports the hypothesis that better housing reduces mosquito entry and in so doing lowers malaria transmission indoors by limiting access to humans as blood meal sources. Subsequent widespread vector control with ITNs and IRS potentially accelerated these changes in behaviours in concert with other, at present, unknown drivers. Mosquito behaviours are dynamic, and the data shows that behaviours changes in ways we do not always expect or understand. WHO guidelines emphasise the use of local data to stratify interventions to be the most effective and our findings support this strategy (233,234).

Supplementary Information

Compiling the dataset

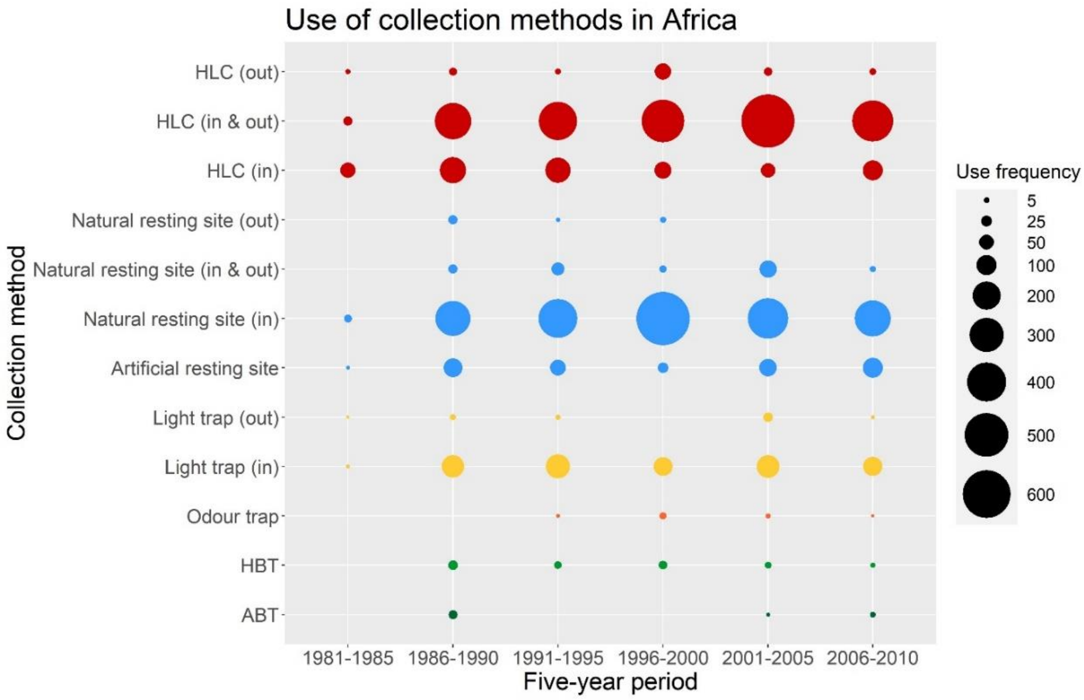
All analyses in this study were based on the bionomics data extracted and collated from literature published between 1981 and 2015 on the global DVS published by Massey et al. (2016) (108) which was based on the Malaria Atlas Project bibliographic database. The MAP bibliographic database was created via extensive searching using "Anopheles" as the keyword. Data was extracted from all references that met the following criteria for inclusion: (i) the reported study was published after December 1984; (ii) the surveys provided location information to a precision of administrative unit level one or higher; (iii) the surveys reported primary data; (iv) the surveys provided species-level information at the studied location; (v) the surveys reported the presence of at least one dominant vector species; and (vi) the surveys reported bionomics data. Each article was searched for relevant bionomics data including indoor and outdoor human biting rate (HBR), indoor and outdoor human blood index (HBI), and biting location (indoor and outdoor), which were extracted as reported in the source document, with no assumptions made.

The database contained 5678 data records from 450 publications from Africa, 1346 data records from 134 publications from the Americas and 3898 data records from 291 publications from the Asia-Pacific region.

