



HAL
open science

Anthropisation et transmission du paludisme dans le Cambodge rural

Anaïs Pepey

► **To cite this version:**

Anaïs Pepey. Anthropisation et transmission du paludisme dans le Cambodge rural. Parasitologie. Université de Montpellier, 2022. Français. NNT : 2022UMONG034 . tel-04189940

HAL Id: tel-04189940

<https://theses.hal.science/tel-04189940>

Submitted on 29 Aug 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

École doctorale GAIA - Biodiversité, Agriculture, Alimentation, Environnement, Terre, Eau

Spécialité : BDI - Biologie des Interactions

Unité de recherche : IPC - Institut Pasteur du Cambodge

Anthropisation et transmission du paludisme dans le Cambodge rural

Présentée par Anaïs Pepey
Le 24 Juin 2022

Sous la direction du Docteur Benoit WITKOWSKI

Devant le jury composé de

Mr Benoit WITKOWSKI, CR & HDR, Institut Pasteur

Mme Amélie VANTAUX, chercheuse contractuelle, Institut Pasteur du Cambodge

Mr Frédéric ARIEY, DR & HDR, Hôpital Cochin

Mme Leanne ROBINSON, professeure & DR, Burnet Institute

Mr Christophe PAUPY, DR & HDR, IRD

Mr Emmanuel BONNET, DR, IRD

Directeur de thèse

Encadrante de thèse

Président du jury, rapporteur

Rapporteure

Examineur

Examineur



UNIVERSITÉ
DE MONTPELLIER

Anthropisation and malaria transmission in rural Cambodia

PhD candidate: Anaïs Pepey

December 2018 – March 2022

Malaria Molecular Epidemiology Unit

Institut Pasteur du Cambodge

Table of Contents

Table of Contents	3
Acknowledgements	7
Abstract – English	9
PhD summary accessible to the general public	12
Abstract – Français	13
Résumé de thèse vulgarisé pour le grand public en français	17
Résumé substantiel en français.....	18
Introduction	18
Biologie et épidémiologie.....	18
Le paludisme au Cambodge	21
Objectifs.....	23
Principaux résultats	23
Discussion	26
Conclusion	27
Références	27
List of Figures.....	2
List of Tables.....	3
List of Appendices	4
List of Abbreviations.....	5
Part I: General introduction.....	7
A. Malaria in a nutshell	8
A.1. Biology and epidemiology of the disease	8
A.1.1. Incidence	8
A.1.2. The life cycle.....	10

A.1.3. The host.....	11
A.1.4. The parasite.....	12
A.1.5. The vector	13
A.1.6. The environment.....	16
A.1.7. Disease control.....	19
A.1.8. Challenges in the way of elimination	21
A.2. Cambodia.....	23
A.2.1. Socio-economic context	26
A.2.2. The epicentre of malaria resistance.....	29
A.2.2. Transmission and incidence	30
A.2.3. Risk population.....	31
A.2.4. Malaria elimination	34
A.2.5. Remaining challenges.....	37
Objectives of this PhD research	39
Research methodologies	41
C.1. Mobility recording.....	41
C.2. Geographic Information Systems.....	42
C.3. Vector collection	45
C.4. Tracking exposure and infections	49
Part II: Research	52
A. Chapter 1: The determination of anthropisation in a rural Cambodian area and the mobility of risk populations	53
1.1. Introduction.....	53
1.2. Anthropisation.....	55
Article: Studying Land Cover Changes in a Malaria-Endemic Cambodian District: Considerations and Constraints.....	55

1.3.	Mobility, space and malaria exposure.....	77
	Article: Forest malaria in Cambodia: the occupational and spatial clustering of <i>Plasmodium vivax</i> and <i>Plasmodium falciparum</i> infection risk in a cross-sectional survey in Mondulakiri province, Cambodia	77
	Article: Mobility evaluation by GPS tracking in a rural, low-income population in Cambodia.....	90
	Article: Comparing malaria risk exposure in rural Cambodia population: GPS tracking versus questionnaires.....	103
	Article: Investigating human-malaria mosquito contact in time and space using GPS data.....	132
1.4.	Discussion	150
Chapter 2:	Ecology and parasite prevalence in vectors	155
2.1.	Introduction	155
2.2.	<i>Anopheles</i> bionomics in Cambodia	156
	Article: <i>Anopheles</i> ecology, genetics and malaria transmission in northern Cambodia	156
	Article: Environmental factors driving <i>Anopheles</i> abundance, diversity and prevalence in Cambodia.....	174
2.3.	Discussion.....	214
Chapter 3:	Characterisation of <i>Plasmodium</i> spp. transmission within human and vector populations	221
3.1.	Introduction	221
3.2.	Application of a deep amplicon sequencing set in <i>P. falciparum</i> samples.....	223
3.2.1.	Material and methods	223
3.2.2.	Results	226
3.3.	Application of a deep amplicon sequencing set in <i>P. vivax</i> samples.....	234
3.4.	Discussion.....	236

General discussion.....	239
References.....	245
Appendices	269

Acknowledgements

I would like to address my sincerest thanks to all the people that supported, guided and helped me through these three years and a half of PhD;

Leanne Robinson and Frédéric Ariey for taking the time to evaluate this manuscript,

Christophe Paupy and Emmanuel Bonnet for accepting the role of examiners and being part of the defence committee,

Vincent Herbreteau, Anna Cohuet and Antoine Berry for helping guiding this project during the yearly committees,

for all of your comments and how you improved the scientific quality of this project and this manuscript.

Benoit Witkowski for your patience and your unerring advice. Working in Malaria Unit under your counsel was a priceless opportunity, a rich and supportive environment in which I could learn how to handle biomolecular techniques, field expeditions, working in a diverse team, trying new approaches and the joys of publishing. Your dark jokes and the drinks on Juniper's rooftop were an essential part of the PhD and the Malaria Unit experience.

Amélie Vantaux, for making it possible and supervising my internship in addition to my PhD, and teaching me so much through four years of PCR disasters, long and dusty days in the field and hours of proofreading my manuscripts. Thank you for being always a support, a sound adviser and to remind me of the true priorities of a PhD. These years have sometimes been a hardship for you too, and I am grateful that you have always found the time, the energy and the words to guide me through my mistakes and my incertitude, allowing me to finally conclude the experience being both sane and prepared for the next step.

Camille and Mélissa, for your friendship, your help and your reassurance through these years. You were the ones to tell me, when I thought everything was failing, that it was not – it is just part of the PhD process. Thank you for the gossip breaks in the culture room, the refreshing excursions and our boxing sessions after a long day at the lab.

All my IPC friends that were present and thoughtful; David always available to cheer me up and take me out, Megu the sweetest friend bringing the most amazing banana bread

and cookies, Béatrice quarrelling your strongest opinions around the lab, Hoa for your apparently limitless energy.

Marc Souris for your patience and helping to ground this project onto solid geostatistics and geographic bases.

Sophy for being a part of this project, helping me through my first PCRs and always answering my questions.

Saorin for gaining the trust of the heads of villages and joining us across the rivers, the villages and the potholes of Kaev Seima.

Baura, Eakpor, Malen, Keng, Bros, Chantrea and all of Malaria Unit for supporting and building this project through the years.

Makara, Koeun and all the field team for your involvement, the delicious food and the friendly evenings in Kaev Seima.

All participants, collectors and volunteers that accepted to join the various studies, follow-ups and collections to make this whole project possible.

Didi, for supporting me through it all, and providing insight where you could, criticising my code and making it slightly better (sorry, still pretty bad) and suggesting new approaches when I could not think of any. We both know your views on academia, and still you helped me without mentioning them (as much as you could have). Thank you for being present and supportive in Phnom Penh, in Kaev Seima or abroad, until the last weeks of redaction.

My parents, for gifting me an interest in biological sciences, the curiosity that accompanies me every day, the will to travel, the inclination to handle bugs, to drive bikes through the mud and to explore forests without apprehension. Thank you for the encouragements throughout it all.

Abstract – English

Malaria cases and mortality are globally decreasing, including in the Great Mekong Subregion, which targets elimination by 2030. Cambodia, after achieving national coverage of insecticide-treated nets, is now facing residual malaria transmission. Currently, the vector-borne disease persists along international borders and in forested areas. Forest-dwelling vectors and forest goers, migrant and mobile populations are responsible for the remaining cases, in spite of the recent village and mobile malaria workers deployments. In a context of rapid deforestation and economic growth, the repercussions of anthropisation remain mostly unknown and undescribed in the area. Indeed, it has not been assessed if the deforestation and urbanisation observed in the kingdom are driving malaria transmission, or, on the contrary, are reducing its incidence. So far, the understanding of the interactions between environmental changes, vectors bionomics and malaria transmission has been neglected in Cambodia, but could help to institute an efficient and durable elimination. Overall, this project places itself at the intersection of geography, epidemiology, entomology and genomics, and aims to decipher some of the remaining unknowns surrounding anthropisation and malaria transmission in rural Cambodia, in order to pinpoint when, where and how human-vector contacts happen.

The first chapter focuses on environmental changes and the populations' mobility in the study area. From high-resolution satellite imagery, we quantified the deforestation of a malaria endemic area in north-eastern Cambodia. We observed the evolution of forest cover, fragmentation and landscape diversity on a span of 30 years, along with the available malaria data from the study area. A cross-sectional study, a cohort and follow-ups of risk populations in the same area were implemented to describe the mobility and behaviours related to malaria infection, by equipping individuals with GPS data-loggers and through questionnaires. We implemented anti-*Anopheles* saliva quantification by ELISA to test its ability to discriminate the subpopulation most exposed to vectors bites.

From this first chapter, we gathered that deforestation is rapidly increasing in the study area, with wooded areas being replaced by fields and villages, while the landscape becomes increasingly fragmented and diverse. Malaria data was inconsistent and unavailable

at a scale relevant to the satellite imagery, and thus it could not be used to determine a potential correlation between anthropisation and malaria dynamics. Through questionnaires only, the most important risk factors for participants to be *P. falciparum* or *P. vivax*-positive were being male and spending time in the forest. Implementing of GPS data-loggers was challenging as a result of climatic conditions, limited study population acceptance and battery life. Spatial data needed curation and pre-processing before interpretation. Overall, malaria risk was not associated with the time spent in forest or any other environment, but was associated to the village of origin, with the villages outside the forest being more exposed than the ones at the fringe or inside the forest. Men were more likely to be infected with *Plasmodium* spp. than women but the *Anopheles* saliva antibody response was similar across men and women, while higher for both during rainy season compared to dry season.

The second chapter addresses the ecology, abundance and prevalence of malaria vectors in the studied area. We deployed three types of traps to collect adult *Anopheles* (human landing catch, human-baited double-net trap and barrier screens) and transects to collect larvae, along an anthropisation gradient through villages, fields, tree plantations and forest. After screening and identifying the collected *Anopheles*, we determined the environmental factors associated with prevalence and abundance of *Anopheles* mosquitoes, and quantified the accuracy of morphological species identification compared to molecular identification. We determined the origin of fed *Anopheles* blood meals to assess if environmental conditions or *Anopheles* species were linked to an anthropophagic behaviour.

This chapter allowed to implement collection methods that were new to Cambodia such as barrier screens, to optimise others such as aquatic habitats transects and baited double-net traps, in addition to human landing catches used as a reference. Overall, over two collections spanning a gradient of environments from villages to deep forest, we collected and identified over 40 *Anopheles* species, of which *Anopheles dirus* was the primary vector. The prevalence of human *Plasmodium* species varied between 3,6% in 2017-2018 and 1,9% in 2020-2021. Higher biting risk was associated to both forests and fields, in addition to sites surrounded by important fragmentation and forest cover. *Anopheles* spp. larvae were more often collected from shallow and turbid breeding sites that hosted other mosquito genera.

The third and last chapter focused on malaria parasite itself. To characterise the parasitic populations circulating in humans and vectors, we attempted to sequence

Plasmodium vivax and *Plasmodium falciparum* samples from forest goers' follow-ups and mosquito collections with the Deep Amplicon Sequencing methodology (AmpSeq). This genetic data could provide crucial information to understand better the context of transmission, *Plasmodium* population genetics and the nature of the clones. However, due to the numerous protocol adjustments that were required and the PhD deadline, the analysis of the genetic structure of *P. falciparum* and *P. vivax* parasitic populations could not be completed. Thus, we present an evaluation of the limitations of AmpSeq implementation in Cambodia using *P. falciparum* field samples. Only half the samples were successfully amplified. Initial DNA concentration did not seem to predict amplification success and the methodology was substantially time, resource and staff-demanding. Based on these preliminary results, AmpSeq does not appear to be the most suitable methodology to characterise genetic structure in low-concentration *Plasmodium falciparum* samples in Cambodia.

This project remains but an introduction to the complexity of the interaction between malaria dynamics and anthropisation. The human-made changes to the environment could decrease malaria transmission by reducing the surface of forests, or, on the opposite, increase it through fields and fragmented environments' expanse. More vector collections are required across the country to refine *Anopheles* ecologies. Additionally, innovative tools will be required to understand risk populations behaviours and molecular tools to efficiently monitor parasitic populations themselves.

Key words: malaria, Cambodia, health ecology, anthropisation, mobility, transmission, *Anopheles*, *Plasmodium*

PhD summary accessible to the general public

Malaria is caused by a parasite transmitted to humans by the bite of an infected mosquito. This disease is linked to environmental changes as they affect parasite survival, mosquito distribution and human exposure to mosquito bites. This project aims to understand the relation between anthropisation (the process of transforming an environment to meet human needs) and malaria transmission in a rural district of Cambodia. The quantification of deforestation and the follow-up of participants with GPS loggers and questionnaires focused on the environment, behaviour and mobility associated to an infection. Mosquito collections identified when and where they transmitted parasites to the human population. Molecular tools identified and characterised the parasites in humans and mosquitoes. To understand the relation between malaria and anthropisation, further research is required on particular aspects of the relation rather than a whole, as anthropisation can influence malaria in many ways.

Abstract – Français

Les cas de paludisme sont globalement en baisse, y compris dans la sous-région du Grand Mékong qui vise l'élimination de la maladie pour 2030. Le Cambodge, après avoir implémenté l'usage de moustiquaires imprégnées d'insecticide sur l'intégralité du territoire, fait maintenant face à une transmission paludique résiduelle se maintenant principalement autour des frontières internationales et des forêts. Les vecteurs habitent les zones boisées et infectent les travailleurs en forêt ainsi que les populations migrantes, maintenant la transmission malgré les efforts des équipes en charge de détecter et traiter les cas de paludisme. Les rapides déforestation et croissance économique pourraient affecter la transmission, mais leurs potentielles répercussions n'ont pas ou peu été étudiées ou décrites dans la sous-région, bien que leur compréhension pourrait être nécessaire à une élimination du paludisme efficace et durable. Particulièrement, il s'avère nécessaire de déterminer si la déforestation et l'urbanisation en hausse au Cambodge pourraient engendrer une augmentation des cas de paludisme dans le pays, ou si, au contraire, seraient responsable d'une diminution de son incidence. Pour ce faire, ce projet se place à l'intersection de la géographie, l'épidémiologie, l'entomologie et la génomique avec l'objectif de compléter les connaissances sur la relation entre anthropisation et transmission paludique au Cambodge, afin de déterminer où, quand et comment se crée le contact entre les populations à risque et les vecteurs.

Le premier chapitre de cette thèse s'intéresse aux changements environnementaux et la mobilité des populations à risque dans la zone d'étude. En utilisant des images satellite à haute résolution, nous avons quantifié la déforestation d'une zone particulièrement affectée par le paludisme au nord-est du pays. Nous avons caractérisé l'évolution de la couverture forestière, la fragmentation ainsi que la diversité du paysage sur 30 ans, et rassemblé les données disponibles sur les cas de paludisme pour cette même période. Une étude transversale, une cohorte et un suivi des populations à risque dans la même zone d'étude ont permis de décrire et mieux comprendre les comportements et la mobilité associés à une infection, au travers de la mise en place de techniques fréquemment utilisées au Cambodge, comme les questionnaires, et innovantes, comme les balises GPS ou la quantification d'anticorps anti-salive d'Anophèles par ELISA.

Les résultats de ce premier chapitre ont permis de quantifier la déforestation rapide affectant la zone d'étude, dans laquelle les champs et les villages remplacent progressivement les zones boisées et le paysage devient plus fragmenté et diversifié. Les données de prévalence du paludisme n'étaient pas toujours cohérentes ou disponibles à une échelle correspondante à l'imagerie satellite utilisée, et pour cette raison, aucune corrélation entre anthropisation et dynamique paludique ne put être définie. Au travers des questionnaires, les principaux facteurs de risque associés à une infection paludique par *P. falciparum* ou *P. vivax* étaient d'être un participant homme et de passer du temps en forêt. L'implémentation des appareils GPS s'avéra difficile à cause des conditions climatiques, l'acceptation limitée au sein de la population et l'autonomie des appareils. Les données spatiales recueillies réclamèrent un travail intensif de curation et de traitement avant de pouvoir être interprétées. Finalement, le risque paludique n'était pas associé au temps passé en forêt ou un environnement en particulier, mais était associé au village d'origine, avec les villages en dehors de la forêt plus affectés que ceux à la lisière ou dans la forêt. Les hommes étaient plus susceptibles d'être infectés par *P. falciparum* ou *P. vivax* que les femmes mais la réponse immunitaire contre la salive d'Anophèles était similaire entre hommes et femmes, et cette réponse était plus élevée pour toute la population d'étude pendant la saison des pluies que la saison sèche.

Le deuxième chapitre se dédia à la diversité, l'abondance et la prévalence des moustiques vecteurs. Trois types de pièges (collecte sur appât humain, piège à double moustiquaire et pièges à interception) et la collecte de larves le long de transects furent employés dans différents environnements suivant un gradient d'anthropisation : villages, champs, plantations d'arbres et forêts. Après identification des espèces d'Anophèles collectées, nous avons déterminé la prévalence de *Plasmodium* dans la population de moustiques, afin d'étudier quels facteurs environnementaux affectaient la présence de *Plasmodium* humains et l'abondance des vecteurs. Nous avons également comparé la précision des identifications morphologiques par rapport aux identifications moléculaires. Enfin, nous avons identifié l'origine des repas sanguins extraits des abdomens des femelles gorgées pour déterminer si certains facteurs environnements ou espèces étaient associés à un comportement anthropophage.

Ce chapitre incluait l'implémentation de méthodes de collecte nouvelles pour le Cambodge comme les pièges à interception, ainsi que l'optimisation de certains jusque-là peu implémentés comme les transects et les pièges à double moustiquaire, en addition des collectes sur appât humain servant de référence. Sur les deux collections réalisées le long d'un gradient environnemental entre village et forêt profonde, plus de 40 espèces d'Anophèles furent collectées et identifiées, avec *Anopheles dirus* comme vecteur primaire. La prévalence de paludisme humain parmi les Anophèles collectées variait de 3,6% en 2017-2018 à 1,9% en 2020-2021. Le risque de piqûre était plus élevé en forêt mais aussi dans les champs, et également positivement associé à la couverture forestière et la fragmentation autour du site de collection. Les larves d'Anophèles étaient plus souvent collectées dans les gîtes larvaires peu profonds, troubles et dans lesquels se trouvaient d'autres genres de larves de moustiques.

Le troisième et dernier chapitre de cette thèse se focalisait sur la nature de la transmission paludique elle-même. Pour caractériser la structure des populations parasites circulant entre les humains et les vecteurs, nous voulions séquencer les échantillons de *Plasmodium falciparum* et *Plasmodium vivax* provenant des suivi GPS des travailleurs forestiers et des Anophèles collectés en utilisant la méthode du séquençage profond d'amplicons (AmpSeq). Les données génétiques associées à ce séquençage permettraient de comprendre la génétique des populations des parasites et la nature des clones composant les infections. Cependant, à cause des nombreux ajustements nécessaires au bon fonctionnement du protocole, ainsi que de l'échéance du doctorat, l'analyse de la structure génétique des populations de *P. falciparum* et de *P. vivax* n'a pas pu être complétée. Ainsi, nous avons choisi de présenter une évaluation des limitations liées à l'implémentation de l'AmpSeq au Cambodge en utilisant des échantillons de terrain de *P. falciparum*. Seulement la moitié des échantillons furent correctement amplifiés par la procédure. La concentration initiale d'ADN dans l'échantillon ne semble pas pouvoir prédire une amplification réussie alors que la méthodologie représentait un investissement considérable de temps, budget et personnel. En se basant sur ces résultats préliminaires, l'AmpSeq n'apparaît pas être un candidat adapté pour déterminer et la structure génétique de *Plasmodium falciparum* au Cambodge pour des échantillons de terrain à faible densité parasitaire.

Ce projet ne représente qu'une introduction à propos de la complexité des interactions entre paludisme et anthropisation. Les changements effectués par l'humain sur son environnement pourraient être la cause d'une baisse du paludisme en conséquence de la déforestation, ou bien entraîner son augmentation au travers de l'amplification des milieux fragmentés et des champs. De nouvelles collectes de vecteurs sont nécessaires à la spécification de l'écologie des nombreuses espèces d'Anophèles présentes au Cambodge. Également, de nouvelles méthodologies innovatives seront nécessaires pour comprendre les comportements des populations à risque, ainsi que des outils moléculaires efficaces pour surveiller la transmission des populations parasitaires elles-mêmes.

Mots-clés : paludisme, Cambodge, écologie de la santé, anthropisation, mobilité, transmission, *Anopheles*, *Plasmodium*

Résumé de thèse vulgarisé pour le grand public en français

Le paludisme, causé par un parasite transmis par une piqûre infectieuse de moustique, est influencé par les changements environnementaux affectant la survie et le développement du parasite, l'écologie des moustiques et le risque d'exposition au parasite des populations humaines. Ce projet de thèse vise à mieux comprendre le lien entre la transmission du paludisme et l'anthropisation (le processus de transformation d'un environnement par l'homme pour l'adapter à ses besoins) dans une région rurale du Cambodge. La quantification de la déforestation et le suivi des populations à risque en utilisant des GPS et des questionnaires ont identifié les environnements et comportements associés à une infection. Des collectes de moustiques ont identifiés où et quand les moustiques transmettent le paludisme, et des outils moléculaires ont caractérisé les parasites présents chez l'homme et le moustique. Le lien entre paludisme et anthropisation peut se manifester de nombreuses manières et pour mieux le comprendre, il sera nécessaire de se concentrer sur des aspects précis de cette interaction.

Résumé substantiel en français

Introduction

Biologie et épidémiologie

Le paludisme est une maladie causée par un parasite du genre *Plasmodium*, transmis lors du repas de sang d'un moustique infecté du genre *Anopheles*. L'organisation mondiale de la santé (WHO) a estimé un total 241 millions de cas et 627,000 décès autour du globe pour l'année 2020, affectant 85 pays principalement dans les zones tropicales et subtropicales [1]. Malgré deux décennies de progrès à partir des années 2000, la lutte pour l'élimination du paludisme a récemment rencontré de nouveaux obstacles. L'émergence de résistances et multi-résistances, l'effet de l'anthropisation globale et la nécessité de renouveler les méthodes antivectorielles ont permis aux cas de paludisme de stagner, voire d'augmenter dans certaines régions [1]. De nombreux facteurs affectent l'exposition des populations au paludisme. Le statut socio-économique et activités qui leur sont associées [2–5], la disponibilité et l'utilisation de protection anti-moustiques [2, 3, 6], l'accessibilité des diagnostics et des traitements [1, 7], la mobilité des individus [4, 8–11] et la connaissance du paludisme et de son mode de transmission [4, 6] sont parmi les facteurs impactant l'exposition des populations au paludisme.

Les parasites responsables du paludisme se transmettent et se reproduisent au travers d'un cycle de vie complexe impliquant des étapes de prolifération, reproduction sexuée et asexuée, dormance et invasion entre un hôte vertébré (décrit comme l'hôte) et un hôte invertébré (décrit comme le vecteur). Le cycle de vie est relativement bien conservé entre les espèces (Figure 1). Cinq espèces de *Plasmodium* sont capables d'infecter et de se multiplier chez l'homme : *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*. Les signes cliniques du paludisme non compliqué sont similaires entre ces cinq espèces : fièvre, nausées, vomissements, courbatures, et anémie. Cependant, ces symptômes sont communs à de nombreuses maladies infectieuses affectant les zones tropicales et subtropicales, telles que la dengue, le chikungunya ou Zika – ce qui complique le diagnostic du paludisme [12]. *Plasmodium falciparum* et *P. vivax* sont les espèces les plus répandues, et sont celles à l'origine des cas les

plus sévères. Les cas sévères peuvent se présenter sous forme de convulsions, anémie sévère, hypoglycémie, lésions rénales, et peuvent évoluer en malaria cérébrale ou en coma [1, 13]. Les sporozoïtes de *P. vivax* et *P. ovale*, en plus de se différencier directement en schizontes dans le foie, peuvent également se différencier en hypnozoïtes, une forme dormante non-délectable qui peut se réactiver des semaines, des mois ou des années après l'infection [14]. Cette reprise du cycle après la réactivation des hypnozoïtes se nomme une reviviscence.

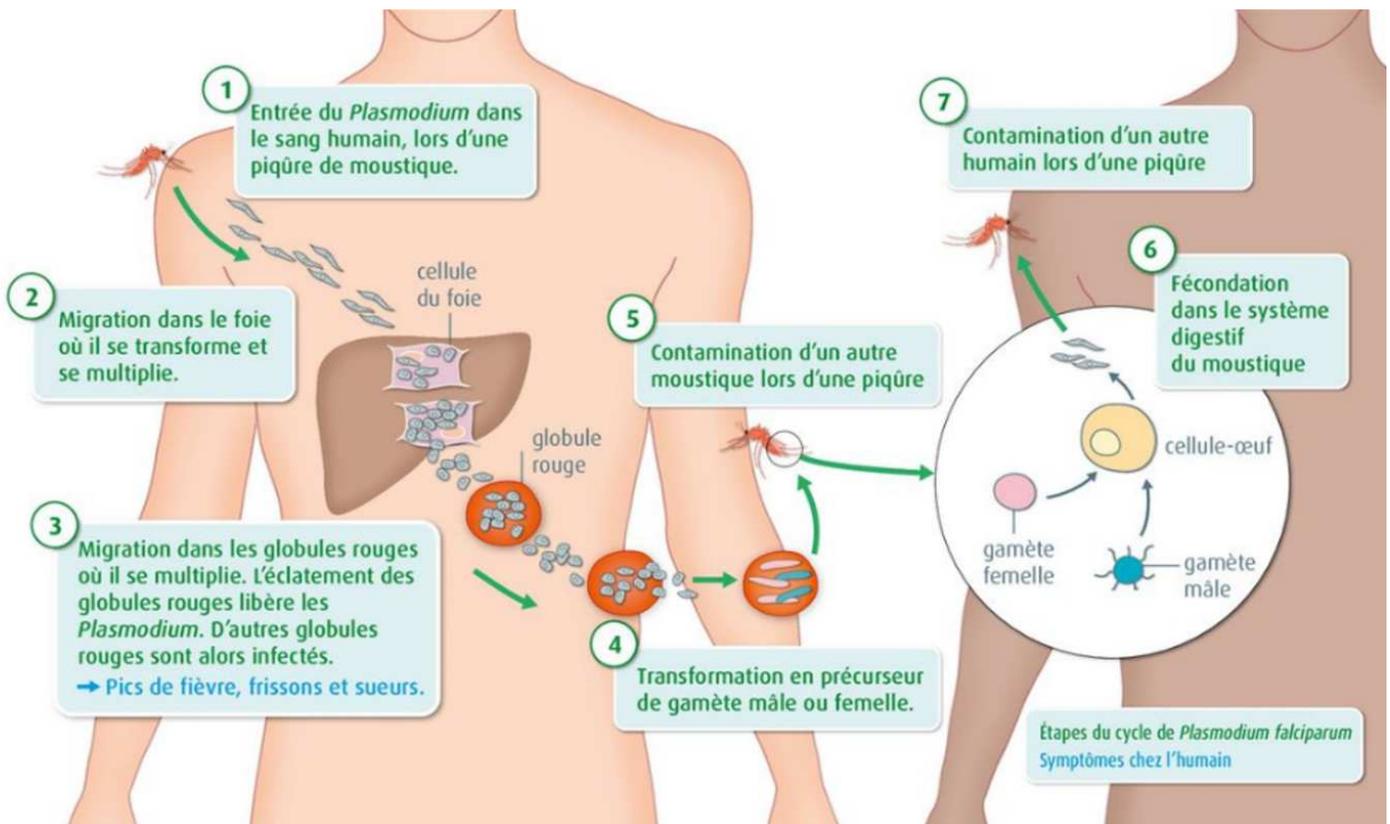


Figure 1 : Cycle de vie du *Plasmodium falciparum* et symptômes chez l'humain 2020 [15].

À ce jour, 465 espèces d'Anophèles sont décrites, desquelles environ 70 sont considérées comme des vecteurs du paludisme [16]. La distinction entre les différentes espèces, sous-espèces et groupes d'Anophèles est fréquemment débattue et actualisée, et des dizaines de groupes se doivent encore d'être officiellement nommés et reconnus [16]. L'abondance et les diversités des vecteurs du paludisme varient grandement d'une région à l'autre. En Afrique, seulement sept vecteurs sont reconnus responsables de maintenir la transmission, dont *Anopheles arabiensis*, *Anopheles gambiae* et *Anopheles funestus* sont au centre des préoccupations des organisations de santé publique. Sur le continent américain, neuf espèces

garantissent la transmission du paludisme, et tout particulièrement *Anopheles darlingi*, répandu sur une large zone du continent sud-américain incluant la majorité du bassin Amazonien. La transmission varie d'une région et d'une saison à l'autre, mais se maintient au travers de nombreux environnements, des forêts tropicales aux zones péri-domestiques et urbaines [17, 18]. La diversité des vecteurs dans la région Asie-Pacifique dépasse de loin celle observée sur les autres continents : pas moins de 19 espèces d'Anophèles y sont des vecteurs dominants [19]. La plupart des pays de cette région ont atteint un stade de transmission résiduelle, correspondant à la transmission se maintenant après l'implémentation de méthodes classiques de protections contre les vecteurs du paludisme : les moustiquaires imprégnées d'insecticide et les pulvérisations intra domiciliaires d'insecticides à effet rémanent [20]. Les vecteurs sont généralement associés aux forêts ou aux environnements en proximité des forêts, et s'adaptent facilement aux hôtes disponibles pour un repas de sang [21]. La transmission du paludisme ne peut se maintenir que si les conditions environnementales permettent à la fois la survie et le développement du parasite, du vecteur et de l'hôte. Des facteurs tels que la température et l'humidité doivent rester dans un intervalle adapté à la survie des vecteurs et peuvent influencer le temps de développement des parasites ou l'abondance et la valeur sélective des vecteurs [22, 23]. La présence de prédateurs ou de compétiteurs peuvent affecter l'abondance des vecteurs [24, 25], et la biodiversité globale de vertébrés peut diluer le risque de piqûre infectante pour un hôte [26, 27]. Des phénomènes se déployant à des échelles plus importantes, comme la déforestation et la fragmentation des paysages, modifient la composition et la répartition des biotopes adaptés aux vecteurs, qui peuvent trouver les nouvelles conditions favorables ou défavorables selon le contexte [23]. Ces changements peuvent également altérer les conditions et la fréquence du contact entre l'hôte et le vecteur.

L'anthropisation, qui correspond à la transformation d'un environnement par l'homme afin de l'adapter à ses besoins, est un phénomène de plus en plus étudié. De toute évidence, anthropisation et transmission du paludisme sont étroitement liés, mais la nature de leur interaction n'est pas toujours évidente. De nombreuses études ont rapportés des résultats contradictoires concernant l'effet de la fragmentation, la déforestation, l'agriculture et autres modifications anthropogéniques sur le paludisme [28]. Ainsi, l'anthropisation d'une région est en effet associée à des variations dans la transmission du paludisme, avec des études

provenant des continents africain [22, 29, 30], américain [31, 32] et asiatique [33, 34], mais la direction de cette association semble varier d'une région à l'autre.

Des stratégies de lutttes sont mises en place pour limiter la prévalence et l'incidence du paludisme. D'une part, par la limitation des piqûres infectantes : la méthodologie la plus implémentée étant l'utilisation de moustiquaires imprégnées d'insecticides durables qui permet de limiter à la fois le contact homme-vecteur et les densités de vecteurs, suivie des pulvérisations intradomiciliaires d'insecticides à effet rémanent [1]. D'autres méthodes antivectorielles existent mais sont en encore en cours de validation : répulsifs [35, 36], larvicides [37], pièges à appâts sucrés toxiques [38], ou encore infection des moustiques avec la bactérie *Wolbachia* [39]. D'autre part, par le traitement médicamenteux des cas de paludisme, la combinaison de drogues utilisée dépendant des pratiques implémentées dans la région et de l'espèce responsable de l'infection [1]. Dans le cas de *P. falciparum*, les combinaisons étant au minimum d'un dérivé d'artémisinine à action rapide et d'une drogue partenaire telles que méfloquine ou pipéraqûine à action prolongée.

Malgré ces méthodes de prévention et les traitements disponibles, l'élimination du paludisme n'est pas garantie. Pour atteindre l'éradication de la maladie, il est primordial de prioriser l'accessibilité du dépistage et des traitements, mais aussi leur disponibilité et leur acceptabilité auprès des populations concernées et la sensibilité des méthodes de détection. Ainsi, le WHO suspecte la pandémie de COVID d'être à l'origine de la récente hausse des cas et des décès associés au paludisme, en affectant l'accessibilité du dépistage et des traitements [1].

[Le paludisme au Cambodge](#)

Le Cambodge est un des six pays inclus dans la Grande Sous-région du Mékong avec le Laos, le Myanmar, la Thaïlande, le Vietnam and la province chinoise du Yunnan. La population s'élève à 16 millions d'habitants, avec une économie basée sur le tourisme et l'export de textile. Le Cambodge a été perçu comme l'un des pays avec le taux de déforestation le plus alarmant des pays d'Asie du sud-est [40]. Cette déforestation est alimentée par l'achat fréquent de larges parcelles de terrain par divers groupes, cambodgiens ou étrangers, qui les transforment en parcelles dédiées à l'agriculture [41].

Le Cambodge est l'épicentre de l'émergence des résistances aux antipaludiques, suspectée de provenir de l'accès sans supervision aux drogues antipaludiques [42], du contexte global de pratiques médicales hasardeuses [43] et d'une transmission de faible intensité.

Les cas de paludisme sont composés par plus de 90% d'infections asymptomatiques [44, 45] et 88% de *P. vivax* [1]. La couverture de moustiquaires par foyer déclarée est très élevée [20], catégorisant la transmission au Cambodge de résiduelle [6]. Leur implémentation a drastiquement réduit la transmission dans les villages, mais les fermes et les forêts, où l'on peut trouver les principaux vecteurs *Anopheles dirus* et *Anopheles minimus* [19, 46], représentent encore un risque paludique important [47–49]. L'épidémiologie au Cambodge est reconnue pour dépendre d'échelles très fines : non seulement le risque d'exposition varie entre villages [50, 51], mais aussi entre différents foyers [52]. Les travailleurs en forêt ou « forest goers » s'exposent fréquemment aux piqûres de moustiques en travaillant et dormant dans la forêt, et sont reconnus comme la principale population à risque [53–56]. Ils traversent des épisodes paludiques plusieurs fois par an, mais ne recherchent que rarement à se faire tester ou à traiter l'infection [20, 57, 58]. Les individus travaillant ou vivant proche de la lisière de la forêt [59] et les populations mobiles à la recherche de travail temporaire dans les zones rurales [60] sont également exposés au paludisme.

Le programme d'élimination mis en place au Cambodge se base sur la détection des cas avec des tests diagnostiques rapides qui sont responsables de nombreux faux négatifs, car leur sensibilité ne leur permet pas de détecter les infections à faible parasitémie, représentant la majorité des cas circulants au Cambodge [61, 62]. L'utilisation de moustiquaires ne permet pas de protéger leurs utilisateurs lors de leurs activités quotidiennes en dehors du foyer [63]. Finalement, les hypnozoïtes et les reviviscences de *P. vivax* sont difficiles à traiter, puisque 2 à 16% des résidents de la région sont atteints d'une déficience en glucose-6-phosphate déshydrogénase qui cause des hémolyses sévères lors de l'ingestion de primaquine, ce qui complique sa prescription [64].

La prévalence du paludisme est en baisse au Cambodge, mais l'élimination de la transmission résiduelle dépend de l'implémentation de nouvelles stratégies basées sur une meilleure compréhension de l'épidémiologie et de la dynamique de la transmission.

Objectifs

Au travers d'une approche multidisciplinaire se concentrant sur la dynamique du paludisme au Cambodge à une très fine échelle, nos travaux visent à :

- 1) Quantifier l'anthropisation et analyser les mouvements des populations d'une zone affectée par le paludisme
- 2) Préciser l'écologie et la prévalence des moustiques vecteurs dans des environnements aux différents niveaux d'anthropisation
- 3) Caractériser la transmission des parasites entre les populations à risque et les vecteurs

En atteignant ces objectifs, la stratégie de contrôle du paludisme pourra s'appuyer sur des données de terrains récentes et quantitatives ainsi que l'utilisation de méthodes innovantes.

Principaux résultats

Les résultats de ce projet de thèse se concentre sur le district de Kaev Seima au nord-est du Cambodge, dans la province du Mondulkiri limitrophe avec le Vietnam. La saison sèche s'étend de Novembre à Avril et la saison des pluies, ou mousson, de Mai à Octobre. La population vit majoritairement de l'agriculture et de l'exploitation des ressources forestières.

En utilisant des images satellite à très haute résolution (SPOT 5/6), nous avons produit une classification d'utilisation du sol de la zone d'étude et complété un jeu de données existant afin d'observer l'évolution du paysage entre les années 1988, 1998, 2008 et 2018. Au cours de ces trente années, la couverture forestière a diminué (1988 : 91% de surfaces boisées, 2018 : 47%) au profit des champs (de 9 à 52%) et des zones construites (de 0.02 à 0.53%). La diversité du paysage a augmenté régulièrement entre 1988, et la fragmentation a globalement doublé, atteignant un sommet en 2008.

Plusieurs études ont été implémentées entre 2017 et 2020 afin de comprendre les facteurs démographiques et comportementaux liés à l'exposition au paludisme. Nous avons pu confirmer que les hommes sont plus affectés que les femmes, que l'utilisation de protection anti-moustique est extrêmement limitée en dehors du foyer, et que des facteurs tels que la couverture forestière du village de résidence ou l'âge impacte le risque de paludisme. Cependant, certains résultats ne correspondaient pas au consensus. Les suivis avec système de géolocalisation (GPS) enregistrant les mouvements des populations à risque ont révélé un temps passé en forêt similaire entre hommes et femmes, et que le temps enregistré en forêt,

qu'il soit durant la nuit (quand les vecteurs sont le plus actifs) ou faible allure (quand le participant est à pied et plus exposé aux piqûres), n'étaient pas associé au risque de paludisme.

Pendant ces suivis, nous avons pu évaluer la validité de certaines méthodes jusque-là peu implémentées au Cambodge. La quantification des anticorps anti-salive d'Anophèles par essai d'immuno-absorption enzymatique (ELISA) s'est avérée robuste pour quantifier l'exposition aux vecteurs ; cependant, en conséquence de la baisse du paludisme, concomitante à la baisse de prévalence chez les moustiques, la méthode ne semble pas adaptée à déterminer l'exposition au paludisme. Les appareils GPS ont mis l'accent sur les désavantages des questionnaires, jusque-là systématiquement utilisés au Cambodge pour caractériser la mobilité d'un participant, ainsi que leurs propres désavantages. Tout comme les ELISA, les appareils GPS n'ont pas pu déterminer les zones ou type d'environnements liés à une exposition au paludisme, probablement à cause de nombreux cas de *P. vivax*, potentiellement des reviviscences, qui proviendraient d'une piqûre infectante ayant eu lieu des semaines ou des mois auparavant.

Une première collecte de moustique a permis de collecter 3,920 Anophèles femelles au cours de la saison humide de 2017 et la saison sèche de 2017-2018. Des pièges à double moustiquaire furent installés par paires, chacune avec un appât humain et un bovin, pour 13 sites, répartis entre forêt profonde (N = 4), forêt proche du village (N = 3), plantations d'hévéa (N = 3) et village (N = 3). La prévalence était de 3.6%, composée principalement d'infections de *P. vivax*. Les femelles étaient principalement zoophages ou généralistes, y compris *An. dirus*. Quatre différentes espèces présentaient des mutations associées à des résistances aux insecticides.

Une collecte de moustique organisée sur trois périodes (un en saison sèche et deux en saison des pluies) et dans plusieurs environnements a permis de mieux déterminer l'écologie des vecteurs du paludisme au Cambodge via l'analyse de l'abondance, la diversité et la prévalence. Trente-sept sites répartis entre villages (N = 7), champs (N = 9), plantations (N = 11) et forêt (N = 10) ont permis la collecte de 6,690 femelles, dont 5,389 femelles capturées par pièges à double moustiquaire sur lesquelles se basent les analyses. Plus de 99% des femelles infectées l'étaient par *P. vivax* ou une infection mixte incluant *P. vivax*. Les prévalences les plus élevées ont été détectées parmi les femelles collectées en forêt pour les

mois de Janvier-Février ($5.13 \pm 0.22\%$) et de Juillet-Août ($3.05 \pm 0.04\%$) mais contre toute attente dans les champs pour les mois d'Octobre-Novembre ($5.35 \pm 0.05\%$). Aucun moustique positif ne fut capturé en village. Vingt-quatre espèces furent identifiées morphologiquement et 29 avec des méthodes moléculaires ($N = 1,252$). Les pièges à interception installés à proximité de parcelles de forêt de 6 villages ont permis la capture de 1,108 femelles se déplaçant entre les villages et les parcelles. Les Anophèles préféraient se gorger sur les vaches au mois d'Octobre-Novembre et les buffles au mois de Juillet-Août. Le reste des repas de sang était d'origine humaine, et aucune femelle gorgée ne fut collectée pendant la saison sèche. Les larves d'Anophèles étaient associées aux habitats aquatiques à l'eau trouble, aux températures élevées, avec des bords profonds et partagés avec d'autres genres de moustiques. Bien qu'*An. dirus* représentait plus de 40% des femelles adultes collectées, aucune larve d'*An. dirus* ne fut collectée.

Bien que peu implémentés au Cambodge, les pièges à double moustiquaire ont permis de capturer davantage d'Anophèles que la référence de la capture sur appât humain. La variation de densité relative et de prévalence ne différait pas de celles de la référence, sans exposer les collecteurs aux piqûres. Les résultats suggèrent que l'exposition aux vecteurs et le risque associé d'une piqûre infectieuse pourrait alterner entre différents environnements en fonction de la saison, avec les champs abritant une concentration de vecteurs infectieux sous-estimée par le consensus scientifique actuel.

L'implémentation d'outils moléculaires comme le séquençage profond d'amplicons permet de comprendre la répartition et la transmission de clones de *Plasmodium* entre différentes populations. L'utilisation de ces méthodes au Cambodge est récente et généralement restreinte aux cas symptomatiques. À partir d'échantillons du terrain provenant de la zone d'étude, en utilisant une RTPCR ciblant le cytochrome b des parasites, nous avons d'une part confirmé la présence de *P. falciparum* et *P. vivax* et d'autre part estimé la concentration en ADN via la valeur de cycle seuil. En créant une librairie d'amplicons basée sur cinq marqueurs moléculaires de *P. falciparum* (*ama1-d3*, *cpmp*, *cpp*, *csp* et *mSP7*), nous sommes parvenus à amplifier entre $56.9 \pm 1.5\%$ (*csp*) et $72.3 \pm 1.3\%$ (*cpp*) d'échantillons selon le marqueur. Après séquençage via la plateforme Illumina MiSeq, $83.4 \pm 5.1\%$ des amplicons produits atteignaient 10,000 *reads*, considéré comme le seuil minimal pour la détection de clones minoritaires. *Ama1-d3* était le seul marqueur pour lequel une concentration initiale d'ADN élevé favorisait

une sortie valide. Les échantillons de *P. vivax* furent amplifiés avec huit marqueurs, pour lesquels l'amplification variait entre $22.6 \pm 5.8\%$ et $44.1 \pm 7\%$ selon le marqueur moléculaire. Les résultats de séquençage de la librairie ne sont pas encore disponibles. L'amplification des échantillons et leur séquençage représentaient un processus lent, coûteux et exigeant de nombreux ajustements. La plateforme d'analyse HaplotypR n'a pour l'instant pas permis d'obtenir de résultats concluants quant aux haplotypes présents dans les échantillons ou à la structure des populations parasites.

Discussion

L'implémentation et la validation de nouvelles méthodes est difficile. D'après le consensus de la littérature, nous savions que l'importance de l'échelle ne devait pas être négligée lors des suivis et des analyses. Les paramètres des appareils, le nombre de participants à suivre, la durée des suivis étaient tout autant de variables pour lesquelles nous devions expérimenter, car des recherches similaires étaient limitées ou inexistantes au Cambodge. L'investissement dans les pièges à double moustiquaire s'est avéré récompensé par des résultats encourageants, mais l'implémentation de méthodes supplémentaires au travers de différentes zones d'étude pourrait aider à mieux caractériser l'écologie des vecteurs du paludisme. Trouver la méthodologie adaptée à une hypothèse peut être un procédé long et fastidieux, pour lequel plusieurs tentatives peuvent s'avérer nécessaires. Tout particulièrement, rechercher et valider une méthodologie lors de recherche observationnelle prend du temps et exige des efforts, en conséquence des ressources et préparations nécessaires, de l'implication des participants et des collecteurs, du traitement des données, et des analyses requises pour obtenir un résultat, même préliminaire.

Pour la comprendre, l'analyse de la diversité des populations de vecteurs et des comportements requière des outils à résolution très fine. A la vue de la faible prévalence désormais observée au Cambodge à la fois dans les populations à risque et les populations vectorielles, la détermination des comportements et de zones à risque se complexifie. Pour compliquer davantage le sujet, l'environnement que nous observons change très rapidement : les résultats de ce projet de thèse provenant d'échantillons collectés entre 2017 et 2020 pourraient devenir graduellement obsolète dans les prochaines années. L'anthropisation augmente au travers de nombreux indicateurs, et la relation entre l'environnement et la dynamique du paludisme pourrait être en cours de changement. En

effet, si pour l'instant les effets de l'anthropisation semblent associés à une baisse de la prévalence au niveau national, cette relation pourrait s'inverser dans le futur. Aussi, les potentiels facteurs de confusion sont nombreux, et l'on pourrait tout aussi bien assister seulement aux effets de la campagne d'élimination.

Conclusion

Seulement une approche transdisciplinaire, basée sur la coopération des acteurs publics et privés, permettra la compréhension de la transmission du paludisme au Cambodge. Dans un contexte d'élimination mais aussi de changements anthropogéniques rapides et radicaux, saisir les causes et le contexte de la transmission représente un défi. Les résultats de ce projet indiquent une variation très fine au travers de différences comportementales dans la population à risque et de l'écologie des vecteurs. Bien que la prévalence soit en baisse, un effort se doit d'être maintenu pour identifier, comprendre et cibler les derniers cas de paludisme, notamment le réservoir asymptomatique.

Références

1. WHO. World Malaria Report 2021. Geneva; 2021.
2. Lowassa A, Mazigo HD, Mahande AM, Mwang'onde BJ, Msangi S, Mahande MJ, et al. Social economic factors and malaria transmission in Lower Moshi, Northern Tanzania. *Parasit Vectors*. 2012;5:129.
3. Ahmed SM, Haque R, Haque U, Hossain A. Knowledge on the transmission, prevention and treatment of malaria among two endemic populations of Bangladesh and their health-seeking behaviour. *Malaria Journal*. 2009;8:173.
4. Martin JA, Hendershot AL, Saá Portilla IA, English DJ, Woodruff M, Vera-Arias CA, et al. Anopheline and human drivers of malaria risk in northern coastal, Ecuador: a pilot study. *Malaria Journal*. 2020;19:354.
5. Manh BH, Clements ACA, Thieu NQ, Hung NM, Hung LX, Hay SI, et al. Social and environmental determinants of malaria in space and time in Viet Nam. *Int J Parasitol*. 2011;41:109–16.
6. Kheang ST, Por I, Sovannaroeth S, Dysoley L, Chea H, Po L, et al. Cambodia malaria indicator survey 2020: Implications for malaria elimination. *Malaria World J*. 2021;12:5.
7. Palafox B, Patouillard E, Tougher S, Goodman C, Hanson K, Kleinschmidt I, et al. Understanding private sector antimalarial distribution chains: a cross-sectional mixed methods study in six malaria-endemic countries. *PLoS ONE*. 2014;9:e93763.

8. Kounnavong S, Gopinath D, Hongvanthong B, Khamkong C, Sichanthongthip O. Malaria elimination in Lao PDR: the challenges associated with population mobility. *Infectious Diseases of Poverty*. 2017;6:81.
9. Chang H-H, Wesolowski A, Sinha I, Jacob CG, Mahmud A, Uddin D, et al. Mapping imported malaria in Bangladesh using parasite genetic and human mobility data. *Elife*. 2019;8.
10. Gryseels C, Peeters Grietens K, Dierickx S, Xuan XN, Uk S, Bannister-Tyrrell M, et al. High Mobility and Low Use of Malaria Preventive Measures among the Jarai Male Youth along the Cambodia–Vietnam Border. *Am J Trop Med Hyg*. 2015;93:810–8.
11. Searle KM, Lubinda J, Hamapumbu H, Shields TM, Curriero FC, Smith DL, et al. Characterizing and quantifying human movement patterns using GPS data loggers in an area approaching malaria elimination in rural southern Zambia. *R Soc Open Sci*. 2017;4.
12. Lee H, Halverson S, Ezinwa N. Mosquito-Borne Diseases. *Prim Care*. 2018;45:393–407.
13. Lindblade KA, Steinhardt L, Samuels A, Kachur SP, Slutsker L. The silent threat: asymptomatic parasitemia and malaria transmission. *Expert Review of Anti-infective Therapy*. 2013;11:623–39.
14. Dayananda KK, Achur RN, Gowda DC. Epidemiology, drug resistance, and pathophysiology of *Plasmodium vivax* malaria. *J Vector Borne Dis*. 2018;55:1–8.
15. Veaux A. Cycle de vie du *Plasmodium falciparum* et symptômes chez l'humain. 2019.
16. Sinka ME. Global Distribution of the Dominant Vector Species of Malaria. *Anopheles mosquitoes - New insights into malaria vectors*. 2013. <https://doi.org/10.5772/54163>.
17. Saavedra MP, Conn JE, Alava F, Carrasco-Escobar G, Prussing C, Bickersmith SA, et al. Higher risk of malaria transmission outdoors than indoors by *Nyssorhynchus darlingi* in riverine communities in the Peruvian Amazon. *Parasit Vectors*. 2019;12:374.
18. Multini LC, Wilke ABB, Marrelli MT. Neotropical *Anopheles* (*Kerteszia*) mosquitoes associated with bromeliad-malaria transmission in a changing world. *Acta Tropica*. 2020;:105413.
19. Sinka ME, Bangs MJ, Manguin S, Rubio-Palis Y, Chareonviriyaphap T, Coetzee M, et al. A global map of dominant malaria vectors. *Parasites & Vectors*. 2012;5:69.
20. Hii J, Hustedt J, Bangs MJ. Residual Malaria Transmission in Select Countries of Asia-Pacific Region: Old Wine in a New Barrel. *J Infect Dis*. 2021;223 Supplement_2.
21. St Laurent B, Burton TA, Zubaidah S, Miller HC, Asih PB, Baharuddin A, et al. Host attraction and biting behaviour of *Anopheles* mosquitoes in South Halmahera, Indonesia. *Malar J*. 2017;16:310.
22. Afrane YA, Zhou G, Lawson BW, Githeko AK, Yan G. Effects of microclimatic changes caused by deforestation on the survivorship and reproductive fitness of *Anopheles gambiae* in western Kenya highlands. *Am J Trop Med Hyg*. 2006;74:772–8.

23. Yasuoka J, Levins R. Impact of deforestation and agricultural development on anopheline ecology and malaria epidemiology. *Am J Trop Med Hyg.* 2007;76:450–60.
24. Olkeba BK, Goethals PLM, Boets P, Duchateau L, Degefa T, Eba K, et al. Mesocosm Experiments to Quantify Predation of Mosquito Larvae by Aquatic Predators to Determine Potential of Ecological Control of Malaria Vectors in Ethiopia. *Int J Environ Res Public Health.* 2021;18:6904.
25. Collins CM, Bonds J a. S, Quinlan MM, Mumford JD. Effects of the removal or reduction in density of the malaria mosquito, *Anopheles gambiae* s.l., on interacting predators and competitors in local ecosystems. *Med Vet Entomol.* 2019;33:1–15.
26. Laporta GZ, Lopez de Prado PIK, Kraenkel RA, Coutinho RM, Sallum MAM. Biodiversity can help prevent malaria outbreaks in tropical forests. *PLoS Negl Trop Dis.* 2013;7:e2139.
27. Ostfeld RS. A Candide response to Panglossian accusations by Randolph and Dobson: biodiversity buffers disease. *Parasitology.* 2013;140:1196–8.
28. Austin KF, Bellinger MO, Rana P. Anthropogenic forest loss and malaria prevalence: a comparative examination of the causes and disease consequences of deforestation in developing nations. *AIMS Environmental Science.* 2017;4:217.
29. Lindblade KA, Walker ED, Onapa AW, Katungu J, Wilson ML. Land use change alters malaria transmission parameters by modifying temperature in a highland area of Uganda. *Trop Med Int Health.* 2000;5:263–74.
30. Kweka EJ, Kimaro EE, Munga S. Effect of Deforestation and Land Use Changes on Mosquito Productivity and Development in Western Kenya Highlands: Implication for Malaria Risk. *Front Public Health.* 2016;4.
31. Tadei WP, Thatcher BD, Santos JM, Scarpassa VM, Rodrigues IB, Rafael MS. Ecologic observations on anopheline vectors of malaria in the Brazilian Amazon. *The American Journal of Tropical Medicine and Hygiene.* 1998;59:325–35.
32. Vittor AY, Gilman RH, Tielsch J, Glass G, Shields T, Lozano WS, et al. The effect of deforestation on the human-biting rate of *Anopheles darlingi*, the primary vector of *Falciparum malaria* in the Peruvian Amazon. *Am J Trop Med Hyg.* 2006;74:3–11.
33. Rerolle F, Dantzer E, Lover AA, Marshall JM, Hongvanthong B, Sturrock HJ, et al. Spatio-temporal associations between deforestation and malaria incidence in Lao PDR. *eLife.* 2021;10:e56974.
34. Vanwambeke SO, Lambin EF, Eichhorn MP, Flasse SP, Harbach RE, Oskam L, et al. Impact of Land-use Change on Dengue and Malaria in Northern Thailand. *EcoHealth.* 2007;4:37–51.
35. Charlwood JD, Nenhep S, Protopopoff N, Sovannaroth S, Morgan JC, Hemingway J. Effects of the spatial repellent metofluthrin on landing rates of outdoor biting anophelines in Cambodia, Southeast Asia. *Medical and Veterinary Entomology.* 2016;30:229–34.

36. Sukkanon C, Nararak J, Bangs MJ, Hii J, Chareonviriyaphap T. Behavioral responses to transfluthrin by *Aedes aegypti*, *Anopheles minimus*, *Anopheles harrisoni*, and *Anopheles dirus* (Diptera: Culicidae). *PLoS ONE*. 2020;15:e0237353.
37. Benelli G, Beier JC. Current vector control challenges in the fight against malaria. *Acta Trop*. 2017;174:91–6.
38. Maia MF, Tenywa FC, Nelson H, Kambagha A, Ashura A, Bakari I, et al. Attractive toxic sugar baits for controlling mosquitoes: a qualitative study in Bagamoyo, Tanzania. *Malaria Journal*. 2018;17:22.
39. Shaw WR, Catteruccia F. Vector biology meets disease control: using basic research to fight vector-borne diseases. *Nat Microbiol*. 2019;4:20–34.
40. Dupuy S, Herbreteau V, Feyfant T, Morand S, Tran A. Land-cover dynamics in Southeast Asia: contribution of object-oriented techniques for change detection. 4th International Conference on GEographic Object-Based Image Analysis (GEOBIA). 2012;:217–22.
41. Davis K, Yu K, Rulli MC, Pichdara L, D’Odorico P. Accelerated deforestation driven by large-scale land acquisitions in Cambodia. *Nat Geosci*. 2015;8:772–5.
42. Thriemer K, Bobogare A, Ley B, Gudo CS, Alam MS, Anstey NM, et al. Quantifying primaquine effectiveness and improving adherence: a round table discussion of the APMEN Vivax Working Group. *Malaria Journal*. 2018;17:241.
43. Gryseels C, Kuijpers LMF, Jacobs J, Grietens KP. When ‘substandard’ is the standard, who decides what is appropriate? Exploring healthcare provision in Cambodia. *Crit Public Health*. 2019;29:460–72.
44. Hoyer S, Nguon S, Kim S, Habib N, Khim N, Sum S, et al. Focused Screening and Treatment (FSAT): A PCR-Based Strategy to Detect Malaria Parasite Carriers and Contain Drug Resistant *P. falciparum*, Pailin, Cambodia. *PLOS ONE*. 2012;7:e45797.
45. Durnez L, Pareyn M, Mean V, Kim S, Khim N, Menard D, et al. Identification and characterization of areas of high and low risk for asymptomatic malaria infections at sub-village level in Ratanakiri, Cambodia. *Malaria Journal*. 2018;17:27.
46. Sinka ME, Bangs MJ, Manguin S, Chareonviriyaphap T, Patil AP, Temperley WH, et al. The dominant *Anopheles* vectors of human malaria in the Asia-Pacific region: occurrence data, distribution maps and bionomic précis. *Parasites & Vectors*. 2011;4:89.
47. Edwards HM, Sriwichai P, Kirabittir K, Prachumsri J, Chavez IF, Hii J. Transmission risk beyond the village: entomological and human factors contributing to residual malaria transmission in an area approaching malaria elimination on the Thailand–Myanmar border. *Malaria Journal*. 2019;18:221.
48. Edwards HM, Chinh VD, Le Duy B, Thanh PV, Thang ND, Trang DM, et al. Characterising residual malaria transmission in forested areas with low coverage of core vector control in central Viet Nam. *Parasites & Vectors*. 2019;12:454.

49. St. Laurent B, Oy K, Miller B, Gasteiger EB, Lee E, Sovannaroth S, et al. Cow-baited tents are highly effective in sampling diverse *Anopheles* malaria vectors in Cambodia. *Malar J.* 2016;15.
50. Okami S, Kohtake N. Spatiotemporal Modeling for Fine-Scale Maps of Regional Malaria Endemicity and Its Implications for Transitional Complexities in a Routine Surveillance Network in Western Cambodia. *Front Public Health.* 2017;5:262.
51. Tripura R, Peto TJ, Veugen CC, Nguon C, Davoeung C, James N, et al. Submicroscopic *Plasmodium* prevalence in relation to malaria incidence in 20 villages in western Cambodia. *Malaria Journal.* 2017;16:56.
52. Bannister-Tyrrell M, Srun S, Sluydts V, Gryseels C, Mean V, Kim S, et al. Importance of household-level risk factors in explaining micro-epidemiology of asymptomatic malaria infections in Ratanakiri Province, Cambodia. *Sci Rep.* 2018;8:11643.
53. Incardona S, Vong S, Chiv L, Lim P, Nhem S, Sem R, et al. Large-scale malaria survey in Cambodia: Novel insights on species distribution and risk factors. *Malar J.* 2007;6:37.
54. Sluydts V, Heng S, Coosemans M, Van Roey K, Gryseels C, Canier L, et al. Spatial clustering and risk factors of malaria infections in Ratanakiri Province, Cambodia. *Malar J.* 2014;13:387.
55. Kar NP, Kumar A, Singh OP, Carlton JM, Nanda N. A review of malaria transmission dynamics in forest ecosystems. *Parasit Vectors.* 2014;7:265.
56. Peeters Grietens K, Gryseels C, Dierickx S, Bannister-Tyrrell M, Trienekens S, Uk S, et al. Characterizing Types of Human Mobility to Inform Differential and Targeted Malaria Elimination Strategies in Northeast Cambodia. *Sci Rep.* 2015;5:16837.
57. Taffon P, Rossi G, Kindermans J-M, Van den Bergh R, Nguon C, Debackere M, et al. "I could not join because I had to work for pay.": A qualitative evaluation of falciparum malaria pro-active case detection in three rural Cambodian villages. *PLoS ONE.* 2018;13:e0195809.
58. Sanann N, Peto TJ, Tripura R, Callery JJ, Nguon C, Bui TM, et al. Forest work and its implications for malaria elimination: a qualitative study. *Malaria Journal.* 2019;18:376.
59. Thomson R, Sochea P, Sarath M, MacDonald A, Pratt A, Poyer S, et al. Rubber plantations and drug resistant malaria: a cross-sectional survey in Cambodia. *Malar J.* 2019;18:379.
60. Parker DM, Tripura R, Peto TJ, Maude RJ, Nguon C, Chalk J, et al. A multi-level spatial analysis of clinical malaria and subclinical *Plasmodium* infections in Pailin Province, Cambodia. *Heliyon.* 2017;3:e00447.
61. Das S, Peck RB, Barney R, Jang IK, Kahn M, Zhu M, et al. Performance of an ultra-sensitive *Plasmodium falciparum* HRP2-based rapid diagnostic test with recombinant HRP2, culture parasites, and archived whole blood samples. *Malaria Journal.* 2018;17:118.

62. Yeung S, McGregor D, James N, Kheang ST, Kim S, Khim N, et al. Performance of Ultrasensitive Rapid Diagnostic Tests for Detecting Asymptomatic Plasmodium falciparum. *Am J Trop Med Hyg.* 2020;102:307–9.
63. Cui L, Yan G, Sattabongkot J, Cao Y, Chen B, Chen X, et al. Malaria in the Greater Mekong Subregion: heterogeneity and complexity. *Acta Trop.* 2012;121:227–39.
64. Bancone G, Menard D, Khim N, Kim S, Canier L, Nguong C, et al. Molecular characterization and mapping of glucose-6-phosphate dehydrogenase (G6PD) mutations in the Greater Mekong Subregion. *Malaria Journal.* 2019;18:20.

List of Figures

Figure 1: Countries with indigenous cases in 2000 and their status in 2020.	9
Figure 2: Life cycle of <i>Plasmodium falciparum</i>	11
Figure 3: A global map showing the distribution of the 34 dominant malaria vectors	13
Figure 4: Twelve species of malaria vectors during a blood meal.	14
Figure 5: Distribution of 16 of the 19 dominant malaria vectors in the Asian-Pacific region.	16
Figure 6: An East African salticid <i>Evarcha culicivora</i> feeding on a blood-fed mosquito.	17
Figure 7: Cambodia and the Great Mekong Subregion.	23
Figure 8: Regional map of malaria incidence in the GMS between 2012 and 2019.	25
Figure 9: Cambodia and all 285 documented ELCs	27
Figure 10: A patch of land being burned during dry season.	28
Figure 11: A forest gradually cleared by the slash-and-burn method.	28
Figure 12: Resistance of malaria vectors to pyrethroids in the GMS (2010-2019).	30
Figure 13: A temporary settlement at a cassava field.	32
Figure 14: A forest goer motorbike loaded with wood.	33
Figure 15: A forest goer building speed with the help of a co-worker.	34
Figure 16: Two GPS devices collected after a few days in the field.	153
Figure 17: Count of mosquitoes collected in CDC light traps	215
Figure 18: Relation between RTPCR Ct and adapter PCR amplification	228
Figure 19: Reads per marker per sample.	229
Figure 20: RTPCR Ct and number of reads per sample per marker.	230
Figure 21: Relation between RTPCR Ct and validity of reads after sequencing	231
Figure 22: Quality profiles of the MiSeq reads from the cohort samples.	232
Figure 23: Quality profiles of the MiSeq reads from the GPS follow-up samples.	233
Figure 24: The environment, the hosts, the vectors and the parasites.	243

List of Tables

Table 1: Twenty-three widely used landscape metrics	44
Table 2: All identified <i>Plasmodium</i> species	218
Table 3: Description of the 5 AmpSeq molecular markers	224
Table 4: Required steps to prepare AmpSeq libraries.	225
Table 5: RTPCR results with Ct details of the <i>P. falciparum</i> samples.	227
Table 6: Percentage of samples amplified with adapter PCR per marker	227
Table 7: GLMs results testing the relation between RTPCR Ct and adapter PCR	228
Table 8: Reads per sample per marker	229
Table 9: GLMs results testing the relation between RTPCR Ct	230
Table 10: Description of the 13 <i>P. vivax</i> AmpSeq molecular markers.	234
Table 11: Percentage of samples amplified with adapter PCR per marker	235
Table 12: Percentage of samples amplified by at least one molecular marker	236

List of Appendices

Appendix 1: REDCap form used to describe breeding sites.	269
---	-----

List of Abbreviations

ACT: Artemisinin-based Combination Therapy

AmpSeq: Deep Amplicon Sequencing

CDC: Centers for Disease Control and Prevention

CI: Confidence Interval

CO₂: Carbon Dioxide

CPMP: Conserved *Plasmodium* Membrane Protein

CSP: Circumsporozoite Protein

DNA: Deoxyribonucleic Acid

DDT: Dichlorodiphenyltrichloroethane

ELC: Economic Land Concession

FAO: Food and Agriculture Organization of the United Nations

FSAT: Focal Screening And Treatment

GIS: Geographic Information System

GLM: Generalised Linear Model

GLMM: Generalised Linear Mixed Model

GMS: Great Mekong Subregion

GPS: Global Positioning System

gSG6: *An. gambiae* Salivary Gland Protein 6

HBR: Human Biting Rate

HDN: Human-Baited Double Net

H_e: Expected Heterozygosity

HLC: Human Landing Catch

HRP2: Histidine Rich Protein 2

IgG: Immunoglobulin G

IPT: Intermittent Preventive Treatment
IRS: Indoor Residual Spraying
ITN: Insecticide-Treated bed Net
LLIN: Long-Lasting Insecticidal Net
MDA: Mass Drug Administration
MMW: Mobile Malaria Workers
MSAT: Mass Screening And Treatment
MSP: Merozoite Surface Protein
OD: Optical Density
OR: Odd Ratio
PCR: Polymerase Chain Reaction
RDT: Rapid Diagnosis Test
RMT: Residual Malaria Transmission
RTPCR: Real-Time Polymerase Chain Reaction
SCR: Barrier Screen Trap
SEA: South-East Asia
SEM: Serological Exposure Markers
SHDI: Shannon's Diversity Index
SIDI: Simpson's diversity Index
SNP: Single-Nucleotide Polymorphism
SOP: Standard Operation Procedure
VMW: Village Malaria Workers
WHO: World Health Organisation

Part I: General introduction

A. Malaria in a nutshell

The word malaria comes from “mal’aria”, Italian for “bad air” deriving from the first impressions that the disease was associated with marshes. Evidence of malaria infection in humans dates back as early as Ancient Egypt [1], implying that the disease has affected humanity for at least three millennia [2]. Charles Louis Alphonse Laveran made the first observation of parasites in the blood of a patient suffering from malaria during the 19th century. Later, Ronald Ross understood that the disease was mosquito-borne, which was then confirmed by Giovanni Battista Grassi in 1898, who demonstrated that *Anopheles* mosquitoes were the vector.

A.1. Biology and epidemiology of the disease

Malaria is a life-threatening, mosquito-borne infectious disease caused by the parasite *Plasmodium*, transmitted to humans by the infectious bite of a female *Anopheles*. The World Health Organisation (WHO) estimated a total of 241 million cases of malaria and 627,000 related fatalities in 2020 [3]. The disease affects mostly low-human development index populations living in tropical and subtropical regions [3].

A.1.1. Incidence

Most would call the 2000s and early 2010s a stage of progress. Indeed, due to increased funding, vector control strategies and an improvement of health systems and their cooperation, malaria burden has globally decreased. Elimination has become the realistic objective of numerous nations for a not-so-distant future. Indeed, between 2000 and 2015, malaria case incidence around the world declined by 27% and a dozen countries managed to obtain the WHO malaria-free status since 2000 (Figure 1) [3].

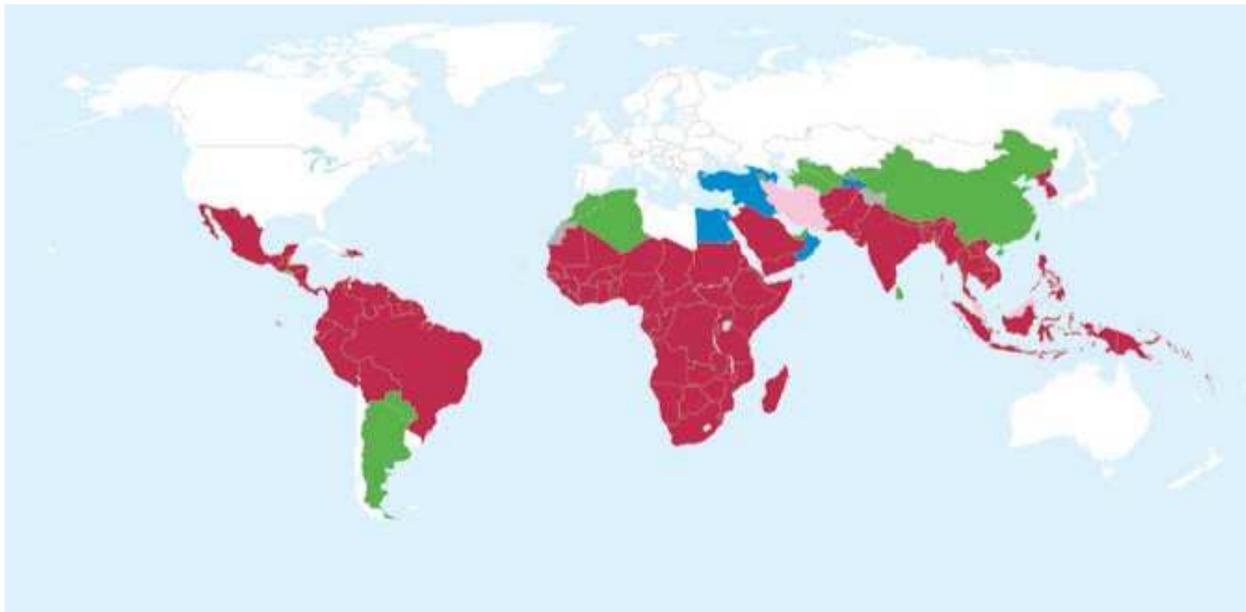


Figure 1: Countries with indigenous cases in 2000 and their status in 2020. To obtain the WHO certification of malaria elimination, a country must have zero indigenous cases for the past three consecutive years [3].

However, between 2015 and 2019, malaria case incidence around the world decreased by only 2% and many countries still harbour indigenous cases, with some territories being on the rise, like Papua New Guinea, Bolivia or the Philippines (>25% malaria case incidence increase since 2015), Sudan, Panama or Colombia (>40% increase) [3]. Malaria epidemiology has become more and more complex, with an augmentation of imported and geographically clustered cases, a rising incidence especially among male, adult and migrant, hard-to reach populations, for which classic control methods are outdated [4].

The host socio-economic status, demographic category and behaviour affect their potential exposure to malaria. Lower education [5–7] or wealth [7–9] and household characteristics [5, 9, 10] have all been associated to heightened malaria exposure. Additionally, social habits [6, 8, 11, 12], treatment and mosquito protection usage [6, 8, 13], mobility [11, 14–17] and knowledge about malaria transmission [11, 13] affect host exposure to the disease.

Malaria, as a mosquito-borne disease, is transmitted between vertebrate and invertebrate hosts through a complex life cycle involving different stages such as proliferation, asexual and sexual reproduction, dormancy and invasion. The environment must provide the opportunity

for them to meet for the transmission to happen, in an acceptable range of biotic and abiotic conditions to grant both the survival and the success of the hosts and the parasites.

A.1.2. The life cycle

Malaria parasites life cycle involves two hosts: an insect and a vertebrate (Figure 2). In medical terms, the invertebrate host is usually called the vector.

The infection of the vertebrate host occurs during a blood meal from an infected female *Anopheles* mosquito, injecting sporozoites into the skin of the human host. The parasites pass through the blood stream and then reach the liver to start the exo-erythrocytic cycle. They infect the hepatocytes and, for most species, start the initial replication: they mature into schizonts, exit the liver as merozoites then burst and release merozoites. Then starts the asexual multiplication in the bloodstream: the merozoites infect the red blood cells, and mature into rings, which can differentiate either into mature trophozoites for asexual development or gametocytes enabling transmission. Mature trophozoites develop into schizonts that burst and release new merozoites. A female *Anopheles* mosquito ingests male and female gametocytes during a blood meal, and the gametes produce zygotes. They develop into an ookinete, which matures into an oocyst in the mosquito midgut to finally rupture and release sporozoites, migrating to the salivary glands, ready for an inoculation and the perpetuation of the malaria life cycle. *Plasmodium vivax* and *Plasmodium ovale* sporozoites, rather than directly maturing into schizonts upon entering the liver, can differentiate into an undetectable dormant stage, the hypnozoite. Hypnozoites can be dormant for weeks to years and their reactivation into schizonts induce relapses.

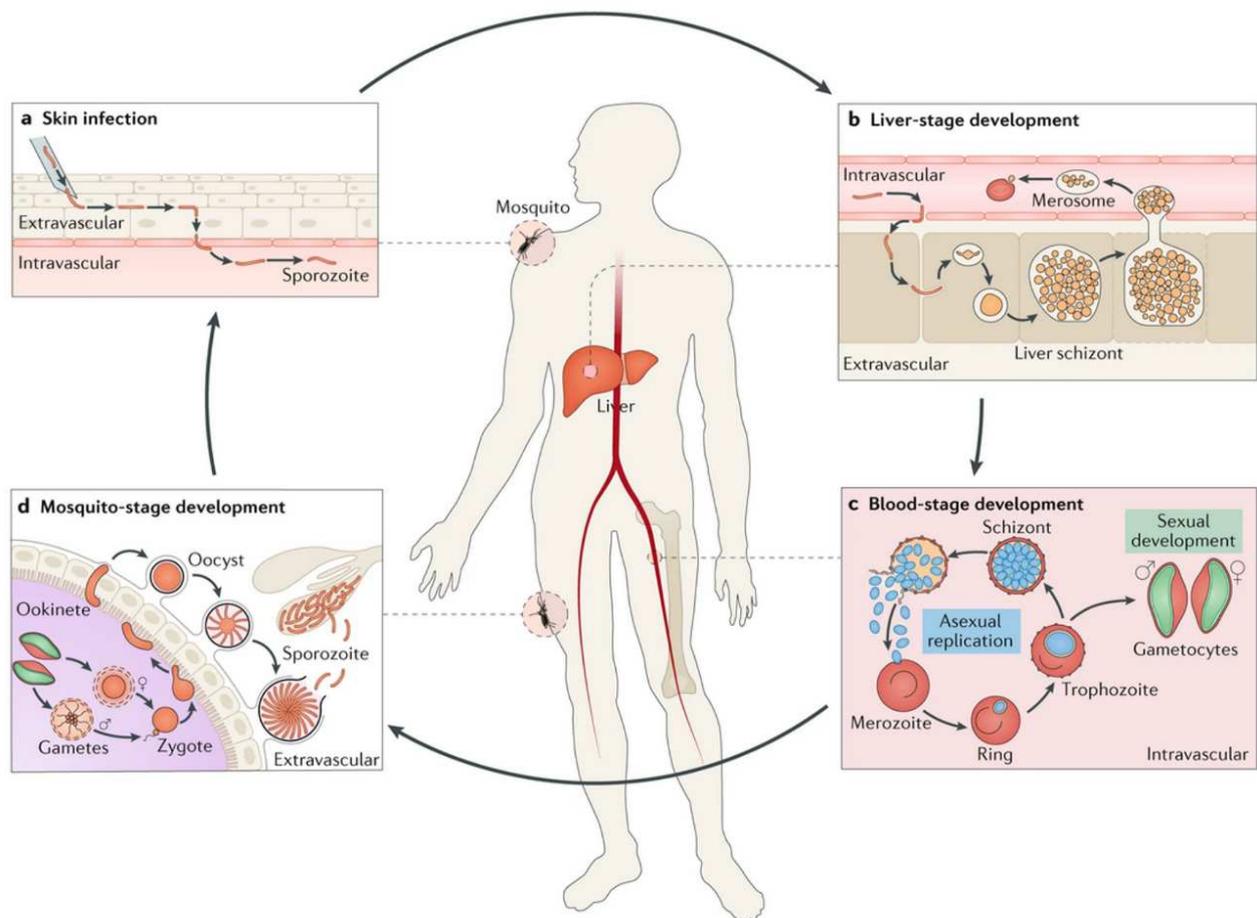


Figure 2: Life cycle of *Plasmodium falciparum* In humans and in female *Anopheles* mosquitoes [18].

A.1.3. The host

Malaria is an acute febrile illness. The asexual development of *Plasmodium* spp. during blood phase induces symptoms including fever, chills, aches, nausea, vomiting and diarrhoea. Severe symptoms can include seizures, coma, cerebral malaria and multi-organ failure [3, 19]. Infection severity depends on immunity and condition, with children under the age of five years old especially at risk of anaemia and accounting for 67% of all malaria-related deaths worldwide [3, 20]. Primiparous women and non-immune individuals are also at higher risk of severe infections [3, 20].

Infection can as well be asymptomatic or paucisymptomatic. As a consequence of extended malaria exposure, individuals can show evidence of clinical immunity, especially in patients with multiple, successive infections – even though this immunity remains partial [21]. Although poorly understood, *P. vivax* acquired immunity appears correlated to age and

exposure intensity in both high [22] and low [23] transmission settings, and can be acquired faster than *P. falciparum* immunity [24].

A.1.4. The parasite

Plasmodium spp. is a genus of parasitic unicellular prokaryotes belonging to the phylum Apicomplexa. Five species can infect and multiply in humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*.

Plasmodium falciparum and *P. vivax*, the two most common species, are responsible for the most severe cases [3]. In 2018, the WHO estimated that *P. falciparum* constituted 99.7% of malaria cases in Africa, 71% of cases in the Eastern Mediterranean, 65% in the Western Pacific and 50% of cases in South-East Asia [3]. *Plasmodium vivax* remains the predominant parasite in the Americas, accounting for an estimated 75% of malaria cases in 2018 [3].

The real incidence of *P. ovale* and *P. malariae* is most probably unknown, as their identification by microscopy or by rapid diagnostic test (RDT) is not optimal [4, 25–27]. The incidence of both *P. malariae* and *P. ovale* was more important than expected when using PCR detection in several locations of Sub-Saharan Africa [28, 29] and in Asia [30]. The proper identification of *P. ovale* cases remains complicated because of its undetectable dormant stage. *Plasmodium malariae* and *P. ovale* infections rarely lead to severe complications, so public health structure invest limited efforts into their detection and treatment [31, 32].

Plasmodium knowlesi, maintained by macaque reservoirs, was considered as a mostly simian malaria parasite until recently – reviews considered the possibilities of transmission to human “unclear” in 2013 [4] or that the parasite “may infect human with a non-episodic frequency” in 2010 [33]. However, in the last few years, this species have been reported within several locations and might be considered widespread in some areas of SEA [34]. The infection can be easily mistaken for *P. malariae* due to their morphological similitudes, but, unlike *P. malariae*, *P. knowlesi* can lead to fatal cases. While human malaria parasites incidence is decreasing in Malaysia and the country is attaining pre-elimination, *P. knowlesi* reported incidence has increased and, with the decrease of *P. vivax* and *P. falciparum* occurrence, accounts now for the majority of human malaria cases [35]. Different studies disclosed high prevalence of *P. knowlesi*, notably in Malaysian Borneo, and support the idea that the species remains dangerously neglected [34, 36–39].

A.1.5. The vector

From the 465 described *Anopheles* species, around 70 of them are confirmed malaria vectors able to transmit the disease to humans, 41 of which being of major importance [40] (Figure 3). Still, more than 50 members of species complexes are yet to be formally named and recognised [40]. The abundance, ecology and diversity of vectors vary greatly from one region to another [41] (Figure 4).

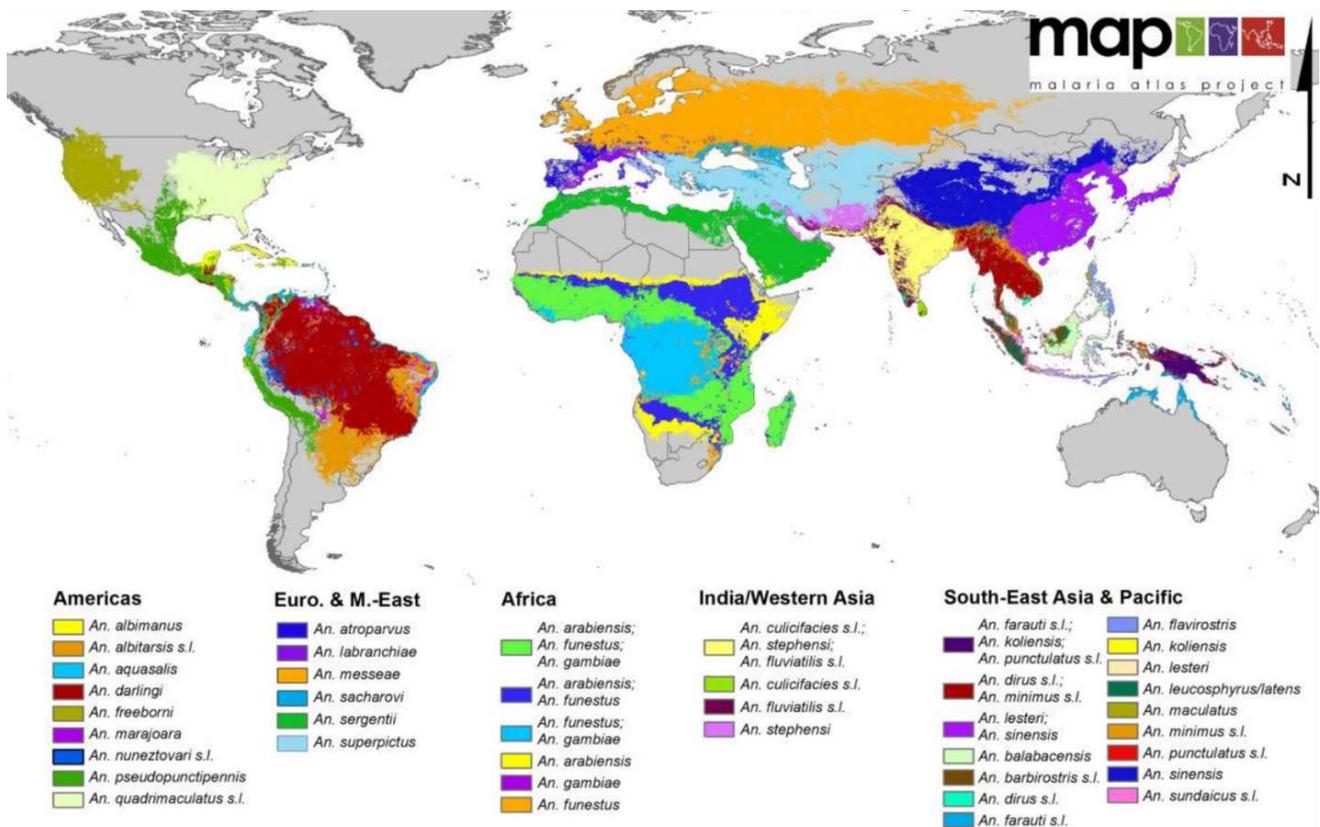


Figure 3: A global map showing the distribution of the 34 dominant malaria vectors, highlighting the variability in vector complexity across regions [40].

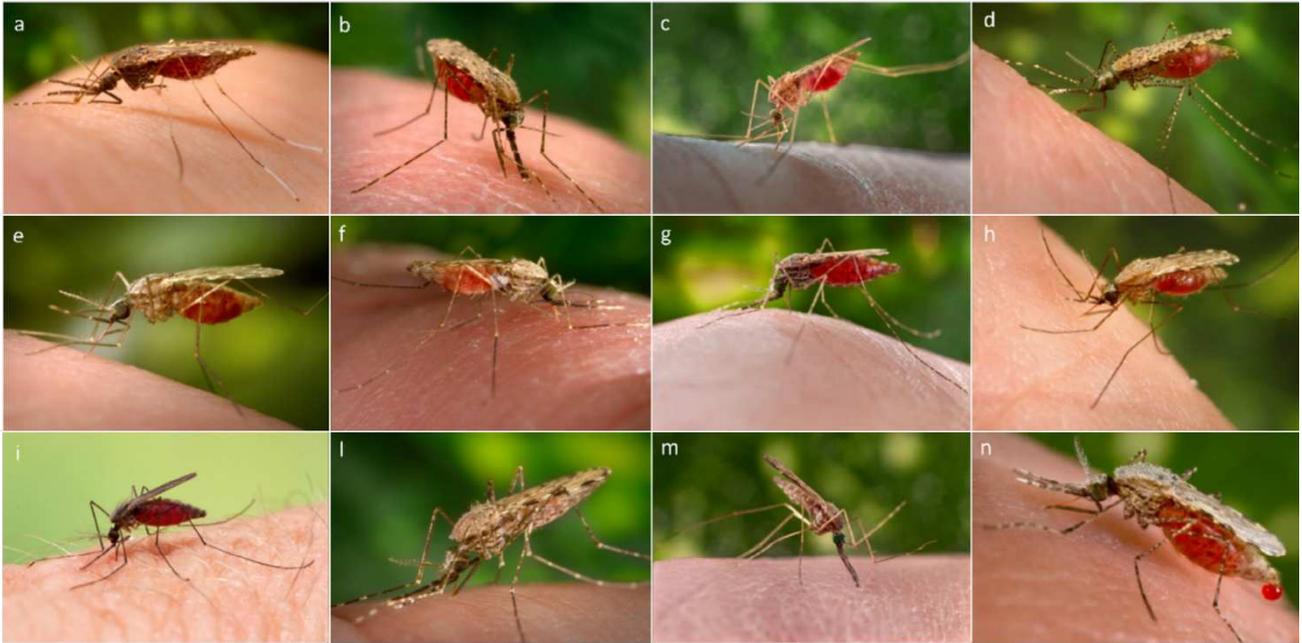


Figure 4: Twelve species of malaria vectors during a blood meal. (a) *Anopheles albimanus*, (b) *Anopheles arabiensis* (c) *Anopheles atroparvus*, (d) *Anopheles farauti*, (e) *Anopheles funestus*, (f) *Anopheles gambiae*, (g) *Anopheles merus*, (h) *Anopheles minimus*, (i) *Anopheles plumbeus*, (l) *Anopheles quadriannulatus*, (m) *Anopheles sinensis*, and (n) *Anopheles stephensi* (photo credit: *A. plumbeus*: ECDC; *A. stephensi*: Dr W. Collins; others: Dr J. Gathany, CDC-PHIL) [42].

On the African continent, seven dominant malaria vectors drive transmission, of which three convey major concern to public health: *Anopheles arabiensis*, *Anopheles gambiae s.l.* and *Anopheles funestus*. These highly anthropophilic vectors are adapted to anthropised environments, making malaria risk highest within densely populated areas and near water bodies [43–45]. Transmission intensity in Africa is highly variable both geographically and seasonally, and depends on many biotic and abiotic factors, such as land cover and use, human densities or socio-economic conditions [46]. Malaria cases are barely recorded in equatorial deep forest for the lack of potential hosts, with an EIR nearing zero [46]. They are also unstable and erratic in desert fringes and highlands [46]. In densely populated and deforested areas, several dominant *Anopheles* vectors ensure intense transmission settings leading to more than a thousand infective bites per person per year, the highest recorded on any continent [47]. *Anopheles gambiae*, *An. arabiensis* and *An. funestus* also drive the

intermediate malaria transmission found in tropical humid savannahs where malaria is rather stable and dry savannahs where it is more unstable [46].

In the Americas, nine dominant vector species and species complexes perpetrate malaria transmission, from which *Anopheles darlingi* (or *Nyssorhynchus darlingi*) occupies a very large area including most of the Amazon basin, while displaying great heterogeneity in seasonal abundance and daily biting activity among locations [40, 48]. This species is highly anthropophilic with crepuscular biting activity [49]. Different malaria transmission dynamics occur in the Americas. The self-explanatory rainforest transmission is responsible for the majority of recent cases in Latin America, with mosquitoes of the *Kerteszia* subgenus as the main vectors [49, 50]. These *Anopheles* can breed in the water bodies collected by bromeliad plants, a monocot widely encountered across the tropical forests of Latin America, leading to the transmission known as bromeliad malaria [50]. These vectors pose great challenges to malaria control as they can dwell in natural, rural as well as periurban areas [50]. In addition to transmission in forested and rural areas, which is responsible for most cases, evidence of urban and periurban transmission has been gathered in various countries [51–53]. In the Peruvian Amazon, malaria risk was recorded to be high in peridomestic areas around villages, with *An. darlingi* being the primary vector [54]. Finally, we can add the specific case of frontier malaria, emerging at the rainforest edge with the building of settlements housing miners, farmers and other nonimmune workers – as discussed in the next section [55].

For unclear reasons, the Asian-Pacific region harbours a substantial diversity of malaria vectors, of which 19 are dominant vectors (Figure 5) [41]. Most countries target elimination by 2030 though many of them are now facing residual malaria transmission (RMT) corresponding to the transmission persisting after full operational coverage with effective ITNs and/or IRS, such as Vietnam or Cambodia [56]. In the GMS, the early and outdoor biting behaviour of primary vectors *Anopheles dirus* and *Anopheles minimus* and secondary vectors varies both geographically and seasonally but vector exposure is highest in and around forested areas [57, 58]. *Anopheles dirus* demonstrates avoidance behaviour towards IRS by not entering sprayed houses and not resting on sprayed surfaces [59]. In archipelagos like the Philippines, Indonesia and Malaysia, transmission risk has been demonstrated to be highest for peridomestic or forested environments [37, 60, 61], with numerous species able to participate actively in transmission through multiple host feeding and flexibility in blood

feeding behaviour [62]. In the Pacific archipelagos such as the Solomon Islands and Papua New Guinea, transmission is driven by species biting in rural, densely forested, lowland districts, with socio-economic factors having an important influence on malaria risk, with the poorer classes being more exposed [61].

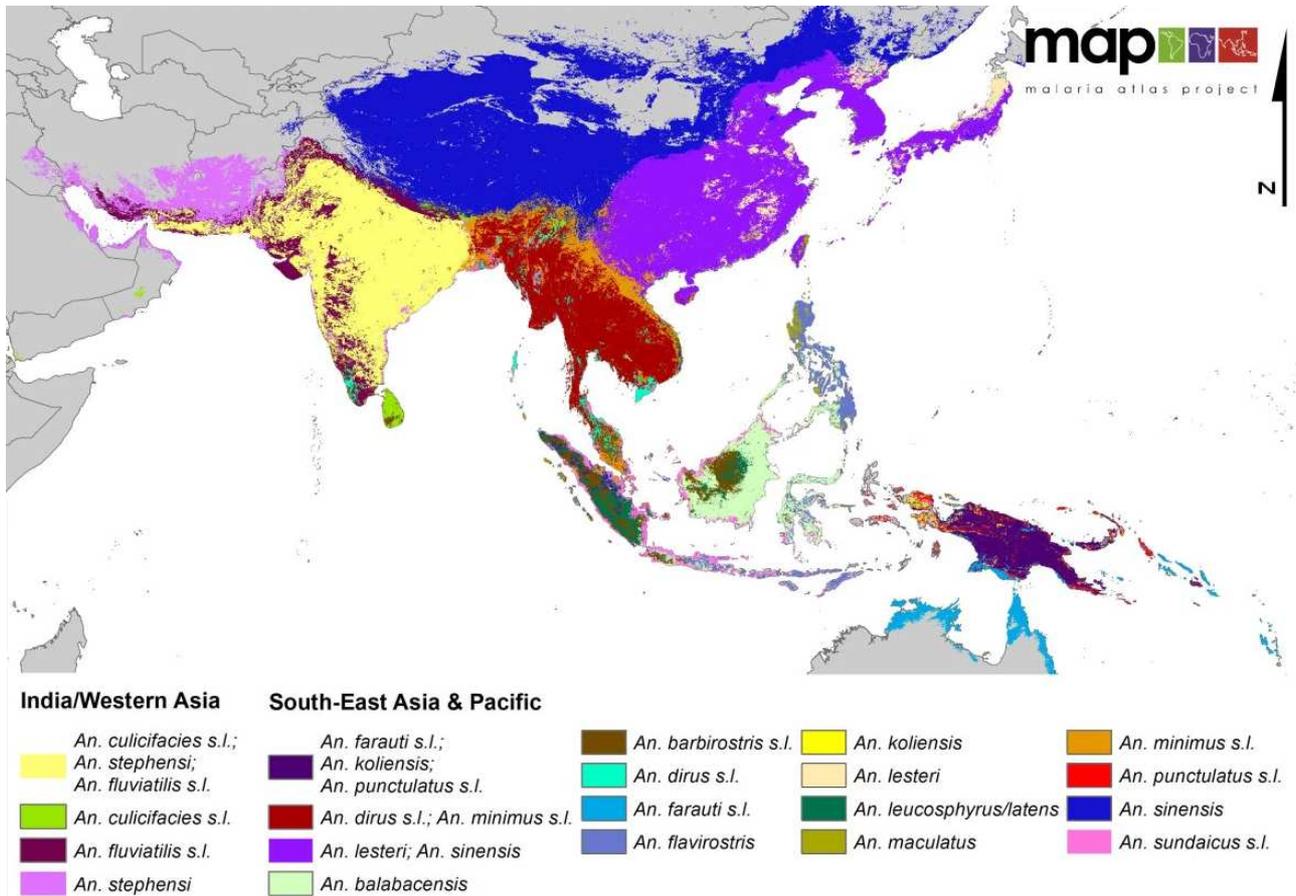


Figure 5: Distribution of 16 of the 19 dominant malaria vectors in the Asian-Pacific region. The GMS is dominated by *An. dirus s.l.* and *An. minimus s.l.* [41].

A.1.6. The environment

Malaria transmission depends on numerous factors such as the presence of potential hosts, predators and competitors. The presence of hosts is required for the parasite to complete its life cycle before the end of the short mosquito lifespan. The interaction with predators and competitors can affect abundance of vectors both at larval and adult stages [63, 64]. For example, Dytiscidae diving beetles prey on mosquito larvae [65] and the jumping spider *Evarcha culicivora* feed on blood-fed adult *Anopheles* [64] (Figure 6). Human densities and

activities – with their associated urbanisation, deforestation and fragmentation – are critical in their impact onto malaria dynamics as they affect both host abundance and availability, if human activities bring them into vectors territory. For instance, the “dilution effect” describes the phenomenon where infection susceptibility varies depending on diversity and quantity of species available to infection by a pathogen: important diversity of hosts would lower the risk of an infectious bite and thus would decrease the pathogen incidence [66, 67]. However, this theory remains controversial, with letters and publications arguing back and forth about the effect legitimacy and its importance in nature [67–70].



Figure 6: An East African salticid *Evarcha culicivora* feeding on a blood-fed mosquito. This species actively chooses and hunts blood-fed *Anopheles* mosquitoes while other East African jumping spiders do not show such specialisation [71]. Photo by Dr Samantha Nixon.

Malaria transmission is also dependent on abiotic environmental conditions such as humidity, temperature or precipitation which are determinant to predict both *Anopheles* and *Plasmodium* presence, density and survival [72–74]. Environmental changes can lead to an increased malaria transmission through effects on mosquito abundance, survival and reproduction [75]. Notably, deforestation can create new water bodies that are used as

breeding sites by *Anopheles* gravid females [76], or affect existing ones by providing them more suitable characteristics for larval development [77]. Higher temperatures caused by the lack of forest cover can create a warmer environment shortening blood meal digestion and egg development, leading to faster development and reproduction of the vectors [78], or create a more attractive environment for vectors which are then found more abundant [79]. Increased temperatures also directly affect the parasite, shortening its life cycle and allowing vectors to become infectious in a reduced time [80–82].

Anthropisation corresponds to the transformation of an environment by humans to meet their needs. The expansion of deforestation, urbanisation and agriculture are demonstrations of this process, driven by human population growth, migration and resources demand escalation. Changes to the environment can affect malaria risk and transmission patterns. Increased local malaria burden has been associated with the installation of new human settlements, changes in land use and deforestation, with numerous descriptions of the phenomenon in Africa [78, 79, 83], the Americas [84, 85] and Asia [86, 87].

The relationship between malaria and anthropisation has been increasingly studied over the last decade, with the effect of deforestation in the tropics being the preferred topic [88, 89]. Of all regions, the Amazon has become the most investigated [89] and the locale happens to harbour a phenomenon tightly linked to anthropisation, defined as frontier malaria. The term describes the type of high transmission settings appearing when workers build settlements along the Amazon rainforest fringes. Before settlements, vector exposure and malaria incidence are limited due to the low human densities in the wild and densely forested area. These regions attract settlers, building encampments or villages at the forest edge, installed for mining, developing agriculture or exploiting timber that creates numerous breeding sites suitable for the dominant vector *An. darlingi* [55]. Vector exposure increases dramatically, given the new availability of water bodies, nonimmune human hosts and vegetation cover [55, 90]. In such settings at the interface between forested and deforested environments, malaria incidence will rise greatly in and around these settlements [55]. As deforestation progresses, transmission decreases, with the remaining forest patches retaining an increased malaria risk [55]. In other cases, land use changes can initiate a decrease or elimination of malaria transmission: various developed countries performed such an achievement as part of their malaria control programmes. Italy, Israel/ Palestine and the southeastern United States

implemented antilarval measures that successfully eliminated malaria from their territories, using extensive drainage and water management [91]. Recently, some controversy arose around conservation efforts that, depending on the source, could induce an increase [92] or a decrease [66, 93] of malaria transmission – or both, as deforestation was associated to a bidirectional causation of increased and decreased malaria transmission [94].

Recent research has allowed a steep advance toward the understanding of environmental changes and their interaction with malaria dynamics. Updates and debates provide material and insights about different settings and geographical areas around the globe, though knowledge paucity remains worrisome in some regions.