Individual data records (rows in the database) showed unique site-collection period-species combinations. Collection sites were recorded with geographical latitude and longitude, without differentiating between the area size. While month and year defined the start as well as the end of an experiment, only the study starting year was used to categorise the study year. Accurate estimates of the number of collection nights for each individual study (the sampling effort) could not be extracted because only start and end month were recorded and the exact number of surveys, traps and workhours were not recorded in the database. The data was extracted with averaged monthly granularity, whenever possible: longitudinal datasets were extracted as multiple datapoints, with one row for each month. Although at times, data was presented as an average across the entire longitudinal study and resultingly the extracted data was presented as such. Data was standardised against the units of the bionomic parameter, for example HBR is always presented as bites per person per night.

Description of the collection methods

Descriptions of the mosquito collection methods used in Africa between 1985-2010 were obtained from a previous publication (Van de Straat et al. 2021) (212). All geo-referenced data records were compiled, summarised by continent, and stratified by year and collection method. The two sampling methods used most often were analysed for potential geospatial use patterns by density-based clustering following the OPTICS algorithm, described in detail by Hahsler et al. (2019) (180). OPTICS starts with a random data point and provides the order in which new points are explored and added to a cluster. Follow-up ξ-extraction is



Supplementary Figure 6.1 - Number of data records per collection method. The number of data records for each collection method used in Africa, presented per five-year time period. ABT = animal-baited trap, HLC = human landing catch, HBT = human-baited double net trap. Figure originally published in (10) and reproduced with permission of the original authors.

required to detect clusters of variable density and to provide the cluster hierarchy. This means that the OPTICS method can detect clusters within clusters. Afterwards, Moran's I for spatial autocorrelation tested whether the detected clustering of the analysed sampling methods was significant, or the locations where a sampling method was deployed were spread randomly across a continent/region.

Global analysis of the data showed that indoor and outdoor human landing catches (HLCs) were by far the most frequently used method to collect anophelines.

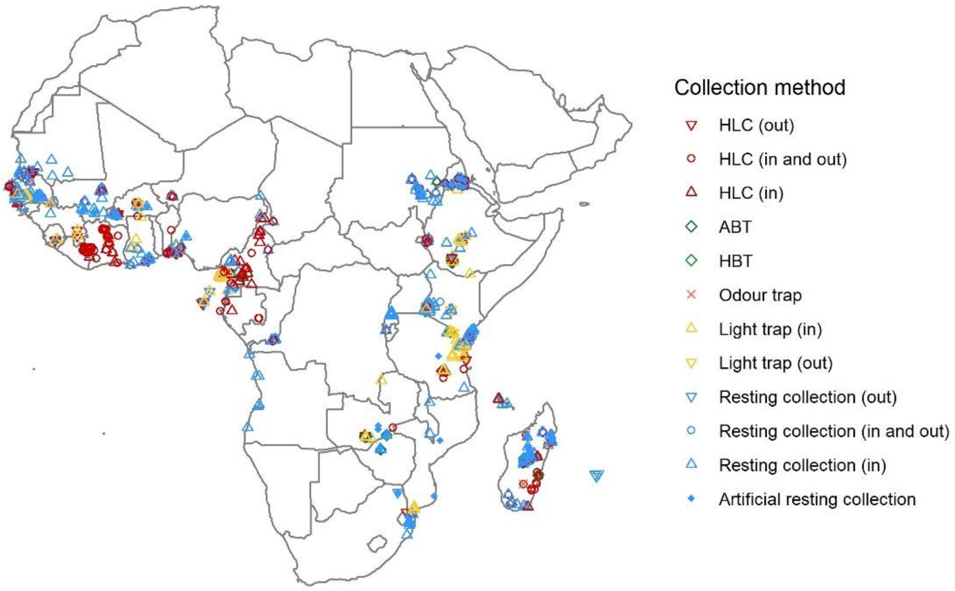
When individual data records were examined in further detail, HLC collections were used in three sampling strategies: indoor-only, outdoor only and simultaneously indoor and outdoor. Simultaneous indoor and outdoor HLCs were common practice in all regions. In the analysed data, 77.4% of HLCs in Africa, 50.1% of HLCs in the Americas and 67.5% of HLCs in the Asia-Pacific region were deployed indoors and outdoors simultaneously.

The collection of anophelines from natural indoor resting sites (defined as structures, including houses and animal shelters, not constructed specifically to lure resting mosquitoes) was the second most used method in both the Asia-Pacific region (29.5%, n = 1718) and Africa (27.1%, n = 2662; Table 3). The small proportion of outdoor resting collections generally reflected the difficulty of collecting mosquitoes outdoors and not the absence of outdoor resting mosquitoes.

In Africa, malaria vector sampling increased in the late 1980s for both host-seeking as well as resting collections. Total sampling effort remained quite stable in the following years to 2010.

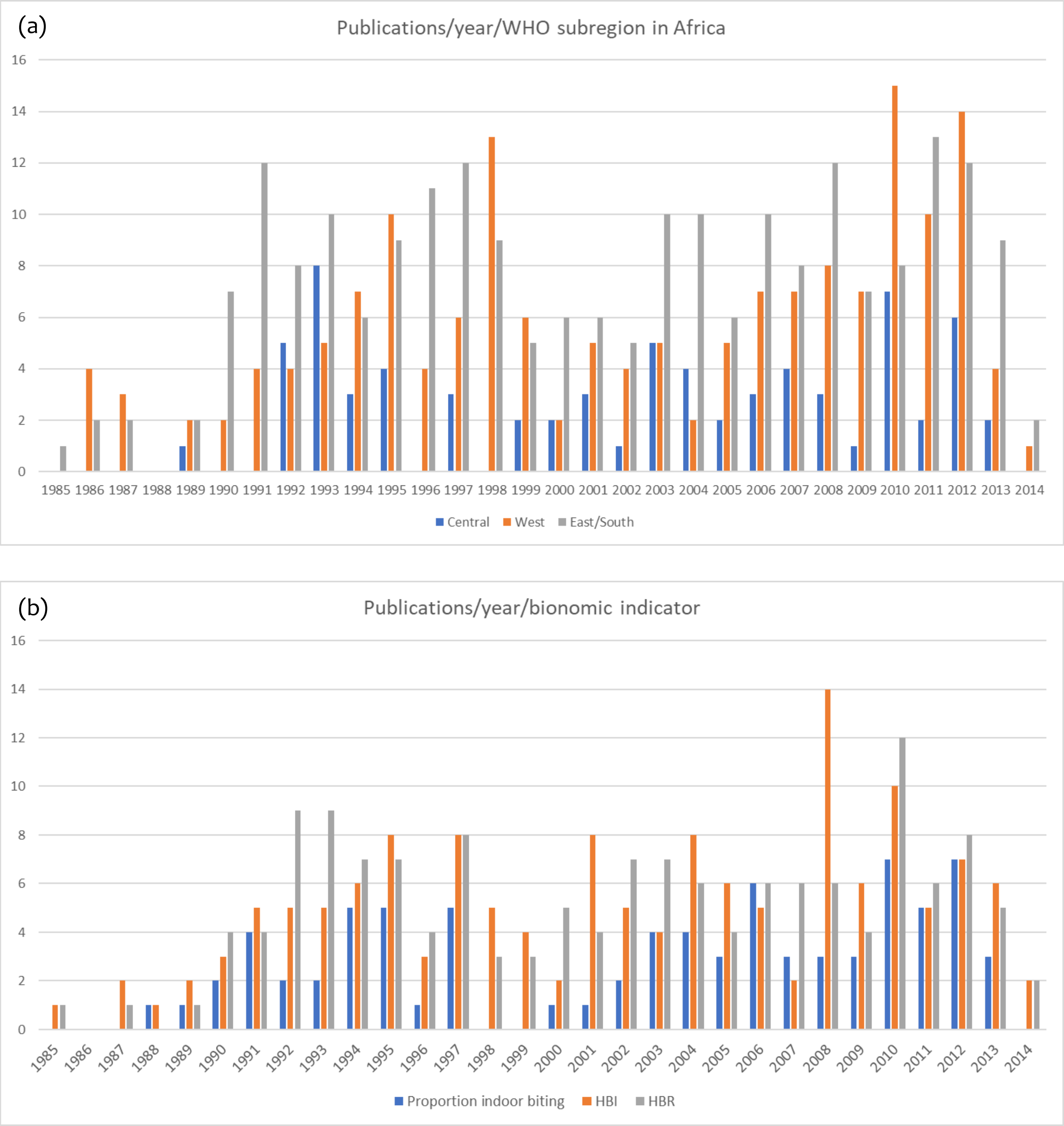
The ratio between host-seeking and resting collections in Africa was the most stable of the three geographic areas analysed. In Africa, HLC (conducted simultaneously indoors and outdoors) and indoor resting collections dominated the database time frame (Supplementary Figure 6.1). Only the data records that include data from either or both methods were used in the follow-up analyses of HBI (resting collection data) and proportion biting indoors (HLC data) in the main article.