A.1.7. Disease control

Malaria control aims to limit its incidence and prevalence through the implementation of a variety of methodologies, targeting either the vectors or the parasites. With the input of an increased funding over the last years, malaria prevention and control attained several major milestones, such as an improved vector control, a more efficient case detection and a generalisation of artemisinin-based combination therapies (ACT) [3].

The most widespread method targeting vectors is long-lasting insecticide-treated nets (LLINs), also called insecticide-treated nets (ITNs), and indoor residual spraying (IRS) of insecticides. In 2019, 253 million ITNs were distributed to malaria endemic countries, an increase of 28% since the previous year [3]. The percentage of the population at risk protected by IRS decreased from 5% (180 million) in 2010 to 2% in 2019 (97 million) [3], as communities tend to prefer LLINs or may refuse IRS over the perceived efficacy or associated inconveniences: smell, bed bugs nuisance increase or political conflicts [95, 96]. These two methodologies, when correctly implemented, can greatly reduce malaria incidence by directly killing the vectors, shortening their life span, or limiting the possibility of infectious bites [97–100]. However, because IRS and LLINs only protect risk populations for a limited timeframe and specific activities such as the time spent within the household and under the net, but usually not during work, social gatherings, commuting, etc., the protection they provide is only partial and their effect on malaria prevention therefore limited. Additionally, their efficacy is threatened by the emergence and spread of insecticide resistance and behavioural avoidance in malaria vectors [101]. The use of other mosquito control tools is still quite limited or under development. Larvicides are efficient but are not recommended in rural areas [101] and

concerns have been raised about the potential deleterious consequences on the environment and on non-target organisms [102, 103]. Topical mosquito repellents (such as DEET or picaridin) can provide efficient, hours-lasting protection against early and outdoor biting, though with important variability between Anopheline vectors [104]. The use of volatile spatial repellents (transfluthrin or metofluthrin) can be moderately to greatly effective at inducing spatial repellency and irritancy responses in malaria vectors, according to both field and laboratory evaluations [105, 106]. Nevertheless, findings agree that these spatial repellents must be complementary to other methods, and that they require improvement and a relevant implementation strategy [105–107]. Additionally, spatial and topical repellents are difficult to institute in rural populations: often, most of the study population would prefer the use of more traditional methods, such as bed nets, even if they accept the repellent during the study [107]. Other control measures exist, but their efficacy is still loosely assessed (as for larvivorous fish, zooprophylaxis [108] and attractive toxic sugar baits [109]) or they can be difficult to implement, such as land bonification, aiming to change the environment into an inhospitable place for mosquitoes [108]. Finally, a new sort of vector control has developed over the recent years, using genetically modified *Anopheles*, manipulating the vector microbiome or infecting the vectors with the bacteria *Wolbachia* spp., which allows interfering with the pathogens development or directly affecting the vector survival [110].

To target *P. falciparum* parasites and ensure their elimination, a variety of drugs can be used, with different implementations depending on the area [3]. In Africa, artemether-lumefantrine (AL), artesunate-amodiaquine (AS-AQ), artesunate-pyronaridine (AS-PY) and dihydroartemisinin-piperaquine (DHA-PPQ) are in use, to this day with low failure rates. First-line treatments in the Americas include AL, artesunate-mefloquine (AS-MQ) and chloroquine (CQ), and in the Eastern Mediterranean are AL and AS+SP, with a high efficacy for these two regions. In the Asia-Pacific region, AL, AS-MQ, AS-PY, artesunate plus sulfadoxine-pyrimethamine (AS+SP) and DHA-PPQ are in use with varying levels of efficacy, inducing occasional policy changes. Primaquine remains the default treatment for a radical cure of *P. vivax* infection [3]. Additionally, in all parts of the world, these drugs are used for chemoprevention, as in intermittent preventive treatment (IPT) and seasonal malaria chemoprevention, targeting people at high risk of malaria such as infants and pregnant

women [111]. This type of intervention, adaptable to different target groups, drugs and delivery approaches, clears any existing and prevents new infections [111].

Vaccination prevails as the most efficient strategy to eliminate a disease. Malaria vaccine research is active and has brought numerous candidates to clinical trials [112, 113], though only one has been validated for large-scale distribution to this day. This vaccine, named Mosquirix with RTS,S as its active substance, was recommended for widespread use in October 2021 by the WHO with the objective of protecting children aged 6 weeks to 17 months from *Plasmodium falciparum* malaria [114]. Nonetheless, the efficacy and the range of implementation of this new vaccine are limited and the need for additional control strategies is still paramount [114].

A.1.8. Challenges in the way of elimination

Not only the efficacy of methods to eradicate malaria is important; so are their accessibility, availability, acceptability and sensibility. Additionally, malaria symptoms are unspecific: other infectious diseases present in the same areas, such as dengue, chikungunya, yellow fever, etc., display the same symptoms, which can hinder the correct identification of malaria cases [115].

Ensuring and maintaining accessibility to all communities at risk of malaria can be challenging. In remote, rural and resource-poor areas, travelling to health centres can represent a long, difficult or expensive journey, which can be sufficient to persuade patients to discard the option of receiving proper care. Recently, the SARS-CoV-2 pandemic and its consequences limited the reach of malaria interventions as people with fever were advised to stay at home. The WHO suspects an increase in malaria deaths corresponding to the disruptions caused by the current global health situation [3]. Other examples exist, such as the 2014-2015 Ebola outbreak that severely affected malaria cases and treatment, in addition to long-term socio-economic and public health impacts still felt to this day, notably a distrust in the health workforce [116, 117]. Wars, conflicts and extremist regimes can equally reduce accessibility. Availability must be regulated too; health workers, medicine and vector control tools are all prone to potential shortages and stock-outs, especially in the public sector, which can cause workers to prescribe inadequate drugs such as antibiotics [118–121]. The private sector suffers fewer shortages but comes with their own issues, notably to over-prescribe and impose a financial stress on their patients [118].

Eliminating a disease also requires acceptability from the target population. Local communities do not always accept antimalarial intervention, with reactions that can be straight refusal [96], removal of vector control tools in the days following their installation [96], or withdrawal from a prescribed treatment.

Detection methods and their sensitivity represent another important factor. The sensitivity of RDTs is limited with a detection threshold around 100 parasites/ μL [122] – meaning they are mostly unfit to detect low density parasitemia cases which are often associated to asymptomatic infections, especially for *P. vivax* cases – and contribute to an important share of false negatives [123, 124]. Additionally, asymptomatic cases can be easily undiagnosed as in the absence of symptoms patients do not seek detection.

Finally, maintaining methods efficacy over time is an important challenge. Disease control heavily relies on antimalarial drugs and vector control tools; however, their efficacy is increasingly compromised by the spread of parasites and vectors resistances, allowing them to escape insecticides and treatments.

Public health systems efficacy itself is important. Not all countries and regions can use a centralised, complete and updated database. Patchy datasets add an extra layer of incertitude to public health effort. Political, economic and environmental events can lead to important data losses. Some data sources can be reliable (satellite imagery is not disturbed by such events – only clouds can hinder their effectiveness) while others depend entirely on the efficacy of local agencies, private health centres or national malaria programmes.

Through these public health systems, studying and understanding transmission patterns becomes possible, which allows to develop tailor-made malaria control, adapted to the local transmission settings. However, such research does not come without challenges and limitations. Numerous societal aspects hinder the success of malaria studies, such as the difficulty to implement research in remote areas and to enrol participants, especially in poor socioeconomic contexts [125, 126], and for cohorts that can involve lengthy questionnaires, blood collection or other interventions requiring time from the participants [127].

A.2. Cambodia

The Kingdom of Cambodia, or Kampuchea, is part of the Great Mekong Subregion (GMS), a trans-national region of the Mekong river basin in South-East Asia, including Laos, Myanmar, Thailand, Vietnam and the Chinese province of Yunnan (Figure 7). Rainy season, characterised by a southwest monsoon bringing heavy precipitations, occurs from May to October and is concomitant to the malaria transmission peak. Dry season lasts from November to April, with temperatures reaching 40°C in April.



Figure 7: Cambodia and the Great Mekong Subregion. The Mekong main stem passing through the GMS is highlighted in blue.

In 2019, 58% of the GMS malaria cases were in Cambodia (Figure 8) [128].

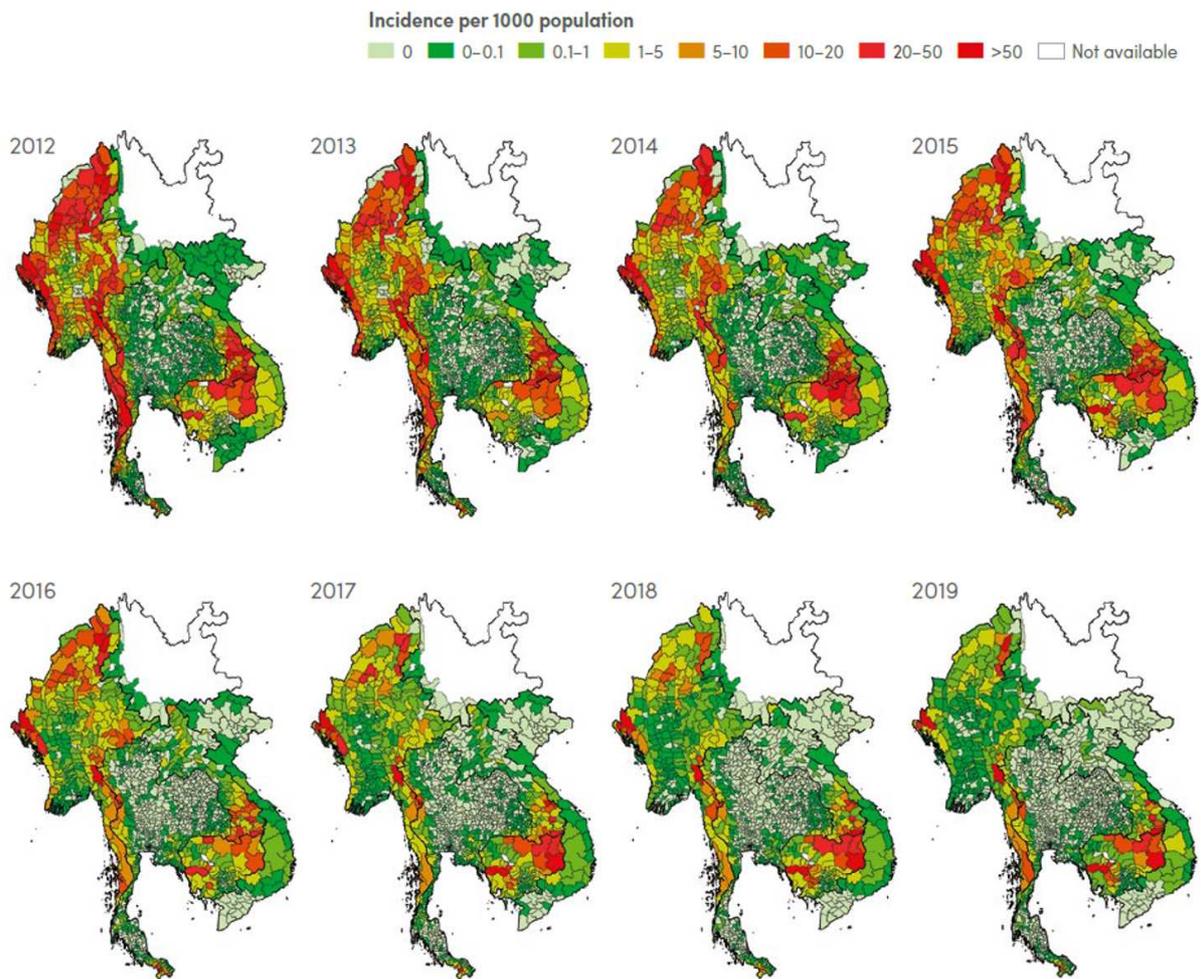


Figure 8: Regional map of malaria incidence in the GMS between 2012 and 2019. Data retrieved from the National Malaria Programme of each country [3].

Cambodia is a tropical country sitting in the centre of the GMS, home to 16 million people, mostly of Khmer ethnicity (around 90%) but also from ethnic minorities such as the Vietnamese, the Khmer Loeu (“Highland Khmer”), the Cham and the Lao. In the 1970s, the country was ruled by the infamous Khmer Rouge, the totalitarian repressive regime whose social, economical and health repercussions are still endured to this day. After years of self-imposed economic isolation and national self-sufficiency imposed by the regime, the Khmer Rouge fell and Cambodia entered decades of economic growth, mostly sustained by tourism and clothing exports. With an average annual growth of 7.7% between 1998 and 2019, the Asian economy has transitioned into one of the fastest-growing in the world, reaching the lower middle-income status in 2015 [129].

A.2.1. Socio-economic context

Among SEA countries – already at the origin of great environmental concern, as its fragmenting, large-scale and accelerating deforestation is rapidly converting natural habitats into monoculture croplands [130–133] – the situation in Cambodia could be the most upsetting [134]. Economic and social development is an important driver of environmental changes that eventually affect malaria transmission at different scales. In Cambodia, foreign groups frequently buy important pieces of land, inducing drastic and sudden changes in land use [135]. These large-scale Economic Land Concessions (ELC) have become very common and occupy now a noticeable surface of the territory (Figure 9) [136]. These ELCs tend to transform forested areas into monocultures, through the intermediate step of slash-and-burn, a very common process used in Cambodia to clear the land and plant new crops in the soil fertilised by the ashes (Figure 10 and Figure 11) [136]. In addition to the direct disastrous environmental effects, these ELCs tend to benefit economically new acquirers rather than local populations, and can lead to human rights violations and evictions [137–139]. Besides, ELCs cause important economic weakness and social inequalities for local populations. On the other hand, when local communities own the land, the forests benefit from better management, less deforestation and show higher forest cover persistence, as they tend to clear only small patches of land and perform selective logging rather than large-scale forest degradation [136].

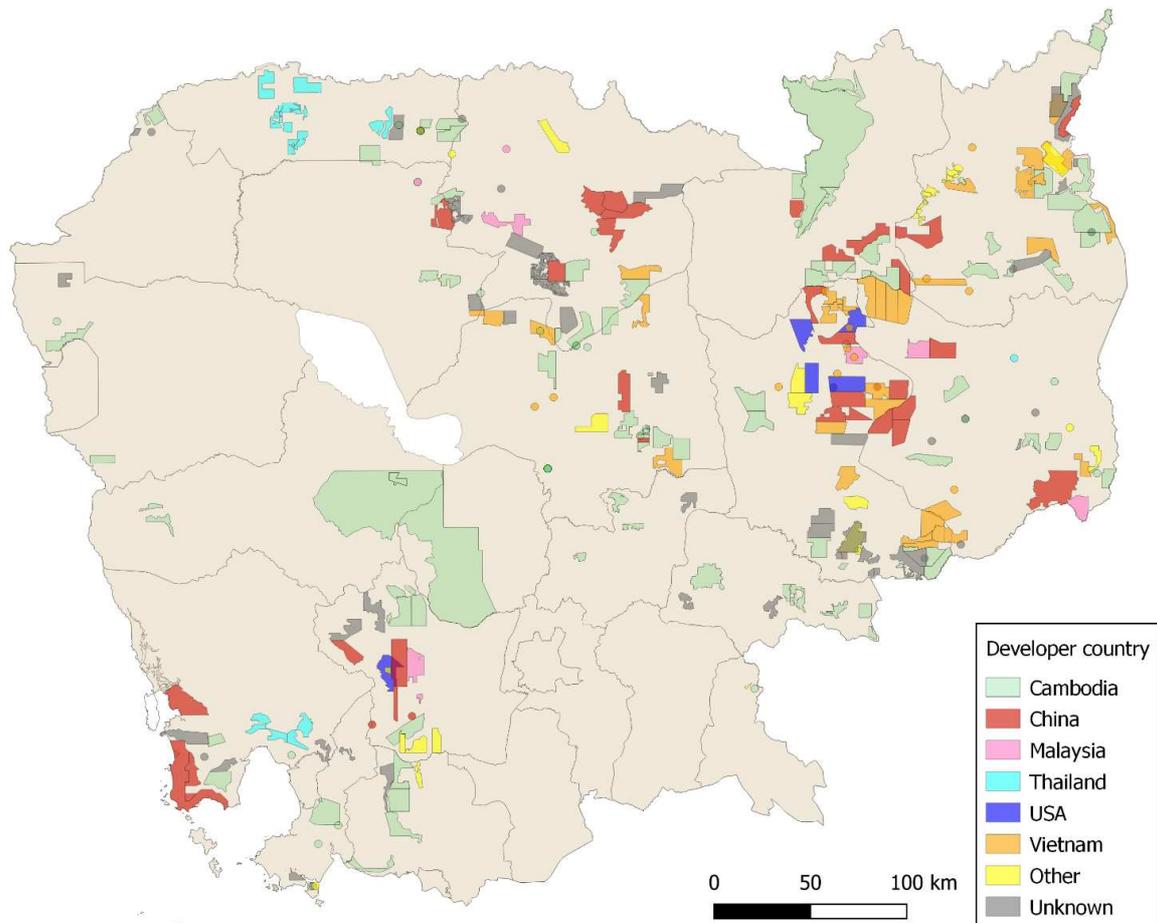


Figure 9: Cambodia and all 285 documented ELCs with their respective developer’s country. The category “other” includes France (two concessions), India (3), Israel (1), South Korea (10), Singapore (2) and Taiwan (1). Fifty-five ELCs developers were of unknown origin. Most of ELCs declared rubber as their intended crop (N = 147, 51.6%), while the others declared projects being cashew, cassava, acacia, palm oil, sugarcane, others crops and/or animal husbandry. Finally, 25.3% of them (N = 72) did not declare their intended crop or project. Data from ODC (Open Development Cambodia), last updated in December 2019 [140].



Figure 10: A patch of land being burned during dry season. Photo by A. Pepey, taken in February 2020 in Kaev Seima district, Mondulkiri province.



Figure 11: A forest gradually cleared by the slash-and-burn method. Photo by A. Pepey, taken in March 2018 in Kaev Seima district, Mondulkiri province.

A.2.2. The epicentre of malaria resistance

As early as 1957, studies were published about the emergence of *P. falciparum* resistance to chloroquine at the Thailand-Cambodia border, which was then the most used antimalarial to treat *P. vivax* and *P. falciparum* [141]. The spread of this resistance led to dreadful effects in Africa, inducing a rise in *P. falciparum* mortality and prevalence during the 1980s. Afterwards, when artemisinin was the principal antimalarial in use, *P. falciparum* resistant strains were identified in Western Cambodia in 2006 [142]. After the implementation of ACT treatments that were developed to limit resistance emergence in *P. falciparum* strains, strains resistant to both artemisinin derivatives and piperazine emerged in Cambodia [143]. Finally, after the official implementation of dihydroartemisinin-piperazine as first-line intervention treatment in 2012, the first treatment failures happened in 2014 and artesunate-mefloquine had to be instituted as first antimalarial response in the country [144, 145]. To this day, although no therapeutic efficacy study has reported mefloquine treatment failure, institutions should not neglect a close monitoring of a resistance emergence.

Unsupervised access to drugs, which can lead to an inappropriate use or to the non-adherence to the prescribed treatment [146], in a context of substandard clinical practices [147], could be a decisive factor in the emergence of drugs resistance. In addition, quite counter-intuitively, resistances tend to emerge and spread rapidly in low transmission settings such as *P. falciparum* is in Cambodia, as infections tend to be monoclonal within a patient. Such low genetic diversity is favourable to mutation fixation because it limits the competition with other strains that do not suffer the fitness cost associated with the resistance.

Historically, the GMS has used dichlorodiphenyltrichloroethane (DDT) to control malaria, then switched to the current pyrethroid-based vector control measures such as ITNs [148]. Exposing vectors to insecticides leads to selection events allowing the emergence and the spread of insecticide-resistant strains of vectors. Susceptibility varies among locations and species but such physiological resistances have been observed in every country of the GMS since the 1980s (Figure 12) [148].

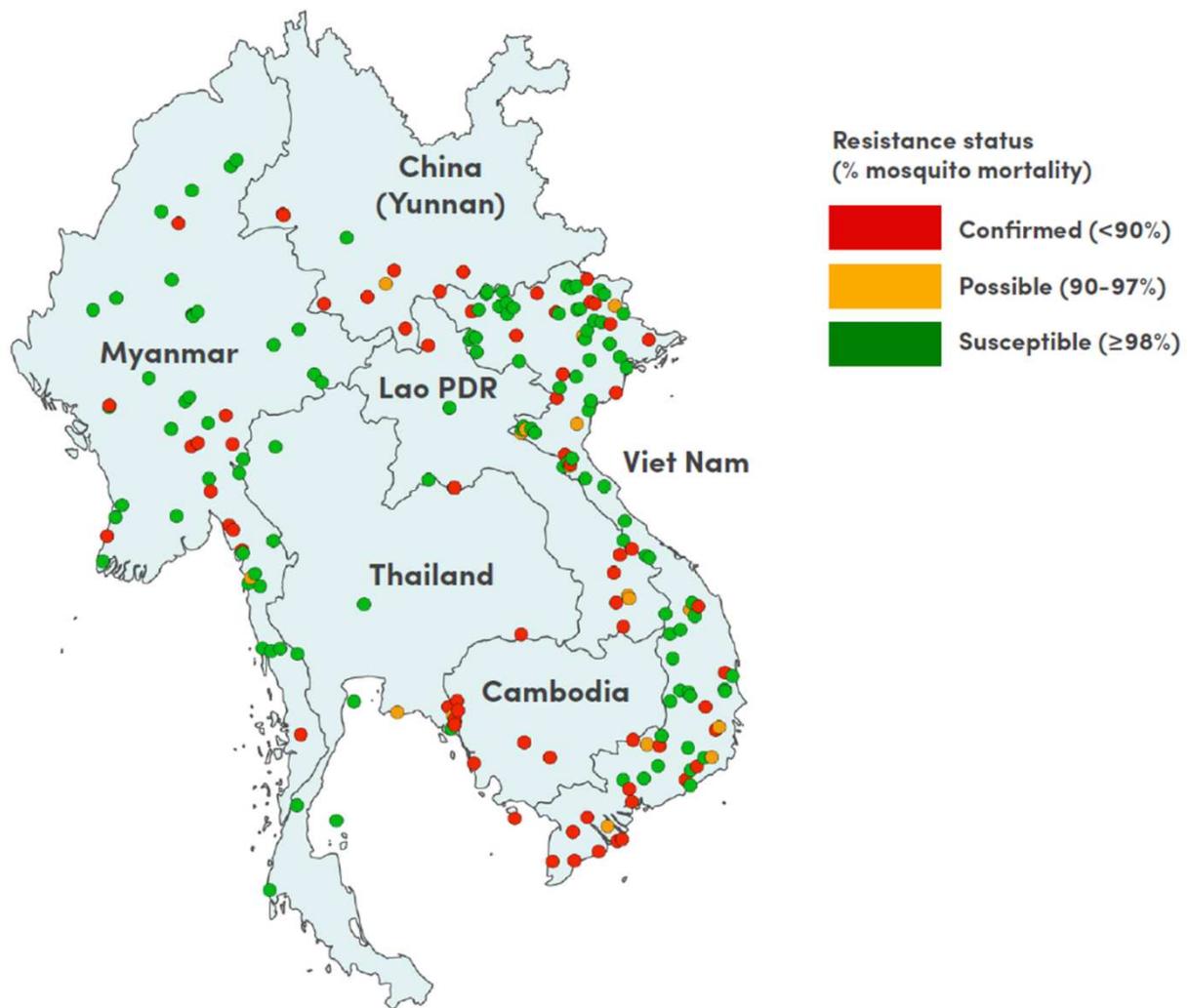


Figure 12: Resistance of malaria vectors to pyrethroids in the GMS (2010-2019). Geographical coverage of resistance monitoring is unevenly distributed, and the variable collection or identification methodologies across locations can affect results. Map from WHO Malaria Threats Map, November 2019. Data on insecticide resistance in the GMS are available on the WHO Malaria Threats Map and are regularly updated [128].

A.2.2. Transmission and incidence

The WHO estimated that Cambodia suffered 140,077 malaria cases and 102 related deaths in 2019, corresponding to a respective decrease of 79% and 94% since 2000 [3]. While *P. vivax* incidence represents only a tiny percentage of the global malaria burden, the species constituted about three quarters of malaria cases in Cambodia in 2019 [128]. Asymptomatic infections form most of these cases: they usually account for more than 90% of PCR-positive participants [149, 150]. They represent a consequential challenge for malaria elimination as they are mostly invisible to conventional detection methods (RDTs and microscopy), making

them more difficult to detect and track, though they significantly participate to malaria transmission [151].

Malaria transmission in Cambodia is considered residual, primarily as a result of the very high nation-wide ITNs coverage [13], with provinces declaring more than one bed net per household on average [56]. While their institution has successfully decreased transmission in the villages, it could however have allowed the relocation of transmission to the farms, in and around forest fringes and forested areas where both primary and secondary vectors can ensure transmission [152–154]. Consequently, the current tools to measure and decipher malaria transmission in Cambodia are outdated and could be leading to a misclassification of the transmission as “residual” while proper transmission intensity stays misevaluated [56].

Malaria risk in Cambodia differs from a village to another adjacent to it [123, 155] or even a household to the next [156], revealing very fine scale epidemiological variations. This local heterogeneity, depending on biotic and abiotic factors, needs to be considered and addressed to ensure elimination [155].

A.2.3. Risk population

Primary vectors *An. dirus* and *An. minimus* are mostly encountered in forest and forest fringes [41, 157]. With deforestation increasing, contact with forest is decreasing for most of the population, but is still part of the daily life of some. Notably, forest goers have consistently been identified as the main risk population, as their hunting and wood logging activities, coupled with recurrent nights in the forest, expose them frequently to infectious *Anopheles* bites [158–161]. In addition, individuals living and working nearby the forest are exposed to malaria, such as farmers owning huts at forest vicinity or plantation workers [162] (Figure 13). Malaria risk exposure is not only linked to forest activities and surroundings. Human movement, subclinical infections and complicated transmission patterns also contribute to the disease steadfastness, making mobile and migrant populations also at risk of malaria [163]. Such populations are common in agricultural areas that require workforce only seasonally. While malaria cases can be detected in every age stratum and for both men and women in the remaining transmission pockets, the most affected usually adult men between 15 and 50 years old [123, 158, 164].



Figure 13: A temporary settlement at a cassava field. Cassava agriculture is very common and an important income source in Kaev Seima. Photo by A. Pepey, taken in March 2018 in Kaev Seima district, Mondulkiri province.

A study from Stung Treng province in Northern Cambodia conducted in-depth interviews with recently positive forest goers [165], illustrating the frequentness of malaria clinical episodes for these workers:

Interviewer: “Have you got malaria before?”

Respondent: “Yes.”

Interviewer: “How many times until now?”

Respondent: “Too many times that I can’t remember...About 100 times. Six times a year or even twice a month.”

Forest goers usually experience multiple episodes of malaria a year and know about associated risks and symptoms, but often, economic realities cannot let them get away from malaria exposure or to afford the time to seek a diagnostic and a adequate treatment [56, 166]. Lack of accessibility also matters: diagnostic and treatment can be far, and travel can be

hard, especially during the rainy season [165]. They are mostly unknowledgeable about the notion of asymptomatic infection [165].

Forest goers work in both forest fringes and deep forest, often at night, in difficult conditions that leave them exposed to mosquito bites. The use of long sleeves, smoking and making fires have been mentioned by these workers to repel and avoid mosquitoes, but the use of nets or repellents is not common [165]. The efficacy of such methods would be limited given their work conditions; forest goers traverse rivers and overcome numerous physical obstacles with motorbikes loaded with wood – conditions rather different from defined places and static activities where nets and repellents would be applicable (Figure 14 and Figure 15). They usually venture deep into forest to find and cut specific high-value trees rather than cleaning whole patches of forest. Depending on ethnicity, the relationship with forest changes; for example, Khmer forest goers would testify: “indigenous people even take their toddlers with them when going to the forest. [...] Unlike us, we don’t do the same.” [165].



Figure 14: A forest goer motorbike loaded with wood. The bundle can reach several hundred kilograms, with wood sections too heavy and too long to allow the motorbike to stand on its own: forest goers would help balance it until the driver builds enough speed to continue on his own. Photo by A. Pepey, taken in March 2018 in Kaev Seima district, Mondulkiri province.



Figure 15: A forest goer building speed with the help of a co-worker. Rainy season makes the terrain strenuous to manoeuvre. Photo by A. Pepey, taken in February 2020 in Kaev Seima district, Mondulkiri province.

A.2.4. Malaria elimination

One of the cornerstone of malaria elimination is the fast detection of malaria cases. Malaria diagnostic was initially carried out by the analysis of thin or thick slides from patient blood with light microscopy. However, this method requires specific material and time, in addition to trained and qualified staff [3]. A more sensitive but cumbersome and expensive methodology is the amplification of parasite DNA through Polymerase Chain Reaction (PCR). Although it is possible to implement PCR detection in the field [167], diagnostic requires expensive material, several hours for sample processing and significant training for staff. Consequently, the most common diagnostic methodology is via RDTs. In 2019, nearly a million RDTs were distributed in Cambodia [3]. These small, cheap and practical devices can provide a diagnostic in a matter of minutes by detecting the parasite antigens from the patient's blood, notably the histidine rich protein (HRP2) expressed by *P. falciparum* and/or *Plasmodium* lactate dehydrogenase (pLDH) expressed by all *Plasmodium* species [168]. As

RDTs sensitivity threshold (around 100 parasites/ μL) does not allow the detection of most asymptomatic cases [122], highly sensitive RDTs specific to *P. falciparum* have been developed, with detection as low as 3 parasites/ μL for HRP2-positive culture strains [122]. Nevertheless, their implementation in the GMS was rather unconvincing: they either allowed only 10% in sensitivity increase for a price twice as expensive [124] or did not demonstrate a sensitivity difference in field settings [169]. Overall, RDTs remain a practical mean of detection that can be implemented anywhere, anytime, with little investment.

Elimination remains a challenge in border and forested areas, as a consequence of highly heterogeneous micro-geographical epidemiology, inaccessible areas, constant movement, and distinct behavioural characteristics of populations [170]. Active and passive detections strategies are implemented in order to diagnose and treat malaria cases in the days following detection [171]. Increasing further access to early diagnosis is part of the country priorities [124]. Since 2009, Cambodia implemented the Village Malaria Workers (VMW) that increase access to testing and treatment, reducing considerably the effort to access these services as the nearest health centre can be difficult to reach [124]. In 2018, the Mobile Malaria Workers (MMW) were deployed to actively approach remote populations, including migrant mobile workers and forest goers, to ultimately provide “test-and-treat services” [124, 170]. Both strategies have been able to reach and diagnose the target populations, with improvement over time as strong community relationships increased service quality and productivity [124, 170]. However, the low sensitivity of RDTs hinders the efficacy of such strategies, and additional strategies are required to ensure elimination [172].

Other “test-and-treat” strategies, either using Mass Screening And Treatment (MSAT) with the whole population targeted or Focal Screening And Treatment (FSAT) restricted to highest risk groups, have been tested, and did not bring any evidence of holding the potential to stop malaria transmission [124]. Mass drug administration (MDA) should be effective and deliver results, though the method is less accepted by populations and is capable of spreading further drug resistance in the field [124]. Methodologies involving malaria prophylaxis have been recently implemented, through projects distributing it to forest goers [172] and the national malaria programme providing IPT and targeted drug administration to travellers visiting risk areas [173], with their effectiveness and results yet to be assessed and published.

First-line treatment courses are exclusively Artemisinin-based Combination Therapies (ACT), for any *Plasmodium* species responsible of infection. Such medicine successfully treated 23% (32,197) of the country malaria cases in 2019 [3]. All infections are systematically treated, regardless of symptoms presence or intensity [174]. Since 2018, a single low dose of primaquine (0,25 mg/kg) has been implemented for *P. falciparum* infections and for mixed infections including *P. falciparum* and another species [174]. Since 2020, a radical cure of primaquine, consisting of 0.25 mg/kg/day for 14 days, has been instituted to clear *P. vivax* and *P. ovale* hypnozoites and prevent relapses [173]. However, this therapeutic scheme is associated with a poor compliance limiting the coverage. Another limitation is the primaquine dose-dependent haemolysis in G6PD (glucose-6-phosphate dehydrogenase) deficient patients. This inherited condition is estimated to be severe enough for 2 to 16% of GMS residents to proscribe them the access to any treatment involving primaquine or a closely related molecule [175]. G6PD deficiency testing prior to primaquine prescription is therefore primordial for a safe prescription and administration but is difficult to implement [174].

In addition to case detection and treatment, vector control represents an important share of the national malaria control strategy. Bed nets remain the most commonly used malaria control tool in Cambodia: in 2017 and 2018 respectively, about 2 million and 1.6 million ITNs were sold and delivered in the country [3]. However, mosquito collections indicate that transmission relies on early and outdoor biting – a major challenge which cannot be resolved through the sole use of bed nets [176]. In addition, *Anopheles* active hours overlap with active time when populations are not under a net [152]. Other factors complicate the matter, such as the multiple residence system of forest goers and farmers, the individual and seasonal variability of bed nets acceptance, the common open housing structures that blur the limit between indoor and outdoor biting, and the considerable vectors diversity in the GMS [124, 177]. All these characteristics of the variable vector exposure of the risk population depend on spatiotemporal variables happening at a very fine scale. In these conditions, targeted interventions are required in order to control and eliminate the disease [176]. Disease control strategies need to consider a better targeting of malaria vectors and consider that transmission happens outdoors and away from home, where ITNs are usually not in use [56, 177, 178].

A.2.5. Remaining challenges

Cambodia situation has greatly improved over the last decade. However, consequential challenges still stand in the way of elimination.

The last years of research have reached a consensus on the importance of the interplay between anthropisation and malaria dynamics. While extensive literature is available about this phenomenon in the Americas and in Africa, such data still lacks in Asia, especially in the GMS – where, of all places, deforestation in Cambodia was deemed alarming and may affect malaria dynamics.

The implementation of malaria workers equipped with RDTs to provide active “test-and-treat” services is a step forward malaria elimination; however, it lacks the sensitivity to detect infections with low parasites densities. As microscopy and PCR diagnoses are not transportable to remote settings where the risk population is encountered, a better understanding of this risk population is required to palliate the problem. The mobile workers and the forest goers are indeed the main risk population. However, the precise dynamics of malaria transmission remain unclear: knowledge of the associated socioeconomic, mobility and behavioural risk factors that lead to a contact with an infectious *Anopheles*, is limited within these populations. Individual heterogeneity within forest goers is not precisely defined, while transmission pockets remain in forested areas. Additionally, the understanding of these risk factors are important not only for malaria elimination, but also for keeping the country malaria-free, once elimination is achieved – especially for the mobile and migrant populations travelling over borders. Clear identification of risk subpopulations and risk behaviours is required to attain effective long-term malaria control in Cambodia.

Vector control tools were implemented to control and eradicate malaria, and they seem to have allowed reaching RMT, but they fail to eliminate the disease. Mostly, malaria control relies on the use of ITNs, but their implementation, even when done correctly, can induce behavioural avoidance [57, 179]. Behavioural avoidance arises from the selection of vectors that bite outside of the timeframe when risk populations are protected by bed nets (*i.e.* when they are sleeping) and are rather active when they are not protected (*i.e.* at dusk and dawn, when socialising or performing tasks around the household). For example, *An. dirus*, as a primary vector that feeds normally indoors late at night unlike most vectors from the Subregion [179], has switched to early outdoor biting, before sleeping time, when it would

exposed to impregnated nets for an extended period of time [180, 181]. In addition, IRS has also been observed to increase outdoor biting from primary vectors such as *An. minimus* and *An. dirus* in Thailand [59, 182, 183]. Because of this evolving behaviour from various *Anopheles* species, in addition to a changing environment potentially favouring secondary vectors, and the great diversity of malaria vectors in the GMS complicating the matter, the current understanding of the contact mosquito-human might be outdated. An update is urgently needed on the nature of the primary vectors, the intensity of transmission and the environments harbouring infectious *Anopheles*. Malaria control needs precise and quantitative data about its entomologic component to implement adequate vector control.

Plasmodium vivax malaria and the associated relapses pose a particular challenge to elimination efforts and require the urgent implementation of new tools, notably a methodology able to define the importance and the role of the asymptomatic reservoir, which curbs cases detection and treatment, and, eventually, compromises elimination [169, 170]. Similarly, we need to understand the parasite epidemiology and assess the efficacy of the elimination strategies. Lastly, genetic markers have been mostly deployed to understand and predict the emergence of resistance phenotypes, but rarely to understand the population genetics of the circulating *P. vivax* and *P. falciparum* clones. The implementation of a surveillance using adapted genetic markers is required to understand *P. vivax* relapses and transmission patterns, to track asymptomatic infections and understand malaria dynamics at the village or the country scale.

A complex interplay of a growing economy and advancing deforestation, the emergence of resistance and multi resistances from both *Plasmodium* spp. parasites and *Anopheles* spp. vectors, the complexity of behavioural and ethnicities diverseness, an impressive diversity of vectors and a fine-scale epidemiology heterogeneity could challenge elimination in Cambodia as it remains poorly understood.

Objectives of this PhD research

To this day, not enough studies documented the importance of fine-scale variations in the spatiotemporal, environmental and behavioural factors affecting malaria transmission in the GMS. With populations spending time outdoors during vectors biting times, mosquito protection poorly suited to the type of housing and activities, it is primordial to understand the context of the human-mosquito contact. A gap of knowledge remains. How can children and women be infected, if only the forest goes venture into the deep forest? Will deforestation decrease malaria transmission intensity? Are rural populations chronically ill with the same parasites or do they acquire frequently new infections? Do the methodologies to answer these questions exist and are they applicable to Cambodia?

Chapter 1: The determination of anthropisation in a rural Cambodian area and the mobility of risk populations

The objectives were to quantify and characterise anthropisation at a very fine scale, notably to measure deforestation and fragmentation of a study area over time, and to determine the potential impact on malaria transmission. In addition, the implementation of GPS trackers provided insights on mobility patterns and the feasibility of implementing this tool in forest worker populations.

Chapter 2: Ecology and parasite prevalence in vectors

Quantifying vector-human contact is crucial to design and implement successful vector control. As such, we aimed to design and implement *Anopheles* collections to measure malaria vectors abundance, diversity and prevalence. We implemented the collections over the most represented land use categories, for different human densities, for all seasons, through baited and non-baited traps and with several collection sites for each of these conditions.

Chapter 3: Characterisation of *Plasmodium* spp. transmission within human and vector populations

Another relevant indicator of malaria transmission will be to analyse the parasites themselves: the initial aim of this third chapter was to differentiate and characterise the *Plasmodium vivax* and *Plasmodium falciparum* genetic clusters that circulate among the study population and the Anopheline vectors. By identifying and comparing the different haplotypes circulating in the human and *Anopheles* populations, we aimed to pinpoint transmission hotspots where both these populations shared the same *Plasmodium* haplotypes. However, considering the difficulties associated with this objective and the PhD deadline, the focus shifted to establishing, optimising and evaluating Deep Amplicon Sequencing as a genetic tool in Cambodia. The actual genetic data and the associated analysis should be available by 2023.

Research methodologies

Research that provides quantitative data about the interactions between the pathogen, the host and the environment is critical to understand transmission in specific settings, which finally allow to model, forecast and control the disease, until elimination. Various methodologies can be applied for field and laboratory work to understand these interactions. We researched, applied and adapted numerous procedures to our study context in order to understand where, when and how malaria transmission persists in Cambodia. For a clearer comprehension of the context and the methodologies applied during this PhD, a description of the most common available methodologies relevant to mobility recording, Geographic Information System (GIS), vectors collections and molecular tools to track infections are described below. After assessing these options, we determined which methods fitted best our study designs and objectives. These selected methodologies are presented in further details below and in the material and methods of the published articles and manuscripts.

C.1. Mobility recording

On both long- and short-distance spatial scales, human movement can be measured at a fine spatiotemporal resolution using commercially available Global Positional System (GPS) data loggers to describe individual movement patterns [184–187]. Their low cost and ease of use make GPS data loggers ideal for tracking human movement to infer risk over specific time periods, including peak transmission seasons and vector biting times [187]. GPS trackers can be chosen from an array of commercially available models or even custom-made to fit the objective of the study [188, 189]. Many of these models have been tested under various situations and environmental conditions [186, 189–192]. However, GPS trackers can be – intentionally or not – forgotten at home rather than brought on daily activities, suffer from inadequate conditions of satellite geometry and signal strength, can be damaged or stolen, are limited by battery life and can lose satellite signal because of surrounding buildings or topography [186, 187, 190]. Data loggers have been used to evaluate the effect of spatiotemporal movements on various infectious diseases, such as malaria [17, 193], but also schistosomiasis [194], dengue [185–187] and influenza [195].

Another source of individual-level geospatial data that has been used in infectious disease research is mobile data [15, 196, 197]. However, this source heavily relies on ownership of

mobile phones, which itself depends on socioeconomic factors [198]. Additionally, this technology cannot measure spatial movements finer than tower-level spatial resolution, and some rural and remote areas can be equipped with a very limited amount of these towers, reducing drastically the spatial resolution [198]. Ownership of multiple SIM cards and calling behaviour can also affect to perceived spatial mobility and individuals density of a certain area [198]. Another way to use mobile phones is to rely on their GPS tracking, but this methodology would be applicable to smartphones only, increasing the socioeconomic bias and potentially infringing users' confidentiality.

The most widespread methodology to record and describe mobility is through questionnaires, as they are logistically and financially easy to implement. They are not subject to socio-economic biases, applicable to all environments and better accepted by communities than tracking devices. Interviews tend to provide more context about locations and their output is easy to process, understand and analyse [187]. However, questionnaires are subject to recall bias, and to respondents that voluntarily omit or change information – especially in the case of illegal activities [163]. Questionnaires can be lengthy and require considerable time and labour to produce and perform the interviews. Translation can be necessary.

Overall, none of these methodologies is the gold standard for mobility recording. They all present their own advantages and drawbacks, and they can be adapted to different contexts to fit specific study designs, objectives and contexts. Novel approaches are proposed, especially since the emergence of SARS-Cov-2, such as modelling airport catchment areas [199], automated digital contact tracing [200], RADAR-based data collection [201] or through dedicated apps [200]. More comparative studies would shed more light on the decisive differences between GPS trackers, mobile data, questionnaires and, and help researchers and public health actors in their choice of a methodology to track individual-level mobility.

C.2. Geographic Information Systems

Much of the true potential of GPS data for health research can be maximized when they are compiled in a GIS to be combined with other spatial data [202–204]. Integration of GPS data into a GIS allows researchers to categorize their data in multiple ways based on location (*e.g.*, using land cover layer to identify GPS data points within one type of environment; creating

density maps; determining geospatial correlations; identifying disease clusters). GIS also allows to construct exposure measures to various environmental characteristics [202].

After curation and processing, satellite imagery can provide such environmental data, and has become more widely available in the recent decades. Various initiatives provide fine scale imagery and associate data at a planetary scale, free of cost. Just to cite a few, the USGS agency has been continuously providing satellite imagery since 1972; the GEOSUD-EQUIPEX project grants access to satellite imagery from suppliers; the Copernicus programme delivers land, atmosphere and marine data captured by the Sentinel satellites; the NASA has been advancing aeronautics research and funding imagery programs since 1958.

Raw satellite imagery is rarely of use; it needs to be processed. Ground and atmosphere temperature, Normalized Difference Vegetation Index, water presence and most of environmental conditions can be extracted from satellite data. Based on these layers, complex data is unearthed, such as land use, land cover or air quality, which can be integrated into weather forecast, disease modelling and climate change predictions.

One way to approach these layers of data is through landscape metrics. They are measurable units of a landscape composition and allow to describe spatial patterns and to measure variation of different spatial variables over time or space, at usually three scales: patch, class and landscape (Table 1). These metrics were designed and made available through the software FRAGSTATS [205], but their use is nowadays common across various software and coding platforms.

Table 1: Twenty-three widely used landscape metrics, applicable to landscape level and effective in quantifying spatial patterns [206].

The basis provided by satellite imagery and the available tools to process them can be actively integrated to public health surveillance or even available to the public, as in the case of [ClimHealth](#) that allows to visualise in real time the risk of leptospirosis transmission in Yangon.

Name	Abbreviation	Category
Number of patches	NP	area, density, edge
Patch density	PD	area, density, edge
Edge density	ED	area, density, edge
Mean patch size	MPS	area, density, edge
Patch size standard deviation	PSSD	area, density, edge
Patch size coefficient of variation	PSCV	area, density, edge
Landscape shape index	LSI	area, density, edge
Largest patch index	LPI	area, density, edge
Mean patch shape index	MSI	shape
Area-weighted mean patch shape index	AWMSI	shape
Perimeter-area fractal dimension	PAFRAC	shape
Mean patch fractal dimension	MFPD	shape
Area-weighted mean patch fractal dimension	AWMPFD	shape
Contagion index	CONT	contagion, interspersion
Aggregation index	AI	contagion, interspersion
Landscape division index	DIVISION	contagion, interspersion
Shannon's diversity index	SHDI	diversity
Simpson's diversity index	SIDI	diversity
Modified Simpson's diversity Index	MSIDI	diversity
Shannon's evenness index	SHEI	diversity
Simpson's evenness index	SIEI	diversity
Modified Simpson's evenness index	MSIEI	diversity
Landscape dominance index	DI	diversity

C.3. Vector collection

To choose accurately a vector collection method, one must consider the entomological parameters, the environmental constraints and the species being studied. Indeed, the type of trap has an influence on the collected species diversity and abundance [207–209] and must be carefully considered and implemented to accurately characterise a vector population.

Adult mosquitoes are attracted to humans through temperature, visual and chemical inputs that are hard to simulate without a human bait [210]. When involving human baits or collectors, they should be trained and know about the inherent risks. In addition, the investigators should randomly assign participants a collection location, to avoid confounding the collector attractiveness to the location attractiveness [211]. Finally, collected mosquitoes can be stored in boxes or cups where they can breathe and stay undamaged, labelled with the date, hour, collection sites and type of trap.

Human Landing Catch (HLC) is considered the “gold standard” of vector collection methods as it corresponds to the direct measurement of mosquitoes seeking a human blood meal [212, 213]. This methodology collects all mosquitoes landing on the bare legs of a collector used as bait. The collector would sit for 30 to 50 minutes per hour, trap the mosquitoes in paper cups using a mouth aspirator or a collection tube, rest for the remaining time and repeat the process every hour. Human Landing Catch is the most frequently used vector collection method in medical entomology research, can be implemented both inside and outside, through extended periods and with minimal material. However, this technique is controversial as it easily exposes collectors to mosquitoes and to potential infectious bites when treatments or chemoprophylaxis and vaccines are not always available or efficient [142]. Also, the task can be strenuous and individual reactivity or attractiveness can induce different results [210]. Finally, the method could overestimate human biting rates, as collectors are more accessible and immobile than in real life settings in which they could be moving, using repellents or be under a net. Overall, HLC is an important tool to estimate *Anopheles* diversity and associated exposure, but might not be relevant to characterise *Anopheles* populations, depending on the research question and the environment [214].

An alternative to HLC is Human-baited Double Net traps (HDN). This variation also includes a human as bait and collector. The trap is made of two mosquito nets: the outer and larger one is raised from the ground (about 20 cm), letting the mosquitoes enter and be trapped

between this net and a second smaller inner net, which completely protects the collector. Every hour, the collector uses a (mouth) aspirator to catch the mosquitoes trapped between the two nets. This technique is easy to implement and require only cheap and widely available materials. As the collectors are not directly exposed to mosquito bites, it also represents a more ethical alternative to HLC, while their efficacy has been estimated to be equivalent [211]. Main concerns about this method is that mosquitoes might escape after not being able to feed and mosquitoes could enter accidentally or by looking for shelter rather than a blood meal [215, 216]. The principle of HDN can be used with animal baits, or can be implemented to test vectors host preference, by using animal and human baits side by side [154].

Another commonly used type of trap is the CDC light trap, which does not require human collectors on site. Usually coupled with an artificial CO₂ production and an odour bait to mimic a human bait, these traps are made of a light that visually attracts mosquitoes. When they are close to the light, a fan pulls the attracted mosquitoes into a net, where they remain trapped until a collector retrieve them. These traps are completely exposure-free, with limited maintenance and easy to install in almost any kind of environment. Being small and less intrusive than other collection methods, CDC traps are usually widely accepted by communities [217]. However, feeding on a human is a process that differs substantially from the CDC traps artificial attraction, which might induce a biased estimation of vectors relative abundance and diversity [218, 219]. Finally, the use of CDC traps in the GMS has encountered little success in catching malaria vectors when compared against other traps [211, 220].

Based on a different principle, the barrier screen (SCR), also called interception trap, is a bait-less trap aiming to determine the mosquito flight periods between two chosen environments – usually, a village and a neighbouring patch of forest. They consist of a long screen, usually about 2 metre high, intercepting the mosquitoes during their movements between environments, while they are looking for a blood meal, a shelter or an oviposition site. They are extremely cheap, usually made from polyethylene shade cloth. Hourly, a collector on each side of the screen examines and collects all intercepted mosquitoes. In addition to the common variables (date, time, etc.), the flight direction (*i.e.* the side of the screen the mosquito is collected from) needs to be recorded, and the height can be informative as well. The screen is usually made of polyethylene or polyester materials fastened on wooden poles, bamboos or other available reinforcement bars. These traps are exposure-free, cheap and

easy to install, while being able to catch a variety of mosquitoes from blood-fed to host-seeking, commuting in and out of the village. They have been tested and implemented numerous times [221–224] and are efficient for the collection of exophilic and exophagic anophelines [222]. Its biggest weakness also resides in its simplicity: mosquitoes can easily fly above it or fly off the screen before being collected.

Numerous other traps can be installed to collect resting mosquitoes, such as pit traps or pit shelters. They can be highly productive in some areas but are relatively permanent, not recommended in floodable areas, difficult to implement and to standardise [225]. Moreover, mosquitoes can fly out of the trap at any time.

Clay pots and other portable artificial resting sites are also used to passively collect shelter looking mosquitoes. They are cheap, easy to set-up and can be deployed in large numbers within a variety of environments. However, mosquitoes entering the pots can easily fly off between or during collection times. A last important drawback is the attractiveness of the pots: to catch mosquitoes, they should be as attractive or more attractive than neighbouring natural resting sites.

Sticky resting traps are another alternative, which come in a variety of designs. Compared to pit shelters and clay pots, sticky traps are easier to standardise, as the collection of mosquitoes does not depend on mosquitoes flying out of the trap or on the skill of the collector. They also have the same advantages as clay pots: cheap and convenient, making them an efficient alternative to other passive traps [225]. Their major inconvenient is that they can damage mosquitoes, proscribing morphological identification [226].

Finally, a range of more or less automated traps can be instituted for vectors collection. Aspiration backpacks can be implemented anywhere easily without collector exposure, but captures mostly gravid mosquitoes and damage them [226]. Host-decoy traps mimics host attractiveness with an array of odours, temperature signals and visual stimuli and can be highly efficient for outdoor feeding mosquitoes with no collector exposure [227]. Mbita traps and Ifakara tent traps have been seen as unreliable compared to other collection methods [228–231]. BG sentinel traps and Suna traps have brought satisfactory results in some areas [232, 233] but are unfit to estimate outdoor biting rates in the GMS [211].

Trap height is an important factor to consider, as different heights can yield different results [234, 235]. In a Peruvian study, the installation of monkey-baited CDC traps highlighted differences between vectors populations captured at ground or canopy level [236].

To understand fully vectors bionomics, collecting adult mosquitoes is insufficient. The capture of adults through the above-cited means cannot provide information about larval densities, oviposition sites and environmental factors affecting larval survival. Methodologies are not as varied as for collecting flying adults. Larval collections mostly rely on catching larvae in their environments, using nets or dippers. For large breeding sites, dipper contents are placed in a white tray, from which larvae can be easily seen. If larvae are not numerous, they are transferred with a pipette into a labelled falcon (with date, location, etc.) – otherwise the full content is transferred. Breeding site geolocalisation and physical characteristics should be noted for further analysis, with pictures, notes and/or questionnaires.

Two approaches can be considered for larval sampling: *i)* the thorough sampling of a specific environment in the study area or *ii)* the evaluation of larval density in all potential breeding sites in a specific area. Even if the first one is definitely implemented [237, 238], the second approach is more frequently used in medical entomology as it can accurately determine vector bionomics (e.g., larval habitats) in chosen transmission settings [239–244]. For the approach focusing on a specific environment, a chosen type of breeding site is sampled (for example, rice paddies) [237, 238]. Mosquito larvae are collected in every site with the same methodology for a certain amount of time. The results can estimate mosquito densities in a specific environment, associated to a human activity or a land use. For the approach focusing on a specific area, it can follow a previously defined transect, or sample all potential breeding sites around an area of interest/collection site. Often, transects are defined as lines (from a collection site to a cardinal direction, for example) on the ground, followed by collectors. To be repeatable among sites and seasons, the transect should not depend on temporary land marks, but rather on a determined set of latitude and longitude [245, 246]. All open water bodies must be searched for larvae, and the descriptions of all breeding sites must be recorded to ensure a valid analysis of environmental factors impacting larvae distribution [246, 247]. Larvae density in the breeding site can be estimated by evaluating the volume of the collected water from the water body and by counting the collected individuals [248].

Mosquito larvae are usually taken care of and fed until emergence [249], as larval stages are more difficult to identify morphologically .

C.4. Tracking exposure and infections

In a context where malaria infections can be dormant and many cases are asymptomatic, follow-ups and characterisation of the parasitic infections are necessary to assess exposure. Being able to determine the recent exposure of a patient to a vector or to a malaria infection is necessary for the precise identification of transmission factors and risk areas.

Recent exposure to vectors can be determined with the quantification of vector-specific anti-mosquito antibodies present in the bloodstream [250, 251]. The immune system produces Immunoglobulins G (IgG) antibodies after *Anopheles* saliva enters the bloodstream during the mosquito blood meal. The salivary peptide gSG6-P1, based on the *An. gambiae* SG6 protein sequence, well conserved among *Anopheles* species, has been validated as a specific biomarker of *Anopheles* exposure in Asia [250], Africa [252, 253] and the Americas [254]. For all these localities, positive correlation between the human exposure level to *Anopheles* bites and human anti-*Anopheles* saliva antibody level has been demonstrated. The only area where the *An. gambiae* peptide failed to estimate vector exposure was the Pacific [255]. A recent systematic review demonstrated that salivary biomarkers measured at the individual level, such as gSG6 IgG, can be used to quantify total vector exposure at the population level, without requiring laborious entomological experiments, but the study could not validate the method's accuracy [256]. Elsewhere, the methodology successfully demonstrated its ability to measure micro-geographical heterogeneity of human exposure to *Anopheles* bites, even in low and seasonal malaria areas, making it an ideal candidate for the GMS transmission settings [250, 257].

While quantifying individual exposure to *Anopheles* is important, quantifying the recent exposure to parasites is equally essential. In particular, attaining malaria elimination will require the capacity to detect and treat relapsing *P. vivax* infections. Indeed, a patient could undergo a *P. vivax* episode months or years after their last infectious bite, due to the species' hypnozoites ability to remain dormant in the host's liver for extended periods [258]. As such, *P. vivax* serological exposure markers (SEM) can provide crucial information about the last episode of a patient. Their efficacy has been assessed in low malaria transmission settings and

its implementation is recommended in areas pursuing malaria elimination. Still, SEM accuracy to identify individuals with recent blood-stage infections greatly decrease in high transmission settings [259].

To grasp the nature and the composition of malaria infections, molecular biology techniques can be implemented to focus on some regions of interest or the whole genome of the parasites.

Microsatellite genotyping has been integrated to malaria research since the 1990s. It is used to investigate the population structure of parasites, to map genetic loci contributing to drug resistance or to distinguish between treatment failures and new infections [260]. Microsatellite genotyping remains a popular approach, but with the increasing availability of whole genome sequencing data enabling effective single nucleotide polymorphism-based panels tailored to a given study question and setting, microsatellite are losing popularity [261].

One common way to analyse these molecular markers is by looking at single nucleotide polymorphisms (SNP) in the genome of the parasite. The method provides a robust, low-cost method of identifying parasite infections through their unique genetic signature. Barcodes including large numbers of SNP markers are accurate and efficient genotyping tools, but they can lack resolution at the local scale if they are not tailored to specific malaria transmission settings [262]. When correctly implemented, SNPs can precisely pinpoint the area of origin of an infection [263], predict the emergence of resistant phenotypes [264] or determine recrudescence from reinfection [265, 266], with applications for *P. falciparum* [264, 265], and to some extent, *P. vivax* [263, 266]. Compared to microsatellite, SNP barcodes revealed greater variability in genetic diversity between populations and geographical population structure [262].

Next-generation sequencing (NGS) methods provide the resolution, scalability, and sensitivity required for high-throughput surveillance of molecular markers. Particularly, deep amplicon sequencing (AmpSeq) has been developed over the last years to investigate markers of drug resistance [267], to quantify clone density over time to study clone competition [268] and overall to understand malaria population genetics. The most important features of AmpSeq are its sensitivity in detecting minority clones and the possibility to multiplex hundreds of samples and dozens of genetic marker on the same flow cell [269]. However, even if in theory,

the amplicon sequencing protocol can be operated with minimal bioinformatics infrastructure to make it suitable to countries with endemic transmission [267], the protocol requires bioinformatics, which is not accessible by all, and important funds for the material, experienced workforce and sequencing itself.

These methodologies provide a detailed genetic profile of the parasites being responsible for the infection. Resistance emergence, selection events and migration can all affect and drive parasites populations and subpopulations survival. Characterising genomic clusters through the identification of unique genetic markers could help determine previous and present genetic pressures that are altering malaria transmission. Such genetic data analysis can provide information on which parasites clusters are actively being transmitted between human and mosquito population, or help identifying bottlenecks events associated with malaria elimination phases.

Part II: Research

A. Chapter 1: The determination of anthropisation in a rural Cambodian area and the mobility of risk populations

1.1. Introduction

The transmission of malaria requires interaction between the host, the vector and the parasite within an adequate environment. Any change in environmental conditions can affect the relationship between these three organisms and consequently impact malaria transmission itself. In an age of anthropisation where resources consumption keeps increasing, their effects may affect malaria dynamics. To forecast and control the effect of human alterations to the environment and to malaria transmission, it appears primordial to understand the nature and the intensity of the interaction between environmental changes and malaria dynamics.

To fully understand the effect of environment, we had to represent the environment in which the study population was living. A land use or land cover classification can represent effectively the repartition and surfaces of forest, agriculture patches and built-up areas over a study area. To produce it, imagery of the area must be acquired then processed. As the use of drones is very restricted in Cambodia, we settled for satellite imagery. Various factors have to be considered. First of all, the nature of the imagery itself: which resolution is needed? Which years or months will provide optimal imagery? In countries including a rainy season, clouds can cover the area every day for several months. Even if progress is being made in analysing infrared imagery, a land cover or a land use classification is easier to produce from images from the visible spectrum and limited cloud cover. In addition, costs and intervals between images have to be considered. Finally, the resolution of the imagery is decisive. What are the smallest objects that we need to include in the map? In Cambodia, there is no description of the importance of smaller patches of forest on vectors densities or malaria transmission. As such, we had to choose a resolution as fine as possible to include even the smaller patches that may or may not play a role in populations mobility, vectors bionomics and overall malaria dynamics in the study area. To create the classification, a range of manual to semi-automated and automated methodologies were considered. After a few attempts with semi-automated classification that mixed tree plantations with primary forest, we opted

for a completely manual delimitation of the land use patches. The process was lengthy but it appeared more accurate than other methods tested until then.

In Cambodia, the main malaria risk factor has been identified numerous times as forest-associated activities. However, the definition of forest across subpopulations and time changes. While the definition of a typical forest goer is a man of working age, it is reported that women and children also visit the forest, but they do not display a prevalence as high as men [30, 158, 159]. To categorise a man as a forest goer, it usually takes a few questions about their main income and their recent mobility to the forest [158, 159, 161]. Because of the limited availability of detailed, actualised maps of the forested areas and the limited spatial literacy of rural populations, pinpointing the exact origin of the vector-host contact is challenging.

Through a cross-sectional study, the socio-economic factors associated with malaria prevalence were determined in the study area. We observed how different strata (men versus women, children versus working age, residing inside or outside a forested village) were affected by the vector-borne disease.

However, we still required a way to objectively and accurately describe the mobility within the study area. We considered the use of questionnaires or use of mobile phone data but finally opted for GPS data-loggers that appeared most adapted to our study design, because they did not rely on spatial literacy, participants recall, socio-economic status and ownership of a phone or the poor mobile coverage. We acquired different trackers and brought them to the study area to test their accuracy and their sturdiness. We wore multiple GPS loggers with different settings non-stop to assess the shortest logging interval that allowed battery life to last a week and that depicted all places we visited.

Once the methodology was chosen for both the land use classification and GPS data loggers' implementation, a great effort was invested into the importance of scale. Without effective data on Cambodian vectors flight range and the importance of forest patches size, it was a difficult process. The final decision of the 100-metre grid resolution was based on a trade-off between GPS tracks processing time and precision of the output.

Finally, we implemented the GPS follow-up among a population of forest goers' description-matching men that were followed during the cross-sectional study, with the objective to understand the prevalence heterogeneity among them. We also implemented a similar GPS

follow-up including both men and women to pinpoint the factors driving the important prevalence difference between them.

This chapter hereinafter details the study designs we developed, data processing and consequent results.

1.2. Anthropisation

Article: Studying Land Cover Changes in a Malaria-Endemic Cambodian District: Considerations and Constraints

Anaïs Pepey, Marc Souris, Amélie Vantaux, Serge Morand, Dysoley Lek, Ivo Mueller, Benoit Witkowski and Vincent Herbreteau

Published in Remote Sensing journal's special issue "Remote Sensing for Health: from Fine-Scale Investigations towards Early-Warning Systems" on the 12th of September 2020.

<https://doi.org/10.3390/rs12182972>



Article

Studying Land Cover Changes in a Malaria-Endemic Cambodian District: Considerations and Constraints

Anaïs Pepey^{1,*}, Marc Souris² , Amélie Vantaux¹ , Serge Morand^{3,4} , Dysoley Lek^{5,6}, Ivo Mueller^{7,8}, Benoit Witkowski¹ and Vincent Herbreteau^{3,4,9}

¹ Malaria Molecular Epidemiology Unit, Institut Pasteur du Cambodge, 5 Blvd Monivong, BP983, Phnom Penh 120 210, Cambodia; avantaux@pasteur-kh.org (A.V.); bwitkowski@pasteur-kh.org (B.W.)

² UMR Unité des Virus Emergents, UVE: Aix-Marseille Univ–IRD 190–Inserm 1207–IHU 5 Méditerranée Infection, 13005 Marseille, France; marc.souris@ird.fr

³ UMR ISEM, CNRS–University of Montpellier–IRD, F-34093 Montpellier, France; serge.morand@umontpellier.fr (S.M.); vincent.herbreteau@ird.fr (V.H.)

⁴ UMR ASTRE, CIRAD, F-34398 Montpellier, France

⁵ National Centre for Parasitology Entomology and Malaria Control (CNM), Phnom Penh 120 801, Cambodia; soleycnm@gmail.com

⁶ School of Public Health, National Institute of Public Health, Phnom Penh 120 104, Cambodia

⁷ Malaria: Parasites and Hosts Unit, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris, France; ivo.mueller@pasteur.fr

⁸ The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, VIC 3052, Australia

⁹ UMR ESPACE-DEV, IRD (IRD, Univ. Antilles, Univ. Guyane, Univ. Montpellier, Univ. Réunion), at Institut Pasteur du Cambodge, 5 Blvd Monivong, BP983, Phnom Penh 120 201, Cambodia

* Correspondence: apepey@pasteur-kh.org

Received: 5 August 2020; Accepted: 1 September 2020; Published: 12 September 2020



Abstract: Malaria control is an evolving public health concern, especially in times of resistance to insecticides and to antimalarial drugs, as well as changing environmental conditions that are influencing its epidemiology. Most literature demonstrates an increased risk of malaria transmission in areas of active deforestation, but knowledge about the link between land cover evolution and malaria risk is still limited in some parts of the world. In this study, we discuss different methods used for analysing the interaction between deforestation and malaria, then highlight the constraints that can arise in areas where data is lacking. For instance, there is a gap in knowledge in Cambodia about components of transmission, notably missing detailed vector ecology or epidemiology data, in addition to incomplete prevalence data over time. Still, we illustrate the situation by investigating the evolution of land cover and the progression of deforestation within a malaria-endemic area of Cambodia. To do so, we investigated the area by processing high-resolution satellite imagery from 2018 (1.5 m in panchromatic mode and 6 m in multispectral mode) and produced a land use/land cover map, to complete and homogenise existing data from 1988 and from 1998 to 2008 (land use/land cover from high-resolution satellite imagery). From these classifications, we calculated different landscapes metrics to quantify evolution of deforestation, forest fragmentation and landscape diversity. Over the 30-year period, we observed that deforestation keeps expanding, as diversity and fragmentation indices globally increase. Based on these results and the available literature, we question the mechanisms that could be influencing the relationship between land cover and malaria incidence and suggest further analyses to help elucidate how deforestation can affect malaria dynamics.

Keywords: malaria; deforestation; remote sensing; GIS; land cover; landscape metrics; Cambodia

1. Introduction

1.1. Foreword

The link between environmental variables and malaria transmission dynamics involves complex interactions, especially between vectors and land use, and its comprehension can be limited by the availability of environmental and epidemiological data. In this article, we examine methods available for understanding these interactions and discuss associated constraints. To do so, we chose to analyse land use in a malaria-endemic area of Cambodia. Based on this example, we further examine the considerations around malaria dynamics linked to anthropization in data-scarce conditions and potential methodologies for understanding vector exposure and its interaction with land use.

1.2. Background

Conflicting reports exist on the effect of deforestation on the transmission of vector-borne diseases [1]. This heterogeneity likely arises from the complexity of vector-borne disease transmission that involves numerous environmental, biological and ecological factors [1]. Modifications to the environment, such as changes in land use and deforestation, greatly alter the density and activity of mosquito populations and as a result the dynamics of associated diseases [2,3].

As malaria is a vector-borne disease, its transmission is closely tied to the environment [4,5]. Deforestation greatly alters the breeding, abundance and species composition of *Anopheles* malaria vectors [5,6]. This is mediated by changes in the availability of breeding sites for the immature stages [7], as well as differences in resources, predation [8,9], survival [10], fecundity [10] and ecological community structures [7]. These factors are known to affect vector species quantitatively more so than nonvector species [1]. Deforestation and associated phenomena, such as forest fragmentation, have also been associated with greater contact between sylvatic mosquito vectors and human hosts [7,11–13]. Indeed, deforestation does not only imply the loss of forest cover but also has an impact on landscape heterogeneity and diversity. Heterogeneity represents the repartition, abundance and size of the different habitat patches within a landscape. Heterogeneity can be affected by fragmentation, the process of a homogenous patch of habitat evolving into smaller and disjointed patches [14]. Another aspect of deforestation is landscape diversity, which corresponds to the proportion of patches from different classes. Usually as deforestation expands, it is replaced by agricultural land or left vacant, increasing landscape diversity [15]. A potentially associated phenomenon is the loss of biodiversity [16], which can be associated with higher malaria burden [17,18] through the decline of the “dilution effect” [19,20] though this theory remains controversial [19,21]. Overall, even with variations among ecological groups and individual species [22], extensive anthropization substantially impacts malaria dynamics [17].

1.3. Malaria and Deforestation

Many studies have investigated the link between deforestation and malaria using remote sensing and landscape analyses. A study that analysed data from 67 nations showed that in developing nations, at country level, a higher deforestation rate is associated with a higher malaria burden [2]. However, this is only a general trend based on global measurements of deforestation using FAO estimates of natural forest areas [2] and may not be valid for all countries. At a more local scale, in the Peruvian Amazon, processed 30-m Landsat imagery showed that deforestation favoured malaria transmission by increasing *Anopheles darlingi* human biting [23]. Furthermore, a controversial publication stated that forest conservation efforts in the Brazilian Amazon could increase malaria burden in humans, based on an annual estimation of forest cover from Landsat imagery [24]. The study found a correlation between greater forest cover in a 20-km radius around cities and higher number of reported malaria cases [24]. However, to perform this analysis, some data was excluded and part of the land use data was too approximate, i.e., land cover classes associated to different malaria risk were aggregated. Consequently, the publication was criticized as this methodology could result in potential ecological fallacies [25]. In a local study in Thailand, land use maps were produced with photointerpretation and their landscape

indices were computed, which allowed to confirm that the primary malaria vectors, *Anopheles maculatus* and *Anopheles minimus*, were more abundant in forests than in agricultural landscapes [26]. Interestingly, a recent report has demonstrated a feedback loop between malaria incidence and deforestation in the Brazilian Amazon whereby forest loss is associated with an increase in malaria incidence, and this increase, in turn, leads to a decrease in the rate of deforestation. The different geographic scales and the quality of land cover data, ranging from global estimates of forest area to precise mapping at high spatial resolution, are important parameters. In addition, studies based on spatial correlations do not indicate cause and effect relationships. Some studies using aggregated data, at a scale including whole jurisdictions rather than population or vector scale, can be subject to biases and ecological fallacies [27]. Publicly available forest cover maps usually have a resolution of 20–30 m, typical of LANDSAT imagery [28,29].

1.4. Issues When Studying the Relationship between Deforestation and Malaria

To produce a land cover classification, a variety of methodologies can be used. Numerous remote sensing sources are available, and their classification can either be automated, supervised or manual, or even done by photointerpretation—all these options are applicable from a wide panel of different software [30,31]. Choices made regarding the methodology, landscape indices or analyses all influence the interpretation of results. Additionally, interpretation can be impacted by the oversimplification of the vector-borne disease paradigm as described in the analyses [1]. Nevertheless, a meaningful analysis of deforestation and associated human risk is fundamental to contribute to the malaria effort.

The link between deforestation and malaria risk in South-East Asia (SEA) has not been completely elucidated [11]. Indeed, the complexity of this linkage arises from the many different vectors adapted to forest or deforested areas, with populations in a steady, close contact to the forest, unlike other malaria-endemic parts of the world [11]. On one hand, deforestation could decrease malaria risk by limiting the availability of vector habitats, thus reducing the abundance of primary vectors [11]. Conversely, deforestation may increase malaria transmission as it encourages contact with primary vectors and induces higher densities of secondary vectors that are often more adapted to deforested areas [32]. Additionally, malaria risk depends on deforestation effect or edge proximity, more than the forest extent. Therefore, intense deforestation can lead to higher fragmentation indices and then augmented malaria transmission. The socioeconomic effects of deforestation also impact malaria risk. Notably, newly arrived low-immunity populations that settle in deforestation sites, with high exposure to vectors but potentially no access to appropriate prevention tools or health services, along with possibly higher transmission due to increased parasites densities [33]. Though, higher wages arising from deforestation profit can allow better vector prevention in these populations [33,34].

Another difficulty lays in the various temporal and spatial scales needed in the analysis of the interaction between forest cover change and malaria dynamics [35]. For example, the Amazon Basin is characterized by frontier malaria [36], where deforestation often takes place for farming and mining, with new populations settling in and creating an environment with high malaria risk in the first years of colonization. The process was shown to be transitory, as social and economic factors eventually cancel out environmental effects, decreasing malaria incidence, usually after 6–8 years [36]. A recent analysis on malaria prevalence, deforestation, land cover, environmental, sociodemographic, health services accessibility and housing data on individuals' data from 17 sub-Saharan countries showed that their "aggregate and survey wave-specific results imply the absence of a geographically generalizable relationship between deforestation and malaria, and between forest cover and malaria, across the countries studied." These results suggest that Africa's deforestation effects likely differ from Asia's and Latin America's [33]. Overall, the SEA paradigm is complex, not yet completely defined, and likely differs from situations described in Africa and the Americas [11].

1.5. Situation in Cambodia

In Cambodia, the decline in malaria cases has come to a halt, and the estimated number of malaria cases has been increasing since 2017 [37]. The rise in case numbers could be attributed to the emergence of various resistant [38,39] and multiresistant [40] *Plasmodium falciparum* strains in the last decade, although these are not the only obstacles in the way of malaria elimination. Substandard clinical practices [41] and vector resistance to insecticides [37] may also contribute to the persistence of malaria.

The main malaria vectors are classically considered to be *An. dirus* and *An. minimus*, usually found in forest and forest fringes [42,43], and to a lesser extent *An. maculatus* which is associated with hilly and mountainous areas [44]. The larval stages of *An. dirus* are typically found in small sites, such as streams, ditches, dips and prints in the ground, whereas *An. minimus*' larvae can be found in small natural sites (pools and streams) or large man-made ones (notably rice paddies). *An. maculatus*' larvae are found in a variety of environments; small or large, man-made or natural sites [42], limiting the effectiveness of landscape analyses to link mosquito risk to standing water.

In Cambodia, forests and their fringes are the primary contact sites for human and vector populations, making forest activities the most important malaria risk factor [45–47], with a disease often referred as “forest malaria.” Thus, the primary at-risk population corresponds to the forest goers that spend a lot of time in and around the forest [48]. However, literature lacks exhaustive and recent data about mosquito ecology and precise epidemiology in Cambodia. Indeed, a substantial gap exists in vector ecology data compared to other parts of the world, notably due to the high diversity of vector species exhibiting a large spatial heterogeneity in distribution and behavioural patterns both between and within species [42,44]. In addition, the type of forest, and notably the distinction between primary and secondary forests, or even between plantation forest and natural forest, is not explored in available studies. Compared to other parts of the world and notably the Amazon, investigations in Cambodia are limited by the absence of detailed data about vectors and epidemiology of the disease, but also by other constraints, such as the difficulty to gather prevalence data over time. The Khmer Rouge regime in the seventies has had a long-lasting influence on the country and prevented the collection of any kind of malaria prevalence records [49]. A national malaria information system was developed in 2009 to gather prevalence data from health centres. In 2013, a network of village malaria workers was implemented to improve malaria detection and treatment access at the village level [50]. Additionally, private health practitioners that are allowed to treat uncomplicated malaria cases are widely spread across the country, although cases are not always reported to the national information system [51]. Overall, an aggregated and homogenous malaria dataset is not available in Cambodia and exhaustive malaria prevalence data at a fine scale is difficult to obtain, increasing the challenges when it comes to studying the disease. In this data-scarce environment, we can assess trends, formulate hypotheses and suggest methods adapted to this specific context. However, the lack of homogenous, small-scale data reduces the possibilities of further analysis.

As deforestation progresses in Cambodia and induces drastic landscape changes [52,53], effects on malaria transmission are to be expected. The alarming deforestation rate is predominantly caused by large land concessions where companies buy large plots of land to make way for agriculture [52]. In particular, this has been observed in the north-eastern province of Mondulkiri. This situation highlights how short-term economic plans lead to long-term impacts on environment stability and are already threatening forest sustainability [52]. Local communities engage in deforestation through “slash and burn” methods (Figure 1), allowing them to extend their agriculture parcels [47]. Another form of forest exploitation that occurs at a slower pace is “selective logging,” where forest goers search for and cut down specific trees without affecting the surrounding trees. This form of deforestation is more difficult to keep track of, particularly by remote sensing [54]. Beneficial effects have been observed from allowing local communities to own and manage nearby forests, compared to large land concessions [55]. Community forests had higher forest cover persistence and lower rates of forest cover loss [55].



Figure 1. Forest recently cut down and burnt to clear space for agriculture, by using the “slash and burn” method, Kaev Seima district, Mondulhiri Province, Cambodia (photo: A.P.).

1.6. Objectives

This article aims to discuss methodologies and constraints associated with studying land cover changes and malaria dynamics using Cambodia as a case study. Available data is used to investigate trends in malaria prevalence over time and space, at country and provincial levels. In addition, we use high resolution satellite imagery of a chosen malaria-endemic area to produce land use/land cover maps from 2018, completing available classifications from 1988 and from 1998 to 2008 of the same locality. Based on these classifications, we quantify land use and land cover characteristics and modifications over these three decades. From the results of this study, we provide insight about appropriate study scale, satellite imagery treatment, landscape metrics interpretation and adapted statistical analysis in such a context and discuss their limitations.

2. Materials and Methods

2.1. Considerations for Methodological Choices

To understand malaria vector ecology, land use/land cover classes and study scales need to be carefully determined. Rather than taking a “traditional” approach to land use classification where a high number of classes are defined, a simpler, more easily attainable classification could better depict *Anopheles* ecology by grouping environments into classes based on their relevance to malaria transmission. Forest is highlighted as the most important risk area in Cambodia as this is the main habitat of malaria vectors [45–47]. Appropriate land use classes include forest, plantations, fields and built-up areas (Figure 2). Forest can include any type of primary or secondary forest cover that is high and dense enough to form a canopy. Separating plantations, referring to tree plantations (notably *Hevea* sp., *Musa* sp. and *Anacardium* sp.) from forests can be beneficial because malaria risk in those

environments differs from that in forests. However, if plantations are separated from forests, they can also be separated from other agricultural areas, as plantations in Mondulkiri have a substantial higher malaria risk in comparison to other kind of crops [56]. Agriculture areas that are not trees, such as rice paddies or cassava fields, should ideally correspond to another class, defined in this study as “Fields.” Lastly, built-up areas stand out as lands that are irreversibly urban in character (houses, buildings, roads, etc.).

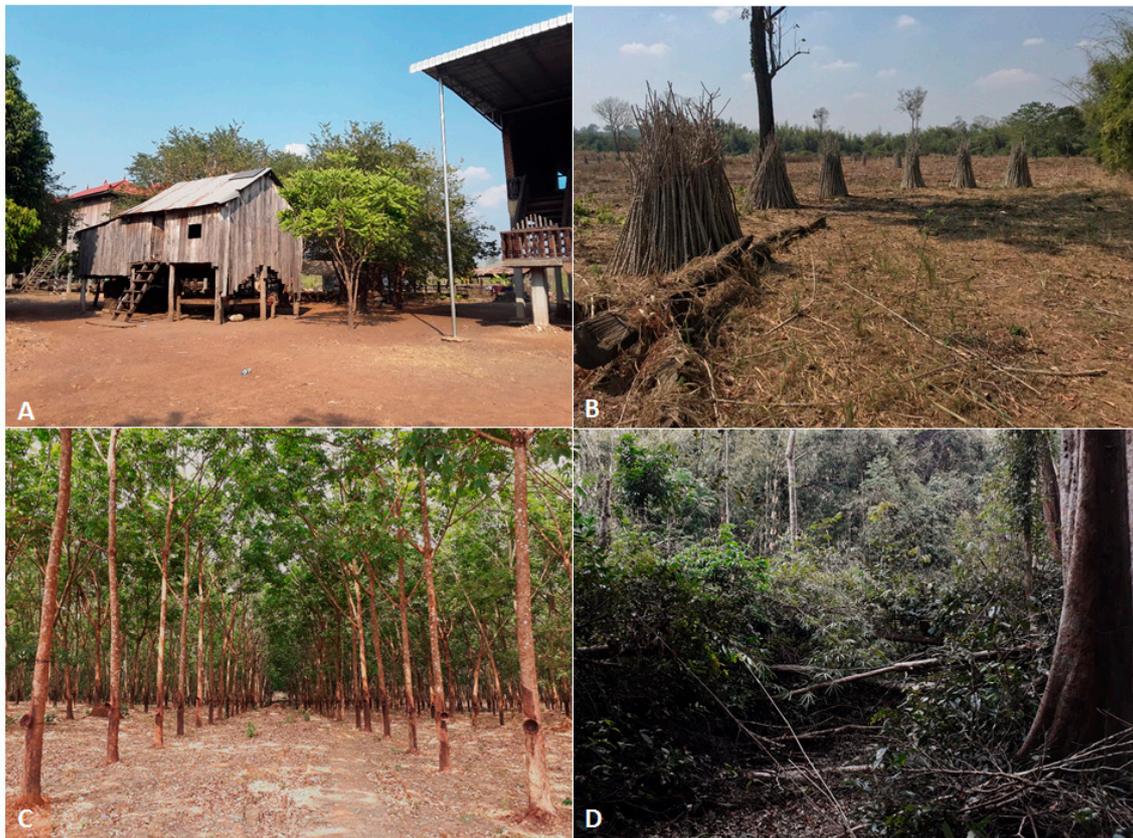


Figure 2. Example of the main environments within the study area. (A) Built-up area in a village. (B) Field used for cassava culture photographed after the collection of cassava plants. (C) Rubber tree plantation. (D) Primary forest.

Scale is also an important parameter to consider; as seen from previous publications, using insufficient resolution or inappropriate scale can lead to fallacies or misinterpretations [27]. To select the optimal scale for analysis, several parameters must be known or estimated, such as the extent of the human and mosquito populations’ mobility. Flight distance of Cambodian vectors remains hypothetical. A study in Malaysia showed that *An. maculatus*’ flight distance could reach 1.6 km [57], but this parameter remains undetermined for the other vectors. Consequently, with such limited knowledge, the minimal resolution needed for delimitating all potential habitat patches for vectors is speculative and dispersal capacity of malaria vectors in the Cambodian context cannot be estimated.

2.2. Study Site

We chose a study area in north-eastern Cambodia where the incidence of malaria is high and the rate of deforestation has been fast. We also had the opportunity to choose an area where deforestation had been previously studied, in 1988 and 1998 and 2008 [53], which gave us the chance to update the situation 10 years later. The study site spans 32 km from north to south and 39 km from west to east, within Kaev Seima district, in Mondulkiri province (Figure 3). The population is mostly engaged in agriculture, notably cassava, rice, cashew nut and rubber production. Forest activities, such as wood

logging and hunting, are part of daily life. Families spend nights at their huts if work requires them to and these huts are typically located in open areas, cultivated areas or forest.

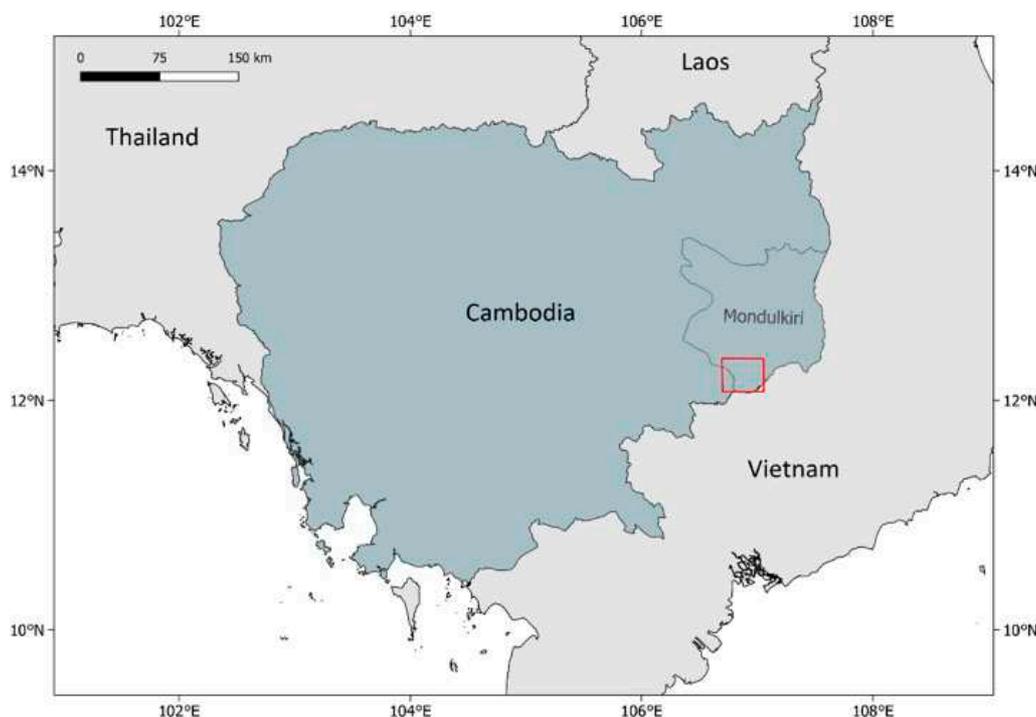


Figure 3. Location of Mondulkiri province and study area (red rectangle).

2.3. Malaria Data

At national level, the estimated number of malaria cases in Cambodia was gathered from the latest annual WHO World Malaria Reports from 2019 [37]. The values from a single report were preferred rather than aggregating data from various sources. At a provincial level, the CNM (National Centre for Parasitology Entomology and Malaria Control) has provided data on malaria incidence from Mondulkiri since 2008 (Dysoley, personal communication). Long-term, provincial-level prevalence data is difficult to access in Cambodia and data prior to 2008 could not be retrieved. District data was too miscellaneous to be presented.

2.4. Satellite Imagery and Processing

In order to compare recent land use with that of the former study, we chose to acquire images in 2018. In the former study, different spatial resolutions were used, the SPOT1 images from 1988 to 1998 having a 20 m resolution and the SPOT5 image from 2008 having a resolution of 2.5 m for panchromatic mode and 10 m for multispectral mode [53]. The land use/land cover maps were produced using object-based image analysis with eCognition software. Since we could not access this software, we chose to describe the 2018 land use/land cover by photointerpretation using the same classes. This method, simple to set up but time consuming, allowed us to find objects that are homogeneous from the point of view of their pixels but with fine contours for comparison with other classifications. This required the acquisition of high spatial resolution images. Therefore, the GEOSUD Equipex project provided SPOT 6/7 satellite images of the study area (© Airbus DS 2018) acquired between 8th and 28th of March 2018. These images have a high spatial resolution of 1.5 m for panchromatic mode and 6 m for multispectral mode. Cloud-free images were chosen as much as possible.

The objective of the 2018 classification was to find the three main classes that allowed for comparisons of the former classifications: forests, plantations, fields and built-up areas. For the purpose

of future studies, we chose to separate wooded areas into forests and plantations. We produced the 2018 land use/land cover map by photointerpreting the satellite images, displayed in true colours using GIS software. We used QGIS software (QGIS Development Team, 2020. QGIS Geographic Information System v3. Open Source Geospatial Foundation Project. qgis.osgeo.org) and SavGIS software (Souris M., 2020. SavGIS Geographic Information System v9. www.savgis.org).

2.5. Ground Truthing and Accuracy Measurement

A ground truth survey was conducted to assess the accuracy of the 2018 land use/land cover map. In the field, a minimum of 32 locations for each class were randomly described. We used the Locus Map (www.locusmap.eu) Android application in order to use a Google Earth background and GIS vector information to better visualize the environment once in the field. This also allowed us to place description points in the middle of the environments (the phone provides the GPS location and the recorded point can be shifted to the location described). These records were imported into QGIS and compared to the land use classification. We computed a confusion matrix and calculated the overall accuracy and the Kappa index to assess the classification accuracy [58].

2.6. Comparison between Maps and Landscape Metrics

In order to compare the 2018 map with those produced from 1988 and between 1998 and 2008, the “Forests” and “Plantations” classes were merged into a single class defined as “Wooded areas.” This step allowed us to incorporate the land use data from 2018 into the dataset built from 1988 and between 1998 and 2008, adding a temporal scale to the spatial observation of the area. We then clipped the extent of all the raster maps at their intersection, in order to compute indices over the same frame of the widest area possible.

To reflect landscape diversity and its evolution since 1988, we computed the same landscape indices over the three decades. These indices are: Shannon’s Diversity Index (SHDI) [59], Simpson’s Diversity Index (SIDI) [60], Edge Density (ED) and Patch Density (PD) at landscape level for all classes. SHDI and SIDI quantify diversity, whereas patch and edge density indices can be interpreted as fragmentation indices quantifying heterogeneity. SHDI, the most popular diversity index, is used as a relative index for comparing different landscapes or the same landscape at different times [61]. SHDI increases as the number of different patch types (i.e., patch richness) increases and/or the proportional distribution of area among patch types becomes more even. SIDI is another popular diversity measure that is less sensitive to the presence of rare types. Specifically, the SIDI value represents the probability that any two cells selected at random would be different. Thus, the higher the value, the greater the likelihood that any two randomly drawn cells would have different values. As SIDI is a probability, it can be interpreted in both absolute and relative terms. SIDI reaches 1 as the number of different patch types (i.e., patch richness) increases and the proportional distribution of area among patch types becomes more equitable [61]. Additionally, the percentage of landscape that belonged to each land use category was computed at a class level.

Once all classifications from 1988 to 2018 were produced and homogenised, the landscape metrics mentioned above were calculated using FRAGSTATS software (McGarigal, Cushman, and Ene, 2012. FRAGSTATS v4: Spatial Pattern Analysis Program for Categorical and Continuous Maps. www.umass.edu/landeco/research/fragstats/fragstats.html).

3. Results

3.1. Malaria Situation in Mondulhiri Province

Although malaria control efforts achieved a diminution of estimated cases in Cambodia until 2016 (124,137 cases or 8 per 1000 inhabitants), a sharp increase occurred in 2017 and 2018 (202,696 cases or 13 per 1000 inhabitants, and 272,272 cases or 17 per 1000 inhabitants, Figure 4) [37]. In Mondulhiri, malaria incidence was stable from 2008 to 2016, ranging from 18 to 28 cases per 1000 inhabitants.

The incidence tripled in 2017 and 2018, reaching 59 and 56 cases per 1000 inhabitants, respectively (National Centre for Parasitology Entomology and Malaria Control, unpublished data).

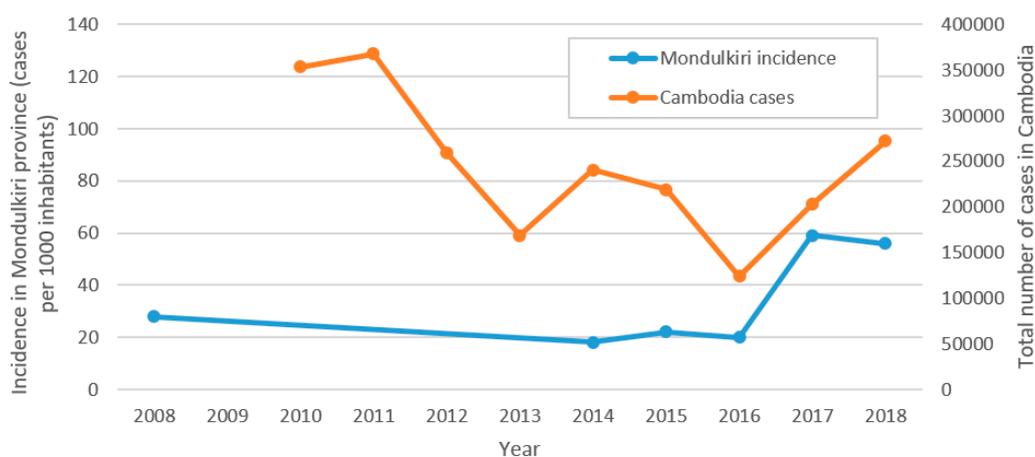


Figure 4. Malaria incidence data for Cambodia (total number of estimated cases) and Mondulakiri province (cases per 1000 inhabitants).

From the available CNM data, Mondulakiri had a malaria prevalence of 17.2‰ in 2016 and 52.3‰ in 2017, while prevalence in other provinces ranged from 1.4‰ to 11.95‰ in 2016 and 2.8‰ to 34.1‰ in 2017, indicating that Mondulakiri experienced the highest malaria burden out of all the Cambodian provinces from 2016 to 2017 (Figure 5). Within Mondulakiri, the Kaev Seima health centre registered the highest number of malaria cases in 2016 and 2017 reporting 839 and 1669 cases, respectively. Comparatively, the average number of malaria cases reported in other districts was 134 and 505 cases per year per health centre in 2016 and 2017, respectively.

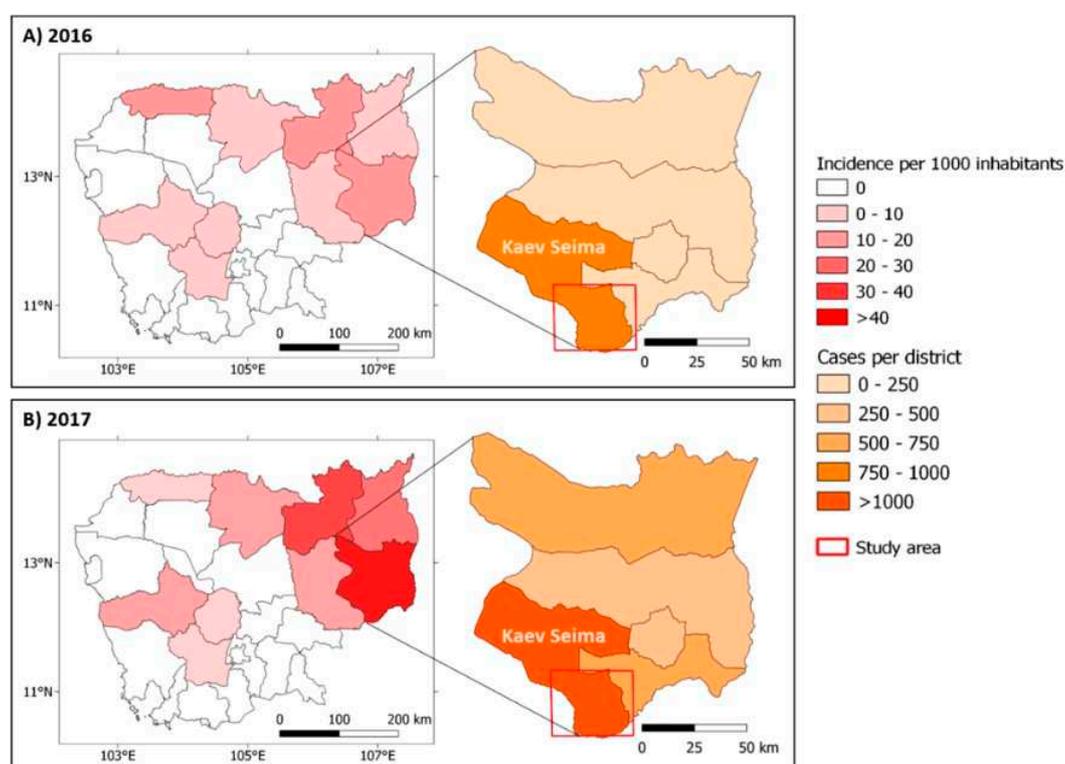


Figure 5. Incidence for 1000 inhabitants per province and number of cases per district of Mondulakiri province for years 2016 (A) and 2017 (B).

3.2. Land Use/Land Cover Classification

The 2018 classification based on forest, plantations, fields and built-up areas classes permitted the production of a malaria-relevant map, a useful resource for potential upcoming investigations in the study area. The land use/land cover classifications from 2018 are represented in Figure 6. The predominant class identified was “Fields” accounting for 47.9% of the study area, followed by “Forests” (38.1%), “Plantations” (13.7%) and finally “Built-up areas” (0.3%).

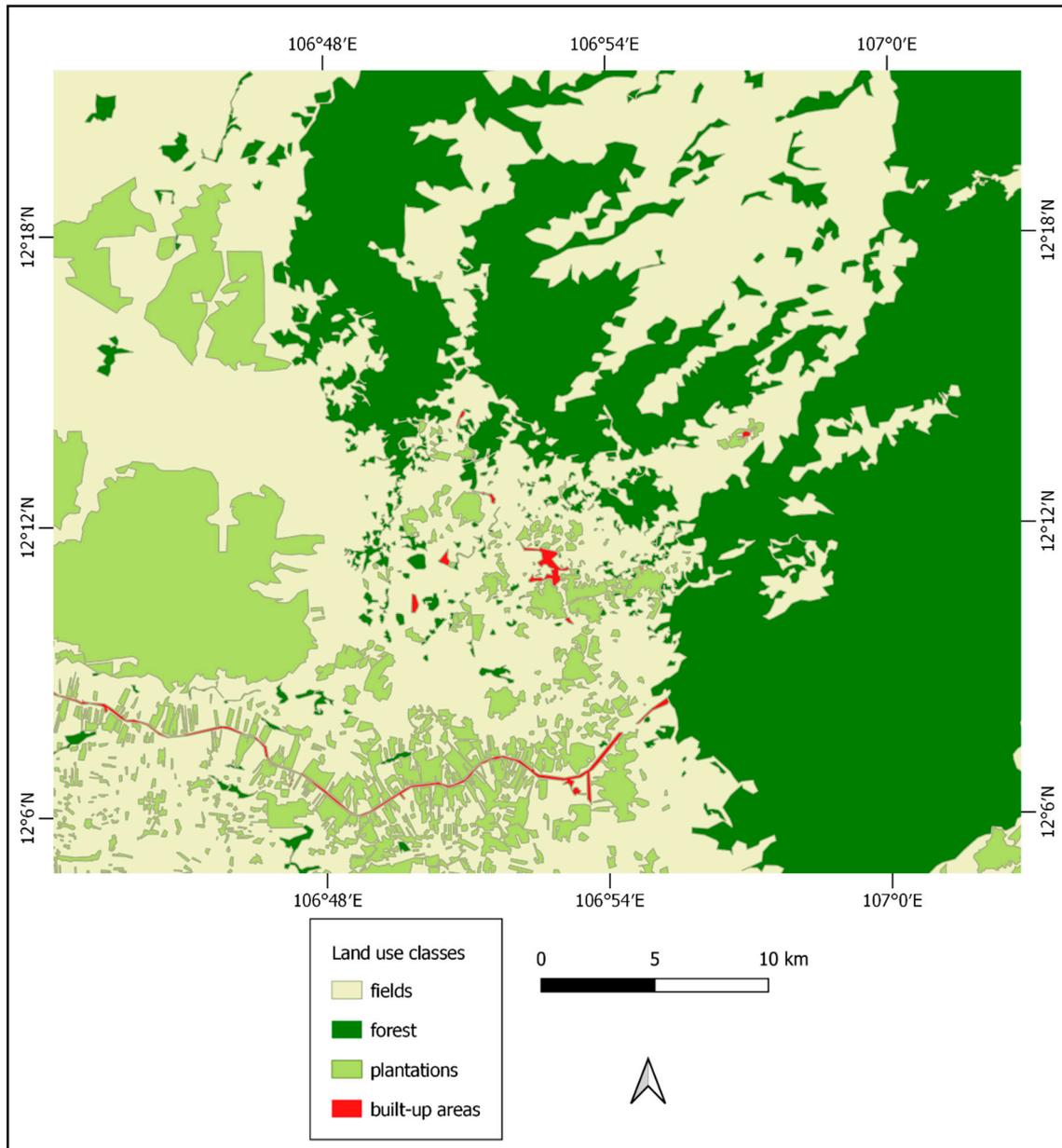


Figure 6. Land use map from 2018 SPOT 6/7 satellite imagery.

In October 2019, we recorded 149 control observations from the study area, attributing 32–43 points to each class (Table 1).

Table 1. Confusion matrix including user’s accuracy, relative omission error, producer’s accuracy and relative commission error.

		Reference Test Accuracy				Total	User’s Accuracy	Relative Omission Error
		Fields	Forests	Plantations	Built-Up Areas			
Remote sensing Classification	Fields	37	3	2	1	43	0.86	0.14
	Forests	11	17	2	2	32	0.53	0.47
	Plantations	8	1	29	0	38	0.76	0.24
	Built-up Areas	7	0	1	28	36	0.78	0.22
	Total	63	21	34	31	149		
	Producer’s Accuracy	0.59	0.81	0.85	0.90			
	Relative Commission Error	0.41	0.19	0.15	0.10			

Overall, accuracy between remote sensing classification and reference test was 74%. From the same values, weighted Kappa’s index was 0.67 (5% confidence interval: 0.55–0.79). Although there is no standardized interpretation of the Kappa’s index, this value indicates that the classification accuracy is “substantial” according to Landis and Koch’s scale (0–0.20 = slight, 0.21–0.40 = fair, 0.41–0.60 = moderate, 0.61–0.80 = substantial, and 0.81–1 = almost perfect) [58].

3.3. Comparison between Maps

Once all maps were clipped at their intersection, the final size of the area spanned 24 km from north to south and 25 km from west to east (Figure 7).

Deforestation keeps expanding (Figure 8); from 1988 to 2018, the extent of wooded areas decreased from 90.78% to 47.41% while the percentage of landscape classified as fields increased from 9.11% to 51.98%. Built-up areas also increased from 0.018% in 1988 to 0.531% in 2018, extending the existing patches as a consequence of urbanization and the development of villages and roads.

SHDI and SIDI diversity indices also increased over time (Figure 9). From 1988 to 2018, SHDI increased from 0.31 to 0.72, whereas SIDI increased from 0.17 to 0.50. PD and ED (Figure 10) demonstrate quite a different pattern as they regularly increased until 2008 (PD: from 0.54 number per 100 hectares in 1988 to 1.36 in 2008 and ED: from 12.31 in 1988 to 31.21 m per hectare in 2008) and then both decreased in 2018 (PD to 0.98 and ED to 26.52).

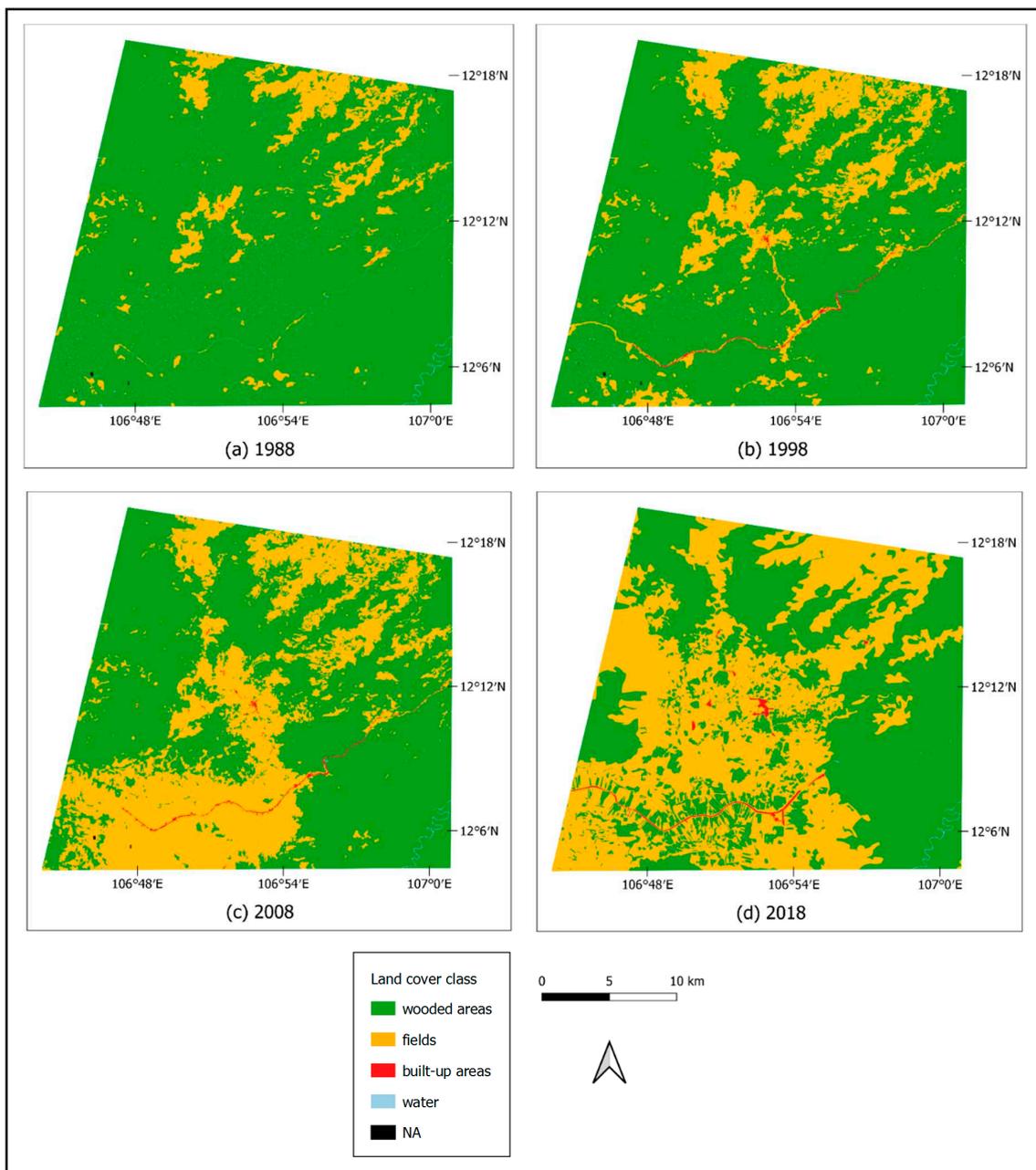


Figure 7. Evolution of land cover between 1988 and 2018 with (a) Land cover in 1988; (b) Land cover in 1998; (c) Land cover in 2008; (d) Land cover in 2018 (NA: not attributed).

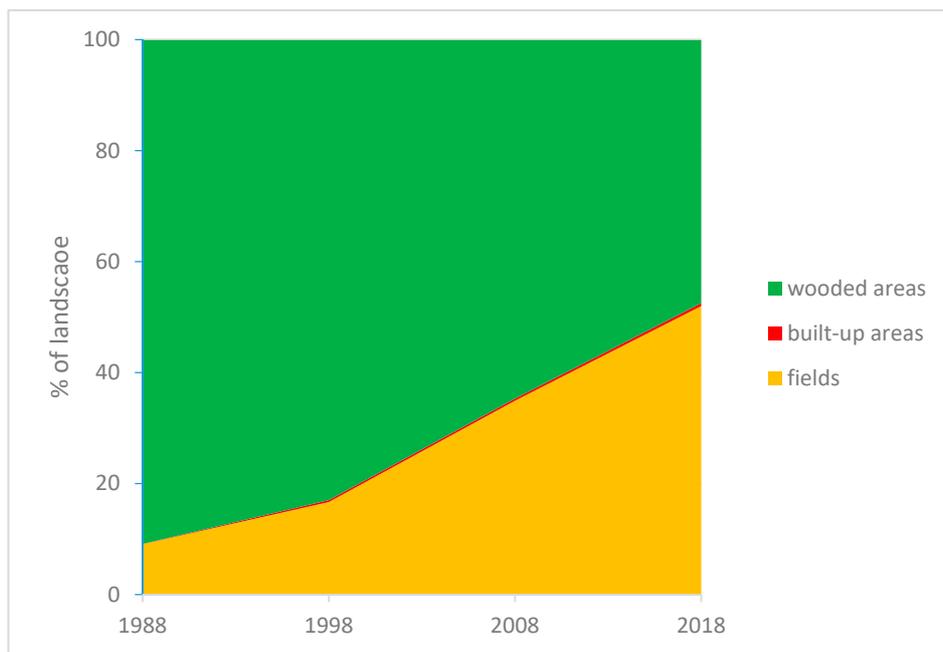


Figure 8. Evolution of landscape percentage for each land use/land cover class over time, between 1988 and 2018.

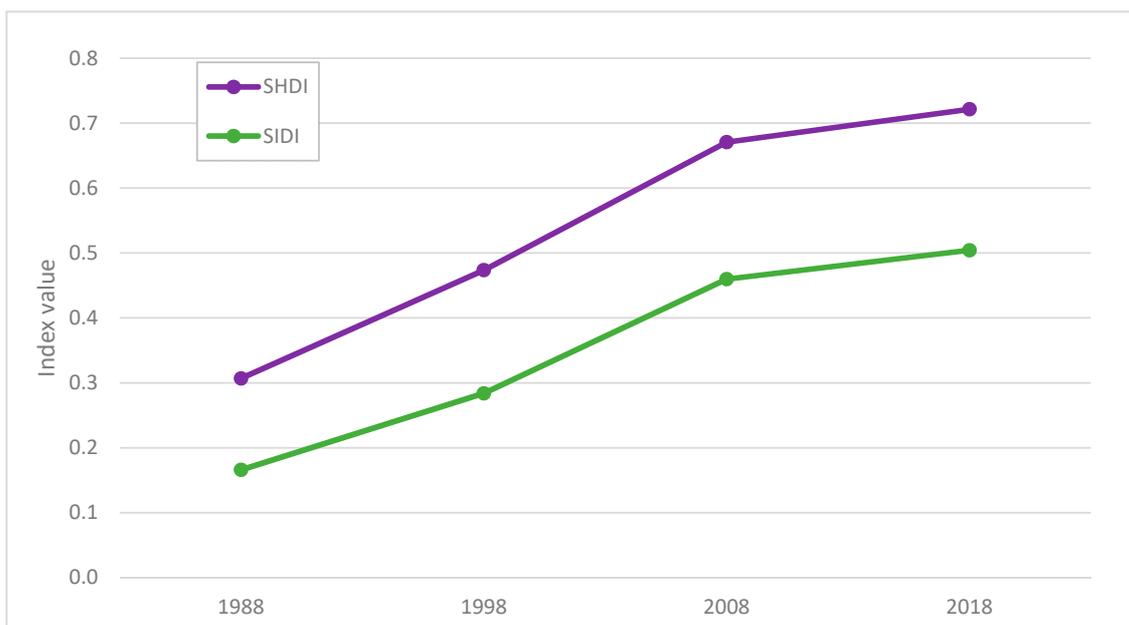


Figure 9. Evolution of the diversity indices, Shannon’s Diversity Index (SHDI) and Simpson’s Diversity Index (SIDI), from 1988 to 2018.

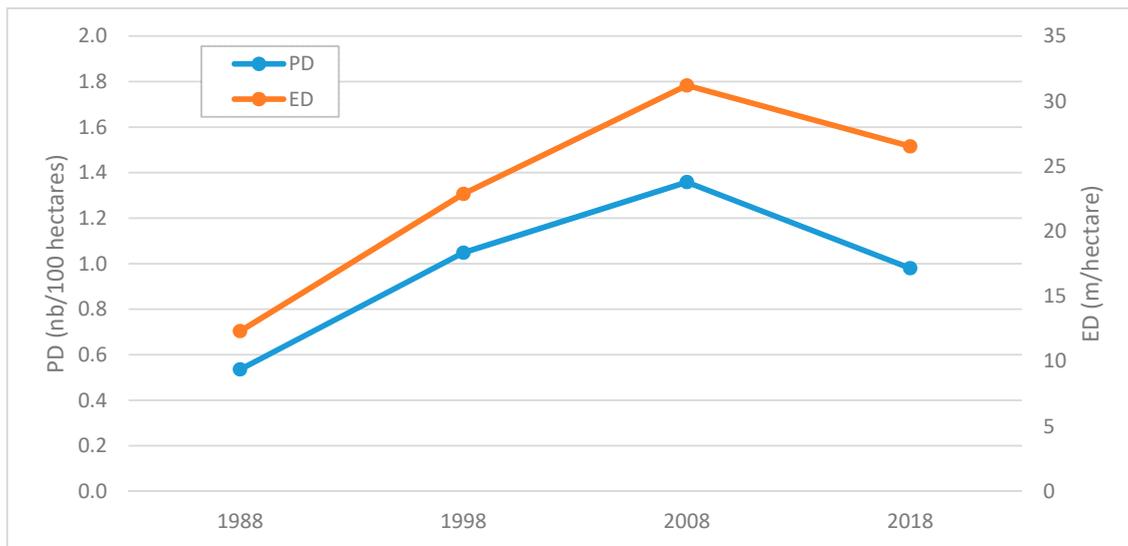


Figure 10. Evolution of the fragmentation indices, Patch Density (PD, number per 100 hectares) and Edge Density (ED, metres per hectares), as a function of time, from 1988 to 2018.

4. Discussion

The spatial resolution of the imagery was fine enough to separate relevant patches at human and mosquito scale. A smaller resolution would be more difficult to obtain and might highlight unnecessary patches, too small to be of biological interest, i.e., groups of trees not extensive enough to represent a suitable environment for a vector population, whereas a larger resolution would not have been fine enough for delimitating all potential patches of interest. Within the confusion matrix, most of the agreement errors arose from the “Fields” class where only 58.7% were rightly attributed (compared with the field control points). Indeed, the producer’s accuracy of this class is particularly low (41.3%). These fields were classified either as Forests (17.5%), Plantations (12.7%) or Built-up areas (11.1%). The incorrect assignment of sites to the “Forests” or “Plantations” classes may have occurred as a consequence of the 18 months delay between the date the satellite images were taken and the collection of control points, particularly in this context where fast pace deforestation occurs, often as the result of “slash and burn” activities. For future classifications, ground truthing and accuracy assessment should be performed immediately after the production of the classification.

Landscape metrics indices showed a consistent evolution of deforestation rate, along with the progress of cultivated areas and built-up areas. Shannon and Simpson’s indices are still increasing through the years, demonstrating an increasing landscape diversity which is emerging from the diversification of patches within the study area. However, even if fragmentation appears to be increasing, the edge density and patch density decreased from 2008 to 2018. This result can usually be linked to (1) a decrease in the number of patches within the landscape (which is not the case here because SHDI is increasing during this time interval) and/or (2) shorter edges on average, arising from more homogenous patches. We can propose at least three hypotheses to explain these results. First, with the cultivated areas extending further every year, an actual homogenization between patches might be occurring, with shorter edges as agriculture spreads and/or as various patches of agriculture that were disconnected now being connected as cultivated areas surfaces expand. Second, the imagery resolution changed in 2018 and might have affected the map output and therefore, the edge density and the patch density values. Lastly, this tendency could arise from the different methodology used in 2018, giving smoother edges, leading to an underestimation of the indices. If the photointerpretation method used in 2018 appears appropriate for quantifying classes’ abundance and repartition, it might estimate fragmentation indices differently than the object-oriented classification. Indeed, during an object-oriented classification, the user defines the parameters for segmentation, which are compactness,

shape and scale. Their values are decisive for edge length, as they determine the level of compactness of the patches and the degree of fineness of the edges. Consequently, the resulting edges might appear differently between object-oriented or photointerpretation methods (Figure 11), leading to a different estimation of fragmentation indices.

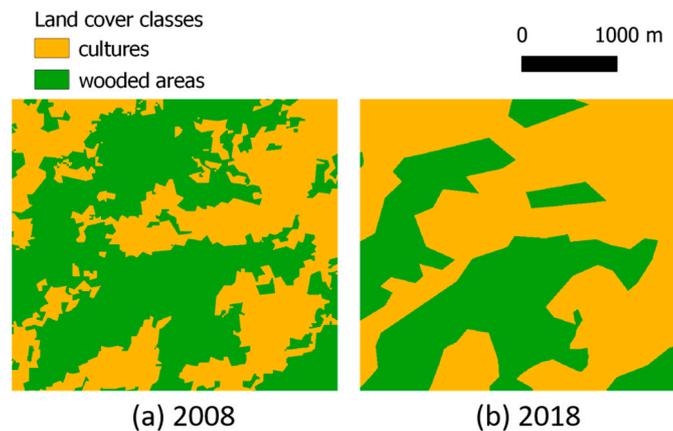


Figure 11. A detail of the land use/land cover map from 2008 with object-oriented method (a) and from 2018 with photointerpretation (b). In addition to the difference of wooded areas surface, edges appear coarser on (a), while on (b), patches are more compact, with shorter edges.

It is worth noting that along with edge density, patch density is also decreasing between 2008 and 2018. The latter index is less sensitive to the method used; as it counts the number of patches and does not take into account the coarseness or shape of these patches. Thus, if patch density is decreasing, we can assume that fragmentation may have decreased between 2008 and 2018, a phenomenon that could be explained through the hypothesis previously mentioned of an actual homogenisation, which might result from the extension of fields that join the existing parcels between them.

Obviously, difficulties arise when attempting to compare images from different dates, and this is amplified by the different methodology used in 2018. For future studies, analysis and interpretation would be eased by using a homogenous classification method. In the case of malaria context and understanding its mechanisms, photointerpretation appears to be a valid approach, notably because it allows the separation between the different wooded areas. Yet, this technique requires very high-resolution satellite imagery, which is not always available. The technique is, in the context of this study, an uncomplicated way to complete an available dataset with free, accessible methodology. Since the 1980s, remote sensing has deeply evolved, as we can observe from the satellite resolutions used between 1988 and 2008 in the study by Dupuy et al. [53], with panchromatic resolution varying from 10 to 2.5 m between SPOT1 and SPOT5. Over the years, satellite technology and their resolution have improved [31], bringing some challenge for studies spanning various decades. As such, depending on the hypothesis, a protocol could focus on (i) getting data with identical resolutions and methodologies from a limited timeframe or (ii) coping with variability within dataset but allowing a larger time frame. In the case of this study, we aim to give an update about deforestation on a rather long timeframe, thus opting for the latter option. In the case of a more detailed analysis, a smaller time frame would probably fit better, with satellite imagery of a consistent resolution.

Over the last decades in Cambodia, it has been assessed and quantified that deforestation has been increasing in every province [15,62]. On the other side, Mondulhiri province has shown a rather slow malaria decrease over time compared to countrywide data, with a rebound in 2017, concomitant to the national increase. Literature shows indeed that malaria does not always follow linearly deforestation expanse. It is important to consider that malaria is a multifactorial dynamic system, not only driven by deforestation, and that temporal scale matters. More variables, and over a smaller time scale, such as yearly data would be needed to allow a thorough multivariate analysis.

Multivariate models have been used on yearly data with variables such as malaria count, human development index, deforestation rate, sociocultural and land cover factors, with spatial scales at the province, municipality or health district levels. Scale has a considerable effect on results of such analyses [63] and consequently, these methods can create ecological fallacies. It has been stated that health district scale would be the most appropriate to conduct such analyses [25,64]—a radically different scale than most studies in literature at province levels.

However, this sort of multivariate model would be hardly applicable to the Cambodian context, due to the lack of data at a relevant scale. Indeed, in the Amazon Basin, organisations such as the SIVEP-Malaria, recording individually malaria cases at municipality level [65], or the Brazil's National Institute for Space Research (INPE), mapping deforestation since 1988, are supporting the design of malaria studies and allowed to explain the frontier malaria paradigm. Such recording is essential to allow in-depth investigation of the effect of deforestation on malaria dynamics. However, Cambodia's geopolitical context makes it difficult to obtain exhaustive malaria data, and almost impossible for long-term data at a relevant scale, as a consequence of the Khmer Rouge regime [49]. Nevertheless, over the last decade, the census data observes net improvements and future studies would probably be able to rely on more complete malaria prevalence. Additionally, future studies must be able to rely on substantial vector data, gathered from mosquito collections. This would ultimately lead to defined transmission-prone environments and allow in-depth investigation on malaria dynamics. Indeed, prevalence data alone is subject to biases—individual immunity, treatment availability and efficacy, control interventions and diagnostic sensibility—and, as such, must be completed by vector data relevant to the study area.

Without formal malaria data, statistical analysis should not be undertaken, but trends can be observed from the observation of land cover changes and the situation of the area under study. First, selective logging might be one reason for the difficulty to formulate a relevant hypothesis. Forest goes create a situation arduous to measure and to model as selective logging induces very low-level to undetectable deforestation [54], while potentially maintaining high malaria prevalence [66]. This highlights the necessity of high geographical precision, attainable with a very fine scale of analysis. A follow-up of this risk population aiming to characterize their mobility and activities within the forest would help to quantify their exposure and their effect on the forest. In addition, an estimation of true forest versus plantations within the wooded areas over time would be a valuable dataset. From the increase in wooded areas near the main road between 2008 and 2018, we can suggest that there was a relative increase of plantation versus forest over the least 10 years. This hypothesis would correlate with the recent investment into rubber and cashew trees observed in the area. Methodologies categorizing the different types of forest are highly valuable in this context, as plantations might have been the land cover category that has extended the most in this landscape. A specific quantification of this increase is required to fully understand malaria dynamics, as some plantations, notably rubber, have been correlated to malaria exposure in Thailand [67] and Cambodia [56].

Second, some forested areas might represent untouched malaria hotbeds that deforestation affects little, where malaria incidence would then mostly depend on other factors. Those include diagnostic efficacy, which might induce bias in the observed incidence, as well as availability of treatments [41], drug efficacy [40] or even long-lasting insecticidal nets real coverage and use [68], which are substantial determinants in malaria dynamics evolution and completely independent from deforestation. In addition, deforestation—even when occurring fast—is a slow process (here observed over 30 years) compared to other factors that could affect malaria incidence (e.g., climatic variation, national control program performance, drug supply, etc.), which can vary from year to year.

Overall, studying the evolution of land use is quite a simple methodology quantifying the forest cover and the anthropogenic changes of the environment. Complementary field approaches would be useful to understand spatiotemporal patterns of exposure to vectors and to ultimately guide efforts towards malaria control and elimination [69]. Techniques relying on active follow-up of populations could favour such an understanding [70]. For example, tracking by GPS data-loggers, coupled with

clinical follow-ups and questionnaires to define at-risk behaviours, have revealed great efficiency to determine the conditions for transmission and helped identify malaria hotspots, risk behaviours and modalities of transmission [71,72]. Following up with Cambodians most exposed to vectors in this area, such as forest goers, coupled with characterization of environments as we did, should provide precious information about malaria transmission modalities and help characterize transmission hotspots. Altogether, predicting the direction of pathogen responses to anthropogenic land use change requires an understanding of the biology and natural history of the pathogen as well as identifying the mechanisms of disease transmission in different epidemiologic situations [73]. Using a GIS approach, some malaria control strategies and elimination programmes have improved their efficacy [74–76]. These approaches must be transdisciplinary and include spatiotemporal, ecological as well as social perspectives in order to develop prevention methods able to break the transmission cycle [16].

5. Conclusions

This investigation illustrates the tremendous anthropogenic land use changes happening in the study area in Cambodia, affecting humans, vectors and their interactions. Indeed, this analysis highlights rapidly growing deforestation in one district of a Cambodian province, along with an increasing diversity of the landscape. The collated malaria data suggests that the interaction between incidence and deforestation is not linear nor easy to decipher. The lack of detailed ecological, environmental and epidemiological data in the area hinders any in-depth analysis. However, the use of fine-scale satellite imagery, photointerpretation, relevant classification and landscape metrics indices not only show their potential but also their limitations for the analysis of the disease dynamics. To further understand malaria transmission dynamics, the temporal and spatial scales should be carefully chosen in order to correctly address the problem. Additionally, multiple factors such as diagnostic efficacy, availability of treatments, drug efficacy or current fit of control strategies must be considered. Future research programs should also utilise remote sensing methodologies with multidisciplinary approaches in order to quantify malaria exposure and eventually grasp how land cover impacts human exposure to vectors.

Author Contributions: Conceptualization, A.P., A.V. and V.H.; data curation, A.P.; formal analysis, A.P.; funding acquisition, A.V., I.M. and B.W.; investigation, A.P.; methodology, A.P., M.S. and V.H.; project administration, A.V.; resources, S.M., D.L., B.W. and V.H.; software, A.P. and M.S.; supervision, A.V. and V.H.; validation, A.V., B.W. and V.H.; visualization, A.P.; writing—original draft, A.P.; writing—review and editing, M.S., A.V., S.M., I.M., B.W. and V.H. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the National Institutes of Health program International Centres of Excellence for Malaria Research, grant 1U19AI129392-01 “Understanding, tracking and eliminating malaria transmission in the Asia-Pacific Region”. A.P. was funded by the Institut Pasteur International Network Calmette and Yersin Ph.D. scholarship, grant MHL/MJ/N°212/18. SPOT images were provided upon request through the GEOSUD Equipex project and are material of © Airbus DS 2018.

Acknowledgments: We want to thank Annika Suttie that accepted to proofread and edit our manuscript as a native English speaker.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

References

1. Burkett-Cadena, N.D.; Vittor, A.Y. Deforestation and vector-borne disease: Forest conversion favors important mosquito vectors of human pathogens. *Basic Appl. Ecol.* **2018**, *26*, 101–110. [[CrossRef](#)]
2. Austin, K.F.; Bellingier, M.O.; Rana, P. Anthropogenic forest loss and malaria prevalence: A comparative examination of the causes and disease consequences of deforestation in developing nations. *AIMS Environ. Sci.* **2017**, *4*, 217. [[CrossRef](#)]
3. Tucker Lima, J.M.; Vittor, A.; Rifai, S.; Valle, D. Does deforestation promote or inhibit malaria transmission in the Amazon? A systematic literature review and critical appraisal of current evidence. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2017**, *372*. [[CrossRef](#)] [[PubMed](#)]

4. Lewis, S.L.; Maslin, M.A. Defining the Anthropocene. *Nature* **2015**, *519*, 171–180. [[CrossRef](#)] [[PubMed](#)]
5. Norris, D.E. Mosquito-borne Diseases as a Consequence of Land Use Change. *EcoHealth* **2004**, *1*, 19–24. [[CrossRef](#)]
6. Takken, W.; Vilarinhos, P.D.T.R.; Schneider, P.; Santos, F.D. Effects of environmental change on malaria in the Amazon region of Brazil. *Frontis* **2005**, *9*, 113–123.
7. Yasuoka, J.; Levins, R. Impact of deforestation and agricultural development on anopheline ecology and malaria epidemiology. *Am. J. Trop. Med. Hyg.* **2007**, *76*, 450–460. [[CrossRef](#)]
8. Kweka, E.J.; Zhou, G.; Gilbreath, T.M.; Afrane, Y.; Nyindo, M.; Githeko, A.K.; Yan, G. Predation efficiency of *Anopheles gambiae* larvae by aquatic predators in western Kenya highlands. *Parasit. Vectors* **2011**, *4*, 128. [[CrossRef](#)]
9. Yanoviak, S.P.; Paredes, J.E.R.; Lounibos, L.P.; Weaver, S.C. Deforestation alters phytotelm habitat availability and mosquito production in the Peruvian Amazon. *Ecol. Appl.* **2006**, *16*, 1854–1864. [[CrossRef](#)]
10. Afrane, Y.A.; Zhou, G.; Lawson, B.W.; Githeko, A.K.; Yan, G. Life-table analysis of *Anopheles arabiensis* in western Kenya highlands: Effects of land covers on larval and adult survivorship. *Am. J. Trop. Med. Hyg.* **2007**, *77*, 660–666. [[CrossRef](#)]
11. Guerra, C.A.; Snow, R.W.; Hay, S.I. A global assessment of closed forests, deforestation and malaria risk. *Ann. Trop. Med. Parasitol.* **2006**, *100*, 189–204. [[CrossRef](#)] [[PubMed](#)]
12. Fischer, J.; Lindenmayer, D.B. Landscape modification and habitat fragmentation: A synthesis. *Glob. Ecol. Biogeogr.* **2007**, *16*, 265–280. [[CrossRef](#)]
13. Taubert, F.; Fischer, R.; Groeneveld, J.; Lehmann, S.; Müller, M.S.; Rödig, E.; Wiegand, T.; Huth, A. Global patterns of tropical forest fragmentation. *Nature* **2018**, *554*, 519–522. [[CrossRef](#)] [[PubMed](#)]
14. Wang, X.; Blanchet, F.G.; Koper, N. Measuring habitat fragmentation: An evaluation of landscape pattern metrics. *Methods Ecol. Evol.* **2014**, *5*, 634–646. [[CrossRef](#)]
15. Hughes, A.C. Understanding the drivers of Southeast Asian biodiversity loss. *Ecosphere* **2017**, *8*, e01624. [[CrossRef](#)]
16. Davidson, G.; Chua, T.H.; Cook, A.; Speldewinde, P.; Weinstein, P. The Role of Ecological Linkage Mechanisms in *Plasmodium knowlesi* Transmission and Spread. *Ecohealth* **2019**. [[CrossRef](#)]
17. Morand, S.; Jittapalpong, S.; Suputtamongkol, Y.; Abdullah, M.T.; Huan, T.B. Infectious diseases and their outbreaks in Asia-Pacific: Biodiversity and its regulation loss matter. *PLoS ONE* **2014**, *9*, e90032. [[CrossRef](#)]
18. Cardinale, B.J.; Duffy, J.E.; Gonzalez, A.; Hooper, D.U.; Perrings, C.; Venail, P.; Narwani, A.; Mace, G.M.; Tilman, D.; Wardle, D.A.; et al. Biodiversity loss and its impact on humanity. *Nature* **2012**, *486*, 59–67. [[CrossRef](#)]
19. Ostfeld, R.S. A Candid response to Panglossian accusations by Randolph and Dobson: Biodiversity buffers disease. *Parasitology* **2013**, *140*, 1196–1198. [[CrossRef](#)]
20. Laporta, G.Z.; Lopez de Prado, P.I.K.; Kraenkel, R.A.; Coutinho, R.M.; Sallum, M.A.M. Biodiversity can help prevent malaria outbreaks in tropical forests. *PLoS Negl. Trop. Dis.* **2013**, *7*, e2139. [[CrossRef](#)]
21. Halsey, S. Defuse the dilution effect debate. *Nat. Ecol. Evol.* **2019**, *3*, 145. [[CrossRef](#)] [[PubMed](#)]
22. Laurance, W.F.; Camargo, J.L.C.; Fearnside, P.M.; Lovejoy, T.E.; Williamson, G.B.; Mesquita, R.C.G.; Meyer, C.F.J.; Bobrowiec, P.E.D.; Laurance, S.G.W. An Amazonian rainforest and its fragments as a laboratory of global change. *Biol. Rev.* **2018**, *93*, 223–247. [[CrossRef](#)] [[PubMed](#)]
23. Vittor, A.Y.; Gilman, R.H.; Tielsch, J.; Glass, G.; Shields, T.; Lozano, W.S.; Pinedo-Cancino, V.; Patz, J.A. The effect of deforestation on the human-biting rate of *Anopheles darlingi*, the primary vector of *Falciparum* malaria in the Peruvian Amazon. *Am. J. Trop. Med. Hyg.* **2006**, *74*, 3–11. [[CrossRef](#)] [[PubMed](#)]
24. Valle, D.; Clark, J. Conservation Efforts May Increase Malaria Burden in the Brazilian Amazon. *PLoS ONE* **2013**, *8*, e57519. [[CrossRef](#)]
25. Hahn, M.B.; Olson, S.H.; Vittor, A.Y.; Barcellos, C.; Patz, J.A.; Pan, W. Conservation Efforts and Malaria in the Brazilian Amazon. *Am. J. Trop. Med. Hyg.* **2014**, *90*, 591–594. [[CrossRef](#)]
26. Overgaard, J.H.; Ekbohm, B.; Suwonkerd, W.; Takagi, M. Effect of landscape structure on anopheline mosquito density and diversity in northern Thailand: Implications for malaria transmission and control. *Landscape Ecol.* **2003**, *18*, 605–619. [[CrossRef](#)]
27. Piantadosi, S.; Byar, D.P.; Green, S.B. The ecological fallacy. *Am. J. Epidemiol.* **1988**, *127*, 893–904. [[CrossRef](#)]

28. Hansen, M.C.; Potapov, P.V.; Moore, R.; Hancher, M.; Turubanova, S.A.; Tyukavina, A.; Thau, D.; Stehman, S.V.; Goetz, S.J.; Loveland, T.R.; et al. High-Resolution Global Maps of 21st-Century Forest Cover Change. *Science* **2013**, *342*, 850–853. [[CrossRef](#)]
29. Saah, D.; Tenneson, K.; Matin, M.; Uddin, K.; Cutter, P.; Poortinga, A.; Nguyen, Q.H.; Patterson, M.; Johnson, G.; Markert, K.; et al. Land Cover Mapping in Data Scarce Environments: Challenges and Opportunities. *Front. Environ. Sci.* **2019**, *7*. [[CrossRef](#)]
30. Palaniyandi, M. The role of remote sensing and GIS for spatial prediction of vector-borne diseases transmission: A systematic review. *J. Vector Borne Dis.* **2012**, *49*, 197–204.
31. Kalluri, S.; Gilruth, P.; Rogers, D.; Szczur, M. Surveillance of arthropod vector-borne infectious diseases using remote sensing techniques: A review. *PLoS Pathog.* **2007**, *3*, 1361–1371. [[CrossRef](#)] [[PubMed](#)]
32. Durnez, L.; Mao, S.; Denis, L.; Roelants, P.; Sochantha, T.; Coosemans, M. Outdoor malaria transmission in forested villages of Cambodia. *Malar. J.* **2013**, *12*, 329. [[CrossRef](#)] [[PubMed](#)]
33. Bauhoff, S.; Busch, J. Does deforestation increase malaria prevalence? Evidence from satellite data and health surveys. *World Dev.* **2020**, *127*, 104734. [[CrossRef](#)]
34. Busch, J.; Ferretti-Gallon, K. What Drives Deforestation and What Stops It? A Meta-Analysis. *Rev. Environ. Econ. Policy* **2017**, *11*, 3–23. [[CrossRef](#)]
35. Singer, B.; de Castro, M.C. Enhancement and suppression of malaria in the Amazon. *Am. J. Trop. Med. Hyg.* **2006**, *74*, 1–2. [[CrossRef](#)]
36. De Castro, M.C.; Monte-Mór, R.L.; Sawyer, D.O.; Singer, B.H. Malaria risk on the Amazon frontier. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 2452–2457. [[CrossRef](#)]
37. WHO. *World Malaria Report 2019*; World Health Organization: Geneva, Switzerland, 2019; ISBN 978-92-4-156572-1.
38. Noedl, H.; Se, Y.; Schaecher, K.; Smith, B.L.; Socheat, D.; Fukuda, M.M. Artemisinin Resistance in Cambodia 1 (ARC1) Study Consortium Evidence of artemisinin-resistant malaria in western Cambodia. *N. Engl. J. Med.* **2008**, *359*, 2619–2620. [[CrossRef](#)]
39. Arieu, F.; Witkowski, B.; Amaratunga, C.; Beghain, J.; Langlois, A.-C.; Khim, N.; Kim, S.; Duru, V.; Bouchier, C.; Ma, L.; et al. A molecular marker of artemisinin-resistant Plasmodium falciparum malaria. *Nature* **2014**, *505*, 50–55. [[CrossRef](#)]
40. Leang, R.; Taylor, W.R.J.; Bouth, D.M.; Song, L.; Tarning, J.; Char, M.C.; Kim, S.; Witkowski, B.; Duru, V.; Domergue, A.; et al. Evidence of Plasmodium falciparum Malaria Multidrug Resistance to Artemisinin and Piperaquine in Western Cambodia: Dihydroartemisinin-Piperaquine Open-Label Multicenter Clinical Assessment. *Antimicrob. Agents Chemother.* **2015**, *59*, 4719–4726. [[CrossRef](#)]
41. Gryseels, C.; Kuijpers, L.M.F.; Jacobs, J.; Grietens, K.P. When ‘substandard’ is the standard, who decides what is appropriate? Exploring healthcare provision in Cambodia. *Crit. Public Health* **2019**, *29*, 460–472. [[CrossRef](#)]
42. Sinka, M.E.; Bangs, M.J.; Manguin, S.; Chareonviriyaphap, T.; Patil, A.P.; Temperley, W.H.; Gething, P.W.; Elyazar, I.R.; Kabaria, C.W.; Harbach, R.E.; et al. The dominant Anopheles vectors of human malaria in the Asia-Pacific region: Occurrence data, distribution maps and bionomic précis. *Parasites Vectors* **2011**, *4*, 89. [[CrossRef](#)] [[PubMed](#)]
43. Sinka, M.E.; Bangs, M.J.; Manguin, S.; Rubio-Palis, Y.; Chareonviriyaphap, T.; Coetzee, M.; Mbogo, C.M.; Hemingway, J.; Patil, A.P.; Temperley, W.H.; et al. A global map of dominant malaria vectors. *Parasites Vectors* **2012**, *5*, 69. [[CrossRef](#)] [[PubMed](#)]
44. Hii, J.; Rueda, L.M. Malaria vectors in the Greater Mekong Subregion: Overview of malaria vectors and remaining challenges. *Southeast Asian J. Trop. Med. Public Health* **2013**, *44* (Suppl. 1), 73–165. [[PubMed](#)]
45. Incardona, S.; Vong, S.; Chiv, L.; Lim, P.; Nhem, S.; Sem, R.; Khim, N.; Doung, S.; Mercereau-Puijalon, O.; Fandeur, T. Large-scale malaria survey in Cambodia: Novel insights on species distribution and risk factors. *Malar. J.* **2007**, *6*, 37. [[CrossRef](#)]
46. Kar, N.P.; Kumar, A.; Singh, O.P.; Carlton, J.M.; Nanda, N. A review of malaria transmission dynamics in forest ecosystems. *Parasit. Vectors* **2014**, *7*, 265. [[CrossRef](#)]
47. Sluydts, V.; Heng, S.; Coosemans, M.; Van Roey, K.; Gryseels, C.; Canier, L.; Kim, S.; Khim, N.; Siv, S.; Mean, V.; et al. Spatial clustering and risk factors of malaria infections in Ratanakiri Province, Cambodia. *Malar. J.* **2014**, *13*, 387. [[CrossRef](#)]
48. Peeters Grietens, K.; Gryseels, C.; Dierickx, S.; Bannister-Tyrrell, M.; Trienekens, S.; Uk, S.; Phoeuk, P.; Suon, S.; Set, S.; Gerrets, R.; et al. Characterizing Types of Human Mobility to Inform Differential and Targeted Malaria Elimination Strategies in Northeast Cambodia. *Sci. Rep.* **2015**, *5*, 16837. [[CrossRef](#)]

49. Kiernan, B. *The Pol Pot Regime: Race, Power, and Genocide in Cambodia under the Khmer Rouge, 1975–1979*, 3rd ed.; Yale University Press: New Haven, CT, USA, 2014; Volume 1, ISBN 978-0-300-14434-5.
50. Fu, C.; Lopes, S.; Mellor, S.; Aryal, S.; Sovannaroth, S.; Roca-Feltrer, A. Experiences from Developing and Upgrading a Web-Based Surveillance System for Malaria Elimination in Cambodia. *JMIR Public Health Surveill.* **2017**, *3*. [[CrossRef](#)]
51. Cox, J.; Sovannaroth, S.; Dy Soley, L.; Ngor, P.; Mellor, S.; Roca-Feltrer, A. Novel approaches to risk stratification to support malaria elimination: An example from Cambodia. *Malar. J.* **2014**, *13*, 371. [[CrossRef](#)]
52. Davis, K.; Yu, K.; Rulli, M.C.; Pichdara, L.; D’Odorico, P. Accelerated deforestation driven by large-scale land acquisitions in Cambodia. *Nat. Geosci.* **2015**, *8*, 772–775. [[CrossRef](#)]
53. Dupuy, S.; Herbreteau, V.; Feyfant, T.; Morand, S.; Tran, A. Land-Cover Dynamics in Southeast Asia: Contribution of Object-Oriented Techniques for Change Detection. In Proceedings of the 4th International Conference on GEographic Object-Based Image Analysis (GEOBIA), Rio de Janeiro, Brazil, 7–9 May 2012; pp. 217–222.
54. Singh, M.; Evans, D.; Chevance, J.-B.; Tan, B.S.; Wiggins, N.; Kong, L.; Sakhoeun, S. Evaluating the ability of community-protected forests in Cambodia to prevent deforestation and degradation using temporal remote sensing data. *Ecol. Evol.* **2018**, *8*, 10175–10191. [[CrossRef](#)] [[PubMed](#)]
55. Singh, M.; Tokola, T.; Hou, Z.; Notarnicola, C. Remote sensing-based landscape indicators for the evaluation of threatened-bird habitats in a tropical forest. *Ecol. Evol.* **2017**, *7*, 4552–4567. [[CrossRef](#)] [[PubMed](#)]
56. Thomson, R.; Sochea, P.; Sarath, M.; MacDonald, A.; Pratt, A.; Poyer, S.; Allen, H.; Kunthy, S.; Chamroeun, S.; Daro, K.; et al. Rubber plantations and drug resistant malaria: A cross-sectional survey in Cambodia. *Malar. J.* **2019**, *18*, 379. [[CrossRef](#)] [[PubMed](#)]
57. Chiang, G.L.; Loong, K.P.; Chan, S.T.; Eng, K.L.; Yap, H.H. Capture-recapture studies with *Anopheles maculatus* Theobald (Diptera: Culicidae) the vector of malaria in peninsular Malaysia. *Southeast Asian J. Trop. Med. Public Health* **1991**, *22*, 643–647. [[PubMed](#)]
58. Foody, G.M. Status of land cover classification accuracy assessment. *Remote Sens. Environ.* **2002**, *80*, 185–201. [[CrossRef](#)]
59. Shannon, C.; Weaver, W. The Mathematical Theory of Communication. *ACM Sigmoblie Mobile Comput. Commun. Rev.* **1949**, *1*, 131.
60. Simpson, E.H. Measurement of Diversity. *Nature* **1949**, *163*, 688. [[CrossRef](#)]
61. McGarigal, K.; Marks, B.J. *FRAGSTATS: Spatial Pattern Analysis Program for Quantifying Landscape Structure*; U.S. Department of Agriculture, Forest Service, Pacific Northwest Research Station: Portland, OR, USA, 1995.
62. Wilcove, D.S.; Giam, X.; Edwards, D.P.; Fisher, B.; Koh, L.P. Navjot’s nightmare revisited: Logging, agriculture, and biodiversity in Southeast Asia. *Trends Ecol. Evol.* **2013**, *28*, 531–540. [[CrossRef](#)]
63. Wiens, J.A. Spatial Scaling in Ecology. *Funct. Ecol.* **1989**, *3*, 385–397. [[CrossRef](#)]
64. Olson, S.H.; Gangnon, R.; Silveira, G.A.; Patz, J.A. Deforestation and malaria in Mâncio Lima County, Brazil. *Emerg. Infect. Dis.* **2010**, *16*, 1108–1115. [[CrossRef](#)]
65. Oliveira-Ferreira, J.; Lacerda, M.V.; Brasil, P.; Ladislau, J.L.; Tauil, P.L.; Daniel-Ribeiro, C.T. Malaria in Brazil: An overview. *Malar. J.* **2010**, *9*, 115. [[CrossRef](#)] [[PubMed](#)]
66. Hahn, M.B.; Gangnon, R.E.; Barcellos, C.; Asner, G.P.; Patz, J.A. Influence of Deforestation, Logging, and Fire on Malaria in the Brazilian Amazon. *PLoS ONE* **2014**, *9*, e85725. [[CrossRef](#)] [[PubMed](#)]
67. Bhumiratana, A.; Sorosjinda-Nunthawarasilp, P.; Kaewwaen, W.; Maneekan, P.; Pimnon, S. Malaria-associated rubber plantations in Thailand. *Travel Med. Infect. Dis.* **2013**, *11*, 37–50. [[CrossRef](#)] [[PubMed](#)]
68. Gryseels, C.; Bannister-Tyrrell, M.; Uk, S.; Set, S.; Sokha, S.; Gerrets, R.; Peeters Grietens, K. A Critical Enquiry into Variability of Insecticidal Net Use in Cambodia: Implications for Assessing Appropriateness of Malaria Elimination Interventions. *Am. J. Trop. Med. Hyg.* **2019**. [[CrossRef](#)] [[PubMed](#)]
69. Sriwichai, P.; Karl, S.; Samung, Y.; Sumruayphol, S.; Kiattibutr, K.; Payakkapol, A.; Mueller, I.; Yan, G.; Cui, L.; Sattabongkot, J. Evaluation of CDC light traps for mosquito surveillance in a malaria endemic area on the Thai-Myanmar border. *Parasit. Vectors* **2015**, *8*, 636. [[CrossRef](#)]
70. Drame, P.M.; Poinsignon, A.; Besnard, P.; Cornelie, S.; Mire, J.L.; Toto, J.-C.; Foumane, V.; Dos-Santos, M.A.; Sembène, M.; Fortes, F.; et al. Human Antibody Responses to the *Anopheles* Salivary gSG6-P1 Peptide: A Novel Tool for Evaluating the Efficacy of ITNs in Malaria Vector Control. *PLoS ONE* **2010**, *5*, e15596. [[CrossRef](#)]

71. Fornace, K.M.; Alexander, N.; Abidin, T.R.; Brock, P.M.; Chua, T.H.; Vythilingam, I.; Ferguson, H.M.; Manin, B.O.; Wong, M.L.; Ng, S.H.; et al. Local human movement patterns and land use impact exposure to zoonotic malaria in Malaysian Borneo. *eLife* **2019**, *8*, e47602. [[CrossRef](#)]
72. Hast, M.; Searle, K.M.; Chaponda, M.; Lupiya, J.; Lubinda, J.; Sikalima, J.; Kobayashi, T.; Shields, T.; Mulenga, M.; Lessler, J.; et al. The use of GPS data loggers to describe the impact of spatio-temporal movement patterns on malaria control in a high-transmission area of northern Zambia. *Int. J. Health Geogr.* **2019**, *18*, 19. [[CrossRef](#)]
73. Gottdenker, N.L.; Streicker, D.G.; Faust, C.L.; Carroll, C.R. Anthropogenic Land Use Change and Infectious Diseases: A Review of the Evidence. *EcoHealth* **2014**, *11*, 619–632. [[CrossRef](#)]
74. Barbieri, A.F.; Sawyer, I.O.; Soares-Filho, B.S. Population and Land Use Effects on Malaria Prevalence in the Southern Brazilian Amazon. *Hum. Ecol.* **2005**, *33*, 847–874. [[CrossRef](#)]
75. Bui, T.Q.; Pham, H.M. Web-based GIS for spatial pattern detection: Application to malaria incidence in Vietnam. *Springerplus* **2016**, *5*. [[CrossRef](#)] [[PubMed](#)]
76. Souris, M.; Marcombe, S.; Laforet, J.; Brey, P.T.; Corbel, V.; Overgaard, H.J. Modeling spatial variation in risk of presence and insecticide resistance for malaria vectors in Laos. *PLoS ONE* **2017**, *12*, e0177274. [[CrossRef](#)] [[PubMed](#)]



© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).

1.3. Mobility, space and malaria exposure

Article: Forest malaria in Cambodia: the occupational and spatial clustering of Plasmodium vivax and Plasmodium falciparum infection risk in a cross-sectional survey in Mondulkiri province, Cambodia

Mirco Sandfort, Amélie Vantaux, Saorin Kim, Thomas Obadia, Anaïs Pepey, Soazic Gardais, Nimol Khim, Dysoley Lek, Michael White, Leanne J. Robinson, Benoit Witkowski and Ivo Mueller

Published in Malaria Journal on the 19th of November 2020.

<https://doi.org/10.1186/s12936-020-03482-4>

RESEARCH

Open Access



Forest malaria in Cambodia: the occupational and spatial clustering of *Plasmodium vivax* and *Plasmodium falciparum* infection risk in a cross-sectional survey in Mondulhiri province, Cambodia

Mirco Sandfort^{1,2*} , Amélie Vantaux³, Saorin Kim³, Thomas Obadia^{1,4}, Anaïs Pepey³, Soazic Gardais¹, Nimol Khim³, Dysoley Lek^{5,6}, Michael White^{1,7}, Leanne J. Robinson^{7,8,9}, Benoit Witkowski^{3†} and Ivo Mueller^{1,7,8†}

Abstract

Background: After a marked reduction in malaria burden in Cambodia over the last decades, case numbers increased again in 2017–2018. In light of the national goal of malaria elimination by 2025, remaining pockets of high risk need to be well defined and strategies well-tailored to identify and target the persisting burden cost-effectively. This study presents species-specific prevalence estimates and risk stratification for a remote area in Cambodia.

Methods: A cross-sectional survey was conducted in 17 villages in the high-incidence province Mondulhiri in the dry season (December 2017 to April 2018). 4200 randomly selected participants (2–80 years old) were tested for *Plasmodium* infection by PCR. Risk of infection was associated with questionnaire-derived covariates and spatially stratified based on household GPS coordinates.

Results: The prevalence of PCR-detectable *Plasmodium* infection was 8.3% (349/4200) and was more than twice as high for *Plasmodium vivax* (6.4%, 268) than for *Plasmodium falciparum* (3.0%, 125, $p < 0.001$). 97.8% (262/268) of *P. vivax* and 92.8% (116/125, $p < 0.05$) of *P. falciparum* infections were neither accompanied by symptoms at the time of the interview nor detected by microscopy or RDT. Recent travels to forest sites (aOR 2.17, $p < 0.01$) and forest work (aOR 2.88, $p < 0.001$) were particularly strong risk factors and risk profiles for both species were similar. Large village-level differences in prevalence of *Plasmodium* infection were observed, ranging from 0.6% outside the forest to 40.4% inside. Residing in villages at the forest fringe or inside the forest compared to outside was associated with risk of infection (aOR 2.14 and 12.47, $p < 0.001$). Villages inside the forest formed spatial hotspots of infection despite adjustment for the other risk factors.

Conclusions: Persisting pockets of high malaria risk were detected in forested areas and in sub-populations engaging in forest-related activities. High levels of asymptomatic infections suggest the need of better case detection plans and the predominance of *P. vivax* the implementation of radical cure. In villages inside the forest, within-village

*Correspondence: msandfor@pasteur.fr

†Benoit Witkowski and Ivo Mueller contributed equally to the work

¹ Malaria: Parasites and Hosts Unit, Institut Pasteur, Paris, France

Full list of author information is available at the end of the article



© The Author(s) 2020. This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

exposure was indicated in addition to risk due to forest activities. Village-level stratification of targeted interventions based on forest proximity could render the elimination efforts more cost-effective and successful.

Keywords: Forest, Occupational risk, Spatial, Vivax, Hotspots, Cambodia, Greater Mekong Subregion

Background

In the last two decades, Cambodia has seen a marked decrease in numbers of reported malaria cases from approximately 140,000 cases in 1999 to 60,000 in 2018 [1]. Likewise, mortality by malaria steadily reduced, with zero deaths reported for the first time in 2018. Similarly remarkable reductions have been observed in the other countries of the Greater Mekong Subregion [2]. Since 2014, these countries have even pursued the goal of elimination of all malaria by 2030 [3].

This ambitious aim however faces several obstacles. After an all-time low of approximately 20,000 reported cases in 2016, numbers have increased again in 2017–2018 [1, 2]. While the annual case numbers increased for both *Plasmodium falciparum* and *Plasmodium vivax* in 2017, they declined for *P. falciparum* in 2018 but increased further for *P. vivax* [1, 3]. Consequently, *P. vivax* now accounts for almost three-quarters of all malaria cases in Cambodia [2, 4].

The national malaria control programme (NMCP) is based on mass distribution campaigns of long-lasting insecticidal nets (LLIN), diagnosis by light microscopy (LM) and rapid diagnostic tests (RDT) in the public health sector, RDT-based testing by village/mobile malaria workers (VMW/MMW), and treatment of blood stage infections with artemisinin-based combination therapy (ACT) [5]. The spread of multi-drug resistant *P. falciparum* malaria in Western Cambodia [6] might explain the resurgence of *P. falciparum* cases in 2017 and the change of first-line treatment from dihydroartemisinin-piperaquine to artesunate-mefloquine their subsequent decline in 2018. For *P. vivax*, such resistance is not reported. However, this species has dormant liver-stages called hypnozoites that escape acute blood stage treatment and cause relapsing infections. Therefore, although LLINs do reduce *P. vivax* transmission and ACT is effective against blood stage *P. vivax* infection, neither intervention targets the hypnozoite reservoir. Hence, there is a less marked effect of these interventions on *P. vivax* than *P. falciparum*.

The only licensed drug with hypnozoitocidal effect is primaquine, typically administered at 0.25 mg/kg daily over 14 days in Cambodia. The roll-out of *P. vivax* radical cure is part of the national treatment guidelines but has not yet been implemented because of concerns over the drug's potential haemolytic effect in patients with G6PD deficiency [7, 8]. In summary, the control and elimination

efforts of the NMCP are successfully targeting clinical *P. falciparum* malaria (understandably in the context of drug-resistant *P. falciparum* [6] in the country) but to a lesser extent to *P. vivax*.

Another obstacle to elimination is the high prevalence of asymptomatic infections [9–12]. Neither health facilities nor VMWs reach these asymptomatic carriers as they do not seek diagnosis or treatment. Although of lower infectivity [13, 14], they remain however a potential source of onward transmission.

Alongside the sustained reduction in countrywide incidence, malaria has also become increasingly fragmented, affecting fewer, usually remote provinces. The highest incidence of malaria in Cambodia and across the Greater Mekong Subregion is now particularly found in the North-Eastern provinces such as Ratanakiri and Mondulakiri [1, 3]. These settings are characterized by a high proportion of ethnic minorities and agricultural or wood-logging activities as the main income sources. Exposure to *Anopheles* mosquitoes and *Plasmodium* in Cambodia is understood to primarily occur in the forest rather than peri-domestically [15–17].

Together with the Western province Pursat, the highest incidence per province in 2018 was reported for Mondulakiri [1]. However, previous studies on population-level burden and risk of malaria in Cambodia focused on the country's West and North [12, 16–21] or the Eastern province of Ratanakiri [10, 11, 15, 20, 22, 23]. Here, using a cross-sectional survey conducted in a rural area of Mondulakiri in the dry season in 2018, estimates of the prevalence of *Plasmodium* infection are shown and key risk factors identified.

Methods

Study area and census

Seventeen villages were selected in the Kaev Seima district, Mondulakiri province, in the Kingdom of Cambodia. The rainy season in Cambodia normally runs from June to October with the high transmission period from June through December [1, 4, 24]. Approx. two-thirds of the population in Mondulakiri is comprised of national ethnic minorities, with the Phnong ethnic group comprising the largest proportion [25, 26]. In November–December 2017, a census was conducted by visiting each household in the 17 villages and collecting basic demographics of household participants such as age and gender. A person's household was defined as location of main residence in

the village according to adult members of the household or the village head. GPS coordinates were collected using Garmin® GPSMAP® 64s devices. When no adult household member could be found, demographic information was obtained from the village head's registry book.

Cross-sectional survey

Based on the census, a random selection of households was drawn oversampling small villages to ensure sufficient coverage (Additional file 1: Table ST1). Selected households were visited from mid-December 2017 until mid-April 2018 and all household members aged 2–80 years who had resided in the study area for at least 3 months were invited to participate in the survey. Upon informed consent, a questionnaire on household variables was administered to the head of household or another adult household member by trained interviewers. A questionnaire on individual-level variables was administered to each consenting household member. Children were interviewed assisted by a parent (or rarely another caregiver in the absence of the parents). Data were collected on tablets and run through automated data quality checks within days after the interview. In case of missing data or discrepancies, the field team was informed for immediate resolution if possible. Finger-prick blood samples were collected as thick and thin film slides and in K⁺EDTA-microtainers. Participants were screened for symptoms, *i.e.* feeling sick or feverish on the day of interview, having felt feverish over the preceding two days, or having an axillary body temperature of at least 37.5 °C. Upon any indication, the participant was administered a standard malaria RDT (Malaria Ag P.f/P.v, Standard Diagnostics Inc., South Korea) and referred to a local health care provider for treatment if positive.

Detection of infection

The microtainer blood samples collected at the interview site were stored in 4 °C ice boxes. At a field laboratory, they were separated into plasma and cell pellet and frozen at –20 °C. Following transport to the main laboratory at Institut Pasteur of Cambodia in Phnom Penh, cell pellets were stored at –20 °C and plasma at –80 °C. Infections with any of the four human *Plasmodium* parasites were determined by real-time PCR [27]. In case of a positive genus-specific result, qPCR specific for *P. falciparum*, *P. vivax*, *Plasmodium malariae*, and *Plasmodium ovale* followed. All positive and a random selection of 10% negative samples were assessed by independent double LM readings of asexual and sexual stages and parasite densities calculated. No discrepancy in parasite densities of above 30% occurred. Parasites were counted per approximately 500 leukocytes and densities were inferred assuming 8000 leukocytes per microlitre blood.

Descriptive analyses

Villages were classified based on the forest cover in a 750 m radius around the households, computed from the land cover analysis in [28] (Additional file 1: Figure S1). Villages with $\geq 50\%$ of households with $\geq 10\%$ forest cover in their vicinity were considered “inside the forest”, with $\geq 30\%$ of households with $\geq 5\%$ forest cover as “at the forest fringe”, or “outside the forest” otherwise. Because of very low sample sizes, the two small, neighbouring villages Beng (11 individuals) and Gaty (95 individuals) were analysed as one. Population prevalence was estimated based on post-sampling weights assigned to each participant according to their representation by village, gender, and 10-year age bins compared to the census population (raw numbers of positive survey samples accompany the estimates in brackets). Categorical covariates were compared using the chi-squared test or the Fisher's exact test when low strata sizes required it.

Risk factor analyses

The association of covariates with infection by *P. vivax*, *P. falciparum*, or all four species was assessed by mixed-effects logistic regression with random intercepts per household and village. Those covariates that were statistically significantly associated with *Plasmodium* infection at two-tailed $\alpha=5\%$ in univariate regressions were included in the multivariate model. The villages' proximity to the forest, work-unrelated overnight travels (incl. to forest sites), and work in the deep forest were considered part of the multivariate model a priori in order to assess the association of forest exposure with risk of infection. Having slept outdoors or under a bed net last night were kept as part of the model based on causal reasoning or prior knowledge due to their association with infection risk in previous publications, *e.g.* [11]. Gender and age were retained in the model as proxies for risk-related behaviour and thus potential confounders. The other covariates comprised potential proxies for socioeconomic status or further exposure variables and their subset significantly associated with infection risk was assessed by backwards variable selection. Akaike Information Criterion (AIC) was used to assess model fit. If collinearities and interactions were identified, covariates were retained comparing fit of the respective models by AIC. Statistical significance in the multivariate regressions was calculated per covariate by likelihood ratio tests. Spatial hotspots were identified by a purely spatial scan statistic via a discrete Poisson model with maximally a third of the population in a scanning window, adjusted for all covariates of the final multivariate model except for the villages' forest proximity [29].

Software

All questionnaires were applied on tablets using the REDCap (Research Electronic Data Capture) software hosted at Institut Pasteur in Paris [30, 31]. Data quality control as well as all descriptive and analytical statistics were performed in R 3.6.3 [32]. The SaTScan™ software version 9.6 was used for the spatial scan statistic [33].

Results

Census, survey representativeness and survey population

From among the 10,053 individuals in 2351 households identified in the census, the survey recruited 4200 participants from 1147 households and oversampled smaller villages on average (Additional file 1: Table ST1). A map of the survey households and the categorization of villages by proximity to the forest is shown in Fig. 1a. Mean age was 26 years in both the census and the survey and with 51% (5135/10,053) and 53% (2231/4200) women, respectively, women were slightly oversampled in the survey. In men, ages of 16 to 40 years were mildly underrepresented (Fig. 1b). The main income source for the survey households was by large majority farming (89.8%, 1030/1147). In terms of mobility, three-quarters (75.9%, 3188/4200) of the survey participants reported work-unrelated trips to forest or field sites in the last month (8.5%, 358/4200) or any work trip in the last two

months (75.8%, 3184/4200). By vast majority, these trips were short and frequent: In only 4.4% (140/3188) of the instances, stays for longer than a week were reported and among those who reported any work trip, 89.4% (2847/3184) went for work at least once a week.

Plasmodium vivax predominated over other human malaria parasites

Infection by *Plasmodium* parasite was detected by PCR in 8.3% (349/4200) of participants. The proportion of samples positive for *P. vivax* was 6.4% (268/4200) compared to 3.0% (125/4200) for *P. falciparum* ($p < 0.001$, Table 1). *Plasmodium vivax* was found in 77% (268/349) of all infections compared to 36% (125/349) for *P. falciparum*. Four samples were positive for *P. malariae* mono-infections and none for *P. ovale*. Speciation by PCR was unsuccessful in 4 samples which were analysed as negative. Extrapolation to the entire Kaev Seima population yielded an estimated prevalence of 8.9% for *Plasmodium* infection, 6.8% for *P. vivax*, and 3.3% for *P. falciparum*. Estimated prevalence was highly heterogeneous across the villages, ranging from 0.6% (3/594) to 36.3% (54/152) and from 0% (0/594) to 25.1% (27/106) for *P. vivax* and *P. falciparum*, respectively (both $p < 0.001$, Table 1). Village-level prevalence of *P. vivax* infection was on average higher in villages inside the forest compared with those

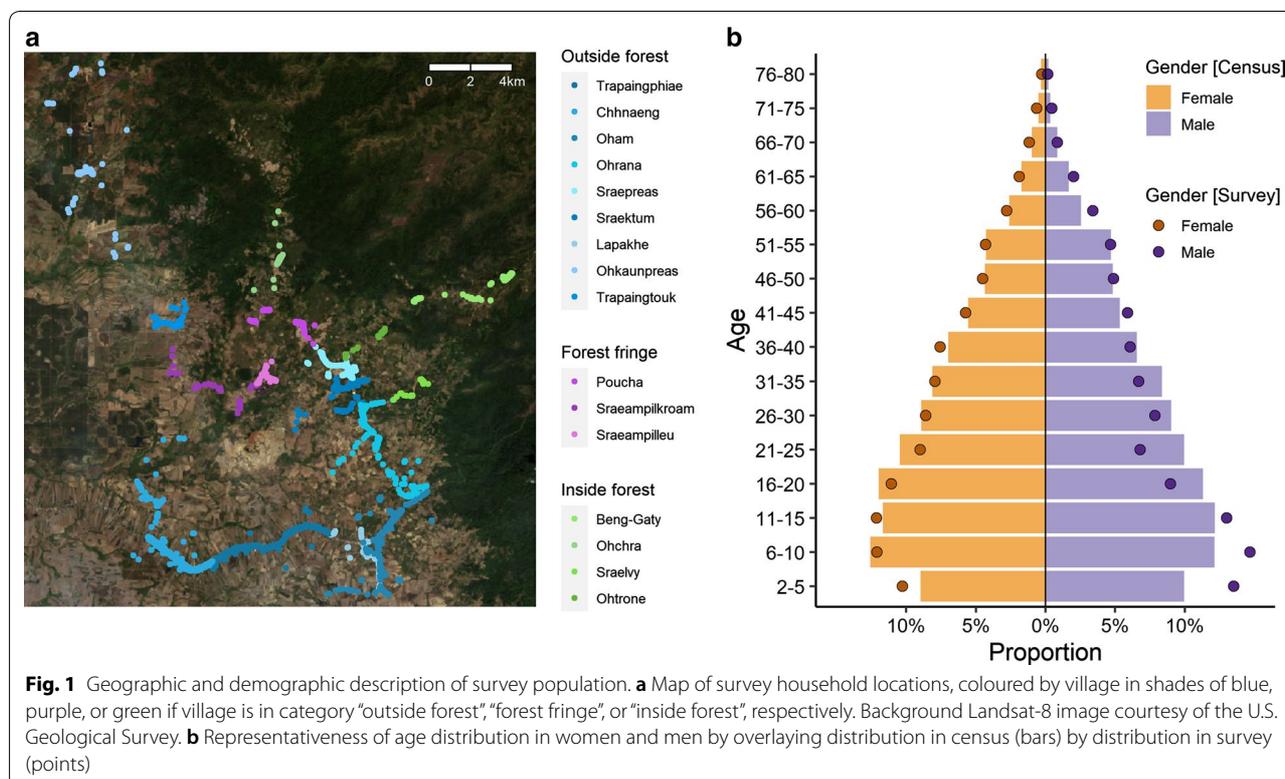


Fig. 1 Geographic and demographic description of survey population. **a** Map of survey household locations, coloured by village in shades of blue, purple, or green if village is in category “outside forest”, “forest fringe”, or “inside forest”, respectively. Background Landsat-8 image courtesy of the U.S. Geological Survey. **b** Representativeness of age distribution in women and men by overlaying distribution in census (bars) by distribution in survey (points)

Table 1 Prevalence of PCR-detected infections

	N	PCR positivity			
		<i>Plasmodium</i>	<i>P. vivax</i>	<i>P. falciparum</i>	Co-infections
Proportion (n) of survey samples	4200	8.3% (349)	6.4% (268)	3.0% (125)	1.1% (48)
Extrapolated population prevalence	10,053	8.9%	6.8%	3.3%	1.3%
Extrapolated prevalence per village					
<i>Outside forest</i>					
Trapaingphiae	594	0.6% (3)	0.6% (3)	0% (0)	0% (0)
Chhnaeng	550	2.7% (12)	1.9% (8)	0.9% (4)	0.3% (1)
Oham	333	8.0% (20)	6.2% (16)	2.9% (7)	1.1% (3)
Ohrana	470	5.7% (25)	4.2% (18)	1.8% (8)	0.2% (1)
Sraepreas	228	6.5% (13)	5.6% (11)	1.7% (3)	0.8% (1)
Sraektum	296	13.1% (36)	8.6% (24)	4.8% (13)	0.8% (2)
Lapakhe	71	10.6% (4)	6.7% (3)	7.8% (2)	3.9% (1)
Ohkaunpreas	317	6.9% (21)	5.9% (18)	1.7% (5)	0.7% (2)
Trapaingtouk	201	1.9% (3)	1.2% (2)	0.6% (1)	0% (0)
<i>Forest fringe</i>					
Poucha	239	9.7% (24)	6.7% (17)	4.3% (10)	1.4% (3)
Sraepilkroam	218	11.0% (23)	7.2% (15)	3.8% (8)	0.6% (1)
Sraepilleu	168	10.6% (17)	8.2% (13)	2.4% (4)	0% (0)
<i>Inside forest</i>					
Beng-Gaty	106	37.9% (40)	30.9% (32)	25.1% (27)	18.1% (19)
Ohchra	152	40.4% (60)	36.3% (54)	10.0% (15)	5.9% (9)
Sraelyy	154	18.7% (28)	15.5% (23)	3.9% (6)	1.3% (2)
Ohtrone	103	19.7% (20)	10.4% (11)	12.4% (12)	3.1% (3)

As proportions of positive blood samples or as census population-level estimates by extrapolation via post-sampling weights (raw numbers of positive samples in brackets). 4 positive *P. malariae* samples omitted

at the forest fringe and outside the forest (medians 23.2, 7.2, 5.6% respectively, Fig. 2; similarly for *P. falciparum* in Additional file 1: Fig. S2).

***Plasmodium vivax* infections were least detectable by the health care system**

Of all PCR-detected infections, 83.2% (223/268) were sub-microscopic for *P. vivax* compared to 74.4% (93/125) for *P. falciparum* ($p \approx 0.056$, Table 2). *Plasmodium vivax* infections coincided less often with reported or measured symptoms at the interview than those by *P. falciparum*, with 6.7% (18/268) and 16.8% (21/125), respectively ($p < 0.01$). Only 2.2% (6/268) of all *P. vivax* infections were symptomatic and also positive by RDT or LM (*i.e.* detectable through the Cambodian health care system), less often than for *P. falciparum* (7.2%, 9/125, $p < 0.05$). Asexual parasite stages were detected in all LM⁺ samples for *P. vivax* (37/37) and in most (81.5%, 22/27) for *P. falciparum* (Table 3). In LM⁺ samples, the geometric mean parasite densities were 149.7 parasites/ μ L and 431.3 parasites/ μ L (*t*-test: $p \approx 0.07$), respectively. While gametocytes were found in only one (2.7%, 1/37) *P. vivax* LM⁺ sample, more than a third (37.0%, 10/27) of LM⁺ samples for *P.*

falciparum were gametocyte-positive with a geometric mean of 51.1 gametocytes/ μ L.

Prevalence was highest in men of working age

Estimated prevalence was more than twice as high in men as in women, *i.e.* 10.4% (188/1969) vs. 3.6% (80/2231) for *P. vivax* ($p < 0.001$), 4.8% (83/1969) vs. 1.9% (42/2231) for *P. falciparum* ($p < 0.001$), and 13.3% (239/1969) vs. 5.0% (110/2231) regardless of species ($p < 0.001$). The patterns across age were similarly heterogeneous for both species (Additional file 1: Figure S3). While there was no difference in genus-wide prevalence across age in women, men showed an elevated risk at working age ($p < 0.001$), regardless of the proximity of the village to the forest (Fig. 3). A fitted interaction term of gender and age was significant in the strata of villages outside the forest ($p < 0.001$), but not across those villages at the forest fringe or inside the forest.

Risk profiles for *P. vivax* and *P. falciparum* infections were similar

Risk of infection was associated with individual covariates for both *P. vivax* and *P. falciparum* in a highly similar

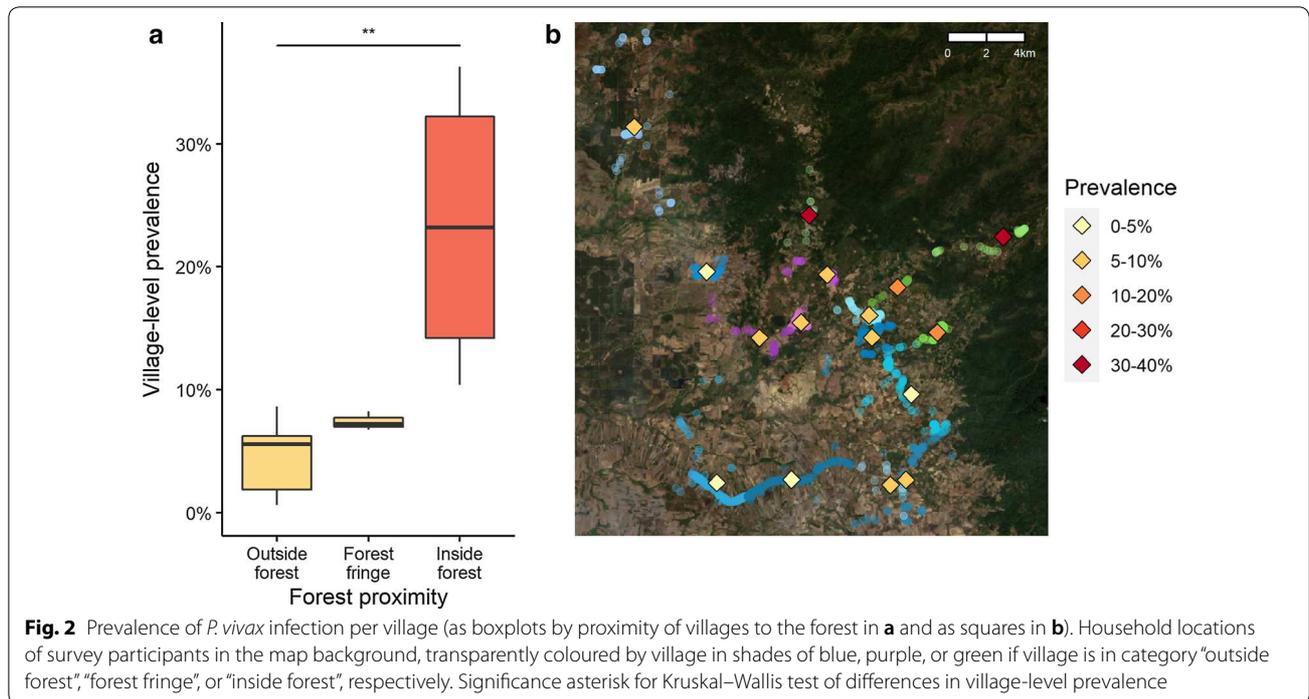


Table 2 Detectability of infections through the public health care system

Measure	PCR positivity	
	<i>P. vivax</i>	<i>P. falciparum</i>
LM ⁺	16.8% (45/268)	25.6% (32/125)
Symptoms at interview	6.7% (18/268)	16.8% (21/125)
Symptoms & LM ⁺	2.2% (6/268)	4.8% (6/125)
Symptoms & RDT ⁺	0.4% (1*/268)	4.0% (5/125)
Symptoms & RDT/LM ⁺	2.2% (6/268)	7.2% (9/125)

Proportion (N) of PCR-positive samples that were also detectable by LM, inspection of symptoms, or by testing of symptomatic individuals with LM or RDTs

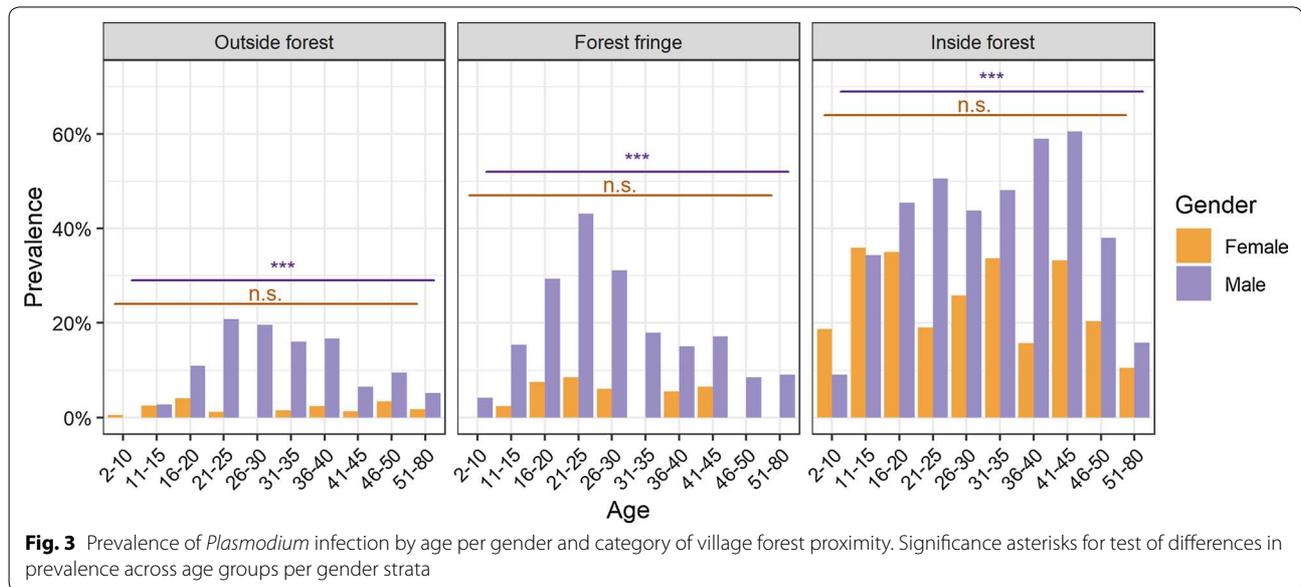
*Actually RDT⁺ for *P. falciparum* in a co-infection with *P. vivax* as by PCR

fashion (Table 4 for behavioural variables, full list of variables with odds ratios in Additional file 1: Table ST2). For both species, prevalence was associated with work-unrelated overnight travels and highest if those occurred to forest sites (22.4%, 60/268, and 14.9%, 40/268, for *P. vivax* and *P. falciparum*, respectively) and lowest for urban destinations (1.7%, 1/59, and 0%, 0/59, for both $p < 0.001$). Infections of both species were also more prevalent among those who reported work trips to sites in the nearby and deep forest (assessed separately). In particular, a significantly higher prevalence was observed in participants who reported work trips into the deep forest (25.5%, 37/145, and 18.6%, 27/145) compared to those who did not (5.7%, 231/4055, and 2.4%, 98/4055,

Table 3 Densities of asexual and sexual stages in LM⁺ samples

	LM positivity	
	<i>P. vivax</i>	<i>P. falciparum</i>
<i>Asexual parasitaemia</i>		
Proportion (N) of LM ⁺ samples with detected asexual parasites	100% (37/37)	81.5% (22/27)
Geometric mean [/ μ l blood]	149.7	431.3
Range [/ μ l blood]	15.4–22,674.7	14.3–41,724.6
<i>Gametocytaemia</i>		
Proportion (N) of LM ⁺ samples with gametocytes	2.7% (1/37)	37.0% (10/27)
Geometric mean [/ μ l blood]	31.9	51.1
Range [/ μ l blood]	31.9–31.9	15.6–175.0

Geometric mean and range always given from among samples with non-zero densities



for *P. vivax* and *P. falciparum*, respectively, $p < 0.001$). Once clustering at household and village-level was taken into account in univariate mixed-effects logistic regression, work in the nearby forest was no longer significantly associated with risk of infection though (Additional file 1: Table ST2). There were fewer infections among those who reported the use of standard protection measures such as sleeping under a bed net (6.1%, 236/3880, and 2.7%, 104/3880, vs. 10.0%, 32/320, and 6.6%, 21/320, if no net was used, $p < 0.01$ and $p < 0.001$ for *P. vivax* and *P. falciparum*, respectively). A higher risk in men, at working age, in villages at the forest fringe or inside the forest, and with indicators of lower socio-economic status was also found similarly for both species (Additional file 1: Table ST2).

Working in and travelling to the forest were strong risk factors of infection

Behavioural covariates related to activities in the forest were statistically significant in the multivariate model of *Plasmodium* infection (Table 5). Having travelled to forest sites (nearby or deep forest) or having worked in the deep forest independently increased the odds of infection two to three-fold (adjusted odds ratio, aOR, 2.17, $p < 0.01$ and aOR 2.88, $p < 0.001$, respectively). Risk of infection was not significantly attributed to the other behavioural covariates that were included in the model a priori, namely having slept outdoors (aOR 1.99, $p \approx 0.08$) and under a bed net (aOR 0.99, $p \approx 0.96$). Risk of infection was increased in males (aOR 3.06, $p < 0.001$) and working age (aOR 7.84 in 21–25 years old compared to children, $p < 0.001$). A fitted interaction term of gender and age

improved the model (AIC 1786 vs. 1802 without interaction, $p < 0.001$, Additional file 1: Table ST3). Other covariates linked higher socio-economic status with lower odds of infection, such as living in a house with a roof built of relatively high-quality material (aOR 0.50, $p < 0.01$). The covariate on recent information on malaria via TV (aOR 0.37, $p < 0.01$) most likely also acts as a proxy for higher socio-economic status, i.e. being able to afford a TV in the first place.

Descriptive characterization of the risk factors forest work and forest travels

Work-unrelated overnight travels to forest sites was a predominantly male domain (reported by 10.7%, 211/1969, of the men vs. 2.6%, 57/2231, of the women, $p < 0.001$) and particularly frequent in male adolescents (with 24.3%, 70/289, highest in 21–30 years old males, $p < 0.001$). Forest work was independently assessed for sites of the nearby and deep forest. Only for the latter, a statistically significant association with risk was observed. As for travels, they were reported more frequently by men than by women (6.9%, 135/1969, vs. 0.4%, 10/2231, $p < 0.001$) and most often by 21–30 years old males (17.6%, 51/289, $p < 0.001$). However, working in the deep forest occurred in both men aged between 8 and 60 years and in 8–43 years old women. Forest travels were reported in 3–62 years old men and 3–63 years old women. Both kind of trips occur usually frequently and of short duration. In 81% (217/268) of the times, the travels to forest sites lasted less than a week. Work trips into the deep forest occurred in 75% (108/145) of the times at least weekly. When going for work several times

Table 4 Univariate association of infection risk and behavioural covariates

Covariate	N	<i>P. vivax</i>		<i>P. falciparum</i>	
		Prevalence	p-value	Prevalence	p-value
<i>Work-unrelated travels overnight in the last month to...</i>					
None	3465	4.9% (171)	< 0.001	2.0% (70)	< 0.001
Field sites	90	3.3% (3)		4.4% (4)	
Forest sites	268	22.4% (60)		14.9% (40)	
A village	177	13.6% (24)		5.6% (10)	
A city	59	1.7% (1)		0% (0)	
Unspecified	123	7.3% (9)		0.8% (1)	
<i>Work in the last two months in...</i>					
... deep forest					
No	4055	5.7% (231)	< 0.001	2.4% (98)	< 0.001
Yes	145	25.5% (37)		18.6% (27)	
... cassava field					
No	2115	5.6% (118)	< 0.05	2.9% (62)	n.s.
Yes	2085	7.2% (150)		3.0% (63)	
No work					
No	3186	7.9% (253)	< 0.001	3.7% (119)	< 0.001
Yes	1014	1.5% (15)		0.6% (6)	
<i>Slept outdoors last night</i>					
Indoors	4124	6.2% (256)	< 0.01	2.9% (118)	< 0.01
Outdoors	76	15.8% (12)		9.2% (7)	
<i>Sprays repellent usually at bedtime</i>					
No	3668	6.7% (247)	< 0.05	3.2% (116)	< 0.10
Yes	524	4.0% (21)		1.7% (9)	
<i>Slept under net last night</i>					
No	320	10.0% (32)	< 0.01	6.6% (21)	< 0.001
Yes	3880	6.1% (236)		2.7% (104)	

Prevalence (n) of PCR-positivity across the strata of those behavioural variables that were statistically significant in univariate logistic regression. "n.s.": Not significant

a month or once a month, the reported duration was also most often less than a week (83%, 15/18, and 53%, 8/15, of the times, respectively). A net was used during overnight work trips into the forest in only 25% (28/113) of the instances. While equally few participants from villages outside the forest, at the forest fringe, and inside the forest reported work in the deep forest (3.4%, 105/3060, 2.9%, 18/625, and 4.3%, 22/515, respectively, $p \approx 0.44$), travelling to forest sites was reported more often in forest fringe and forest villages (8.6%, 54/625, and 11.5%, 59/515, respectively, compared to 5.1%, 155/3060, outside the forest, $p < 0.001$).

Residing in a village inside the forest was an independent spatial risk factor

Living in a village inside the forest remained associated with risk of infection when adjusting for the significant

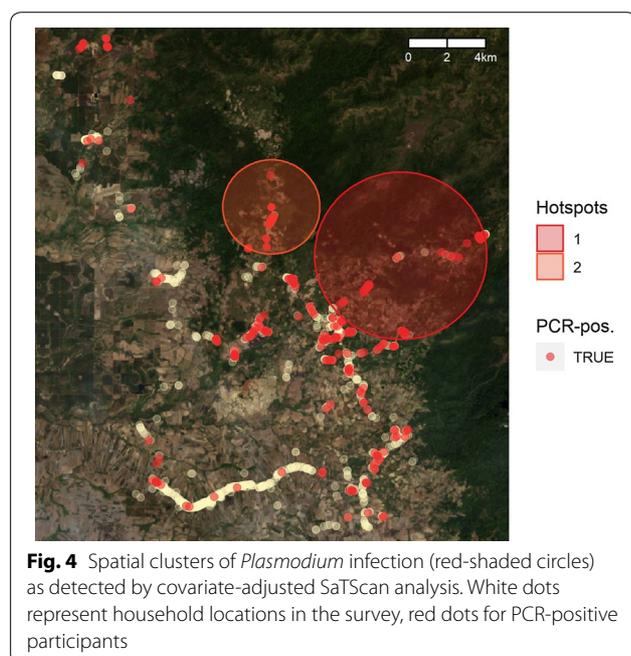
Table 5 Risk factors after multivariate mixed-effects logistic regression for *Plasmodium* infection as detected by PCR

Covariate	N	aOR	95% CI	p
Gender				
Female	2231	Reference		< 0.001
Male	1969	3.06	[2.26–4.13]	
Age [years]				
2–10	1054	Reference		< 0.001
11–15	527	4.51	[2.52–8.06]	
16–20	424	7.47	[4.14–13.47]	
21–25	335	7.84	[4.20–14.63]	
26–30	347	8.08	[4.36–14.94]	
31–35	309	7.71	[4.05–14.70]	
36–40	289	7.15	[3.76–13.58]	
41–45	244	5.33	[2.67–10.65]	
46–50	197	4.16	[1.92–8.99]	
51–80	474	2.68	[1.36–5.29]	
Forest proximity of village				
Outside forest	3060	Reference		< 0.001
Forest fringe	625	2.14	[1.02–4.52]	
Inside forest	515	12.47	[6.29–24.71]	
Material of roof				
Grass/leaves	81	0.96	[0.26–3.55]	< 0.01
Tent	79	3.43	[1.36–8.64]	
Corrugated iron	3301	Reference		
Wood planks/cement/tiles	739	0.50	[0.30–0.84]	
Household owns a toilet				
No	2383	Reference		< 0.05
Yes	1816	0.69	[0.48–1.00]	
Household owns buffaloes				
No	3977	Reference		< 0.01
Yes	222	2.11	[1.21–3.69]	
Household head had received information on malaria via TV in the past 3 months				
No	3784	Reference		< 0.01
Yes	415	0.37	[0.16–0.81]	
Insecticides had been sprayed inside the house in the past year				
No	2935	Reference		< 0.01
Yes	1264	0.62	[0.43–0.89]	
Slept outdoors last night				
No	4124	Reference		0.08
Yes	76	1.99	[0.93–4.25]	
Slept under net last night				
No	320	Reference		0.96
Yes	3880	0.99	[0.61–1.59]	

demographic, socio-economic, and behavioural covariates (aOR 12.47, $p < 0.001$, Table 5). Households from all four villages inside the forest also formed hotspots of *Plasmodium* infection, *i.e.* purely spatial clusters of elevated risk of infection that cannot be explained by

Table 5 (continued)

Covariate	N	aOR	95% CI	p
Work-unrelated travels overnight in the last month to...				
None	3465	Reference		< 0.01
Field sites	90	0.63	[0.25–1.59]	
Forest sites	268	2.17	[1.41–3.35]	
A village	177	1.28	[0.75–2.17]	
A city	59	0.27	[0.03–2.13]	
Unspecified	123	1.23	[0.56–2.74]	
Work in the last two months in...				
...deep forest				
No	4055	Reference		< 0.001
Yes	145	2.88	[1.69–4.93]	
...cassava field				
No	2115	Reference		< 0.05
Yes	2085	1.37	[1.01–1.85]	



the other covariates (Fig. 4, Additional file 1: Tables ST4–ST5).

Discussion

This is the first detailed study on the prevalence of *Plasmodium* infection and associated risk factors on population-level in Mondulakiri province in Cambodia. The overall prevalence of *Plasmodium* infection is consistent with that observed in the neighbouring province of Ratanakiri [10, 11, 22] or other endemic provinces in the West of the country [16].

Plasmodium falciparum has long been the predominant species countrywide [9, 34]. However, following a scale-up in the VMW programmes, a steady decrease in reported *P. falciparum* cases was observed in 2009–2011, while numbers of reported *P. vivax* cases increased [24]. In recent years, cases of both species were reported at an almost equal share [4] and in 2018 *P. vivax* accounted for approximately three quarters of reported cases [2]. That *P. vivax* started to predominate over *P. falciparum* in Cambodia even earlier is suggested by other studies in high-incidence provinces where molecular and serological diagnosis identified a higher prevalence of *P. vivax* than *P. falciparum* infections [11, 12, 15–19], consistent with the presented study in Mondulakiri.

7.2% of the *P. falciparum* infections could be identified by symptoms and a positive result by LM or RDTs, i.e. the diagnostics used by the health system, compared to only 2.2% for *P. vivax*. Low sensitivity of RDTs for *P. vivax* is a commonly reported problem [12, 35–38]. In addition, higher proportions of asymptomatic infections for *P. vivax* are regularly observed as the relapses can lead to higher levels of immunity and thus overall lower parasite densities [39, 40]. Data from studies using membrane feeding assays suggest that the densities of asexual stages and gametocytes in the asymptomatic, LM-positive samples in this survey are infectious to mosquitoes [14, 41–45]. It can thus not be ruled out that these subclinical infections contribute to the ongoing transmission in this area.

More asymptomatic infections, lower diagnostic sensitivity for *P. vivax*, high transmissibility, and the lack of radical cure by primaquine all might add up as possible explanations why the control efforts have been less successful against *P. vivax* than against *P. falciparum*. In order to address this high burden of *P. vivax*, Cambodia is now piloting the use of primaquine against *P. vivax* infections in four provinces. While an essential step, the effect of radical cure will be limited without point-of-care diagnostics that are more sensitive for *P. vivax* infections and ideally a test allowing the detection of hypnozoite carriage (irrespective of blood stage parasitaemia). New advances in the use of multiple antigens to detect antibodies as serological markers for recent exposure and thus for potential hypnozoite carriers are promising in this respect [46].

With as many as 40% of the inhabitants infected in the highest-prevalence village in this survey, it may be necessary to complement the current malaria control efforts by active or reactive case detection schemes [47, 48] to reduce the infected (and potentially infectious) reservoir and eventually reach the goal of malaria elimination. Targeting such resource-intensive interventions to high-risk groups will render them significantly more cost-efficient.

The similarity of the risk profiles for both *P. falciparum* and *P. vivax* presented here is encouraging as it suggests that such targeted interventions would appropriately address both of the two species.

This study emphasizes the predominant role of forest malaria transmission in explaining elevated risk of infection in remote rural areas of Southeast Asia. Previous cross-sectional surveys have also identified an association of infection risk with either work in the forest [15, 17, 49] or time spent therein [16, 50]. However, this study is unique in retaining them both as statistically significant risk factors in one multivariate model while also adjusting for gender, age, socio-economic proxies, and other behavioural covariates. While work in and travels into the forest are most frequent in adolescent and adult men who are usually considered the forest-related risk group, this behaviour also occurred in women and from children to participants in their sixties and thus actually extends to a much broader range of the population.

On the relatively small spatial scale of 17 villages in an area of $22 \times 26 \text{ km}^2$, the prevalence ranged from less than 1% to above 40% in the dry season. The trend towards higher prevalence in villages at the forest fringe and inside the forest compared to those outside the forest is in line with the widely accepted notion for forest-related mosquito abundance and exposure in Cambodia. Other studies have also identified a higher prevalence in villages at close proximity to the forest [21, 49, 50]. It is apparent that the association of risk with living near or inside the forest was retained in this study even if adjusting for demographic, socio-economic, occupational, and other behavioural differences.

Not more than 60% of infections could be attributed to the strong behavioural, forest-related risk factors. In villages outside the forest or at the forest fringe, most infections indeed occurred in working age men. This predominance of this occupational risk group was much lower in forest fringe villages and entirely lost in villages situated inside the forest. Taking gender and age as proxies for risk-related behaviour and occupational activities, this could indicate that in villages outside the forest, risk can be explained almost completely by such occupational exposure. By contrast, for villages within the forest, the more homogeneous presence of infection across all age groups in both males and females indicates additional exposure inside the village. This is in line with other studies in which household-level risk factors in forested areas were important to explain infection risk [10] or infections clustered in households over 2 years [51] in Ratanakiri, the other Eastern province of Cambodia.

The current WHO report on malaria eradication calls for subnational stratification of intervention programmes [52, 53]. This study suggests that such a stratification of interventions could happen along a gradient of villages inside or outside the forest. Population-oriented interventions such as targeted mass drug administration or test and treat programmes with sensitive molecular or serological diagnostics [46, 54] (together with their costs and risk of overtreatment) could be justified in forest villages. By contrast, approaches targeted at risk groups only may be effective at lower costs for the NMCP and the local population in residential settings outside of the forest.

Conclusion

Despite a substantial reduction in malaria burden in Cambodia over the last decades, this study demonstrates that pockets of high malaria prevalence persist in the country. Given that the vast majority of infections were asymptomatic, the study strengthens the argument to enhance malaria elimination efforts by measures of (re-)active detection of also asymptomatic infections. *Plasmodium vivax* infections were detected at a higher prevalence than *P. falciparum* infections, more often asymptomatic, and less detectable by RDT and LM. Consequently, novel tools for the identification of hypnozoite carriers could play a key role in the presented setting as well as the roll-out of routine radical cure treatment. The study corroborates the notion of forest malaria in the Greater Mekong Subregion by demonstrating the independent association of travels to the forest, of forest work, and of living in close proximity to forest with malaria infection risk. However, the study also demonstrates that infection risk is less confined to forest-goers in sub-populations that already live in forested areas and suggests within-village transmission therein. A focus of interventions solely on forest-goers could thus be insufficient to reach the goal of nation-wide malaria elimination in due time. In the light of the presented results, interventions could be targeted at whole villages inside the forest, and targeted at stratified risk groups for villages outside the forest.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s12936-020-03482-4>.

Additional file 1. Additional tables ST1–ST5 and additional figures S1–S3.

Abbreviations

ACT: Artemisinin-based combination therapy; AIC: Akaike Information Criterion; aOR: Adjusted odds ratio; G6PD: Glucose-6-phosphate dehydrogenase; GPS: Global positioning system; K⁺EDTA: Potassium ethylenediaminetetraacetic acid; LLIN: Long-lasting insecticidal net; LM: Light microscopy; MMW: Mobile malaria worker; N: Sample size; NMCP: National malaria control programme; PCR: Polymerase chain reaction; RDT: Rapid diagnostic test; VMW: Village malaria worker; WHO: World Health Organization.

Acknowledgements

The authors express their gratitude to the study participants, village heads, VMWs, local health facilities, and officials for making this study possible. We also thank the interviewers, technicians, and other study staff for their support.

Authors' contributions

AV, LR, BW, and IM conceived and designed the study and were overall responsible for the study. SK, AV, and BW implemented and managed the data collection, with support by SG, NK, and DL, and supervised the sample processing. SK conducted the data collection, supported by data management by MS and TO. TO, MS, SK, AV, LR, and IM designed the questionnaires. MW contributed to the statistical analysis. AP contributed the land cover analysis. MS analysed the data and wrote the first draft of the manuscript. AV, MW, TO, BW, LR, and IM contributed to data interpretation and writing. All authors read and approved the final manuscript.

Funding

This study is part of the International Centers of Excellence for Malaria Research programme "Understanding, tracking and eliminating malaria transmission in the Asia-Pacific Region", funded by the National Institutes of Health, MD, US (grant 1U19AI129392-01) and received additional funding by NHMRC (Australia, GNT1092789). IM is supported by an NHMRC Principal Research Fellowship (GNT1155075). LJR is supported by an NHMRC Career Development Fellowship (GNT1161627). MS is part of the PhD programme of the doctoral school ED393 Pierre Louis de santé publique and supported by the Sorbonne Université (contract n°2695/2017). AP is supported by the Pasteur Institute International Network PhD fellowship programme "Calmette & Yersin". The funding bodies had no role in the design of the study, the collection, analysis, and interpretation of data, and in writing the manuscript.

Availability of data and materials

The de-identified dataset analysed for this study is being made publicly available in ClinEpiDB repository, <https://clinepidb.org/ce/app/>. In the meantime, it is available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the National Ethics Committee for Health Research, Ministry of Health, Kingdom of Cambodia (reference 239NECHR) and by the Institut Pasteur Institutional Review Board (reference 2017-03). Written informed consent to participate in the study was obtained from all participants (or their parent or legal guardian).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹ Malaria: Parasites and Hosts Unit, Institut Pasteur, Paris, France. ² Sorbonne Université, Collège doctoral, Paris, France. ³ Malaria Molecular Epidemiology Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia. ⁴ Hub de Bioinformatique et Biostatistique, Département Biologie Computationnelle, Institut Pasteur, USR 3756 CNRS, Paris, France. ⁵ National Centre for Parasitology, Entomology, and Malaria Control, Phnom Penh, Cambodia. ⁶ School of Public Health, National Institute of Public Health, Phnom Penh, Cambodia. ⁷ Population Health & Immunity, Walter and Eliza Hall Institute, Melbourne, Australia. ⁸ University of Melbourne, Melbourne, Australia. ⁹ Burnet Institute, Melbourne, Australia.

Received: 24 June 2020 Accepted: 4 November 2020

Published online: 19 November 2020

References

- National Center for Parasitology, Entomology and Malaria Control (CNM). Annual Report 2018. Phnom Penh; 2019.
- WHO. World malaria report 2019. Geneva: World Health Organization; 2019a.
- WHO. Countries of the Greater Mekong zero in on falciparum malaria. Geneva: World Health Organization; 2019b.
- National Center for Parasitology, Entomology and Malaria Control (CNM). Annual Report 2017. Phnom Penh; 2018.
- Ministry of Health, Kingdom of Cambodia. Cambodia Malaria Elimination Action Framework 2016–2020. Phnom Penh; 2016.
- Duru V, Witkowski B, Ménard D. *Plasmodium falciparum* resistance to artemisinin derivatives and piperazine: a major challenge for malaria elimination in Cambodia. *Am J Trop Med Hyg*. 2016;95:1228–38.
- President's malaria initiative. Malaria Operational Plan FY 2018, Cambodia. Phnom Penh; 2018.
- National Center for Parasitology, Entomology and Malaria Control (CNM). National Strategic Plan for Elimination of Malaria in Cambodia (2011–2025). Phnom Penh; 2011.
- Lek D, Popovici J, Arie F, Vinjamuri SB, Meek S, Bruce J, et al. National malaria prevalence in Cambodia: microscopy versus polymerase chain reaction estimates. *Am J Trop Med Hyg*. 2016;95:588–94.
- Bannister-Tyrrell M, Srun S, Sluydts V, Gryseels C, Mean V, Kim S, et al. Importance of household-level risk factors in explaining micro-epidemiology of asymptomatic malaria infections in Ratanakiri Province, Cambodia. *Sci Rep*. 2018;8:11643.
- Durnez L, Pareyn M, Mean V, Kim S, Khim N, Menard D, et al. Identification and characterization of areas of high and low risk for asymptomatic malaria infections at sub-village level in Ratanakiri, Cambodia. *Malar J*. 2018;17:27.
- Imwong M, Nguyen TN, Tripura R, Peto TJ, Lee SJ, Lwin KM, et al. The epidemiology of subclinical malaria infections in South-East Asia: findings from cross-sectional surveys in Thailand-Myanmar border areas, Cambodia, and Vietnam. *Malar J*. 2015;14:381.
- Vantaux A, Samreth R, Piv E, Khim N, Kim S, Berne L, et al. Contribution to malaria transmission of symptomatic and asymptomatic parasite carriers in Cambodia. *J Infect Dis*. 2018;217:1561–8.
- Kiattibutr K, Roobsoong W, Sriwichai P, Saesue T, Rachaphaew N, Suan-somjit C, et al. Infectivity of symptomatic and asymptomatic *Plasmodium vivax* infections to a Southeast Asian vector, *Anopheles dirus*. *Int J Parasitol*. 2017;47:163–70.
- Kerkhof K, Sluydts V, Heng S, Kim S, Pareyn M, Willen L, et al. Geographical patterns of malaria transmission based on serological markers for falciparum and vivax malaria in Ratanakiri, Cambodia. *Malar J*. 2016;15:510.
- Tripura R, Peto TJ, Veugen CC, Nguon C, Davoeung C, James N, et al. Submicroscopic *Plasmodium* prevalence in relation to malaria incidence in 20 villages in western Cambodia. *Malar J*. 2017;16:56.
- Parker DM, Tripura R, Peto TJ, Maude RJ, Nguon C, Chalk J, et al. A multi-level spatial analysis of clinical malaria and subclinical *Plasmodium* infections in Pailin Province, Cambodia. *Heliyon*. 2017;3:e00447.
- Tripura R, Peto TJ, Chalk J, Lee SJ, Sirithiranon P, Nguon C, et al. Persistent *Plasmodium falciparum* and *Plasmodium vivax* infections in a western Cambodian population: implications for prevention, treatment and elimination strategies. *Malar J*. 2016;15:181.
- Bosman P, Stassijns J, Nackers F, Canier L, Kim N, Khim S, et al. *Plasmodium* prevalence and artemisinin-resistant falciparum malaria in Preah Vihear Province, Cambodia: a cross-sectional population-based study. *Malar J*. 2014;13:394.
- Cook J, Speybroeck N, Sochant T, Somony H, Sokny M, Claes F, et al. Sero-epidemiological evaluation of changes in *Plasmodium falciparum* and *Plasmodium vivax* transmission patterns over the rainy season in Cambodia. *Malar J*. 2012;11:86.