In Africa, the locations of indoor/outdoor HLCs and indoor natural resting collections were confined to West and East Africa, with limited sampling in Central Africa. However, the data did not show a geospatial pattern in the spread of both methods (Supplementary Figure 6.2) (212).



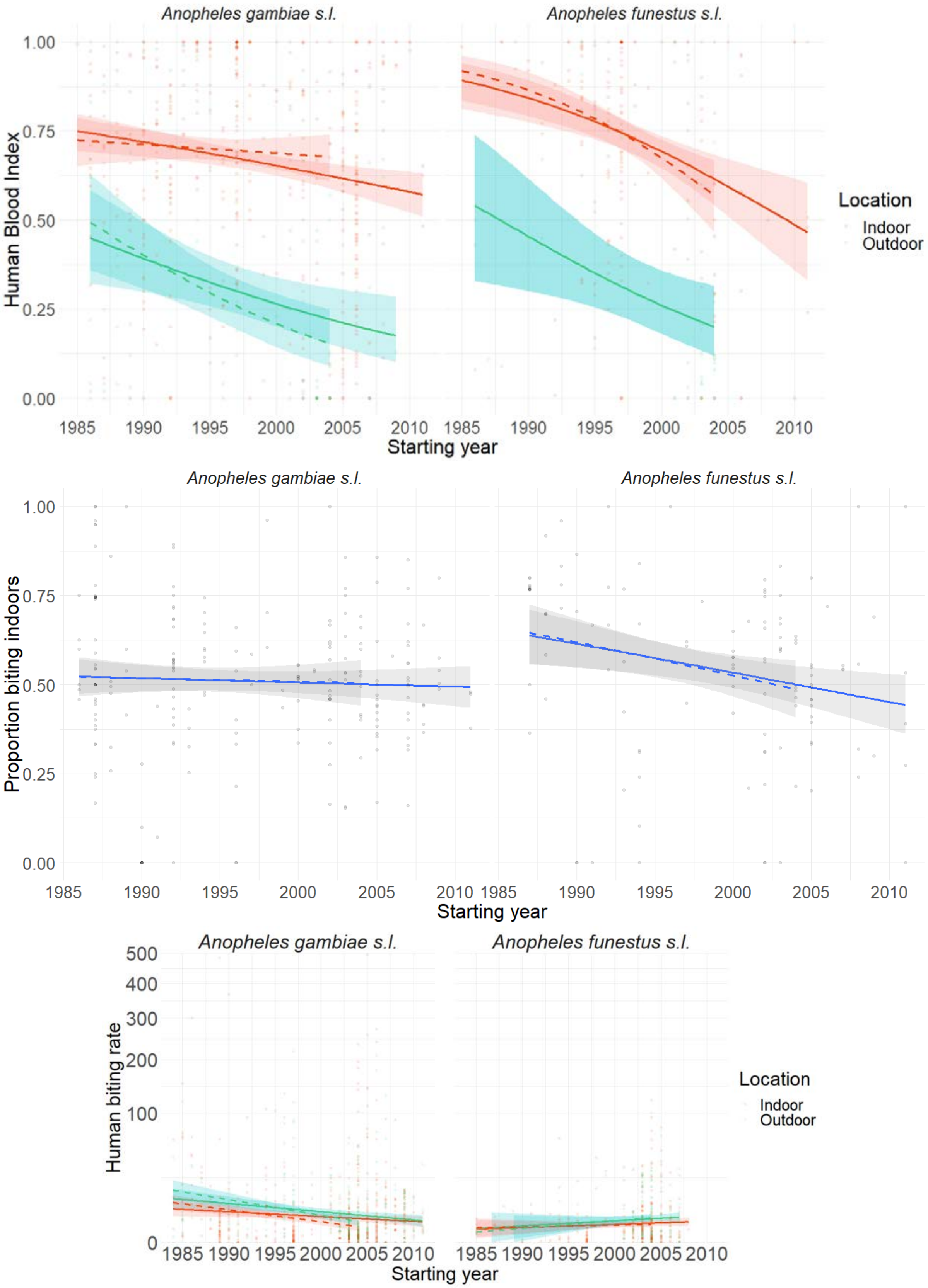
Supplementary Figure 6.2 - Geographical distribution of the different collection methods used in Africa to collect malaria vectors. ABT = animal-baited trap, HLC = human landing catch, HBT = human-baited double net trap. Maps were made with R statistical software (R version 4.0.2), packages 'tidyverse' and 'maps'. Figure originally published in (10) and reproduced with permission of the original authors.