21. Incardona S, Vong S, Chiv L, Lim P, Nhem S, Sem R, et al. Large-scale malaria survey in Cambodia: novel insights on species distribution and risk factors. *Malar J*. 2007;6:37.
22. Sluydts V, Heng S, Coosemans M, Van Roey K, Gryseels C, Canier L, et al. Spatial clustering and risk factors of malaria infections in Ratanakiri Province, Cambodia. *Malar J*. 2014;13:387.
23. Steenkeste N, Rogers WO, Okell L, Jeanne I, Incardona S, Duval L, et al. Sub-microscopic malaria cases and mixed malaria infection in a remote area of high malaria endemicity in Ratanakiri province, Cambodia: implication for malaria elimination. *Malar J*. 2010;9:108.
24. Maude RJ, Nguon C, Ly P, Bunkea T, Ngor P, Canavati de la Torre SE, et al. Spatial and temporal epidemiology of clinical malaria in Cambodia 2004–2013. *Malar J*. 2014;13:385.
25. Asian Development Bank. Indigenous peoples/ethnic minorities and poverty reduction: Cambodia. Phnom Penh; 2002.
26. Moul P, Seng S. Country Technical Note on Indigenous Peoples' Issues: Kingdom of Cambodia. Phnom Penh: IFAD; 2012.
27. Canier L, Khim N, Kim S, Sluydts V, Heng S, Dourng D, et al. An innovative tool for moving malaria PCR detection of parasite reservoir into the field. *Malar J*. 2013;12:405.
28. Pepey A, Souris M, Vantaux A, Morand S, Lek D, Mueller I, et al. Studying land cover changes in a malaria-endemic Cambodian district: considerations and constraints. *Remote Sens*. 2020;12:2972.
29. Kulldorff M. A spatial scan statistic. *Commun Stat Theory Methods*. 1997;26:1481–96.
30. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)—a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform*. 2009;42:377–81.
31. Harris PA, Taylor R, Minor BL, Elliott V, Fernandez M, O'Neal L, et al. The REDCap consortium: Building an international community of software platform partners. *J Biomed Inform*. 2019;95:103208.
32. R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2019. <https://www.R-project.org/>
33. Kulldorff M, Information Management Services, Inc. SaTScan™ v9.6: Software for the spatial and space-time scan statistics. 2018. www.satscan.org
34. World Health Organization. World malaria report 2018. Geneva: World Health Organization; 2018.
35. Cho SJ, Lee J, Lee HJ, Jo H-Y, Sinniah M, Kim H-Y, et al. A novel malaria Pf/Pv Ab rapid diagnostic test using a differential diagnostic marker identified by network biology. *Int J Biol Sci*. 2016;12:824–35.
36. Landier J, Parker DM, Thu AM, Lwin KM, Delmas G, Nosten FH. Effect of generalised access to early diagnosis and treatment and targeted mass drug administration on *Plasmodium falciparum* malaria in Eastern Myanmar: an observational study of a regional elimination programme. *Lancet*. 2018;391:1916–26.
37. Tadesse E, Workalemahu B, Shimelis T. Diagnostic performance evaluation of the SD Bioline Malaria Antigen AG Pf/Pan test (05FK60) in a malaria endemic area of southern Ethiopia. *Rev Inst Med Trop São Paulo*. 2016;58:59.
38. Kim SH, Nam M-H, Roh KH, Park HC, Nam DH, Park GH, et al. Evaluation of a rapid diagnostic test specific for *Plasmodium vivax*. *Trop Med Int Health*. 2008;13:1495–500.
39. White NJ. Determinants of relapse periodicity in *Plasmodium vivax* malaria. *Malar J*. 2011;10:297.
40. Olliaro PL, Barnwell JW, Barry A, Mendis K, Mueller I, Reeder JC, et al. Implications of *Plasmodium vivax* biology for control, elimination, and research. *Am J Trop Med Hyg*. 2016;95:4–14.
41. Tadesse FG, Slater HC, Chali W, Teelen K, Lanke K, Belachew M, et al. The relative contribution of symptomatic and asymptomatic *Plasmodium vivax* and *Plasmodium falciparum* infections to the infectious reservoir in a low-endemic setting in Ethiopia. *Clin Infect Dis*. 2018;66:1883–91.
42. Churcher TS, Bousema T, Walker M, Drakeley C, Schneider P, Ouedraogo AL, et al. Predicting mosquito infection from *Plasmodium falciparum* gametocyte density and estimating the reservoir of infection. *eLife*. 2013;2:e00626.
43. Ouedraogo AL, Bousema T, Schneider P, de Vlas SJ, Ilboudo-Sanogo E, Cuzin-Ouattara N, et al. Substantial contribution of submicroscopical *Plasmodium falciparum* gametocyte carriage to the infectious reservoir in an area of seasonal transmission. *PLoS ONE*. 2009;4:e8410.
44. Gonçalves BP, Kapulu MC, Sawa P, Guelbéogo WM, Tiono AB, Grignard L, et al. Examining the human infectious reservoir for *Plasmodium falciparum* malaria in areas of differing transmission intensity. *Nat Commun*. 2017;8:1133.
45. Da DF, Churcher TS, Yerbanga RS, Yaméogo B, Sangaré I, Ouedraogo JB, et al. Experimental study of the relationship between *Plasmodium* gametocyte density and infection success in mosquitoes; implications for the evaluation of malaria transmission-reducing interventions. *Exp Parasitol*. 2015;149:74–83.
46. Longley RJ, White MT, Takashima E, Brewster J, Morita M, Harbers M, et al. Development and validation of serological markers for detecting recent *Plasmodium vivax* infection. *Nat Med*. 2020;26:741–9.
47. Rossi G, Vernaeve L, Van den Bergh R, Nguon C, Debackere M, Abello Peiri C, et al. Closing in on the reservoir: proactive case detection in high-risk groups as a strategy to detect *Plasmodium falciparum* asymptomatic carriers in Cambodia. *Clin Infect Dis*. 2018;66:1610–7.
48. Lover AA, Dantzer E, Hongvanthong B, Chindavongsa K, Welty S, Reza T, et al. Prevalence and risk factors for asymptomatic malaria and genotyping of glucose 6-phosphate (G6PD) deficiencies in a vivax-predominant setting, Lao PDR: implications for sub-national elimination goals. *Malar J*. 2018;17:218.
49. Dysoley L, Kaneko A, Eto H, Mita T, Socheat D, Börkman A, et al. Changing patterns of forest malaria among the mobile adult male population in Chumkiri District, Cambodia. *Acta Trop*. 2008;106:207–12.
50. National Center for Parasitology, Entomology and Malaria Control (CNM). Cambodia malaria survey 2013. Phnom Penh; 2013.
51. Bannister-Tyrrell M, Krit M, Sluydts V, Tho S, Sokny M, Mean V, et al. Households or hotspots? Defining intervention targets for malaria elimination in Ratanakiri province, Eastern Cambodia. *J Infect Dis*. 2019;220:1034–43.
52. Strategic Advisory Group on Malaria Eradication. Malaria eradication: benefits, future scenarios & feasibility. Geneva: World Health Organization; 2020.
53. Stresman G, Bousema T, Cook J. Malaria hotspots: is there epidemiological evidence for fine-scale spatial targeting of interventions? *Trends Parasitol*. 2019;35:822–34.
54. Lek D, Callery JJ, Nguon C, Debackere M, Sovannaroth S, Tripura R, et al. Tools to accelerate falciparum malaria elimination in Cambodia: a meeting report. *Malar J*. 2020;19:151.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Article: Mobility evaluation by GPS tracking in a rural, low-income population in Cambodia

Anaïs Pepey, Thomas Obadia, Saorin Kim, Siv Sovannaroth, Ivo Mueller, Benoit Witkowski,
Amélie Vantaux, Marc Souris

Published in PLoS ONE on the 13th of May 2022.

<https://doi.org/10.1371/journal.pone.0266460>

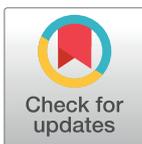
RESEARCH ARTICLE

Mobility evaluation by GPS tracking in a rural, low-income population in Cambodia

Anaïs Pepey^{1*}, Thomas Obadia^{2,3}, Saorin Kim¹, Siv Sovannaroth⁴, Ivo Mueller^{5,6}, Benoit Witkowski¹, Amélie Vantaux¹, Marc Souris⁷

1 Malaria Molecular Epidemiology Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia, **2** Department of Parasites and Insect Vectors, Infectious Diseases Epidemiology and Analytics, Institut Pasteur, Paris, France, **3** Département de Biologie Computationnelle, Hub de Bioinformatique et Biostatistique, Institut Pasteur, Paris, France, **4** National Centre for Parasitology Entomology and Malaria Control (CNM), Phnom Penh, Cambodia, **5** The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia, **6** Department of Medical Biology, University of Melbourne, Melbourne, Victoria, Australia, **7** UMR Unité des Virus Emergents, UVE: Aix-Marseille Univ–IRD 190–Inserm 1207–IHU 5 Méditerranée Infection, Marseille, France

* apepey@posteo.net



OPEN ACCESS

Citation: Pepey A, Obadia T, Kim S, Sovannaroth S, Mueller I, Witkowski B, et al. (2022) Mobility evaluation by GPS tracking in a rural, low-income population in Cambodia. *PLoS ONE* 17(5): e0266460. <https://doi.org/10.1371/journal.pone.0266460>

Editor: Andrés Viña, Michigan State University, UNITED STATES

Received: August 19, 2021

Accepted: March 21, 2022

Published: May 13, 2022

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: <https://doi.org/10.1371/journal.pone.0266460>

Copyright: © 2022 Pepey et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: The datasets are available on figshare at the following DOI: <https://doi.org/10.6084/m9.figshare.19538020.v1>.

Abstract

Global Positioning System (GPS) technology is an effective tool for quantifying individuals' mobility patterns and can be used to understand their influence on infectious disease transmission. In Cambodia, mobility measurements have been limited to questionnaires, which are of limited efficacy in rural environments. In this study, we used GPS tracking to measure the daily mobility of Cambodian forest goers, a population at high risk of malaria, and developed a workflow adapted to local constraints to produce an optimal dataset representative of the participants' mobility. We provide a detailed assessment of the GPS tracking and analysis of the data, and highlight the associated difficulties to facilitate the implementation of similar studies in the future.

Introduction

The study of individuals' daily movement patterns is important for understanding the temporal and spatial scales of infectious disease transmission [1,2]. The use of Global Positioning System (GPS) technology to assess these movement patterns has become increasingly common in infectious disease research projects due to its accessibility and convenience, for example in the context of various vector-borne transmission studies for dengue [3,4] and malaria [5–7]. The validity of various models has been assessed under different environmental conditions [8]. These devices offer various advantages compared to other methodologies, like cell phones, maps or questionnaires. Indeed, GPS data loggers are not subject to recall bias and do not depend on spatial literacy in the study population or on the existence of addresses and maps in the study area, which can be limited in resource-poor and low-income populations [9]. Compared to GPS devices, cell phones are another option but are more expensive and more prone to theft. Relying on personal cellular devices might furthermore result in biased sampling of the population [10]. In addition, the encryption of cell phone positional data is harder to

Funding: This study was funded by the National Institutes of Health program International Centres of Excellence for Malaria Research, grant 1U19AI129392-01 “Understanding, tracking and eliminating malaria transmission in the Asia-Pacific Region”. A.P. was funded by the Pasteur Institute International Network Calmette and Yersin Ph.D. scholarship, grant MHL/MJ/N°212/18. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

institute [3] and the mobile network in remote or rural areas is often partial [11], which would deteriorate spatial data recording [10].

Though GPS tracking is an effective methodology for the collection of spatial data for vector-borne disease research, it also comes with limitations: timing and orbital errors, inadequate satellite geometry and signal inconsistencies can generate bias or errors [8,12]. Human-induced error is also to be expected, as participants could forget to carry the device.

Some recommendations already exist for handling GPS devices and data processing, but do not include the difficulties of implementing the devices in the study population [13]. A previous study described a GPS follow-up implementation to compare their efficacy to semi-structured interviews in a population sampled in urban South America [4]. The best practices to implement GPS tracking to follow forest goers, in accordance to their working conditions and to a rural environment, are not yet available in the literature.

While Cambodia aims to eliminate malaria by 2025, several pockets of transmission remain in the country, with the highest incidence rate found in Monduliri, Eastern Cambodia [14]. Forest goers—represented by young and adult males [15–17]—have repeatedly been shown to be the most at-risk population for malaria in Cambodia [15,18–21]. Malaria vectors (*Anopheles spp.*) are found mostly in forest environments [22]: forest activities are therefore the most important risk factor. Malaria vectors also inhabit forest fringes [22], putting individuals in farmer huts and engaging in plantation work at risk of exposure to infectious mosquito bites [23]. *Anopheles* vectors are mostly nocturnal species, but they can also be active during dusk and dawn, outside the hours of bed net use [24,25]. Malaria risk exposure can vary from one village to another [16,26] but also between households [27], highlighting the need for more precise tools to measure such local heterogeneity. Individuals’ traversal of a study area, in and around forest patches at different times of day, needs to be assessed and analysed to estimate individual exposure risk.

In Cambodia, the standard methodology to measure mobility has been through questionnaires [15,19–21]. To understand the role of daily movement patterns on its transmission, we implemented a GPS follow-up study to track the population at risk. We described and characterised individuals’ mobility patterns, and integrated the data into a Geographic Information System (GIS) to be combined and analysed with environmental data. We furthermore documented the methodology and the limitations of the implementation to facilitate the design of future GPS tracking studies in similar rural and resource-poor environments.

Material and methods

Study site and sample population

The study took place in Kaev Seima district, Monduliri province, North-East Cambodia. Here, the climate is divided into rainy (May–October) and dry (November–April) seasons. Kaev Seima district is a rural, low-income environment where populations mainly live from agriculture and forest products exploitation. Study participants were men between the age of 13 and 60 years old to match the main malaria risk population [15,18–21]. The enrolment occurred in two rounds to characterise both seasons: first round during the rainy season (April to September 2018) with 160 participants, and second round during the dry season (February to April 2019) with 200 participants.

Study design

We implemented GPS tracking in a rural area of Cambodia, following all participants’ movements for two weeks by providing them a GPS data-logger that recorded their position. In accordance with previous guidelines [13], after the two weeks, participants were asked to fill

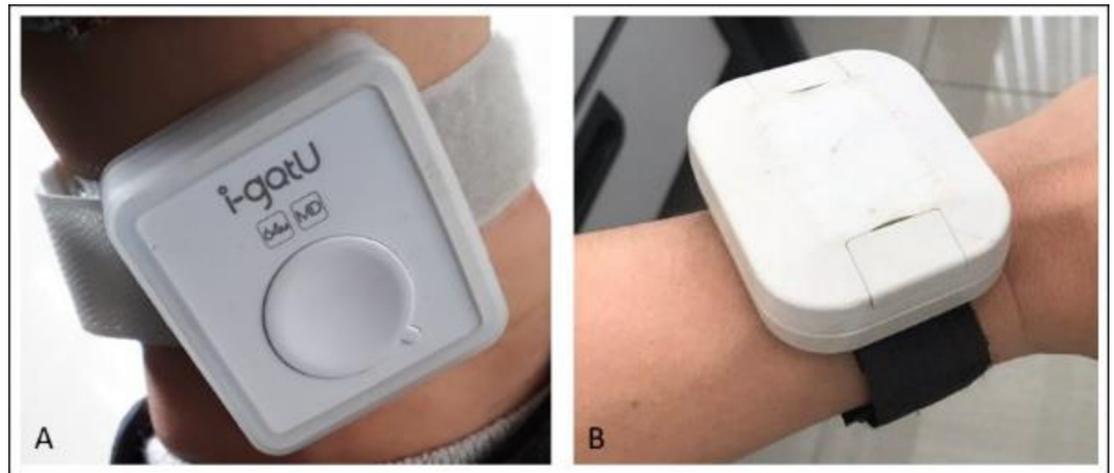


Fig 1. Devices distributed to the study population. A: I-gotU GT600, distributed for rainy season (here worn on an individual's ankle), B: Takachi waterproof enclosure containing the I-gotU GT600 distributed for dry season (here worn on an individual's wrist). The waterproof enclosure was not yet used during rainy season because the devices were sold as "water-resistant" and were expected to be adapted to study conditions.

<https://doi.org/10.1371/journal.pone.0266460.g001>

out a questionnaire containing questions related to their use of GPS devices. Similar to other studies of this kind [5,6], the I-gotU GT600 (Mobile Action Technology Inc., Taipei, Taiwan, Fig 1) was used to record participants' mobility. Its battery life (up to a week), data storage (262,000 waypoints), price (< 50 USD), precision (< 10m) and unobtrusive design (37g) corresponded best to our study design. Each participant was assigned two GPS data loggers over course of the study (one for each of the two 7 day tracking periods). Serial numbers of the GPS data loggers were matched to participant unique identification numbers. The GPS loggers were password-protected, allowing only the research team to access the data. The settings intentionally prohibited the devices to be turned off, thus allowing the devices to record the mobility of the field team between households and base. Curation of the spatial data was performed with R [28].

Geographical coordinates were logged every 30 seconds. A smart tracking mode allowed for changing of logging intervals to 10 seconds when an individual's speed exceeded 10 km/h. The GPS data loggers were programmed to be motion activated and to hibernate when not in motion to better preserve battery life.

Waterproof enclosures (Takachi IP67 enclosures, reference WP5-7-2G) were used during the second round of collection to protect the loggers from precipitation and immersion (Fig 1). We fixed a Velcro strap on the plastic enclosure of the device to allow it to be worn on one's wrist or ankle.

GPS accuracy and precision tests

To quantify the spatial exactness of the GPS data loggers, two values should be measured: accuracy and precision. Accuracy represents the closeness between the measurement and a true value, while precision describes the closeness between different measurements of a same location. Typically, GPS accuracy is determined by comparing the positional coordinates recorded by a GPS receiver to that of a known location [8]. Such location could not be found in our study area, we thus had to rely on other methods, such as comparing records from the GPS

data loggers and the most accurate GPS device available, a Garmin GPSMAP[®] 64s. Both precision and accuracy may depend on the type of surrounding environment.

The manufacturer did not provide any information about the device accuracy or precision. However, Duncan *et al.* (2013) tested the I-gotU GT600's stationary accuracy under various environmental conditions and found a mean error of 19.6m (SD +/- 30.9m) and a mean circular error of 10.8m, which varied from 3.3m (open sky) to 47.1m (between high-rise buildings). Vazquez-Prokopec *et al.* [3] also tested the device's spatial accuracy and obtained a stationary root mean square error of 4.4m for their stationary test and of 10.3m for their motion test along a known horizontal linear path.

To control for GPS accuracy and precision, we selected four geodic sites, one per different type of environment. In each, two to four units were left side by side at ground level to record their position for 30 minutes. The devices' hibernation mode was deactivated and their location was sampled using the Garmin GPS device. We repeated the recording process on eight occasions for each land environment category. The selected sites were: i) under a traditional Khmer house elevated three metres from the ground, where members of the households meet for social interactions; ii) in an open space next to the road in a village; iii) in a rubber plantation where trees reached three to four metres in height; and iv) in the primary forest under a sparse six-metre tall canopy.

To measure the precision, we computed the mean and standard deviation of distances to the mean centre of the recorded points. To measure the accuracy, we computed the mean distance and standard deviation to a reference point in addition to the percentage of logged points that were included in a 5-metre, 10-metre or 20-metre radius around the reference point [29]. Calculations were performed in R [28], using the `distGeo` function from the `geosphere` package [30].

Data management

From the coordinates downloaded from the data loggers, GPS points were assembled into segments and segments into routes (or tracks). Data curation was conducted along this process, removing all points falling outside the study area or segments exceeding 150 km/h, a speed assumed not attainable within the study area and most probably arising from device recording errors (concordant with the speed threshold value recommended in [31]).

Segments were categorized based on time of capture: day (between 06h00 to 17h59) or night (between 18h00 and 05h59). Segment speeds were categorized as "slow" (≤ 5 km/h, i.e. when participant was motionless or walking) and "fast" (when speed was > 5 km/h, i.e. movement in a vehicle).

Each GPS data track was categorised depending on the logged duration (hours) and distance (kilometres). The total duration of GPS track was classified as suboptimal (< 48 h) or optimal (≥ 48 h) depending on the total hours logged over one week. Similarly, the average distance per day was classified as suboptimal (< 10 km) or optimal (≥ 10 km). Finally, only tracks categorized as optimal for both the total duration and the average distance per day were categorised as optimal. These categories allowed to dismiss the data from: i) devices for which the cumulative duration of records could fail to provide sufficient insights into a participant's mobility and ii) participants that left the device at home rather than taking it with them.

Using SavGIS [32], the time spent into each type of land use (described in Pepey *et al.* [33]) was calculated for each track. A 100m-resolution grid was produced to cover the study area, with each cell attributed to the major corresponding land use category (Fig 2).

We calculated for each dataset (optimal and suboptimal) the proportion of time spent: i) at slow speed versus fast speed; ii) during day time versus night time; iii) in each land use

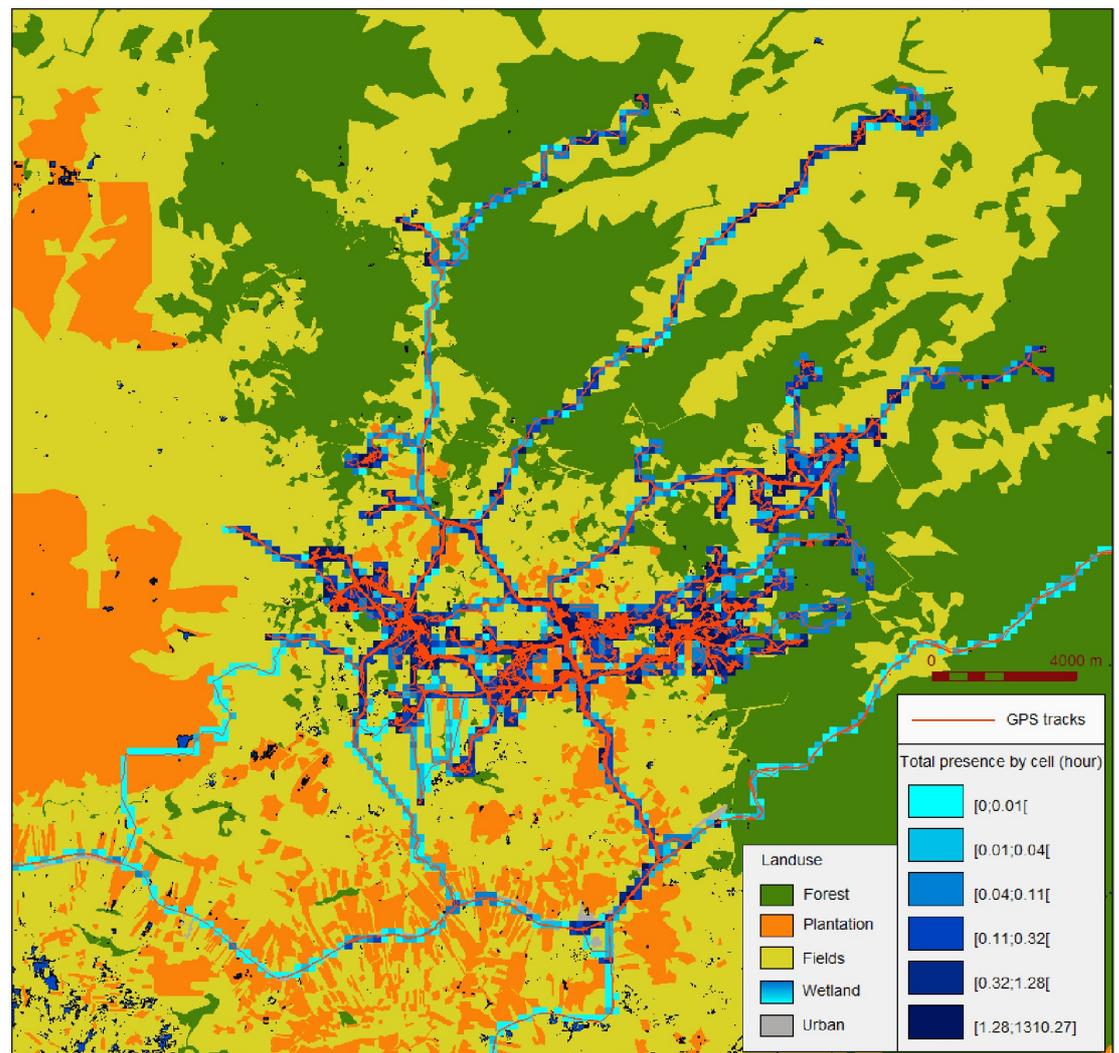


Fig 2. GPS tracks and total presence by cell from all participants during rainy season ($N_{\text{tracks}} = 249$, $N_{\text{participants}} = 151$). Land use reprinted from [33] under a CC BY license, with permission from creators of the map A. Pepey *et al*, original copyright 2020.

<https://doi.org/10.1371/journal.pone.0266460.g002>

category. These distributions were compared using Pearson's Chi-squared test and accounting for multiple comparisons using the Bonferonni correction.

Ethics approval and consent to participate

Ethical approvals were obtained from the Cambodian National Ethics Committee for Health Research (045NECHR & 312NHECR) and from the National Institutes of Health (DMID Protocol Number: 17-0036). The protocols conformed to the Helsinki Declaration on ethical principles for medical research involving human subjects (version 2002) and informed written consent was obtained for all volunteers. Parental assent was obtained for every participant between 13 and 18 years old.

Table 1. Distance and duration logged by participants over the 2-week GPS follow-up.

Variable	Unit	Value	Rainy season	Dry season
Distance	km	Min	0	0
		Q1	68.2	74.6
		Median	142.1	127.8
		Q3	234.4	205.5
		Max	767.1	488.6
Time	days	Min	0	0
		Q1	3.6	4.9
		Median	6.5	8.4
		Q3	9.4	11
		Max	14.8	16.7
Distance/day	km	Min	0	0
		Q1	16.3	11.8
		Median	23.6	18.1
		Q3	32.7	29.4
		Max	74.3	78.3

Durations exceeding 14 days are due to the unavailability of participants on the scheduled date, leading to postponed visits.

<https://doi.org/10.1371/journal.pone.0266460.t001>

Results

Overview

Over the two seasons 4,383,582 GPS data points were logged, 59.9% of which were retained after data curation (S1 Table). Median distance logged by participants were consistent across seasons but varied greatly across participants (Table 1).

The average battery life of GPS devices was 3 day and 7 hours in the rainy season, and 4 days and 1 hour in the dry season, with great variability ranging from total malfunction (0m logged) to 16 days and 18 hours. The minimal non-null duration recorded by a GPS device was 1m and 1s, recording a total of 3 GPS points, during rainy season.

GPS unit performance

Across all sites, the GPS devices were accurate within 20m for at least 90% of points. The stationary error of devices left in open space (accuracy: 6.1m, precision: 5.6m) or under house (accuracy: 5.9m, precision: 5m) were similar to values reported previously [3,8]. The GPS devices' logged coordinates varied between environments, with the greatest average error in plantations, for which the percentage of accurate points within a 20m radius was less than 80%. Accuracy and precision measurements for each land type can be found in S2 and S3 Tables, respectively.

Data loss

Of the 360 enrolled participants, 261 returned both devices, allowing for a complete dataset to be downloaded (Table 2).

For the other 99 participants, an incomplete dataset was obtained. Data loss was mostly due to water infiltration and other dysfunctions from devices (77 devices, representing 69.3% of missing data, Table 3). However, data loss also arose from participants losing the device,

Table 2. Count of participants' available GPS tracks per season and for all data.

Participants GPS datasets	Rainy season	Dry season	All
2 weeks logged	98	163	261
1 week logged	53	34	87
None	9	3	12
Total collected GPS tracks	249	360	609

<https://doi.org/10.1371/journal.pone.0266460.t002>

withdrawal or being unreachable after baseline. Eight participants withdrew because they or their families were against pursuing the study (Table 3).

In the first round of collection, 54 GPS data loggers were heavily damaged by water entering the device. This resulted in 22.2% of the data being unusable. By using waterproof enclosures to protect the GPS devices during the second round of collection, we minimised the data loss to 10%.

Optimal and suboptimal datasets

We assigned 393 tracks (64.5%) as optimal (S4 Table). Optimal data averaged 7 days of logging per participant (range: 2 to 15 days) for a mean of 176.6 km per participants over the follow-up (25.4 to 767.1 km). Logged optimal GPS data totalised 48,399 km and 46,337 hours. The percentage of time logged in built-up areas was significantly lower for the optimal dataset, and higher for the other land use categories (S5 Table). Optimal dataset also had a significantly higher proportion of points logged during day time and above 5km/h (S5 Table).

Questionnaire

Only 35 (9.7%) participants declared at least one removal of the device during the follow-up (21 during the rainy season and 14 during the dry season), with an average of two removals per participant per follow-up. Five participants declared that the removal was due to the discomfort, one because of its broken strap, while the 29 others did not indicate a reason. According to the number of removals and estimated time of removal given by the participants, we estimated that the data loss averaged 4 hours 36 minutes per participant per 2-week follow-up (min = 4 minutes, median = 2 hours, max = 20 hours). However, the data recorded from these participants was more complete than the global dataset: only 11.5% of them had a suboptimal length per day. Meanwhile, 16.4% of the GPS data from the global dataset had a suboptimal distance per day.

There was discrepancy between the data retrieved from questionnaire on day 14 and GPS data (optimal data, standardised time): participants could declare a visit in a specific

Table 3. Missing GPS tracks and reasoning.

Missing GPS tracks	Rainy season	Dry season	All
Lost device	7	8	15
Damaged device	54	23	77
Participant withdrawal	4	4	8
Participant out of reach	5	5	10
Participant death	1	0	1
Total missing GPS tracks	71	40	111
Total expected GPS tracks	320	400	720
GPS data loss (%)	22.2	10	15.4

<https://doi.org/10.1371/journal.pone.0266460.t003>

environment, without any corresponding visit in their GPS data, and vice-versa (S6 Table). Overall, data discrepancy between GPS and questionnaire data varied from 26.9% (forest visits; over 197 participants, 53 had contradictory data between GPS and questionnaire) to 48.2% (fields visits, S7 Table).

Discussion

Curated data provides useful insight about GPS tracking implementation to a rural Cambodian population, though results interpretation can be subject to numerous biases. Recall bias during questionnaires may have led to erroneous estimations of device removals. The data loggers may attribute time spent in a given land to a participant incorrectly, as accuracy depends on the land use surrounding the device, although the grid definition (100-metre cells) mitigates the effects from accuracy error (maximum = 17.2m) and precision error (maximum = 15.4m).

Though participants who declared device removal did not constitute a significant proportion of suboptimal data, over the whole dataset, most of the suboptimal data seems to have come from participants that did not wear their GPS data loggers during their daily activities and did not declare these removals during the questionnaire. This is suggested by the 129 answers that did not declare a visit to a specific environment while the corresponding GPS data did include such a visit (S6 Table).

The differences between suboptimal and optimal datasets suggest that most of the removed points were from stationary positions at night in built-up areas. Performing mobility analyses on the complete dataset, including the suboptimal data, would not be representative of the true population mobility, as time spent in built-up areas would be over-represented.

The proportion of suboptimal data from devices left at home, in addition to GPS malfunctions and hardware damage, highlights the importance of designing a study that considers this substantial data loss, with a methodology that accounts only for optimal data. The sample size should also account for this data depletion, in addition to participants leaving the study. For instance, Hast et al. [5] observed an important drop-out from their participants which limited the interpretation of their results.

The selection of optimal versus suboptimal data is also potentially subject to bias. Even though the study population habits were taken into account, the threshold of optimal data quality was defined artificially as a minimum of 48 hours logged over the week and 10 km traversed per day for each participant. We also assumed that the GPS tracks with a logged duration of less than 48 hours did not fully depict participants' movements, though it is still possible that a participant travelled only short distances that week. In addition, any participant who stayed at home most of the week, keeping his device on him at all times (the device would therefore go into hibernation mode), would be categorized as suboptimal even though his mobility would be adequately represented.

Participants' compliance to use GPS data loggers is often partial—participants are not always willing to carry the GPS data loggers at all times—as reported in previous studies using the devices for tracking populations at risk of malaria [7] and in other contexts [4,34,35]. In our study, we observed that most declared removals were minor and did not significantly affect the quality of the data. However, the data suggests that there were removals not declared during the questionnaire, enough to categorise 35.5% of retrieved data as suboptimal. As for participants unwilling to take the GPS devices with them for certain activities, we hypothesise that they would not acknowledge removing their loggers. However, the declared compliance of the participants was very high, with 90.3% reporting no removal of the GPS data logger during the follow-up. Vazquez-Prokopec et al. [35] also observed that participants can declare a very high

compliance (>80%) even though they did not carry the GPS loggers at all times. Consequently, it is essential to consider both technical issues and participant-induced biases, and to retain only the optimal data needed to estimate the mobility at individual level. These results highlight the need for a full data analysis and cleaning methodology with GIS when considering the use of GPS data loggers.

In the GMS, malaria exposure risk is associated with activities in and around forested areas [15,20,21]. The estimation of time spent in these environments at night is important for estimating individuals' exposure risk. The use of GPS tracking imported into a GIS environment can provide quantitative and precise data about mosquito exposure at a fine resolution [36]. Coupled with a follow-up to track recent [37] and active [38] malaria infections, this method can help pinpoint hotspot transmission areas that might require special attention to eradicate the disease.

Our study had two main limitations: participants' compliance and devices' suitability. Firstly, the representativeness of the GPS data was affected by participants withdrawing from the study or being out of reach, as well as by participants who did not take their devices with them for their daily mobility. Revising the enrolment strategy, or initiating education and awareness of local populations about malaria transmission would likely improve this aspect. Another option would be to further increase the sample size although this would come at a cost of time and funds. Secondly, the quality of the GPS devices was below expectations, despite manufacturers' specifications and even after some preliminary testing that validated the model and the settings. The I-gotU GT600 battery was not suited to log one week of GPS points according to the selected settings. The device robustness and water resistance were also not satisfactory. Different models should therefore be tested in order to find a device able to fulfil both the battery life requirements and sturdiness expectations under the given conditions. Higher-quality GPS tracking devices can be found on the market; however users have to balance the cost, sturdiness and size acceptable by the volunteers.

Conclusion

The implementation of GPS follow-up for infectious disease research holds promising results. Our study showed what limitations need to be considered during study design and data analysis. Here, GPS trackers were decently accepted by the population. The accuracy and precision of GPS data loggers were concordant with the findings from other studies and corresponded to our requirements. After curating the data and accounting for suboptimal and missing tracks, it effectively described participants' daily movements. We insist on anticipating harsh climatic and working conditions, in addition to the necessity for thorough data pre-processing to remove GPS tracks from participants unwilling to bring their devices to their daily activities while declaring no removals during the questionnaire. Thus, GPS follow-ups with careful curation have the potential to generate detailed daily data at the individual level for rural populations. Such movement patterns can be used to refine epidemiological models and to adapt public health intervention strategies to focus on populations of interest.

Supporting information

S1 Table. Count of logged and analysed GPS points.

(DOCX)

S2 Table. GPS devices' accuracy controlled as the average error between individual recorded positions and true georeference given by a precise device.

(DOCX)

S3 Table. GPS devices' precision controlled as the average error between individual recorded positions and centroid of the units' recorded positions.
(DOCX)

S4 Table. Proportions of tracks belonging to insufficient, poor and optimal categories, allowing selecting optimal GPS tracks for analysis.
(DOCX)

S5 Table. Percentage of every land use, speed and time variables attributed to suboptimal and optimal datasets. Every land use category was compared to the total time logged minus the time in this land use category using a Chi-squared test. According to Bonferonni correction, p-value was significant when $p < 0.05/6 = 0.0083$.
(DOCX)

S6 Table. Count of data discrepancies between questionnaire and GPS datasets about participants visits in forest, plantations and fields, for all participants with a complete dataset for questionnaire at week 2 and GPS follow-up (N = 197). Discordant data is highlighted in yellow.
(DOCX)

S7 Table. Total discrepancies between GPS and questionnaire data for participants with a complete dataset (N = 197).
(DOCX)

Acknowledgments

The authors express their gratitude to the study participants, village heads, VMWs and officials for making this study possible. The authors thank Anthony Ruberto for proofreading the manuscript.

Author Contributions

Conceptualization: Anaïs Pepey, Benoit Witkowski, Amélie Vantaux.

Data curation: Anaïs Pepey, Thomas Obadia, Marc Souris.

Formal analysis: Anaïs Pepey, Amélie Vantaux, Marc Souris.

Funding acquisition: Ivo Mueller, Benoit Witkowski.

Investigation: Anaïs Pepey, Saorin Kim, Amélie Vantaux, Marc Souris.

Methodology: Anaïs Pepey, Thomas Obadia, Amélie Vantaux, Marc Souris.

Project administration: Ivo Mueller, Benoit Witkowski, Amélie Vantaux.

Resources: Benoit Witkowski.

Software: Marc Souris.

Supervision: Ivo Mueller, Benoit Witkowski, Amélie Vantaux, Marc Souris.

Validation: Siv Sovannaroth, Ivo Mueller, Benoit Witkowski, Amélie Vantaux, Marc Souris.

Writing – original draft: Anaïs Pepey.

Writing – review & editing: Anaïs Pepey, Thomas Obadia, Benoit Witkowski, Amélie Vantaux, Marc Souris.

References

1. Stoddard S.T.; Forshey B.M.; Morrison A.C.; Paz-Soldan V.A.; Vazquez-Prokopec G.M.; Astete H.; et al. House-to-House Human Movement Drives Dengue Virus Transmission. *PNAS* 2013, 110, 994–999, <https://doi.org/10.1073/pnas.1213349110> PMID: 23277539
2. Stoddard S.T.; Morrison A.C.; Vazquez-Prokopec G.M.; Paz Soldan V.; Kochel T.J.; Kitron U.; et al. The Role of Human Movement in the Transmission of Vector-Borne Pathogens. *PLoS Negl Trop Dis* 2009, 3, e481, <https://doi.org/10.1371/journal.pntd.0000481> PMID: 19621090
3. Vazquez-Prokopec G.M.; Stoddard S.T.; Paz-Soldan V.; Morrison A.C.; Elder J.P.; Kochel T.J.; et al. Usefulness of Commercially Available GPS Data-Loggers for Tracking Human Movement and Exposure to Dengue Virus. *Int J Health Geogr* 2009, 8, 68, <https://doi.org/10.1186/1476-072X-8-68> PMID: 19948034
4. Paz-Soldan V.A.; Stoddard S.T.; Vazquez-Prokopec G.; Morrison A.C.; Elder J.P.; Kitron U.; et al. Assessing and Maximizing the Acceptability of Global Positioning System Device Use for Studying the Role of Human Movement in Dengue Virus Transmission in Iquitos, Peru. *Am. J. Trop. Med. Hyg.* 2010, 82, 723–730, <https://doi.org/10.4269/ajtmh.2010.09-0496> PMID: 20348526
5. Hast M.; Searle K.M.; Chaponda M.; Lupiya J.; Lubinda J.; Sikalima J.; et al. The Use of GPS Data Loggers to Describe the Impact of Spatio-Temporal Movement Patterns on Malaria Control in a High-Transmission Area of Northern Zambia. *Int J Health Geogr* 2019, 18, 19, <https://doi.org/10.1186/s12942-019-0183-y> PMID: 31426819
6. Searle K.M.; Lubinda J.; Hamapumbu H.; Shields T.M.; Curriero F.C.; Smith D.L.; et al. Characterizing and Quantifying Human Movement Patterns Using GPS Data Loggers in an Area Approaching Malaria Elimination in Rural Southern Zambia. *R Soc Open Sci* 2017, 4, <https://doi.org/10.1098/rsos.170046> PMID: 28573009
7. Fornace K.M.; Alexander N.; Abidin T.R.; Brock P.M.; Chua T.H.; Vythilingam I.; et al. Local Human Movement Patterns and Land Use Impact Exposure to Zoonotic Malaria in Malaysian Borneo. *eLife* 2019, 8, e47602, <https://doi.org/10.7554/eLife.47602> PMID: 31638575
8. Duncan S.; Stewart T.I.; Oliver M.; Mavoja S.; MacRae D.; Badland H.M.; et al. Portable Global Positioning System Receivers: Static Validity and Environmental Conditions. *Am J Prev Med* 2013, 44, e19–29, <https://doi.org/10.1016/j.amepre.2012.10.013> PMID: 23332343
9. Paz-Soldan V.A.; Reiner R.C.; Morrison A.C.; Stoddard S.T.; Kitron U.; Scott T.W.; et al. Strengths and Weaknesses of Global Positioning System (GPS) Data-Loggers and Semi-Structured Interviews for Capturing Fine-Scale Human Mobility: Findings from Iquitos, Peru. *PLoS Negl Trop Dis* 2014, 8, e2888, <https://doi.org/10.1371/journal.pntd.0002888> PMID: 24922530
10. Wesolowski A.; Buckee C.O.; Engø-Monsen K.; Metcalf C.J.E. Connecting Mobility to Infectious Diseases: The Promise and Limits of Mobile Phone Data. *J. Infect. Dis.* 2016, 214, S414–S420, <https://doi.org/10.1093/infdis/jiw273> PMID: 28830104
11. Caceres N.; Wideberg J.P.; Benitez F.G. Review of Traffic Data Estimations Extracted from Cellular Networks. *IET Intelligent Transport Systems* 2008, 2, 179–192, <https://doi.org/10.1049/iet-its:20080003>
12. Enge P.; Misra P. Special Issue on Global Positioning System. *Proceedings of the IEEE* 1999, 87, 3–15, <https://doi.org/10.1109/JPROC.1999.736338>
13. Kerr J.; Duncan S.; Schipperijn J.; Schipperijn J. Using Global Positioning Systems in Health Research: A Practical Approach to Data Collection and Processing. *Am J Prev Med* 2011, 41, 532–540, <https://doi.org/10.1016/j.amepre.2011.07.017> PMID: 22011426
14. WHO *Countries of the Greater Mekong Zero in on Falciparum Malaria*; WHO: Geneva, 2019;
15. Incardona S.; Vong S.; Chiv L.; Lim P.; Nhem S.; Sem R.; et al. Large-Scale Malaria Survey in Cambodia: Novel Insights on Species Distribution and Risk Factors. *Malar. J.* 2007, 6, 37, <https://doi.org/10.1186/1475-2875-6-37> PMID: 17389041
16. Tripura R.; Peto T.J.; Veugen C.C.; Nguon C.; Davoeung C.; James N.; et al. Submicroscopic Plasmodium Prevalence in Relation to Malaria Incidence in 20 Villages in Western Cambodia. *Malaria Journal* 2017, 16, 56, <https://doi.org/10.1186/s12936-017-1703-5> PMID: 28143518
17. Rossi G.; Vernaev L.; Van den Bergh R.; Nguon C.; Debackere M.; Abello Peiri C.; et al. Closing in on the Reservoir: Proactive Case Detection in High-Risk Groups as a Strategy to Detect Plasmodium Falciparum Asymptomatic Carriers in Cambodia. *Clin Infect Dis* 2018, 66, 1610–1617, <https://doi.org/10.1093/cid/cix1064> PMID: 29361021
18. Kar N.P.; Kumar A.; Singh O.P.; Carlton J.M.; Nanda N. A Review of Malaria Transmission Dynamics in Forest Ecosystems. *Parasit Vectors* 2014, 7, 265, <https://doi.org/10.1186/1756-3305-7-265> PMID: 24912923

19. Peeters Grietens K.; Gryseels C.; Dierickx S.; Bannister-Tyrrell M.; Trienekens S.; Uk S.; et al. Characterizing Types of Human Mobility to Inform Differential and Targeted Malaria Elimination Strategies in Northeast Cambodia. *Sci Rep* 2015, 5, 16837, <https://doi.org/10.1038/srep16837> PMID: 26593245
20. Sandfort M.; Vantaux A.; Kim S.; Obadia T.; Pepey A.; Gardais S.; et al. Forest Malaria in Cambodia: The Occupational and Spatial Clustering of Plasmodium Vivax and Plasmodium Falciparum Infection Risk in a Cross-Sectional Survey in Mondulakiri Province, Cambodia. *Malaria Journal* 2020, 19, 413, <https://doi.org/10.1186/s12936-020-03482-4> PMID: 33213471
21. Sluydts V.; Heng S.; Coosemans M.; Van Roey K.; Gryseels C.; Canier L.; et al. Spatial Clustering and Risk Factors of Malaria Infections in Ratanakiri Province, Cambodia. *Malar. J.* 2014, 13, 387, <https://doi.org/10.1186/1475-2875-13-387> PMID: 25269827
22. Sinka M.E.; Bangs M.J.; Manguin S.; Chareonviriyaphap T.; Patil A.P.; Temperley W.H.; et al. The Dominant Anopheles Vectors of Human Malaria in the Asia-Pacific Region: Occurrence Data, Distribution Maps and Bionomic Précis. *Parasites & Vectors* 2011, 4, 89, <https://doi.org/10.1186/1756-3305-4-89> PMID: 21612587
23. Thomson R.; Sochea P.; Sarath M.; MacDonald A.; Pratt A.; Poyer S.; et al. Rubber Plantations and Drug Resistant Malaria: A Cross-Sectional Survey in Cambodia. *Malar. J.* 2019, 18, 379, <https://doi.org/10.1186/s12936-019-3000-y> PMID: 31775755
24. Hii J.; Hustedt J.; Bangs M.J. Residual Malaria Transmission in Select Countries of Asia-Pacific Region: Old Wine in a New Barrel. *J Infect Dis* 2021, 223, S111–S142, <https://doi.org/10.1093/infdis/jiab004> PMID: 33906222
25. Vantaux A.; Riehle M.M.; Piv E.; Farley E.J.; Chy S.; Kim S.; et al. Anopheles Ecology, Genetics and Malaria Transmission in Northern Cambodia. *Scientific Reports* 2021, 11, 6458, <https://doi.org/10.1038/s41598-021-85628-1> PMID: 33742030
26. Okami S.; Kohtake N. Spatiotemporal Modeling for Fine-Scale Maps of Regional Malaria Endemicity and Its Implications for Transitional Complexities in a Routine Surveillance Network in Western Cambodia. *Front Public Health* 2017, 5, 262, <https://doi.org/10.3389/fpubh.2017.00262> PMID: 29034229
27. Bannister-Tyrrell M.; Verdonck K.; Hausmann-Muela S.; Gryseels C.; Muela Ribera J.; Peeters Grietens K. Defining Micro-Epidemiology for Malaria Elimination: Systematic Review and Meta-Analysis. *Malar J* 2017, 16, 164, <https://doi.org/10.1186/s12936-017-1792-1> PMID: 28427389
28. R Core Team *R: A Language and Environment for Statistical Computing*. [Www.R-Project.Org](http://www.R-project.org); R: A language and environment for statistical computing; R Foundation for Statistical Computing, Vienna, Austria, 2020.
29. McGranahan D.A.; Geaumont B.; Spiess J.W. Assessment of a Livestock GPS Collar Based on an Open-source Datalogger Informs Best Practices for Logging Intensity. *Ecology and Evolution* 2018, <https://doi.org/10.1002/ece3.4094> PMID: 29938081
30. Hijmans, R.J. *Geosphere: Spherical Trigonometry*; R package version 1.5–5, 2016;
31. Schuessler N.; Axhausen K.W. Processing Raw Data from Global Positioning Systems without Additional Information. *Transportation Research Record* 2009, 2105, 28–36, <https://doi.org/10.3141/2105-04>
32. Souris, M. *SavGIS Geographic Information System*. [Www.Savgis.Org](http://www.savgis.org); 2018.
33. Pepey A.; Souris M.; Vantaux A.; Morand S.; Lek D.; Mueller I.; et al. Studying Land Cover Changes in a Malaria-Endemic Cambodian District: Considerations and Constraints. *Remote Sensing* 2020, 12, 2972, <https://doi.org/10.3390/rs12182972>
34. Cho G.-H.; Rodríguez D.A.; Evenson K.R. Identifying Walking Trips Using GPS Data. *Med Sci Sports Exerc* 2011, 43, 365–372, <https://doi.org/10.1249/MSS.0b013e3181ebec3c> PMID: 20581721
35. Vazquez-Prokopec G.M.; Bisanzio D.; Stoddard S.T.; Paz-Soldan V.; Morrison A.C.; Elder J.P.; et al. Using GPS Technology to Quantify Human Mobility, Dynamic Contacts and Infectious Disease Dynamics in a Resource-Poor Urban Environment. *PLoS ONE* 2013, 8, e58802, <https://doi.org/10.1371/journal.pone.0058802> PMID: 23577059
36. Bharati K.; Ganguly N.K. Tackling the Malaria Problem in the South-East Asia Region: Need for a Change in Policy? *Indian J Med Res* 2013, 137, 36–47. PMID: 23481050
37. Greenhouse B.; Daily J.; Guinovart C.; Goncalves B.; Beeson J.; Bell D.; et al. Priority Use Cases for Antibody-Detecting Assays of Recent Malaria Exposure as Tools to Achieve and Sustain Malaria Elimination. *Gates Open Res* 2019, 3, 131, <https://doi.org/10.12688/gatesopenres.12897.1> PMID: 31172051
38. Canier L.; Khim N.; Kim S.; Sluydts V.; Heng S.; Dourng D.; et al. An Innovative Tool for Moving Malaria PCR Detection of Parasite Reservoir into the Field. *Malaria Journal* 2013, 12, 405, <https://doi.org/10.1186/1475-2875-12-405> PMID: 24206649

Article: Comparing malaria risk exposure in rural Cambodia population: GPS tracking versus questionnaires

Anaïs Pepey, Marc Souris, Saorin Kim, Thomas Obadia, Sophy Chy, Malen Ea, Sivkeng Ouk, Frank Remoue, Siv Sovannaroth, Ivo Mueller, Benoit Witkowski, Amélie Vantaux

Manuscript under preparation.

24 **ABSTRACT**

25 **Background:** Countries of the Great Mekong Subregion have attained a major decline in their malaria
26 cases and fatalities over the last years, but some pockets of transmission remain, supposedly
27 maintained by the forest workers and migrant populations. This study aims to: *i)* understand the fine-
28 scale mobility of forest goers to understand how and when their daily movement patterns may affect
29 malaria transmission by using parasites detection via RTPCR and anti-*Anopheles* saliva antibodies
30 quantification via ELISA; *ii)* compare the respective efficacy of questionnaires and GPS data loggers for
31 measuring mobility.

32 **Methods:** Two 28-day follow-ups during dry and rainy seasons, including a GPS tracking,
33 questionnaires and health examinations, were performed on male forest goers to match the main risk
34 population. We analysed their time spent in different land use categories and demographic data to
35 understand the risk factors driving malaria in the study area.

36 **Results:** Participants risk exposure varied according to their village forest coverage, all within a few
37 kilometres of each other: participants from outside the forest were significantly more exposed than
38 participants from the forest fringes. The GPS data did not highlight risk factors linked to the time spent
39 in a specific environment, particularly the time spent in forest was not associated with a higher
40 probability to detect malaria. The levels of antibody response among participants were significantly
41 higher in the rainy season and for participants using mosquito protection in the deep forest. We
42 conclude that the exposure to *Anopheles* infectious bites is similar across study participants, as they
43 all belong to the risk population sharing the same mobility habits and socio-economic characteristics.
44 *Plasmodium vivax* relapses and participants' immunity may interfere with the observed malaria
45 prevalence, explaining the lack of evidence of risk heterogeneity. The proportion of participants
46 visiting each environment was different according to questionnaire and GPS data estimates, with a
47 concordance of only 61.9% between the two datasets.

48 **Conclusions:** The comparison of questionnaire and GPS datasets underline the limitations of both
49 methods and the need to reconsider the systematic use of questionnaires to quantify mobility in
50 health studies.

51

52 **KEYWORDS**

53 Cambodia; GPS data loggers; mobility; anti-*Anopheles* saliva antibodies; land use; malaria;
54 transmission.

55 **BACKGROUND**

56 Despite an international effort towards elimination, malaria persists with an estimated 241 million
57 cases in 2020 [1]. In 2014, the Greater Mekong Subregion (GMS) aimed to end malaria transmission
58 by 2030 and, since then, has witnessed considerable progress [2]. However, if *Plasmodium falciparum*
59 incidence has drastically diminished, *Plasmodium vivax* infections remain important and challenge
60 elimination [2], due to the existence of its dormant stage causing potential relapses [3] as well as its
61 ability to circulate unnoticed and untreated, with a substantial amount of asymptomatic cases
62 participating to transmission [4].

63 In 2019, 85% of Cambodian malaria cases were represented by *P. vivax* [2]. Mondulkiri province held
64 the highest recorded malaria incidence in the GMS, with more than 50 cases per 1000 inhabitants
65 from January to July 2019 [2]. Forest-related activities have been repeatedly shown to be the main
66 malaria risk factor as the main malaria vectors (*Anopheles* spp.) are forest-associated. Vector control
67 tools to accelerate malaria elimination such as insecticide impregnated clothing or spatial repellents
68 have still not shown effectiveness in the subregion [5]. Larviciding efficacy which requires landscape
69 analyses to determine mosquito risk and its association to water bodies' presence is limited by the
70 large diversity of larval habitats type and size [6, 7].

71 Therefore and beside passive case detection (PCD), tools to accelerate malaria elimination are limited
72 to active case detection (ACD), such as Mass Screening And Treatment (MSAT) with the whole
73 population targeted or Focal Screening And Treatment (FSAT) restricted to high risk groups, usually
74 forest goers [8]. Reactive case detection has also been tested in different provinces but the very low
75 detection rates and diagnostic sensitivity for asymptomatic participants did not demonstrate sufficient
76 efficacy to be continued [9, 10].

77 Both ACD and PCD strategies in Cambodia rely on immunochromatography-based Rapid Diagnostic
78 Tests (RDT). However, their sensitivity threshold is about 100 parasites/ μL [11] meaning they are unfit
79 to detect most of asymptomatic infections [5]. This limitation contributes to an important share of
80 false negatives and restrains the coverage of ACD and PCD interventions [5, 12].

81 Simulations suggest that mass drug administration to entire communities or sub-populations at the
82 highest risk would be effective, but could induce the emergence of multi-drug resistant *P. falciparum*
83 parasites [8, 13]. This elimination strategy could also face insufficient coverage as high risk individuals
84 live and work in remote areas, like deep forest, and can be difficult to reach [14, 15].

85 In Cambodia, primaquine is used as both a single dose treatment for *P. falciparum* since 2018 and a
86 14 day radical cure for *P. vivax* and *Plasmodium ovale* hypnozoites under certain conditions since 2020
87 [16]. This treatment implies rigorous adherence to be efficient and includes an important risk as it can

88 trigger dose-dependent haemolysis in glucose-6-phosphate dehydrogenase deficient patients,
89 accounting for 2 to 16% of the GMS residents [17]. Deficiency testing prior to primaquine use is
90 therefore primordial for a safe prescription and administration but is difficult to implement [16].

91 The risk of false negatives, resistance emergence, insufficient coverage or haemolysis could be
92 reduced by prescribing malarial drugs only after a precise targeting aiming at identifying and
93 diagnosing efficiently whom to treat. To target the adequate risk population, a population-oriented
94 study could determine individual exposure and characterise the vector-host contact in transmission
95 areas.

96 Although epidemiological questionnaires help characterise risk factors, other tools such as serology or
97 GPS tracking are being used in health studies to circumvent some of their limitations. Notably, because
98 *P. vivax* induces relapses, an individual can suffer a malaria episode arising from an infective bite that
99 occurred weeks or months prior [3]. For such cases, immunologic markers detection can provide
100 insights about recent *Anopheles* exposure. The immune system produces Immunoglobulins G (IgG)
101 antibodies after *Anopheles* saliva enters the bloodstream during the mosquito blood meal, which can
102 be detected from blood samples from exposed populations [18]. The intensity of the immune
103 response and the concentration of anti-*Anopheles* saliva antibodies is proportional to the amount of
104 bites received by the exposed individuals [19], hence the gSG6 peptide and the immune response it
105 triggers has been used to measure individual exposure [18, 20].

106 Malaria transmission depends on fine spatial scales [21, 22], and daily mobility patterns around areas
107 with different malaria epidemiology settings can lead to imported cases and an increase of
108 heterogeneity in individual disease risk [23, 24]. Tracking the daily movements of the risk populations
109 and understanding the zones responsible for malaria infections allows determining tailor-made
110 interventions and thus achieving more effective elimination [25]. The standard methodology for
111 measuring risk populations mobility in Cambodia has been through questionnaires [15, 26–28], which
112 are likely to generate bias: a study from 2017 showed that participants were reluctant to admit some
113 of their travels, for example to illegal wood logging locations [29]. The implementation of a GPS follow-
114 up to a population at risk of malaria would measure individual fine-scale mobility and identify the
115 differences between GPS tracking and questionnaire to help define which one to prefer in this context.
116 Indeed, limitations arising from behavioural, technical and analytical aspects can lead to errors for
117 both methods and need to be quantified [30].

118 The current epidemiological situation of malaria in Cambodia points out the necessity to focus efforts
119 on population involved in residual transmission. Therefore, we investigated whether complementary
120 characterisation of a high risk population based on GPS tracking, questionnaires and vector exposure

121 serological marker quantification could enable to focus elimination strategies on the last pockets of
122 transmission.

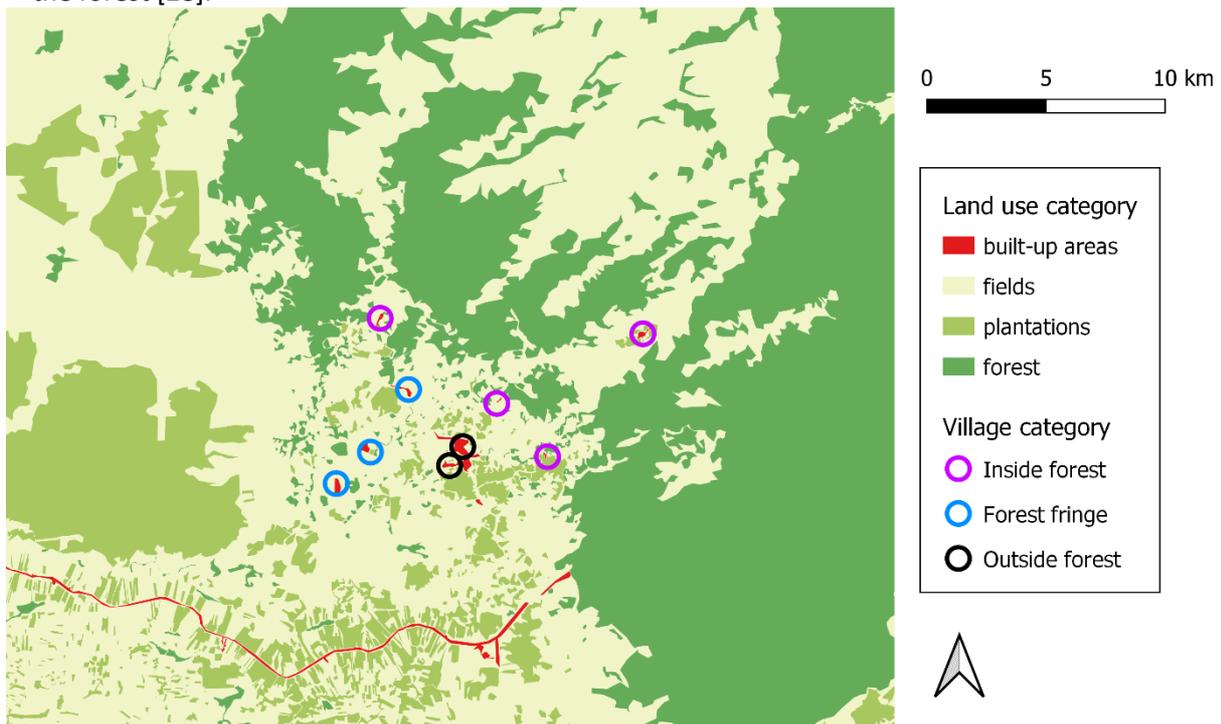
123

124 MATERIAL AND METHODS

125 1) Study site and population sample

126 The study site is located in Kaev Seima district, Mondulkiri province, North-eastern Cambodia. Year is
127 divided into rainy (May-October) and dry (November-April) seasons. Kaev Seima district is a rural, low-
128 income environment where populations mainly live from agriculture and forest products exploitation.

129 The land use of the study site was determined previously from high resolution satellite imagery (SPOT
130 6/7) captured in March 2018 (Figure 1) [31]. The classification categorised the land into built-up areas
131 (villages and roads), fields (rice paddies, cassava culture, etc.), plantations (banana, cashew nut,
132 rubber trees, etc.) and forest. The nine villages were attributed to three categories according to their
133 forest coverage around the households, following a previously defined gradient from inside to outside
134 the forest [28].



135 Figure 1: Land use and villages of the study area.

136 The study was conducted on the most at risk of malaria population group *i.e.* men between 13 and 60
137 years old [12, 26, 28]. The enrolment included two rounds to characterise both seasons, with rainy
138 season's round lasting from April to September 2018 and dry season from February to April 2019.
139 Participants were enrolled according to their status during the cross-sectional study in the same
140 district in 2017 [28], if they were available and willing to participate in the study, with half of them

141 from malaria-negative and the other half from malaria-positive participants at the time of the cross-
142 sectional study. A total of 160 participants for rainy season and 200 participants for dry season
143 volunteered.

144 **2) Study design**

145 Household visits were scheduled on days 0, 7, 14 and 28 and included a short health examination, and
146 questionnaires at day 0 (demographic data) and day 14 (mobility during the last 2 weeks). A GPS data
147 logger was provided from day 0 to day 14. At day 7, the device was exchanged for a fully charged
148 logger.

149 The questionnaire, designed in English and translated by a native Khmer speaker, gathered
150 demographic data and information about recently visited environments. The comments were
151 translated by a bilingual native Khmer speaker and experienced field coordinator. First round of data
152 was collected on paper questionnaires and second round of data on the REDCap mobile app, an
153 electronic data capture secure, web-based software platform hosted at Pasteur Institute Paris [32].

154 Health examination consisted of axillary temperature measure and a capillary blood collection. In case
155 of fever (temperature above 37.5°C), a RDT was performed – if positive, the participant was referred
156 to a health centre to receive an antimalarial treatment.

157 The Igot-U GT600 (Mobile Action Technology Inc., Taipei, Taiwan) was selected to record participants'
158 mobility [33], as its battery life, data storage, price and unobtrusive design corresponded best to our
159 study design and has proven a certain efficacy in other studies with comparable study designs [34, 35].

160 **3) Laboratory procedures**

161 DNA was extracted from filter paper blood spots with Instagene® Matrix (Bio-Rad, Courtaboeuf,
162 France) according to the manufacturer's instructions. Real-Time Polymerase Chain Reactions (RTPCR)
163 were performed to detect malaria parasites and to carry out parasites speciation, as previously
164 described [36]. Briefly, a first RTPCR targeted the *Plasmodium* cytochrome b gene and determined the
165 positive samples. Then, five nested RTPCR assays targeted the same gene specific to each species: *P.*
166 *falciparum*, *P. knowlesi*, *P. malariae*, *P. ovale* and *P. vivax* (assay parameters and primers sequences
167 are detailed in Table S1).

168 Quantification of anti-*Anopheles* saliva antibodies in participants' serum was done by enzyme-linked
169 immunosorbent assay (ELISA) on blood samples from the last day of follow-up (week 4) as described
170 in [19, 37] with some modifications (see supplementary material [Protocol S1]). The intensity of the
171 IgG response was measured at the individual level and was expressed as the Δ OD, calculated as the
172 difference between the mean of the individual optical densities (OD) in 2 antigen wells and the OD in
173 1 blank well containing no gSG6-P1 antigen. As a negative control, the specific anti-gSG6-P1 IgG

174 response was also assayed in non-*Anopheles* exposed individuals volunteer residing in an urban area
175 with limited *Anopheles* mosquitoes populations (Phnom Penh city) for more than three months: to
176 quantify the nonspecific background antibody level and to calculate the cut-off value (calculated as
177 the mean $\Delta OD_{neg} + 3$ SDs) [19, 38, 39]. Based on our findings, a participant was classified as an immune
178 responder if their ΔOD was > 0.539 .

179 **4) Data processing and analysis**

180 The GPS tracking data was curated and an optimal GPS dataset was produced, including only
181 participants willing to take the device on their daily mobility and for GPS trackers that logged enough
182 time to be representative of their mobility [33]. The time spent in each land use category was
183 extracted from participants GPS data along with the locations metadata (day or night time and speed
184 for each location) [33].

185 A binomial variable representing infection status was attributed to each participant: 1 represented a
186 participant with at least one RTPCR positive for any species over the 4-week follow-up, and 0 never
187 positive. Sociodemographic variables (at baseline) and declared mosquito protection use for the two
188 previous weeks in every environment (at day 14) were extracted from the questionnaire and coded as
189 factor variables. The time spent by a participant in an environment, for the 2-week GPS follow-up, was
190 both computed as the “total hours” (total time logged by the device) and the “standardised time”
191 (proportion of time logged extrapolated to a 2-week logging). Analyses were performed under two
192 conditions: night data, when *Anopheles* vectors are most active and slow data (<5 km/h) when
193 individuals are not driving a vehicle and are more exposed to mosquito bites, in order to determine
194 which conditions led to increased malaria exposure for a participant. We tested the effect of the time
195 spend in the different environments, sociodemographic factors (age, village and main income) and
196 declared mosquito protection use using univariate Generalized Linear Mixed-effects Models (GLMMs)
197 with participant ID coded as a random factor to account for repeated measurements on the same
198 individual.

199 A binomial variable representing anti-*Anopheles* saliva seropositivity was attributed to each
200 participant: 1 represented a seropositive participant anti-*Anopheles* saliva antibody OD $> \Delta OD_{neg} + 3$
201 SDs, and 0 a seronegative participant. The effects of season, age, village, declared mosquito protection
202 use and time spent in different environments (extracted from the optimal GPS data) were tested on
203 participants’ seropositivity (qualitative binomial model) and on their ΔOD value (quantitative Gaussian
204 model, with log transformation of the variable to satisfy normality assumptions) using univariate
205 Generalized Linear Mixed-effects Models (GLMMs) with participant ID coded as a random factor. All
206 factors with a P value <0.2 were included in a multivariate GLMM including all biologically relevant

207 interactions (between *i*) season and village category, ST in forest, ST in fields, ST in village, malaria
208 status and *ii*) village category and ST in forest, ST in fields, ST in village and malaria status).

209 The questionnaire at day 14 enquired participants about any visit in the following environments during
210 the two previous weeks: nearby forest, deep forest, cashew nut plantations, rubber plantations, rice
211 fields and cassava fields. We compared the percentage of participants that had a visit logged in their
212 GPS data (corresponding to at least 30 minutes logged at slow speed in the corresponding land use
213 category) and participants that declared a visit during the questionnaire at day 14, for each type of
214 environment. By comparing these two datasets, we assessed the total discordance between them by
215 visited environment and attributed a binomial variable to each participant depending on their data
216 discordance (1: discordance, 0: no discordance). We also used GLMMs to assess the potential
217 association between dataset discordance and participants' malaria status using, with participant ID
218 coded as a random factor.

219 We compared the groups of participants with fever, with a positive RTPCR result and with a
220 symptomatic malaria infection from each season with Pearson's Chi-squared tests.

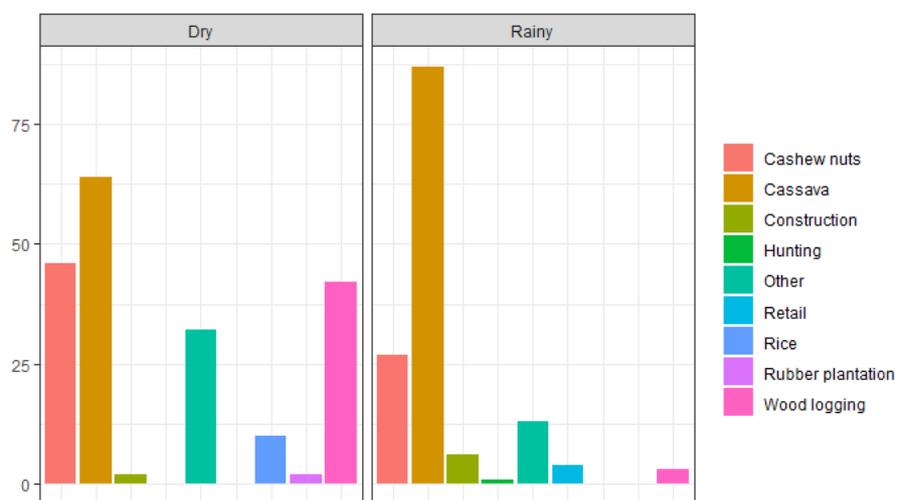
221 For model selection, we used the stepwise removal of terms, followed by likelihood ratio tests. Term
222 removals that significantly reduced explanatory power ($P < 0.05$) were retained in the minimal
223 adequate model [40]. All statistical analyses were conducted in R version 4.0 [41].

224

225 RESULTS

226 1) Study population

227 Based on the 339 participants that answered sociodemographic questions, participants' median age
228 was 29 years old (SD = 13.4, Figure S1), and the most common main income was related to agriculture
229 ($69.6 \pm 4.9\%$ of participants, Figure 2).



230 Figure 2: Main income declared by participants for each season during baseline questionnaire.

231 Based on the 184 participants informing of their visits of the last two weeks during the questionnaire
 232 at day 14, the most visited environment was nearby forest, during short trips (only one stay reported
 233 to last more than one day, Table 1). Deep forest visits corresponded to stays from less than a day to
 234 more than week. The preferred way of transportation was by motorbike, except for accessing the
 235 nearby forest and rice fields, for which walking most often was declared by the participants (Table S2).
 236 Although participants mainly declared to go to the deep forest with men from their family ($37.8 \pm$
 237 11.1%) or co-workers ($50 \pm 11.4\%$), they were also sometimes accompanied by women ($8.1 \pm 6.2\%$)
 238 and children ($4.1 \pm 4.5\%$, Table S2). Women and children were often joining participants in cashew
 239 plantations ($44.4 \pm 9.4\%$ and $21.3 \pm 7.7\%$) and cassava fields ($59.2 \pm 13.8\%$ and $18.4 \pm 10.8\%$, Table
 240 S2).

241 |Table 1: Visits in the different environments during the past two weeks declared by participants during
 242 the questionnaire at day 14, for all participants with a complete questionnaire at day 14 (N = 184).

Visits in the past 2 weeks	N	%	95% CI
Nearby forest	141	76.6	6.1
Deep forest	74	40.2	7.1
Rubber plantation	3	1.6	1.8
Cashew plantation	108	58.7	7.1
Cassava field	49	26.6	6.4
Rice field	15	8.2	4.0

243 2) Health examination

244 Based on data from all participants (N = 360), malaria prevalence at baseline (rainy season: $31.9 \pm$
 245 7.2% , dry season: $28.5 \pm 6.3\%$) and over follow-up (rainy: $50.6 \pm 7.7\%$, dry: $43.5 \pm 6.9\%$) were not
 246 significantly different among seasons ($\chi^2_1 = 0.33$, $P = 0.56$ and $\chi^2_1 = 1.54$, $P = 0.21$, respectively; Table
 247 2). The proportion of symptomatic cases over follow-up (rainy: $18.8 \pm 9.6\%$, dry: $10.3 \pm 6.4\%$) did not
 248 significantly differ by season either ($\chi^2_1 = 1.53$, $P = 0.22$), but more participants had fever (related or
 249 unrelated to malaria) in the rainy season compared to the dry season ($22.7 \pm 7.3\%$ vs. $12.5 \pm 4.6\%$; χ^2_1
 250 $= 5.14$, $P = 0.02$).

251 Table 2: Malaria prevalence in rainy and dry seasons. "Over follow-up" corresponds to individuals that
 252 have been positive at least once over the follow-up.

Season Week Value	Rainy season						Dry season					
	Baseline			Over follow-up			Baseline			Over follow-up		
	N	%	95% CI	N	%	95% CI	N	%	95% CI	N	%	95% CI
<i>Plasmodium</i> spp.	51	31.9	7.2	81	50.6	7.7	57	28.5	6.3	87	43.5	6.9
<i>Plasmodium vivax</i>	44	27.5	6.9	64	40	7.6	47	23.5	5.9	78	39	6.8
<i>Plasmodium falciparum</i>	4	2.5	2.4	4	2.5	2.4	2	1	1.4	2	1	1.4
<i>P. vivax/P. falciparum</i>	2	1.3	1.7	4	2.5	2.4	4	2	1.9	5	2.5	2.2
Undetermined	1	0.6	1.2	9	5.6	3.6	4	2	1.9	2	1	1.4

253 Over follow-up, *Plasmodium vivax* was the most common parasite species for both rainy and dry
254 seasons representing respectively $79 \pm 8.9\%$ and $89.7 \pm 6.7\%$ of the infections. *Plasmodium falciparum*
255 infections respectively added up to $4.9 \pm 4.7\%$ and $2.3 \pm 1.4\%$ of total infections, and mixed infections
256 to $4.9 \pm 4.7\%$ and $5.7 \pm 4.9\%$ of infections. Undetermined infections represented $11.1 \pm 6.8\%$ and 2.3
257 $\pm 1.4\%$ of infections. No carriage of *P. ovale*, *P. malariae* or *P. knowlesi* was detected.

258 **3) GPS data**

259 From the collected tracks (N = 609), the GPS devices logged on average 159.9 km by participant over
260 the complete follow-up. The mobility as depicted by distance/day, varied greatly among participants
261 but was consistent among seasons and averaged 22.2 km per day per participant (median: 20.8 km,
262 minimum: 0 km, maximum: 78.3 km per day per participant). Battery life averaged 3 days and 7 hours
263 per device in the rainy season, and 4 days and 1 hour in the dry season, with a great variability ranging
264 from a few minutes to 16 days and 18 hours.

265 **4) Anti-Anopheles saliva antibodies quantification**

266 Analyses relative to ELISA antibodies were performed on a subset of participants that has complete
267 malaria status, serology and GPS datasets (N = 270). In both qualitative and quantitative univariate
268 models, seroprevalence and average ΔOD were significantly higher during rainy season compared to
269 dry season ($\chi^2_1 = 1.2$, $P = 0.27$ and $\chi^2_1 = 1.2$, $P = 0.27$ respectively; Table S3). The villages on the forest
270 fringe had the lowest average ΔOD , followed by outside the forest and finally inside the forest but
271 were not significantly different (Table S3). All other factors such as malaria status, age and time spent
272 in different environments had no significant effects (Table S3). When all the factors showing a
273 potentially positive or negative association in the univariate models were tested in two multivariable
274 models, only season was significant with a higher seroprevalence (dry season: $30.5 \pm 0.6\%$, rainy
275 season: 66.4 ± 0.8 ; $P < 0.001$) and a higher average ΔOD (dry season: 0.48 ± 0.2 , rainy season: $0.98 \pm$
276 0.6 ; $P < 0.001$) during the rainy season compared to the dry season (Table S4).

277 **5) Risk factors**

278 Risk factors analysis was performed on a subset of participants having complete socioeconomic,
279 malaria prevalence and optimal GPS data (N = 258). Season was not associated to malaria prevalence
280 variation in the sample over follow-up ($\chi^2_1 = 1.2$, $P = 0.27$) nor was the main income declared by the
281 participant during baseline questionnaire ($\chi^2_8 = 9.5$, $P = 0.3$). Adults between 21 and 39 years old had
282 higher malaria prevalence ($57.4 \pm 9.3\%$) than younger ($42.5 \pm 10.8\%$) and older men ($44.3 \pm 11.6\%$)
283 over follow-up, but the difference was not significant ($\chi^2_2 = 5.3$, $P = 0.07$). Participants visited different
284 parts of the study area depending on the village of origin (Figure S2) and their malaria prevalence was
285 associated to the forest cover category their village belonged to ($\chi^2_2 = 7.4$, $P = 0.02$). However, post-

286 hoc comparisons showed only a significantly lower malaria prevalence in the participants from villages
287 from the forest fringe ($36.4 \pm 10.7\%$) compared to participants from villages outside the forest ($55.1 \pm$
288 8.4% ; Table S5). Malaria prevalence in participants belonging to villages from inside the forest was
289 $53.3 \pm 14.6\%$.

290 The malaria positive participants travelled greater distances at night time on a vehicle (speed > 5 km/h)
291 with an average of 37.1 ± 46.6 km (adjusted distance per follow-up) than the negative participants
292 (averaging 27.6 ± 36.7 km) but the difference was not significant ($\chi^2_1 = 3.43$, $P = 0.06$).

293 When considering either the total hours or the standardised time, the time spent in a specific land use
294 category by an individual was not associated to an increased malaria risk, both for data recorded by
295 night and at slow speed (Table S6).

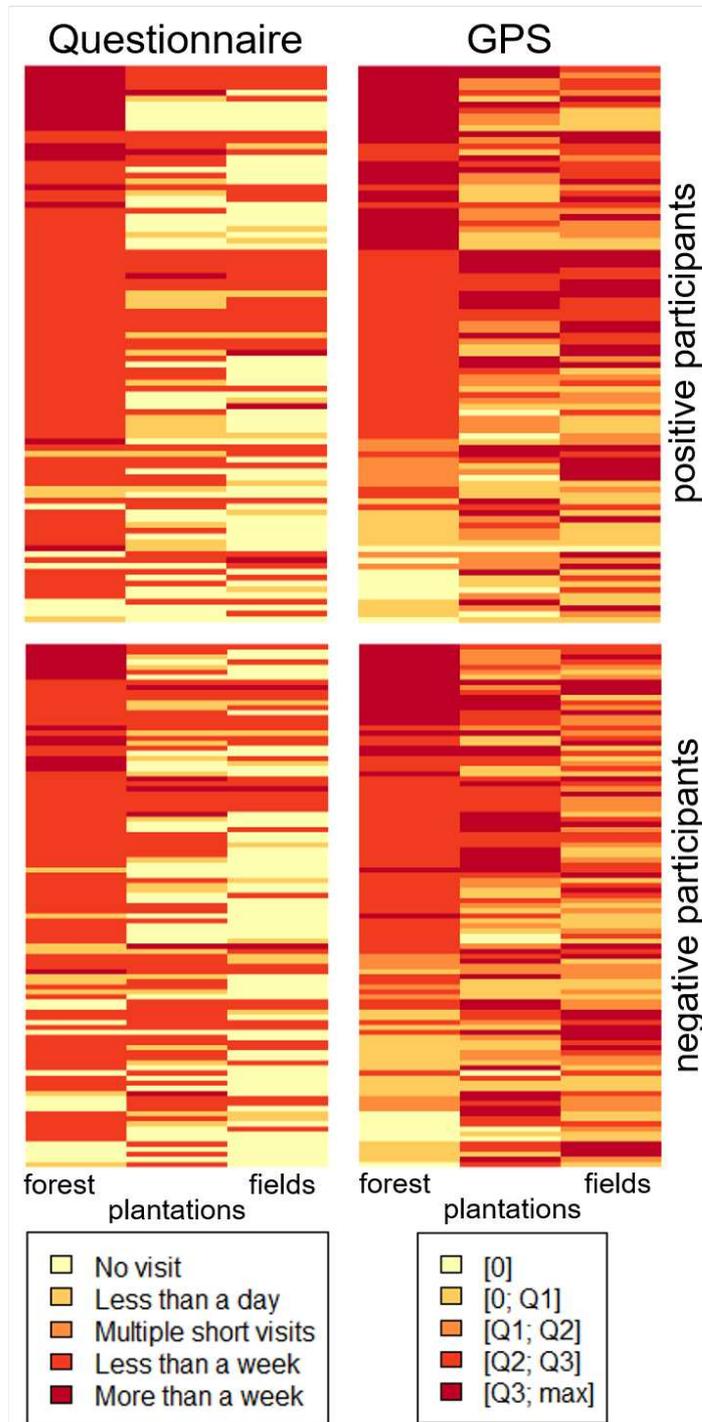
296 Based on the 184 participants informing of their visits of the last two weeks during the questionnaire
297 at day 14, participants that visited the deep forest between day 0 and day 14 of the follow-up had a
298 higher malaria prevalence ($51.4 \pm 11.4\%$) than the participants that did not ($39.1 \pm 9.1\%$) but the
299 correlation was not significant ($\chi^2_1 = 2.7$, $P = 0.1$).

300 **6) Mosquito protection use**

301 Most participants would not describe their use of mosquito protection for the two first weeks of
302 follow-up during the questionnaire at day 14: depending on the environment, only $56.5 \pm 5.4\%$ (rubber
303 plantations) to $61.7 \pm 5.3\%$ (deep forest) of interrogated participants would answer (Table S7). From
304 these answers, mosquito protection use was minimal in all environments (from $0.5 \pm 1.1\%$ in both
305 nearby forest and rubber plantations to $4.3 \pm 2.9\%$ in cassava fields) but mosquito protection use
306 reached $24.5 \pm 6.0\%$ in the deep forest (Table S7). From these participants answering about their
307 mosquito use in deep forest, 197 had complete malaria and serology results. Mosquito protection use
308 in deep forest was not associated to malaria prevalence ($\chi^2_1 = 0.9$, $P = 0.34$). Average ΔOD was
309 significantly higher among participants that declared using mosquito protection in the deep forest
310 than the ones that did not ($\Delta OD_{\text{protection}} = 0.68 \pm 0.49$ versus $\Delta OD_{\text{no protection}} = 0.47 \pm 0.21$, $\chi^2_1 = 9.9$, $P =$
311 0.002 , Figure S3) and their seroprevalence was higher ($42.9 \pm 13.9\%$ versus $38.3 \pm 9.2\%$), but the
312 difference was not significant ($\chi^2_1 = 3.8$, $P = 0.052$). The limited sample size in the other environments
313 precluded more in-depth analyses.

314 **7) Comparison between questionnaire and GPS data**

315 Overall, 197 participants had both a complete optimal GPS dataset and gave an answer for every type
316 of visit during the questionnaire at day 14 (Figure 3).



317 Figure 3: Cumulative time spent in the different environments declared by participants at day 14 during
 318 the two previous weeks and logged by the GPS devices, categorised by quartiles of standardised time.
 319 Only participants with complete GPS and questionnaire datasets were included (N = 197).

320 Compared to GPS, questionnaires underestimated the time spent in fields (48.7% declared a visit
 321 versus 95.9% observed in GPS data) and overestimated the time spent in forest (91.4% versus 70.6%)
 322 and plantations (71.1% versus 61.4%). We determined for each participant if their data was
 323 concordant (both GPS and questionnaire data demonstrated at least a visit or no visit at all) or
 324 discordant (only one dataset had evidence of a visit in the considered environment, Table S8). The

325 total concordance between the two datasets was 61.9%, with the highest concordance between
326 datasets for visits in the forest (73.1%), followed by plantations (60.9%) and fields (51.8%).

327 For participants that had both a complete questionnaire dataset and optimal GPS (N = 197),
328 discordance between the questionnaire and GPS datasets was not associated to malaria prevalence
329 variation over follow-up in the sample, being any discordance ($\chi^2_1 = 1.03$, P = 0.31), or between fields
330 ($\chi^2_1 = 2.27$, P = 0.13), plantations ($\chi^2_1 = 0.08$, P = 0.78) or forest datasets ($\chi^2_1 = 0.57$, P = 0.49).

331

332 **DISCUSSION**

333 The main objective of this study was to identify the factors associated with a gradation in infections
334 at a fine scale to ultimately precise the transmission hotspots in Cambodia. As such, our study design
335 aimed to determine whether the time spent in a certain land use category would correlate with
336 malaria positivity, to ultimately enable identifying more accurately transmission areas. Notably, we
337 expected to observe that the participants spending more time in the forest, at peak biting hours and
338 low speed, would be exposed to more infectious mosquito bites, resulting to an increased positivity
339 rate. However, data from both GPS and questionnaire did not demonstrate any correlation. The main
340 reason to explain this outcome would be contextual. The continuous and swift decrease in *P.*
341 *falciparum* prevalence, associated with *P. vivax* becoming responsible for the major share of malaria
342 infections at the time of the study, is likely to make this metric poorly relevant. Indeed, the aim was
343 to associate malaria infection to the recent individual mobility data, which is not possible with *P. vivax*
344 as the majority of infections are due to relapses that occur at a distant time from the initial infection
345 [42].

346 The questionnaire and GPS data were concordant for only 61.9% of the visits they included. Notably,
347 the fields were overrepresented in the GPS data. That could be a consequence of the satellite imagery
348 processing into a land use classification, which might interpret fallow lands as fields. Such difference
349 between the two datasets suggest that a complementary approach could be necessary to capture all
350 aspects of malaria risk factors in Cambodia.

351 The quantification of anti-*Anopheles* antibodies assessed that the exposure to vectors was more
352 important during the rainy season. Although not significantly different, the levels of anti-*Anopheles*
353 antibodies intensity response followed the trend of the malaria prevalence. Interestingly, the
354 classification of the villages, depending on the percentage of forest surrounding the households,
355 highlighted that participants from villages outside the forest had a greater associated malaria risk than
356 from villages at the forest fringes. Although, the sample size of the 3 categories were different (185
357 participants from outside the forest, 116 from forest fringes and 59 from inside the forest), these

358 results suggest that individual mobility, rather than immediate household surroundings, remains
359 determinant to predict *Anopheles* exposure.

360 Residual malaria transmission corresponds to transmission still occurring when full coverage of
361 classical malaria control tools (bed nets, IRS) have been attained. Cambodia scores high in terms of
362 bed net use, with provinces declaring more than one bed net per household on average [43]. However,
363 the questionnaires from this follow-up highlights that mosquito protection use, including bed nets, is
364 not common outside the household. Mosquito protection was almost not declared in most nearby
365 forest, fields and plantations, while only about a quarter of participants declared using it in the deep
366 forest. However, the limited sample size prevents to draw any further conclusions.

367 Despite the initial sample size, results were often limited by the amount of data available post-
368 collection and post-curation. Limited sample sizes can produce misleading, non-representative results
369 which could explain some of the counterintuitive results we obtained during our analysis such as
370 higher levels of anti-*Anopheles* antibodies in participants declaring using mosquito protection in the
371 deep forest. Future studies should account for data curation removing substantial volumes of
372 suboptimal data, material defect or participants quitting the study, and target large sample sizes.

373 The three methodologies that we implemented during this study – questionnaires, GPS loggers and
374 vector exposure serological marker – could not identify the mobility patterns and environments
375 responsible for malaria cases in the study area. However, we can assume that rather than the methods
376 themselves, the context is not suitable to their implementation. The ΔOD values followed the
377 expected seasonal abundance of *Anopheles* and most of the GPS data was optimal. Both
378 methodologies were successfully implemented elsewhere to characterise vector exposure [18, 20]
379 and mobility associated to malaria transmission [34, 44, 45]. However, malaria prevalence is sharply
380 decreasing in Cambodia. *Plasmodium falciparum* cases represent only a fraction of infections,
381 meaning that GPS trackers are not suitable to characterise the origin of most malaria infections.
382 Infectious bites constitute only a very small percentage of mosquito bites, making vector exposure
383 quantification less relevant. In addition, mosquito protection use is limited outside the household and
384 could be responsible for some variation in vector exposure but not often or long enough to
385 significantly reduce malaria risk in this context of very low transmission.

386 As such, both GPS tracking and vector exposure serological marker quantification do not appear to
387 hold the resolution required to determine the risk factors associated to malaria cases, mostly relapsing
388 *P. vivax* infections, in the current Cambodian context. An FSAT approach targeting people with fever
389 would have limited efficacy as only 12.5% (21 symptomatic cases for 168 positive RTPCRs) participants
390 with an ongoing malaria infection had fever. Since many subclinical *P. vivax* cases stay undetected,

391 testing for recent *P. vivax* infections via the use of serological exposure markers could be a more
392 adapted elimination strategy [46]. The modelling of *P. vivax* serological testing and treatment
393 (PvSeroTAT) demonstrated that it could significantly reduce *P. vivax* transmission while avoiding
394 overtreatment, thus preventing the risk of haemolysis in G6PD deficient patients and the spread of
395 resistant parasites [47]. The PvSeroTAT strategy, targeting both blood and liver stages parasites,
396 appears to be a safer and efficient alternative to MDA, while also more efficient than MSAT [47].

397

398 **CONCLUSION**

399 In a context of residual malaria transmission which is mainly depicted by *P. vivax* asymptomatic
400 infections, the implementation of questionnaires, GPS data-loggers and ELISA quantification of anti-
401 *Anopheles* antibodies detection on the high-risk group were not powerful enough to detect malaria
402 risk factors associated with different mobility behaviours or time spent in various environments. The
403 conjoint implementation of GPS trackers and questionnaires allowed to highlight the limitations of
404 both methodologies and the benefits of using them together. The collected data characterised forest
405 goers mobility, their mosquito protection use, the social context of their visits in different
406 environments, in addition to quantitative data about the time and distance spent in these
407 environments. New detection and follow-up strategies are still called for.

408

409 **LIST OF ABBREVIATIONS**

410 ACD: Active Case Detection
411 CI: Confidence Interval
412 DBS: Dried Blood Spot
413 df: degrees of freedom
414 ELISA: Enzyme-Linked Immunosorbent Assay
415 FSAT: Focal Screening and Treatment
416 IgG: Immunoglobulin G
417 IRS: Indoor Residual Spraying
418 GLMM: Generalized Linear Mixed Models
419 GMS: Great Mekong Subregion
420 GPS: Global Positioning System
421 MSAT: Mass Screening and Treatment
422 OD: Optical Density
423 OR: Odd Ratio
424 PCD: Passive Case Detection

425 PvSeroTAT: *Plasmodium vivax* serological testing and treatment
426 RDT: Rapid Diagnostic Test
427 REDCap: Research Electronic Data Capture
428 RTPCR: Real Time Polymerase Chain Reaction
429 ST: Standardised time
430 WHO: World Health Organisation

431

432 **DECLARATIONS**

433 **Ethics approval and consent to participate**

434 Ethical approvals were obtained from the Cambodian National Ethics Committee for Health Research
435 (045NECHR & 312NHECR) and from the National Institutes of Health (DMID Protocol Number: 17-
436 0036). The protocols conform to the Helsinki Declaration on ethical principles for medical research
437 involving human subjects (version 2002) and informed written consent was obtained for all
438 volunteers. Parental assent was obtained for every participant between 13 and 18 years old.

439 **Consent for publication**

440 Not applicable.

441 **Availability of data and materials**

442 The de-identified datasets generated and/or analysed during the current study is being made publicly
443 available in a Figshare repository. In the meantime, it is available from the corresponding author on
444 reasonable request.

445 **Competing interests**

446 The authors declare that they have no competing interests.

447 **Funding**

448 This study was funded by the National Institutes of Health program International Centres of Excellence
449 for Malaria Research, grant 1U19AI129392-01 "Understanding, tracking and eliminating malaria
450 transmission in the Asia-Pacific Region". A.P. was funded by the Pasteur Institute International
451 Network Calmette and Yersin Ph.D. scholarship, grant MHL/MJ/N°212/18. The funding bodies had no
452 role in the design of the study, the collection, analysis, and interpretation of data, and in writing the
453 manuscript.

454 **Authors' contribution**

455 IM, BW and AV, conceived and designed the study and were overall responsible for the study. AP
456 contributed to data curation, statistical analysis and wrote the first draft, supported by TO and AV.

- 492 11. Das S, Peck RB, Barney R, Jang IK, Kahn M, Zhu M, et al. Performance of an ultra-sensitive
493 Plasmodium falciparum HRP2-based rapid diagnostic test with recombinant HRP2, culture parasites,
494 and archived whole blood samples. *Malaria Journal*. 2018;17:118.
- 495 12. Tripura R, Peto TJ, Veugen CC, Nguon C, Davoeung C, James N, et al. Submicroscopic Plasmodium
496 prevalence in relation to malaria incidence in 20 villages in western Cambodia. *Malaria Journal*.
497 2017;16:56.
- 498 13. Maude RJ, Nguon C, Dondorp AM, White LJ, White NJ. The diminishing returns of atovaquone-
499 proguanil for elimination of Plasmodium falciparum malaria: modelling mass drug administration
500 and treatment. *Malaria Journal*. 2014;13:380.
- 501 14. Dysoley L, Kaneko A, Eto H, Mita T, Socheat D, B rkman A, et al. Changing patterns of forest
502 malaria among the mobile adult male population in Chumkiri District, Cambodia. *Acta Trop*.
503 2008;106:207–12.
- 504 15. Sluydts V, Heng S, Coosemans M, Van Roey K, Gryseels C, Canier L, et al. Spatial clustering and
505 risk factors of malaria infections in Ratanakiri Province, Cambodia. *Malar J*. 2014;13:387.
- 506 16. WHO’s Mekong Malaria Elimination Programme. Countries of the Greater Mekong ready for the
507 “last mile” of malaria elimination. 2020.
- 508 17. Bancone G, Menard D, Khim N, Kim S, Canier L, Nguong C, et al. Molecular characterization and
509 mapping of glucose-6-phosphate dehydrogenase (G6PD) mutations in the Greater Mekong
510 Subregion. *Malaria Journal*. 2019;18:20.
- 511 18. Sagna AB, Sarr JB, Gaayeb L, Drame PM, Ndiath MO, Senghor S, et al. gSG6-P1 salivary biomarker
512 discriminates micro-geographical heterogeneity of human exposure to Anopheles bites in low and
513 seasonal malaria areas. *Parasit Vectors*. 2013;6:68.
- 514 19. Remoue F, Cisse B, Ba F, Sokhna C, Herve J-P, Boulanger D, et al. Evaluation of the antibody
515 response to Anopheles salivary antigens as a potential marker of risk of malaria. *Trans R Soc Trop
516 Med Hyg*. 2006;100:363–70.
- 517 20. Ya-Umphun P, Cerqueira D, Parker DM, Cottrell G, Poinsignon A, Remoue F, et al. Use of an
518 Anopheles Salivary Biomarker to Assess Malaria Transmission Risk Along the Thailand-Myanmar
519 Border. *J Infect Dis*. 2017;215:396–404.
- 520 21. Bannister-Tyrrell M, Srun S, Sluydts V, Gryseels C, Mean V, Kim S, et al. Importance of household-
521 level risk factors in explaining micro-epidemiology of asymptomatic malaria infections in Ratanakiri
522 Province, Cambodia. *Sci Rep*. 2018;8:11643.
- 523 22. Okami S, Kohtake N. Spatiotemporal Modeling for Fine-Scale Maps of Regional Malaria
524 Endemicity and Its Implications for Transitional Complexities in a Routine Surveillance Network in
525 Western Cambodia. *Front Public Health*. 2017;5:262.
- 526 23. Stoddard ST, Morrison AC, Vazquez-Prokopec GM, Paz Soldan V, Kochel TJ, Kitron U, et al. The
527 role of human movement in the transmission of vector-borne pathogens. *PLoS Negl Trop Dis*.
528 2009;3:e481.
- 529 24. Prosper O, Ruktanonchai N, Martcheva M. Assessing the role of spatial heterogeneity and human
530 movement in malaria dynamics and control. *J Theor Biol*. 2012;303:1–14.