Supplementary Figure 6.3



Supplementary Figure 6.3 - (a) the number of publications included in the dataset, grouped by World Health Organization subregion in Africa and year of publication; (b) the number of publications included in the dataset per bionomic indicator analysed.

Supplementary Figure 6.4



Supplementary Figure 6.4 – the mean predicted values of the analysis of the full dataset (solid lines) and the pre-vector control period data subset (1985-2004); top two panels show human blood index, middle two panels show the proportion of biting indoors, and the bottom two panels show the human biting rate. Shaded areas represent the 95% CI.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are available upon request from the authors. Original databases can also be found in Massey NC, Garrod G, Wiebe A, Henry AJ, Huang Z et al. *Sci Data*. A global bionomic database for the dominant vectors of human malaria. 2016;3:1-13. Doi: 10.1038/sdata.2016.14.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

BvdS, TLR and TRB conceived the research. MES compiled the dataset. BvdS analysed the data and wrote the original draft of the manuscript. TLR provided input on the data analyses. All authors reviewed and approved the final manuscript.

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Chapter 7

This thesis defined core behaviours of the An. leucosphyrus *complex and* An. maculatus *Group of malaria vectors in North Sumatra, Indonesia, and their distribution in different land-use types on vector behaviours. However, the global COVID-19 pandemic necessitated expanding the thesis to a global analysis of vector sampling methods and vector behaviours. This provided the broad context for the studies conducted in Indonesia. In this chapter, the key findings are integrated, and their implications are discussed along with recommendations for future research.*

1
- Introduction to the thesis

2
- Behaviours of simian malaria vectors

3
- Sampling methods used to define malaria vector behaviours across geographic regions

4
- Evaluating sampling methods for malaria vectors in North Sumatra, Indonesia

5
- Defining malaria vector behaviours in North Sumatra

6
- Trends in malaria vector behaviours across geographic regions

7
- General discussion, future research, conclusions

Key findings

Anthropogenic drivers of habitat changes are affecting ecosystems across the globe, including malaria vector occurrence and abundance and in so doing, changing malaria transmission dynamics (124,137). Some ecosystem changes are associated with reductions in disease incidence in humans, while in other cases the disease incidence is amplified (235). In all cases, effective malaria control remains largely dependent on interventions targeting specific vector behaviours to reduce contact between humans and mosquito vectors (8). Human-vector contact is affected by vector behavioural changes in response to vector control and land-use type changes. Surveillance of vectors to detect behavioural changes remains essential to track how the effectiveness of known specific vector control measures are impacted by vector behaviours and bionomics.

The key findings of each research component in this thesis are:

1. Chapter 2 compiles the knowledge on the vectors transmitting simian malaria parasites to humans, showing that our knowledge on the behaviours of the vectors of *P. knowlesi* malaria is limited, being based on a small number of studies, and consequently, the strength of the evidence is low.
2. Chapter 3 shows that although similar malaria vector collection methods were used globally, their frequency of use varied among regions. This may reflect biases in the perceived behaviours of the dominant vector species in each region with collection methods selected to maximise the numbers captured. Adherence to current dogmas and expert opinions to design vector sampling strategies may reinforce biases in surveillance data and can delay the detection of behavioural shifts of vectors, which could diminish the effectiveness of deployed vector control strategies. A tailor-made surveillance effort integrating multiple collection methods for specific regions can provide better insights into vector behaviours and enable more rapid detection of behavioural shifts.
3. *Anopheles leucosphyrus* complex females can be collected effectively by methods using humans or cows as lures. *Anopheles maculatus* Group females were mainly attracted to cows but will also bite humans, albeit in lower numbers. Passive box traps and CDC miniature light traps were not sensitive to host-seeking anophelines in North Sumatra, while the BG-Sentinel 2 trap varied in its sensitivity relative to HLC.
4. Peak biting of potential simian malaria vectors in the *An. leucosphyrus* complex occurred around 22.00h, well before most people went inside their houses. The highest biting rates of *Anopheles leucosphyrus* complex mosquitoes were observed in a small-scale subsistence agriculture area close to a village, while its lowest biting rates were observed in a large-

scale oil palm plantation. *Anopheles maculatus* Group females showed substantially different behaviours, with highest biting rates on humans in the oil palm plantation.

5. From 1985 to 2011, the proportion of blood meals on humans and the proportion of bites indoors by dominant malaria vectors in Africa diminished, thus lowering the vectorial capacity of these malaria vectors. Such behaviour shifts usually define behavioural resistance to insecticide-based interventions like ITNs and IRS in which vectors avoid contact with the insecticides in ITNs and IRS inside houses. However, the behavioural changes across Africa started before the onset of the scale-up in ITNs and IRS in the early 2000s suggested that other widespread drivers were present during this time. House improvements and untreated bed nets may have been exerting pressure to prevent vector house entry and feeding on humans in an analogous manner to ITNs and IRS. Similar selection pressure may be occurring in other geographic regions, but current surveillance data is inadequate to detect subtle behavioural changes in malaria vectors in Asia or the Americas.

The work that was done for this thesis was strongly affected by the Covid-19 pandemic and the resulting global restrictions on travel. The field study sites were only accessible after June 2022, which limited the time for field data collections. The lower quantity of field data makes interpretation of these data more difficult, and currently it is not possible to distinguish individual habitat-type effects from Dusun-level effects. Thus, this confounding factor requires careful consideration when interpreting the field study results. The chapters in which long-term trends were analysed were based on a single dataset on mosquito bionomics (the Malaria Atlas Project dataset). As in all cross-study meta-analyses, there is a risk of bias in compiling a large dataset. Bionomics data have been recorded by many researchers, often using varying methodologies, and as such, sampling effort was likely variable across sampling locations. To minimise bias, the data was extracted using a defined standardised process. Additionally, the size of this dataset and the use of standardised collection methods to study and report the bionomic indicators analysed reduced the impact of bias across the time frame and geographic scope of the data.