- 531 25. Chaix B, Kestens Y, Perchoux C, Karusisi N, Merlo J, Labadi K. An interactive mapping tool to
532 assess individual mobility patterns in neighborhood studies. *Am J Prev Med.* 2012;43:440–50.
- 533 26. Incardona S, Vong S, Chiv L, Lim P, Nhem S, Sem R, et al. Large-scale malaria survey in Cambodia:
534 Novel insights on species distribution and risk factors. *Malar J.* 2007;6:37.
- 535 27. Peeters Grietens K, Gryseels C, Dierickx S, Bannister-Tyrrell M, Trienekens S, Uk S, et al.
536 Characterizing Types of Human Mobility to Inform Differential and Targeted Malaria Elimination
537 Strategies in Northeast Cambodia. *Sci Rep.* 2015;5:16837.
- 538 28. Sandfort M, Vantaux A, Kim S, Obadia T, Pepey A, Gardais S, et al. Forest malaria in Cambodia:
539 the occupational and spatial clustering of *Plasmodium vivax* and *Plasmodium falciparum* infection
540 risk in a cross-sectional survey in Mondulkiri province, Cambodia. *Malaria Journal.* 2020;19:413.
- 541 29. Parker DM, Tripura R, Peto TJ, Maude RJ, Nguon C, Chalk J, et al. A multi-level spatial analysis of
542 clinical malaria and subclinical *Plasmodium* infections in Pailin Province, Cambodia. *Heliyon.*
543 2017;3:e00447.
- 544 30. Paz-Soldan VA, Reiner RC, Morrison AC, Stoddard ST, Kitron U, Scott TW, et al. Strengths and
545 weaknesses of Global Positioning System (GPS) data-loggers and semi-structured interviews for
546 capturing fine-scale human mobility: findings from Iquitos, Peru. *PLoS Negl Trop Dis.* 2014;8:e2888.
- 547 31. Pepey A, Souris M, Vantaux A, Morand S, Lek D, Mueller I, et al. Studying Land Cover Changes in
548 a Malaria-Endemic Cambodian District: Considerations and Constraints. *Remote Sensing.*
549 2020;12:2972.
- 550 32. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture
551 (REDCap)--a metadata-driven methodology and workflow process for providing translational
552 research informatics support. *J Biomed Inform.* 2009;42:377–81.
- 553 33. Pepey A, Obadia T, Kim S, Sovannaroth S, Mueller I, Witkowski B, et al. Mobility evaluation by
554 GPS tracking in a rural, low-income population in Cambodia. *PLOS ONE.* 2022;:accepted manuscript.
- 555 34. Hast M, Searle KM, Chaponda M, Lupiya J, Lubinda J, Sikalima J, et al. The use of GPS data loggers
556 to describe the impact of spatio-temporal movement patterns on malaria control in a high-
557 transmission area of northern Zambia. *Int J Health Geogr.* 2019;18:19.
- 558 35. Searle KM, Lubinda J, Hamapumbu H, Shields TM, Curriero FC, Smith DL, et al. Characterizing and
559 quantifying human movement patterns using GPS data loggers in an area approaching malaria
560 elimination in rural southern Zambia. *R Soc Open Sci.* 2017;4.
- 561 36. Canier L, Khim N, Kim S, Sluydts V, Heng S, Dourng D, et al. An innovative tool for moving malaria
562 PCR detection of parasite reservoir into the field. *Malaria Journal.* 2013;12:405.
- 563 37. Poinsignon A, Cornelié S, Mestres-Simon M, Lanfrancotti A, Rossignol M, Boulanger D, et al.
564 Novel Peptide Marker Corresponding to Salivary Protein gSG6 Potentially Identifies Exposure to
565 *Anopheles* Bites. *PLoS One.* 2008;3.
- 566 38. Pollard EJM, Patterson C, Russell TL, Apairamo A, Oscar J, Arcà B, et al. Human exposure to
567 *Anopheles farauti* bites in the Solomon Islands is not associated with IgG antibody response to the
568 gSG6 salivary protein of *Anopheles gambiae*. *Malar J.* 2019;18:334.

569 39. Drame PM, Poinsignon A, Besnard P, Cornelié S, Mire JL, Toto J-C, et al. Human Antibody
570 Responses to the Anopheles Salivary gSG6-P1 Peptide: A Novel Tool for Evaluating the Efficacy of
571 ITNs in Malaria Vector Control. PLOS ONE. 2010;5:e15596.

572 40. Crawley MJ. The R book. John Wiley & Sons; 2012.

573 41. R Core Team. R: A language and environment for statistical computing. www.R-project.org. R. R
574 Foundation for Statistical Computing, Vienna, Austria; 2020.

575 42. Adekunle AI, Pinkevych M, McGready R, Luxemburger C, White LJ, Nosten F, et al. Modeling the
576 dynamics of Plasmodium vivax infection and hypnozoite reactivation in vivo. PLoS Negl Trop Dis.
577 2015;9:e0003595.

578 43. Hii J, Hustedt J, Bangs MJ. Residual Malaria Transmission in Select Countries of Asia-Pacific
579 Region: Old Wine in a New Barrel. J Infect Dis. 2021;223 Supplement_2.

580 44. Fornace KM, Alexander N, Abidin TR, Brock PM, Chua TH, Vythilingam I, et al. Local human
581 movement patterns and land use impact exposure to zoonotic malaria in Malaysian Borneo. eLife.
582 2019;8:e47602.

583 45. Chang H-H, Wesolowski A, Sinha I, Jacob CG, Mahmud A, Uddin D, et al. Mapping imported
584 malaria in Bangladesh using parasite genetic and human mobility data. Elife. 2019;8.

585 46. Longley RJ, White MT, Takashima E, Brewster J, Morita M, Harbers M, et al. Development and
586 validation of serological markers for detecting recent Plasmodium vivax infection. Nat Med.
587 2020;26:741–9.

588 47. Obadia T, Nekkab N, Robinson LJ, Drakeley C, Mueller I, White MT. Developing sero-diagnostic
589 tests to facilitate Plasmodium vivax Serological Test-and-Treat approaches: modeling the balance
590 between public health impact and overtreatment. BMC Medicine. 2022;20:98.

591

592

593 **SUPPLEMENTARY MATERIAL**

594 Table S1: Primers sequences and real-time PCR conditions used to detect *Plasmodium* species.

PCR	Primer	Sequence (5'-3')	Master Mix	Assay parameters
Real-time PCR screening				
Screening PCR	RTPCRScreening2_F	TGGAGTGGATGGTGTTTTAGA	Hot FirePol EvaGreen qPCR Mix Solis Biodyne 1X (#08-24-00020), Primers 150 nM, 5 µl DNA template. Total volume 20 µl	95°C-15 min; 45 cycles: 95°C-15 sec/60°C-20 sec/72°C-20 sec 95°C-2 min 68°C-2 min
	RTPCRScreening2_R	TTGCACCCCAATARCTCATTT		
Nested real-time PCR species				
Primary PCR	RTPCRScreening2_F	TGGAGTGGATGGTGTTTTAGA	Hot FirePol DNA Pol. Solis BioDyne 1.25 U (#01-02-01000), dNTP 200 µM, MgCl2 2.5mM, Primers 250 nM, 5 µl DNA template. Total volume 20 µl	94°C –15 min 20 cycles: 94°C-30 sec/ 58°C –1 min/ 72°C-1 min 72°C-10 min
	RTPCRScreening3_R	ACCCTAAAGGATTTGTGCTACC		
Nested real-time PCR Pf	Pf_RTPCR_F	ATGGATATCTGGATTGATTTTATT TATGA		
	Pf_RTPCR_R	TCCTCCACATATCCAAATTACTGC		
Nested real-time PCR Pv	Pv_RTPCR_F	TGCTACAGGTGCATCTCTGTATTC		
	Pv_RTPCR_R	ATTTGTCCCAAGGTAAAACG		
Nested real-time PCR Pm	Pm_RTPCR_F	ACAGGTGCATCACTGTATTTTTTC	Hot FirePol EvaGreen HRM Mix Solis Biodyne 1X (#08-33-00001), Primers 250 nM, 5 µl template Primary PCR products 1:10. Total volume 20 µl	95°C-15 min 35–45 cycles*: 95°C-10 sec/62°C-20 sec/72°C-25 sec 95°C-1 min 40°C-1 min
	Pm_RTPCR_R	TGCTGGAATTGAAGATAATAAATTA GTAATAACT		
Nested real-time PCR Po	Po_RTPCR_F	GTTATATGGTTATGTGGAGGATATA CTGTT		
	Po_RTPCR_R	CGAATGGAAGAATAAAAATGTAGTA CG		
Nested real-time PCR Pk	Pk_RTPCR_F	CTCCAGAAATTTCTTACGCATACTAC		
	Pk_RTPCR_R	CCCAAGGTAAAACATAACCTATAA AA		

595 *35 cycles when nested real-time PCR, 45 cycles when real-time PCR alone.

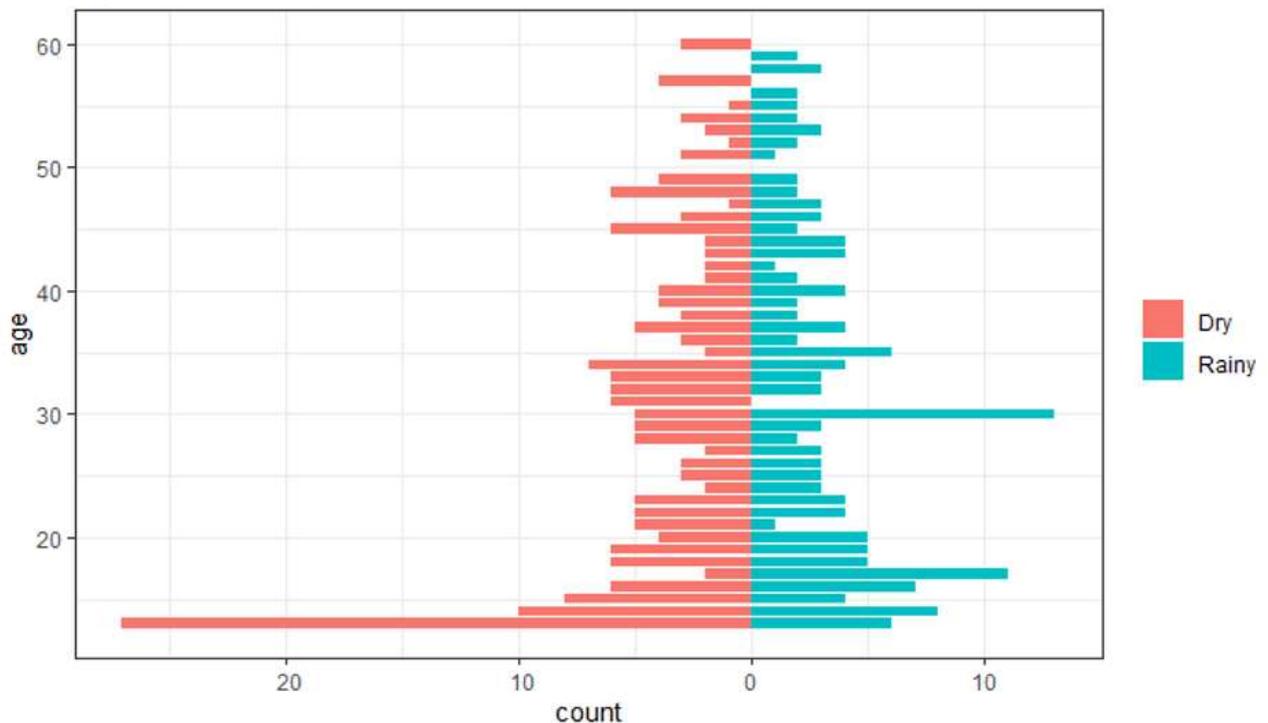
596

597 Protocol S1: Anti-*Anopheles* saliva quantification by ELISA:

598 The wells were coated with 100 μ L of gSG6-P1 peptide solution (20 μ g/mg) for 2h30min at 37°C. Plate
599 wells were then blocked for 1h at 37°C using 200 μ L of blocking buffer, then washed. Individual sera
600 were incubated in 100 μ L duplicate at 4°C overnight at a 1/10 dilution for DBS eluate and 1/40 for
601 serum (in PBS—Tween 0.1%). After washing the plate, secondary human IgG in PBS-tween 1% (final
602 concentration 1/1000) was added and incubated at 37°C for 1h30min, then was washed again. The
603 signal peptide (final concentration 1/1000) was incubated at 37°C for 1h, then washed. Colorimetric
604 development was carried out using ABTS in 0.05M citrate buffer (pH=4) containing 0.003% H₂O₂ and
605 incubated at room temperature for 2h. Absorbance (OD) was measured at 415 nm.

606

607 Figure S1: Age distribution among study population for dry and rainy season (N = 360).



608

609

610 Table S2: Transportation and relatives going to the different environments with the participants at
 611 least once. Each category respectively encompasses *i*) children: kids and grandkids; *ii*) women: all
 612 women from family but daughters (sisters, wives, mothers, etc.); *iii*) men: all men from the same family
 613 but sons (brothers, fathers, etc.); *iv*) co-workers: unrelated adult people working with them. Data from
 614 all participants declaring their visits at day 14 (N = 184).

Visits	N	%	95% CI	Relatives	N	%	95% CI	Transportation	N	%	95%CI
Nearby forest	141	76.6	6.1	Children	5	3.5	3.1	Walking	123	87.2	5.5
				Women	7	5	3.6	Bike	17	12.1	5.4
				Men	9	6.4	4	Car	0	0	0
				Coworkers	11	7.8	4.4				
Deep forest	74	40.2	7.1	Children	3	4.1	4.5	Walking	4	5.4	5.2
				Women	6	8.1	6.2	Bike	65	87.8	7.4
				Men	28	37.8	11.1	Car	4	5.4	5.2
				Coworkers	37	50	11.4				
Rubber plantation	3	1.6	1.8	Children	0	0	0	Walking	1	33.3	53.3
				Women	0	0	0	Bike	2	66.7	53.3
				Men	1	33.3	53.3	Car	0	0	0
				Coworkers	0	0	0				
Cashew plantation	108	58.7	7.1	Children	23	21.3	7.7	Walking	25	23.1	8.0
				Women	48	44.4	9.4	Bike	79	73.1	8.4
				Men	19	17.6	7.2	Car	1	0.9	1.8
				Coworkers	3	2.8	3.1				
Cassava field	49	26.6	6.4	Children	9	18.4	10.8	Walking	8	16.3	10.3
				Women	29	59.2	13.8	Bike	41	83.7	10.3
				Men	14	28.6	12.6	Car	0	0	0
				Coworkers	3	6.1	6.7				
Rice field	15	8.2	4	Children	1	6.7	12.6	Walking	9	60	24.8
				Women	2	13.3	17.2	Bike	6	40	24.8
				Men	1	6.7	12.6	Car	0	0	0
				Coworkers	1	6.7	12.6				

615

616 Table S3: Anti-*Anopheles* saliva antibodies univariate models results and their significance value (N =
 617 197). All variables included in final multivariate model ($P < 0.2$) are in italic and all variables with
 618 significant P-value ($P < 0.05$) are indicated in bold.

Qualitative	Prevalence (%)	95% CI	χ^2	df	P-value
Season			35.36	1	2.74E-09
<i>dry</i>	30.5	7.3			
<i>rainy</i>	66.4	8.5			
Age category			0.37	2	0.83
<i>younger [≤ 20 years old]</i>	43.9	10.7			
<i>mid [21-39 years old]</i>	48.2	9.2			
<i>older [≥ 40 years old]</i>	49.5	11.4			

Village category			3.41	2	0.18
<i>inside forest</i>	57.8	14.4			
<i>forest fringe</i>	40.7	10.7			
<i>outside forest</i>	45.8	8.1			
Malaria status			1.76	1	0.19
<i>positive</i>	50.4	8.5			
<i>negative</i>	42.3	8.3			
ST in forest slow	-	-	1.45	1	0.23
ST in forest at night	-	-	1.71	1	0.19
ST in fields slow	-	-	2.05	1	0.15
ST in fields at night	-	-	1.82	1	0.18
ST in plantations slow	-	-	0.21	1	0.64
ST in plantations at night	-	-	0.14	1	0.70
ST in village slow	-	-	2.49	1	0.11
ST in village at night	-	-	2.60	1	0.11
Quantitative	Average ΔOD	SD	χ^2	df	P-value
Season			58.48	1	2.05E-14
<i>dry</i>	0.48	0.20			
<i>rainy</i>	0.98	0.61			
Age category			0.75	2	0.68
<i>younger [≤ 20 years old]</i>	0.66	0.47			
<i>mid [21-39 years old]</i>	0.72	0.53			
<i>older [≥ 40 years old]</i>	0.69	0.49			
Village category			5.55	2	0.06
<i>inside forest</i>	0.87	0.58			
<i>forest fringe</i>	0.62	0.42			
<i>outside forest</i>	0.69	0.50			
Malaria status			0.3	1	0.61
<i>positive</i>	0.72	0.52			
<i>negative</i>	0.68	0.48			
ST in forest slow	-	-	1.35	1	0.24
ST in forest at night	-	-	1.65	1	0.20
ST in fields slow	-	-	2.99	1	0.08
ST in fields at night	-	-	2.57	1	0.11
ST in plantations slow	-	-	0.82	1	0.36
ST in plantations at night	-	-	0.74	1	0.39
ST in village slow	-	-	2.58	1	0.11
ST in village at night	-	-	2.43	1	0.12

619

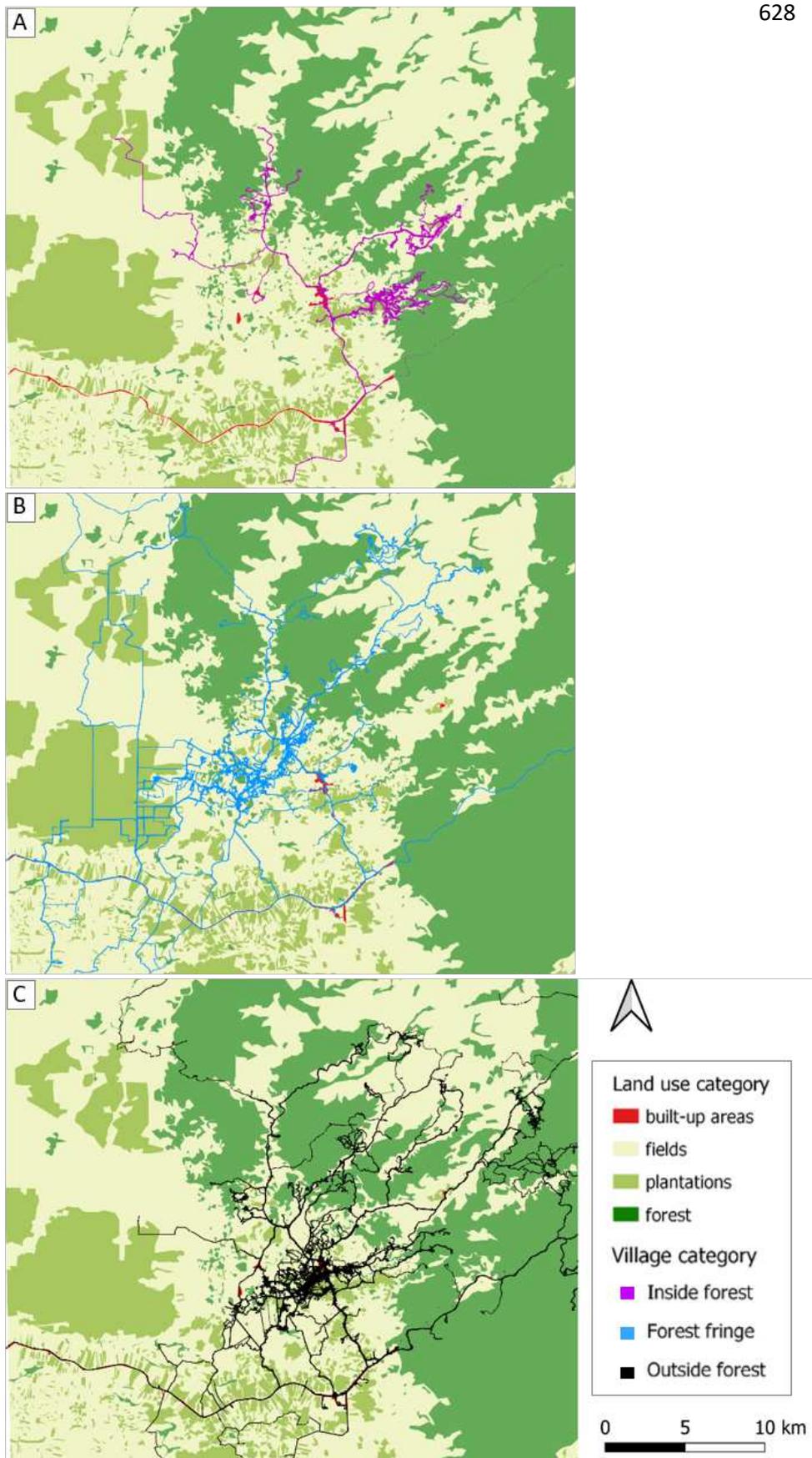
620

621 Table S4: Anti-*Anopheles* saliva antibodies multivariate models. Significance value of factors during
622 stepwise removal of terms for both qualitative (tested factor: seroprevalence) and quantitative
623 (average Δ OD) multivariate models (N = 270). The original quantitative model could not converge
624 because of three variables with unusually large Z statistics, which were removed from mentioned
625 model before starting the stepwise removal of terms.

Variable	χ^2	df	P
Qualitative model: prevalence			
Season:Plasmodium	0.00	1	0.96
Season:ST night fields	0.05	1	0.82
Season:ST night village	0.87	1	0.35
Village category:ST slow fields	3.50	2	0.17
Village category:ST slow village	2.93	2	0.23
Season:ST night forest	1.71	1	0.19
Season:ST slow fields	1.33	1	0.25
Village category:Season	4.08	2	0.13
Village category:ST night forest	4.74	2	0.09
Village category:ST night village	0.99	2	0.61
Village category:ST night fields	2.04	2	0.36
Season:ST slow village	2.86	1	0.09
Village category	0.43	2	0.81
ST slow fields	0.18	1	0.67
ST night forest	0.87	1	0.35
ST night fields	0.37	1	0.55
Plasmodium	1.56	1	0.21
ST slow village	2.35	1	0.13
ST night village	0.11	1	0.74
Season	35.36	1	<0.001
Quantitative model: average ΔOD			
Season:ST night fields	0.24	1	0.62
Season:ST slow fields	0.07	1	0.79
Village category:ST night forest	1.67	2	0.43
Village category:ST slow fields	5.21	3	0.16
Village category:ST night fields	0.95	2	0.62
Village category:ST night village	2.80	2	0.25
Village category:ST slow village	1.74	2	0.42
Village category:Season	3.63	2	0.16
Season:ST slow village	3.56	1	0.06
Village category	1.04	2	0.60
ST night village	0.70	1	0.39
ST night forest	1.41	1	0.23
ST slow village	2.35	1	0.13
ST night fields	0.89	1	0.35
Season	58.48	1	<0.001

626 Figure S2: Optimal GPS points of participants from villages A: inside the forest (N = 59); B: in forest
627 fringes (N = 116); C: outside the forest (N = 185).

628



629 Table S5: Tukey post-hoc test results on the prevalence over follow-up of each village age category.
 630 Significant results are in bold.

Interaction	OR	OR 95% CI	P-value
Forest fringe - Inside forest	2.17	[1.32 – 3.02]	0.17
Inside forest - Outside forest	1.08	[0.33 – 1.83]	0.98
Forest fringe - Outside forest	2.34	[1.67 – 3]	0.03

631

632 Table S6: GLMMs comparisons of PCR status and time spent in a category of land use on optimal data,
 633 using all data, night (18:00 to 5:59) data and slow (<5 km/h) data for all participants with a valid
 634 optimal GPS dataset (N = 258).

635

636

Dataset	Land use category	Time variable	χ^2_1	P-value
night	forest	Standardised time	0.02	0.89
	fields		0.04	0.84
	plantations		0.37	0.54
	village		0.07	0.79
	forest	Total hours	1.74	0.19
	fields		0.56	0.45
	plantations		0.24	0.63
	village		0.09	0.77
slow	forest	Standardised time	0.01	0.93
	fields		1.02	0.31
	plantations		0.04	0.85
	village		0.39	0.53
	forest	Total hours	0.34	0.56
	fields		3.04	0.08
	plantations		0.30	0.58
	village		0.29	0.59

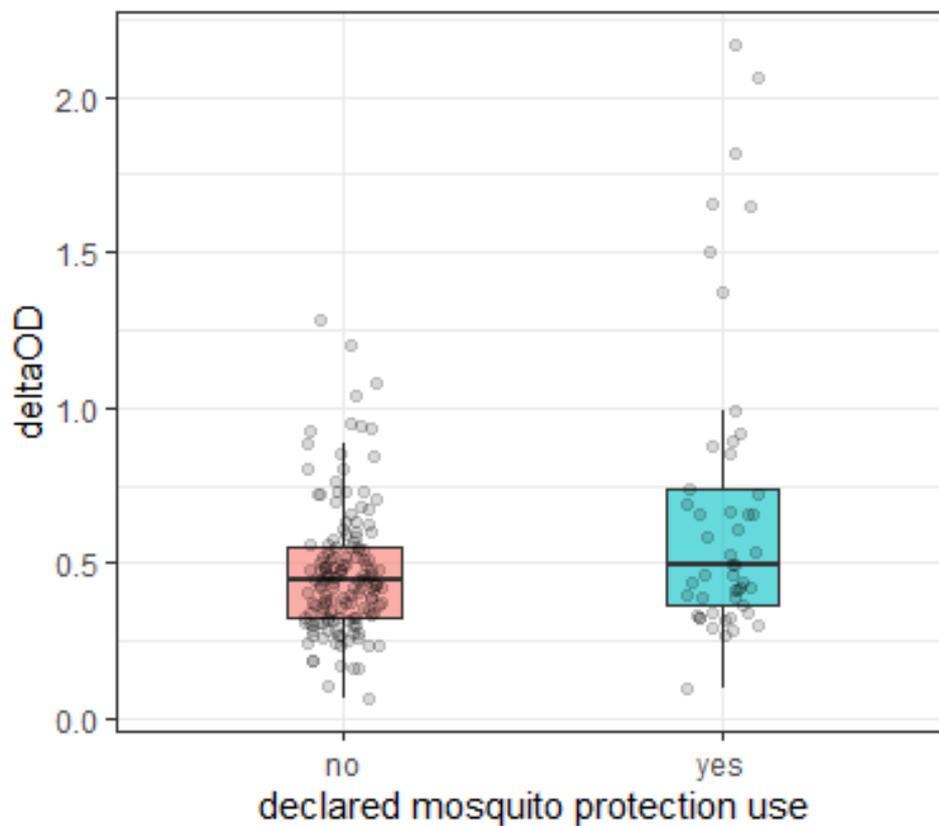
637 Table S7: Declared use of mosquito protection at week 2 of follow-up (N = 324 questionnaire entries).

Environment	N answers	Protection use (%)	95% CI	Protection type
nearby forest	184	0.5	1.1	treated bed net
deep forest	200	24.5	6.0	treated and non-treated bed net, treated and non-treated hammock, coils and fire
rubber plantation	183	0.5	1.1	treated bed net and fire
cashew plantation	188	3.2	2.5	treated and non-treated bed net, non-treated hammock and fire
cassava fields	188	4.3	2.9	treated and non-treated bed net, treated and non-treated hammock, fire
rice fields	186	1.6	1.8	treated bed net

638

639

640 Figure S3: Anti-*Anopheles* saliva antibody response and mosquito protection use in deep forest
 641 declared during questionnaire at day 14 (N = 197).



642

643 Table S8: Comparison of participants' responses to questionnaire to their GPS data, for all land use
 644 categories (N = 197). Underlined numbers correspond to count of participants with discordant data
 645 between GPS and questionnaire.

Dataset from GPS device	Data from questionnaire		
	visiting	not visiting	
visiting	133	<u>6</u>	forest
not visiting	<u>47</u>	11	
visiting	92	<u>29</u>	plantations
not visiting	<u>48</u>	28	
visiting	95	<u>94</u>	fields
not visiting	<u>1</u>	7	

646

Article: Investigating human-malaria mosquito contact in time and space using GPS data

Anaïs Pepey, Marc Souris, Saorin Kim, Thomas Obadia, Sophy Chy, Malen Ea, Sivkeng Ouk,
Frank Remoue, Siv Sovannaroth, Ivo Mueller, Benoit Witkowski, Amélie Vantaux

Manuscript under preparation.

Investigating human-malaria mosquito contact in time and space using GPS data loggers

Anaïs Pepey¹, Marc Souris², Saorin Kim¹, Thomas Obadia^{3,4}, Sophy Chy¹, Malen Ea¹, Sivkeng Ouk¹, Frank Remoue⁵, Siv Sovannaroeth⁶, Ivo Mueller^{7,8}, Benoit Witkowski^{1*}, Amélie Vantaux^{1*}

AFFILIATIONS

¹ Malaria Molecular Epidemiology Unit, Institut Pasteur du Cambodge, 5 Blvd Monivong, BP983, Phnom Penh 120 210, Cambodia

² UMR Unité des Virus Emergents, UVE: Aix-Marseille Univ–IRD 190–Inserm 1207–IHU 5 Méditerranée Infection, 13005 Marseille, France

³ Infectious Diseases Epidemiology and Analytics, Department of Parasites and Insect Vectors, Institut Pasteur, Paris, France

⁴ Hub de Bioinformatique et Biostatistique, Département de Biologie Computationnelle, Institut Pasteur, Paris, France

⁵ UMR MIVEGEC, University of Montpellier, IRD, CNRS, Montpellier, France

⁶ National Centre for Parasitology Entomology and Malaria Control (CNM), Phnom Penh 120 801, Cambodia

⁷ The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia

⁸ Department of Medical Biology, University of Melbourne, Melbourne, Victoria, Australia

* These authors contributed equally to the manuscript.

Corresponding author: Anaïs Pepey

ABSTRACT

Background. Malaria prevalence is decreasing in Cambodia but elimination is staggering due to the relative increase of relapsing *Plasmodium vivax* cases and numerous asymptomatic infections. Malaria control strategies need to evolve to be able to target these cases. Methodologies such as GPS tracking, RTPCR parasite detection and vector exposure serological markers open up possibilities on characterising the remaining malaria cases in Cambodia.

30 **Material and methods.** We enrolled participants from Kaev Seima district in Mondulhiri province to
31 collect blood samples and track their mobility with GPS data loggers twice with a three-month interval.
32 We used molecular techniques to determine malaria prevalence (RTPCR screening and *Plasmodium*
33 species determination) and vector exposure (ELISA quantification of anti-*Anopheles* antibodies).

34 **Results.** We followed 301 participants over six months. Malaria prevalence was significantly higher in
35 men ($22.5 \pm 0.4\%$) than women ($5.4 \pm 0.2\%$), with 93.8% of cases being *Plasmodium vivax*. Malaria
36 status did not appear to be associated to the time spent in a particular type of land use.
37 Seroprevalence was higher for participants that spend more time in village and less time in fields.

38 **Conclusions.** The saturation of *P. vivax* does not allow defining precisely the land use associated with
39 newly acquired infections. The important proportion of participants being exposed to *Anopheles* bites
40 suggest that infectious bites are rare but *Anopheles* mosquitoes are present in all types of land use.
41 New tools are required to understand the *P. vivax* reservoir in Cambodia.

42 **KEYWORDS**

43 Cambodia; Great Mekong Subregion; GPS data loggers; mobility; anti-*Anopheles* saliva antibodies;
44 land use; forest; malaria.

45

46 **INTRODUCTION**

47 Since 2014, when the Greater Mekong Subregion (GMS) aimed to end malaria transmission by 2030,
48 malaria elimination has progressed [1]. *Plasmodium falciparum* incidence have drastically diminished,
49 but *Plasmodium vivax* infections remain important and challenge elimination [1]. *Plasmodium vivax*
50 can infect the liver as a dormant stage causing potential relapses [2], allowing the parasite to circulate
51 unnoticed and untreated, with a substantial amount of asymptomatic cases participating to
52 transmission [3], carried by individuals which, in the absence of symptoms, are not seeking diagnosis
53 or treatment.

54 In 2019, the WHO estimated that 85% of malaria cases occurring in Cambodia were *P. vivax* infections
55 [1]. Mondulhiri province held the highest recorded malaria incidence in the GMS, with more than 50
56 cases per 1000 inhabitants from January to July 2019 [1]. The main risk group correspond to male,
57 adult population engaging in forest-associated activities [4–8], as the principal malaria vectors from
58 the *Anopheles* genus are forest-associated [9, 10].

59 Currently, Cambodia bases its malaria elimination programme on long-lasting insecticidal nets and
60 test-and treat services with mobile and village malaria workers relying on immunochromatography-

61 based Rapid Diagnostic Tests (RDT) for diagnosis [11]. Other elimination tools exist, such as mass
62 screening and treatment, focal screening and treatment and reactive case detection, however the
63 very low detection rates and diagnostic sensitivity for asymptomatic participants make their
64 implementation not cost effective enough [12, 13]. Mass drug administration (MDA) is also not
65 considered an appropriate elimination strategy, because of its high risk to spread further resistance
66 and multi resistance in Cambodia [14, 15]. Furthermore, MDA would probably not attain the majority
67 of the highest risk population of forest goers that are hard to reach [8, 16].

68 Because of their detection threshold around 100 parasites/ μL [17], RDTs often fail to detect low-
69 parasitemia infections, often corresponding to asymptomatic infections, and misrepresent the actual
70 prevalence in the tested population [11, 18]. To detect asymptomatic, submicroscopic or low-
71 parasitemia infections, molecular techniques are often required. Polymerase Chain Reaction (PCR) is
72 an effective tool able to detect such infections [19].

73 As *P. vivax* hypnozoites can cause relapses weeks to months after an infectious bite, and that parasites
74 are only detectable with PCR during their blood stage, methodologies based on *Anopheles* spp.
75 exposure can be a surrogate to gauge risk of malaria infection. Immunologic markers detection can
76 provide insights about recent *Anopheles* exposure. The immune system produces Immunoglobulins G
77 (IgG) antibodies after *Anopheles* saliva enters the bloodstream during the mosquito blood meal, which
78 can be detected from blood samples from exposed populations [20]. The intensity of the immune
79 response and the concentration of anti-*Anopheles* saliva antibodies is proportional to the amount of
80 bites received by the exposed individuals [21], hence the gSG6 peptide and the immune response it
81 triggers has been used to measure individual exposure [20, 22].

82 The standard methodology for measuring risk populations mobility in Cambodia has been through
83 questionnaires [4, 6–8]. However, their efficacy can be limited by the lack of spatial literacy in rural
84 populations or recall bias [23]. In Cambodia, previous research demonstrated that bias arises from the
85 refusal to declare mobility associated with illegal work [24] and lack of correspondence between
86 questionnaire outputs and GPS data loggers [25]. However, understanding daily mobility patterns
87 within areas with circulating malaria can help precise the time and place of malaria exposure [26, 27].
88 Tracking the daily movements of the risk populations and understanding the zones responsible for
89 malaria infections allows determining tailor-made interventions and thus achieving more effective
90 elimination [28]. Measuring precisely the mobility of individuals and implementing corresponding
91 malaria elimination measures has been achieved in the past with the implementation of GPS data
92 loggers [29–31]. Previously, the GPS follow-up we implemented over a rural Cambodian population
93 did not allow to determine associations between mobility and malaria risk factors, however it focused
94 on the high risk group only *i.e.* men between 13 and 49 years old [32]. Thus, based alongside a cohort

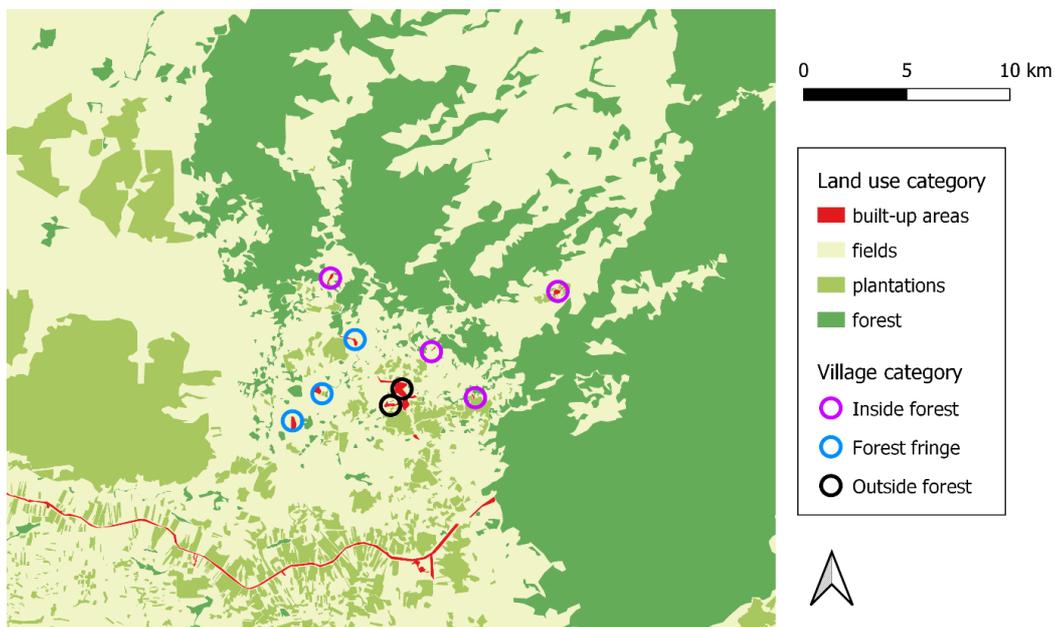
95 study of the rural population in the study area, we designed a study with a larger sample size to
96 compensate for data loss observed in the previous study, which included women to investigate
97 possible mobility behaviors differences. Therefore, we estimated the prevalence of *Plasmodium*
98 infections in a rural population and we investigated the key risk factors with GPS tracking and vector
99 exposure serological marker quantification.

100 MATERIAL AND METHODS

101 Study site and study population

102 The study site is located in Kaev Seima district, Mondulkiri province, northeastern Cambodia. Year is
103 divided into rainy (May-October) and dry (November-April) seasons. The land use of the study site was
104 determined previously from high resolution satellite imagery (SPOT 6/7) captured in March 2018
105 (Figure 1) [33]. The classification categorised the land into built-up areas (villages and roads), fields
106 (rice paddies, cassava culture, etc.), plantations (banana, cashew nut, rubber trees, etc.) and forest.
107 Kaev Seima district is a rural, low-income environment where populations mainly live from agriculture
108 and forest products exploitation.

109 Figure 1: Land use and villages of the study area.



110 Study design

111 We aimed to recruit 300 volunteers, with 100 participants enrolled per month, both male and female,
112 with a 0.5 sex ratio. When they were recruited (time point T1), we collected their sociodemographic
113 data, a capillary blood sample and we equipped participants with a GPS data logger that tracked their
114 mobility for a complete week [25]. Three months after their enrolment (time point T2), a second blood
115 sample was collected and a GPS logger provided. Over the study, each participant was expected to
116 provide two blood samples and two 7-day GPS tracks.

117 **GPS data collection**

118 The GPS data loggers were i-gotU GT-600 worn on the wrist with a custom strap and waterproof case.
119 The GPS tracking data was curated and an optimal GPS dataset was produced, including only
120 participants willing to take the device on their daily mobility and for GPS trackers that logged enough
121 time to be representative of their mobility [25]. The time spent in each land use category was
122 extracted from participants GPS data along with the locations metadata (day or night time and speed
123 for each location) [25].

124 **Laboratory procedures**

125 **RTPCR.** DNA was extracted from blood pellets with Qiagen DNA extraction kits according to the
126 manufacturer's instructions. Real-Time Polymerase Chain Reactions (RTPCR) were performed to
127 detect malaria parasites and to carry out parasites speciation [19]. Briefly, a first RTPCR targeted the
128 *Plasmodium* cytochrome b gene and determined the positive samples. Then, five nested RTPCR assays
129 targeted the same gene specific to each species: *P. falciparum*, *P. knowlesi*, *P. malariae*, *P. ovale* and
130 *P. vivax* (Table S1).

131 **ELISA.** Quantification of anti-*Anopheles* saliva antibodies in participants' serum was done by enzyme-
132 linked immunosorbent assay (ELISA) on blood samples from the last day of follow-up (week 4) as
133 described in [21, 34] with some modifications (Protocol S1). The intensity of the IgG response was
134 measured at the individual level and was expressed as the ΔOD , calculated as the difference between
135 the mean of the individual optical densities (OD) in 2 antigen wells and the OD in 1 blank well
136 containing no gSG6-P1 antigen. As a negative control, the specific anti-gSG6-P1 IgG response was also
137 assayed in non-*Anopheles* exposed volunteers residing in an urban area with limited *Anopheles*
138 mosquitoes populations (Phnom Penh city) for more than three months. The OD values from the
139 negative participants determined the nonspecific background antibody level, used to calculate the
140 cut-off value, calculated as the mean $\Delta OD_{neg} + 3 SDs$ [21, 35, 36]. Based on our findings, a participant
141 was classified as an immune responder if their ΔOD was > 0.54 .

142 **Analysis**

143 **Sociodemographic data.** Sociodemographic variables from the T1 questionnaire were coded as factor
144 variables. We previously categorised villages of the study area according to the forest cover
145 surrounding the households in three categories: outside forest, forest fringe or inside forest [7].
146 Sociodemographic included in models variables were: age category, sex, and village category.

147 **Mobility data.** The time spent in different environments was extracted from the optimal GPS data.
148 Mobility data was then calculated under two conditions: night data, when *Anopheles* vectors are most
149 active and slow data (<5 km/h) when individuals are not driving a vehicle and are more exposed to
150 mosquito bites, in order to determine which conditions led to increased malaria exposure for a

151 participant. Mobility variables were represented as standardised hours (proportion of data logged
152 extrapolated to a complete 1-week logging) per type of environment and coded as numeric variables.

153 **Prevalence data.** A binomial variable representing infection status was attributed to each participant
154 for every time point: 1 represented a participant with a positive RTPCR for any *Plasmodium* species
155 and 0 negative.

156 **ELISA data.** A binomial variable representing anti-*Anopheles* saliva seropositivity was attributed to
157 each participant for every time point: 1 represented a seropositive participant with an OD of anti-
158 *Anopheles* saliva antibody response $> \Delta OD_{neg} + 3 SDs$, and 0 a seronegative participant.

159 **Models.** We tested the effect of sociodemographic and mobility variables on malaria status and
160 serostatus using univariate Generalized Linear Mixed-effects Models (GLMMs) with participant ID
161 coded as a random factor to account for repeated measurements on the same participant.
162 Additionally, we tested the effect of the participant's sex on the total standardised time spent in each
163 environment using univariate GLMMs with participant ID coded as a random factor.

164 All percentages were calculated with their 95% confidence interval (CI), means with their standard
165 error (SE) and ΔOD with their standard deviation. Significance threshold was set at $p = 0.05$. All
166 statistical analyses were conducted in R version 4.0 [37].

167 **RESULTS**

168 **Overview**

169 The study lasted from June to November 2019, with 301 participants recruited at baseline, of which
170 150 ($49.6 \pm 5.6\%$) were women. Participants' age ranged from 13 to 49 years old, with a median of 29
171 years old (Figure S1) and came from ten different villages (Figure S2). Of them, 298 ($99 \pm 1.1\%$) were
172 present for follow-up two months later. Over the 527 downloaded GPS tracks, 503 ($95.8 \pm 1.8\%$) were
173 optimal and included in the analysis. We calculated malaria prevalence and seroprevalence and
174 implemented models over a unique subset of 470 entries with complete sociodemographic, PCR, ELISA
175 and optimal GPS datasets.

176 **Malaria prevalence**

177 Malaria prevalence did not differ significantly between time point T1 ($12.9 \pm 0.2\%$) and time point T2
178 ($15 \pm 0.3\%$, $\chi^2_1 = 1.43$, $p = 0.23$). Of the 65 positive RTPCRs, 61 ($93.8 \pm 5.8\%$) were *P. vivax*, one ($1.5 \pm$
179 3%) was *P. falciparum* and three ($4.6 \pm 5.1\%$) were unidentified. The only case of *P. falciparum* was a
180 38 year old male living in a village inside the forest, seropositive for anti-*Anopheles* saliva antibodies
181 ($\Delta OD = 1.85 \pm 0.12$, in the top 2% of the subset ΔOD values), having spent more than 150 standardised
182 hours ($89.4 \pm 4.7\%$ of recorded time) in village environment. The prevalence was higher in men (22.5

183 $\pm 0.4\%$) than women ($5.4 \pm 0.2\%$) but the difference was not significant ($\chi^2_1 = 2.88$, $p = 0.09$). Malaria
 184 prevalence was not significantly different among age categories ($\chi^2_2 = 0.04$, $p = 0.98$, Table S2) or
 185 village categories ($\chi^2_2 = 0.54$, $p = 0.76$, Table S2). The time spent in a specific environment (at night or
 186 at slow speed) was not significantly associated to malaria prevalence (Table S3).

187 Seroprevalence

188 Seroprevalence varied significantly between time point T1 ($44.3 \pm 0.4\%$) and time point T2 ($35.4 \pm$
 189 0.5% , $\chi^2_1 = 4.23$, $p = 0.04$). Seroprevalence was not significantly different between men ($43.3 \pm 0.4\%$)
 190 and women ($37.7 \pm 0.4\%$, $\chi^2_1 = 1.7$, $p = 0.19$) or across age categories ($\chi^2_2 = 3.6$, $p = 0.17$) and village
 191 categories ($\chi^2_2 = 3.1$, $p = 0.22$, Table S4). The time spent in an environment (at night and at slow speed)
 192 was significantly associated to seroprevalence: participants that were seropositive spent more time in
 193 villages and less time in fields (Table 1).

194 Table 1: Average standardised hours spent in every environment at slow speed ($< 5\text{km/h}$) and at night
 195 (18:00 to 6:00) with standard error (SE) by participants' serostatus. Significant results are highlighted in
 196 bold.

	Average standardised hours				GLMMs results		
	Seronegative participants	SE	Seropositive participants	SE	χ^2	df	p
Mobility at night							
village	75.9	4.6	97.3	5.5	6.76	1	0.009
fields	68.8	4.4	48.9	4.9	6.11	1	0.013
plantations	12.5	2.4	10.4	2.7	0.27	1	0.60
forest	10.7	2.2	11.2	2.7	0.005	1	0.95
Mobility at slow speed							
village	74.4	4.3	94.4	5.2	6.91	1	0.009
fields	70.1	4.1	50.9	4.5	6.80	1	0.009
plantations	11.9	2.2	9.8	2.6	0.43	1	0.51
forest	11.6	2.0	12.6	2.4	0.02	1	0.90

197 Mobility

198 The average time spent in village, fields, plantations or forest was not significantly different between
 199 men and women (Table S5). Nine participants spent more than 100 hours in forest for both of their
 200 GPS follow-up (standardised hours, optimal GPS data). None of these participants was from villages
 201 inside the forest and 5 were women (Table S6).

202 DISCUSSION

203 As most *Plasmodium* cases are identified as *P. vivax*, the detected infections could be the consequence
 204 of relapsing hypnozoites originating from an infectious bite that could have occurred weeks or months
 205 prior to the observed infection. In such case, these infections are not dependent of the recent mobility

206 or vector exposure and the time spent in forest is not associated to the observed prevalence in the
207 study population.

208 We observed that malaria prevalence is 5 times higher in men than in women, even if the difference
209 was not significant and that the average time spent in forest was not different between men and
210 women. Moreover, the participants spending more time in the forest were often women, which does
211 not correspond with the expected forest goers demographic that are adult men. Because men are not
212 apparently spending more time in forest areas but are more affected by malaria, we can suggest that
213 the type of activities or the type of forest is crucial to determine what makes a forest goer and its
214 exposure to infectious mosquito bites. The task of logging wood, an activity mostly done by men [38],
215 can prevent them to use mosquito protection and may bring them further into the deep forest, where
216 *Anopheles* densities or vector competence may be higher than on the forest edges. Women, on the
217 other hand, could spend more time in nearby forest to collect wood or forest products, or accompany
218 the men into deep forest without engaging in logging wood. Previous studies have reported that most
219 men and women go frequently into the nearby and deep forest, possibly at night or for several days,
220 for activities such as fishing, hunting, collecting vegetables or simply going to the toilet [39, 40].
221 Declared activities can happen in the forest vicinity, such as farming around the forest fringes or
222 attending to newly slashed-and-burnt crops [39, 40], which would appear as time spent in forest in
223 our results, if forest was cleared after producing the land use category. The definition of a forest,
224 perceived malaria risk and reported activities done within the forest differ across households,
225 ethnicities and subpopulations [40, 41], highlighting the importance of defining which populations
226 visit the nearby or the deep forest. Another hypothesis could be that both men and women spending
227 an important share of their time in the forest are hypnozoites carriers, with higher densities in men
228 leading to higher chance of detection during a PCR screening. In such a case, both RDT and PCR
229 screening would often diagnose women as false negatives, meaning that elimination programmes
230 could be overlooking a potential transmission reservoir.

231 The seroprevalence of anti-*Anopheles* antibodies in the study population suggested that all
232 sociodemographic strata were in contact with *Anopheles*. Participants spending more time in village
233 environments had higher antibodies levels and were significantly more likely to be seropositive than
234 the participants spending more time in fields. Interestingly, mosquito collections in the GMS have
235 reported contrasting results with *Anopheles* densities lower in villages compared to other
236 environments [42–45] or on the contrary similar densities in forest and village sites [46]. Thus, village
237 environments could harbour higher *Anopheles* densities (not necessarily linked to higher primary or
238 overall competent vectors densities) or the activities done in these types of land use could increase
239 individual bites exposure. Mosquito collections in various environments would help the understanding

240 of *Anopheles* vectors prevalence and abundance, and help decipher antibodies levels variation in the
241 study population.

242 Overall, malaria risk seems heterogeneous across the population, with men more affected than
243 women, but *Anopheles* bites seem quite prevalent across the population, meaning the risk of an
244 infectious bite, over all potential *Anopheles* bites, is very low. To pinpoint accurately the time and the
245 context of transmission, innovative tools and strategies are required. Notably, the development of
246 methodologies able to characterise the context of *P. vivax* infections, such as serological markers and
247 targeted amplicons sequencing, could help determine the nature of the *P. vivax* reservoir and
248 determine accordingly the elimination strategy to deploy.

249 **CONCLUSIONS**

250 Bites from *Anopheles* mosquitoes appear to affect both men and women but prevalence is higher in
251 men, while both men and women can spend considerable amounts of time in forested environments.
252 The saturation of cases by relapse-inducing *P. vivax* infections is making the determination of mobility
253 risk factors difficult. New tools are required to characterise and understand the *P. vivax* reservoir.

254

255 **DECLARATIONS**

256 **Ethics approval and consent to participate**

257 Ethical approvals were obtained from the Cambodian National Ethics Committee for Health Research
258 (136NECHR & 262NHECR) and from the National Institutes of Health (DMID Protocol Number: 17-
259 0036). The protocols conform to the Helsinki Declaration on ethical principles for medical research
260 involving human subjects (version 2002) and informed written consent was obtained for all
261 volunteers. Parental assent was obtained for every participant between 13 and 18 years old.

262 **Consent for publication**

263 Not applicable.

264 **Availability of data and materials**

265 The de-identified datasets generated and/or analysed during the current study is being made publicly
266 available in a Figshare repository, <https://figshare.com/>. In the meantime, it is available from the
267 corresponding author on reasonable request.

268 **Competing interests**

269 The authors declare that they have no competing interests.

270 **Funding**

271 This study was funded by the National Institutes of Health program International Centres of Excellence
272 for Malaria Research, grant 1U19AI129392-01 “Understanding, tracking and eliminating malaria
273 transmission in the Asia-Pacific Region”. A.P. was funded by the Pasteur Institute International
274 Network Calmette and Yersin Ph.D. scholarship, grant MHL/MJ/N°212/18. The funding bodies had no
275 role in the design of the study, the collection, analysis, and interpretation of data, and in writing the
276 manuscript.

277 **Authors' contribution**

278 IM, BW and AV, conceived and designed the study and were overall responsible for the study. AP
279 contributed to data curation, statistical analysis and wrote the first draft, supported by TO and AV.
280 MS, BW and AV contributed to data interpretation and writing. AP and SK conducted the data
281 collection, supported by SS, BW and AV, and supervised the sample processing. AP, SC, ME, SO, FR,
282 BW and AV contributed to molecular biology procedures and laboratory protocols development. All
283 authors have read and agreed to the published version of the manuscript.

284 **Corresponding author**

285 Correspondence to Anaïs Pepey.

286 **Acknowledgements**

287 The authors express their gratitude to the study participants, village heads and officials for making this
288 study possible. We also thank the interviewers, technicians, and other study staff for their work.

289

290 **REFERENCES**

- 291 1. WHO. Countries of the Greater Mekong zero in on falciparum malaria. Geneva: WHO; 2019.
- 292 2. Dayananda KK, Achur RN, Gowda DC. Epidemiology, drug resistance, and pathophysiology of
293 Plasmodium vivax malaria. J Vector Borne Dis. 2018;55:1–8.
- 294 3. Vantaux A, Samreth R, Piv EP, Khim N, Kim S, Berne L, et al. Contribution to malaria transmission
295 of symptomatic and asymptomatic parasite carriers in Cambodia. J Infect Dis. 2018.
296 <https://doi.org/10.1093/infdis/jiy060>.
- 297 4. Incardona S, Vong S, Chiv L, Lim P, Nhem S, Sem R, et al. Large-scale malaria survey in Cambodia:
298 Novel insights on species distribution and risk factors. Malar J. 2007;6:37.
- 299 5. Kar NP, Kumar A, Singh OP, Carlton JM, Nanda N. A review of malaria transmission dynamics in
300 forest ecosystems. Parasit Vectors. 2014;7:265.
- 301 6. Peeters Grietens K, Gryseels C, Dierickx S, Bannister-Tyrrell M, Trienekens S, Uk S, et al.
302 Characterizing Types of Human Mobility to Inform Differential and Targeted Malaria Elimination
303 Strategies in Northeast Cambodia. Sci Rep. 2015;5:16837.

- 304 7. Sandfort M, Vantaux A, Kim S, Obadia T, Pepey A, Gardais S, et al. Forest malaria in Cambodia: the
305 occupational and spatial clustering of *Plasmodium vivax* and *Plasmodium falciparum* infection risk in
306 a cross-sectional survey in Mondulakiri province, Cambodia. *Malaria Journal*. 2020;19:413.
- 307 8. Sluydts V, Heng S, Coosemans M, Van Roey K, Gryseels C, Canier L, et al. Spatial clustering and risk
308 factors of malaria infections in Ratanakiri Province, Cambodia. *Malar J*. 2014;13:387.
- 309 9. Sinka ME, Bangs MJ, Manguin S, Rubio-Palis Y, Chareonviriyaphap T, Coetzee M, et al. A global
310 map of dominant malaria vectors. *Parasites & Vectors*. 2012;5:69.
- 311 10. Sinka ME, Bangs MJ, Manguin S, Chareonviriyaphap T, Patil AP, Temperley WH, et al. The
312 dominant *Anopheles* vectors of human malaria in the Asia-Pacific region: occurrence data,
313 distribution maps and bionomic précis. *Parasites & Vectors*. 2011;4:89.
- 314 11. Lek D, Callery JJ, Nguon C, Debackere M, Sovannaroth S, Tripura R, et al. Tools to accelerate
315 *falciparum* malaria elimination in Cambodia: a meeting report. *Malar J*. 2020;19:151.
- 316 12. Yeung S, McGregor D, James N, Kheang ST, Kim S, Khim N, et al. Performance of Ultrasensitive
317 Rapid Diagnostic Tests for Detecting Asymptomatic *Plasmodium falciparum*. *Am J Trop Med Hyg*.
318 2020;102:307–9.
- 319 13. Rossi G, Vernaev L, Van den Bergh R, Nguon C, Debackere M, Abello Peiri C, et al. Closing in on
320 the Reservoir: Proactive Case Detection in High-Risk Groups as a Strategy to Detect *Plasmodium*
321 *falciparum* Asymptomatic Carriers in Cambodia. *Clin Infect Dis*. 2018;66:1610–7.
- 322 14. Hoyer S, Nguon S, Kim S, Habib N, Khim N, Sum S, et al. Focused Screening and Treatment (FSAT):
323 A PCR-Based Strategy to Detect Malaria Parasite Carriers and Contain Drug Resistant *P. falciparum*,
324 Pailin, Cambodia. *PLOS ONE*. 2012;7:e45797.
- 325 15. Maude RJ, Nguon C, Dondorp AM, White LJ, White NJ. The diminishing returns of atovaquone-
326 proguanil for elimination of *Plasmodium falciparum* malaria: modelling mass drug administration
327 and treatment. *Malaria Journal*. 2014;13:380.
- 328 16. Dysoley L, Kaneko A, Eto H, Mita T, Socheat D, Börkman A, et al. Changing patterns of forest
329 malaria among the mobile adult male population in Chumkiri District, Cambodia. *Acta Trop*.
330 2008;106:207–12.
- 331 17. Das S, Peck RB, Barney R, Jang IK, Kahn M, Zhu M, et al. Performance of an ultra-sensitive
332 *Plasmodium falciparum* HRP2-based rapid diagnostic test with recombinant HRP2, culture parasites,
333 and archived whole blood samples. *Malaria Journal*. 2018;17:118.
- 334 18. Tripura R, Peto TJ, Veugen CC, Nguon C, Davoeung C, James N, et al. Submicroscopic *Plasmodium*
335 prevalence in relation to malaria incidence in 20 villages in western Cambodia. *Malaria Journal*.
336 2017;16:56.
- 337 19. Canier L, Khim N, Kim S, Sluydts V, Heng S, Dourng D, et al. An innovative tool for moving malaria
338 PCR detection of parasite reservoir into the field. *Malaria Journal*. 2013;12:405.
- 339 20. Sagna AB, Sarr JB, Gaayeb L, Drame PM, Ndiath MO, Senghor S, et al. gSG6-P1 salivary biomarker
340 discriminates micro-geographical heterogeneity of human exposure to *Anopheles* bites in low and
341 seasonal malaria areas. *Parasit Vectors*. 2013;6:68.

- 342 21. Remoue F, Cisse B, Ba F, Sokhna C, Herve J-P, Boulanger D, et al. Evaluation of the antibody
343 response to Anopheles salivary antigens as a potential marker of risk of malaria. *Trans R Soc Trop*
344 *Med Hyg.* 2006;100:363–70.
- 345 22. Ya-Umphon P, Cerqueira D, Parker DM, Cottrell G, Poinsignon A, Remoue F, et al. Use of an
346 Anopheles Salivary Biomarker to Assess Malaria Transmission Risk Along the Thailand-Myanmar
347 Border. *J Infect Dis.* 2017;215:396–404.
- 348 23. Paz-Soldan VA, Reiner RC, Morrison AC, Stoddard ST, Kitron U, Scott TW, et al. Strengths and
349 weaknesses of Global Positioning System (GPS) data-loggers and semi-structured interviews for
350 capturing fine-scale human mobility: findings from Iquitos, Peru. *PLoS Negl Trop Dis.* 2014;8:e2888.
- 351 24. Parker DM, Tripura R, Peto TJ, Maude RJ, Nguon C, Chalk J, et al. A multi-level spatial analysis of
352 clinical malaria and subclinical Plasmodium infections in Pailin Province, Cambodia. *Heliyon.*
353 2017;3:e00447.
- 354 25. Pepey A, Obadia T, Kim S, Sovannaroth S, Mueller I, Witkowski B, et al. Mobility evaluation by
355 GPS tracking in a rural, low-income population in Cambodia. *PLOS ONE.* 2022;:accepted manuscript.
- 356 26. Stoddard ST, Morrison AC, Vazquez-Prokopec GM, Paz Soldan V, Kochel TJ, Kitron U, et al. The
357 role of human movement in the transmission of vector-borne pathogens. *PLoS Negl Trop Dis.*
358 2009;3:e481.
- 359 27. Prosper O, Ruktanonchai N, Martcheva M. Assessing the role of spatial heterogeneity and human
360 movement in malaria dynamics and control. *J Theor Biol.* 2012;303:1–14.
- 361 28. Chaix B, Kestens Y, Perchoux C, Karusisi N, Merlo J, Labadi K. An interactive mapping tool to
362 assess individual mobility patterns in neighborhood studies. *Am J Prev Med.* 2012;43:440–50.
- 363 29. Chang H-H, Wesolowski A, Sinha I, Jacob CG, Mahmud A, Uddin D, et al. Mapping imported
364 malaria in Bangladesh using parasite genetic and human mobility data. *Elife.* 2019;8.
- 365 30. Fornace KM, Alexander N, Abidin TR, Brock PM, Chua TH, Vythilingam I, et al. Local human
366 movement patterns and land use impact exposure to zoonotic malaria in Malaysian Borneo. *eLife.*
367 2019;8:e47602.
- 368 31. Hast M, Searle KM, Chaponda M, Lupiya J, Lubinda J, Sikalima J, et al. The use of GPS data loggers
369 to describe the impact of spatio-temporal movement patterns on malaria control in a high-
370 transmission area of northern Zambia. *Int J Health Geogr.* 2019;18:19.
- 371 32. Pepey A, Souris M, Kim S, Obadia T, Mueller I, Witkowski B, et al. Comparing malaria risk
372 exposure in rural Cambodia population: GPS tracking versus questionnaires. submitted manuscript.
373 2022.
- 374 33. Pepey A, Souris M, Vantaux A, Morand S, Lek D, Mueller I, et al. Studying Land Cover Changes in
375 a Malaria-Endemic Cambodian District: Considerations and Constraints. *Remote Sensing.*
376 2020;12:2972.
- 377 34. Poinsignon A, Cornelié S, Mestres-Simon M, Lanfrancotti A, Rossignol M, Boulanger D, et al.
378 Novel Peptide Marker Corresponding to Salivary Protein gSG6 Potentially Identifies Exposure to
379 Anopheles Bites. *PLoS One.* 2008;3.

380 35. Pollard EJM, Patterson C, Russell TL, Apairamo A, Oscar J, Arcà B, et al. Human exposure to
381 Anopheles farauti bites in the Solomon Islands is not associated with IgG antibody response to the
382 gSG6 salivary protein of Anopheles gambiae. *Malar J.* 2019;18:334.

383 36. Drame PM, Poinsignon A, Besnard P, Cornélie S, Mire JL, Toto J-C, et al. Human Antibody
384 Responses to the Anopheles Salivary gSG6-P1 Peptide: A Novel Tool for Evaluating the Efficacy of
385 ITNs in Malaria Vector Control. *PLOS ONE.* 2010;5:e15596.

386 37. R Core Team. R: A language and environment for statistical computing. www.R-project.org. R. R
387 Foundation for Statistical Computing, Vienna, Austria; 2022.

388 38. Sanann N, Peto TJ, Tripura R, Callery JJ, Nguon C, Bui TM, et al. Forest work and its implications
389 for malaria elimination: a qualitative study. *Malaria Journal.* 2019;18:376.

390 39. Gryseels C, Durnez L, Gerrets R, Uk S, Suon S, Set S, et al. Re-imagining malaria: heterogeneity of
391 human and mosquito behaviour in relation to residual malaria transmission in Cambodia. *Malar J.*
392 2015;14:165.

393 40. Masunaga Y, Muela Ribera J, Nguyen TT, Tesfazghi K, Peeters Grietens K. In search of the last
394 malaria cases: ethnographic methods for community and private-sector engagement in malaria
395 elimination in Vietnam, Laos, and Cambodia. *Malar J.* 2021;20:370.

396 41. Bannister-Tyrrell M, Krit M, Sluydts V, Tho S, Sokny M, Mean V, et al. Households or Hotspots?
397 Defining Intervention Targets for Malaria Elimination in Ratanakiri Province, Eastern Cambodia. *The
398 Journal of Infectious Diseases.* 2019;220:1034–43.

399 42. Edwards HM, Chinh VD, Le Duy B, Thanh PV, Thang ND, Trang DM, et al. Characterising residual
400 malaria transmission in forested areas with low coverage of core vector control in central Viet Nam.
401 *Parasites & Vectors.* 2019;12:454.

402 43. Edwards HM, Sriwichai P, Kirabittir K, Prachumsri J, Chavez IF, Hii J. Transmission risk beyond the
403 village: entomological and human factors contributing to residual malaria transmission in an area
404 approaching malaria elimination on the Thailand–Myanmar border. *Malaria Journal.* 2019;18:221.

405 44. Van Bortel W, Trung HD, Hoi LX, Van Ham N, Van Chut N, Luu ND, et al. Malaria transmission and
406 vector behaviour in a forested malaria focus in central Vietnam and the implications for vector
407 control. *Malar J.* 2010;9:373.

408 45. Vantaux A, Riehle MM, Piv E, Farley EJ, Chy S, Kim S, et al. Anopheles ecology, genetics and
409 malaria transmission in northern Cambodia. *Scientific Reports.* 2021;11:6458.

410 46. Durnez L, Mao S, Denis L, Roelants P, Sochantha T, Coosemans M. Outdoor malaria transmission
411 in forested villages of Cambodia. *Malaria Journal.* 2013;12:329.

412

413

414 **SUPPLEMENTARY MATERIAL**415 Table S1: Primers sequences and real-time PCR conditions used to detect *Plasmodium* species.

PCR	Sequence (5'-3')	Master Mix	Assay parameters
Screening RTPCR	TGGAGTGGATGGTGTTTTAGA TTGCACCCCAATARCTCATTT	Hot FirePol EvaGreen qPCR Mix Solis Biodyne 1X (#08-24-00020), Primers 150 nM, 5 µL DNA template. Total volume 20 µL.	95°C-15 min; 45 cycles: 95°C-15 sec/60°C-20 sec/72°C-20 sec 95°C-2 min 68°C-2 min
Primary PCR	TGGAGTGGATGGTGTTTTAGA ACCTAAAGGATTTGTGCTACC	Hot FirePol DNA Pol. Solis BioDyne 1.25 U (#01-02-01000), dNTP 200 µM, MgCl ₂ 2.5mM, Primers 250 nM, 5 µl DNA template. Total volume 20 µL.	94°C -15 min 20 cycles: 94°C-30 sec/ 58°C -1 min/ 72°C-1 min 72°C-10 min
Nested RTPCR <i>P. falciparum</i>	ATGGATATCTGGATTGATTTTATTTATGA TCCTCCACATATCCAAATTACTGC		
Nested RTPCR <i>P. vivax</i>	TGCTACAGGTGCATCTCTTGTATTC ATTTGTCCCAAGGTAACG	Hot FirePol EvaGreen HRM Mix Solis Biodyne 1X (#08-33-00001), Primers 250 nM, 5 µL template Primary PCR products 1:10.	95°C-15 min 35–45 cycles*: 95°C-10 sec/62°C-20 sec/72°C-25 sec 95°C-1 min 40°C-1 min
Nested RTPCR <i>P. malariae</i>	ACAGTGCATCACTTGTATTTTTC TGCTGGAATTGAAGATAATAAATTAGTAATAACT		
Nested RTPCR <i>P. ovale</i>	GTTATATGGTTATGTGGAGGATATACTGTT CGAATGGAAGAATAAAATGTAGTACG	Total volume 20 µL.	
Nested RTPCR <i>P. knowlesi</i>	CTCCAGAAATTTCTTACGCATACTAC CCCAAGGTAAAACATAACCTATAAAA		

416 *35 cycles when nested real-time PCR, 45 cycles when real-time PCR alone.

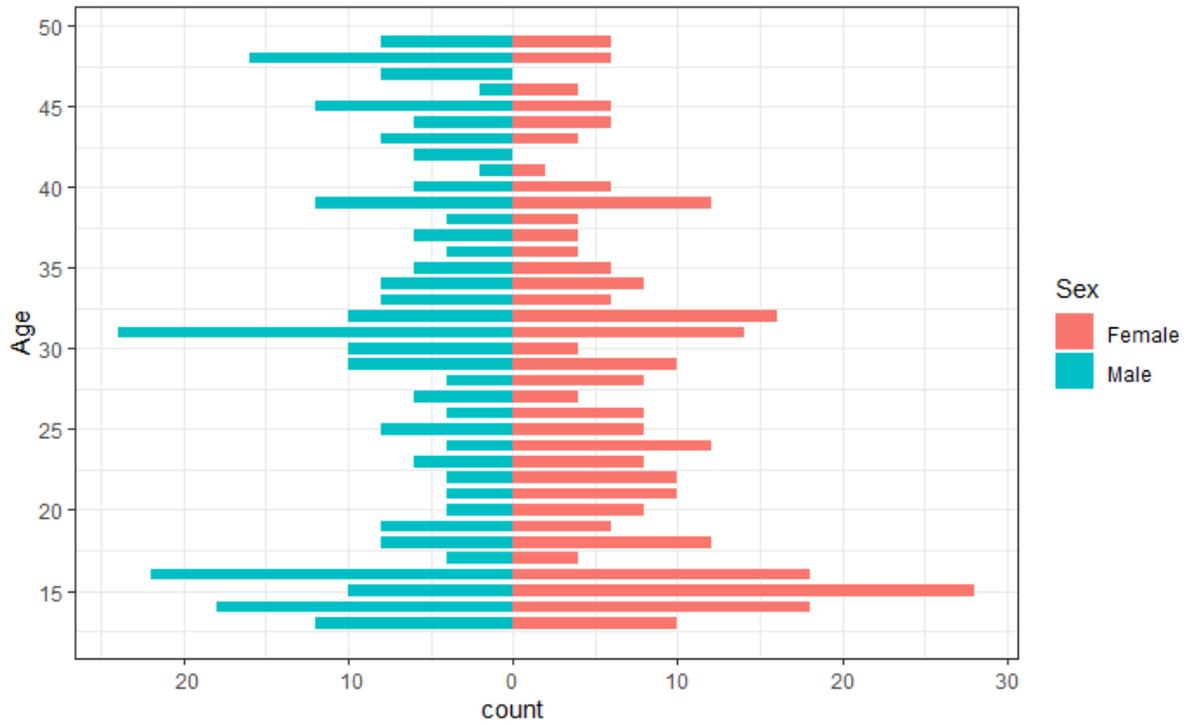
417

418 Protocol S1: Anti-*Anopheles* saliva quantification by ELISA.

419 The wells were coated with 100 µL of gSG6-P1 peptide solution (20µg/mg) for 2h30min at 37°C. Plate
420 wells were then blocked for 1h at 37°C using 200 µL of blocking buffer, then washed. Individual sera
421 were incubated in 100 µL duplicate at 4°C overnight at a 1/10 dilution for DBS eluate and 1/40 for
422 serum (in PBS—Tween 0.1%). After washing the plate, secondary human IgG in PBS-tween 1% (final
423 concentration 1/1000) was added and incubated at 37°C for 1h30min, then was washed again. The
424 signal peptide (final concentration 1/1000) was incubated at 37°C for 1h, then washed. Colorimetric
425 development was carried out using ABTS in 0.05M citrate buffer (pH = 4) containing 0.003% H₂O₂ and
426 incubated at room temperature for 2h. Absorbance (OD) was measured at 415 nm.

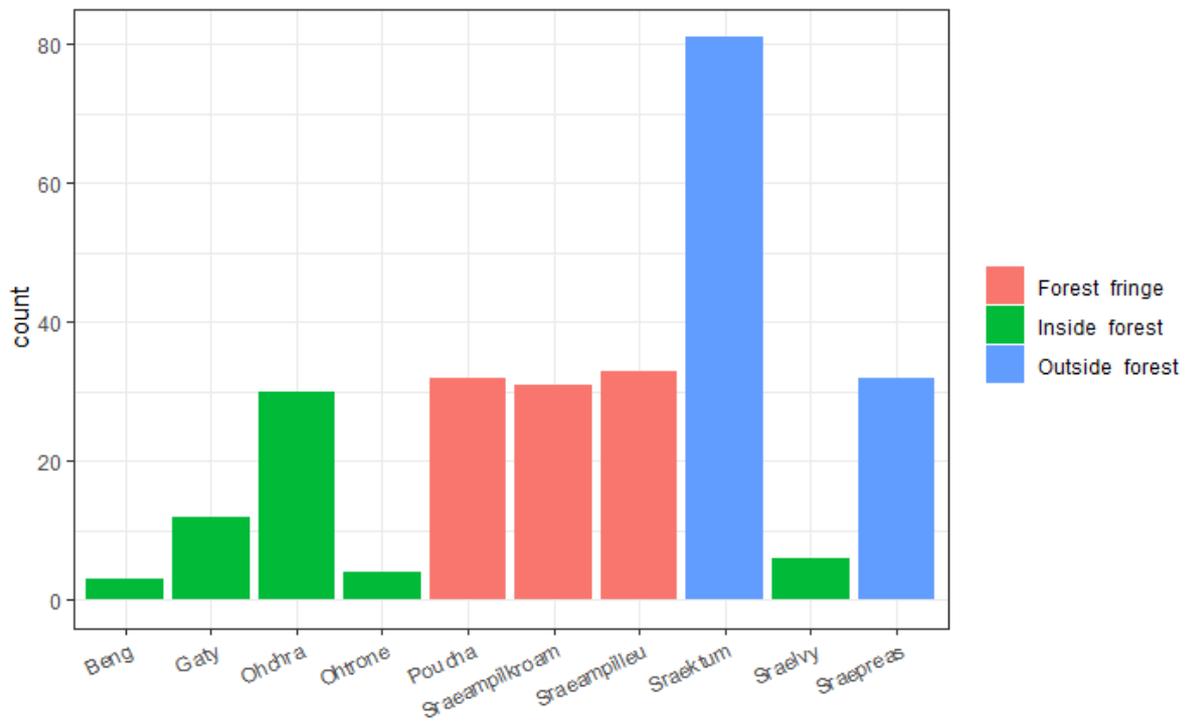
427

428 Figure S1: Age pyramid from baseline data (N = 301).



429

430 Figure S2: Count of participants per village of origin at baseline (N =301).



431

432 Table S2: Malaria prevalence and GLMMs results on sociodemographic variables.

Variable	N	Malaria prevalence	95% CI	χ^2	df	p
Sex	-	-	-	2.9	1	0.09
<i>female</i>	239	5.4	0.2	-	-	-
<i>male</i>	231	22.5	0.4	-	-	-
Age	-	-	-	0.0	2	0.98
<i>younger [≤ 20 years old]</i>	136	14	0.5	-	-	-
<i>mid [21-39 years old]</i>	250	13.2	0.3	-	-	-
<i>older [≥ 40 years old]</i>	84	15.5	0.8	-	-	-
Village category	-	-	-	0.5	2	0.76
<i>inside forest</i>	98	21.4	0.8	-	-	-
<i>forest fringe</i>	168	10.1	0.4	-	-	-
<i>outside forest</i>	204	13.2	0.3	-	-	-

433

434 Table S3: Average standardised hours spent in every environment at slow speed (< 5km/h) and at night
435 (18:00 to 6:00) with standard error (SE) by participants' malaria status and GLMMs results.

	Average standardised hours				GLMMs results		
	Malaria negative participants	SE	Seropositive participants	SE	χ^2	df	p
Mobility at night							
village	86.1	3.9	74.8	9.0	0.01	1	0.92
fields	59.3	3.6	69.8	9.0	0.26	1	0.61
plantations	11.8	1.9	11.0	4.9	0.001	1	0.98
forest	10.7	1.8	12.5	4.4	0.73	1	0.39
Mobility at slow speed							
village	83.8	3.6	73.9	8.5	0.01	1	0.94
fields	61.4	3.3	68.4	7.9	0.11	1	0.74
plantations	11.2	1.8	10.2	4.3	0.03	1	0.87
forest	11.4	1.7	15.4	4.3	0.48	1	0.49

436

437 Table S4: Seroprevalence and GLMMs results on sociodemographic variables.

Variable	N	Seroprevalence	95% CI	χ^2	df	p
Sex	-	-	-	1.7	1	0.19
<i>female</i>	239	37.7	0.4	-	-	-
<i>male</i>	231	43.3	0.4	-	-	-
Age	-	-	-	3.6	2	0.17
<i>younger [≤ 20 years old]</i>	136	37.5	0.7	-	-	-
<i>mid [21-39 years old]</i>	250	38.4	0.4	-	-	-
<i>older [≥ 40 years old]</i>	84	51.2	1.2	-	-	-
Village category	-	-	-	3.1	2	0.22
<i>inside forest</i>	98	45.9	1.0	-	-	-
<i>forest fringe</i>	168	42.3	0.6	-	-	-
<i>outside forest</i>	204	36.3	0.5	-	-	-

438

439 Table S5: Average standardised hours spent in every environment and GLMMs results.

	Female		Male		GLMMs		
	standardised hours	SE	standardised hours	SE	χ^2	df	p
village	87.9	4.7	76.3	4.7	0.70	1	0.40
fields	59.5	4.3	65.7	4.3	0.26	1	0.61
plantations	9.0	2.1	13.5	2.5	0.73	1	0.39
forest	11.3	2.3	12.5	2.2	0.002	1	0.97

440

441 Table S6: Characteristics of the nine participants that spent more than 100 standardised hours in the
 442 forest during both baseline and follow-up GPS tracking. Positive malaria status and serostatus are
 443 highlighted in bold. “(Sero)negative” and “(sero)positive” corresponds to participants that had the same
 444 (sero)status at both baseline and follow-up. A hundred hours represent 59.5% of a week.

Sex	Age	Village category	Malaria status	Serostatus
Female	14	Forest fringe	negative	seronegative
Female	24	Inside forest	negative	became negative
Female	29	Forest fringe	negative	seropositive
Female	29	Forest fringe	negative	seronegative
Female	29	Inside forest	became positive	became negative
Male	16	Forest fringe	negative	seronegative
Male	17	Forest fringe	positive	seropositive
Male	31	Forest fringe	negative	seronegative
Male	49	Forest fringe	negative	became positive

445

1.4. Discussion

This chapter tied together numerous approaches to understand the spatial complexity of fine-scale malaria transmission. First of all, by approaching the environment itself with the classification of land use, then the risk population through questionnaires and finally their interaction with the implementation of GPS loggers.

A land use or land cover classification was absolutely necessary to understand the impact of the environment on malaria risk: it was the most practical approach to obtain quantitative environmental data over the entirety of the study area. However, the land use we produced and used as a basis for our analyses was not optimal. Indeed, the land use classification was not categorising separately the different types of tree plantations, such as rubber and cashew nut or the types of fields, like cassava and rice. In most available literature, land cover classifications also aggregate these tree plantations into a single category, as in “forest plantation” or “orchard”. Also, the distinction between different types of fields can be difficult to define from satellite imagery alone: the variables detected by satellite imagery and used to determine the land cover and land uses classes (such as normalised difference vegetation index, surface reflectance, brightness temperature) can be very similar for different types of crops, making the discrimination between them possible at a cost of subsequent data availability, curated methodologies and dedicated workforce [270, 271]. However, the distinction might be critical: a specific type of crop could be linked to a higher exposure. Indeed, malaria parasite infections have been linked to rubber plantations [162, 272] and rice paddies [273] but not particularly to other types of tree plantations or crops.

Despite the limitations induced by the lack of distinction between plantations or fields types, a land use classification allowed to quantify environmental variables such as forest cover, agriculture surfaces or landscape fragmentation. Land use and land cover classifications require a better integration into epidemiological research in order to answer the important questions about the role of environmental variables on malaria transmission, among other vector-borne diseases [274]. For example, the standard definition of a forest comes from the Food and Agriculture Organisation of the United Nations (FAO) and applies to all regions: “A land area that is more than 0.5 hectares which contains trees higher than 5 metres and a canopy cover of more than 10%” [275]. However, forests around the world differ in their

structure and their exploitation, resulting in different malaria transmission characteristics: as such, the term “forest malaria”, which has been applied to Sub Saharan Africa, the Amazon, the Western Pacific and South East Asia, conveys different meanings [160, 276, 277]. In addition, key concepts such as “fringes areas” or “forest fringes” still lack an official – or at least standard – definition, while it is frequently used in malaria research without proper description of the intended meaning [34, 176, 278, 279].

Based on our results and our observations in Kaev Seima, a standard definition of a forest fringe could be defined based on, rather than a fixed size (such as the first 100 metres within a forest patch), the activities in said forest. As such, the fringes would correspond to the areas used for latrines or that are used to travel from a point to another. The deep forest would correspond to the area dedicated to foraging, hunting and wood logging. Determining these areas on a land use map would be challenging as it would require a combination of GPS points, questionnaires and, more importantly, could not be defined from satellite imagery alone. Overall, the term “forest fringes” might be misleading and inappropriate, at least in the context of this study. Objective and quantitative approaches such as forest patch size or individual diaries filled by participants could be more appropriate to categorise the forest type or the participants activity.

Questionnaires represented a major data acquisition tool throughout this chapter. They are considered a standard practice by many, as they are relatively cheap and easy to integrate into a field study, and produce a data type easy to process and analyse. If our results challenge their accuracy about individual mobility, questionnaires remain an irreplaceable tool to quantify other variables. Other than the obvious social, demographic and economic variables, questionnaires are able to gather data about behaviours and habits.

Mosquito protection use is central in malaria control in the GMS and its use by risk populations must be monitored. Although it is assumed that rural populations have access to and use mosquito nets in their household, it is equally important to know if they are used outside of the households, where individuals are exposed to exophilic vectors bites [56, 124]. During the years 2018-2019, participants were mostly not declaring mosquito protection in environments outside the village, as it had been observed before [152]. However, in the rare cases when the forest goers declared their use of protection in the deep forest, it appeared

that these participants were more likely to be seropositive for anti-*Anopheles* saliva antibodies. This quite counterintuitive result could be explained if the declared use of mosquito protection was associated to participants being more aware of mosquito bites, engaging in activities linked to increased bites exposure or simply being hosts that are more attractive to *Anopheles* mosquitoes. A similar association was found in Indonesian Papua where being malaria-positive was associated to the use of treated bed nets [61]. This association could arise from the potential over-reporting of patients' sickness in individuals more knowledgeable about malaria symptoms, a phenomenon also described in Africa [280–282]. A study from Malawi observed a similar association as the consequence of using the nets for different purposes such as fishing – an activity that can greatly increase risk exposure [283].

This result could be worrying, as the use of mosquito nets should decrease exposure. Vector control strategies could need to consider the mosquito net distribution and use to assess if it is effective. However, considering the very small sample size of this observation, further data on the matter should be collected before drawing any solid conclusion.

Finally, an important aspect of this chapter was the novel implementation of GPS data loggers to follow Cambodian populations at risk of malaria. Because resources were limited on their use on the GMS, and inexistent in Cambodia, using GPS trackers represented a challenge.

Firstly, choosing the study design and sample size was difficult, but was ultimately based on a trade-off between budget, volunteers' availability, feasibility and high population coverage. Then, before even analysing the content of the GPS trackers, getting them to withstand the fields conditions and to provide usable data required lots of efforts and experimentation. Indeed, when reviewing the literature before the GPS tracking implementation, no study mentioned the use of cases to protect the devices from external factors such as impacts or precipitations, and we had to note it ourselves. After our first implementation during the rainy season in 2018, with no protective enclosures, many devices came back full of water to the point of becoming rusty, or had broken upon impacts – one had also been chewed by a dog, but was still working (Figure 16).

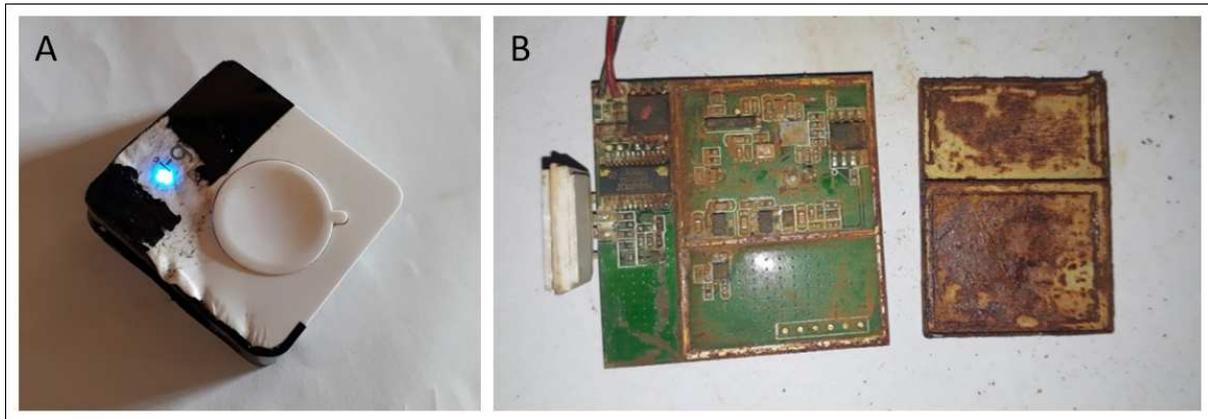


Figure 16: Two GPS devices collected after a few days in the field. A) A device still working after a brutal encounter with a dog. The black tape was used to hide the blinking lights, which was reported to be troublesome for participants trying to sleep. B) The components of a device after a week with a participant. Data could not be retrieved, even after thorough cleaning and numerous attempts.

After resolving the impermeability and suboptimal data issues, we had to understand how to use and analyse the spatial data itself. The hypothesis tested with the GPS follow-ups was that the time spent in forest was associated to malaria exposure and the observed malaria status of a participant. In both GPS follow-ups, such correlation was not found. Several hypotheses might explain this result. The first follow-up included only high-risk individuals, the forest goers. The study population was only composed of men between 13 and 60 years old, so the exposure to an infectious mosquito bite might be similar for all participants. Additionally, the GPS devices might simply not be representative of true mobility of many forest goers, which could purposely don't use them when going to specific forest locations, in a similar fashion than they avoid to disclose their visits to illegal wood logging locations during questionnaires [163]. Also, because the rate of deforestation is very important in Cambodia, and particularly in the study area [134], we can suggest that some of the patches represented as forest on our land use classification have already been cleared and replaced by crops. Finally, a finer scale might be required to make sense of the fragmented landscape including small fields, plantations and forest patches very close to each other.

Despite our results limitations, GPS trackers have demonstrated their ability to characterise mobility elsewhere and successfully helped to update control strategies. In Malaysia, Fornace

et al. [284], suggested that control programmes should include larval source management and target outdoor transmission rather than relying on treated bed nets and indoor residual spraying (IRS). In Zambia, the mobility patterns observed by Hast *et al.* [193] also indicated that they may decrease the efficacy of the focal IRS strategy. In Bangladesh, Chang *et al.* [15] pinpointed the region requiring a control strategy in complement to the interventions in forested areas. Therefore, the methodology still merits attention; it could be able to provide important fine-scale spatial data, but could be easier to interpret with another land cover/land use classification or by focusing on areas rather than types of land use.

Overall, this chapter was a quite ambitious part focusing on environmental, spatial and mobility variables through the lens of different methodologies, some completely new to Cambodia. Even if the outcome did not perfectly match out hypotheses, the documentation of the implementation process and the fine-scale description of rural mobility will provide a solid basis for future experiments taking on the challenge to understand the complex relationship between malaria, environment and mobility.

Across these five manuscripts and the generated data, we can assess that while *P. falciparum* malaria is rapidly decreasing in Cambodia, *P. vivax* prevalence remains difficult to estimate and to control, even with a national malaria elimination strategy focusing on testing and treating risk populations. Considering that more than 90% of infections are asymptomatic and risk factors are difficult to determine, elimination might require a considerable effort aiming at massive radical cure targeting *P. vivax* infections. To be effective, such intervention should be aimed at more than the usual “forest goers” demographic and across the whole GMS rather than locally. This could however induce or sustain drug resistance in *P. falciparum* parasites for which the “test-and-treat” approach remains relevant and efficient. Malaria in Cambodia has become a complex and twofold challenge, for which *P. falciparum* and *P. vivax* eliminations will respectively require different approaches for testing, tracking and treating across distinct timelines.

Chapter 2: Ecology and parasite prevalence in vectors

2.1. Introduction

Malaria vectors survival, fitness and reproduction depend on the ecology of their habitat, so environmental changes can drastically affect their abundance and their infectivity. As deforestation alters environmental factors such as vegetation composition, human population density, water bodies abundance, air and water temperature, etc., the indigenous *Anopheles* vectors are affected. Some species can adapt to the changing ecology or they can be replaced by a different, opportunist species that occupy the vacated niche.

The intensifying deforestation and the elimination measures implemented in Cambodia may affect the diversity, prevalence and abundance of malaria vectors. By conducting mosquito collections, we can measure these characteristics and attempt to determine to which environmental conditions they are associated.

Before the start of this PhD project, a mosquito collection had already provided important insights on the bionomics of vectors in the study area. Indeed, in 2017 and 2018, HDN traps were implemented for the first time in Cambodia, in Kaev Seima district. The collection aimed to determine vectors host preference between cattle and human baits, to measure their density and their prevalence across various environments and to identify insecticides resistance markers in the *Anopheles* population. These outcomes represented important data on their own; however, with the start of this project, we became interested in determining if there was a relation between vectors bionomics and anthropisation metrics. As such, we used a land cover classification to quantify the cover of different categories of environments (forest, cropland, tree plantations, etc.), the fragmentation and the diversity surrounding the different collection sites. This analysis provided a solid baseline that motivated the design of a second mosquito collection in the same area, with the objective to describe the evolution between these two time points. Additionally, this other collection included more collection sites across a greater variety of environments, with additional traps and an extended HDN collection. Besides, within the framework of our overall project in this area we were interested in linking the human densities and passage identified from the previous GPS follow-ups to the choice of mosquito collection sites. Finally, to grasp better the ecology of our studied vectors, we designed reproducible transects to collect mosquito larvae and

characterise which potential breeding are favourable for *Anopheles* reproduction (Appendix 1). Most of the methodologies we used during this mosquito collection were innovative in Cambodia and required extended testing and validation. Some of them, and especially the transects, needed an important investment of time for sometimes no exploitable results.

Still, these two mosquito collections provided consequential amounts of entomological data, most of it still being analysed.

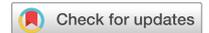
2.2. *Anopheles* bionomics in Cambodia

Article: *Anopheles* ecology, genetics and malaria transmission in northern Cambodia

Amélie Vantaux, Michelle M. Riehle, Eakpor Piv, Elise J. Farley, Sophy Chy, Saorin Kim, Anneli G. Corbett, Rachel L. Fehrman, Anaïs Pepey, Karin Eiglmeier, Dysoley Lek, Sovannaroth Siv, Ivo Mueller, Kenneth D. Vernick, Benoit Witkowski

Published in Scientific Reports on the 19th of March 2021.

<https://doi.org/10.1038/s41598-021-85628-1>



OPEN

Anopheles ecology, genetics and malaria transmission in northern Cambodia

Amélie Vantaux^{1,8}, Michelle M. Riehle^{2,8}, Eakpor Piv¹, Elise J. Farley², Sophy Chy¹, Saorin Kim¹, Anneli G. Corbett², Rachel L. Fehrman², Anais Pepey¹, Karin Eiglmeier^{3,4}, Dysoley Lek^{5,6}, Sovannaroeth Siv⁵, Ivo Mueller⁷, Kenneth D. Vernick^{3,4,9} & Benoit Witkowski^{1,9}

In the Greater Mekong Subregion, malaria cases have significantly decreased but little is known about the vectors or mechanisms responsible for residual malaria transmission. We analysed a total of 3920 *Anopheles* mosquitoes collected during the rainy and dry seasons from four ecological settings in Cambodia (villages, forested areas near villages, rubber tree plantations and forest sites). Using odor-baited traps, 81% of the total samples across all sites were collected in cow baited traps, although 67% of the samples attracted by human baited traps were collected in forest sites. Overall, 20% of collected *Anopheles* were active during the day, with increased day biting during the dry season. 3131 samples were identified morphologically as 14 different species, and a subset was also identified by DNA amplicon sequencing allowing determination of 29 *Anopheles* species. The investigation of well characterized insecticide mutations (*ace-1*, *kdr*, and *rdl* genes) indicated that individuals carried mutations associated with response to all the different classes of insecticides. There also appeared to be a non-random association between mosquito species and insecticide resistance with *Anopheles peditaeniatus* exhibiting nearly fixed mutations. Molecular screening for *Plasmodium sp.* presence indicated that 3.6% of collected *Anopheles* were positive, most for *P. vivax* followed by *P. falciparum*. These results highlight some of the key mechanisms driving residual human malaria transmission in Cambodia, and illustrate the importance of diverse collection methods, sites and seasons to avoid bias and better characterize *Anopheles* mosquito ecology in Southeast Asia.

Malaria remains a major public health challenge with an estimated 229 million cases recorded in 2019¹. However, while considerable progress has been made in the last decade in decreasing malaria burden, the emergence and spread of parasite drug and mosquito insecticide resistance threaten this achievement²⁻⁶. To counteract the problem of antimalarial drug resistance, the six countries of the Greater Mekong Subregion aim at eliminating malaria by 2030⁷. Since the launching of the Mekong Malaria Elimination in 2012, a six fold reduction in malaria cases has occurred with Cambodia accounting for 58% of the 239,000 malaria cases reported in 2019¹.

Malaria control is based on two pillars of rapid diagnostic tests and artemisinin-based combination therapies, and indoor vector control by using insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS). Consequently, residual malaria transmission, defined as the forms of transmission that can persist after achieving full universal coverage with effective ITNs and IRS⁸, can be maintained due to mosquito behavioral variation for example, malaria vectors biting mainly outdoors or when people are active. Thus, even an improved coverage and efficacy of IRS and ITNs will not achieve malaria elimination across most of the tropics due to a non-negligible proportion of vectors avoiding fatal contact with these interventions⁸. Residual transmission also maintains the possibility to cause a resurgence of malaria when the vector control program is weakened or withdrawn.

¹Malaria Molecular Epidemiology Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia. ²Department of Microbiology and Immunology, Medical College of Wisconsin, Milwaukee, WI, USA. ³Unit of Insect Vector Genetics and Genomics, Department of Parasites and Insect Vectors, Institut Pasteur, Paris, France. ⁴CNRS Unit of Evolutionary Genomics, Modeling, and Health (UMR2000), Institut Pasteur, Paris, France. ⁵National Center for Parasitology, Entomology and Malaria Control Program, Phnom Penh, Cambodia. ⁶School of Public Health, National Institute of Public Health, Phnom Penh, Cambodia. ⁷Malaria: Parasites and Hosts Unit, Institut Pasteur, Paris, France. ⁸These authors contributed equally: Amélie Vantaux and Michelle M. Riehle. ⁹These authors jointly supervised this work: Kenneth D. Vernick and Benoit Witkowski. ✉email: amelie.vantaux@gmail.com

Five classes of insecticides are currently recommended for vector control measures: carbamates, organochlorines, organophosphates, neonicotinoids and pyrethroids, with only the latter being approved for use in ITNs⁹. Following reports of insecticide resistance in malaria vectors, routine resistance monitoring has been set up in several countries. Two types of resistances can be found in vectors: behavioural avoidance and physiological or metabolic resistance. Among the mechanisms involved in physiological resistance, alteration of target site nerve receptors such as *kdr*, *Rdl* and *Ace1.R* have well characterized mutations allowing for efficient assessment of allelic frequencies in local vector populations¹⁰.

In the Greater Mekong Subregion, malaria cases have significantly decreased and are now mainly confined to pockets of transmission occurring along international borders and in forest or forest fringes¹¹. These residual foci of transmission are often populated by ethnic minorities, local populations and rural migrants exploiting new economic opportunities such as rubber plantations, mining and agriculture¹¹. In this context, prolonged visits to forest farms increase malaria exposure. Indeed, an overnight stay at the forest plot is one of the main malaria risk factors likely aided by often partially or completely open forest huts as well as variable sleeping time depending on economic activity^{12–17}. Since malaria entomological surveys classically collect vectors in villages, only a handful of studies have collected *Anopheles* mosquitoes at forest-fringes or forest sites^{16–20}. While a single study found similar mosquito densities at forest and village collection sites when comparing 12 coupled sites in three Cambodian provinces¹⁹, *Anopheles* mosquito densities are more typically reported as higher in the forest plots compared to the villages^{16–18}. Moreover, the number of infected mosquitoes is usually higher in or even exclusive to forest sites^{16,18,19}, supporting the increased malaria risk in forest sites.

The complexity of malaria transmission in the Greater Mekong Subregion is further amplified by a high diversity of vector species, including several sibling species only distinguishable molecularly, exhibiting a large spatial heterogeneity in distribution and behavioural patterns both between and within species and species complexes^{21–23}. Consequently, a deep understanding of the vectors involved in malaria transmission is extremely difficult without molecular species identifications, even though many studies have relied solely on morphological identifications likely due to the cost involved. Further blurring the pictures of malaria transmission is the tendency to limit malaria parasite screening only to presumptive malaria vectors, and to collections only on humans, or to forego screening altogether^{16,24–27}. These short-cuts also tend to underestimate the species involved in malaria transmission in the Greater Mekong Subregion. Nevertheless, on the rare occasions when thorough sampling and screening occurs, the results highlight a high diversity of malaria vectors including presumed zoophilic vectors^{28,29}, which is expected since most of the *Anopheles* mosquitoes in the Greater Mekong Subregion often exhibit zoophilic or opportunistic feeding behavior^{16,17,25}. In addition, zoophilic preferences are also reported in populations of the main malaria vectors such as *A. dirus* or *A. minimus*^{21,23,30–32}. Thus, in an era of malaria elimination in the Greater Mekong Subregion, embedded in a fast-changing environment, limiting screening to anthropophilic and/or classical primary vectors is likely to underestimate the importance of behaviourally opportunistic mosquitoes that could maintain residual malaria transmission, thereby decreasing the effectiveness of malaria elimination programs.

In sampling for malaria vectors, mosquitoes are traditionally collected between the times of 18:00 and 06:00 h, even though Southeast Asian (SEA) malaria vectors are mainly early- and outdoor biters^{14,22,33,34}. Nonetheless, some studies extended the collection time, usually between villages and forest sites^{18,19} or in the forest sites¹⁷ and even sometimes in villages¹⁴, to better understand human exposure to mosquito bites. Comparison of outdoor collection traps during day and night collections resulted in sampling only a few *Anopheles* during daytime³⁵, although a longitudinal study with 24 h collections found most of *A. barbirostris s.l.* mosquitoes collected during daytime²⁰. Altogether, these results suggest that longer sampling time of malaria vectors could produce a more comprehensive overview of human exposure to malaria mosquito bites.

Although efficient human disease vectors usually exhibit anthropophilic behavior (i.e. preference for humans as a source of blood) strong spatial, temporal and population variations are observed^{36,37}. Host-choice tests using either human or cow odor as alternative attractants allow researchers to assess the inherent host preference of mosquito vector populations. Paired-odor entry traps have been successfully used in the field to study host preferences^{37–40} and comparisons with odor baited double net traps showed similar anthropophily indices⁴¹. In addition, a study in the Lao PDR confirmed that human-baited double net traps collected similar number of *Anopheles* mosquitoes as the ‘gold standard’ human-landing catch method³⁵ which led us to use odor-baited traps to investigate mosquito bionomics in a Cambodian district. We investigated *Anopheles* mosquito bionomics in four ecological settings (villages, forested areas near villages, rubber tree plantations and forest sites) during the 2017 rainy season and the 2017–2018 dry season. Using paired odor-baited traps in a choice arrangement, we assessed odor-mediated mosquito host preference. We explored *Anopheles* activity rhythms by conducting 24 h collections. We screened the collected mosquitoes for carriage of malaria parasites to determine their role in disease transmission. Finally, we complemented morphological identification with DNA sequence-based identification on a subsample of mosquitoes and we investigated mutation patterns in known insecticide resistance genes.

Methods

Collection sites. The study was conducted in Kaev Seima district, Mondulkiri Province, in northeastern Cambodia. This province is characterized by hilly-forested areas and hotspots of ongoing malaria transmission. The main source of income in the district is based on agricultural activity such as subsistence farming and commercial plantations, details on malaria epidemiology were previously published⁴². Mosquito collections were carried out during the rainy season in July–August 2017 and during the dry season in December 2017–January 2018. Four different types of sites were sampled: villages (3 sites), forested areas near the villages (within approximately 200 m from the village, 3 sites), rubber tree plantations (3 sites) and forest sites (4 sites, but one site, initially forest site 3, was changed to forest site 4 during the dry season collection as logistics prevented access to

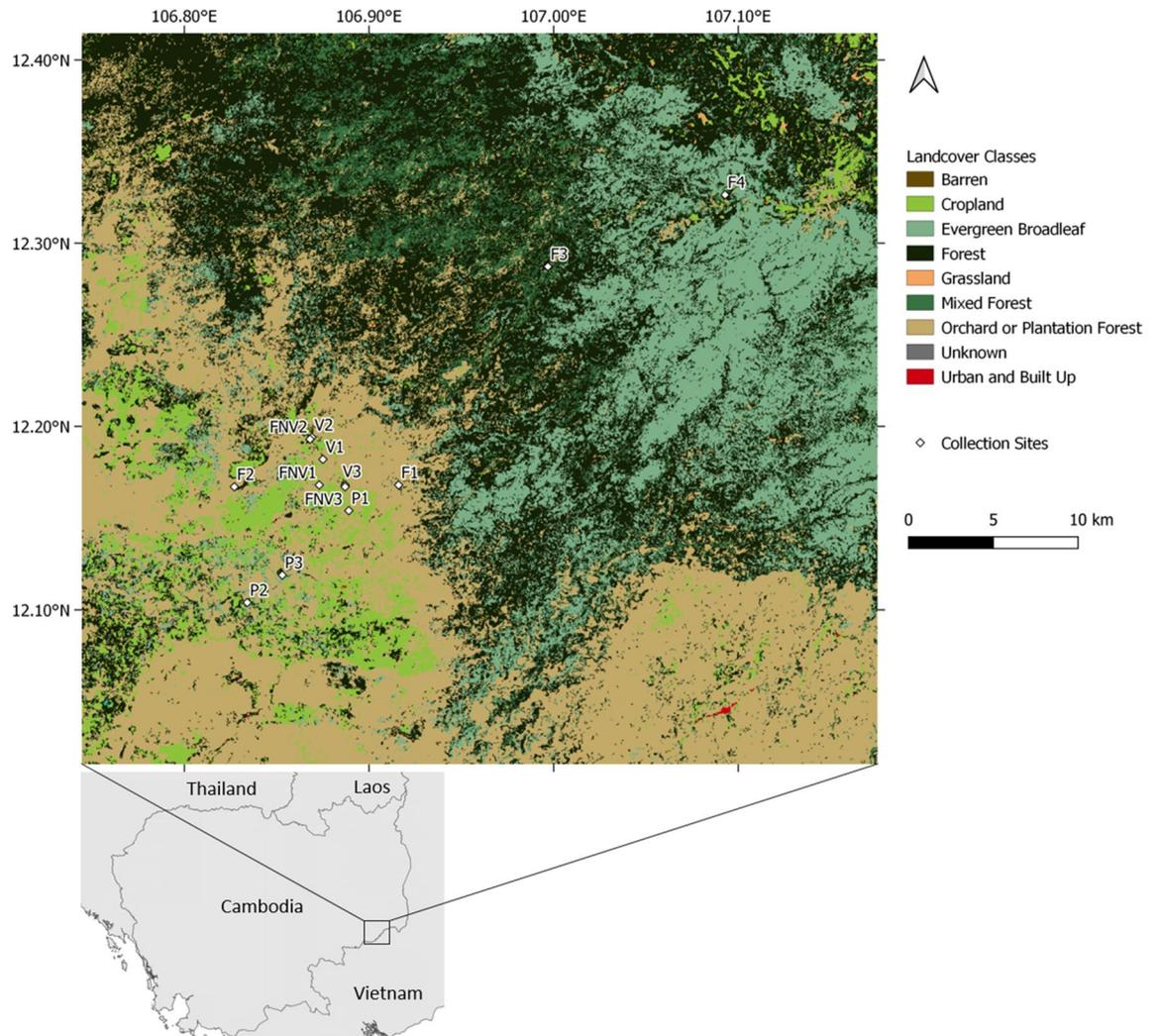


Figure 1. Map of the mosquito collection sites in Kaev Seima district, Mondulakiri, Cambodia. Land use map from Landsat satellite imagery retrieved from SERVIR Mekong (<https://landcovermapping.org/en/>). Map created in QGIS version 3.14.0 Pi (<https://www.qgis.org/en/site/>). Collection sites: F = forest site, V = village, FNV = forest area near the village, P = rubber tree plantation.

the original site) (Fig. 1). The forest sites were chosen based on interviews with recent *Plasmodium falciparum* symptomatic cases, all males between 14 and 47 years old who had spent time in the forest two weeks before their malaria symptoms.

Collection trap. Two odor-baited double net traps (BNT) as in Tangena et al.³⁵ baited with calf and human odors were used to assess the host preference of field populations of mosquitoes in the villages, forest sites and the forested areas near the villages. The two BNTs consisted of an outer untreated bed net (200 × 200 × 250 cm for the human BNT (HBNT) and 300 × 300 × 300 cm for the cow BNT (CBNT) from which each corner was raised 20 cm above ground and a smaller untreated inner bed net (180 × 180 × 250 cm for the HBNT and 280 × 280 × 280 cm for the CBNT). In the HBNTs, the two human volunteers rested on metal-framed beds (180 × 60 cm) and were protected from mosquito bites by the inner net. HBNTs and CBNTs were operated for 24 h for 2 days in each collection sites and were set up at the same place with the same volunteers. Trapped mosquitoes were retrieved every hour for 10 min using a mouth aspirator. Mosquitoes were transferred to paper cups covered with netting and labelled by date, type of trap, location and hour of collection. Cups were kept with a humid towel on top and brought back every day to the laboratory for further processing (see below). Only HBNTs were set up in the plantation sites to not interfere with rubber tree tapping. Two HBNTs per plantation site were set-up to investigate edge effects.

Laboratory processing of samples. All *Anopheles* sp. mosquitoes were morphologically identified using a taxonomic key⁴³. Following morphological identification, samples were preserved in 70% ethanol until molecular biology analyses. The mosquito heads and thoraxes were crushed individually and DNA was extracted using

the QIAamp DNA Mini kit, (Qiagen, Germany), according to the manufacturer's instructions. A two-step semi quantitative real-time PCR was performed to detect malaria parasites, as previously described⁴⁴.

Amplicon sequencing. To benchmark the accuracy of our morphological species called, as well as to refine species assignments within morphologically identical taxa, a subset of samples was sequenced to determine mosquito species. To generate the subset, we randomly selected an equal number of samples collected in the CBNTs to match the sample size of the HBNTs ($n=734$ samples). Among these 1468 (734 from HBNT and 734 from CBNT) samples we randomly selected a final set of 844 to represent the diversity of trap type, collection site and season. In addition, we used the same sequencing technique to complete the molecular species determination for an additional 79 *Plasmodium sp.* infected samples which were not part of the random selection, bringing to 923 the total number of samples molecularly screened.

Sequencing of ITS2 for molecular determination of species. Mosquito species was determined molecularly via PCR amplification and Sanger sequencing of the ribosomal DNA internal transcribed spacer region (ITS2; see supplementary information for PCR conditions). All sequence reads were obtained using standard Sanger sequencing methods and resulting sequence traces were visually verified prior to being submitted to nucleotide blast. Resulting sequences were blasted against the NCBI GenBank database. If a given sample gave at least 250 bps of clean sequence and the percent identity of the input ITS2 sequence with a single published (preferably voucher) sequence was 98% or higher, a species determination was made. We were able to confidently assign species for 774 of the 844 samples this way.

In the rare event samples where species determination was not possible (~8% of total, 67 samples/841 total samples with species calls) either using ITS2 sequence ($n=2$), or obtaining ITS2 sequence was technically problematic ($n=65$), amplification and sequencing of the mitochondrial cytochrome oxidase subunit 1 (CO1) gene was carried out using Sanger sequencing with quality control as for ITS2, discussed above (see supplementary information for PCR conditions). Two samples gave high quality ITS2 sequence and high quality CO1 sequence, but did not have a match in the database meeting species assignment parameters.

Sequencing of known insecticide resistance mutations. Of the 844 samples that were molecularly typed for species, a randomly chosen subset of 192 samples representative of species and collection location were sequenced for the presence of known insecticide resistance mutations for *ace-1* G119S, *rdl* A296S, and *kdr* L1014F (see supplementary information for PCR and sequencing details). These mutations were chosen as they are well described and have been associated with insecticide resistance^{45–47}. All sequence reads were obtained using standard Sanger sequencing methods and resulting sequence traces were visually verified prior to examination of known insecticide resistance mutations. All heterozygote calls were verified with a second PCR amplification and sequencing. The G119S mutation within the acetylcholinesterase subunit 1 (*ace-1*) gene has been previously shown to be associated with insecticide resistance⁴⁵. The A296S mutation in exon seven of the *rdl* gene has previously been shown to be associated with dieldrin resistance. The L1014F mutation in the Voltage Gated Sodium Channel (VGSC) gene has been previously shown to be associated with pyrethroid and organochlorine insecticide resistance⁴⁷.

This subset of 192 individuals represented the most abundant species (≥ 20 samples per species) in the original molecular typing of species in 844 samples. To examine insecticide mutation rates in rarer species we also typed the same three mutations in 68 individual samples representing 14 species and 2 unknown anopheline samples.

Landscape indices. Land use classification over the Keav Seima district was based on Landsat imagery (Landsat 4–8, 30 m resolution, 2017; <https://landcovermapping.org/en/>). A buffer zone of 2 km around each mosquito collection site was used to determine the percentage of the different land cover classes defined as: cropland, evergreen broadleaf, forests, mixed forests, plantation forests (see supplementary information for land cover class description).

Landscape metrics computation was performed on Fragstats software, version 4.2.1⁴⁸. To reflect landscape diversity and heterogeneity, we computed Shannon's diversity index (SHDI)⁴⁹, Simpson's diversity index (SIDI)⁵⁰, patch density (PD) and edge density (ED) at landscape level. SHDI and SIDI allow quantification of diversity (see supplementary information for details), whereas PD and ED indices are fragmentation indices quantifying heterogeneity. In addition, the percentage of landscape of each land cover category was computed at class level.

Human behaviour indicators. A cross-sectional survey of 4200 inhabitants from Kaev Seima district was carried out between November 2017 and April 2018⁴². A questionnaire was administered to each consenting household members from the randomly selected households. The set of questions regarding sleeping habits, bed nets usage and overnight sleeping in the working areas is summarized below.

Statistical analyses. *Day biting rate*—We tested the effect of collection site (forest, village, forest near the village, plantation), season (dry vs. rainy) and their interactions on the day biting rate (number of *Anopheles* collected between 6 am and 6 pm over the total number of females collected over 24 h) using a General Linearized Mixed Model (GLMM) with a binomial error structure and Site coded as a random factor to account for repeated measurements. Data for the CBNTs and HBNTs were analysed separately.

Malaria infection rates—We tested the effect of collection site, season and daytime biting as well as the interaction between collection site and season and the interaction between collection site and daytime on the prevalence of infectious mosquitoes in the CBNTs using a GLMM with a binomial error structure and Site coded as a random

Season	Dry		Rainy	
Trap	CBNT	HBNT	CBNT	HBNT
Forest				
IR	0.08	0.01	0.03	0.09
BR	96	47	96	35
AI	NA	0.67	NA	3
Forest near village				
IR	0.01	0	0.03	0.11
BR	25	5	162	9
AI	NA	0	NA	1
Plantation				
IR	NA	0	NA	0.05
BR	NA	3	NA	7
AI	NA	0	NA	0.33
Village				
IR	0.01	0	0.01	0.08
BR	15	1	138	6
AI	NA	0	NA	0.5

Table 1. Biting and infectious rates of *Anopheles* mosquitoes collected by HBNTs and CBNTs across sites and seasons. IR = infection rate (number of infectious mosquitoes/total number of mosquitoes), BR = average biting rate per trap per day, AI = average number of infectious bites per day (IRxBR). NA = non applicable.

factor to account for repeated measurements. The same GLMM was used for the HBNTs without testing for the interactions as an almost complete segregation of the data was observed (no infectious mosquitoes collected in all sites except the forest during the dry season) preventing the model from converging.

Mosquito host preference—The anthropophily index (AI) was expressed as the number of *Anopheles* sp. caught in the human-baited trap over the total number of mosquitoes caught in both human- and cow- baited traps. We assessed statistically the anthropophily indices only in the two species having a sufficient number of collected samples as well as a good agreement between morphological and molecular identifications (96.5% and 98.7% agreement for *A. dirus* and *A. kochi* respectively) for meaningful assessment since morphological identifications often encompass several species in the same group. We tested the effect of infection status (uninfected vs. infected with the sporozoite transmissible stages), season (dry vs. rainy) and time (day vs. night) on AI using a GLMM with a binomial error structure and Site coded as a random factor.

Frequencies of the molecularly identified species and the insecticide resistance mutations were compared using Chi-square contingency table.

For model selection, we used the stepwise removal of terms, followed by likelihood ratio tests. Term removals that significantly reduced explanatory power ($p < 0.05$) were retained in the minimal adequate model⁵¹. All analyses were performed in R v.3.0.3. Results are presented as proportion \pm 95% confidence interval.

Ethics statement. For the odor-baited double net traps capture of mosquitoes, the study protocol was reviewed and approved by the National Ethics Committee for Health Research, Ministry of Health, Kingdom of Cambodia (161NECHR). The study procedures, benefits and risks were explained to collectors and their informed consent was obtained. All collectors were followed and symptomatic subjects were administered a standard malaria rapid diagnostic test and referred to a local health care provider for treatment if positive.

The questionnaire from the cross-sectional survey was approved by the National Ethics Committee for Health Research, Ministry of Health, Kingdom of Cambodia (239NECHR) and by the Institut Pasteur Review Board (2017-03). Written informed consent to participate in the study was obtained from all participants or their parent or legal guardian.

All experiments were performed in accordance with relevant guidelines and regulations.

Results

Mosquito abundance, biting rate and morphological identifications. A total of 3920 *Anopheles* sp. females, 1167 and 2753 during the dry and rainy seasons respectively, were captured on a total of 60 collection days. Overall 81% (3187/3920) of the samples were collected in the cow odor-baited double net traps (CBNTs) and while this relative abundance was rather consistent between different collection sites for the CBNTs, 67% (490/733) of the *Anopheles* from the human odor-baited double net traps (HBNTs) were collected in the forest sites (Table S1).

The biting rate (# of females/trap/day) for the HBNTs was consistently higher in the forest sites compared to all other locations during both the rainy and the dry seasons (Table 1). However, for the CBNTs, while the biting rate was the highest in the forest sites during the dry season, the tendency changed during the rainy season with a higher biting rate in the villages and the forests near the villages compared to the forest sites (Table 1).

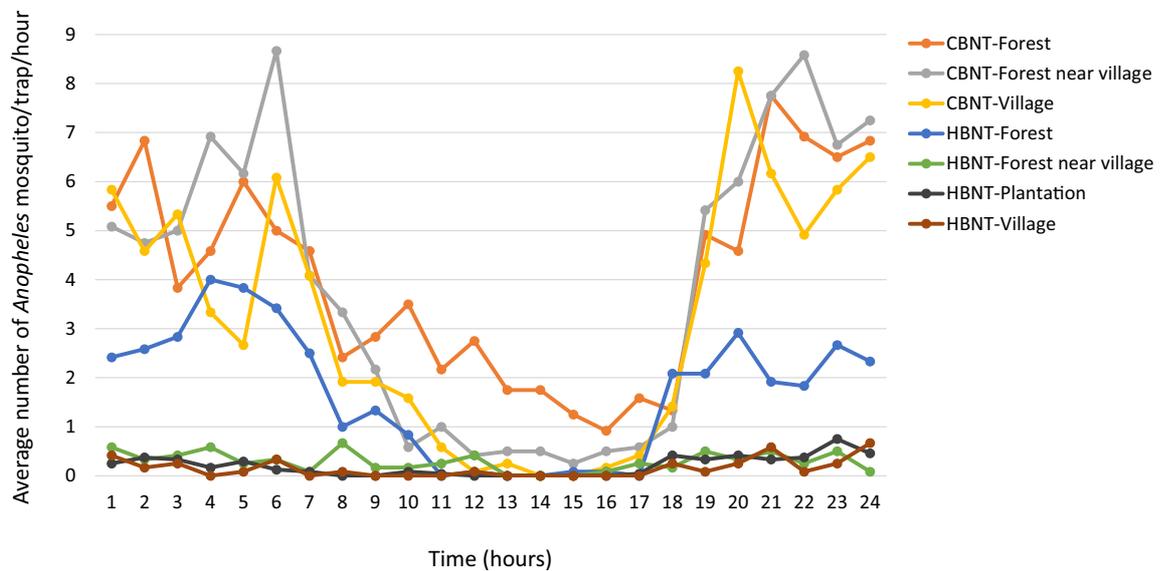


Figure 2. Average number of *Anopheles* females collected per trap per hour in the different collection sites in the HBNTs and the CBNTs.

A total of 3131 females were morphologically identified as 14 different *Anopheles* species or complexes of morphologically indistinguishable sibling species. Based on these morphological identifications, species thought to be primary vectors comprised only 10.2% of the collected mosquitoes: *Anopheles dirus* s.l. (8.1%, n = 319), *A. minimus* s.l. (0.4%, n = 15) and *A. maculatus* s.l. (1.7%, n = 67). The most abundant species (represented by more than a hundred individuals in our collection) constituted 75.8% of the collected *Anopheles* mosquitoes and were represented by 6 species complexes: *A. barbirostris* (21.2%, n = 831), *A. philippinensis* (14.6%, n = 571), *A. hyrcanus* (13.6%, n = 535), *A. kochi* (10.5%, n = 412), *A. dirus* (8.1%), *A. aconitus* (7.7%, n = 303).

Molecular determination of mosquito species. A total of 844 females were molecularly characterized for species in the random subset and represent 26 distinct *Anopheles* species as determined by ITS2 and CO1 (Table S2). The most abundant species (representing $\geq 5\%$ of the samples; $n \geq 42$) comprise 77.8% of the molecularly typed individuals and represent 8 species from 6 different species complexes. These most abundant species included *A. dirus* (13.2%, n = 112) from the Dirus complex. From the Barbirostris complex; *A. dissidens* (13.2%, n = 112), and *A. campestris-wejchochotei* (8.1%, n = 69). From the Hyrcanus Group, *A. peditaeniatus* (12.8%, n = 108), and *A. nitidus* (5.7%, n = 48). The Annularis, Funestus, and Kochi Groups were each represented by a single species *A. nivipes* (9.2%, n = 78), *A. aconitus* (6.7%, n = 57), and *A. kochi* (8.7%, n = 74), respectively. The 18 less abundant species, represent by fewer than 42 samples and in many cases just a handful of samples included *A. philippinensis* (n = 17) and *A. annularis* (n = 1) from the Annularis Group, *A. jamesii* (n = 16), *A. pseudojamesii* (n = 1), and *A. splendidus* (n = 1) from the Jamesii Group and *A. saeungae* (n = 29) and *A. barbirostris* (n = 2) from the Barbirostris Group. From the Hyrcanus Group *A. crawfordi* (n = 40), *A. argyropus* (n = 1), *An. nigerrimus* (n = 28), and *A. sinensis* (n = 3) were sampled. *Anopheles maculatus* (n = 22), *A. sawadwongporni* (n = 4), and *A. rampae* (n = 2) from the Maculatus Group. *Anopheles tessellatus* from the Tessellatus Group and *A. interruptus* from the Asiaticus Group were each sampled once. *A. vagus* (n = 12) and *A. karwari* (n = 3) were also present. There were 2 mosquitoes that had 99.9% identical ITS2 and 99.4% identical CO1 sequences but matched no species in the NCBI database. In addition to the random subset, 79 *Plasmodium* sp. infected samples were molecularly characterized for species which resulted in a total of 29 *Anopheles* species as determined by ITS2 and CO1.

Day biting rate. Overall $20.2 \pm 1.2\%$ of the *Anopheles* females were captured during the daytime (between 06:00 and 18:00). Indeed, while the majority of *Anopheles* mosquitoes bite at night, an important proportion was active during the day (Fig. 2). Excluding species with extremely low sample sizes and unidentified samples, day biting behaviour was observed for all the *Anopheles* species and varied from 13 to 68% (Table S3).

The day biting rate in the HBNTs was not different across collection sites ($19.6 \pm 2.9\%$; $\chi^2 = 3.6$, df = 3, $p = 0.3$; Fig. 3a) but was higher during the dry season ($25.9 \pm 4.6\%$) compared to the rainy season ($13.8 \pm 3.5\%$; $\chi^2 = 19.08$, df = 1, $p < 0.001$). There was an overall trend for a higher day biting rate during the dry season compared to the rainy season for all sites except in the forest near the villages where an opposite tendency was observed, however the interaction between collection sites and seasons was not significant ($\chi^2 = 7.5$, df = 3, $p = 0.057$).

The day biting rate in the CBNTs was higher during the dry season ($37.7 \pm 3.3\%$) compared to the rainy season ($14.5 \pm 1.4\%$; $\chi^2 = 115.53$, df = 1, $p < 0.0001$). There was no significant difference across the collection sites ($20.4 \pm 1.4\%$; $\chi^2 = 0.65$, df = 2, $p = 0.72$; Fig. 3b) nor of the interaction between collection sites and seasons ($\chi^2 = 1.97$, df = 2, $p = 0.37$).

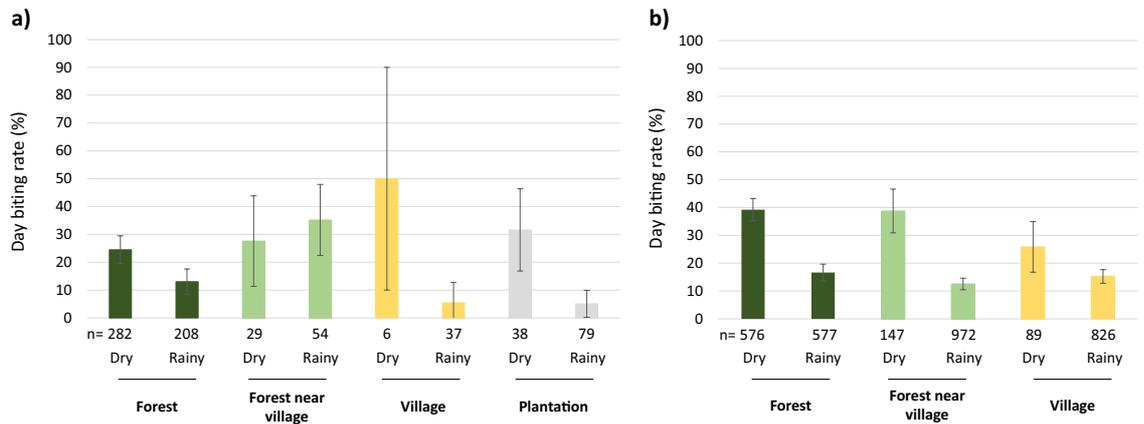


Figure 3. Day biting rate (proportion of *Anopheles* females collected between 6am and 6 pm) of mosquitoes captured in the different collection sites and seasons in (a) HBNTs and (b) CBNTs. The day biting rate was significantly higher during the dry season compared to the rainy season in both kind of traps (HBNTs $\chi^2 = 19.08$, $df = 1$, $p < 0.001$ and CBNTs $\chi^2 = 115.53$, $df = 1$, $p < 0.0001$). Data show average proportion \pm 95% confidence intervals.

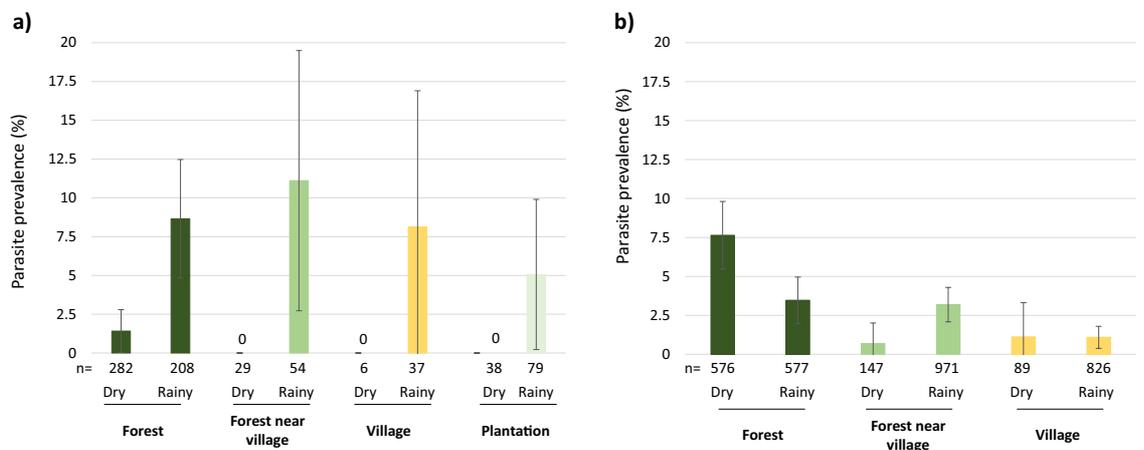


Figure 4. Malaria parasite prevalence in mosquitoes captured across different collection sites and seasons in (a) HBNTs and (b) CBNTs. Data show average proportion \pm 95% confidence intervals. Numbers below the bars indicate the total number of mosquitoes. Zero in the graph indicates no infected mosquitoes. Malaria parasite prevalence was significantly higher during the dry season compared to the rainy season in the HBNTs ($\chi^2 = 8.57$, $df = 1$, $p = 0.003$) whereas the opposite was observed in the CBNTs ($\chi^2 = 5.44$, $df = 1$, $p = 0.02$). In the CBNTs, malaria parasite prevalence was significantly affected by daytime ($\chi^2 = 8.13$, $df = 1$, $p = 0.004$) and the interaction between collection sites and seasons ($\chi^2 = 6.94$, $df = 2$, $p = 0.03$).

Malaria infection rates. Samples were screened to detect *Plasmodium* sp. in the head and thorax, thus indicating mosquitoes likely to be infectious due to carriage of sporozoites in the salivary glands (Fig. 4). Over the 3919 mosquitoes tested, 141 positive samples were found (3.6%; 122 *P. vivax*, 13 *P. falciparum*, 4 mixed *P. falciparum* and *P. vivax*, 1 mixed *P. falciparum* and *P. vivax* and *P. ovale*, 1 mixed *P. falciparum* and *P. inui*). In addition, 2 mosquitoes were found infected by *P. cynomologi* and 3 by *P. inui*, these non-human malaria parasites were not included in the following analyses. Malaria infection rate in HBNTs ($4.77 \pm 1.5\%$) was not significantly higher than in CBNTs ($3.33 \pm 0.62\%$; $\chi^2 = 3.19$, $df = 1$, $p = 0.07$). In the HBNTs, malaria infection rate was significantly higher during the rainy season ($8.2 \pm 2.8\%$) compared to the dry season ($1.13 \pm 1.1\%$; $\chi^2 = 8.57$, $df = 1$, $p = 0.003$; Odds Ratio = 5.04 [1.53–16.53]). *Plasmodium* sp. prevalence was not significantly correlated with collection site ($\chi^2 = 1.89$, $df = 3$, $p = 0.60$), nor with time of sampling ($\chi^2 = 0.22$, $df = 1$, $p = 0.64$).

In the CBNTs, the prevalence of *Plasmodium*-positive mosquitoes was significantly higher during the dry season ($5.66 \pm 1.6\%$) compared to the rainy season ($2.53 \pm 0.6\%$; $\chi^2 = 5.45$, $df = 1$, $p = 0.02$). Malaria infection rate was not significantly different across collection sites ($\chi^2 = 5.87$, $df = 2$, $p = 0.053$), although more infectious mosquitoes were collected in the forests ($5.55 \pm 1.32\%$) compared to the forest near the villages ($2.86 \pm 1\%$) and the villages ($1.1 \pm 0.7\%$). Malaria infection rate was higher in mosquitoes collected during daytime ($6 \pm 1.8\%$) compared to night time ($2.64 \pm 0.6\%$; $\chi^2 = 8.13$, $df = 1$, $p = 0.004$) regardless of the collection sites (daytime: collection site interaction: $\chi^2 = 3.28$, $df = 2$, $p = 0.19$). Malaria infection rate was significantly influenced by the interaction between collection sites and seasons ($\chi^2 = 6.89$, $df = 2$, $p = 0.03$) with opposite patterns in the malaria infection

Trap	Morphological identification	Molecular identification	Number of <i>Plasmodium</i> sp. positive samples	Season	Collection site and season
HBNTs	<i>A. barbiostris</i>	<i>A. dissidens</i>	2	Rainy	Forest near village rainy (2)
		<i>A. nigerrimus</i>	1	Rainy	Plantation rainy (1)
	<i>A. dirus</i>	<i>A. dirus</i>	21	Dry (4), rainy (17)	Forest dry (4), forest rainy (14), forest near village rainy (2), plantation rainy (1)
		<i>A. nitidus</i>	1	Rainy	Forest rainy (1)
		Unidentified	2	Rainy	Forest rainy (2)
	<i>A. hyrcanus</i>	<i>A. peditaeniatus</i>	3	Rainy	Forest near village rainy (1), village rainy (2)
	<i>A. kochi</i>	<i>A. kochi</i>	1	Rainy	Forest rainy (1)
	<i>A. maculatus</i>	<i>A. maculatus</i>	1	Rainy	Plantation rainy (1)
	Unidentified	<i>A. maculatus</i>	1	Rainy	Plantation rainy (1)
		<i>A. nitidus</i>	1	Rainy	Forest near village rainy (1)
<i>A. sinensis</i>		1	Rainy	Village rainy (1)	
CBNTs	<i>A. aconitus</i>	<i>A. vagus</i>	2	Rainy	Village rainy (1), forest near village rainy (1)
		<i>A. aconitus</i>	9	Dry	Forest dry (8), village dry (1)
	<i>A. barbiostris</i>	<i>A. campestris—wejchoochotei</i>	4	Dry	Forest dry (4)
		<i>A. dissidens</i>	17	Dry (11), rainy (6)	Forest dry (11), forest rainy (2), forest near Village rainy (3), village rainy (1)
		<i>A. peditaeniatus</i>	2	Rainy	Forest near village rainy (2)
	<i>A. dirus</i>	<i>A. saeungae</i>	1	Dry	Forest (1)
		<i>A. dirus</i>	3	Rainy	Forest rainy (3)
	<i>A. hyrcanus</i>	<i>A. aconitus</i>	1	Rainy	Forest near village rainy (1)
		<i>A. crawfordi</i>	6	Dry	Forest dry (6)
		<i>A. nigerrimus</i>	4	Dry (1), rainy (3)	Forest near village rainy (1), forest dry (1), forest rainy (2)
		<i>A. nitidus</i>	4	Dry (3), rainy (1)	Forest dry (3), forest rainy (1)
		<i>A. peditaeniatus</i>	11	Rainy	Forest rainy (3), forest near village rainy (7), village rainy (1)
	<i>A. insulaeflorum</i>	<i>A. peditaeniatus</i>	2	Rainy	Forest near village rainy (2)
	<i>A. jamesii</i>	<i>A. jamesii</i>	1	Rainy	Forest near village rainy (1)
		<i>A. pallidus</i>	2	Rainy	forest rainy (2)
	<i>A. kochi</i>	<i>A. kochi</i>	11	Dry (5), rainy (6)	Forest dry (5), forest rainy (5) forest near village rainy (1)
		<i>A. karwari</i>	1	Rainy	Forest rainy (1)
	<i>A. maculatus</i>	<i>A. maculatus</i>	2	Dry (1), rainy (1)	Forest near village dry (1), forest near village rainy (1)
		<i>A. rampae</i>	1	Dry	Forest dry (1)
		<i>A. sawadwongporni</i>	2	Dry	Forest dry (2)
	<i>A. philippinensis</i>	<i>A. jamesii</i>	2	Rainy	Forest near village rainy (1), village rainy (1)
		<i>A. nivipes</i>	7	Rainy	Forest near village rainy (3), village rainy (4)
	Unidentified	<i>A. dirus</i>	1	Rainy	Forest rainy (1)
		<i>A. dissidens</i>	1	Rainy	Forest rainy (1)
		<i>A. minimus</i>	1	Dry	Forest dry (1)
		<i>A. nigerrimus</i>	2	Rainy	Forest near village rainy (2)
		<i>A. peditaeniatus</i>	6	Rainy	Forest near village (5), village (1)

Table 2. Individual *Plasmodium* sp.-positive *Anopheles* specimens collected by HBNTs and CBNTs across sites and seasons. Sample sizes are given in parentheses.

rates observed during the dry and the rainy seasons in the forests and the forest near the villages ($\chi^2 = 6.51$, $df = 1$, $p = 0.032$, all other post-hoc comparisons were non-significant; Fig. 4).

The infectious mosquitoes collected in the HBNTs belonged to 8 different mosquito species (molecular identification; Table 2). Interestingly, an additional 12 *Anopheles* species were also found infected by human malaria parasites among the mosquitoes collected in the CBNTs (*A. aconitus*, *A. campestris-wejchoochotei*, *A. crawfordi*, *A. jamesii*, *A. karwari*, *A. minimus*, *A. nivipes*, *A. pallidus*, *A. rampae*, *A. saeungae*, *A. sawadwongporni*, *A. vagus*),

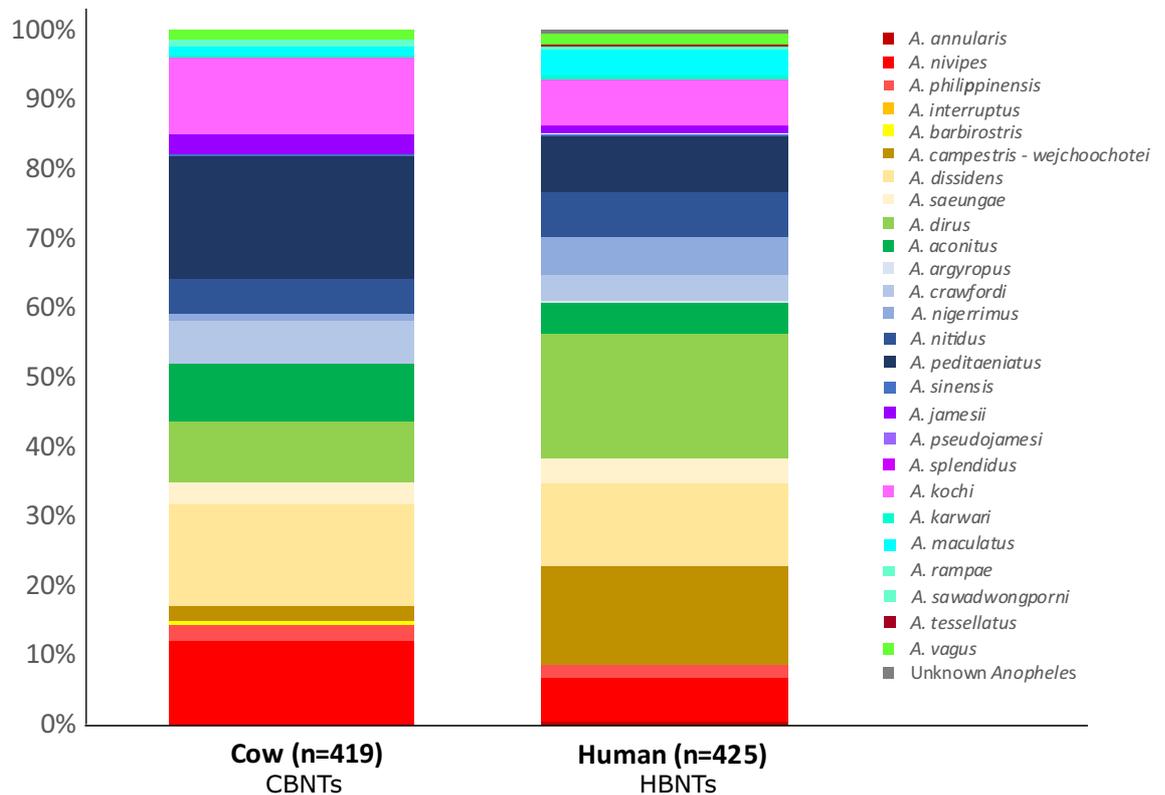


Figure 5. Proportions of the molecularly identified samples in the CBNTs and HBNTs.

bringing to 20 the total number of malaria vectors collected in this study among the 29 species molecularly identified (Table 2).

Mosquito host preferences. We assessed the inherent mosquito host preference by calculating the anthropophily index (AI) for the different species, defined as the number of *Anopheles sp.* individuals caught in the human-baited trap over the total number of mosquitoes caught in both human- and calf-baited traps (see Methods). Most of the collected mosquitoes were zoophilic (AI < 50) or generalist (AI ~ 50) including *A. dirus* or *A. maculatus* mosquitoes (Table S4).

Anopheles dirus—A total of 319 *An. dirus* females were collected, mainly in the forest sites ($n = 303$). Human *Plasmodium sp.* prevalence was $8.46 \pm 3\%$ overall and $7.9 \pm 3\%$ in the forest sites. The anthropophily index was calculated in the forest sites only and was $48.51 \pm 5.63\%$. Infectious mosquitoes were significantly more anthropophilic ($87.5 \pm 13.23\%$) than uninfected mosquitoes ($45.16 \pm 5.84\%$; $\chi^2 = 21.56$, $df = 1$, $p < 0.0001$). *Anopheles dirus* females were more anthropophilic during the dry season ($51.83 \pm 7.65\%$) compared to the rainy season ($44.6 \pm 8.26\%$; $\chi^2 = 7.19$, $df = 1$, $p = 0.008$). There was no difference in the anthropophily index of *A. dirus* collected during the day or night time ($\chi^2 = 3.39$, $df = 1$, $p = 0.066$).

Anopheles kochi—A total of 412 *A. kochi* females were collected with more than half collected in the forest sites ($n = 257$). Human *Plasmodium sp.* prevalence was $3.15 \pm 1.69\%$ overall and $4.67 \pm 2.58\%$ in the forest sites. There was no significant effect of season ($\chi^2 = 1.98$, $df = 1$, $p = 0.16$) or infection status ($\chi^2 = 0.67$, $df = 1$, $p = 0.41$) on the anthropophily index of *A. kochi*. *Anopheles kochi* females were significantly more anthropophilic during the daytime ($25.64 \pm 9.69\%$) compared to night time ($8.98 \pm 3.07\%$; $\chi^2 = 8.53$, $df = 1$, $p = 0.003$).

Out of the random subset 26 species were molecularly identified, among which 19 species were collected in both HBNTs and CBNTs although in variable proportions (Fig. 5). Indeed, *A. annularis* ($n = 2$), *A. argyropus* ($n = 1$), *A. splendidus* ($n = 1$) and *A. tessellatus* ($n = 1$) were only observed in the CBNTs, whereas *A. barbirostris* ($n = 2$), *A. interruptus* ($n = 1$) and *A. pseudojamesi* ($n = 1$) were only observed in the HBNTs. Among the 19 species collected in both type of traps, 3 species were enriched in the CBNTs—*A. nivipes* ($n = 78$, $\chi^2 = 8.332$, $df = 1$, $p = 0.039$), *A. peditaeniatus* ($n = 108$, $\chi^2 = 17.334$, $df = 1$, $p < 0.0001$), *A. kochi* ($n = 74$, $\chi^2 = 4.944$, $df = 1$, $p = 0.026$)—whereas 2 species were enriched in the HBNTs—*A. campestris-wejchoochetei* ($n = 69$, $\chi^2 = 40.678$, $df = 1$, $p < 0.0001$), *A. dirus* ($n = 110$, $\chi^2 = 16.413$, $df = 1$, $p < 0.0001$).

Insecticide resistance mutations. 192 mosquitoes were genotyped by amplicon sequencing for known mutations associated with resistance to different classes of insecticides: pyrethroids, carbamates, organochlorides, and organophosphates. We genotyped previously characterized mutations in *ace-1*, *kdr*, and *rdl* genes, G119S⁴⁵, L1014F⁴⁵ and A296S⁵², respectively. Of the 192 individuals, 187 were successfully genotyped for G119S, 189 for L1014F, and 191 for A296S. The few missing genotypes were due to PCR amplification failure. Twenty-

seven individuals (14%) carried the G119S mutation, none of which were heterozygotes. Nineteen (10%) individuals carried the L1014F mutation, five of which were heterozygotes. Twenty-nine (15%) individuals carried the A296S mutation, seven of which were heterozygotes. There were four species (of 109 total) that carried resistant mutations. Two *A. dirus* (of 31 total) carried the *rdl* A296S mutation, a single *A. kochi* individual carried the *ace-1* G119S mutation and an additional *A. kochi* individual carried the *rdl* A296S mutation (18 *A. kochi* individuals total). Two *A. nivipes* individuals carried the *rdl* A296S mutation (21 total). Interestingly, a single species, *A. peditaeniatus* was significantly overrepresented for all resistant genotypes (*ace-1*: $\chi^2 = 179$, $df = 1$, $p = 8.01 \times 10^{-41}$, *kdr*: $\chi^2 = 132.4$, $df = 1$, $p = 1.22 \times 10^{-30}$, *rdl*: $\chi^2 = 168.1$, $df = 1$, $p = 1.92 \times 10^{-38}$). *A. peditaeniatus* accounted for 96% of individuals carrying the *ace-1* mutation (26/27), 100% of individuals with the *kdr* mutation (19/19), and 83% of individuals with the *rdl* mutation (24/29); despite the fact that it comprised only 13% (26/192) of the sample set genotyped. To gain a better understanding of this apparent enrichment of known insecticide resistance mutations in *A. peditaeniatus*, we returned to the larger sample set ($n = 844$) and genotyped the additional 82 individuals not included in the representation plates, for a total of 108 genotyped *A. peditaeniatus*. When resistant allele frequencies were compared between the *A. peditaeniatus* in the representation plates ($n = 26$) and all other *A. peditaeniatus* ($n = 82$), there was no statistically significant difference (*ace-1*: $\chi^2 = 1.69$, $df = 1$, $p = 0.2$, *kdr*: $\chi^2 = 0.01$, $df = 1$, $p = 0.91$, *rdl*: $\chi^2 = 0.78$, $df = 1$, $p = 0.38$), indicating that the representation plates accurately represented the larger dataset.

Of the 108 total *A. peditaeniatus*, 106 carried the *ace-1* G119S mutation (3 heterozygotes; 103 homozygotes), 78 carried the *kdr* L1014F mutation (22 heterozygotes; 56 homozygotes), and 104 carried the *rdl* A296S mutation (12 heterozygotes, 92 homozygotes; Fig. 6). The presence of the *ace-1* mutation, either homozygote or heterozygous was also a strong predictor of whether that individual was also resistant to other insecticides as 97% of individuals that carried the *ace* G119S mutation also had at least one other resistance mutation, homozygote or heterozygote. For the L1014F mutation, the distribution of alleles by ecological site was not uniform (Fig. 7). The L1014F *kdr* resistant allele was significantly more common in the Forests Near Village 2 and 3 ($\chi^2 = 4.0$, $df = 1$, $p = 0.04$ and $\chi^2 = 5.1$, $df = 1$, $p = 0.02$ respectively) as compared to all other sites. Conversely, Forest 2 had a significantly greater proportion of the susceptible *kdr* L1014F allele ($\chi^2 = 4.5$, $df = 1$, $p = 0.03$). The frequency of the *ace-1* G119S mutation was significantly greater than the other mutations (*ace-1* vs *kdr*: $\chi^2 = 76$, $df = 1$, $p = 2.76 \times 10^{-18}$, *ace* vs *rdl*: $\chi^2 = 7.8$, $df = 1$, $p = 5.3 \times 10^{-3}$). Additionally, the frequency of the A296S *rdl* resistant allele was greater than the frequency of the L1014F *kdr* resistant allele ($\chi^2 = 45.2$, $df = 1$, $p = 1.8 \times 10^{-11}$). All heterozygotes were verified by amplifying and resequencing the necessary fragment and visualizing the resultant sequence trace files.

We also examined the frequency of the *kdr* L1014F, *ace-1* G119S and *rdl* A296S insecticide resistance mutations in rarer mosquito species that were detected in the random subset of 844 samples molecularly typed for species. These 68 mosquito samples of rarer species represent 14 species and 2 unknown Anopheline samples (each species represented by 1–17 individuals; median = 2; Supplementary Table S5). In these rare species, rates of resistance alleles were high for *rdl* A296S with resistance being present in 8 of the 14 species and in the unknown Anopheline. In contrast no mosquitoes carried the L1014F mutation in *kdr* and only *A. sinensis* carried the *ace-1* G119S mutation.

Landscape indices. A 2 km buffer zone around each mosquito collection site was used to determine the percentage of the different land cover classes present and showed high variability between sites (Fig. 8a). Patches of evergreen forest, forest and plantation forest were observed in all sites. Croplands were observed in all buffer zones, from 0.16% in Forest 3 up to 40.2% in Plantation 1, however the largest human-made land cover class was represented by plantation forests, from 7.5% in Forest 4–89.7% in Forest 1. While more *A. dirus* were observed in Forests 3 and 4 ($n = 122$ and $n = 92$ respectively), it was still present in the more degraded landscapes of Forests 1 and 2 ($n = 71$ and $n = 18$). The most conserved forest sites (Forests 3 and 4) were also associated with lower densities and lower diversities of mosquitoes as also observed in Plantations 2 and 3 (Fig. 8b).

Although Forests 3 and 4 had high proportions of natural forests, their high PD and ED values indicate more fragmented landscapes than Forests 1 and 2. Patch richness only varies between 4 and 5 types of land cover classes therefore an increase value of SHDI and SIDI corresponded to an increase evenness between patch types, with the highest patch evenness observed in Forests 2, 3 and 4 as well as Plantations 2 and 3. Villages and forest near the villages exhibited uneven patch distribution associated with fragmented landscapes.

Human behaviour indicators. More than half of the villagers reported going to bed between 19:00 and 20:00 pm and using a bed net the previous night (92%; Table S6). However, only 41% were insecticide-treated bed nets. More than three quarters of the villagers were working in cashew plantations and cassava fields at the time of the study (77%, Table S6). Interestingly, sleeping overnight in the field sites was reported in 3.7–7.81% of the cases, with a vast majority reporting the use of bed nets, whereas 77.9% of people working in the forest sites also slept there with only 25% of them using a bednet (Table S6).

Discussion

This mosquito collection across ecological zones in northern Cambodian shows a high *Anopheles* mosquito diversity with morphological identification of 14 species complexes and 29 species in the subset of genotyped and molecularly identified mosquitoes ($n = 923$). A recent study in Cambodia found similarly high mosquito diversity although the 27 species were collected over three different provinces²⁸. Three species were unique to the latter collection while 5 other species were unique to our collection, further highlighting the high *Anopheles* mosquito diversity in Cambodia. Here, despite sampling from only 13 sites across a 25 × 30 km area in Monduliri district, our results indicate both high mosquito diversity as well as highly variable mosquito densities. Although the sites chosen for mosquito sampling were in close proximity (average distance between any two

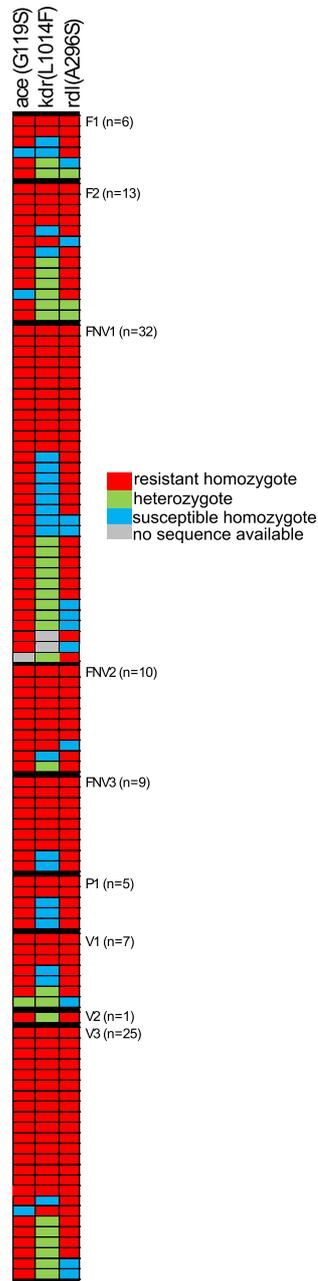


Figure 6. Insecticide resistance mutations are very frequent in *Anopheles peditaeniatus*. All *A. peditaeniatus* ($n = 108$) in the molecularly typed set of 844 are depicted here sorted by their collection location and within collection site by their insecticide resistance genotypes. Each row of three boxes represents genotype data from a single *A. peditaeniatus* individual. Out of 108 *A. peditaeniatus*, 106 carried the G119S mutation (3 heterozygotes, 103 homozygotes), 78 carried the L1014F mutation (22 heterozygotes, 56 homozygotes), and 104 carried the A296S mutation (12 heterozygotes, 92 homozygotes). Forty nine percent (53/108) of individuals have homozygous resistant genotypes for all 3 loci and an additional 23% (25/108) carry at least one resistant allele for each of the 3 insecticide loci typed. Of the remaining 28% of individuals (30/108), 28 carried the homozygous susceptible allele at the L1014F *kdr* mutation (2 individuals with no available sequence), 17 were homozygous for the resistant allele at both *ace-1* and *rdl*, another 7 individuals were homozygous for *ace* G119S and heterozygous for *rdl* A296S. A single individual was homozygous for the *rdl* resistant mutation and heterozygous for the *ace-1* mutation. Five individuals carried a resistance mutation at only one of the 3 loci tested, 3 were *ace-1* mutation homozygotes, 1 was heterozygous for *rdl* resistance and 1 was homozygous for *rdl* resistance with no sequence available for *ace-1* resistance.

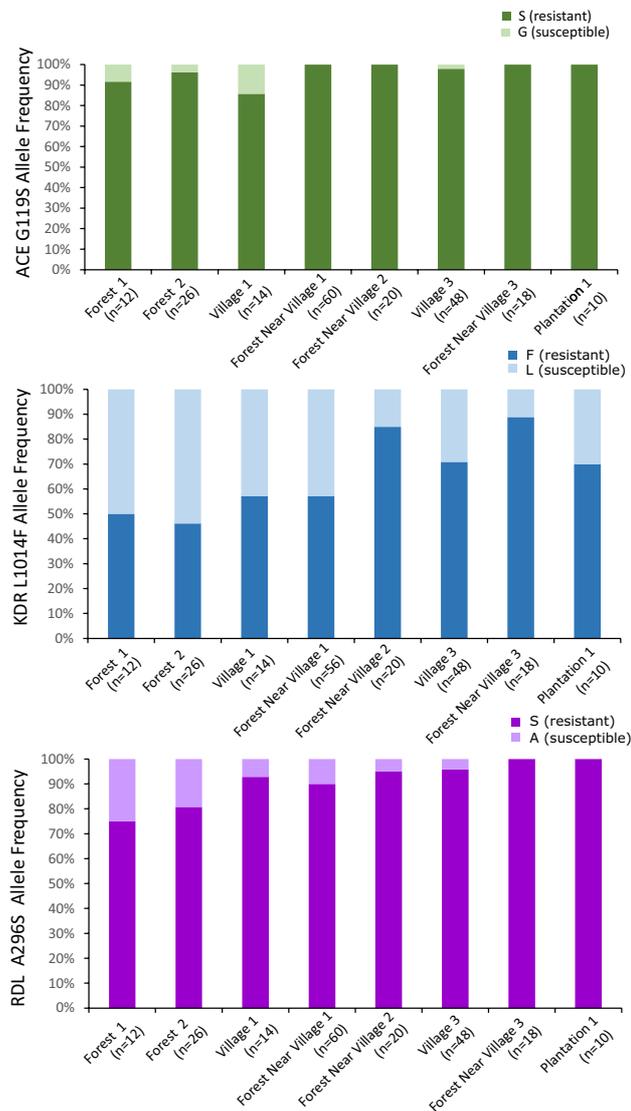


Figure 7. Geographic distribution of insecticide resistance mutations in *A. peditaeniatus*. Allele frequencies for *ace-1* G119S (top), *kdr* L1014F (middle) and *rdl* A196S (bottom) are shown as a function of sampling location. For *kdr* there is a detectable geographic pattern with the Forest site 2 exhibiting significantly ($p=0.03$) higher susceptible allele frequency than all other sites combined, while Forest near Village 2 ($p=0.04$) and 3 ($p=0.02$) exhibit significantly higher resistant allele frequencies than all other sites combined. A similar pattern is observed for *rdl*, but here the total number of susceptible alleles does not provide the power to detect significant differences. For locations: F = Forest, FNV = Forest Near Village, P = Plantation, and V = Village. Only those 8 sites with at least 5 *A. peditaeniatus* (10 alleles) are shown here. Resistant (res) and susceptible (sus) alleles are colored according to the legends, with resistant alleles in the darker shade.

sites = 10.8 km, min = 0.011 km, max = 28.7 km) especially for the 3 pairs of villages and the forest near the village samples (0.011 km, 0.015 km and 1.56 km), large variations in mosquito species abundance can be observed on a microscale (Fig. 8a). *Anopheles dirus* mosquitoes were collected even in highly degraded and fragmented forest sites, suggesting that they can adapt to human-altered habitats. Interestingly, mosquito densities were similar in the dry and the rainy seasons in the forest sites whereas densities strongly dropped in the dry season in the other sampled sites. Thus, patches of forests might represent refuges for the mosquito populations during the dry season until they disperse again to the field and villages in the rainy season, as has been suggested previously for *A. minimus*⁵³ or *A. dirus*⁵⁴. Consequently, these forest refuges might not only maintain *Anopheles* populations, but also serve as a transmission reservoir during the dry season since human activity is also high in the forest sites during this period. This is in line with the local epidemiology where although malaria cases exhibit a seasonal pattern in Cambodia, symptomatic cases are still presenting at the health centers during the dry season.

A higher proportion of mosquitoes were collected in CBNTs as compared to HBNTs (81% of the total collection caught on CBNT) and screening them for infection with malaria parasites added 12 species to the 8 mosquito species already found infected in the HBNTs, for a total of 20 of the 29 species molecularly identified

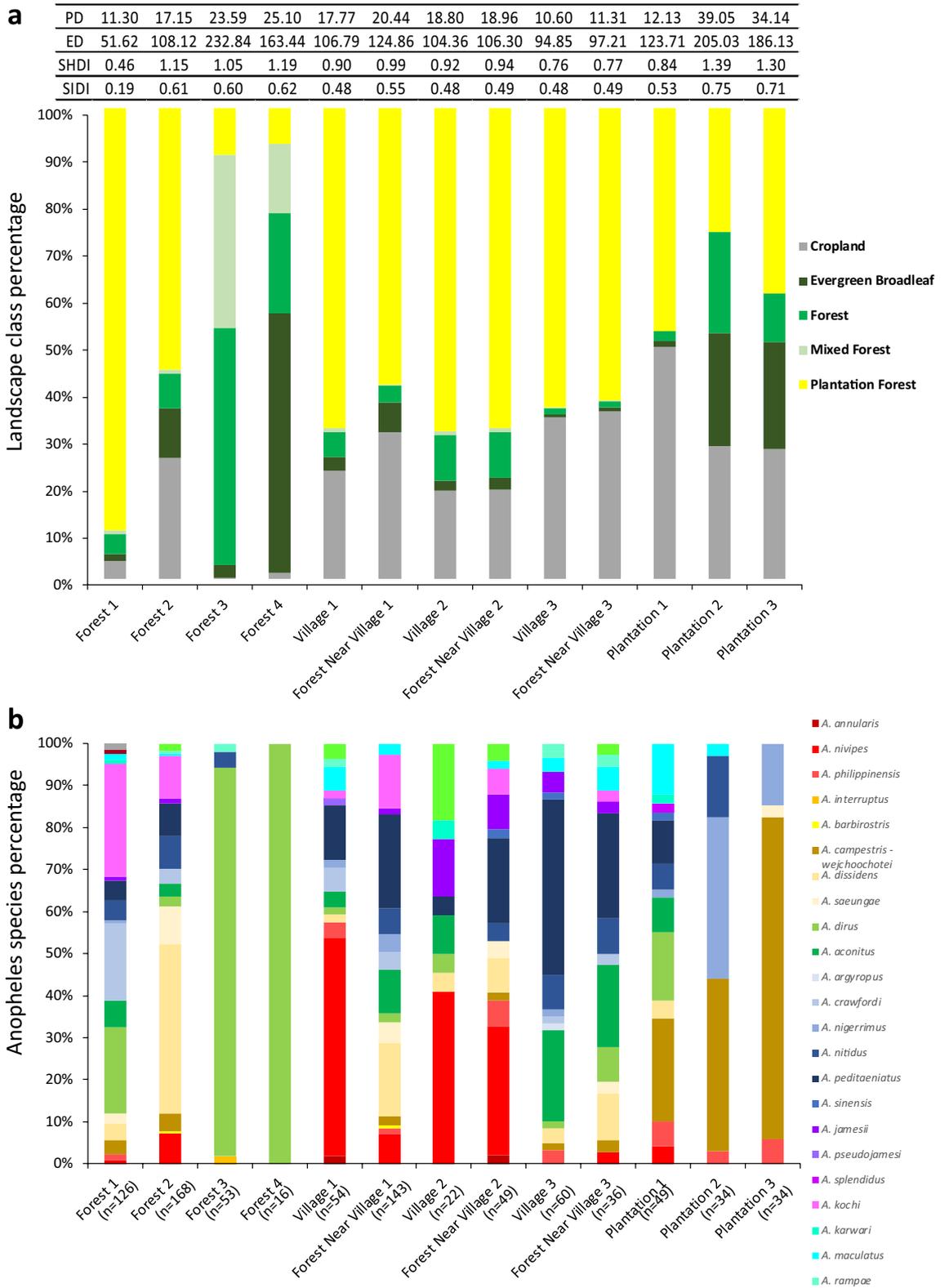


Figure 8. (a) Percentage of each land cover category per collection site determined by a 2 km buffer zone and based on Landsat imagery (Landsat 4–8, 30 m resolution, 2017). Land cover classes defined as: cropland (land with herbaceous and shrubby crops with harvesting and bare soil periods), evergreen broadleaf (land dominated by trees >60% canopy cover with a tree above 5 m, evergreen broadleaf trees make up >60% of the total tree cover), forest (land spanning more than 0.5 hectares with trees higher than 5 m and a canopy cover of more than 10%), mixed forests (land with >60% tree canopy cover, tree height is greater than 5 m, and the forest composition is mixed such that no single forest type makes up >60% of the total tree cover), plantation forests (land cultivated with perennial crops that reach heights above 5 m and occupy the land for long periods). The table is indicating patch density (PD), edge density (ED), Shannon's diversity index (SHDI), and Simpson's diversity index (SIDI) for each buffer zone. (b) Percentage of each mosquito species (molecular identification) sampled in each collection site. Sample sizes are given in parentheses.

having at least one individual positive for malaria parasites. The efficiency of cow baited traps, either collecting resting mosquitoes from the interior of a surrounding tent or directly on the surrounding vegetation, in collecting large number of mosquitoes has been shown previously^{28,29}. Historically the CBNT sampling technique has been dismissed as unrepresentative of malaria parasite vectors. Here, we investigated the innate host-preference of mosquitoes using exactly the same trapping techniques for both human and cow. We showed that the collected species of mosquitoes were all generalist or zoophilic in their preferences. The random subset of molecularly identified species showed that among the 19 species collected in both type of traps, *A. nivipes*, *A. peditaeniatus* and *A. kochi* were enriched in the CBNTs whereas *A. campestris-wejchoochotei* and *A. dirus* were enriched in the HBNTs. These 5 species enriched in one type of trap represented 47 and 25 samples for the CBNTs and the HBNTs respectively, out of the 139 infected samples which were typed (Table 2). Furthermore, the overall malaria parasite prevalence in HBNTs ($4.77 \pm 1.5\%$) and CBNTs ($3.33 \pm 0.6\%$) were not significantly different with the CBNTs still collecting an important proportion of infectious mosquitoes (75%) represented by 19 different *Anopheles* species. Moreover, as CBNTs are a more efficient means of capturing mosquitoes, a greater numbers of species of mosquitoes are able to be studied and the risk to human volunteers is eliminated. Overall, these results are of importance for two reasons. First, screening for malaria parasites only in mosquitoes collected on humans is likely to miss species involved in malaria parasite transmission unless very large collection efforts are carried out. Second, it suggests that many of these mosquito populations are largely maintained by feeding on animals, and that, different from African malaria vectors, the notion of highly preferential human feeding may be inaccurate in the SEA region. Knowing that opportunistic feeding is very common could inform the design of new vector control tools to control malaria infection levels. Labeling of a particular mosquito species as zoophilic or anthropophilic does not represent the reality of more opportunistic feeding preferences.

We found a total of 20 different *Anopheles* species infected with malaria parasites (Table 2). St. Laurent et al²⁶ found 5 different species infected with *P. falciparum* and together both studies identify a total of 22 different *Anopheles* species being able to transmit malaria parasites in Cambodia. These two studies are in strong contrast with another collection in Cambodia which found malaria parasites in only three mosquito species: *A. dirus* s.s., *A. minimus* s.s. and *An. barbirostris* s.s.¹⁹. However it should be noted that only human landing catches were used in Durnez et al.¹⁷, whereas all the infected mosquitoes collected in St. Laurent et al²⁶ were captured in cow baited tents and 75.2% of infective samples in this study were collected in CBNTs, further highlighting the importance of opportunistic feeding behaviour in SEA mosquitoes. In addition, high malaria parasite prevalence was observed in the local human population (8.3%) with important heterogeneity exemplified by malaria infection prevalence rates of 5.7%, 6.5% and 13.1% in the three villages corresponding to the mosquito collection sites⁴². These persisting pockets of high malaria risk as well as our selection of forest sites based on recent *P. falciparum* cases are likely to explain the high malaria parasite prevalence as well as the high diversity of infected mosquitoes observed compared to previous collections.

Anopheles dirus and *Anopheles minimus* are usually considered the main malaria vectors in the Greater Mekong Subregion^{21,22}. Whereas *A. dirus* represented 8.1% ($n = 319$) of the collected mosquitoes in this study and were concentrated in forest sites ($n = 303$), very few *A. minimus* (0.4%, $n = 15$) were captured in our study. The collection carried out in 2013 by St Laurent et al²⁸ found less than 2% of *A. dirus* ($n = 29$) and *A. minimus* ($n = 7$) mosquitoes. Interestingly, the work from Durnez et al¹⁹ carried out in 2005 in three Cambodian provinces found 10.3% of *A. dirus* mosquitoes and 24.8% of *A. minimus* s.l. with a majority being *A. minimus* s.s.. Several hypotheses could explain these differences in species composition. First, collections using cow baited traps in our study and the study by St. Laurent and colleagues likely increased species diversity and numbers, consequently decreasing individual species relative abundance. Secondly, variability between sampling sites is likely to be an important factor influencing the relative abundance of mosquito species collected. Thirdly, the increased human influence upon Cambodian landscapes including the studied area^{55–57} is likely to impact mosquito ecology over time and space resulting in large changes in *Anopheles* communities. Finally, malaria vector control measures might have shifted the *Anopheles* species involved in malaria parasite transmission as observed in Africa^{58,59}.

It is generally accepted that the vast majority of mosquito feeding happens in the period between dusk and dawn. Despite this, we collected ~20% of *Anopheles* mosquito between 6 am and 6 pm, with light conditions in the forest which might resemble dusk or night conditions and might explain the higher density of females collected in this environment (Fig. 2). Malaria vectors are traditionally collected between 6 pm and 6 am since these 12 h usually encompass their peak biting times. Unlike their African counterparts, SEA *Anopheles* are known to be mainly early-biters and extended collections have sometimes been carried out^{14,17–19}, with two studies sampling mosquitoes over 24 h^{20,35}. Although these studies did collect small numbers of *Anopheles* during the day, the overall sample sizes were considerably lower than those we report here^{20,35}. The non-negligible proportion of *Anopheles* mosquito biting during the daytime, when people are active and not protected by a bed net, highlights the importance of residual transmission in this area. Recent estimates of residual transmission in Africa found that between 5 and 40% of mosquito bites occur when people are out and not protected by a bed net⁶⁰. Similar estimates would be needed to adequately quantify the importance of residual transmission in SEA, still, with local vectors being mainly early and outdoor -biters, SEA estimates are likely to be at least equal if not largely higher than those reported for Africa.

Malaria infection rate in the HBNTs was not significantly correlated with the different collection sites, which is likely due to the comparatively low numbers of mosquitoes sampled from villages, forest near the villages and plantations as compared to the forest sites as well as the absence of infectious mosquitoes collected in these sites during the dry season (Fig. 4). There were 6 times more infectious bites per day in the forests compared to the villages during the rainy season (Table 1) further supporting the higher malaria risk in forests in at least northern Cambodia. Few people reported sleeping overnight when working in their field or plantation sites (3.7–18.9%) and when they did, most of them used bed nets (89–100%; Table S6). However, an opposite tendency was observed in the forest sites, with ~78% reporting sleeping overnight in the forest when working there with

only a quarter of them using a bed net (Table S6). Coupled with the fact that a non-negligible proportion of vectors bite during the day time, these observations call for new malaria vector control tools adapted to forest work activities. These control tools would ideally be easy to use and carry, and provide protection during both day and night hours.

The random subset of mosquitoes genotyped for known insecticide resistance mutations (*ace-1* G119S, *rdl* S196, and *kdr* L1014F) indicated that individuals carried mutations associated with response to all the different classes of insecticides. Indeed, among the 192 mosquitoes genotyped only 4 out of the 12 species represented were found carrying any of the three genotyped insecticide resistance mutations: *A. dirus*, *A. kochi*, *A. nivipes* and *A. peditaeniatus*. Interestingly, two *A. dirus*, two *A. nivipes* and one *A. kochi* individuals had an *rdl* mutation associated with dieldrin resistance, one *A. kochi* carried an *ace-1* mutation associated with carbamate and organophosphate cross-resistances, while all other mutations were observed in *A. peditaeniatus* (26 *ace-1* mutations, 19 *kdr* mutations and 24 *rdl* mutations in 26 *A. peditaeniatus*). Since *A. dirus* is considered a forest mosquito, insecticide exposure and thus development of resistance may be less likely, although potential resistance to DDT was noted at one site in Cambodia⁶¹. However, with fragmentation and degradation of forests, human activities are increasingly in closer contact with sites harbouring forest mosquitoes leading to potential insecticide resistance emergence even in forest-associated species. Interestingly, insecticide resistance genotypes were overrepresented in *A. peditaeniatus* (Fig. 6). This mosquito species is mainly zoophilic, outdoor biting and breeding in ponds and rice fields^{29,62–66}. Therefore exposure to agricultural pesticides could explain the observed pattern of insecticide resistance mutations. Even though *A. peditaeniatus* is not considered a primary malaria vector due to its zoophilic behaviour, several occurrences, including this collection, of *Plasmodium* sp. infection have been found^{28,29,67–69}. In addition, entomological collections during a malaria outbreak in Thailand found a very high prevalence of this mosquito species, with ~ half of the tested individuals being resistant to at least one insecticide class, further suggesting a potential involvement of *A. peditaeniatus* in malaria transmission⁴⁶. Thus, *A. peditaeniatus* might play a more important role than suspected in maintaining residual malaria transmission and knowledge of its insecticide resistance mutations is important as malaria control tools are optimized.

Overall, this study illustrates the importance of diverse collection methods, sites and seasons to better characterize *Anopheles* mosquito ecology in SEA. In addition, the increased patchiness and landscape changes following economy-driven human activities is likely to result in large changes in *Anopheles* communities over time and space. The frequency of opportunistic feeding and day-biting behavior highlights key mechanisms driving residual human malaria transmission in Cambodia. Further investigations on the importance of day-biting in various *Anopheles* species and ecological sites would be interesting to better quantify its frequency. Finally, the low frequency of known insecticide resistance mutations with the exception of a single vector species suggest that new integrated vector control methods could be added as a component in the malaria vector control toolbox, which would help accelerate malaria elimination in the Greater Mekong sub region. Nevertheless, any increase in insecticide usage should be carefully monitored by frequent measurement of insecticide resistance mutations and mosquito phenotypic assays.

Data availability

Novel Anopheline sequence has been submitted to Genbank with accession numbers <https://www.ncbi.nlm.nih.gov/nuccore/MW237766> and <https://www.ncbi.nlm.nih.gov/nuccore/MW307220>. The dataset generated during the current study is included in supplementary material.

Received: 21 December 2020; Accepted: 4 March 2021

Published online: 19 March 2021

References

- World Health Organization. World malaria report 2020. 299 (2020).
- Hemingway, J. *et al.* Averting a malaria disaster: Will insecticide resistance derail malaria control?. *Lancet* **387**, 1785–1788. [https://doi.org/10.1016/S0140-6736\(15\)00417-1](https://doi.org/10.1016/S0140-6736(15)00417-1) (2016).
- Noedl, H. *et al.* Evidence of artemisinin-resistant malaria in Western Cambodia. *N. Engl. J. Med.* **359**, 2619–2620. <https://doi.org/10.1056/NEJMc0805011> (2008).
- Leang, R. *et al.* Evidence of *Plasmodium falciparum* malaria multidrug resistance to artemisinin and piperaquine in Western Cambodia: Dihydroartemisinin-piperaquine open-label multicenter clinical assessment. *Antimicrob. Agents Chemother.* **59**, 4719–4726. <https://doi.org/10.1128/aac.00835-15> (2015).
- Imwong, M. *et al.* The spread of artemisinin-resistant *Plasmodium falciparum* in the Greater Mekong subregion: A molecular epidemiology observational study. *Lancet Infect. Dis.* **17**, 491–497. [https://doi.org/10.1016/S1473-3099\(17\)30048-8](https://doi.org/10.1016/S1473-3099(17)30048-8) (2017).
- Ranson, H. & Lissenden, N. Insecticide resistance in African *Anopheles* mosquitoes: A worsening situation that needs urgent action to maintain malaria control. *Trends Parasitol.* **32**, 187–196. <https://doi.org/10.1016/j.pt.2015.11.010> (2016).
- World Health Organization. Strategy for malaria elimination in the Greater Mekong Subregion (2015–2030). (2015).
- Killeen, G. Characterizing, controlling and eliminating residual malaria transmission. *Malar. J.* **13**, 330 (2014).
- World Health Organization. Guidelines for malaria vector control. 171 (2019).
- World Health Organization. Global report on insecticide resistance in malaria vectors: 2010–2016. 72 (2018).
- Cui, L. *et al.* Malaria in the Greater Mekong Subregion: Heterogeneity and complexity. *Acta Trop.* **121**, 227–239. <https://doi.org/10.1016/j.actatropica.2011.02.016> (2012).
- Peeters Grietens, K. *et al.* Characterizing types of human mobility to inform differential and targeted malaria elimination strategies in Northeast Cambodia. *Sci. Rep.* **5**, 16837. <https://doi.org/10.1038/srep16837> (2015).
- Erhart, A. *et al.* Forest malaria in Vietnam: A challenge for control. *Am. J. Trop. Med. Hyg.* **70**, 110–118. <https://doi.org/10.4269/ajtmh.2004.70.110> (2004).
- Gryseels, C. *et al.* Re-imagining malaria: Heterogeneity of human and mosquito behaviour in relation to residual malaria transmission in Cambodia. *Malar. J.* **14**, 165 (2015).
- Sluydts, V. *et al.* Spatial clustering and risk factors of malaria infections in Ratanakiri Province Cambodia. *Malar. J.* **13**, 387 (2014).

16. Edwards, H. M. *et al.* Characterising residual malaria transmission in forested areas with low coverage of core vector control in central Viet Nam. *Parasit. Vectors*. **12**, 454. <https://doi.org/10.1186/s13071-019-3695-1> (2019).
17. Edwards, H. M. *et al.* Transmission risk beyond the village: Entomological and human factors contributing to residual malaria transmission in an area approaching malaria elimination on the Thailand-Myanmar border. *Malar. J.* **18**, 221. <https://doi.org/10.1186/s12936-019-2852-5> (2019).
18. Van Bortel, W. *et al.* Malaria transmission and vector behaviour in a forested malaria focus in central Vietnam and the implications for vector control. *Malar. J.* **9**, 373. <https://doi.org/10.1186/1475-2875-9-373> (2010).
19. Durnez, L. *et al.* Outdoor malaria transmission in forested villages of Cambodia. *Malar. J.* <https://doi.org/10.1186/1475-2875-12-329> (2013).
20. Tangena, J. A., Thammavong, P., Lindsay, S. W. & Brey, P. T. Risk of exposure to potential vector mosquitoes for rural workers in Northern Lao PDR. *PLoS Negl. Trop. Dis.* **11**, e0005802. <https://doi.org/10.1371/journal.pntd.0005802> (2017).
21. Sinka, M. E. *et al.* The dominant *Anopheles* vectors of human malaria in the Asia-Pacific region: occurrence data, distribution maps and bionomic precis. *Parasit. Vectors*. **4**, 89. <https://doi.org/10.1186/1756-3305-4-89> (2011).
22. Hii, J. & Rueda, L. M. Malaria vectors in the Greater Mekong Subregion: Overview of malaria vectors and remaining challenges. *Southeast Asian J. Trop. Med. Public Health*. **44**(Suppl 1), 73–165; discussion 306–167 (2013).
23. Marcombe, S. *et al.* New insights into malaria vector bionomics in Lao PDR: A nationwide entomology survey. *Malar. J.* **19**, 396. <https://doi.org/10.1186/s12936-020-03453-9> (2020).
24. Trung, H. D. *et al.* Malaria transmission and major malaria vectors in different geographical areas of Southeast Asia. *Trop. Med. Int. Health*. **9**, 230–237. <https://doi.org/10.1046/j.1365-3156.2003.01179.x> (2004).
25. Kwansomboon, N. *et al.* Vector bionomics and malaria transmission along the Thailand-Myanmar border: A baseline entomological survey. *J. Vector Ecol.* **42**, 84–93. <https://doi.org/10.1111/jvec.12242> (2017).
26. Sumarnrote, A. *et al.* Plasmodium infections in *Anopheles* mosquitoes in Ubon Ratchathani province, Northeastern Thailand during a malaria outbreak. *J. Am. Mosq. Control Assoc.* **34**, 11–17 (2018).
27. Marasri, N. *et al.* Abundance and distribution of *Anopheles* mosquitoes in a malaria endemic area along the Thai-Lao border. *J. Vector Ecol.* **42**, 325–334. <https://doi.org/10.1111/jvec.12273> (2017).
28. St. Laurent, B. *et al.* Cow-baited tents are highly effective in sampling diverse *Anopheles* malaria vectors in Cambodia. *Malar. J.* **15**, 1–11. <https://doi.org/10.1186/s12936-016-1488-y> (2016).
29. Do Manh, C. *et al.* Vectors and malaria transmission in deforested, rural communities in north-central Vietnam. *Malar. J.* **9**, 259. <https://doi.org/10.1186/1475-2875-9-259> (2010).
30. Tananchai, C. *et al.* Species diversity and biting activity of *Anopheles dirus* and *Anopheles baimaii* (Diptera: Culicidae) in a malaria prone area of western Thailand. *Parasit. Vectors* **5**, 211. <https://doi.org/10.1186/1756-3305-5-211> (2012).
31. Sungvornyothin, S. *et al.* Trophic behavior and biting activity of the two sibling species of the *Anopheles minimus* complex in western Thailand. *J. Vector Ecol.* **31**, 252–261. [https://doi.org/10.3376/1081-1710\(2006\)31\[252:tbabao\]2.0.co;2](https://doi.org/10.3376/1081-1710(2006)31[252:tbabao]2.0.co;2) (2006).
32. Sungvornyothin, S. *et al.* Seasonal abundance and bloodfeeding activity of *Anopheles dirus* sensu lato in Western Thailand. *J. Am. Mosq. Control Assoc.* **25**, 425–430. <https://doi.org/10.2987/09-5907.1> (2009).
33. Chareonviriyaphap, T., Prabaripai, A., Bangs, M. J. & Aum-Aung, B. Seasonal abundance and blood feeding activity of *Anopheles minimus* Theobald (Diptera: Culicidae) in Thailand. *J. Med. Entomol.* **40**, 876–881. <https://doi.org/10.1603/0022-2585-40.6.876> (2003).
34. Smithuis, F. M. *et al.* Entomological determinants of insecticide-treated bed net effectiveness in Western Myanmar. *Malar. J.* **12**, 364. <https://doi.org/10.1186/1475-2875-12-364> (2013).
35. Tangena, J.-A.A., Thammavong, P., Hiscoc, A., Lindsay, S. W. & Brey, P. T. The human-baited double net trap: An alternative to human landing catches for collecting outdoor biting mosquitoes in Lao PDR. *PLoS ONE* **10**, e0138735. <https://doi.org/10.1371/journal.pone.0138735> (2015).
36. Day, J. F. Host-seeking strategies of mosquito disease vectors. *J. Am. Mosq. Control Assoc.* **21**, 17–22. [https://doi.org/10.2987/8756-971x\(2005\)21\[17:hsomdv\]2.0.co;2](https://doi.org/10.2987/8756-971x(2005)21[17:hsomdv]2.0.co;2) (2005).
37. Besansky, N. J., Hill, C. A. & Costantini, C. No accounting for taste: Host preference in malaria vectors. *Trends Parasitol.* **20**, 249–251. <https://doi.org/10.1016/j.pt.2004.03.007> (2004).
38. Lefèvre, T. *et al.* Beyond nature and nurture: Phenotypic plasticity in blood-feeding behavior of *Anopheles gambiae* s.s. when humans are not readily accessible. *Am. J. Trop. Med. Hyg.* **81**, 1023–1029. <https://doi.org/10.4269/ajtmh.2009.09-0124> (2009).
39. Costantini, C. *et al.* Odor-mediated host preferences of West African mosquitoes, with particular reference to malaria vectors. *Am. J. Trop. Med. Hyg.* **58**, 56–63 (1998).
40. Duchemin, J. B. *et al.* Zoophily of *Anopheles arabiensis* and *An. gambiae* in Madagascar demonstrated by odour-baited entry traps. *Med. Veterinary Entomol.* **15**, 50–57. <https://doi.org/10.1046/j.1365-2915.2001.00276.x> (2001).
41. Vantaux, A. *et al.* Field evidence for manipulation of mosquito host selection by the human malaria parasite, *Plasmodium falciparum*. *bioRxiv*, 207183, ver. 6 recommended and peer-reviewed by PCI Evol Biol. <https://doi.org/10.1101/207183> (2018).
42. Sandfort, M. *et al.* Forest malaria in Cambodia: the occupational and spatial clustering of *P. vivax* and *P. falciparum* infection risk in a cross-sectional survey in Mondulakiri province Cambodia. *Malar. J.* **19**, 413. <https://doi.org/10.1186/s12936-020-03482-4> (2020).
43. Rattarithikul, R. *et al.* Illustrated keys to the mosquitoes of Thailand. IV. *Anopheles*. *Southeast Asian J. Trop. Med. Public Health*. **37**(Suppl 2), 1–128 (2006).
44. Canier, L. *et al.* An innovative tool for moving malaria PCR detection of parasite reservoir into the field. *Malar. J.* **12**, 405 (2013).
45. Fang, Y. *et al.* Resistance to pyrethroid and organophosphate insecticides, and the geographical distribution and polymorphisms of target-site mutations in voltage-gated sodium channel and acetylcholinesterase 1 genes in *Anopheles sinensis* populations in Shanghai China. *Parasit. Vectors* **12**, 396. <https://doi.org/10.1186/s13071-019-3657-7> (2019).
46. Sumarnrote, A. *et al.* Species diversity and insecticide resistance within the *Anopheles hyrcanus* group in Ubon Ratchathani Province Thailand. *Parasit. Vectors* **13**, 525. <https://doi.org/10.1186/s13071-020-04389-4> (2020).
47. Sumarnrote, A. *et al.* Status of insecticide resistance in *Anopheles* mosquitoes in Ubon Ratchathani province Northeastern Thailand. *Malar. J.* **16**, 299. <https://doi.org/10.1186/s12936-017-1948-z> (2017).
48. McGarigal, K., Marks, B. & Ene, E. FRAGSTATS v4: Spatial Pattern Analysis Program for Categorical and Continuous Maps. Computer software program produced by the authors at the University of Massachusetts, Amherst. <http://www.umass.edu/landeco/research/fragstats/fragstats.html>. (2012).
49. Shannon, C. E. A mathematical theory of communication. *Bell Syst. Tech.* **27**, 379–423 (1948).
50. Simpson, E. H. Measurement of diversity. *Nature* **163**, 688–688. <https://doi.org/10.1038/163688a0> (1949).
51. Crawley, M. J. *The R book* (Wiley, 2007).
52. Du, W. *et al.* Independent mutations in the Rdl locus confer dieldrin resistance to *Anopheles gambiae* and *An. arabiensis*. *Insect Mol. Biol.* **14**, 179–183. <https://doi.org/10.1111/j.1365-2583.2005.00544.x> (2005).
53. Overgaard, H. J., Ekbohm, B., Suwonkerd, W. & Takagi, M. Effect of landscape structure on anopheline mosquito density and diversity in northern Thailand: Implications for malaria transmission and control. *Landsc. Ecol.* **18**, 605 (2003).
54. Wilkinson, R. N., Gould, D. J., Boonyakanist, P. & Segal, H. E. Observations on *Anopheles balabacensis* (Diptera: Culicidae) in Thailand. *J. Med. Entomol.* **14**, 666–671. <https://doi.org/10.1093/jmedent/14.6.666> (1978).

55. Davis, K. F., Yu, K., Rulli, M. C., Pichdara, L. & D'Odorico, P. Accelerated deforestation driven by large-scale land acquisitions in Cambodia. *Nat. Geosci.* **8**, 772. <https://doi.org/10.1038/ngeo2540> (2015).
56. Singh, M. *et al.* Evaluating the ability of community-protected forests in Cambodia to prevent deforestation and degradation using temporal remote sensing data. *Ecol. Evol.* **8**, 10175–10191. <https://doi.org/10.1002/ece3.4492> (2018).
57. Pepey, A. *et al.* Studying land cover changes in a malaria-endemic Cambodian district: Considerations & constraints. *Remote Sens.* **12**, 2972 (2020).
58. Bayoh, M. N. *et al.* *Anopheles gambiae*: historical population decline associated with regional distribution of insecticide-treated bed nets in western Nyanza Province, Kenya. *Malar. J.* **9**, 62. <https://doi.org/10.1186/1475-2875-9-62> (2010).
59. Mwangangi, J. M. *et al.* Shifts in malaria vector species composition and transmission dynamics along the Kenyan coast over the past 20 years. *Malar. J.* **12**, 13. <https://doi.org/10.1186/1475-2875-12-13> (2013).
60. Sherrard-Smith, E. *et al.* Mosquito feeding behavior and how it influences residual malaria transmission across Africa. *Proc. Natl Acad. Sci. USA* **116**, 15086–15095. <https://doi.org/10.1073/pnas.1820646116> (2019).
61. Van Bortel, W. *et al.* The insecticide resistance status of malaria vectors in the Mekong region. *Malar. J.* **7**, 102. <https://doi.org/10.1186/1475-2875-7-102> (2008).
62. Zhang, S.-S. *et al.* Monitoring of malaria vectors at the China-Myanmar border while approaching malaria elimination. *Parasit. Vectors* **11**, 511. <https://doi.org/10.1186/s13071-018-3073-4> (2018).
63. Amerasinghe, F. P. & Ariyasena, T. G. Larval survey of surface water-breeding mosquitoes during irrigation development in the Mahaweli Project, Sri Lanka. *J. Med. Entomol.* **27**, 789–802. <https://doi.org/10.1093/jmedent/27.5.789> (1990).
64. Gunathilaka, N., Hapugoda, M., Wickremasinghe, R. & Abeyewickreme, W. A comprehensive analysis on abundance, distribution, and bionomics of potential malaria vectors in Mannar district of Sri Lanka. *Malar. Res. Treat.* **2019**, 1650180. <https://doi.org/10.1155/2019/1650180> (2019).
65. Bashar, K., Rahman, M. S., Nodi, I. J. & Howlader, A. J. Species composition and habitat characterization of mosquito (Diptera: Culicidae) larvae in semi-urban areas of Dhaka, Bangladesh. *Pathog. Glob. Health.* **110**, 48–61. <https://doi.org/10.1080/20477724.2016.1179862> (2016).
66. Tananchai, C. *et al.* Diversity and biting patterns of *Anopheles* species in a malaria endemic area, Umphang valley, Tak Province Western Thailand. *Acta Trop.* **190**, 183–192. <https://doi.org/10.1016/j.actatropica.2018.11.009> (2019).
67. Amerasinghe, P. H., Amerasinghe, F. P., Konradsen, F., Fonseka, K. T. & Wirtz, R. A. Malaria vectors in a traditional dry zone village in Sri Lanka. *Am. J. Trop. Med. Hyg.* **60**, 421–429. <https://doi.org/10.4269/ajtmh.1999.60.421> (1999).
68. Gingrich, J. B., Weatherhead, A., Sattabongkot, J., Pilakasiri, C. & Wirtz, R. A. Hyperendemic malaria in a Thai village: dependence of year-round transmission on focal and seasonally circumscribed mosquito (Diptera: Culicidae) habitats. *J. Med. Entomol.* **27**, 1016–1026. <https://doi.org/10.1093/jmedent/27.6.1016> (1990).
69. Sugiarto, Kesumawati Hadi, U., Soviana, S. & Hakim, L. Confirmation of *Anopheles peditaeniatus* and *Anopheles sundaicus* as Malaria Vectors (Diptera: Culicidae) in Sungai Nyamuk Village, Sebatik Island North Kalimantan, Indonesia Using an Enzyme-Linked Immunosorbent Assay. *J. Med. Entomol.* **53**, 1422–1424. <https://doi.org/10.1093/jme/tjw100> (2016).

Acknowledgements

This study is part of the International Centres of Excellence for Malaria Research program from the National Institutes of Health “Understanding, tracking and eliminating malaria transmission in the Asia-Pacific Region” (Grant 1U19AI129392-01). A.P. is funded by the Institut Pasteur International Network Calmette and Yersin Ph.D. scholarship (MHL/MJ/N°212/18). This work was supported by NIH R01 AI145999-01A1 to MMR; and to KDV from the European Commission, Horizon 2020 Infrastructures #731060 Infracvec2, French Laboratoire d'Excellence "Integrative Biology of Emerging Infectious Diseases" #ANR-10-LABX-62-IBEID, and French Agence Nationale de la Recherche, #ANR-19-CE35-0004 ArboVec.

Author contributions

Conceptualization: A.V., K.D.V., K.E., D.L., S.S., I.M. and B.W. Design: A.V., M.M.R., K.D.V. and B.W. Data acquisition: A.V., M.M.R., E.P., E.J.F., S.C., S.K., A.G.C., R.L.F., A.P. Data analyses and original draft writing: A.V., M.M.R., E.J.F. Data interpretation: A.V., M.M.R., K.D.V. and B.W. All authors reviewed, edited and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-021-85628-1>.

Correspondence and requests for materials should be addressed to A.V.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2021, corrected publication 2021

Article: Environmental factors driving *Anopheles* abundance, diversity and prevalence in Cambodia

Anaïs Pepey, Baura Tat, Eakpor Piv, Chansophea Chhin, Sophy Chy, Malen Ea, Saorin Kim, Olivier Roux, Dysoley Lek, Ivo Mueller, Benoit Witkowski and Amélie Vantaux

Manuscript under preparation.

1 Environmental factors driving *Anopheles* abundance, diversity and prevalence in Cambodia

2

3 Anaïs Pepey¹, Baura Tat¹, Eakpor Piv¹, Chansophea Chhin¹, Sophy Chy¹, Malen Ea¹, Saorin Kim¹, Olivier
4 Roux⁴, Dysoley Lek^{2,3}, Ivo Mueller⁴, Benoit Witkowski^{1*} and Amélie Vantaux^{1*}

5

6 ¹ Malaria Molecular Epidemiology Unit, Institut Pasteur du Cambodge, Phnom Penh, Cambodia

7 ² National Center for Parasitology, Entomology and Malaria Control Program, Phnom Penh, Cambodia

8 ³ School of Public Health, National Institute of Public Health, Phnom Penh, Cambodia

9 ⁴ Institute of Research for Development, Infectious Diseases and Vectors: Ecology, Genetics, Evolution and
10 Control (MIVEGEC), Montpellier, France

11 ⁴ The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia

12

13 * These authors contributed equally to the manuscript.

14

15 **ABSTRACT**

16 The Great Mekong Subregion aims to eliminate malaria by 2030. The limitations of traditional collection
17 methods and the knowledge paucity about the numerous malaria vectors of the region hinders the
18 possibility to implement efficient and up-to-date vector control methods. To understand better the
19 different vector species ecology and their respective role within malaria transmission, we implemented
20 thirty-seven collection sites and four collection methods across a land use gradient in a district with an
21 important malaria prevalence and deforestation rate in Mondulkiri province, north-eastern Cambodia.
22 We collected mosquitoes from 24 to 72 hours per site, using Human-baited Double Net traps, Human-
23 Land Catching, Barrier Screens and CDC light traps. We implemented transects to describe aquatic habitats
24 and collect mosquito larvae. We detected no significant difference between HLC and HDN relative
25 abundance and prevalence, but the abundance and the diversity were greater in HDN traps. We collected
26 5,389 female *Anopheles* with HDN traps and determined the environmental variables associated to their
27 prevalence or their abundance. We recorded that during the first round, prevalence was highest in fields
28 (October-November, rainy season: 5.35 ± 0.05%) and during the two other rounds in forest (January-

29 February, dry season: $5.13 \pm 0.22\%$; July-August, rainy season: $3.05 \pm 0.04\%$). Prevalence was positively
30 associated to forest cover in a 2 km radius and negatively associated to fragmentation in a 2 km radius. In
31 addition, there were more *Anopheles* in fields and forest, compared to tree plantations and village.
32 Abundance was also positively associated with cropland and forest cover in a 2 km radius. Less *Anopheles*
33 were collected during the dry season round compared to the two rainy season rounds. The HDN traps
34 collected $15 \pm 1\%$ of *Anopheles* during daytime. We collected 1,108 female *Anopheles* with SCR traps.
35 Seventeen percent of identified blood meals were of human origin, behind cattle (25%) and buffaloes
36 (58%). Larvae were associated to turbid aquatic habitats with shallow edges, tended to cohabitate with
37 *Culex* larvae, and preferred warmer sites. We identified 24 species morphologically and 28 with molecular
38 techniques. Overall, deforestation, which is associated to increased temperatures and the expansion of
39 fields, might increase vectors abundance and diversity, or on the opposite, drive malaria elimination as
40 forest areas diminish and fragmentation increases. From a public health perspective, further attention
41 and effort is required in fields and agricultural areas in addition to forest.

42

43 **KEYWORDS**

44 *Anopheles*, *Anopheles dirus*, malaria vectors, *Plasmodium*, Human-baited Double Net trap, barrier screen,
45 Cambodia

46

47 **INTRODUCTION**

48 Even after almost a century of malaria control and a decade of progress, this mosquito-borne disease still
49 represents a serious public health issue. The World Health Organisation (WHO) estimated 241 million
50 cases and 627,00 associated deaths around the globe in 2020, respectively a 6% and 12% increase since
51 2019 [1]. Even if globally more intense at night, and affecting the lower economic classes, malaria
52 transmission follows different dynamics across continents [2, 3]. Malaria in Africa is driven by three
53 primary vectors, biting often indoors [2, 4, 5]. In the Americas, seven primary vectors dwell the continent,
54 but transmission is mostly sustained by the highly adaptable vector *Anopheles darlingi*, supporting malaria
55 transmission from rainforest to peridomestic and urban environments [2, 6, 7]. The Asia-Pacific region is
56 renowned for an extended diversity of vectors with 19 primary malaria vectors, mostly forest or forest
57 fringes dwellers [2, 8] with frequent zoophilic or opportunistic feeding behaviour [9–11]. The identification
58 of these vectors can represent a challenge: some taxa are very similar, with overlapping morphological

59 identification clues, and molecular methodologies is required to accurately identify cryptic species [12–
60 14].

61 In a global context of increasing anthropisation, where populations are altering their environments to
62 adapt it to their needs, numerous studies have tried to understand its influence on malaria transmission
63 [15]. Environmental changes can lead to a variation in malaria transmission through effects on mosquito
64 abundance, survival and reproduction [15, 16]. Deforestation generally create attractive environment for
65 vectors [17]. It affects humidity and temperature, which in turn can affect *Anopheles* survival and density
66 [18–20], creates new water bodies [21] or affects existing ones [22] and induce an increase of *Anopheles*
67 densities. Deforestation is associated to higher temperatures that minimise blood meal digestion and egg
68 development time, leading to faster development and reproduction [23]. The construction of new
69 settlements dedicated to forest exploitation along forest fringes heighten vector exposure to nonimmune
70 populations, as in the case of frontiers malaria from the Amazon basin [24, 25]. Anthropisation can also
71 lessen the dilution effect, by reducing the biodiversity and thus the availability of potential non-human
72 hosts and increasing the human biting rate [26, 27]. Of all regions, the Amazon has become the most
73 investigated, followed by Africa that harbours most of malaria cases [15]. The extent of knowledge about
74 the effect of anthropisation of vectors in the Pacific and in Asia is more limited.

75 Over the last decade, the countries of the Great Mekong Subregion (GMS, composed of Cambodia, Laos,
76 Myanmar, Thailand, Vietnam and the Chinese province of Yunnan) have been able to reduce both malaria
77 prevalence and fatal cases [1, 28]. The GMS aims to eliminate the disease by 2030 [1, 28]. To achieve their
78 goals, these countries will have to target the forest-associated and border cases.

79 In Cambodia, malaria control relays on test-on-treat services, such as rapid diagnosis tests and
80 artemisinin-combination therapies and primaquine treatments [1]. Vector control consists of using
81 insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS) that reduce transmission by
82 both reducing vectors densities and limiting the physical contact between the host and the vector.
83 However, even after attaining full universal coverage with ITNs and IRS, transmission can subsist through
84 residual malaria transmission when vectors bite the hosts outside the range of action of these methods.
85 Additionally, the emergence and spread of both behavioural and metabolic resistance in vectors is likely
86 to restrain the effectiveness of current methods [8, 14, 29, 30].

87 Vector collections are implemented to understand vector ecology and estimate exposure. Multiple
88 methods exist to capture mosquitoes, but the gold standard remains the Human Landing Catch (HLC). This

89 technique replicates the settings in which populations are exposed to vectors bites, by capturing
90 mosquitoes on the exposed legs of a collector, which is labour intensive and exposes the collectors to
91 potential infective bites [31]. Other promising methods exist and can replicate these conditions more or
92 less accurately while protecting the collector, such as Human-baited Double Net traps (HDN) [14, 32] or
93 paired-odour entry traps [31, 33]. Other traps, rather than mimicking bites exposure, can inform on
94 mosquito mobility or resting habits [31, 34, 35]. Recently, the implementation of interception traps, or
95 barrier screens (SCR), have characterised *Anopheles* flight activity and host-seeking behaviour in several
96 Pacific locations [36–38] and in Asia [39]. Quantifying larval abundance and diversity requires the sampling
97 and description of aquatic habitats, for lack of other options.

98 Collection methods are not exempt of bias, both in their nature and in their implementation [40–42].
99 Paired-odour entry traps cannot exactly replicate the thermal, visual and chemical manifestations
100 detected by mosquitoes [43]. In human-baited traps, collector attractiveness can confound the location
101 attractiveness [32]. While *Anopheles* spp. are known to be also active at dusk and dawn in the GMS [8, 12,
102 44], collections often cover nighttime only, during the classical collection time of malaria vectors:18:00 to
103 6:00[10, 13, 42, 45–48].

104 We investigated the *Anopheles* population across an anthropisation gradient of an area affected by both
105 malaria and deforestation to attain several objectives. Firstly, we aimed to complete and update the
106 current entomological knowledge of Cambodia vectors; hence, malaria control strategies can rely on
107 recent and quantitative data. Secondly, we wanted to assess the efficacy of traps alternatives to HLC such
108 as HDN and SCR. Lastly, we aimed at determining anthropisation effects on vectors bionomics. Altogether,
109 this mosquito collection was established to describe finely the context of malaria transmission in a
110 Cambodian district and provide data to ground a tailor-made control intervention.

111 **MATERIAL AND METHODS**

112 **1) Study site**

113 The study site was located in Kaev Seima district, Mondulkiri province, Eastern Cambodia. Year divides
114 into rainy (May-October) and dry (November-April) seasons. The area, mostly occupied by farmers and
115 forest goers, displayed the highest malaria incidence of the GMS in 2019 [28]. The expansion of rice,
116 cassava, cashew nut and rubber plantations has led to increasing deforestation with wooded areas
117 decreasing from 91% of the study area in 1988 to 47% in 2018 [49].

118 **2) Study design**

119 **Collection sites.** Three collections, one in dry season and two in rainy season, were established over a
120 year, each of them with the same traps installed at the same coordinates, permitting direct comparison
121 across ecologies and time. The sites of collection were chosen to represent four types of environment
122 from a previous determination of land use [49]: village, forest, fields and plantations. Half of the non-
123 village sites were selected with low human passage and the other half with high human passage, which
124 was determined using the mobility data collected in a previous study [50]. Village locations were all heavily
125 frequented. Prior to the survey implementation, the field coordinator visited all heads of village relevant
126 figures to inform them about the upcoming study and ask for approval to conduct the survey. Ethical
127 approval was obtained from the Cambodian National Ethics Committee for Health Research (NECHR228).

128 **Human-baited Double Net traps.** As described in Tangena et al. [32] and Vantaux et al. [14], each HDN
129 consisted of an outer untreated net (200*200*250cm) raised 20 centimetres above ground and an inner
130 untreated net (180*180*250cm). Inside, two collectors could rest in hammocks and operated for a 72-
131 hour period. Each hour involved 15 minutes of catching the mosquitoes trapped between the nets and 45
132 minutes of resting inside the inner net.

133 **Human Land Catching.** Described by previous studies as the golden standard [31, 43, 51], HLC was
134 implemented as a reference to be “calibrated against” [51]. Each HLC consisted of a 24-hour collection,
135 and each hour involved 45 minutes of catching and 15 minutes of resting. The HLC was operated at the
136 same site than an HDN within its collection period, to compare their respective collection. We kept a
137 distance of at least 100m between them to prevent interference.