Implications of findings

Surveillance and representative sampling of malaria vectors

Surveillance of malaria vectors remains an essential component of malaria control strategies in all malarious regions across the globe as recommended by the World Health Organization (15,233). The coverage of targeted interventions like ITNs and IRS (or 'active vector control') has been increasing over the past twenty years and will continue to do so in the future, as more and more countries are setting goals for, or are already approaching, malaria elimination (40). Besides the active control of malaria vectors, anthropogenic factors can also unintentionally affect malaria vectors, e.g., changing house designs and construction materials can reduce the house entry of malaria vectors (hereafter called 'passive vector control'). Although many of these anthropogenic factors may have an initially positive effect on malaria incidence, malaria vector populations and behaviours are likely to change to circumvent the exposure to both active (programmatic) and passive vector control. For example, malaria vectors are shifting from late-night, indoor biting to earlier, outdoor biting, when their preferred human blood hosts are not yet protected by bed nets. Thus, both active and passive vector control can ultimately lose effectiveness and result in increases in residual malaria transmission (210,236,237).

To detect behavioural shifts of malaria vectors early and to adapt targeted interventions for control when required, it is important to implement representative sampling in malaria vector surveillance to monitor changes in dominant as well as minority behaviours. Representative sampling using multiple sampling methods provides epidemiologically relevant data to simultaneously capture indoor and outdoor biting and resting behaviours. When possible, sampling efforts should be conducted across different micro-habitats in an area, because vectors can display highly heterogenic behaviours, as was observed in this thesis [Ch. 4, Ch. 5]. Tailor-made surveillance programmes can provide better insights in malaria vector behaviour and further help optimise the efficacy of vector control strategies by more proactive early detection of changes in vector behaviours.

Malaria vector behaviours and anthropogenic land-use change

Behaviours and distributions of malaria vectors are intrinsically heterogeneous, depending on the species and the suitability of habitats (23,238). Humans can significantly change the characteristics of micro-habitats in an area, i.e., by deforestation, agricultural development, and urbanisation, encroaching on existing habitats. This creates selection pressure, which can affect human and simian malaria vector behaviours. In sub-Saharan Africa, selection pressure has resulted in a behavioural shift towards outdoor biting in *An. gambiae* s.s., one of the dominant malaria vectors in the region, thereby potentially lowering the efficacy of current vector control methods [Ch. 6]. In Southeast Asia including Indonesia, many dominant malaria vectors are naturally exophagic and originally forest-dwelling (99). Here, agricultural development is often preceded by logging or deforestation, and forests are largely replaced by extensive oil palm plantations with remnant forest patches in areas unsuitable for agriculture, resulting in habitat fragmentation and biodiversity loss (138). In agricultural areas, human activities can reduce any diluting effect on the biting and infectivity rates of malaria vectors with less frequent feeding on non-reservoir hosts while simultaneously increasing the number of human blood hosts (133). Malaria vectors like *An. leucosphyrus* were originally forest-dwelling and predominantly blood-fed on monkeys like long-tailed and pig-tailed macaques, reservoir hosts of many simian malaria species that can also infect humans (70,239–241). However, both macaques and mosquitoes can adapt quickly to the presence of humans, with macaques feeding on human food scraps and oil palm fruits (131) while the mosquitoes bite macaques and humans. The lasting presence of macaques (reservoir hosts), mosquitoes, and humans (competent hosts) in an area can thus create opportunities for spill-over transmission of simian malaria species to humans.