138 **Barrier Screens.** The SCR were placed between the village perimeter and a surrounding forest
139 environment. Each barrier screen was positioned with one side of the screen facing the village, hereafter
140 referred to as “inner side” (SCR-In), and the other facing away from the village, hereafter referred to as
141 “outer side” (SCR-Out). The barrier screen intercepts host-seeking and blood-fed mosquitoes as they
142 commute in and out of the village. Barrier screens were used for 48 hours at each site. Every hour, two
143 collectors (one for each side of the SCR) with mouth aspirators and torches sampled the mosquitoes
144 resting on the screen for 15 minutes. For the 45 remaining minutes, collectors could rest. Barrier screens
145 were designed as such: i) comparable to those described elsewhere in literature [36, 39], ii) made from
146 resources available in Cambodia, iii) with an optimised physical design [38]. Each barrier screen consisted
147 of a 20m long and 2m wide polyethylene shade cloth (forest green, 60% shading grade, no eaves) tightly
148 fastened on bamboo poles with staples.

149 All collections were performed by trained and volunteer adults relaying each other, with two collectors
150 for each HDN and HLC traps and four collectors for barrier screens. Groups of participants were randomly
151 assigned to a location, so that variation in attractiveness between collectors were compounded with
152 location attractiveness. Mosquitoes were collected by mouth aspirators, then transferred into plastic cups
153 covered with netting and labelled by date, location and hour of collection or in 15mL tubes for HLC
154 collections stored in bags with the same information as the cups.

155 ***Aquatic habitats transects.*** Eleven collection sites were selected for larval collection among all types of
156 studied environments. From the transect centre, a 100-metre rope was extended in every cardinal
157 direction. Collectors looked for any aquatic habitat that could be a breeding site (e.g. any body of water,
158 temporary to permanent) over one metre on each side of the rope. Any body of water was geo-referenced
159 and various ecologic observations were made (type of body of water, turbidity, current, presence of
160 predators and mosquito larvae, etc.). This data was collected and managed using REDCap electronic data
161 capture tools hosted at Pasteur Institute Paris [52]. For every aquatic habitat, water was collected and
162 emptied into a large white tray in order to find the potential mosquito larvae, which were removed with
163 a pipette and stored in 50mL falcons with unique identification for each aquatic habitat. The sampling of
164 mosquito immatures was done using standard 0.5-litre dippers. Habitats too small to be sampled with the
165 dipper were directly sampled with a pipette. Collected larvae were brought back to the field base where
166 they were installed in emergence boxes stored at ambient temperature until they grew into adults for
167 identification.

168 **3) Laboratory procedures**

169 ***Morphological species identification.*** *Anopheles* mosquitoes were identified to the species level using the
170 taxonomic key from Rattanarithikul et al. [53]. Non-*Anopheles* were identified to genus level.

171 ***Molecular species identification.*** We used sampling weights to select a representative subsample of the
172 collections for molecular screening. To this aim, we implemented a modified random sampling with
173 sampling rates depending on the number of individuals from each species to prevent under-sampling
174 species less represented in the dataset. Briefly, minimal sample size per species was fixed at 10 or at total
175 size for smaller clusters, and for species that contributed more than 10 mosquitoes under a normal
176 sampling regime, sampling rates decreased linearly with increasing species cluster size. Slope was fixed
177 by condition to reach predetermined final sample size corresponding to Slovin's formula sample size
178 estimation ($N = 1,291$). In addition, all mosquitoes positive for at least one species of human malaria

179 parasite was also sequenced (N = 127). Mosquito species was determined molecularly via sequencing of
180 the internal transcribed spacer 2 (ITS2) region, and if the determination was not possible, via sequencing
181 of the mitochondrial cytochrome oxidase subunit 1 (CO1) gene.

182 **Malaria prevalence.** Heads and thoraces were subjected to DNA extraction using the commercial
183 extraction kit QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's
184 instruction. A semi quantitative real-time polymerase chain reaction (RT-PCR) was performed to detect
185 malaria parasites, as previously described [54]. For all positive samples, the *Plasmodium* species was
186 determined molecularly via sequencing of the cytochrome b region (cytb). If both screening and at least
187 one of the five human *Plasmodium* species returned positive, the sample was considered positive.

188 **Blood meal identification.** Abdomen of fed mosquitoes captured with barrier screens were subjected to
189 DNA extraction using the commercial extraction kit QIAamp DNA mini kit (Qiagen, Hilden, Germany)
190 according to the manufacturer's instruction. The blood meal origin was determined molecularly via semi-
191 nested polymerase chain reaction (PCR) amplification and sequencing of the vertebrates' CO1 gene [55].

192 **Sequence assignment.** All sequence reads were obtained using standard Sanger sequencing methods and
193 resulting sequence traces were visually verified prior to being submitted to nucleotide blast. Resulting
194 sequences were blasted against the National Centre for Biotechnology Information GenBank database. If
195 a given sample gave at least 250 bps of clean sequence and the percent identity of the input gene
196 sequence with a single published (preferably voucher) sequence was 98% or higher, a species
197 determination was made. In the rare event samples where species determination was not possible either
198 using the gene sequence, or obtaining a gene sequence was technically problematic, amplification and
199 sequencing of a different gene was carried out using Sanger sequencing with quality control as discussed
200 above.

201 **4) Data analyses**

202 **HDN-HLC comparison.** The efficacy of HDN to HLC was compared using the subset of HDN collections that
203 completely overlapped HLC locations and days of collections. The malaria parasite prevalence (infected
204 with any human *Plasmodium* species or not) was analysed with a binomial generalized linear mixed model
205 (GLMM). Abundance was coded as a discrete variable corresponding to the total count of *Anopheles*
206 captured in a day at a specific site and was analysed with a negative binomial GLMM. Relative abundance
207 was coded as a discrete variable corresponding to the count of *Anopheles* captured in a day at a specific
208 site divided by the total yield of that site during this round of collection, allowing to consider trap

209 variability but preserving daily variability, and was analysed with a Gaussian GLMM. In all these models,
210 trap type, land use and round of collection were coded as fixed factors and collection site was coded as a
211 random factor to account for repeated measurements in the same site. Land use category was determined
212 visually as the most prevalent category around the trap between forest (primary or secondary),
213 plantations (such as rubber or cashew nut trees), fields (crops, shrubs, etc. such as rice paddies or cassava
214 crops) or village. Diversity was compared between the traps by calculating the Bray-Curtis distance
215 between HDN and HLC count of species per day per site of collection.

216 **Abundance.** We aggregated the abundance of *Anopheles* captured with HDN by collection sites and round.
217 In a first model, we assessed the effects of several environmental variables on mosquito abundance with
218 round of collection, land use category, passage intensity, the interaction round of collection by land use,
219 and the interaction round of collection by passage intensity coded as fixed factors and collection site
220 coded as a random factor in a negative binomial GLMM. Passage intensity was determined using the GPS
221 data collected in a previous study [50]. In a second model, we analysed the effects of several landscapes
222 variables on mosquito abundance including distance to the closest registered household, percentage of
223 cropland, percentage of forest and percentage of plantations, patch density and Simpson's Diversity Index
224 (SID) in a 2 km radius coded as fixed factors and collection site coded as random factor using a negative
225 binomial GLMM. Households positions were determined from a previous cross-sectional study [56].
226 Landscape metrics were produced with Fragstats [57] and extracted from the land use map based on
227 Landsat satellite imagery retrieved from SERVIR Mekong [58].

228 **Malaria parasite prevalence.** We tested the same sets of environmental and landscape variables as for
229 the abundance models to determine their effect on malaria parasite prevalence (*Anopheles* positive for
230 any *Plasmodium* species or not) using binomial GLMMs with collection site coded as a random factor.

231 **Blood meals.** A binomial variable representing blood meal origin was attributed to each *Anopheles*, with
232 1 corresponding to a human blood meal and 0 a different blood meal. We tested the effect of several
233 explanatory variables using GLMMs: landscape variables, time of collection (day or night time), side of
234 screen the mosquito was captured on, round of collection and species. Collection site was coded as a
235 random factor. Data analysis was restricted to the subset of *Anopheles* for which a vertebrate could be
236 identified as the origin of the blood meal with a >98% match.

237 **Larvae.** Due to the multitude of described variables per aquatic habitat, we tested their effect on the
238 presence/absence of *Anopheles* larvae in the aquatic habitat individually in univariate binomial GLMMs,

239 with transect site coded as a random factor to account for repeated measurements in the same area. All
240 variables with a $p < 0.2$ were included in a multivariate binomial GLMM.

241 Percentages were calculated with their 95% confidence interval (CI) and means with their standard
242 deviation (sd). Significance threshold was set at $p = 0.05$. For model selection, we used the stepwise
243 removal of terms, followed by likelihood ratio tests. Term removals that significantly reduced explanatory
244 power ($p < 0.05$) were retained in the minimal adequate model [59]. All statistical analyses were
245 conducted in R version 4.0 [60]. DHARMA package was used to assess all models' goodness of fit [61].

246 RESULTS

247 1) Adults

248 Thirty-seven locations were found to be eligible for trap installation (Figure Supp Mat S1), collecting
249 34,966 mosquitoes over three rounds. The traps collected 6,690 *Anopheles* females, of which 2,380
250 during round 1 (rainy season, October-November 2019), 1,189 during round 2 (dry season, January-
251 February 2020) and 3,121 during round 3 (rainy season, July-August 2020). We collected 866 *Anopheles*
252 by daytime ($12.9 \pm 0.8\%$).

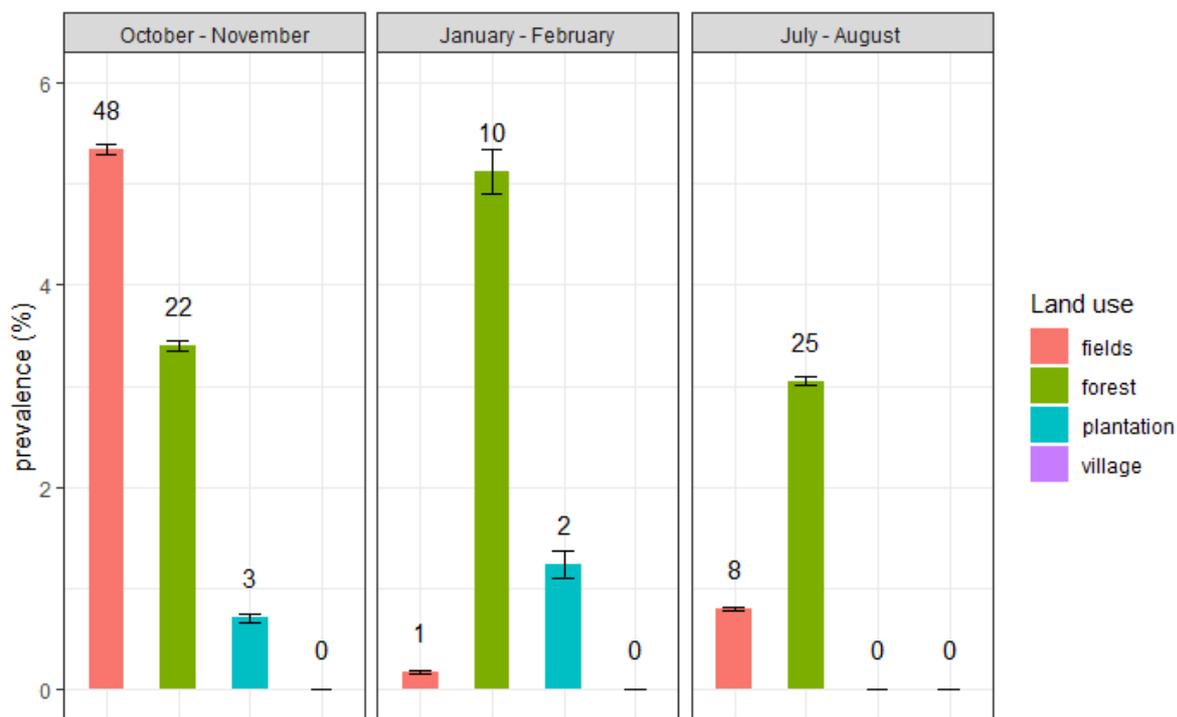
253 **Trap efficacy.** Over the same days and sites of collection, the 10 HDN traps collected 491 *Anopheles* and
254 the 10 HLC collected 191 *Anopheles*. The HDN collected significantly more *Anopheles* mosquitoes ($16.4 \pm$
255 21.9 *Anopheles* per trap per day) than the HLC (6.4 ± 9.8 ; $\chi^2_1 = 6.90$, $p = 0.009$). Abundance significantly
256 varied by land use ($\chi^2_3 = 17.28$, $p < 0.001$), with a higher abundance in forest (28.3 ± 31.5), followed by
257 fields (14.4 ± 8.9), plantations (6.7 ± 7.3) and villages (2.8 ± 4.4). Abundance did not vary significantly
258 across rounds of collection ($\chi^2_1 = 1.25$, $p = 0.26$). Relative abundance varied only by land use ($\chi^2_3 = 13.03$,
259 $p = 0.005$), following the same pattern as abundance, with forest having the highest relative abundance
260 (7.6 ± 7.8), followed by fields (5.0 ± 4.2), plantations (2.0 ± 1.8) and village sites (0.7 ± 0.9). Relative
261 abundance was not significantly associated to the round of collection ($\chi^2_1 = 0.52$, $p = 0.47$) or trap type
262 ($\chi^2_1 = 3.10$, $p = 0.08$). The two types of traps collected equivalent proportion of infected mosquitoes (χ^2_1
263 $= 0.08$, $p = 0.77$) during the different rounds of collection ($\chi^2_1 = 0.51$, $p = 0.47$). Malaria parasite prevalence
264 per round per site varied significantly among land use categories ($\chi^2_3 = 8.40$, $p = 0.04$), with highest
265 prevalence in forest (over this subset: 6.19 ± 0.17), followed by fields (2.31 ± 0.14), plantations (0) and
266 finally villages (0). However, no significant pairwise post-hoc comparisons were found. Twelve species
267 were present in both traps, whereas *Anopheles karwari* was found only in HLC traps ($N = 1$) and *Anopheles*

268 *aconitus* (N = 2), *Anopheles interruptus* (N = 1), *Anopheles jamesii* (N = 1) and *Anopheles vagus* (N = 1)
269 were present only in HDN traps.

270 **Prevalence.** There were 127 *Anopheles* that were identified positive for malaria over the whole collection,
271 of which 114 (89,8 ± 5,3%) were molecularly identified as *An. dirus*. The other species were *Anopheles*
272 *campestris* (N = 1), *Anopheles maculatus* (N = 3), *Anopheles nigerrimus* (N = 1, the only *P. falciparum*
273 infection), *Anopheles saeungae* (N = 1), *Anopheles tessellatus* (N = 1), *Anopheles vagus* (N = 2) and
274 *Anopheles wejchoochotei* (N = 4). Of these 127 *Plasmodium*, 120 (94.5 ± 4.0%) were *P. vivax* only, 1 (0.8 ±
275 1.5%) was *P. falciparum*, 1 (0.8 ± 1.5%) was a co-infection with *P. falciparum* and *P. vivax* and 5 (3.9 ±
276 3.4%) were co-infections with *P. vivax* and *P. ovale*.

277 Over the HDN collection, the prevalence of RT-PCR positive *Anopheles* was 2.2 ± 0.4% (119/ 5,389).
278 Daytime prevalence (1.6 ± 0.9%) was not significantly different from nighttime prevalence (2.3 ± 0.4%,
279 Chi-squared test, $\chi^2_1 = 1.52$, p = 0.22). Prevalence was associated to round ($\chi^2 = 12.81$, p < 0.001) and to
280 the interaction between round and land use ($\chi^2_3 = 24.59$, p < 0.001, Table Supp Mat S1): it was highest
281 during October-November round in fields (5.35 ± 0.05%) then in forest for January-February round (5.13
282 ± 0.22%) and July-August round (3.05 ± 0.04%, Figure 1, Figure Supp Mat S2). Infected *Anopheles* were
283 positively associated with forest cover in a 2-km radius ($\chi^2_1 = 4.02$, p = 0.04) and negatively associated
284 with SIDI in a 2-km radius ($\chi^2_1 = 14.3$, p < 0.001, Table Supp Mat S2).

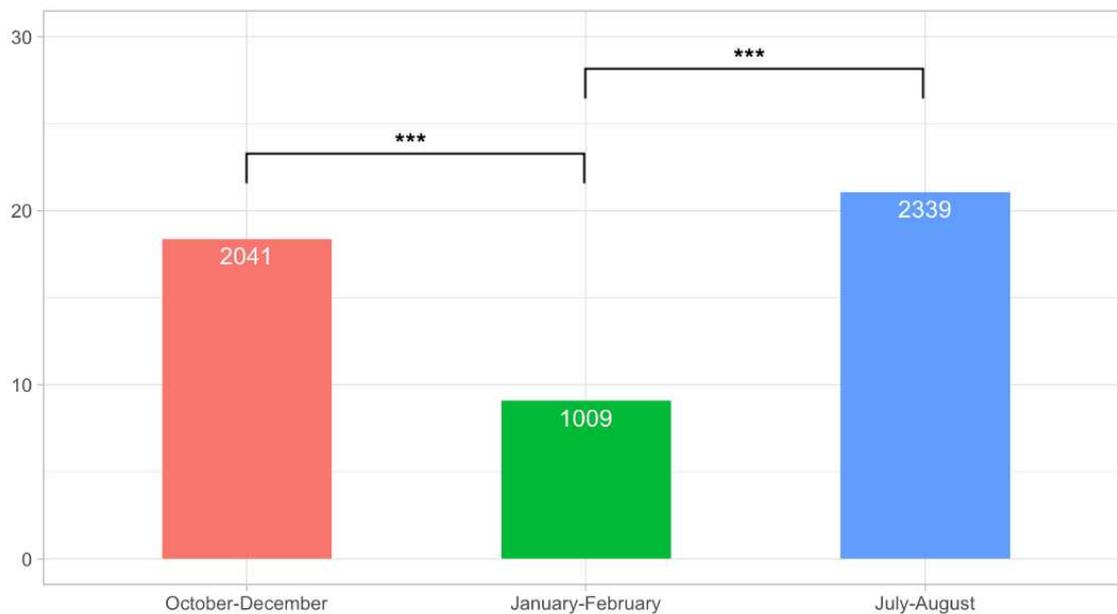
285 Figure 1: Prevalence per round and per land use with 95% CI, with the count of positive *Anopheles*.



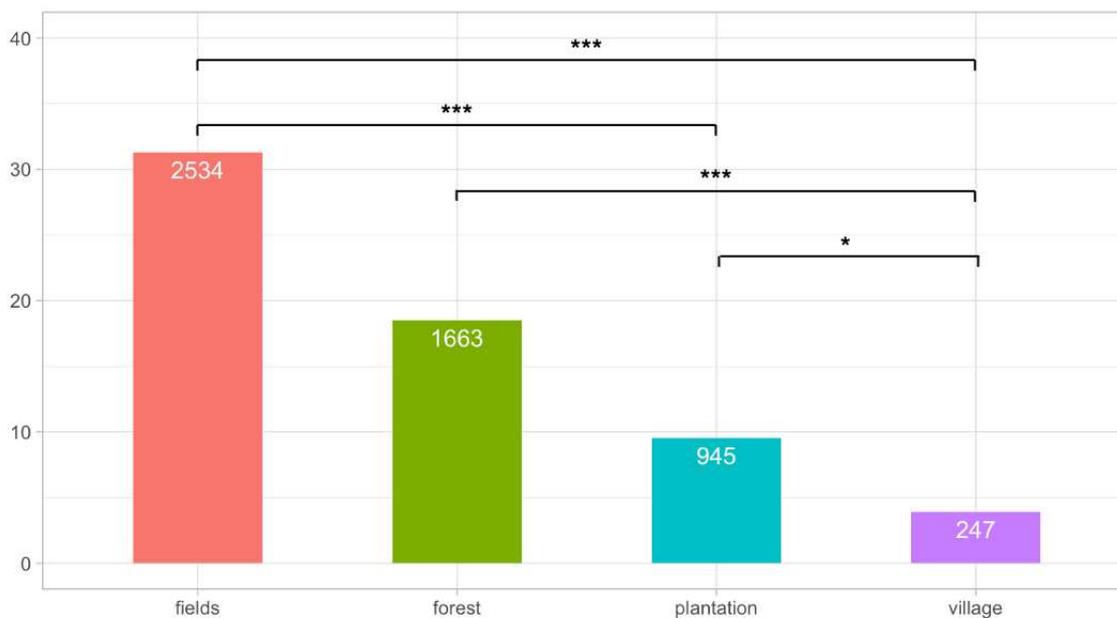
286 **Abundance.** Overall, 5,389 *Anopheles* were captured with HDN traps with an average of 16 individuals per
 287 day per site (median = 6). The minimum was zero with 38 collection events collecting no *Anopheles* (at
 288 least once for every round, passage intensity and land use type) and the maximum was 145 individuals,
 289 at a deep forest site with high passage during October-November. Abundance was associated with round
 290 ($\chi^2_2 = 22.43$, $p < 0.001$, Figure 2), and land use category ($\chi^2_3 = 27.81$, $p < 0.001$, Figure 3, Table Supp Mat
 291 S3, Table Supp Mat S4, Table Supp Mat S5). Passage intensity ($\chi^2_1 = 1.91$, $p = 0.17$) and the interaction
 292 between passage and round ($\chi^2_2 = 3.23$, $p = 0.20$) were not significant (Table Supp Mat S1). The interaction
 293 between land use and round was not significant, as abundance followed the same trend for all rounds of
 294 collections, with fields having the highest, followed by forest, plantations and finally village ($\chi^2_6 = 4.18$, p
 295 = 0.65, Figure 4, Table Supp Mat S1).

296

297 Figure 2: Average count of trapped *Anopheles* per HDN collection event, displayed per round of collection,
 298 with Tukey's post-hoc comparisons (***) $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). White numbers refer to the total
 299 count of *Anopheles* collected in that category (N = 5,389).



300
 301 Figure 3: Average count of trapped *Anopheles* per HDN collection event, displayed per land use category,
 302 with Tukey's post-hoc comparisons (***) $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). White numbers refer to the total
 303 count of *Anopheles* collected in that category (N = 5,389).



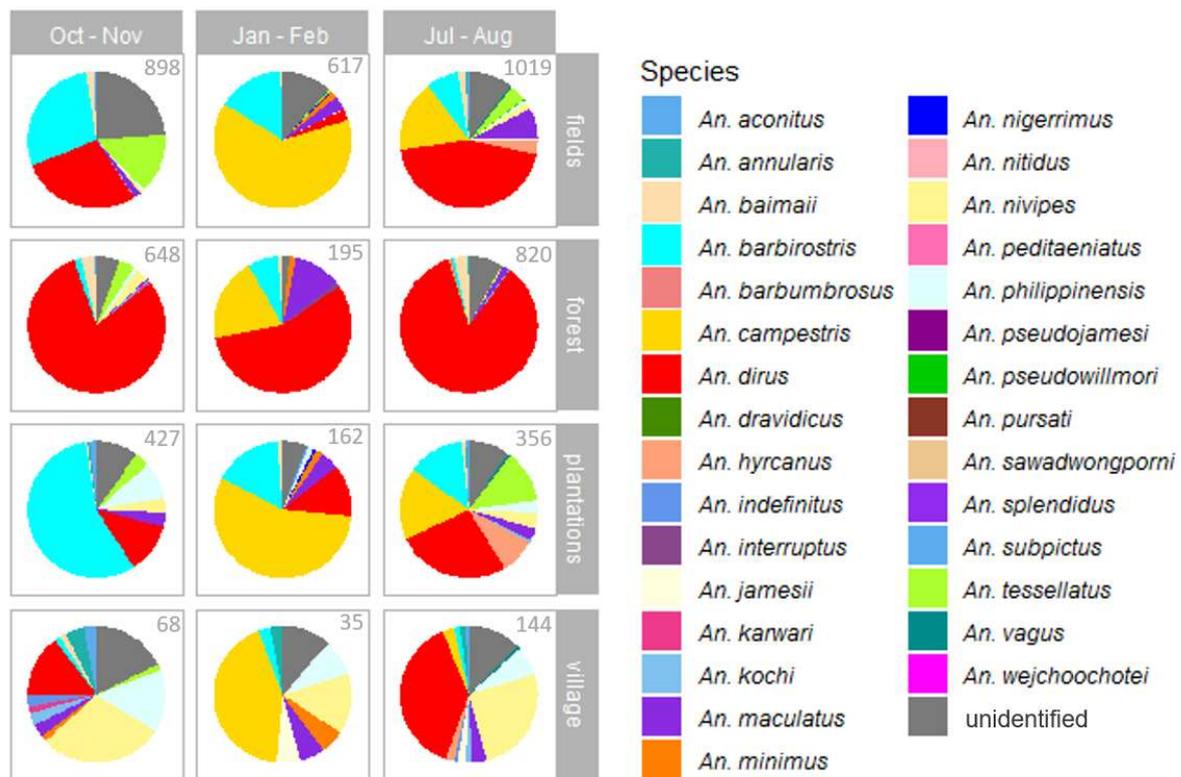
305 Figure 4: Average count of trapped *Anopheles* per HDN collection event, displayed per land use categories
 306 and round of collection (N = 5,389).



307
 308 Abundance was also positively associated to the percentage of cropland ($\chi^2_1 = 5.44$, $p = 0.02$) and forest
 309 cover ($\chi^2_1 = 7.66$, $p = 0.006$) in a 2 km radius (Table Supp Mat S6). Abundance was not associated with
 310 passage intensity, distance to closest registered household, percentage of plantations, patch density and
 311 SIDI in a 2 km radius (Tables Supp Mat S6).

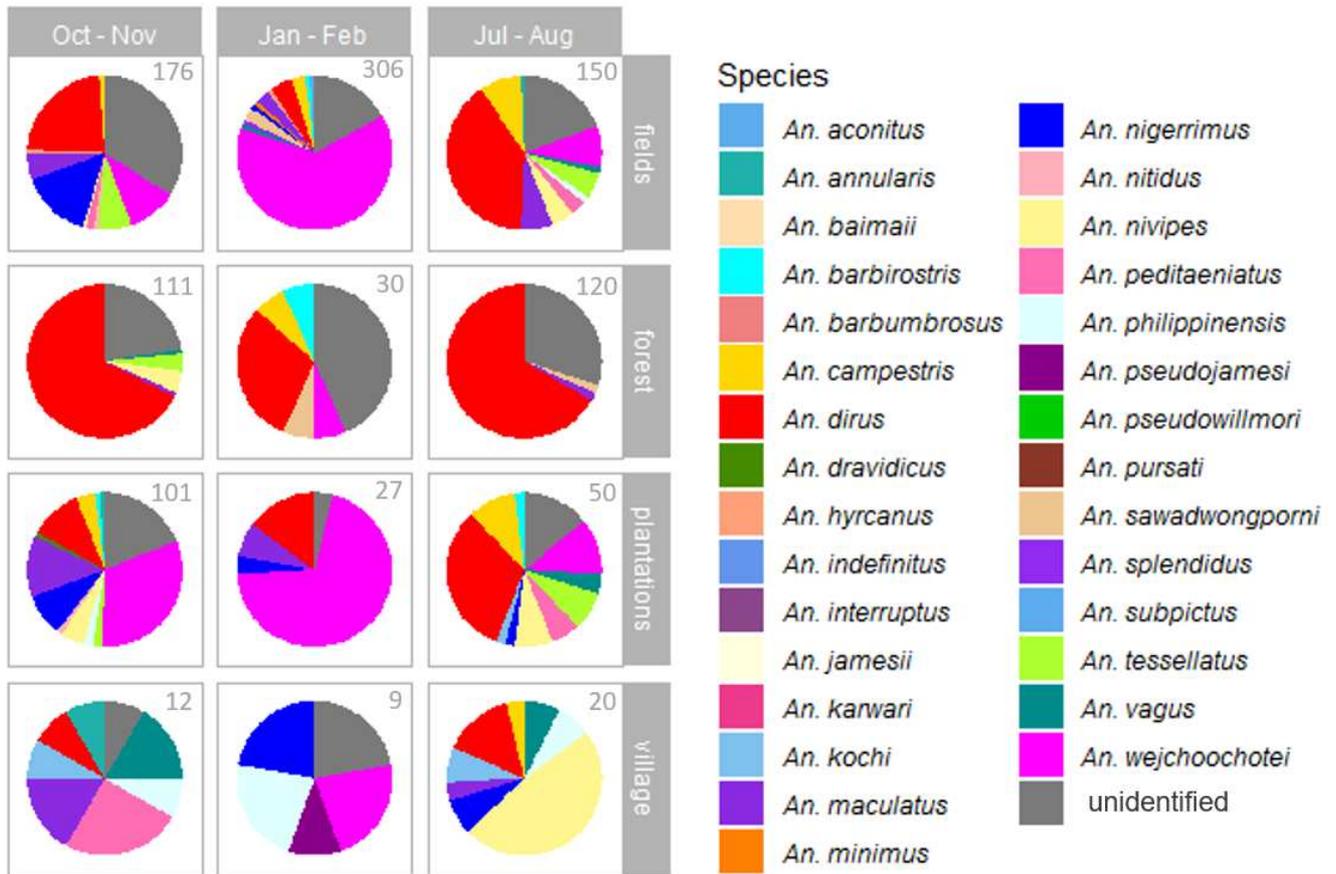
312 **Diversity.** Twenty-four species of *Anopheles* were morphologically identified from HDN collections (Figure
 313 5). Of the 4,776 morphologically identified mosquitoes, the most represented species were *Anopheles*
 314 *dirus* ($47.7 \pm 1.4\%$), followed by *Anopheles barbirostris* and *Anopheles campestris* (both $16.3 \pm 1.0\%$).

315 Figure 5: *Anopheles* species distribution among collection rounds and land use type, collected by HDN and
 316 morphologically identified (N = 5,389). Total number of *Anopheles* females per category is indicated in
 317 grey.



318 Over the whole follow-up, 1,362 samples were successfully amplified with the ITS2 or CO1 PCRs, of which
 319 1,061 (77.9 ± 2.2%) could produce a consensus sequence that was correctly matched to an identification.
 320 Over the HDN collection, 919 samples were amplified and 708 (77 ± 2.7%) were successfully identified,
 321 representing 28 species (Figure 6). The most represented species were *An. dirus* (43.2 ± 3.6%), followed
 322 by *Anopheles wejchoochotei* (22.5 ± 3.1%), which was not identified morphologically, even for non-HDN
 323 collection events, and finally *An. maculatus* (6.2 ± 1.8%).

324 Figure 6: *Anopheles* species distribution among collection rounds and land use type, collected by HDN
 325 and molecularly identified (N = 919). Total *Anopheles* per category is indicated in grey.

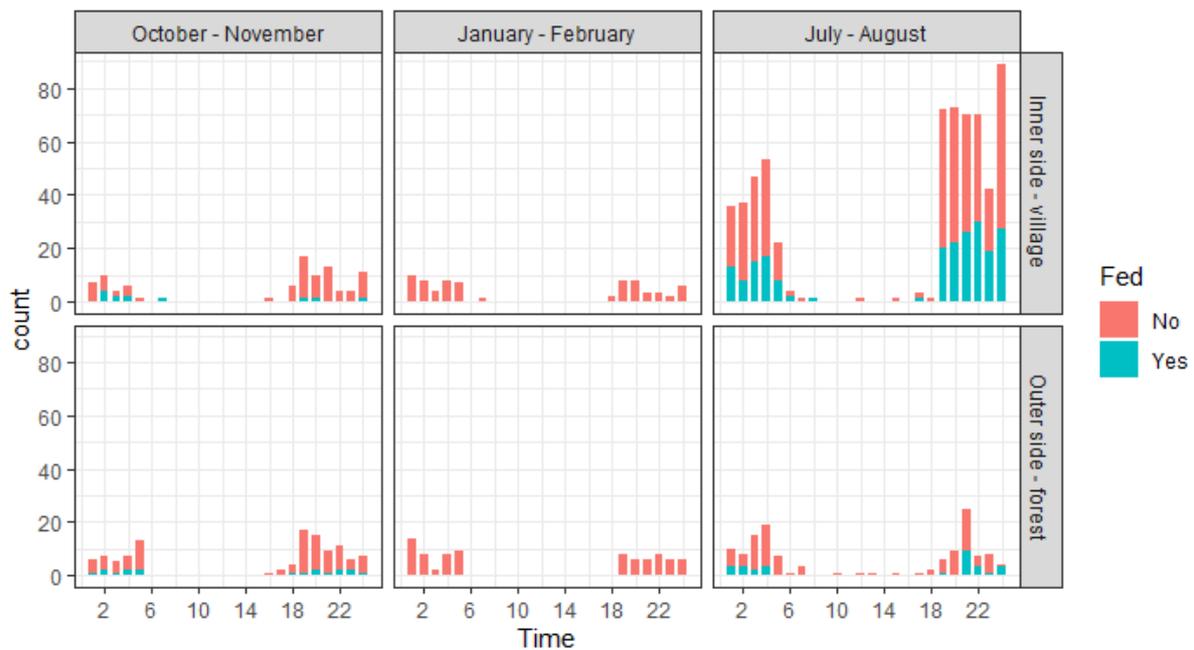


326 **Identification accuracy.** Over the whole follow-up, 28 species were molecularly identified. Seven species
 327 were always correctly identified morphologically: *Anopheles aconitus*, *An. dirus*, *Anopheles kochi*,
 328 *Anopheles nigerrimus*, *Anopheles pseudojamesi*, *Anopheles tessellatus* and *Anopheles vagus*. Seven species
 329 were never correctly identified: *Anopheles baimaii*, *Anopheles hyrcanus*, *Anopheles indefinitus*, *Anopheles*
 330 *jamesii*, *Anopheles pseudowillmori*, *Anopheles pursati* and *Anopheles subpictus*. The accuracy of the
 331 identification of the remaining seven species ranged between 9.5% (*Anopheles barbirostris*) and 93.8%
 332 (*Anopheles annularis*, Table Supp Mat S7).

333 **Barrier screens.** SCR traps collected 205 *Anopheles* during October-November (30 fed, $14.6 \pm 4.8\%$), 151
 334 during January-February (none fed) and 752 during July-August (228 fed, $30.0 \pm 3.3\%$, Figure 7). The
 335 species that was most captured on the barrier screen varied by round of collection, but not by side of the
 336 screen. *Anopheles philippinensis* was the main species during round 1 ($29.8 \pm 6.3\%$), *An. barbirostris* during

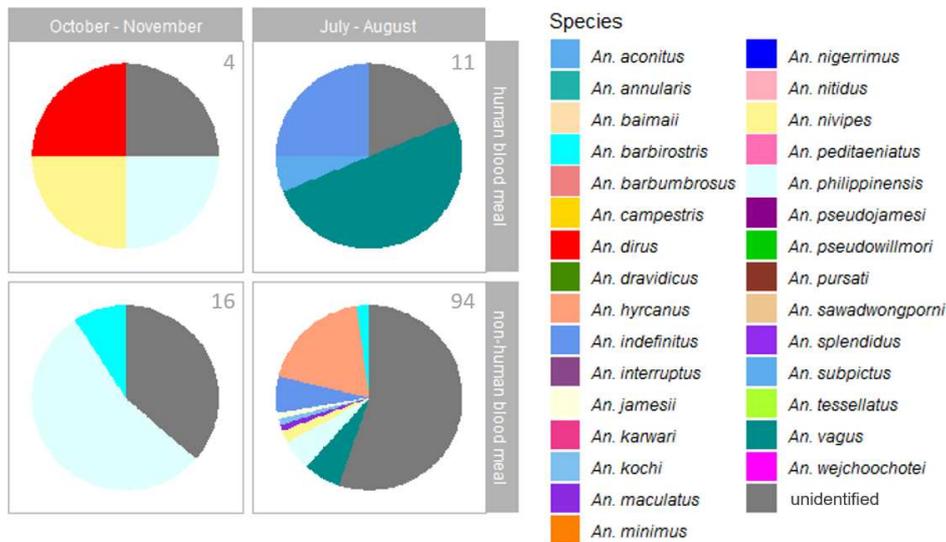
337 round 2 ($44.4 \pm 7.9\%$) and *An. vagus* during round 3 ($18.1 \pm 2.8\%$). Seventeen species were morphologically
 338 identified from SCR traps (16 on the inner side, 14 on the outer side, Figure Supp Mat S3).

339 Figure 7: Count of trapped *Anopheles* on both sides of the SCR traps per round and hour of collection
 340 (Time by 24:00 format, N = 1,108).



341 **Feeding patterns.** Fed *Anopheles* were mostly collected at night on the inner side of the interception trap.
 342 Of the 258 fed *Anopheles*, 125 ($48.8 \pm 6.1\%$) could have their blood meal extracted and processed to
 343 obtain the blood meal origin. Over the 126 identified blood meals, $16.7 \pm 6.5\%$ were from humans, while
 344 $25.4 \pm 7.6\%$ from cattle and $57.9 \pm 8.6\%$ from water buffaloes. A blood meal was found to originate from
 345 a dog, but the identity match was only 96.5%. Cattle was the main blood meal in October-November (N =
 346 11, $73.3 \pm 22.4\%$) whereas mosquitoes mainly fed on water buffalo in July-August (N = 73, $66.4 \pm 8.8\%$).
 347 From the 125 *Anopheles* with an identifiable blood meal, 11 species were morphologically identified
 348 (Figure 8). Four species were fed on both human and other vertebrates (*An. indefinitus*, *An. nivipes*, *An.*
 349 *philippinensis* and *An. vagus*), while two species fed only on humans (*An. dirus* and *An. subpictus*) and five
 350 never on humans (*An. barbirostris*, *An. hyrcanus*, *An. jamesii*, *An. kochi*, *An. maculatus*).

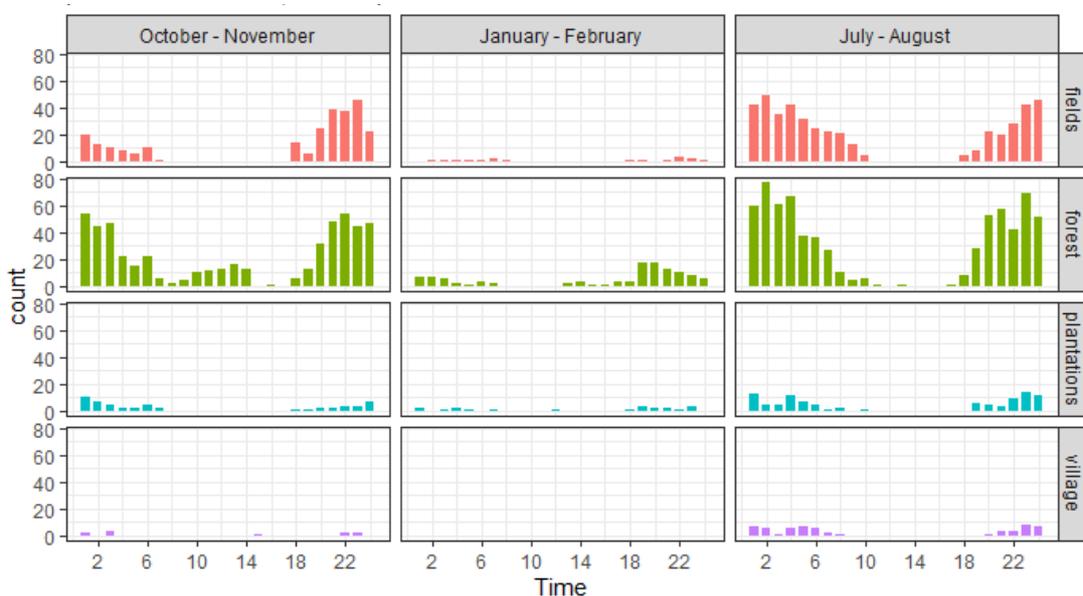
351 Figure 8: Blood meal origin for the different morphologically identified *Anopheles* species collected with
 352 SCR traps, during October-November (N = 20) and July-August (N = 95) rounds of collection.



353 Human blood meals were not associated with the time of the day (none in daytime, $16.1 \pm 6.5\%$ in
 354 nighttime, Fisher test, $\chi^2_1 = 1.15$, $p = 0.28$), with the side of the screen the *Anopheles* was collected (14.4
 355 $\pm 6.8\%$ on the inner side, $23.8 \pm 18.2\%$ on the outer side, $\chi^2_1 = 1.15$, $p = 0.28$) or with the round of collection
 356 ($26.7 \pm 22.4\%$ in October-November, $14.5 \pm 6.6\%$ in July-August, $\chi^2_1 = 1.44$, $p = 0.23$).

357 ***Anopheles dirus***. The most captured *Anopheles* species was *An. dirus*, with 2,356 specimens captured over
 358 all traps (of which 2,279 with HDN), corresponding to $41.5 \pm 1.3\%$ of all morphologically identified
 359 *Anopheles*. Over all HDN collection events, the time *An. dirus* was most captured was between 23h and
 360 midnight, but the time of the peak varied among land use category and round of collection (Figure 9). No
 361 *An. dirus* was found in villages in the dry season.

362 Figure 9: Count of captured *An. dirus* among land use category and round of collection, identified
 363 morphologically, with HDN traps (N = 2,279).



364 **2) Larvae**

365 Over rainy season rounds, we described 244 aquatic habitats (October-November: 72, July-August: 172),
366 from which 2,020 mosquito larvae successfully emerged, including 295 *Anopheles* (respectively N = 166
367 and N = 129). During the dry season round, we could describe only nine aquatic habitats, without any
368 mosquito larva, that were not included in the analysis.

369 **Aquatic habitats.** Over rainy season rounds, 240 aquatic habitats had an exhaustive description. The
370 univariate analyses determined that 12 variables were significantly associated to *Anopheles* larvae
371 presence in an aquatic habitat (Table Supp Mat S8). We collected more larvae in aquatic habitats from
372 fields or villages, but rarely in plantations and never in forest. In addition, large and very large (Table Supp
373 Mat S9), deep (>10 cm) aquatic habitats with medium to high turbidity and none to partial shading were
374 more susceptible to host *Anopheles* larvae. The presence of multiple species of predators, *Culex* larvae,
375 mud or composite matter at the bottom of the aquatic habitat (rather than leaves or bare) and shallow
376 edges were associated with larvae presence. The average temperature of aquatic habitats with larvae was
377 higher than the ones without larvae. Finally, stagnant temporary water bodies, rather than any other
378 habitat, were significantly associated with larvae presence. Otherwise, the presence of emerged
379 vegetation or multiple types of vegetation were associated with larvae absence in the aquatic habitat.

380 Without significance but with a p-value < 0.2, we observed that a higher proportion of aquatic habitats
381 contained larvae during the first round of collection than the last round, and that aquatic habitats with no
382 evidence of pollution were associated to larvae presence (Table Supp Mat S8). These two variables were
383 also included in the multivariate model.

384 Only four variables remained significantly associated with *Anopheles* larvae presence in the multivariate
385 model (Table Supp Mat S10, Figure 10): turbidity ($\chi^2_3 = 11.11$, $p = 0.01$), shallow edges ($\chi^2_1 = 7.22$, $p =$
386 0.007), water temperature ($\chi^2_1 = 12.39$, $p < 0.001$) and other mosquito genera in the aquatic habitat (χ^2_3
387 $= 34.59$, $p < 0.001$). Average water temperature was $30.6 \pm 3.0^\circ\text{C}$ in the habitats with *Anopheles* larvae
388 and $28.3 \pm 2.4^\circ\text{C}$ without.

389

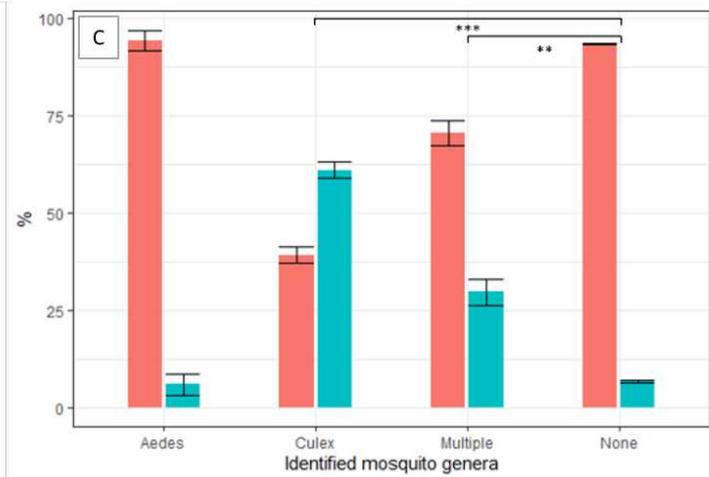
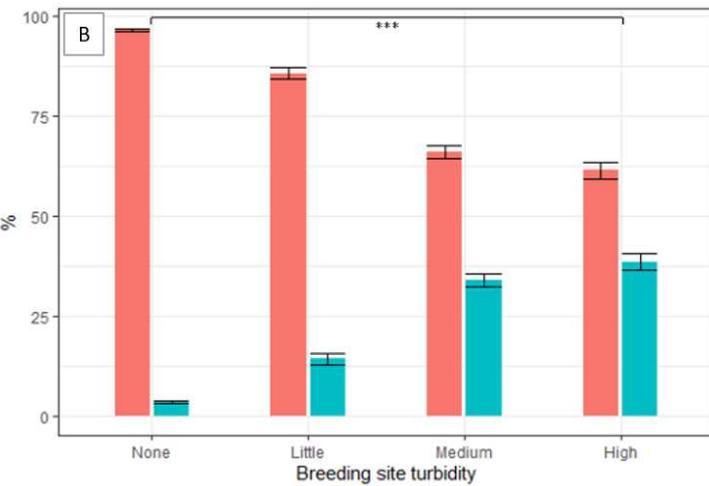
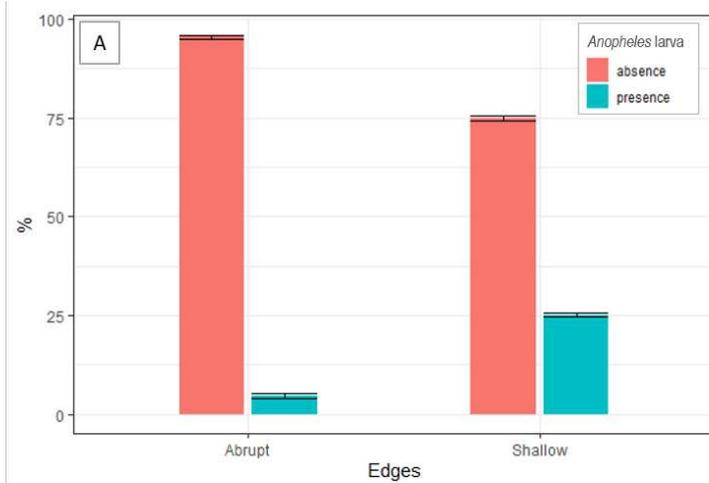
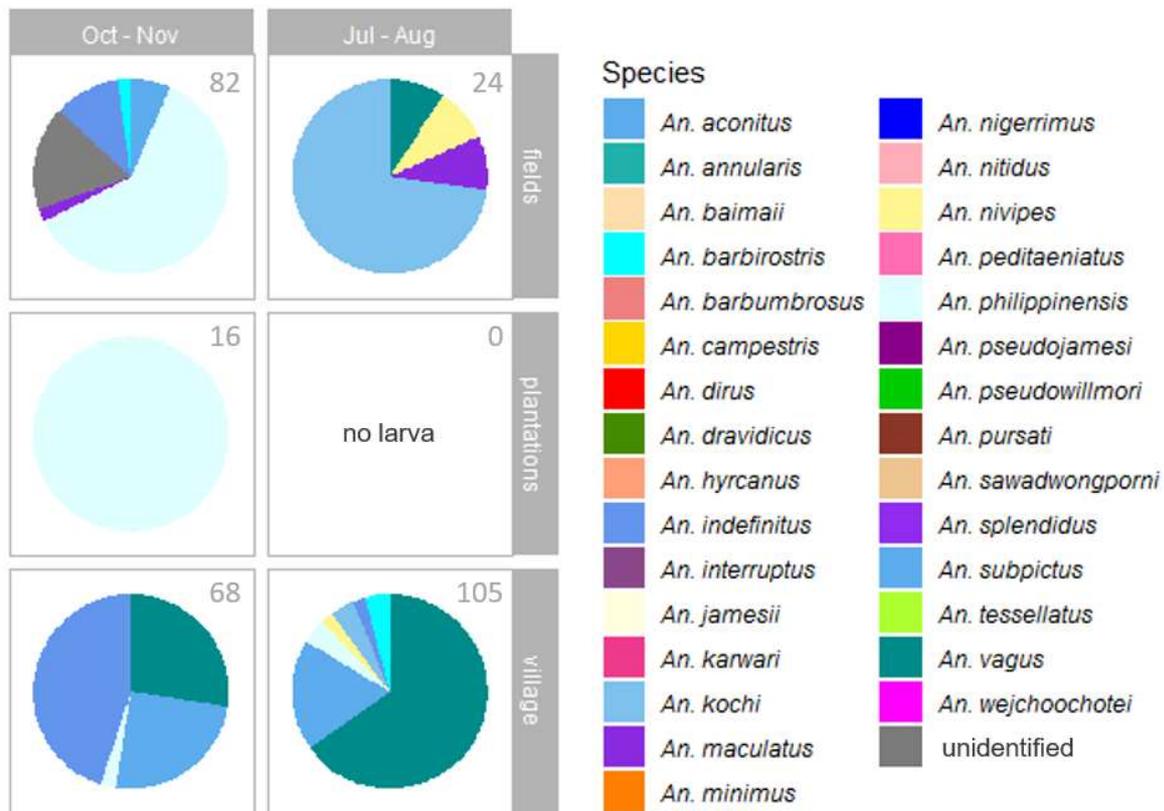


Figure 10: Variables significantly associated to *Anopheles* larvae presence with Tukey post-hoc significant difference (***) $p < 0.001$, ** $p < 0.01$.

A) *Anopheles* larvae presence by edges type in the aquatic habitat. A well or a water reservoir would have abrupt edges while a road track or a pond would have shallow edges. B) Percentage of aquatic habitats with or without *Anopheles* larvae by turbidity. C) *Anopheles* larvae presence and other identified genera in the aquatic habitat.

403 **Diversity.** Nine species were morphologically identified from the 157 emerged *Anopheles* females (Figure
 404 11). The most represented species were *Anopheles vagus* ($28.0 \pm 7.0\%$), *Anopheles philippinensis* ($23.6 \pm$
 405 6.6%) and *Anopheles indefinitus* ($15.3 \pm 5.6\%$). No larva of *An. dirus* was found. No larva was found in
 406 forest environments, even if 29 aquatic habitats were described in forest sites (8 during October-
 407 November and 21 during July-August).

408 Figure 11: *Anopheles* species distribution among collection rounds and land use type, emerged. Only
 409 females (N = 157) are displayed. No larva was found during forest transects investigations.



410

411 DISCUSSION

412 The implementation of this extensive mosquito collection highlighted important features about the
 413 ecology of Cambodian malaria vectors. Indeed, while we confirmed the importance of malaria exposure
 414 in forested areas, we also highlighted the importance of a rather unsuspected environment: the fields.
 415 Malaria exposure risk has been justifiably linked to the forests, as the primary vectors are forest dwellers
 416 and forest-associated activities are associated to a heightened malaria risk [2, 8, 56, 62], but not to fields.

417 On one hand, the outcome of this collection corroborates the forest-associated risk consensus: *Anopheles*
418 prevalence was associated to preserved habitats, as positive *Anopheles* were significantly more collected
419 in sites with lower SIDI values and higher forest coverage. SIDI values, ranging between 0 and 1, are
420 positively associated to fragmented landscapes. Also, abundance was positively associated to forest cover
421 in a 2 km radius. Thus, *Anopheles* abundance and prevalence appear to be higher in preserved habitats
422 with lower anthropisation.

423 On other hand, these results also challenge the current consensus. *Anopheles* abundance was also
424 positively associated to the fields cover in a 2 km radius and was higher in fields than in forest during the
425 October-November round. This outcome can convey multiple meanings. Firstly, the prevalence and
426 abundance observed in fields could arise from the proximity with forest patches, as observed with rubber
427 plantations [63]. Secondly, an increased malaria risk could be a consequence of the adaptability of *An.*
428 *dirus* and other primary vectors leading them to venture into fields, or because secondary vectors can
429 exploit this available niche to find blood meals, including from infected humans, and maintain malaria
430 transmission. However, given that almost 90% of malaria-positive *Anopheles* were molecularly identified
431 as *An. dirus*, the potential importance of secondary vectors remains minimal. Not a single *An. minimus*,
432 supposedly a primary vector, was molecularly identified. In a previous collection implementing only
433 human-baited traps, the diversity of infected species was similarly low (N = 3 [64]), while collections using
434 both cow and human-baited traps identified one [10], five [47] or twenty infected species [14]. These
435 contrasting results highlight the importance of the trap chosen to test a hypothesis and underline the
436 opportunistic feeding behaviour observed in GMS vectors.

437 The results of this collection suggest that *An. dirus* is the sole primary vector maintaining transmission.
438 The species was the most represented, both with morphological and molecular identifications, through
439 all rounds of collection and land use categories. *Anopheles dirus* was present in both preserved
440 environments and anthropised setting, as seen in the previous collection in the same study area [14],
441 which hints towards some adaptiveness of the species to anthropised environments. The typical *An. dirus*
442 breeding sites are described are small, shaded, shallow, normally temporary and stagnant aquatic habitats
443 in forests, orchards and rubber plantations [11]. Over the whole collection, we could not identify a single
444 *An. dirus* larva. We can speculate that *An. dirus* females lay eggs in inaccessible breeding sites, such as in
445 tall trees axils and leaves, in bromeliads plants [65] or other canopy sites not attainable during a transect.
446 *Anopheles dirus* breeding sites could also indeed correspond to small temporary aquatic sites in areas that
447 were not sampled, while the adults disperse effectively around the original breeding site, explaining the

448 important *An. dirus* abundance in all sites. In addition, despite the high *Anopheles* spp. densities observed
449 in forest, no larva was collected in any of the nine forest sites over the three rounds of collection.
450 Identifying *An. dirus* breeding sites and other *Anopheles* spp. forest breeding sites would provide insights
451 on potential vector control strategies.

452 Even if informative, the efficacy of the SCR traps was limited by the identification of blood meals. We can
453 expect that a proportion of blood meals cannot be identified if digestion has started more than 24 hours
454 before collection [66, 67], but our low percentage of successful identifications calls for optimisation. An
455 ELISA approach could yield better identifications, in addition to be better suited for multiple hosts feeding.
456 Due to the small sample size of the identified blood meals, no solid conclusion can be drawn.

457 These collections covered a small geographic area, with the farthest traps separated by only 27 kilometres.
458 While focusing on a limited study area was important to grasp the fine-scale of malaria risk as observed
459 in Cambodia [68–70], it also limits the generalisation of our findings. Mosquito collections implementing
460 the same tools would be necessary to differentiate the vector-habitat association from the *Anopheles*
461 bionomics characteristics to Kaev Seima district. Differences in the association between the vector and
462 the habitat can arise from site-specific factors rather than habitat [71], highlighting the need of replicated
463 sampling over wide geographical areas for robust habitat prediction.

464 As suggested by the previous 24 hour-round collection implemented in the area [14], capturing *Anopheles*
465 during daytime was not irrelevant: daytime prevalence is comparable to nighttime prevalence. Further
466 analysis of daily rhythms will provide important data about risk exposure throughout the day for different
467 environments.

468 Overall, mosquito identification was quite inaccurate, with over the molecularly identified *Anopheles*, 16.5
469 $\pm 2.2\%$ unidentified *Anopheles* and $31.6 \pm 2.8\%$ misidentified. Molecular identification, although more
470 costly, might be required in Cambodia or the Asia-Pacific region in general, because of the diverse and
471 overlapping *Anopheles* taxa.

472 Once again, it is not obvious if anthropisation is associated to increased *Anopheles* densities and facilitated
473 contact between the host and the vector, or is actually partly responsible for the decrease of malaria in
474 Cambodia. Anthropisation could drive malaria prevalence, as we collected significantly more infected
475 *Anopheles* in and around cultivated areas like fields. Deforestation is linked to higher temperatures that
476 would create more suitable breeding sites, as we observed more larvae in warmer aquatic habitats.

477 However, *Anopheles* densities are higher in and around less anthropised environments, with higher forest
478 cover and lower fragmentation.

479 **CONCLUSION**

480 The relation between anthropisation and *Anopheles* abundance, prevalence and diversity in Cambodia
481 appears to be driven by multiple environmental factors. We observed increased prevalence in forests but
482 also in fields, and we suggest that more collections should be implemented in fields to ensure a real
483 entomological surveillance. The implementation of HDN and SCR is encouraged, as they protect collectors
484 from bites and are suited to the exophilic vectors of the GMS. We suggest that 24-hour collections,
485 implemented multiple times a year through different provinces, might be necessary to grasp entirely
486 vectors bionomics in Cambodia and eventually in the GMS, to attain the elimination of residual malaria
487 transmission.

488

489 **ABBREVIATIONS**

490 CI: Confidence Interval

491 CO1: Cytochrome Oxidase subunit 1

492 cytb: cytochrome b

493 GLMM: Generalized Linear Mixed Models

494 GMS: Great Mekong Subregion

495 HDN: Human-baited Double Net Trap

496 HLC: Human Landing Catch

497 IRS: Indoor Residual Spraying

498 ITS: Insecticide-Treated Net

499 ITS2: Internal Transcribed Spacer 2

500 PCR: Polymerase Chain Reaction

501 REDCap: Research Electronic Data Capture

502 RTPCR: Real-Time Polymerase Chain Reaction

503 SCR: Barrier Screen

504 SCR-In: Inner side of the Barrier Screen

505 SCR-Out: Outer side of the Barrier Screen

506 sd: standard deviation

507 WHO: World Health Organisation

508

509 **DECLARATIONS**

510 **Funding**

511 This study is part of the International Centres of Excellence for Malaria Research program from the
512 National Institutes of Health “Understanding, tracking and eliminating malaria transmission in the Asia-
513 Pacific Region” (grant 1U19AI129392-01). A.P. is funded by the Pasteur Institute International Network
514 Calmette and Yersin Ph.D. scholarship (MHL/MJ/N°212/18).

515 **Competing interests**

516 The authors declare no competing interests.

517 **Corresponding author**

518 Correspondence to Anaïs Pepey.

519 **Acknowledgements**

520 The authors express their gratitude to the collectors, village heads, VMWs and officials for making this
521 study possible. We also thank the technicians and field staff for their work.

522 **REFERENCES**

523 1. WHO. World Malaria Report 2021. Geneva; 2021.

524 2. Sinka ME. Global Distribution of the Dominant Vector Species of Malaria. Anopheles mosquitoes - New
525 insights into malaria vectors. 2013. <https://doi.org/10.5772/54163>.

526 3. Sinka ME, Bangs MJ, Manguin S, Rubio-Palis Y, Chareonviriyaphap T, Coetzee M, et al. A global map
527 of dominant malaria vectors. *Parasites & Vectors*. 2012;5:69.

528 4. Endo N, Eltahir EAB. Environmental Determinants of Malaria Transmission Around the Koka Reservoir
529 in Ethiopia. *GeoHealth*. 2018;2:104–15.

530 5. Ferrão JL, Earland D, Novela A, Mendes R, Ballat MF, Tungaza A, et al. Mapping Risk of Malaria as a
531 Function of Anthropogenic and Environmental Conditions in Sussundenga Village, Mozambique. *Int J Environ
532 Res Public Health*. 2021;18.

- 533 6. Multini LC, Wilke ABB, Marrelli MT. Neotropical *Anopheles* (*Kerteszia*) mosquitoes associated with
534 bromeliad-malaria transmission in a changing world. *Acta Tropica*. 2020;:105413.
- 535 7. Saavedra MP, Conn JE, Alava F, Carrasco-Escobar G, Prussing C, Bickersmith SA, et al. Higher risk of
536 malaria transmission outdoors than indoors by *Nyssorhynchus darlingi* in riverine communities in the
537 Peruvian Amazon. *Parasit Vectors*. 2019;12:374.
- 538 8. Hii J, Hustedt J, Bangs MJ. Residual Malaria Transmission in Select Countries of Asia-Pacific Region:
539 Old Wine in a New Barrel. *J Infect Dis*. 2021;223 Supplement_2.
- 540 9. Edwards HM, Chinh VD, Le Duy B, Thanh PV, Thang ND, Trang DM, et al. Characterising residual
541 malaria transmission in forested areas with low coverage of core vector control in central Viet Nam.
542 *Parasites & Vectors*. 2019;12:454.
- 543 10. Edwards HM, Sriwichai P, Kirabittir K, Prachumsri J, Chavez IF, Hii J. Transmission risk beyond the
544 village: entomological and human factors contributing to residual malaria transmission in an area
545 approaching malaria elimination on the Thailand–Myanmar border. *Malaria Journal*. 2019;18:221.
- 546 11. Sinka ME, Bangs MJ, Manguin S, Chareonviriyaphap T, Patil AP, Temperley WH, et al. The dominant
547 *Anopheles* vectors of human malaria in the Asia-Pacific region: occurrence data, distribution maps and
548 bionomic précis. *Parasites & Vectors*. 2011;4:89.
- 549 12. Hii J, Rueda LM. Malaria vectors in the Greater Mekong Subregion: overview of malaria vectors and
550 remaining challenges. *Southeast Asian J Trop Med Public Health*. 2013;44 Suppl 1:73–165; discussion
551 306-307.
- 552 13. Marcombe S, Maithaviphet S, Bobichon J, Phommavan N, Nambanya S, Corbel V, et al. New insights
553 into malaria vector bionomics in Lao PDR: a nationwide entomology survey. *Malaria Journal*. 2020;19:396.
- 554 14. Vantaux A, Riehle MM, Piv E, Farley EJ, Chy S, Kim S, et al. *Anopheles* ecology, genetics and malaria
555 transmission in northern Cambodia. *Scientific Reports*. 2021;11:6458.
- 556 15. Austin KF, Bellinger MO, Rana P. Anthropogenic forest loss and malaria prevalence: a comparative
557 examination of the causes and disease consequences of deforestation in developing nations. *AIMS*
558 *Environmental Science*. 2017;4:217.
- 559 16. Yasuoka J, Levins R. Impact of deforestation and agricultural development on anopheline ecology and
560 malaria epidemiology. *Am J Trop Med Hyg*. 2007;76:450–60.
- 561 17. Lindblade KA, Walker ED, Onapa AW, Katungu J, Wilson ML. Land use change alters malaria
562 transmission parameters by modifying temperature in a highland area of Uganda. *Trop Med Int Health*.
563 2000;5:263–74.
- 564 18. Rossati A, Bargiacchi O, Kroumova V, Zaramella M, Caputo A, Garavelli PL. Climate, environment
565 and transmission of malaria. *Infez Med*. 2016;24:93–104.
- 566 19. Matthew OJ. Investigating climate suitability conditions for malaria transmission and impacts of climate
567 variability on mosquito survival in the humid tropical region: a case study of Obafemi Awolowo University
568 Campus, Ile-Ife, south-western Nigeria. *Int J Biometeorol*. 2020;64:355–65.

- 569 20. Coutinho PEG, Candido LA, Tadei WP, da Silva Junior UL, Correa HKM. An analysis of the influence
570 of the local effects of climatic and hydrological factors affecting new malaria cases in riverine areas along
571 the Rio Negro and surrounding Puraquequara Lake, Amazonas, Brazil. *Environ Monit Assess.*
572 2018;190:311.
- 573 21. Walsh JF, Molyneux DH, Birley MH. Deforestation: effects on vector-borne disease. *Parasitology.*
574 1993;106 Suppl:S55-75.
- 575 22. Tuno N, Okeka W, Minakawa N, Takagi M, Yan G. Survivorship of *Anopheles gambiae* sensu stricto
576 (Diptera: Culicidae) larvae in western Kenya highland forest. *J Med Entomol.* 2005;42:270–7.
- 577 23. Afrane YA, Zhou G, Lawson BW, Githeko AK, Yan G. Effects of microclimatic changes caused by
578 deforestation on the survivorship and reproductive fitness of *Anopheles gambiae* in western Kenya
579 highlands. *Am J Trop Med Hyg.* 2006;74:772–8.
- 580 24. Barros FSM, Honório NA. Deforestation and Malaria on the Amazon Frontier: Larval Clustering of
581 *Anopheles darlingi* (Diptera: Culicidae) Determines Focal Distribution of Malaria. *Am J Trop Med Hyg.*
582 2015;93:939–53.
- 583 25. da Silva-Nunes M, Moreno M, Conn JE, Gamboa D, Abeles S, Vinetz JM, et al. Amazonian malaria:
584 asymptomatic human reservoirs, diagnostic challenges, environmentally driven changes in mosquito vector
585 populations, and the mandate for sustainable control strategies. *Acta Trop.* 2012;121:281–91.
- 586 26. Laporta GZ, Lopez de Prado PIK, Kraenkel RA, Coutinho RM, Sallum MAM. Biodiversity can help
587 prevent malaria outbreaks in tropical forests. *PLoS Negl Trop Dis.* 2013;7:e2139.
- 588 27. Ostfeld RS. A Candide response to Panglossian accusations by Randolph and Dobson: biodiversity
589 buffers disease. *Parasitology.* 2013;140:1196–8.
- 590 28. WHO. Countries of the Greater Mekong zero in on falciparum malaria. Geneva: WHO; 2019.
- 591 29. Cui L, Yan G, Sattabongkot J, Cao Y, Chen B, Chen X, et al. Malaria in the Greater Mekong Subregion:
592 heterogeneity and complexity. *Acta Trop.* 2012;121:227–39.
- 593 30. Suwonkerd W, Amg-Ung B, Rimwangtrakul K, Wongkattiyakul S, Kattiyamongkool B, Chitprarop U,
594 et al. A field study on the response of *Anopheles dirus* to DDT and fenitrothion sprayed to huts in
595 Phetchabun Province, Thailand. *Tropical Medicine (Nagasaki).* 1990;32:1–5.
- 596 31. Service MW. *Mosquito Ecology: Field Sampling Methods.* Springer Netherlands; 1993.
- 597 32. Tangena J-AA, Thammavong P, Hiscox A, Lindsay SW, Brey PT. The Human-Baited Double Net Trap:
598 An Alternative to Human Landing Catches for Collecting Outdoor Biting Mosquitoes in Lao PDR. *PLoS*
599 *One.* 2015;10.
- 600 33. Sriwichai P, Karl S, Samung Y, Sumruayphol S, Kiattibutr K, Payakkapol A, et al. Evaluation of CDC
601 light traps for mosquito surveillance in a malaria endemic area on the Thai-Myanmar border. *Parasit*
602 *Vectors.* 2015;8:636.
- 603 34. Davidson JR, Wahid I, Sudirman R, Makuru V, Hasan H, Arfah AM, et al. Comparative field evaluation
604 of kelambu traps, barrier screens and barrier screens with eaves for longitudinal surveillance of adult
605 *Anopheles* mosquitoes in Sulawesi, Indonesia. *Parasites & Vectors.* 2019;12:399.

- 606 35. Williams CR, Long SA, Russell RC, Ritchie SA. Field efficacy of the BG-Sentinel compared with CDC
607 Backpack Aspirators and CO₂-baited EVS traps for collection of adult *Aedes aegypti* in Cairns,
608 Queensland, Australia. *J Am Mosq Control Assoc.* 2006;22:296–300.
- 609 36. Burkot TR, Russell TL, Reimer LJ, Bugoro H, Beebe NW, Cooper RD, et al. Barrier screens: a method
610 to sample blood-fed and host-seeking exophilic mosquitoes. *Malaria Journal.* 2013;12:49.
- 611 37. Keven JB, Katusele M, Vinit R, Koimbu G, Vincent N, Thomsen EK, et al. Species abundance,
612 composition, and nocturnal activity of female *Anopheles* (Diptera: Culicidae) in malaria-endemic villages
613 of Papua New Guinea: assessment with barrier screen sampling. *Malar J.* 2019;18.
- 614 38. Pollard EJM, Russell TL, Burkot TR. Maximising mosquito collections from barrier screens: the
615 impacts of physical design and operation parameters. *Parasit Vectors.* 2019;12:31.
- 616 39. Davidson JR, Sukowati S, Shinta, Asih PBS, Syafruddin D, Baskin RN, et al. Using barrier screens to
617 characterize mosquito composition, flight activity, and abdominal status in South Lampung, Indonesia.
618 *Parasit Vectors.* 2018;11.
- 619 40. Wagman J, Grieco JP, Bautista K, Polanco J, Briceño I, King R, et al. A comparison of two commercial
620 mosquito traps for the capture of malaria vectors in northern Belize, Central America. *J Am Mosq Control*
621 *Assoc.* 2014;30:175–83.
- 622 41. Obenauer PJ, Abdel-Dayem MS, Stoops CA, Villinski JT, Tageldin R, Fahmy NT, et al. Field
623 Responses of *Anopheles gambiae* Complex (Diptera: Culicidae) in Liberia using Yeast-Generated Carbon
624 Dioxide and Synthetic Lure-Baited Light Traps. *Journal of Medical Entomology.* 2013;50:863–70.
- 625 42. Ponlawat A, Khongtak P, Jaichapor B, Pongsiri A, Evans BP. Field evaluation of two commercial
626 mosquito traps baited with different attractants and colored lights for malaria vector surveillance in
627 Thailand. *Parasit Vectors.* 2017;10:378.
- 628 43. Achee NL, Youngblood L, Bangs MJ, Lavery JV, James S. Considerations for the use of human
629 participants in vector biology research: a tool for investigators and regulators. *Vector Borne Zoonotic Dis.*
630 2015;15:89–102.
- 631 44. Gryseels C, Durnez L, Gerrets R, Uk S, Suon S, Set S, et al. Re-imagining malaria: heterogeneity of
632 human and mosquito behaviour in relation to residual malaria transmission in Cambodia. *Malar J.*
633 2015;14:165.
- 634 45. Andrews ES, Schoeler GB, Gozalo AS, Carbajal F, Lopez-Sifuentes V, Turell MJ. Species Diversity,
635 Seasonal, and Spatial Distribution of Mosquitoes (Diptera: Culicidae) Captured in Aotus Monkey-Baited
636 Traps in a Forested Site Near Iquitos, Peru. *J Med Entomol.* 2014;51:1127–35.
- 637 46. Nguyen TQ, Nguyen MD, Pham VX, Ro HM, Edstein MD, Chow WK, et al. Entomological survey in
638 two communes with residual malaria transmission in Gia Lai Province in the central highlands of Vietnam.
639 *Malaria Journal.* 2021;20:403.
- 640 47. St. Laurent B, Oy K, Miller B, Gasteiger EB, Lee E, Sovannaroeth S, et al. Cow-baited tents are highly
641 effective in sampling diverse *Anopheles* malaria vectors in Cambodia. *Malar J.* 2016;15.

- 642 48. Sumarnrote A, Corbel V, Overgaard HJ, Celhay O, Marasri N, Fustec B, et al. Plasmodium Infections
643 in Anopheles Mosquitoes in Ubon Ratchathani Province, Northeastern Thailand During a Malaria
644 Outbreak. *J Am Mosq Control Assoc.* 2018;34:11–7.
- 645 49. Pepey A, Souris M, Vantaux A, Morand S, Lek D, Mueller I, et al. Studying Land Cover Changes in a
646 Malaria-Endemic Cambodian District: Considerations and Constraints. *Remote Sensing.* 2020;12:2972.
- 647 50. Pepey A, Obadia T, Kim S, Sovannaroth S, Mueller I, Witkowski B, et al. Mobility evaluation by GPS
648 tracking in a rural, low-income population in Cambodia. *PLOS ONE.* 2022; accepted manuscript.
- 649 51. Githeko AK, Mbogo CNM, Curtis CF, Lines J, Lengeler CP. Entomological monitoring of large-scale
650 vector-control interventions. *Parasitology Today.* 1996;12:127–8.
- 651 52. Harris PA, Taylor R, Minor BL, Elliott V, Fernandez M, O’Neal L, et al. The REDCap consortium:
652 Building an international community of software platform partners. *J Biomed Inform.* 2019;95:103208.
- 653 53. Rattanarithikul R, Harrison BA, Harbach RE, Panthusiri P, Coleman RE, Panthusiri P. Illustrated keys
654 to the mosquitoes of Thailand. IV. Anopheles. *Southeast Asian J Trop Med Public Health.* 2006;37 Suppl
655 2:1–128.
- 656 54. Canier L, Khim N, Kim S, Sluydts V, Heng S, Dourng D, et al. An innovative tool for moving malaria
657 PCR detection of parasite reservoir into the field. *Malaria Journal.* 2013;12:405.
- 658 55. Townzen JS, Brower AVZ, Judd DD. Identification of mosquito bloodmeals using mitochondrial
659 cytochrome oxidase subunit I and cytochrome b gene sequences. *Med Vet Entomol.* 2008;22:386–93.
- 660 56. Sandfort M, Vantaux A, Kim S, Obadia T, Pepey A, Gardais S, et al. Forest malaria in Cambodia: the
661 occupational and spatial clustering of Plasmodium vivax and Plasmodium falciparum infection risk in a
662 cross-sectional survey in Mondulkiri province, Cambodia. *Malaria Journal.* 2020;19:413.
- 663 57. McGarigal K, Marks BJ. FRAGSTATS: spatial pattern analysis program for quantifying landscape
664 structure. General technical report PNW (USA). 1995.
- 665 58. SERVIR Mekong. 2020.
- 666 59. Crawley MJ. *The R book.* John Wiley & Sons; 2012.
- 667 60. R Core Team. *R: A language and environment for statistical computing.* www.R-project.org. R. R
668 Foundation for Statistical Computing, Vienna, Austria; 2022.
- 669 61. Hartig F. *DHARMA: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models.*
670 2021.
- 671 62. Incardona S, Vong S, Chiv L, Lim P, Nhem S, Sem R, et al. Large-scale malaria survey in Cambodia:
672 Novel insights on species distribution and risk factors. *Malar J.* 2007;6:37.
- 673 63. Thomson R, Sochea P, Sarath M, MacDonald A, Pratt A, Poyer S, et al. Rubber plantations and drug
674 resistant malaria: a cross-sectional survey in Cambodia. *Malar J.* 2019;18:379.
- 675 64. Durnez L, Mao S, Denis L, Roelants P, Sochantha T, Coosemans M. Outdoor malaria transmission in
676 forested villages of Cambodia. *Malaria Journal.* 2013;12:329.

- 677 65. Ikhsan M, Hadi UK, Soviana S. Diversity and distribution bromeliads plants as breeding habitat for
678 mosquito larvae (Diptera: Culicidae) in Bogor, Indonesia. *Biodiversitas Journal of Biological Diversity*.
679 2020;21.
- 680 66. Escobar D, Ascencio K, Ortiz A, Palma A, Sánchez A, Fontecha G. Blood Meal Sources of *Anopheles*
681 spp. in Malaria Endemic Areas of Honduras. *Insects*. 2020;11.
- 682 67. Kent RJ, Norris DE. *Am J Trop Med Hyg*. 2005;73:336–42.
- 683 68. Bannister-Tyrrell M, Srun S, Sluydts V, Gryseels C, Mean V, Kim S, et al. Importance of household-
684 level risk factors in explaining micro-epidemiology of asymptomatic malaria infections in Ratanakiri
685 Province, Cambodia. *Sci Rep*. 2018;8:11643.
- 686 69. Okami S, Kohtake N. Spatiotemporal Modeling for Fine-Scale Maps of Regional Malaria Endemicity
687 and Its Implications for Transitional Complexities in a Routine Surveillance Network in Western
688 Cambodia. *Front Public Health*. 2017;5:262.
- 689 70. Tripura R, Peto TJ, Veugen CC, Nguon C, Davoeung C, James N, et al. Submicroscopic *Plasmodium*
690 prevalence in relation to malaria incidence in 20 villages in western Cambodia. *Malaria Journal*.
691 2017;16:56.
- 692 71. Brown R, Chua TH, Fornace K, Drakeley C, Vythilingam I, Ferguson HM. Human exposure to zoonotic
693 malaria vectors in village, farm and forest habitats in Sabah, Malaysian Borneo. *PLoS Negl Trop Dis*.
694 2020;14:e0008617.

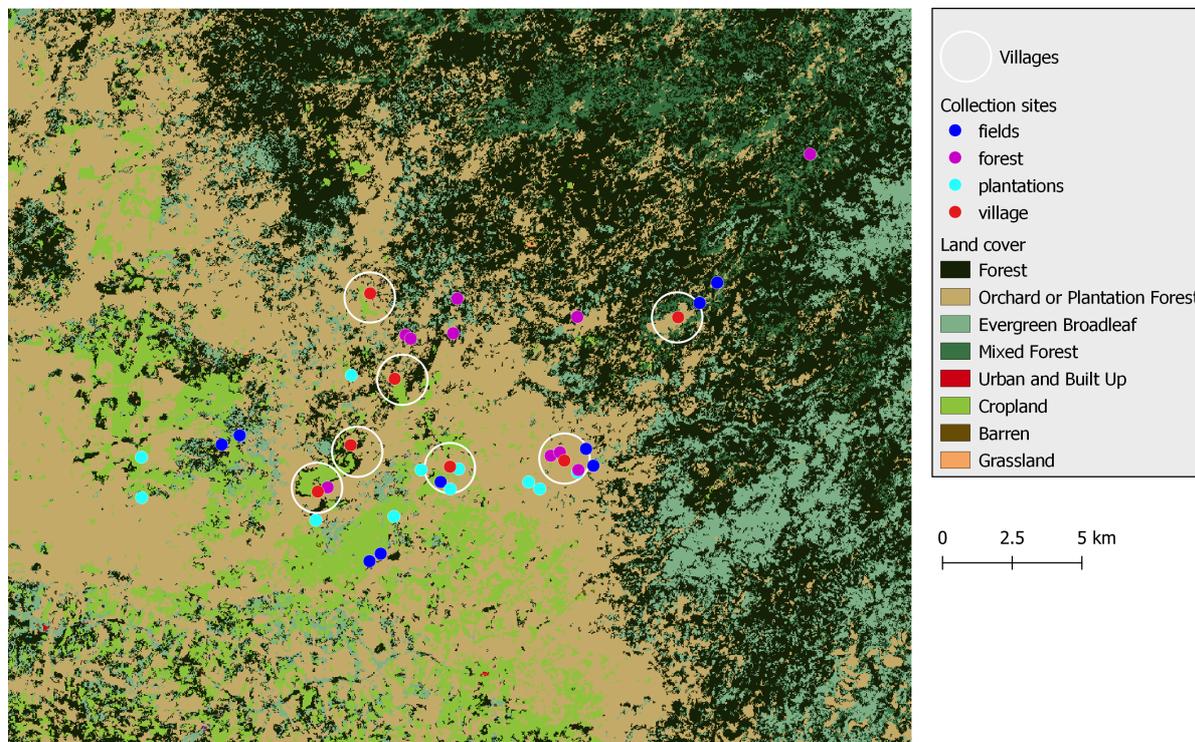
695

696

697 Supplementary material

698

699 Figure S1: Study area and collections sites. Land cover produced by the SERVIR Mekong Project [1].



700

701

702 Table S1: *Anopheles* prevalence multivariate models results (environmental variables model). Significant
 703 results are highlighted in bold.

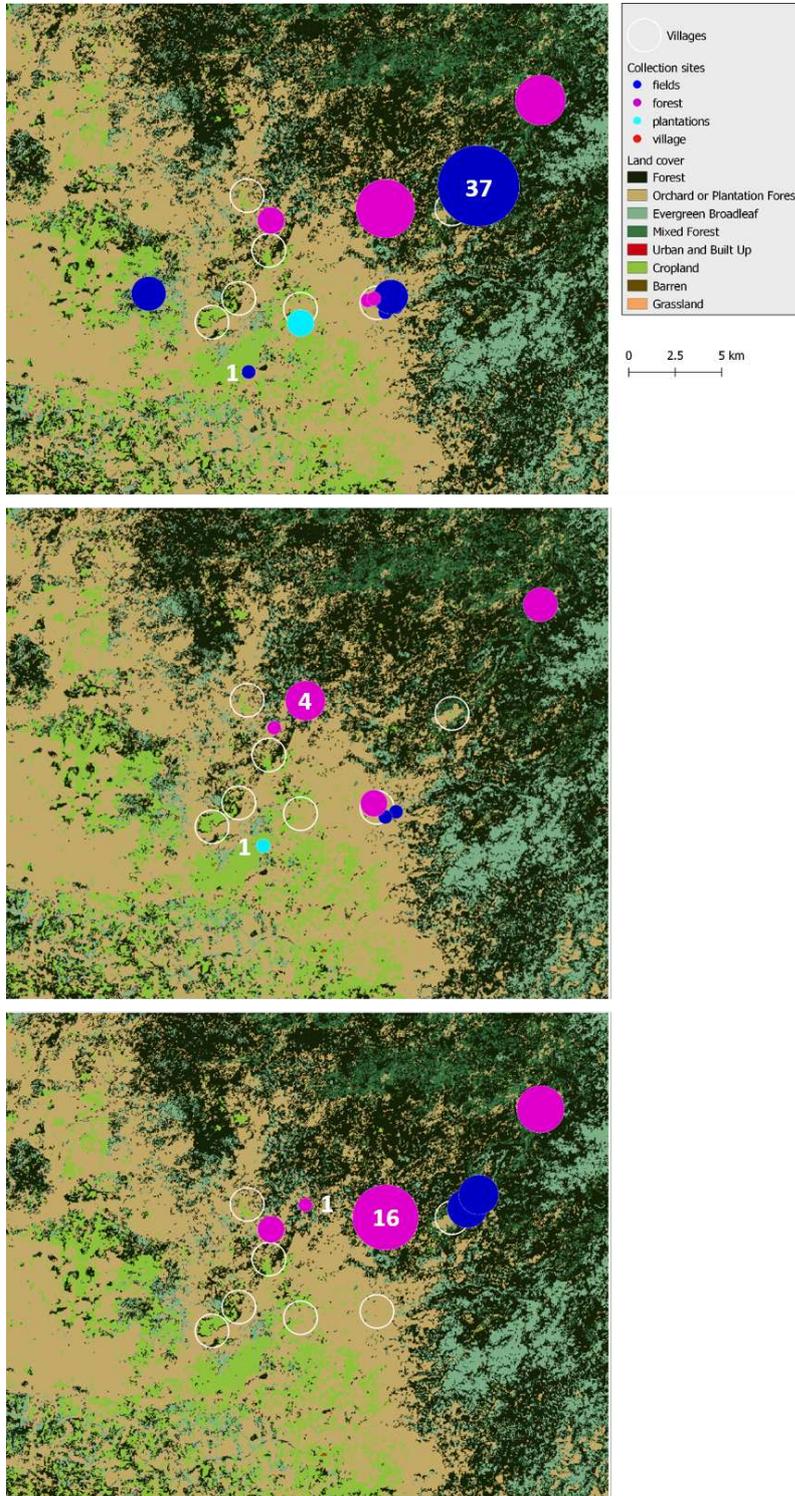
Variable	χ^2	df	p
LU	1.99	3	0.57
Passage	0.73	1	0.39
Round:Passage	1.13	1	0.29
Round:LU	24.59	3	<0.001
Round	12.81	1	<0.001

704

705 Table S2: *Anopheles* prevalence multivariate models results (landscape variables model). Significant
 706 results are highlighted in bold.

Variable	χ^2	df	p
Plantations cover	0.51	1	0.48
Distance to nearest household	0.80	1	0.37
Cropland cover	1.55	1	0.21
Patch density	3.19	1	0.07
SIDI	4.02	1	0.04
Forest cover	14.30	1	<0.001

707 Figure S2: Localisation of the identified infected *Anopheles* over the study area. The minimum and
 708 maximum per site per round is indicated next to the corresponding location. Land cover produced by the
 709 SERVIR Mekong Project [1].



710 Table S3: *Anopheles* abundance multivariate model results (environment variables model).

Variable	χ^2	df	p
Round:LU	4.18	6	0.65
Round:Passage	3.23	2	0.20
Passage	1.91	1	0.17
Round	22.43	2	<0.001
Land use	27.81	3	<0.001

711

712 Table S4: Abundance per land use type with Tukey post-hoc results and significance. Significant
713 differences are highlighted in bold.

Variable	n/day/site	Odd ratios				p			
Land use		<i>fields</i>	<i>forest</i>	<i>plantation</i>	<i>village</i>	<i>fields</i>	<i>forest</i>	<i>plantation</i>	<i>village</i>
<i>fields</i>	31		2.1	3.6	8.7	0.09	<0.001	<0.001	
<i>forest</i>	18			1.7	4.1		0.34	<0.001	
<i>plantation</i>	10				2.5				0.045
<i>village</i>	4								

714

715 Table S5: Abundance per collection round with Tukey post-hoc results and significance. Significant
716 differences are highlighted in bold.

Variable	n/day/site	Odd ratios			p		
Round		<i>Oct-Nov</i>	<i>Jan-Feb</i>	<i>Jul-Aug</i>	<i>Oct-Nov</i>	<i>Jan-Feb</i>	<i>Jul-Aug</i>
<i>Oct-Nov</i>	18		2.2	1.2		<0.001	0.5
<i>Jan-Feb</i>	9			2.7			<0.001
<i>Jul-Aug</i>	21						

717

718 Table S6: *Anopheles* abundance multivariate models results (landscape variables model). Significant
 719 results are highlighted in bold.

Variable	χ^2	df	p
SIDI	3.62	1	0.06
Patch density	1.70	1	0.19
Plantations cover	2.89	1	0.09
Distance to nearest household	3.29	1	0.07
Cropland cover	5.44	1	0.02
Forest cover	7.66	1	<0.01

720

721 Table S7: Correspondence between morphological and molecular identification, all traps (N = 1,061 valid
 722 morphological identifications).

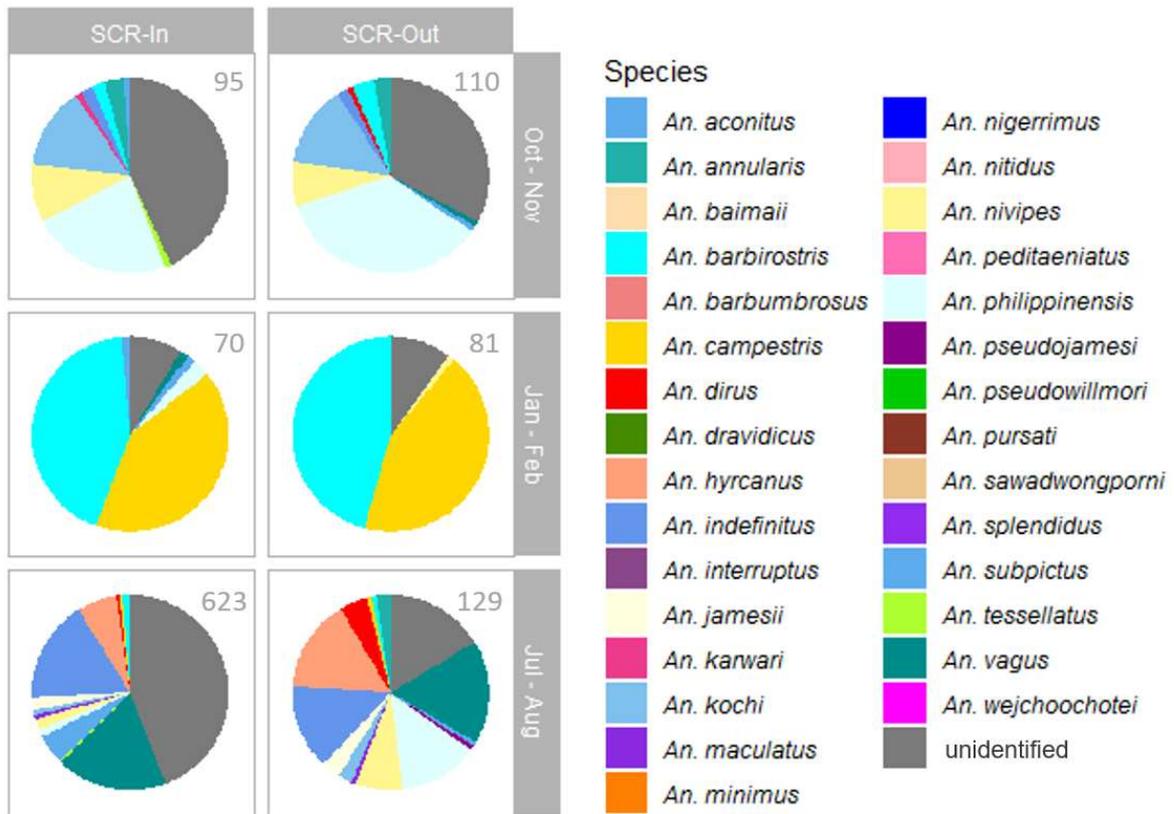
Morphological identification	Molecular identification	n	Accuracy (%)
<i>An. aconitus</i>	<i>An. aconitus</i>	1	100
<i>An. annularis</i>	<i>An. pseudojamesi</i>	1	93.8
	<i>An. annularis</i>	15	
<i>An. baimaii</i>	<i>An. dirus</i>	10	0
<i>An. barbirostris</i>	<i>An. wejchoochotei</i>	121	9.5
	<i>An. nigerrimus</i>	2	
	<i>An. campestris</i>	11	
	<i>An. barbirostris</i>	14	
<i>An. campestris</i>	<i>An. wejchoochotei</i>	88	18.6
	<i>An. saeungae</i>	1	
	<i>An. maculatus</i>	1	
	<i>An. campestris</i>	21	
	<i>An. barbirostris</i>	2	
<i>An. dirus</i>	<i>An. dirus</i>	320	100
<i>An. hyrcanus</i>	<i>An. sinensis</i>	2	0
	<i>An. peditaeniatus</i>	12	
	<i>An. nigerrimus</i>	2	

<i>An. indefinitus</i>	<i>An. vagus</i>	25	0
<i>An. jamesii</i>	<i>An. splendidus</i>	1	0
	<i>An. philippinensis</i>	8	
	<i>An. pallidus</i>	5	
	<i>An. nivipes</i>	3	
<i>An. kochi</i>	<i>An. kochi</i>	12	100
<i>An. maculatus</i>	<i>An. sawadwongporni</i>	7	77.6
	<i>An. rampae</i>	4	
	<i>An. maculatus</i>	45	
	<i>An. dravidicus</i>	1	
	<i>An. dirus</i>	1	
<i>An. minimus</i>	<i>An. pampanai</i>	1	50
	<i>An. minimus</i>	1	
<i>An. nigerrimus</i>	<i>An. nigerrimus</i>	2	100
<i>An. nivipes</i>	<i>An. nivipes</i>	30	90.9
	<i>An. annularis</i>	3	
<i>An. philippinensis</i>	<i>An. philippinensis</i>	17	58.6
	<i>An. nivipes</i>	12	
<i>An. pseudojamesi</i>	<i>An. pseudojamesi</i>	1	100
<i>An. pseudowillmori</i>	<i>An. maculatus</i>	3	0
<i>An. pursati</i>	<i>An. hyrcanus</i>	1	0
<i>An. subpictus</i>	<i>An. vagus</i>	7	0
<i>An. tessellatus</i>	<i>An. tessellatus</i>	35	100
<i>An. vagus</i>	<i>An. vagus</i>	37	100
unidentified	<i>An. wejchoochotei</i>	9	NA
	<i>An. vagus</i>	10	
	<i>An. tessellatus</i>	2	
	<i>An. sawadwongporni</i>	3	
	<i>An. philippinensis</i>	9	
	<i>An. peditaeniatatus</i>	21	
	<i>An. pampanai</i>	1	

	<i>An. pallidus</i>	1	
	<i>An. nivipes</i>	14	
	<i>An. nitidus</i>	2	
	<i>An. nigerrimus</i>	55	
	<i>An. maculatus</i>	2	
	<i>An. jamesii</i>	2	
	<i>An. hyrcanus</i>	1	
	<i>An. dissidens</i>	3	
	<i>An. dirus</i>	33	
	<i>An. campestris</i>	2	
	<i>An. barbirostris</i>	3	
	<i>An. annularis</i>	2	

723

724 Figure S3: *Anopheles* species distribution among collection rounds and side of the barrier screen (SCR-In:
 725 inner side facing the village, SCR-Out: outer side facing the forest), morphological identification (N =
 726 1,108).



727

728 Table S8: Univariate models results of breeding sites characteristics selected to create the *Anopheles*
 729 larvae presence multivariate model. Significant results are highlighted in bold, results with $p < 0.2$ included
 730 in multivariate model in italic.

Variable	χ^2	df	p
Visible people	2.65	2	0.27
Pollution	8.84	4	<i>0.06</i>
Round	3.41	1	<i>0.06</i>
Land use	9.15	3	0.03
Depth	6.31	1	0.01
Edges	9.74	1	<0.01
Turbidity	16.39	3	<0.001
Shading	14.03	2	<0.001
Bottom layer	17.93	3	<0.001
Predators	26.60	3	<0.001
Water temperature	20.36	1	<0.001
Vegetation	29.23	3	<0.001
Dimensions	33.57	4	<0.001
Habitat class	51.45	4	<0.001
Presence of other mosquito genus	52.73	3	<0.001

731

732 Table S9: Correspondence between size category and estimated surface, with examples observed in the
 733 field. Surface was estimated from dimensions measured on site with measuring tape.

Dimension category	Estimated surface (cm²)	Examples
Very Small	≤ 100	bamboo hole, plastic bottle, plant's axil
Small	101-2,000	jar, road tracks, puddle
Medium	2,001-40,000	water reservoir, well
Large	40,001-400,000	ditches, ponds
Very Large	$\geq 401,000$	river, rice paddy, stream

734

735 Table S10: *Anopheles* larvae presence multivariate model results. Land use, shading and habitat class
 736 variables could not be included as model would not converge. Significant results are highlighted in bold.

Variable	χ^2	df	p
Dimensions	8.79	4	0.67
Depth	0.40	1	0.53
Predators	3.18	3	0.36
Vegetation	3.79	3	0.29
Pollution	5.32	4	0.26
Bottom layer	4.67	3	0.20
Round of collection	1.95	1	0.16
Turbidity	11.11	3	0.01
Edges	7.22	1	<0.001
Water temperature	12.39	1	<0.001
Non- <i>Anopheles</i> larvae	34.59	3	<0.001

737

2.3. Discussion

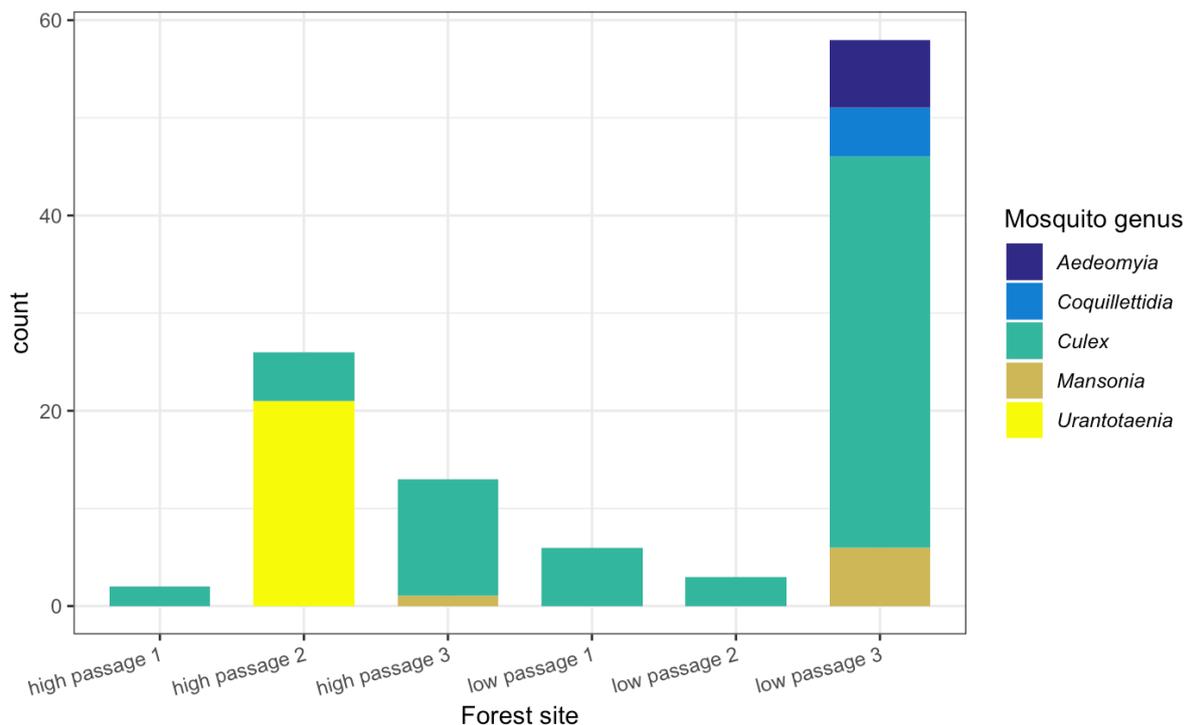
The main rationale for carrying out this chapter was to focus on what is known and what remains to be known about malaria vectors in Cambodia. The primary vectors are the forest-associated *An. dirus* and *An. minimus*, and a dozen other species participate to malaria transmission [57, 157]. These vectors are exophilic, are most active at dusk, night or dawn and can bite actively outside of the hours of mosquito net use, when risk populations are resting or socialising in the evenings [177]. While bed nets are an effective tool to protect from infectious bites when correctly implemented, they are not efficient outside of the households, where individuals scarcely use bed nets [152]. To understand the conditions of exposure outside the household, we implemented collection mosquitoes in village, fields, plantations and forest. To fully grasp the conditions of the host-vector contact, we both aimed to target mosquito looking for a blood meal (baited traps) and the flight activity and abdominal status of mosquitoes travelling between environments.

Because of the risk and labour inherently linked to HLC collections, we wanted to substitute them with another type of human-baited trap, that could replicate natural vector exposure conditions. In that matter, the HDN confirmed that it can and should be implemented to estimate *Anopheles* densities and prevalence in Cambodia, as it was successfully validated against the HLC. To intercept mosquitoes, barrier screens revealed an important variability in terms of abundance and fed mosquitoes between seasons. As it was the first implementation in Cambodia, a replication of the study in another province could help defining if the seasonal variability was related to mosquito travel patterns variations or was a consequence of interfering factors that were not considered during collection.