Implications for zoonotic simian malaria control

Simian malaria species, notably *Plasmodium knowlesi* and *Plasmodium cynomolgi*, routinely contribute to clinical malaria in Southeast Asia (242–245). In 2021, more than 4000 simian malaria cases in humans were reported, most in Malaysia with Indonesia ranking second (4). Simian malaria cases in humans will prevent national and regional efforts to eliminate malaria in humans. Therefore, it is critical to establish targeted malaria interventions for simian malarias. However, successful malaria interventions are challenged by changes in malaria vector behaviours and distributions resulting from changing land-use. Anthropogenic land-use changes are not likely to cease, with a growing human population and its increasing pressure on the natural ecosystems in Indonesia. Hence, controlling simian malaria transmission to humans will be a major challenge. None of the *An. leucosphyrus* complex females that were screened for *P. knowlesi* were positive for *Plasmodium*, which prevents accurate predictions if and where transmission takes place. However, human surveillance data resulted in four human infections with *P. knowlesi* between March 2022 and March 2023 in Ujung Bandar (I. N. D. Lubis, personal communication, November 2023), showing that transmission to humans takes place in the study area. Therefore, the lack of *Plasmodium*-positive *An. leucosphyrus* complex mosquitoes sampled was likely due to a low infection rate, which was also observed in Sabah, Malaysian Borneo (39,81) The results from this thesis suggest that the presence of large-scale oil palm plantations around Dusun 2 does not increase the risk of *An. leucosphyrus* complex bites inside these plantations, although more data from a larger number of sampling sites across North Sumatra is necessary to support these observations. Nevertheless, biting rates of simian malaria vectors on humans in a large-scale oil palm plantation were low, while biting rates were much higher in a mixed-crop agriculture area with remaining forest patches and larger areas of forest nearby. This habitat fragmentation increases human interactions with both macaques and simian malaria vectors, especially in forest edges. Finding a balance between agricultural and economic development and the preservation of the unique ecosystem and biodiversity in North Sumatra will likely lead to further fragmentation of the forests outside national parks and other protected areas, and thus to even more interactions between humans, macaques, and malaria vectors. Due to the exophilic and exophagic biting behaviour of the vectors, traditional interventions like ITNs or IRS will likely be limited in effectiveness. Additionally, the parasite infection rate in macaques is often high, which makes them a highly competent reservoir that is challenging for infection prevention or treatment (240,241). Educating people that live or work in high-risk areas about the infection risks of mosquito bites and encouraging the use of personal protection measures like repellents and long-sleeved clothing may be the best presently available intervention methods to control emerging simian malaria cases in humans.

Future research

In this study, large heterogeneity was observed in *Anopheles* abundance across different land-use types in North Sumatra. Predicting the risk of simian malaria infections in specific land-use types remains a challenge, requiring knowledge on the bionomics and infection rates of vectors, and behaviours of humans and macaques. Research on core behaviours of potential simian and human malaria vectors needs to be expanded to a larger scale to better explain differences in vector densities across land-use types. Additionally, in-depth behavioural studies of both macaques and humans need to define areas of overlap between malaria vectors and their hosts, and in so doing would help determine areas of high transmission risk where control efforts should be focussed.

The high diversity of potential simian malaria vector species in Indonesia may vary strongly between islands, which could restrict the direct translation of the results of this study to other areas in Indonesia (99). To accurately determine the risk of simian malaria transmission to humans in other regions, additional, local studies of anophelines, macaques and human movements are needed. The Kalimantan region on Borneo should be a region of prime interest, since it borders on the Malaysian states of Sarawak and Sabah, which form the main regions of simian malaria transmission to humans. Kalimantan shares many ecological characteristics with its neighbouring Malaysian states, possibly including the high simian malaria transmission rates to humans observed in Sarawak and Sabah. The current relocation of the capital of Indonesia to Kalimantan will change human land-use patterns in the region and could increase exposure of humans to simian malaria vectors (246). Hence, it is paramount that malaria vector densities and behaviours as well as human-macaque interactions are defined in areas of increasing anthropogenic influence and development.

Conclusions

The aim of this thesis was to provide novel insights in the behaviours and bionomics of malaria vectors in landscapes affected by anthropogenic factors. Anthropogenic drivers of vector behaviour changes can act locally, as was seen in North Sumatra, where the occurrence and density of *Anopheles* species, including the potential malaria vectors in the *An. leucosphyrus* complex and *An. maculatus* Group, varied based on land-use type. Additionally, behavioural changes in dominant malaria vectors were observed on a continental scale in Africa, thus providing evidence that anthropogenic factors which are not targeted at malaria vectors can drive behavioural change in these vectors. To effectively control malaria cases in humans, including emerging simian malaria, epidemiologically relevant and unbiased surveillance of malaria vectors remains vital.

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Bram van de Straat[1*], **Tanya L. Russell**[1], **Kyran M. Staunton**[1], **Marianne E. Sinka**[2] & **Thomas R. Burkot**[1]

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Malaria Journal

REVIEW **Open Access**

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Zoonotic malaria transmission and land use change in Southeast Asia: what is known about the vectors

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