In addition to HDN, HLC and SCR traps, other traps were deployed during mosquito collections but failed to collect any *Anopheles*, so their installation was not mentioned in the corresponding manuscript draft. Clay pots were installed around the field base, with various collection intervals (3h, 6h and 12h), to collect shelter-looking mosquitoes. Over the few days of testing, no mosquito was collected. After humidifying the pots to make them more hospitable for potential mosquitoes, the collection remained unsuccessful. More importantly, CDC light traps were installed in seven forest sites during the first round of collection (October, corresponding to the end of rainy season). The traps were installed in pairs: one at ground level (1.5 metres from the ground) and one at canopy level (6 to 12 metres high,

levelling with the highest trees). The traps did not include CO₂ baits, as the temperatures in Mondulkiri are too low during night time to allow yeast fermentation and setup with dry ice would be difficult to implement in the forest canopy. To mimic human scent, a cotton shirt that had been worn by a volunteer for 24h was placed next to the entry of the trap. A slingshot was used to pass a rope over the highest branches of the canopy, which could pull the CDC traps and their batteries at canopy level. Ground level traps were installed in the lower branches, below the other trap. Every twelve hours for 48h, mosquitoes trapped inside the CDC net were collected. No *Anopheles* was collected, neither at ground or canopy levels, but mosquitoes from other genera could be identified (Figure 17). Given the considerable effort and time invested in the installation of canopy traps, the CDC traps were not instituted in the other collection rounds.

Figure 17: Count of mosquitoes collected in CDC light traps during the October-November round. Over the six forest locations, traps collected mostly moths, ants and flies. No mosquito was trapped at canopy level.



Precautions were taken to optimise larvae collections and their raising into adults. During transects, the dipping into the breeding site was very carefully made to avoid casting shadow in the habitat as larvae are very sensitive and would dive to the bottom if a shadow was to be cast over the water. Mosquito larvae can predate other larvae, such as some *Culex* or *Toxorhynchites*¹ species that can predate on smaller individuals from a different or the same species [285, 286]. As such, larvae were separated by breeding sites but also by genus (easy to determine morphologically on live larvae) to limit interspecific and intergeneric predation. Intraspecific predation is more difficult to prevent, but was not observed between the larvae we collected. In such a case, a separation of larvae of different sizes or stages could have helped.

With *Anopheles* being considered a vector mostly active at night, a majority of collections in SEA do not include day time collection [152, 154, 209, 236, 287–289]. In a study from 2019 at the Thai-Myanmar border [152], a significant amount of bites occurred between 05:00 and 08:00 for all three primary vectors (13%, 10% and 8% of *An. minimus*, *An. maculatus* and *An. dirus*, respectively). However, the collections were not extended after 8:00, and further daytime exposure was not quantified. As our collections captured between 15 and 20% of *Anopheles* during daytime, depending on the year of collection, we can suggest that an important share of *Anopheles* is neglected and that daily rhythms are misunderstood. The exposure of rural populations at dusk and dawn, when bed nets do not protect them, is a source of frequent malaria exposure [177]. However, without further investigating the daytime abundance, our understanding of mosquito exposure is limited and may be missing key exposure hours throughout the day, that could vary from an environment to another, based on difference in light, temperature and humidity.

¹ We collected a *Toxorhynchites* larva from a tree hole in the deep forest in Kaev Seima. While kept in its emergence box, it ate twelve *Anopheles* larvae in less than 12 hours.

The study design is not exempt of biases or limitations. The mosquito collection from 2019-2020 was designed with a collection every three months over a year, with two rounds per season. The rounds of collection would have been October-November, January-February, April-May and July-August. However, the third round of collection had to be cancelled due to travel restrictions associated to the COVID pandemic. This imbalance between dry and rainy seasons collections most probably affected the results and their interpretation. However, since the 2019-2020 collection, routine collections have been implemented on a subsample of the sites and continue to this day. This follow-up of *Anopheles* populations over several years, while the country aims to achieve malaria elimination soon and that prevalence is greatly decreasing, should provide meaningful data about *Anopheles* reservoirs and prevalence evolution within vectors subpopulations. The monitoring of simian parasites within the mosquito populations should not be neglected during this timeframe, to avoid their relative increase in malaria cases such as *P. knowlesi* in Malaysia.

By looking at the RTPCR results of the 2019-2020 mosquito collection, we observed a decrease in malaria prevalence since 2017, corresponding to the observed decrease in malaria cases. This RTPCR amplifying the cytochrome b from *Plasmodium* parasites DNA, have been routinely used since 2012 in the laboratory. By using this protocol, over the entire collection (including all trap types, N = 6,688 tested *Anopheles*) we detected 205 positive *Anopheles* for any species of *Plasmodium*, and 127 *Anopheles* positive for at least human *Plasmodium* species, or a prevalence of $1.9 \pm 0.3\%$ for human malaria parasites. Recently, another protocol was developed to assess malaria prevalence, by using standard Sanger sequencing of the same cytochrome b region and blasting the nucleotide sequence against the National Centre for Biotechnology Information Genbank database. Prevalence changed drastically when applying this methodology. Rather than 127, only six human parasites, all *P. vivax*, were identified, corresponding to a prevalence of $0.1 \pm 0.1\%$. Additionally, six other simian species were identified (BLAST identity >98%, sequence length > 900bp, Table 2).

Table 2: All identified *Plasmodium* species (N = 72) from *Anopheles* samples identified with cytochrome b sequencing, with their prevalence and 95% CI (N = 6,688). We can note that *P. knowlesi*, of increasing concern in other SEA countries such as Malaysia, was not detected in the collected *Anopheles* mosquitoes, neither through RTPCR nor cytochrome b sequencing.

Species	N	Prevalence	95% CI
<i>Plasmodium coatneyi</i>	17	0.3	0.1
<i>Plasmodium cynomolgi</i>	8	0.1	0.1
<i>Plasmodium fieldi</i>	4	0.1	0.1
<i>Plasmodium hylobati</i>	5	0.1	0.1
<i>Plasmodium inui</i>	32	0.5	0.2
<i>Plasmodium vivax</i>	6	0.1	0.1

The analysis of the full cytochrome b sequences suggest that this identification of simian parasites might be correct, but this new protocol for sequencing has still to be validated. A major drawback is the limited number of resources available to compare our sequences to a reference. Detailed data about these simian species is rare and often barely described. Some of the uploaded simian sequences could be *vivax* or *vivax*-like. We may need to develop a second PCR for amplification on another conserved part of the genome (such as ITS2 or CO1) to cross-validate the current identification.

Interestingly, the selection of samples to include in the *P. vivax* AmpSeq library (see Chapter 3) was done before the identification of the simian species in *Anopheles* samples. Of the eleven *Anopheles* samples that were successfully amplified using the *P. vivax* AmpSeq molecular markers, four were effectively identified as *P. vivax* by *cytb* sequencing. Five amplicons were not identified because the *cytb* PCR amplification was not successful (N = 4) or because the output sequence was of poor quality and could not produce a consensus sequence (N = 1). Lastly, two amplicons were identified as *P. hylobati*, with 98.5% et 99.9% of identity with a BLAST sequence. One of the *P. hylobati* samples was amplified by two markers (chr02 and chr07) while the other sample by one marker (chr11). We can hypothesise that the *P. vivax* AmpSeq molecular markers were not tested for other *Plasmodium* species and might amplify simian species taxonomically close to *P. vivax*. Otherwise, we can suggest that

the sequencing of the cyt b region cannot discriminate finely between different *Plasmodium* species.

If the cyt b sequencing were accurate at identifying *Plasmodium* species, the prevalence observed during the years 2019 and 2020 would be extremely low, with 0.1% of *Anopheles* infected with a human *Plasmodium* over the whole collection. Even if this proportion might appear very low to maintain transmission, it would not be the first time that such numbers are observed in the GMS. In neighbouring Laos, where malaria transmission resembles Cambodia's, Marcombe et al. [287] found only two positive *Anopheles* over 4,000 tested. In Thailand, no infected mosquitoes could be found over 2,240 tested individuals in the Kanchanburi province during a malaria outbreak [289]. We can hypothesise that malaria prevalence is effectively low in wild populations as a consequence of malaria control and residual transmission settings, or that populations are hyperimmune to malaria. However, as *An. dirus* mosquitoes have showed in the past their ability to avoid mosquito control tools after their implementation [59] and that we do not have quantitative data about their flight range, collections could as well be missing the space and time they are active and biting.

While Cambodia vector control relies on bed nets and IRS that are mostly used within the village, the elimination strategy fails to target the environments in which we identified an important *Anopheles* exposure, in fields and in the forest. New vector control tools may be required. Spatial and topical repellents may protect populations from bites while being compatible with the working conditions of farmers and forest goers, but could induce or increase insecticides resistance in mosquito populations. Alternatively, as the GMS vectors are highly opportunistic, zooprophyllaxis could be successful and well accepted by rural populations. Finally, the deployment of predators such as *Toxorhynchites* could be tested to assess their efficacy as a larvae control tool with limited deleterious effects on the biodiversity. Overall, vector control will require innovative tools targeting the *Anopheles* populations outside the village.

Describing the environment associated to an increased exposure to mosquito bites is essential to determine risk factors. However, observing the environment, the host and the vector, without understanding the nature of the parasites themselves, would set aside some important features about the disease dynamics. The screening and the Sanger sequencing of the parasites cannot provide information about the nature of the clones, nor the dynamics and the distribution of parasites associated with the asymptomatic reservoir. To achieve a complete understanding of malaria transmission in the study area, a deeper analysis into the *P. falciparum* and *P. vivax* clones detected in both humans and mosquitoes is necessary.

Through the thousands of *Anopheles* that were collected and the consequent database that was produced, one important conclusion stands out: Cambodia urgently requires new vector control tools that can be used when villagers are working or are outside of the household. The methods implemented so far, ITNs and IRS, do not protect rural populations effectively against opportunistic day-time biting and are mostly not suited for farmers, forest goers and their families sleeping in the forest, the fields and isolated farming huts. Raising awareness among local populations about biting exposure outside of the forest and during daytime, or increasing the distribution of ITNs would probably not be effective, as bed nets ownership and use assured by a vast majority of households. The use of spatial repellents or ivermectin would be promising in that context, but would require an adjustment phase to allow the rural populations to understand and accept these interventions.

Chapter 3: Characterisation of *Plasmodium* spp. transmission within human and vector populations

3.1. Introduction

Understanding the genetic structure of pathogens populations provides insight into the adaptive processes that have affected those populations, such as migration, transmission or selection processes. Such information can have important applications in public health [290]. Indeed, during and after elimination, the monitoring of the genetic structure of the parasite populations can help pinpoint areas to target for a successful and definitive elimination and differentiate local from imported cases [268, 290, 291].

While we can observe through classic monitoring the decrease of symptomatic and *P. falciparum* cases in Cambodia since roughly a decade, the effects of the elimination programme on asymptomatic cases are harder to grasp.

A broader understanding of *Plasmodium* population genetics in the GMS, based on multiple molecular markers, is required to understand malaria dynamics. Previous genetic studies on *Plasmodium falciparum* have been done at country [292] and GMS level [293], focusing on *P. falciparum* drugs resistance markers only. Resistance markers are very informative on the potential spread of resistance strains, changes in drug efficacy and provide crucial information about the implementation of alternative therapies [290], however they do not allow detailed population genetics. Thus, over the last years, molecular markers not related to resistance markers have also been developed [269, 294], holding the potential to describe and understand malaria dynamics at a finer scale. Besides, the detection of *P. vivax* haplotypes and the understanding of its population dynamics is an equally, if not more, important task, as the parasite species is now representing most of the cases in the GMS [3] and seems less affected than *P. falciparum* by elimination measures [295].

The detection and quantification of the different clones harboured in a single individual provide precious knowledge about the disease epidemiology and dynamics, but minority clones can be difficult to detect with traditional methods. Indeed, cut-off requirements associated with microsatellites does not enable the detection of minority clones below a certain threshold. Length polymorphic markers are not suited to detect low-density clones in

polyclonal infections and they often exhibit significant bias depending on the length of fragments, with shorter fragments outperforming larger fragments, and larger fragments being potentially lost [296, 297]. Whole genome sequencing (WGS) offers very high resolution results, but the process is lengthy and costly [290]. However, the identification of parasite clones is crucial to measure the acquisition, elimination and persistence of individual clones in a human host and a population. Deep sequencing of highly polymorphic amplicons (AmpSeq) allows the sensitive detection of minority clones and improves discriminatory power for genotyping multi-clone infections [297]. AmpSeq methods are able to track specific genes across clones, infections and populations [267, 298, 299] and can assess the efficacy of vaccines [300].

As such, AmpSeq can decipher multi-clones infections without overlooking minority clones from *Plasmodium falciparum* infections [297], which can convey important information about transmission dynamics in the elimination settings of Cambodia. This methodology may be capable of assessing if the asymptomatic reservoir is distinct from the symptomatic one and if some clones are associated to more active transmission dynamics. The protocol has been developed for *P. falciparum* genotyping to be implemented both in epidemiological studies and drug efficacy trials [269]. The main advantage of AmpSeq over existing methods is the increased ability to detect low densities *Plasmodium* clones from routine blood samples [301]. The methodology is also tailored to provide accurate drug failure estimates [297]. In addition, the protocol has been optimised to be performed on an Illumina MiSeq, which has overall smaller error rate compared to other sequencing platforms [302]. The libraries should be created with samples duplicates [269] or even triplicates [294]. Indeed, if sample size is very small, during reads analysis, the cut-off for singleton exclusion can remove true haplotypes in samples without replicates. However, we expect a true haplotype to be present in all replicates: the haplotype would be amplified in more than one sample and the singleton exclusion would not discard it.

In 2019, Mondulakiri province held the highest recorded malaria incidence in the GMS, with more than 50 cases per 1000 inhabitants from January to July 2019 [128]. By implementing the AmpSeq methodology using field samples from Mondulakiri province, we aimed at estimating the cost-effectiveness and the feasibility of the method at a local scale. We used samples from two epidemiological studies (GPS follow-up and cohort studies) and vector

collections carried out in our field site in Mondulhiri province. We determined the proportion of *P. falciparum* and *P. vivax* field samples that were effectively amplified with AmpSeq methodology. For *P. falciparum* samples, we determined if RT-CPR cycle threshold, as a proxy of samples DNA concentration, can be used to determine which samples will amplify and provide valid sequencing output (an amplicon with >10,000 reads for both forward and reverse reads). We also discuss the investment required in this methodology when implemented in a developing country such as Cambodia.

3.2. Application of a deep amplicon sequencing set in *P. falciparum* samples

3.2.1. Material and methods

Study design

Blood samples from a cohort and a follow-up study implemented in Kaev Seima district, Mondulhiri province, Northeastern Cambodia, were used to produce AmpSeq libraries. The cohort corresponded to 950 individuals aged 5 to 66 years old, followed monthly for one year and then three-monthly for nine months. The GPS follow-up study constituted of two 28-day follow-ups of respectively 160 and 200 forest goers. Every follow-up of the cohort and on day 0, day 7, day 14 and day 28 of the GPS follow-up study, participants underwent capillary blood withdrawal and a Rapid Diagnosis Test (RDT). If the RDT was positive, they were referred to appropriate structures to receive treatment.

Laboratory procedures

DNA was extracted from blood pellets with Qiagen DNA extraction kits according to the manufacturer's instructions. Real-Time Polymerase Chain Reactions (RT-PCR) were performed to detect malaria parasites and to carry out parasites speciation, as previously described [167]. Briefly, a first RT-PCR targeted the *Plasmodium* cytochrome b gene and determined the positive samples. Then, a nested RT-PCR assays targeted the same gene specific to *P. falciparum*. All *P. falciparum* positive samples were selected to prepare the Deep Amplicon Sequencing libraries. Blood containing culture strains of 3D7 were extracted to be used as positive controls and parasite-free blood as negative control. Samples were processed to produce amplicons from the five genetic markers *ama1-d3*, *cpmp*, *csp*, *cpp* and *msp7*, placed on different chromosomes (Table 3), recognisable with a sample-specific eight nucleotide barcode combination, following previous protocols [269, 294].

Table 3: Description of the 5 AmpSeq molecular markers and their characteristics in 3411 *P. falciparum* isolates from MalariaGEN database [294].

Marker ID	Marker name	Protein	Size (bp)	Haplotypes	H _e
PF3D7_1133400	ama1-D3	Apical membrane antigen1	477	79	0.93
PF3D7_0104100	cpmp	Conserved Plasmodium protein	329	82	0.93
PF3D7_0304600	csp	Circumsporozoite protein	403	77	0.85
PF3D7_1475800	cpp	Conserved Plasmodium protein	394	77	0.92
PF3D7_1335100	msp7	Merozoite protein7	374	67	0.91

Several rounds of PCRs using high fidelity Taq polymerase, purifications with magnetic beads, quantification with DNA-bound fluorescent reagents, normalisation and pooling are required to prepare the amplicon libraries. The workflow is described in Table 4.

Table 4: Required steps to prepare AmpSeq libraries.

Step	Key reagents	Equipment	Aim
DNA extraction	Qiagen kit	-	Isolating <i>P. falciparum</i> DNA from blood
RTPCR	PCR MasterMix	PCR cycler	Assessing <i>P. falciparum</i> DNA presence
Primary PCR	HiFi Taq polymerase, primers	PCR cycler	Binding primers
Secondary PCR	HiFi Taq polymerase, linkers	PCR cycler	Binding linkers
Agarose gel	-	Electrophoresis tank and transilluminator	Assessing amplicons presence and size
Purification	NucleoMag NGS bead suspension	Magnetic beads	Removing amplicons outside of desired size range
Quantification	Quant-iT PicoGreen assay kit, TE buffer	Fluorometer	dsDNA quantification
Normalisation	Buffer	-	Diluting DNA to 20 ng/uL
Adapter PCR	HiFi Taq polymerase, barcodes		Binding adapters
Agarose gel	-	Electrophoresis tank and transilluminator	Assessing amplicons presence and size
Quantification	Quant-iT PicoGreen assay kit, TE buffer	Fluorometer	dsDNA quantification
Normalisation	Buffer	-	Diluting DNA to 40 ng/uL
Pooling	-	-	Pooling all samples per marker
Purification	NucleoMag NGS bead suspension		Removing amplicons outside of desired size range
Quantification	Qubit assay kit	Qubit fluorometer	dsDNA quantification
Pooling	-	-	Pooling all samples in a single library
Sequencing	flow cell, PhiX	Illumina MiSeq	Library sequencing

Sequencing

Sequencing was done by Macrogen (Seoul, South Korea) with an Illumina MiSeq platform in paired-end mode (2 x 250 bp) using Illumina MiSeq reagent kit v3 (500-cycles) with 30% of enterobacteria phage PhiX control (Illumina, PhiXControl v3).

Analysis

A valid output should include at least 10,000 reads per amplicon [269]. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to exceed

background level. Ct levels are inversely proportional to the amount of DNA in the sample and are automatically known when the RT-CPR results are available.

A binomial variable representing amplification per molecular marker was attributed to every sample: 1 represented a successful sample amplification (*i.e.* the presence of a clear band on the agarose gel after the last PCR: the adapter PCR), and 0 no amplification. We tested if there was an association between RT-CPR Ct (Cycle threshold) and adapter PCR amplification of the sample, using binomial generalised linear models (GLMs).

We attributed a binomial variable to amplicons depending on the validity of the output of each amplicon type, with 1 for valid outputs (N reads > 10,000 for both forward and reverse reads) and 0 invalid. We tested if there was a relation between the Ct value the validity of the output of each amplicon using binomial GLMs.

All percentages were calculated with their 95% confidence interval (CI) and means with standard deviation (sd). Significance threshold was set at $p = 0.05$. We log-transformed the Ct variable to satisfy normality assumptions. All statistical analyses were conducted in R version 4.1 [303]. DHARMA package was used to assess all models' goodness of fit [304]. To visualise the raw output from the MiSeq platform and determine reads quality, a data analysis pipeline was developed, available at <https://github.com/lerch-a/HaplotypR> [269].

3.2.2. Results

Samples

Over the 9,928 tested cohort samples, 1,010 ($10.2 \pm 0.6\%$) were positive for *Plasmodium* spp. and 33 ($0.3 \pm 0.1\%$) were positive for *P. falciparum*. Over the 1,391 tested GPS follow-up samples, 424 ($30.5 \pm 2.4\%$) were positive for *Plasmodium* spp. and 32 ($2.3 \pm 0.8\%$) were positive for *P. falciparum* (Table 5). Two independent libraries, one with cohort samples and the other with GPS follow-up samples, were prepared for sequencing.

Table 5: RTPCR results with Ct details of the *P. falciparum* samples.

collection	N processed	N <i>Plasmodium spp</i>	N <i>P. falciparum</i>	% <i>P. falciparum</i>	95% CI <i>P. falciparum</i>
cohort	9,928	1,010	33	0.33	0.11
GPS follow-up	1,391	424	32	2.30	0.79

collection	min Ct	average Ct	median Ct	max Ct
cohort	19.4	28.4	28.5	34.1
GPS follow-up	17.2	22.5	22.3	29.2

Amplification

A successful amplification of five genetic markers from sixty-five samples generates 325 amplicons. Here, only 205 amplicons ($63.1 \pm 5.2\%$) were successfully retrieved during adapter PCR. Amplification varied by marker, from *cpp* amplifying $72.3 \pm 1.3\%$ of the 65 RTPCR-positive samples to *csp* amplifying $56.9 \pm 1.5\%$ of them (Table 6). All 3D7 positive controls were successfully amplified but were not included in the analysis.

Table 6: Percentage of samples amplified with adapter PCR per marker (all RTPCR-positive samples excluding controls, N = 325).

Marker	n	%	95% CI
ama1-d3	40	61.5	1.5
cpmp	39	60	1.5
cpp	47	72.3	1.3
csp	37	56.9	1.5
msp7	42	64.6	1.4

There was no significant relation between RTPCR Ct value and the successful amplification of a sample during adapter PCR (Table 7, Figure 18).

Table 7: GLMs results testing the relation between RTPCR Ct and adapter PCR (N = 325).

marker	average Ct				GLMs results	
	no amplification	sd	amplification	sd	χ^2_1	p
ama1-d3	26.2	4.4	25	4.4	1.30	0.25
cpmp	25.4	3.7	25.6	4.9	0.002	0.96
cpp	25.3	3.6	25.6	4.8	0.002	0.97
csp	26.6	4.1	24.7	4.6	3.33	0.07
mcp7	25.4	3.6	25.5	4.9	0.01	0.94

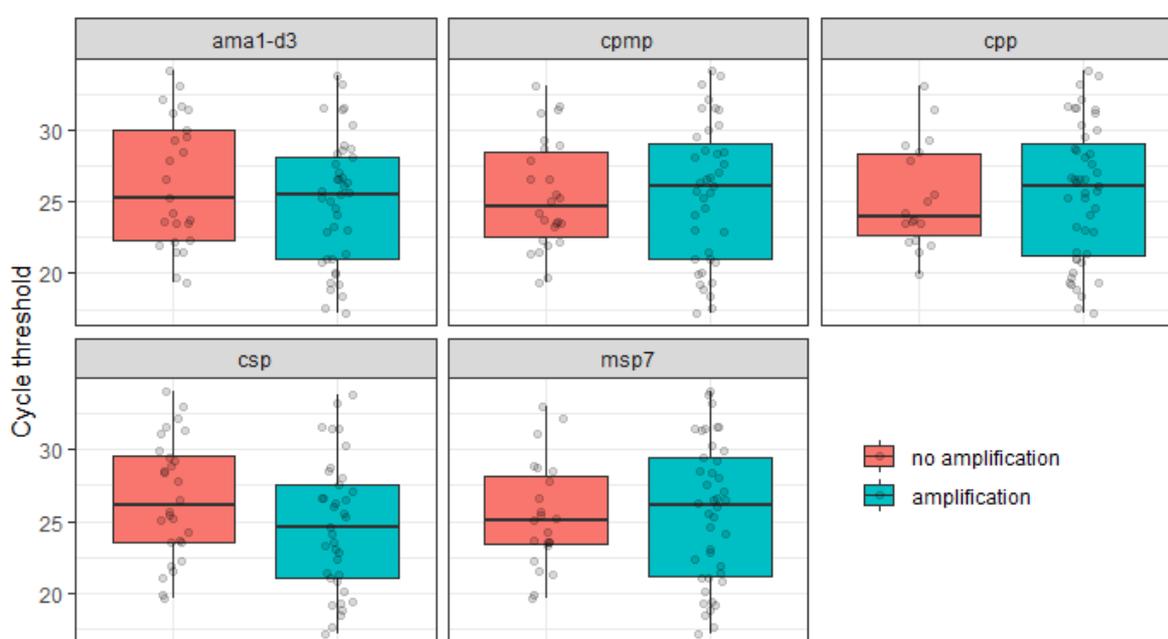


Figure 18: Relation between RTPCR Ct and adapter PCR amplification for all five markers (N = 325, 65 per marker).

Sequencing

MacroGen received the first library constituted of the cohort samples on the 27th of September 2021 and sent the raw MiSeq output on the 22nd of December 2021. The flow cell was shared between cohort samples and other clients' samples. MacroGen received the second library constituted of the GPS follow-up samples on the 26th of October 2021 and sent the raw output on the 4th of March 2022. The flow cell was not shared with other clients' samples. Respectively, the time elapsed between the reception of samples and raw reads data sharing was 86 and 129 days.

Reads output

From the 205 amplicons that were successfully amplified during the preparation of AmpSeq libraries, 171 (83.4 ± 5.1%) had a valid read output (both forward and reverse reads > 10,000). This proportion represents 52.6 ± 5.4% of the 325 amplicon that could be amplified from the 65 RTPCR-positive samples. The amount of forward and reverse reads varied greatly by sample and by marker (Figure 19, Table 8).

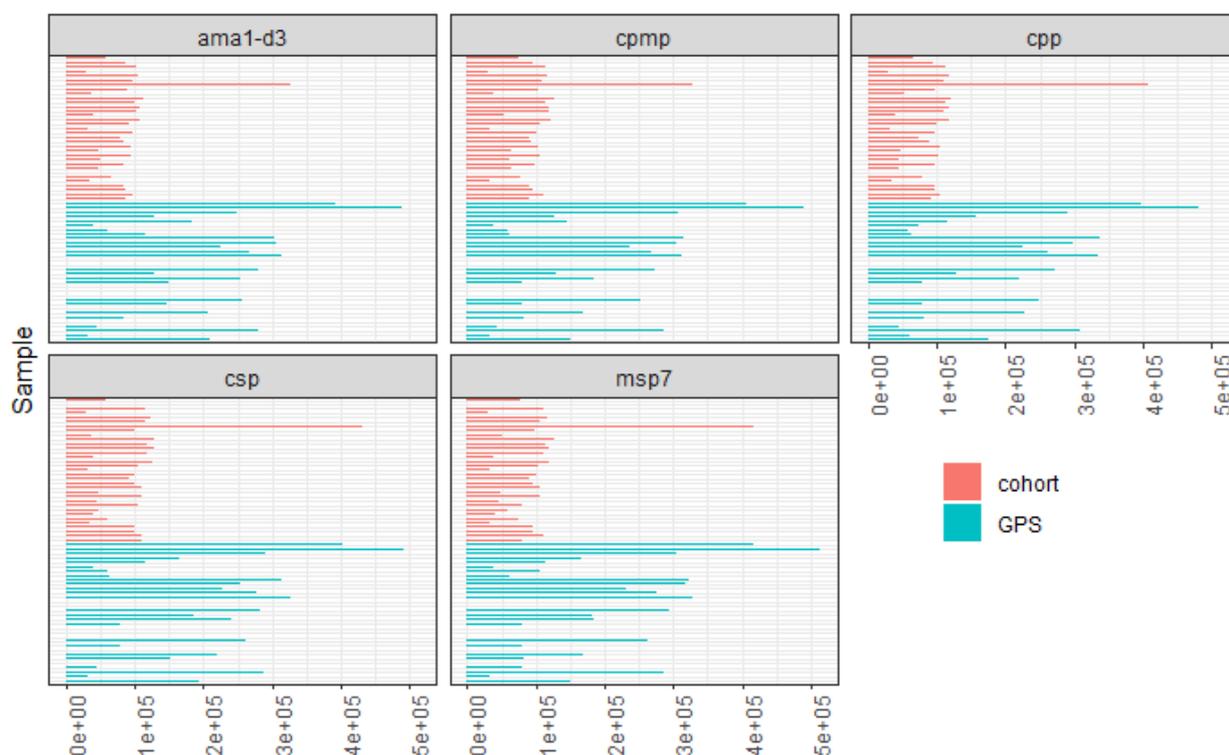


Figure 19: Reads per marker per sample (N = 325, 65 per marker).

Table 8: Reads per sample per marker (N = 325).

reads per sample per marker	forward	reverse
minimum	21	0
median	82,604	13,877
mean	107,326	17,348
maximum	422,595	103,196

Even if the data suggested that a lower Ct was associated to an increased number of reads per sample per marker for every molecular marker (Figure 20), there was a significant association between Ct and a valid read output only for ama1-d3 ($\chi^2_1 = 16.38$, $p < 0.001$, Table 9, Figure 21). Indeed, for samples amplified with ama1-d3 marker, the average RTPCR Ct for samples with a valid read output was significantly lower ($Ct = 22.1 \pm 3.5$) than the samples that did not have a valid output ($Ct = 26.8 \pm 4.1$).

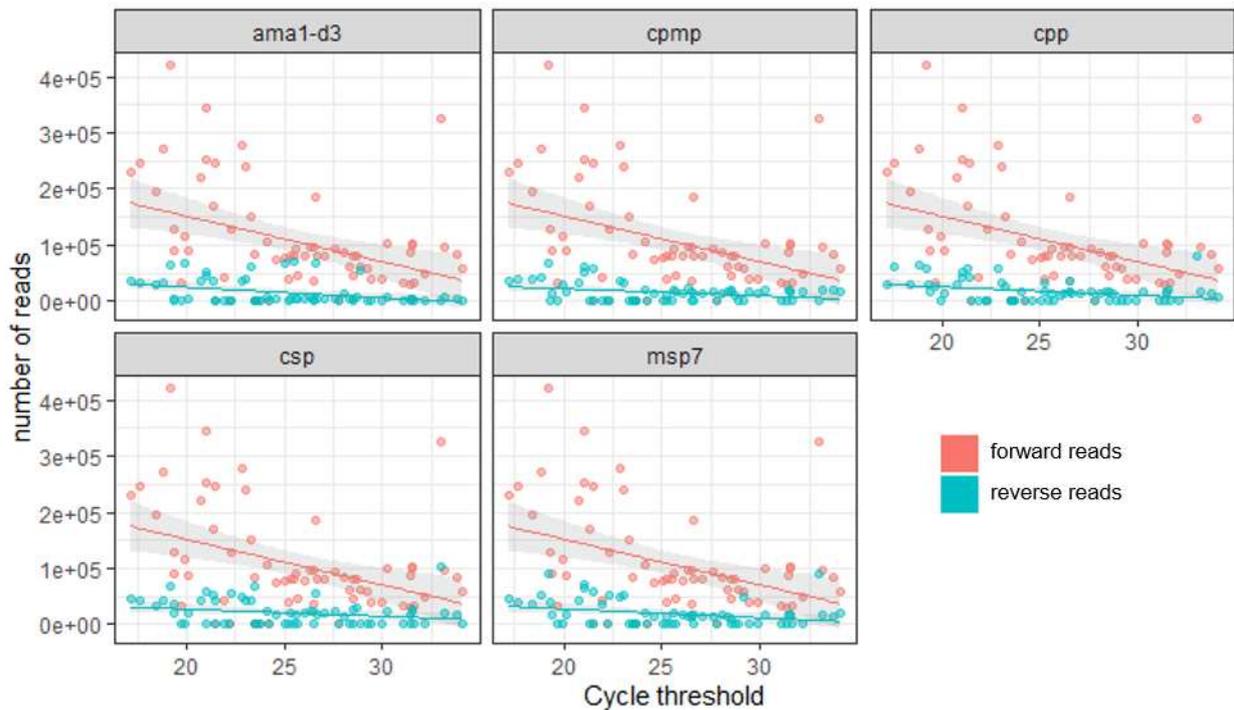


Figure 20: RTPCR Ct and number of reads per sample per marker.

Table 9: GLMs results testing the relation between RTPCR Ct and validity of reads after sequencing (N = 325, 65 per marker). Significant results are highlighted in bold.

marker	invalid read output	average Ct		GLMs results		
		sd	valid read output	sd	χ^2_1	p
ama1-d3	26.8	4.1	22.1	3.5	16.35	5.3E-05
cpmp	25.3	3.8	25.6	4.9	0.02	0.88
cpp	26.4	4	24.9	4.7	2.06	0.15
csp	26.4	3.9	24.9	4.7	2.27	0.13
msp7	25.9	3.7	25.2	4.9	0.67	0.41

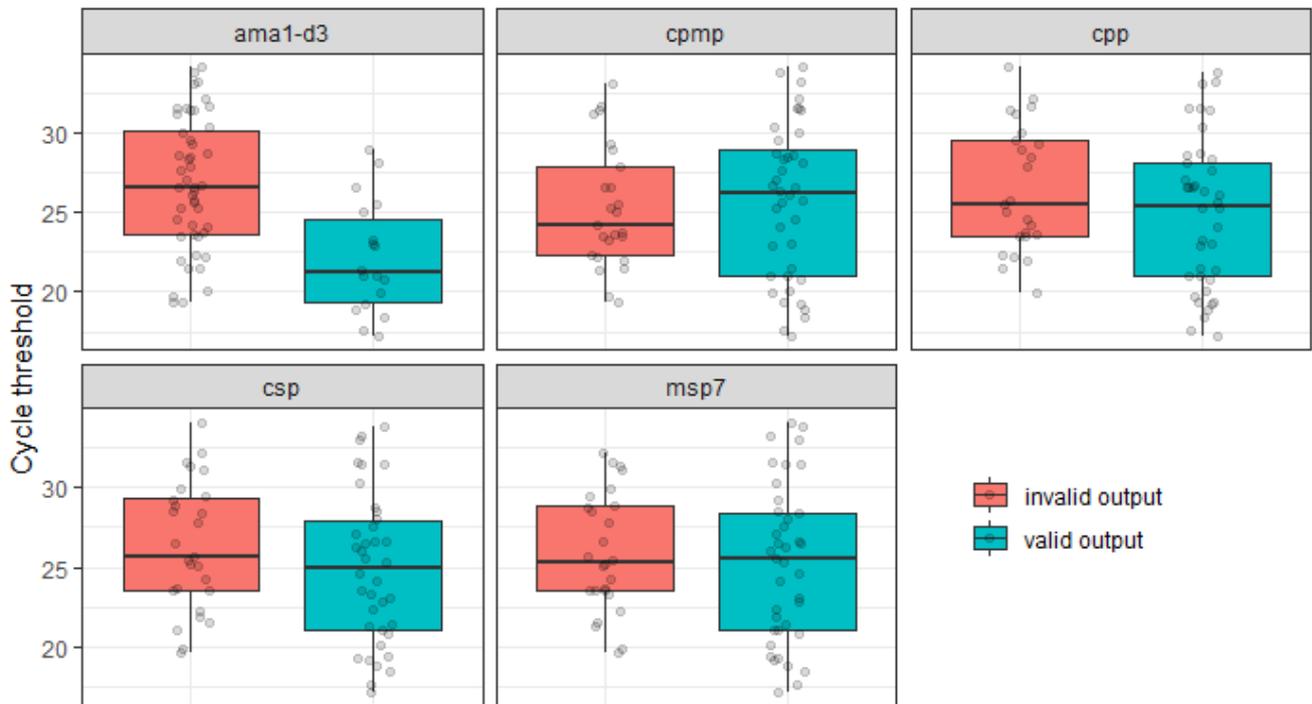


Figure 21: Relation between RTPCR Ct and validity of reads after sequencing (N = 325, 65 per marker).

Overall, the forward reads were of better quality than the reverse reads and the GPS follow-up library reads were of better quality than the cohort library reads (Figures 22 and 23). From the cohort reads, 11 amplicons had a very low number of reverse reads (< 200 reads), including four with no reverse read at all. The four outputs with no reverse read were cpp amplicons, and the other 7 were either cpp (N = 3) or msp7 amplicons (N = 4).

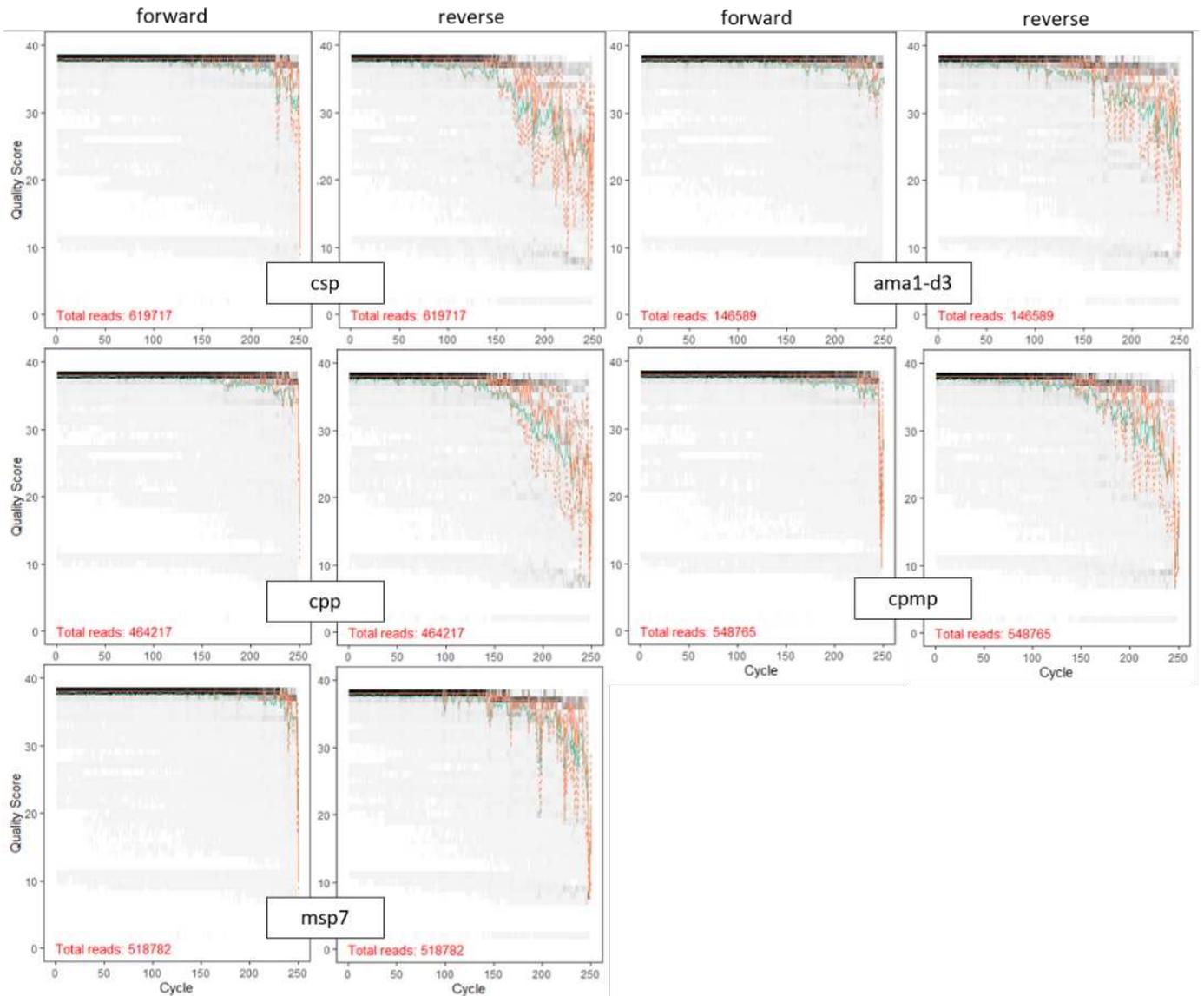


Figure 22: Quality profiles of the MiSeq reads from the cohort samples. In grey-scale is a heat map of the frequency of each quality score at each base position. The green line shows the mean quality score at each position, and the quartiles of the quality score distribution by the orange lines. The red line shows the scaled proportion of reads that extend to at least that position.

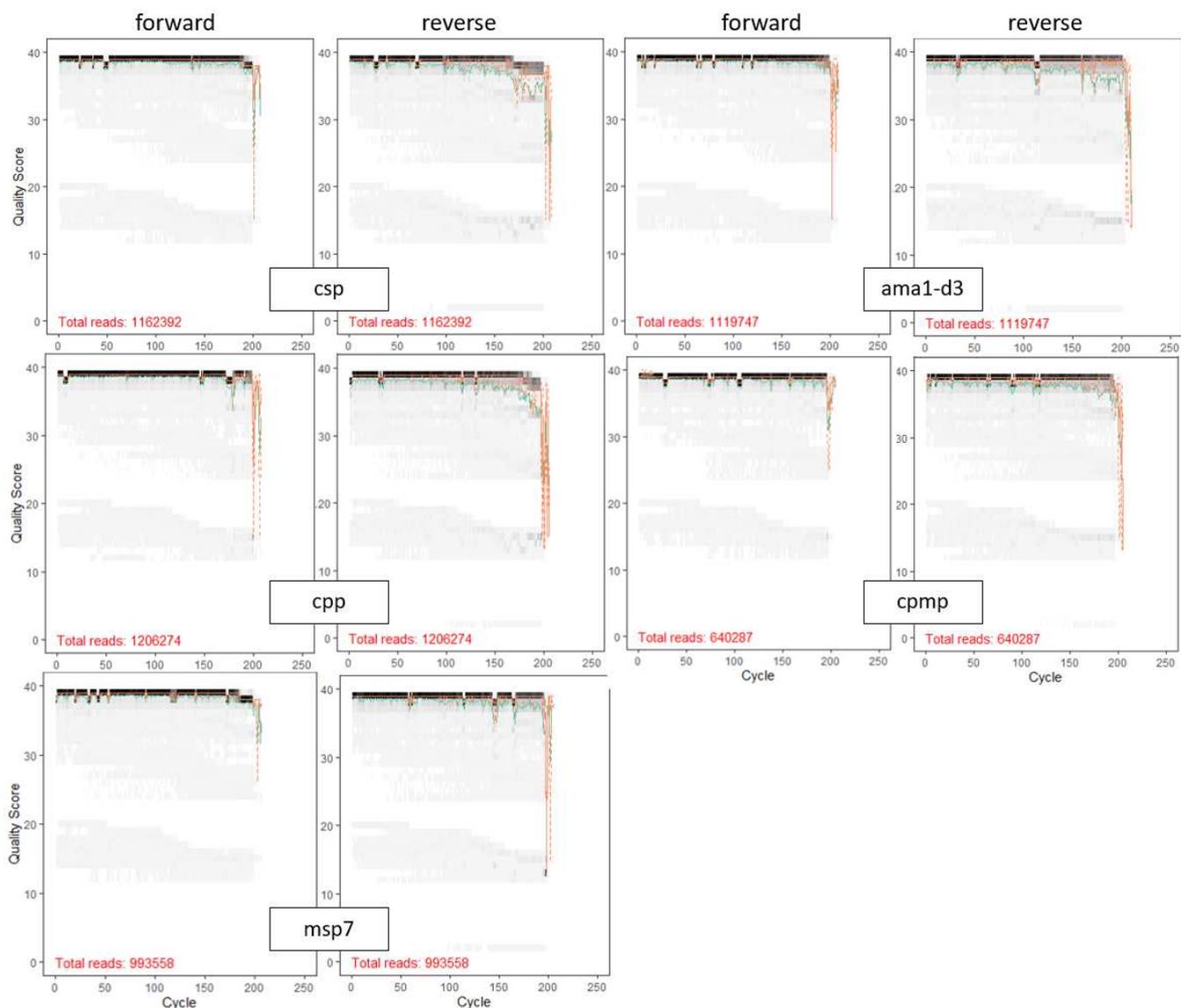


Figure 23: Quality profiles of the MiSeq reads from the GPS follow-up samples. In grey-scale is a heat map of the frequency of each quality score at each base position. The green line shows the mean quality score at each position, and the quartiles of the quality score distribution by the orange lines. The red line shows the scaled proportion of reads that extend to at least that position.

3.3. Application of a deep amplicon sequencing set in *P. vivax* samples

A novel AmpSeq set targeting thirteen short but highly polymorphic regions across thirteen *P. vivax* chromosomes (Table 10) was developed by a collaboration between the Swiss TPH, the Walter and Eliza Hall Institute and the Pasteur Institute [305]. The protocol was shared with the Institute Pasteur in Cambodia in the context of the Asia-Pacific International Centre of Excellence (ICEMR) project “Understanding, tracking and eliminating malaria transmission in the Asia-Pacific Region”.

Table 10: Description of the 13 *P. vivax* AmpSeq molecular markers.

Molecular marker	Size (bp)	Target start	Target end	N variants
Chr_01 - Protein Tyrosine phosphatase putative	200	612222	612358	6
Chr_02 - Lysophospholipase, putative	216	62503	62625	11
Chr_03 - conserved Plasmodium protein	214	129741	129866	9
Chr_04 - Serine-repeat antigen 5	187	710052	710165	21
Chr_05 - STP1 protein	188	1412445	1412544	5
Chr_06 - PIR protein	205	30825	30957	17
Chr_07 - Merozoite surface protein 1	194	1219847	1219964	36
Chr_08 - Plasmodium exported protein	221	1610537	1610675	9
Chr_09 - Apical Membrane antigen 1	210	1459600	1459728	17
Chr_10 - Merozoite surface protein 3	196	1354022	1354110	15
Chr_11 - Glyoxalase I-like protein	119	1872223	1872351	6
Chr_13 - conserved Plasmodium protein	202	2030854	2030957	6
Chr_14 - Reticulocyte binding protein 2a	202	114293	114419	14

The protocol implemented to produce an AmpSeq library of *P. vivax* samples follows the same workflow, and it requires the same reagents and material. However, we had to alter the original protocol, which had been developed mostly on samples from Peru, Papua New Guinea and Salomon islands, to be able to amplify Cambodian field samples. Indeed, proceeding with the PCR conditions of the original protocol was producing amplicons of different sizes, resulted in a smear on the agarose gel, or were not producing any amplicon. After determining the optimal conditions (by singleplexing and changing temperature or

reagents mix of primary and secondary PCRs) that produced amplicons of the expected size, we discarded five molecular markers (chr01, chr04, chr05, chr06 and chr09) for which we could not produce satisfying amplicons. From the original 13 molecular markers, we validated eight markers (chr02, chr03, chr07 cr08, chr10, chr11, chr13 and chr14).

The samples used to build the library were selected from *P. vivax* positive samples from cohort, mosquito collections and symptomatic cases originating from Kaev Seima district. Samples from each category were collected over three periods (October 2019, January 2020 and August-September 2020) in order to compare the genetic structure of the parasite populations across different collections and time points. We selected a total of 195 samples to build the library: 71 from the cohort, 92 from the mosquito collections and 31 symptomatic cases.

We assessed the success of a sample amplification as the presence of a clear band after migration on the agarose gel of the adapter PCR products. Amplification success varied across molecular markers, ranging from $22.6 \pm 5.8\%$ to $44.1 \pm 7\%$ (Table 11). The origin of the sample also appeared to have an effect on the success of amplification: the percentage of samples successfully amplified by at least one marker ranged from $12 \pm 6.6\%$ for mosquito samples to 100% for symptomatic cases samples (Table 12).

Table 11: Percentage of samples amplified with adapter PCR per marker (all RTPCR-positive samples, N = 195).

Molecular marker	n	%	95% CI
Chr_02 - Lysophospholipase, putative	84	43.1	7.0
Chr_03 - conserved Plasmodium protein	85	43.6	7.0
Chr_07 - merozoite surface protein 1	83	42.6	6.9
Chr_08 - Plasmodium exported protein	81	41.5	6.9
Chr_10 - Merozoite surface protein 3	58	29.7	6.4
Chr_11 - Glyoxalase I-like protein	86	44.1	7.0
Chr_13 - conserved Plasmodium protein	55	28.2	6.3
Chr_14 - Reticulocyte binding protein 2a	44	22.6	5.8

Table 12: Percentage of samples amplified by at least one molecular marker and average count of successfully amplified markers, per origin of sample (N = 195).

category	amplified in at least one marker				average amplified markers			
	N	n	%	95% CI	min	median	average	max
cohort	71	61	85.9	8.1	0	5	4.3	8
mosquito collection	92	11	12	6.6	0	0	0.3	6
symptomatic	31	31	100	0	7	8	7.8	8

3.4. Discussion

These results provided quantitative data about the success of 5 *P. falciparum* and 8 *P. vivax* AmpSeq markers, in addition to an estimation of the methodology cost-effectiveness for field isolates. We could determine which markers were better suited for amplifying Cambodian samples (such as *cpp* for *P. falciparum* markers or *chr03* and *chr11* for *P. vivax* markers) and that the success of the *P. falciparum* marker *ama1-d3* was proportional to the initial DNA concentration in the samples.

The quality of the reads was better for forward reads than for reverse reads. Even if this is often observed with Illumina sequencing [306–309], we can also observe that the quality difference between forward and reverse reads appeared to vary between libraries, with the second library having a better quality output. The Illumina paired end sequencing involves a temporal separation and a mechanistic process. The amplicons are attached to the flow cell by their forward end for a first read, then bent and reattached for the reverse reads. Depending on the protocol and the workflow, the second step may occur days later, which can reduce reverse reads quality. Sharing the flow cell with samples with different concentrations could also affect reads output if normalisation is not properly undertaken before sequencing.

Most of the AmpSeq data is still being analysed, but some limitations were already observed from the preliminary data.

Amplification was not successful in about half of samples, for both *P. falciparum* and *P. vivax*. Because most of the samples were from *Anopheles* vectors (low volumes) and from

asymptomatic cases (low parasite density), we can expect that the amount of available parasite DNA was not suitable for amplification. Previous implementation of amplicons sequencing through the GenRe-Mekong in the GMS was based on samples from symptomatic patients only, confirmed by microscopy or rapid diagnostic test [290]. They assessed that low blood volumes are associated to low genotyping success rates from sub-microscopic infections, without providing data about their success rate. Lerch *et al* [269] could produce sequence reads from samples with a parasitaemia of only 5 parasites/ μ l, but the coverage was extremely low (<100 reads). Another hypothesis is that circulating parasites from Cambodia are not suited for the selection of molecular markers that were developed on samples from other countries, with different transmission dynamics and intensity.

Because of the added stress in workforce and budget that they would represent, samples were not amplified as duplicates or triplicates. Once the pipeline issues are resolved, results would still be limited by the absence of replicates; we may dismiss low-density true haplotypes as singletons. The production of new libraries using the same samples can however still be considered in the future.

Although AmpSeq is an extremely promising methodology as it allows to sequence several markers simultaneously at a lower cost [294, 297], it is not that simple to implement. Indeed, along the difficulties inherent to the creation of set of markers with sufficient resolution and limited primers interference [269, 310], it requires a consequent workforce (at least 3 PCRs, 2 gels, 3 DNA quantifications, 3 normalisations and 2 purifications per sample), material (such as high fidelity Taq polymerase not used in classical PCRs) and budget (more expensive reagents than classical PCRs) that can be challenging to obtain in developing countries. Importing the reagents to Phnom Penh also represented a challenge, as some reagents, such as the high fidelity Taq polymerase (KAPA HiFi HotStart MasterMix) needed months to arrive and clear immigration. Also, acquiring and maintaining a sequencer such as a MiSeq is often not possible for laboratories in developing countries, which then need to send the libraries to third parties for sequencing. In addition to the associated price increase, results can be delayed from days (as it could take in the laboratory) to weeks or even months before obtaining a raw reads output. Third parties can share a flow cell for different projects, which can decrease the amount and the quality of reads attributed per sample. Moreover, recommendations to produce the libraries using duplicates [269] or even triplicates [294]

increase even further the financial stress of the experiment. Finally, performing the experiment required a trained workforce able to dedicate potential weeks of labour to the production of a single library. These limitations have been mentioned previously by Jones et al. [297], and that AmpSeq implementation in areas like SSA or SEA is very challenging in the short term, but would be possible and sustainable on the long-term.

Further work is required to understand better which samples can be efficiently processed using AmpSeq, if some markers or DNA concentrations are more suitable to the methodology, and to analyse the reads with the HaplotypR pipeline. The sequence analysis pipeline HaplotypR was developed under R, with an associated public GitHub repository sharing resources. The pipeline aims to remove the background noise caused by sequencing and amplification errors, call haplotypes and understand the population genetics of the parasite population. The pipeline is supposedly functional and accessible to all. However, even for staff familiar with the programming language R, the use of the pipeline is cumbersome. Errors are numerous, and the resources limited, with little to no description of the called functions within the pipeline. Overall, the pipeline is hard to navigate; a more completed and accessible version would have allowed the presentation of sequenced haplotypes in this manuscript.

The Deep Amplicon Sequencing methodology is a promising tool to understand malaria transmission dynamics in elimination settings, but the efforts associated with its implementation make it difficult to use in developing countries. For *P. falciparum*, only half of samples successfully provided a valid output, and amplification success did not seem to be associated to samples DNA density. For *P. vivax*, some molecular markers were not suitable to amplify amplicons, and amplification success remained low for the validated molecular markers. To use routinely parasitic data through AmpSeq in the GMS, a more adapted panel of molecular markers and a more accessible pipeline could be necessary. Overall, in this context, the method suitability and cost-efficacy appear to be limited.

General discussion

The interactions between ecosystems alteration, biodiversity, and infectious diseases are complex. Even if the theme of deforestation and malaria dynamics had been thoroughly studied in some parts of the world, contradictory results are still frequently published. This data in Cambodia is unfortunately extremely limited. From a public health perspective, it is essential to propose a comprehensive mechanism of these interactions in order to propose tailored recommendations. The remaining gaps regarding this topic where the rationale to conduct this PhD project, focusing on the association between anthropisation and malaria dynamics in an understudied part of the world, in Cambodia.

This PhD project allowed to provide recent and quantitative data about key components of malaria transmission in Cambodia over a single district. Additionally, we tested and implemented tools that were new to Cambodia: the ones that were a success, such as HDN traps and barrier screens, and the ones that were more difficult to operate in this low transmission settings, such as GPS data loggers and deep amplicon sequencing.

By using satellite imagery, we confirmed that deforestation and landscape fragmentation were increasing in the study area. We also realised that inferring malaria transmission variations from landscape changes is a tricky task, for which the study scale matters as much as the quality of the land use classification.

From the environmental data provided by the land use classification, we had the opportunity to observe in more details the mobility of the notorious forest goers and the risk population of the study area. While the characteristics of their mobility had been only described through questionnaires before, we could finally describe some of their limitations by implementing GPS trackers to compare them to. Unsurprisingly, forest goers were often and recurrently affected by malaria, but our methodology could not tell apart the recently acquired infections from the relapses, and we suggest that serological tools will be necessary to understand better forest goers exposure to malaria. By following all of the risk population, we observed that men are effectively more affected than women by malaria, as emphasised by the literature consensus. However, the GPS data loggers could not identify a difference in the amounts of time they respectfully spent in the forest – the environment in which men are supposedly acquiring new infections. Participant gender might not be a risk factor in itself but

a confounding factor of the gender-biased forest activities, highlighting that malaria risk could depend of socio-cultural habits and practices more than a fixed demographic such as the forest goers.

The implementation of mosquito collections including both conventional and innovative traps yielded a considerable number of specimens and associated data, of which much are yet to be analysed. From the thousands of collected *Anopheles*, we could capture a clearer picture of *Anopheles* bionomics in Cambodia, across an anthropisation gradient including villages, fields, plantations and forests. The abundance and the prevalence of malaria vectors appeared to be significantly higher in non-anthropised environments than in anthropised ones, such as forest sites or collection sites surrounded by a high forest coverage and low fragmentation. At the same time, abundance and prevalence were associated to field sites with important cropland cover in the surroundings, which are anthropised environments. The primary vector *An. dirus* could be dwelling fields, in addition to forest, as a consequence of forest cover diminution. An estimation of *An. dirus* flight range in addition to the description of *An. dirus* breeding sites would be very informative as it could inform if the long-lived is regularly travelling from the forests to the fields or simply resides in the fields.

Finally, the implementation of the AmpSeq methodology in Cambodia could not provide as many results as we expected. Firstly, because the biomolecular process itself was complex to prepare and execute. The assay had to be delayed for literal months, waiting for the reagents to be delivered. Even with all the required material and reagents at hand, the production of amplicons was further delayed by the numerous adjustments needed to produce satisfying results. Secondly, the analysis was equally, if not more, demanding: using HaplotypR took a lot of trying, without providing the results we expected. Developing another pipeline is a laborious and long commitment which we had not the opportunity nor the time to consider. We expect to resolve the analysis pipeline issues as soon as possible and finally characterise the genetic structure of our samples.

Still, the results we obtained through the GPS follow-ups – forest goers are not so straightforward to identify, malaria exposure depends on fine sociodemographic variations – and the mosquito collections – malaria exposure happens both in forests and anthropised environments, notably fields – could have consequences for malaria control and elimination strategy in Cambodia. Malaria control and elimination measures are largely implemented

based on the evidence that transmission continues amongst hard-to-reach populations widely (mis)categorised as forest-goers or mobile and migrant populations who face difficulties in accessing malaria diagnosis and treatment [318]. However, even if the demographic corresponding to forest goers is more affected by malaria than the rest of the population and efforts towards an effective detection of their infections is required, it might not be enough to eliminate malaria. Because women are also affected by malaria and infected *Anopheles* are present in fields, an elimination strategy should focus on cases detection in anthropised environments and not only among forest goers.

However, before influencing malaria control strategy, these results should be corroborated by similar experiments replicated in other provinces of the country. The certainty of these results may never be complete, because investigating malaria transmission in Cambodia is not an easy task. Incidence records are incomplete, cases are difficult to identify, the deforestation is extremely fast and many methodologies have not been tested and validated in the country yet. Because quantitative entomological and mobility data is so scarce, practically non-existent, we cannot determine if the important *Anopheles* prevalence in the fields or the similar time spent in forest by men and women arise from a recent change induced by elimination strategies or anthropisation, or if these numbers have not evolved in decades.

The full understanding of the relationship between anthropisation and malaria transmission in Cambodia will likely never be complete, and this PhD project is still missing additional data and results that remain to be obtained. However, we have come closer to an understanding of the relationship between anthropisation and malaria transmission in rural Cambodia has emerged through these three years and a half of research, and how to understand it further. This whole project aimed to understand how the interaction of three entities – environment, vectors, hosts – maintained malaria transmission after attaining residual malaria transmission (Figure 24).

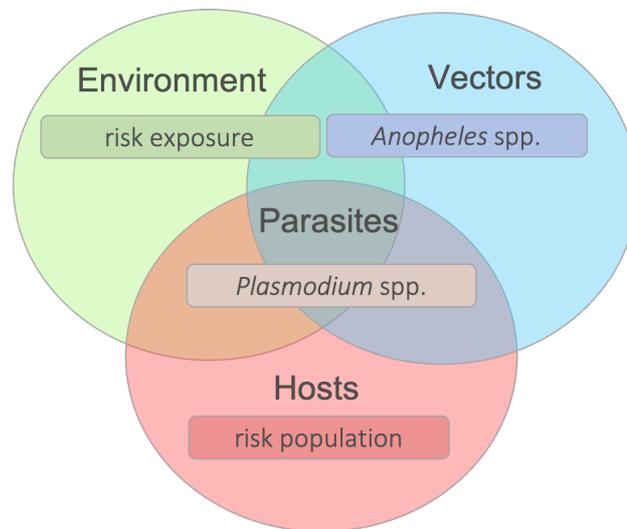


Figure 24: The environment, the hosts, the vectors and the parasites. Parasitic population is maintained only if the conditions in the environment, the vector and the host populations are adapted to *Plasmodium* survival, reproduction and transmission.

Nevertheless, this Venn diagram is only a very simplistic view of the actual relationships between parasites, mosquitoes, humans and environmental conditions. Numerous other interactions should be added. Between the host and the vector, the implementation of IRS and LLINs can induce mosquitoes to bite outside of their original nocturnal activity timeframe, and become more active at dusk and dawn, when individuals are not protected. Anthropisation will affect mosquitoes' habitats and densities, and in turn, transmission. Environmental changes can also affect mosquito fitness, induce mosquito populations changes or replacements, which could affect vector competence. At the intersection of hosts and parasites, clinical practices and antimalarial treatment chosen by the host will select resistance mutations in the *Plasmodium* populations [143, 147]. When the malaria prevalence increases, forest logging and deforestation can decrease, decreasing the effect of human populations on their immediate environments and their exposure to mosquito bites [94]. Most probably, anthropisation affects malaria in ways that we have not identified yet and which largely depend on the local context.

Because “everything is related to everything else, but near things are more related than distant things” [311] and the previous evidence of very fine scale malaria transmission in Cambodia, we studied environmental characteristics and human mobility at a very fine scale rather than trying to understand transmission at the subregional, national or province levels. Previous research justified the need to avoid aggregating data from different study scales,

because of the risk of ecological fallacy [274, 312]. The use of such a small scale is also justifiable on account of previous observations: in the Amazon, the relationship between deforestation and malaria incidence was undetectable at county level [313], but could be described and analysed at a smaller scale [314]. As such, for malaria studies smaller scales perhaps should be prioritised: trying to understand the relationship between two very specific events – for instance landscape fragmentation and *An. dirus* abundance at a specific locale – then replicate it across geographical locations, and finally, piece after, understand the relationship between anthropisation and malaria dynamics.

Rather than trying to find a way to understand anthropisation as a whole, the dissection of anthropisation into spatial and temporal subparts could help its understanding. Fragmentation, deforestation and urbanisation are all wholly included into anthropisation but, they could result in very different outcomes regarding malaria dynamics. Overall, we can suggest that it is unlikely that a single, unidirectional link exists between anthropisation and malaria, even at a single locality. To answer “will anthropisation increase or decrease malaria transmission?”, too simple an answer such as “yes” or “no” are probably misleading.

References

1. Al-Khafif GD, El-Banna R, Khattab N, Gad Rashed T, Dahesh S. The Immunodetection of Non-Falciparum Malaria in Ancient Egyptian Bones (Giza Necropolis). *Biomed Res Int.* 2018;2018:9058108.
2. Nerlich A. Paleopathology and Paleomicrobiology of Malaria. *Microbiol Spectr.* 2016;4.
3. WHO. World Malaria Report 2021. Geneva; 2021.
4. Cotter C, Sturrock HJW, Hsiang MS, Liu J, Phillips AA, Hwang J, et al. The changing epidemiology of malaria elimination: new strategies for new challenges. *Lancet.* 2013;382:900–11.
5. Ernst KC, Lindblade KA, Koech D, Sumba PO, Kuwuor DO, John CC, et al. Environmental, socio-demographic and behavioural determinants of malaria risk in the western Kenyan highlands: a case-control study. *Trop Med Int Health.* 2009;14:1258–65.
6. Lowassa A, Mazigo HD, Mahande AM, Mwang'onde BJ, Msangi S, Mahande MJ, et al. Social economic factors and malaria transmission in Lower Moshi, Northern Tanzania. *Parasit Vectors.* 2012;5:129.
7. Mr B, R K, Cj S. Risk Factors for Infectious Diseases in Urban Environments of Sub-Saharan Africa: A Systematic Review and Critical Appraisal of Evidence. *Tropical medicine and infectious disease.* 2019;4.
8. Ahmed SM, Haque R, Haque U, Hossain A. Knowledge on the transmission, prevention and treatment of malaria among two endemic populations of Bangladesh and their health-seeking behaviour. *Malaria Journal.* 2009;8:173.
9. Goesch JN, Schwarz NG, Decker M-L, Oyakhrome S, Borchert LB, Kombila UD, et al. Socio-economic status is inversely related to bed net use in Gabon. *Malaria Journal.* 2008;7:60.
10. Abe T, Honda S, Nakazawa S, Tuong TD, Thieu NQ, Hung LX, et al. Risk factors for malaria infection among ethnic minorities in Binh Phuoc, Vietnam. *Southeast Asian J Trop Med Public Health.* 2009;40:18–29.
11. Martin JA, Hendershot AL, Saá Portilla IA, English DJ, Woodruff M, Vera-Arias CA, et al. Anopheline and human drivers of malaria risk in northern coastal, Ecuador: a pilot study. *Malaria Journal.* 2020;19:354.
12. Manh BH, Clements ACA, Thieu NQ, Hung NM, Hung LX, Hay SI, et al. Social and environmental determinants of malaria in space and time in Viet Nam. *Int J Parasitol.* 2011;41:109–16.
13. Kheang ST, Por I, Sovannaroth S, Dysoley L, Chea H, Po L, et al. Cambodia malaria indicator survey 2020: Implications for malaria elimination. *Malaria World J.* 2021;12:5.

14. Kounnavong S, Gopinath D, Hongvanthong B, Khamkong C, Sichanthongthip O. Malaria elimination in Lao PDR: the challenges associated with population mobility. *Infectious Diseases of Poverty*. 2017;6:81.
15. Chang H-H, Wesolowski A, Sinha I, Jacob CG, Mahmud A, Uddin D, et al. Mapping imported malaria in Bangladesh using parasite genetic and human mobility data. *Elife*. 2019;8.
16. Gryseels C, Peeters Grietens K, Dierickx S, Xuan XN, Uk S, Bannister-Tyrrell M, et al. High Mobility and Low Use of Malaria Preventive Measures among the Jarai Male Youth along the Cambodia–Vietnam Border. *Am J Trop Med Hyg*. 2015;93:810–8.
17. Searle KM, Lubinda J, Hamapumbu H, Shields TM, Curriero FC, Smith DL, et al. Characterizing and quantifying human movement patterns using GPS data loggers in an area approaching malaria elimination in rural southern Zambia. *R Soc Open Sci*. 2017;4.
18. Venugopal K, Hentzschel F, Valkiūnas G, Marti M. Plasmodium asexual growth and sexual development in the haematopoietic niche of the host. *Nat Rev Microbiol*. 2020;18:177–89.
19. Lindblade KA, Steinhardt L, Samuels A, Kachur SP, Slutsker L. The silent threat: asymptomatic parasitemia and malaria transmission. *Expert Review of Anti-infective Therapy*. 2013;11:623–39.
20. White N, Pukrittayakamee S, Hien T, Faiz M, Mokuolu O. Malaria. *Lancet*. 2014;383:723–35.
21. Antonelli LR, Junqueira C, Vinetz JM, Golenbock DT, Ferreira MU, Gazzinelli RT. The immunology of Plasmodium vivax malaria. *Immunological Reviews*. 2020;293:163–89.
22. Lin E, Kiniboro B, Gray L, Dobbie S, Robinson L, Laumaea A, et al. Differential Patterns of Infection and Disease with *P. falciparum* and *P. vivax* in Young Papua New Guinean Children. *PLOS ONE*. 2010;5:e9047.
23. Phimpraphi W, Paul RE, Yimsamran S, Puangsa-art S, Thanyavanich N, Maneeboonyang W, et al. Longitudinal study of Plasmodium falciparum and Plasmodium vivax in a Karen population in Thailand. *Malaria Journal*. 2008;7:99.
24. Adams JH, Mueller I. The Biology of Plasmodium vivax. *Cold Spring Harb Perspect Med*. 2017;7:a025585.
25. Tanizaki R, Kato Y, Iwagami M, Kutsuna S, Ujiie M, Takeshita N, et al. Performance of Rapid Diagnostic Tests for Plasmodium ovale Malaria in Japanese Travellers. *Tropical Medicine and Health*. 2014;42:149.
26. Tang J, Tang F, Zhu H, Lu F, Xu S, Cao Y, et al. Assessment of false negative rates of lactate dehydrogenase-based malaria rapid diagnostic tests for Plasmodium ovale detection. *PLoS Neglected Tropical Diseases*. 2019;13.
27. Oriero EC, Olukosi AY, Oduwale OA, Djimde A, D’Alessandro U, Meremikwu MM, et al. Seroprevalence and Parasite Rates of Plasmodium malariae in a High Malaria Transmission

Setting of Southern Nigeria. *The American Journal of Tropical Medicine and Hygiene*. 2020;103:2208.

28. Oguike MC, Betson M, Burke M, Nolder D, Stothard JR, Kleinschmidt I, et al. *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* circulate simultaneously in African communities. *Int J Parasitol*. 2011;41:677–83.

29. Baltzell KA, Shakely D, Hsiang M, Kemere J, Ali AS, Björkman A, et al. Prevalence of PCR Detectable Malaria Infection among Febrile Patients with a Negative *Plasmodium falciparum* Specific Rapid Diagnostic Test in Zanzibar. *The American Journal of Tropical Medicine and Hygiene*. 2013;88:289–91.

30. Steenkeste N, Rogers WO, Okell L, Jeanne I, Incardona S, Duval L, et al. Sub-microscopic malaria cases and mixed malaria infection in a remote area of high malaria endemicity in Rattanakiri province, Cambodia: implication for malaria elimination. *Malaria journal*. 2010;9:1–11.

31. Mueller I, Zimmerman PA, Reeder JC. *Plasmodium malariae* and *Plasmodium ovale*--the “bashful” malaria parasites. *Trends Parasitol*. 2007;23:278–83.

32. Kotepui M, Kotepui KU, Milanez GD, Masangkay FR. Global prevalence and mortality of severe *Plasmodium malariae* infection: a systematic review and meta-analysis. *Malaria Journal*. 2020;19.

33. Sabbatani S, Fiorino S, Manfredi R. The emerging of the fifth malaria parasite (*Plasmodium knowlesi*): a public health concern? *Braz J Infect Dis*. 2010;14:299–309.

34. Davidson G, Chua TH, Cook A, Speldewinde P, Weinstein P. Defining the ecological and evolutionary drivers of *Plasmodium knowlesi* transmission within a multi-scale framework. *Malaria Journal*. 2019;18:66.

35. Chin AZ, Maluda MCM, Jelip J, Jeffree MSB, Culleton R, Ahmed K. Malaria elimination in Malaysia and the rising threat of *Plasmodium knowlesi*. *Journal of Physiological Anthropology*. 2020;39:36.

36. Fornace KM, Nuin NA, Betson M, Grigg MJ, William T, Anstey NM, et al. Asymptomatic and Submicroscopic Carriage of *Plasmodium knowlesi* Malaria in Household and Community Members of Clinical Cases in Sabah, Malaysia. *J Infect Dis*. 2016;213:784–7.

37. Fornace KM, Herman LS, Abidin TR, Chua TH, Daim S, Lorenzo PJ, et al. Exposure and infection to *Plasmodium knowlesi* in case study communities in Northern Sabah, Malaysia and Palawan, The Philippines. *PLOS Neglected Tropical Diseases*. 2018;12:e0006432.

38. Barber BE, William T, Grigg MJ, Menon J, Auburn S, Marfurt J, et al. A prospective comparative study of *knowlesi*, *falciparum*, and *vivax* malaria in Sabah, Malaysia: high proportion with severe disease from *Plasmodium knowlesi* and *Plasmodium vivax* but no mortality with early referral and artesunate therapy. *Clin Infect Dis*. 2013;56:383–97.

39. Yusof R, Lau YL, Mahmud R, Fong MY, Jelip J, Ngian HU, et al. High proportion of knowlesi malaria in recent malaria cases in Malaysia. *Malar J.* 2014;13:168.
40. Sinka ME. Global Distribution of the Dominant Vector Species of Malaria. *Anopheles mosquitoes - New insights into malaria vectors.* 2013. <https://doi.org/10.5772/54163>.
41. Sinka ME, Bangs MJ, Manguin S, Rubio-Palis Y, Chareonviriyaphap T, Coetzee M, et al. A global map of dominant malaria vectors. *Parasites & Vectors.* 2012;5:69.
42. Benelli G. Managing mosquitoes and ticks in a rapidly changing world – Facts and trends. *Saudi Journal of Biological Sciences.* 2019;26:921–9.
43. Endo N, Eltahir EAB. Environmental Determinants of Malaria Transmission Around the Koka Reservoir in Ethiopia. *GeoHealth.* 2018;2:104–15.
44. Ferrão JL, Earland D, Novela A, Mendes R, Ballat MF, Tungaza A, et al. Mapping Risk of Malaria as a Function of Anthropogenic and Environmental Conditions in Sussundenga Village, Mozambique. *Int J Environ Res Public Health.* 2021;18.
45. Robert V, Le Goff G, Toto JC, Mulder L, Fondjo E, Manga L, et al. Anthropophilic mosquitoes and malaria transmission at Edea, Cameroon. *Trop Med Parasitol.* 1993;44:14–8.
46. Fontenille D, Simard F. Unravelling complexities in human malaria transmission dynamics in Africa through a comprehensive knowledge of vector populations. *Comparative Immunology, Microbiology and Infectious Diseases.* 2004;27:357–75.
47. Beier JC, Killeen GF, Githure JJ. Entomologic inoculation rates and *Plasmodium falciparum* malaria prevalence in Africa. *The American journal of tropical medicine and hygiene.* 1999;61:109–13.
48. Zimmerman RH. Ecology of malaria vectors in the Americas and future direction. *Mem Inst Oswaldo Cruz.* 1992;87 Suppl 3:371–83.
49. Carlos BC, Rona LDP, Christophides GK, Souza-Neto JA. A comprehensive analysis of malaria transmission in Brazil. *Pathog Glob Health.* 2019;113:1–13.
50. Multini LC, Wilke ABB, Marrelli MT. Neotropical *Anopheles* (*Kerteszia*) mosquitoes associated with bromeliad-malaria transmission in a changing world. *Acta Tropica.* 2020;:105413.
51. Padilla JC, Chaparro PE, Molina K, Arevalo-Herrera M, Herrera S. Is there malaria transmission in urban settings in Colombia? *Malaria Journal.* 2015;14:453.
52. Arróspide N, Miranda E, Casas JV. Malaria urbana por *Plasmodium vivax* en La Molina, Lima. *Revista Peruana de Medicina Experimental y Salud Publica.* 2007;24:192–3.
53. Herrera S, Quiñones ML, Quintero JP, Corredor V, Fuller DO, Mateus JC, et al. Prospects for malaria elimination in non-Amazonian regions of Latin America. *Acta Tropica.* 2012;121:315–23.

54. Saavedra MP, Conn JE, Alava F, Carrasco-Escobar G, Prussing C, Bickersmith SA, et al. Higher risk of malaria transmission outdoors than indoors by *Nyssorhynchus darlingi* in riverine communities in the Peruvian Amazon. *Parasit Vectors*. 2019;12:374.
55. Barros FSM, Honório NA. Deforestation and Malaria on the Amazon Frontier: Larval Clustering of *Anopheles darlingi* (Diptera: Culicidae) Determines Focal Distribution of Malaria. *Am J Trop Med Hyg*. 2015;93:939–53.
56. Hii J, Hustedt J, Bangs MJ. Residual Malaria Transmission in Select Countries of Asia-Pacific Region: Old Wine in a New Barrel. *J Infect Dis*. 2021;223 Supplement_2.
57. Hii J, Rueda LM. Malaria vectors in the Greater Mekong Subregion: overview of malaria vectors and remaining challenges. *Southeast Asian J Trop Med Public Health*. 2013;44 Suppl 1:73–165; discussion 306-307.
58. Kwansomboon N, Chaumeau V, Kittiphanakun P, Cerqueira D, Corbel V, Chareonviriyaphap T. Vector bionomics and malaria transmission along the Thailand-Myanmar border: a baseline entomological survey. *Journal of Vector Ecology*. 2017;42:84–93.
59. Suwonkerd W, Amg-Ung B, Rimwangtrakul K, Wongkattiyakul S, Kattiyamongkool B, Chitprarop U, et al. A field study on the response of *Anopheles dirus* to DDT and fenitrothion sprayed to huts in Phetchabun Province, Thailand. *Tropical Medicine (Nagasaki)*. 1990;32:1–5.
60. Brown R, Chua TH, Fornace K, Drakeley C, Vythilingam I, Ferguson HM. Human exposure to zoonotic malaria vectors in village, farm and forest habitats in Sabah, Malaysian Borneo. *PLoS Negl Trop Dis*. 2020;14:e0008617.
61. Hanandita W, Tampubolon G. Geography and social distribution of malaria in Indonesian Papua: a cross-sectional study. *International Journal of Health Geographics*. 2016;15:13.
62. St Laurent B, Burton TA, Zubaidah S, Miller HC, Asih PB, Baharuddin A, et al. Host attraction and biting behaviour of *Anopheles* mosquitoes in South Halmahera, Indonesia. *Malar J*. 2017;16:310.
63. Olkeba BK, Goethals PLM, Boets P, Duchateau L, Degefa T, Eba K, et al. Mesocosm Experiments to Quantify Predation of Mosquito Larvae by Aquatic Predators to Determine Potential of Ecological Control of Malaria Vectors in Ethiopia. *Int J Environ Res Public Health*. 2021;18:6904.
64. Collins CM, Bonds J a. S, Quinlan MM, Mumford JD. Effects of the removal or reduction in density of the malaria mosquito, *Anopheles gambiae* s.l., on interacting predators and competitors in local ecosystems. *Med Vet Entomol*. 2019;33:1–15.
65. Onen H, Odong R, Chemurot M, Tripet F, Kayondo JK. Predatory and competitive interaction in *Anopheles gambiae sensu lato* larval breeding habitats in selected villages of central Uganda. *Parasit Vectors*. 2021;14:420.

66. Laporta GZ, Lopez de Prado PIK, Kraenkel RA, Coutinho RM, Sallum MAM. Biodiversity can help prevent malaria outbreaks in tropical forests. *PLoS Negl Trop Dis*. 2013;7:e2139.
67. Ostfeld RS. A Candide response to Panglossian accusations by Randolph and Dobson: biodiversity buffers disease. *Parasitology*. 2013;140:1196–8.
68. Halsey S. Defuse the dilution effect debate. *Nature Ecology & Evolution*. 2019;3:145.
69. Randolph SE, Dobson ADM. Pangloss revisited: a critique of the dilution effect and the biodiversity-buffers-disease paradigm. *Parasitology*. 2012;139:847–63.
70. Salkeld DJ, Padgett KA, Jones JH. A meta-analysis suggesting that the relationship between biodiversity and risk of zoonotic pathogen transmission is idiosyncratic. *Ecol Lett*. 2013;16:679–86.
71. Jackson RR, Nelson XJ. *Evarcha culicivora* chooses blood-fed *Anopheles* mosquitoes but other East African jumping spiders do not. *Medical and Veterinary Entomology*. 2012;26:233–5.
72. Rossati A, Bargiacchi O, Kroumova V, Zaramella M, Caputo A, Garavelli PL. Climate, environment and transmission of malaria. *Infez Med*. 2016;24:93–104.
73. Matthew OJ. Investigating climate suitability conditions for malaria transmission and impacts of climate variability on mosquito survival in the humid tropical region: a case study of Obafemi Awolowo University Campus, Ile-Ife, south-western Nigeria. *Int J Biometeorol*. 2020;64:355–65.
74. Coutinho PEG, Candido LA, Tadei WP, da Silva Junior UL, Correa HKM. An analysis of the influence of the local effects of climatic and hydrological factors affecting new malaria cases in riverine areas along the Rio Negro and surrounding Puraquequara Lake, Amazonas, Brazil. *Environ Monit Assess*. 2018;190:311.
75. Yasuoka J, Levins R. Impact of deforestation and agricultural development on anopheline ecology and malaria epidemiology. *Am J Trop Med Hyg*. 2007;76:450–60.
76. Walsh JF, Molyneux DH, Birley MH. Deforestation: effects on vector-borne disease. *Parasitology*. 1993;106 Suppl:S55-75.
77. Tuno N, Okeka W, Minakawa N, Takagi M, Yan G. Survivorship of *Anopheles gambiae* sensu stricto (Diptera: Culicidae) larvae in western Kenya highland forest. *J Med Entomol*. 2005;42:270–7.
78. Afrane YA, Zhou G, Lawson BW, Githeko AK, Yan G. Effects of microclimatic changes caused by deforestation on the survivorship and reproductive fitness of *Anopheles gambiae* in western Kenya highlands. *Am J Trop Med Hyg*. 2006;74:772–8.
79. Lindblade KA, Walker ED, Onapa AW, Katungu J, Wilson ML. Land use change alters malaria transmission parameters by modifying temperature in a highland area of Uganda. *Trop Med Int Health*. 2000;5:263–74.

80. Afrane YA, Little TJ, Lawson BW, Githeko AK, Yan G. Deforestation and Vectorial Capacity of *Anopheles gambiae* Giles Mosquitoes in Malaria Transmission, Kenya. *Emerg Infect Dis.* 2008;14:1533–8.
81. Thomas S, Ravishankaran S, Justin NAJA, Asokan A, Kalsingh TMJ, Mathai MT, et al. Microclimate variables of the ambient environment deliver the actual estimates of the extrinsic incubation period of *Plasmodium vivax* and *Plasmodium falciparum*: a study from a malaria-endemic urban setting, Chennai in India. *Malar J.* 2018;17:201.
82. Afrane YA, Lawson BW, Githeko AK, Yan G. Effects of microclimatic changes caused by land use and land cover on duration of gonotrophic cycles of *Anopheles gambiae* (Diptera: Culicidae) in western Kenya highlands. *J Med Entomol.* 2005;42:974–80.
83. Kweka EJ, Kimaro EE, Munga S. Effect of Deforestation and Land Use Changes on Mosquito Productivity and Development in Western Kenya Highlands: Implication for Malaria Risk. *Front Public Health.* 2016;4.
84. Tadei WP, Thatcher BD, Santos JM, Scarpassa VM, Rodrigues IB, Rafael MS. Ecologic observations on anopheline vectors of malaria in the Brazilian Amazon. *The American Journal of Tropical Medicine and Hygiene.* 1998;59:325–35.
85. Vittor AY, Gilman RH, Tielsch J, Glass G, Shields T, Lozano WS, et al. The effect of deforestation on the human-biting rate of *Anopheles darlingi*, the primary vector of *Falciparum* malaria in the Peruvian Amazon. *Am J Trop Med Hyg.* 2006;74:3–11.
86. Rerolle F, Dantzer E, Lover AA, Marshall JM, Hongvanthong B, Sturrock HJ, et al. Spatio-temporal associations between deforestation and malaria incidence in Lao PDR. *eLife.* 2021;10:e56974.
87. Vanwambeke SO, Lambin EF, Eichhorn MP, Flasse SP, Harbach RE, Oskam L, et al. Impact of Land-use Change on Dengue and Malaria in Northern Thailand. *EcoHealth.* 2007;4:37–51.
88. Gottdenker NL, Streicker DG, Faust CL, Carroll CR. Anthropogenic Land Use Change and Infectious Diseases: A Review of the Evidence. *EcoHealth.* 2014;11:619–32.
89. Austin KF, Bellinger MO, Rana P. Anthropogenic forest loss and malaria prevalence: a comparative examination of the causes and disease consequences of deforestation in developing nations. *AIMS Environmental Science.* 2017;4:217.
90. da Silva-Nunes M, Moreno M, Conn JE, Gamboa D, Abeles S, Vinetz JM, et al. Amazonian malaria: asymptomatic human reservoirs, diagnostic challenges, environmentally driven changes in mosquito vector populations, and the mandate for sustainable control strategies. *Acta Trop.* 2012;121:281–91.
91. Kitron U, Spielman A. Suppression of Transmission of Malaria Through Source Reduction: Antianopheline Measures Applied in Israel, the United States, and Italy. *Reviews of Infectious Diseases.* 1989;11:391–406.

92. Valle D, Clark J. Conservation Efforts May Increase Malaria Burden in the Brazilian Amazon. *PLOS ONE*. 2013;8:e57519.
93. Hahn MB, Olson SH, Vittor AY, Barcellos C, Patz JA, Pan W. Conservation Efforts and Malaria in the Brazilian Amazon. *Am J Trop Med Hyg*. 2014;90:591–4.
94. MacDonald AJ, Mordecai EA. Amazon deforestation drives malaria transmission, and malaria burden reduces forest clearing. *PNAS*. 2019;116:22212–8.
95. Sakeni M, Khorram A, Majdzadeh R, Raiesi A. Indoor Residual Spraying Coverage and Acceptability Rates to Control Malaria and the Householders' Reasons of Acceptance or Rejection of Spraying, in South-East of Iran. *Int J Infect*. 2015;2.
96. Opiyo MA, Paaijmans KP. 'We spray and walk away': wall modifications decrease the impact of indoor residual spray campaigns through reductions in post-spray coverage. *Malaria Journal*. 2020;19:30.
97. Chizema-Kawesha E, Miller JM, Steketee RW, Mukonka VM, Mukuka C, Mohamed AD, et al. Scaling up malaria control in Zambia: progress and impact 2005-2008. *Am J Trop Med Hyg*. 2010;83:480–8.
98. Fegan GW, Noor AM, Akhwale WS, Cousens S, Snow RW. Effect of expanded insecticide-treated bednet coverage on child survival in rural Kenya: a longitudinal study. *Lancet*. 2007;370:1035–9.
99. Sharp BL, Ridl FC, Govender D, Kuklinski J, Kleinschmidt I. Malaria vector control by indoor residual insecticide spraying on the tropical island of Bioko, Equatorial Guinea. *Malaria Journal*. 2007;6:52.
100. Townson H, Nathan MB, Zaim M, Guillet P, Manga L, Bos R, et al. Exploiting the potential of vector control for disease prevention. *Bull World Health Organ*. 2005;83:942–7.
101. Benelli G, Beier JC. Current vector control challenges in the fight against malaria. *Acta Trop*. 2017;174:91–6.
102. Devillers J. Fate and ecotoxicological effects of pyriproxyfen in aquatic ecosystems. *Environ Sci Pollut Res Int*. 2020;27:16052–68.
103. Russell TL, Kay BH, Skilleter GA. Environmental effects of mosquito insecticides on saltmarsh invertebrate fauna. *Aquatic Biology*. 2009;6:77–90.
104. Van Roey K, Sokny M, Denis L, Van den Broeck N, Heng S, Siv S, et al. Field Evaluation of Picaridin Repellents Reveals Differences in Repellent Sensitivity between Southeast Asian Vectors of Malaria and Arboviruses. *PLoS Negl Trop Dis*. 2014;8.
105. Charlwood JD, Nenhep S, Protopopoff N, Sovannaroth S, Morgan JC, Hemingway J. Effects of the spatial repellent metofluthrin on landing rates of outdoor biting anophelines in Cambodia, Southeast Asia. *Medical and Veterinary Entomology*. 2016;30:229–34.

106. Sukkanon C, Nararak J, Bangs MJ, Hii J, Chareonviriyaphap T. Behavioral responses to transfluthrin by *Aedes aegypti*, *Anopheles minimus*, *Anopheles harrisoni*, and *Anopheles dirus* (Diptera: Culicidae). *PLoS ONE*. 2020;15:e0237353.
107. Liverani M, Charlwood JD, Lawford H, Yeung S. Field assessment of a novel spatial repellent for malaria control: a feasibility and acceptability study in Mondulkiri, Cambodia. *Malar J*. 2017;16.
108. Gachelin G, Garner P, Ferroni E, Verhave JP, Opinel A. Evidence and strategies for malaria prevention and control: a historical analysis. *Malaria Journal*. 2018;17:96.
109. Maia MF, Tenywa FC, Nelson H, Kambagha A, Ashura A, Bakari I, et al. Attractive toxic sugar baits for controlling mosquitoes: a qualitative study in Bagamoyo, Tanzania. *Malaria Journal*. 2018;17:22.
110. Shaw WR, Catteruccia F. Vector biology meets disease control: using basic research to fight vector-borne diseases. *Nat Microbiol*. 2019;4:20–34.
111. WHO. Getting malaria control back on track: Making chemoprevention contribute. 2019.
112. Laurens MB. The Promise of a Malaria Vaccine-Are We Closer? *Annu Rev Microbiol*. 2018;72:273–92.
113. Duffy PE, Patrick Gorres J. Malaria vaccines since 2000: progress, priorities, products. *NPJ Vaccines*. 2020;5:48.
114. Laurens MB. RTS,S/AS01 vaccine (Mosquirix™): an overview. *Hum Vaccin Immunother*. 2019;16:480–9.
115. Lee H, Halverson S, Ezinwa N. Mosquito-Borne Diseases. *Prim Care*. 2018;45:393–407.
116. Elston JWT, Cartwright C, Ndumbi P, Wright J. The health impact of the 2014-15 Ebola outbreak. *Public Health*. 2017;143:60–70.
117. Check Hayden E. Ebola outbreak shuts down malaria-control efforts. *Nature*. 2014;514:15–6.
118. Kamal-Yanni MM, Potet J, Saunders PM. Scaling-up malaria treatment: a review of the performance of different providers. *Malar J*. 2012;11:414.
119. Noor AM, Rage IA, Moonen B, Snow RW. Health service providers in Somalia: their readiness to provide malaria case-management. *Malar J*. 2009;8:100.
120. Kangwana BB, Njogu J, Wasunna B, Kedenge SV, Memusi DN, Goodman CA, et al. Malaria drug shortages in Kenya: a major failure to provide access to effective treatment. *Am J Trop Med Hyg*. 2009;80:737–8.
121. Zurovac D, Guintran J-O, Donald W, Naket E, Malinga J, Taleo G. Health systems readiness and management of febrile outpatients under low malaria transmission in Vanuatu. *Malar J*. 2015;14:489.

122. Das S, Peck RB, Barney R, Jang IK, Kahn M, Zhu M, et al. Performance of an ultra-sensitive Plasmodium falciparum HRP2-based rapid diagnostic test with recombinant HRP2, culture parasites, and archived whole blood samples. *Malaria Journal*. 2018;17:118.
123. Tripura R, Peto TJ, Veugen CC, Nguon C, Davoeung C, James N, et al. Submicroscopic Plasmodium prevalence in relation to malaria incidence in 20 villages in western Cambodia. *Malaria Journal*. 2017;16:56.
124. Lek D, Callery JJ, Nguon C, Debackere M, Sovannaroth S, Tripura R, et al. Tools to accelerate falciparum malaria elimination in Cambodia: a meeting report. *Malar J*. 2020;19:151.
125. Kaji A, Thi SS, Smith T, Charunwatthana P, Nosten FH. Challenges in tackling tuberculosis on the Thai-Myanmar border: findings from a qualitative study with health professionals. *BMC Health Services Research*. 2015;15:464.
126. Dräger S, Gedik G, Dal Poz MR. Health workforce issues and the Global Fund to fight AIDS, Tuberculosis and Malaria: an analytical review. *Human Resources for Health*. 2006;4:23.
127. Wong ML, Chia KS, Yam WM, Teodoro GR, Lau KW. Willingness to donate blood samples for genetic research: a survey from a community in Singapore. *Clin Genet*. 2004;65:45–51.
128. WHO. Countries of the Greater Mekong zero in on falciparum malaria. Geneva: WHO; 2019.
129. The World Bank. The World Bank - Cambodia. World Bank. <https://www.worldbank.org/en/country/cambodia/overview>. Accessed 1 Nov 2021.
130. Hughes AC. Understanding the drivers of Southeast Asian biodiversity loss. *Ecosphere*. 2017;8:e01624.
131. Sodhi NS, Koh LP, Brook BW, Ng PKL. Southeast Asian biodiversity: an impending disaster. *Trends Ecol Evol (Amst)*. 2004;19:654–60.
132. Wilcove DS, Giam X, Edwards DP, Fisher B, Koh LP. Navjot's nightmare revisited: logging, agriculture, and biodiversity in Southeast Asia. *Trends Ecol Evol (Amst)*. 2013;28:531–40.
133. Bordes F, Morand S, Pilosof S, Claude J, Krasnov BR, Cosson J-F, et al. Habitat fragmentation alters the properties of a host-parasite network: rodents and their helminths in South-East Asia. *J Anim Ecol*. 2015;84:1253–63.
134. Dupuy S, Herbreteau V, Feyfant T, Morand S, Tran A. Land-cover dynamics in Southeast Asia: contribution of object-oriented techniques for change detection. 4th International Conference on GEographic Object-Based Image Analysis (GEOBIA). 2012;:217–22.
135. von Seidlein L, Peto TJ, Tripura R, Pell C, Yeung S, Kindermans JM, et al. Novel Approaches to Control Malaria in Forested Areas of Southeast Asia. *Trends Parasitol*. 2019;35:388–98.
136. Davis K, Yu K, Rulli MC, Pichdara L, D'Odorico P. Accelerated deforestation driven by large-scale land acquisitions in Cambodia. *Nat Geosci*. 2015;8:772–5.

137. D’Odorico P, Rulli MC. The land and its people. 2014;7:324–5.
138. Deininger KB Derek. Rising Global Interest in Farmland. The World Bank; 2011.
139. Golay C, Biglino I. Human Rights Responses to Land Grabbing: a right to food perspective. *Third World Quarterly*. 2013;34:1630–50.
140. Open Development Cambodia. ELCs: All economic land concessions. 2019.
141. Payne D. Spread of chloroquine resistance in *Plasmodium falciparum*. *Parasitol Today*. 1987;3:241–6.
142. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM, et al. Evidence of artemisinin-resistant malaria in western Cambodia. *N Engl J Med*. 2008;359:2619–20.
143. Duru V, Witkowski B, Ménard D. *Plasmodium falciparum* Resistance to Artemisinin Derivatives and Piperaquine: A Major Challenge for Malaria Elimination in Cambodia. *Am J Trop Med Hyg*. 2016;95:1228–38.
144. Leang R, Taylor WRJ, Bouth DM, Song L, Tarning J, Char MC, et al. Evidence of *Plasmodium falciparum* Malaria Multidrug Resistance to Artemisinin and Piperaquine in Western Cambodia: Dihydroartemisinin-Piperaquine Open-Label Multicenter Clinical Assessment. *Antimicrob Agents Chemother*. 2015;59:4719–26.
145. Saunders DL, Vanachayangkul P, Lon C. Dihydroartemisinin–Piperaquine Failure in Cambodia. *New England Journal of Medicine*. 2014;371:484–5.
146. Thriemer K, Bobogare A, Ley B, Gudo CS, Alam MS, Anstey NM, et al. Quantifying primaquine effectiveness and improving adherence: a round table discussion of the APMEN Vivax Working Group. *Malaria Journal*. 2018;17:241.
147. Gryseels C, Kuijpers LMF, Jacobs J, Grietens KP. When ‘substandard’ is the standard, who decides what is appropriate? Exploring healthcare provision in Cambodia. *Crit Public Health*. 2019;29:460–72.
148. Van Bortel W, Trung HD, Thuan LK, Sochantha T, Socheat D, Sumrandee C, et al. The insecticide resistance status of malaria vectors in the Mekong region. *Malaria Journal*. 2008;7:102.
149. Hoyer S, Nguon S, Kim S, Habib N, Khim N, Sum S, et al. Focused Screening and Treatment (FSAT): A PCR-Based Strategy to Detect Malaria Parasite Carriers and Contain Drug Resistant *P. falciparum*, Pailin, Cambodia. *PLOS ONE*. 2012;7:e45797.
150. Durnez L, Pareyn M, Mean V, Kim S, Khim N, Menard D, et al. Identification and characterization of areas of high and low risk for asymptomatic malaria infections at sub-village level in Ratanakiri, Cambodia. *Malaria Journal*. 2018;17:27.
151. Vantaux A, Samreth R, Piv EP, Khim N, Kim S, Berne L, et al. Contribution to malaria transmission of symptomatic and asymptomatic parasite carriers in Cambodia. *J Infect Dis*. 2018. <https://doi.org/10.1093/infdis/jiy060>.

152. Edwards HM, Sriwichai P, Kirabittir K, Prachumsri J, Chavez IF, Hii J. Transmission risk beyond the village: entomological and human factors contributing to residual malaria transmission in an area approaching malaria elimination on the Thailand–Myanmar border. *Malaria Journal*. 2019;18:221.
153. Edwards HM, Chinh VD, Le Duy B, Thanh PV, Thang ND, Trang DM, et al. Characterising residual malaria transmission in forested areas with low coverage of core vector control in central Viet Nam. *Parasites & Vectors*. 2019;12:454.
154. St. Laurent B, Oy K, Miller B, Gasteiger EB, Lee E, Sovannaroth S, et al. Cow-baited tents are highly effective in sampling diverse *Anopheles* malaria vectors in Cambodia. *Malar J*. 2016;15.
155. Okami S, Kohtake N. Spatiotemporal Modeling for Fine-Scale Maps of Regional Malaria Endemicity and Its Implications for Transitional Complexities in a Routine Surveillance Network in Western Cambodia. *Front Public Health*. 2017;5:262.
156. Bannister-Tyrrell M, Srun S, Sluydts V, Gryseels C, Mean V, Kim S, et al. Importance of household-level risk factors in explaining micro-epidemiology of asymptomatic malaria infections in Ratanakiri Province, Cambodia. *Sci Rep*. 2018;8:11643.
157. Sinka ME, Bangs MJ, Manguin S, Chareonviriyaphap T, Patil AP, Temperley WH, et al. The dominant *Anopheles* vectors of human malaria in the Asia-Pacific region: occurrence data, distribution maps and bionomic précis. *Parasites & Vectors*. 2011;4:89.
158. Incardona S, Vong S, Chiv L, Lim P, Nhem S, Sem R, et al. Large-scale malaria survey in Cambodia: Novel insights on species distribution and risk factors. *Malar J*. 2007;6:37.
159. Sluydts V, Heng S, Coosemans M, Van Roey K, Gryseels C, Canier L, et al. Spatial clustering and risk factors of malaria infections in Ratanakiri Province, Cambodia. *Malar J*. 2014;13:387.
160. Kar NP, Kumar A, Singh OP, Carlton JM, Nanda N. A review of malaria transmission dynamics in forest ecosystems. *Parasit Vectors*. 2014;7:265.
161. Peeters Grietens K, Gryseels C, Dierickx S, Bannister-Tyrrell M, Trienekens S, Uk S, et al. Characterizing Types of Human Mobility to Inform Differential and Targeted Malaria Elimination Strategies in Northeast Cambodia. *Sci Rep*. 2015;5:16837.
162. Thomson R, Sochea P, Sarath M, MacDonald A, Pratt A, Poyer S, et al. Rubber plantations and drug resistant malaria: a cross-sectional survey in Cambodia. *Malar J*. 2019;18:379.
163. Parker DM, Tripura R, Peto TJ, Maude RJ, Nguon C, Chalk J, et al. A multi-level spatial analysis of clinical malaria and subclinical *Plasmodium* infections in Pailin Province, Cambodia. *Heliyon*. 2017;3:e00447.
164. Rossi G, Vernaeve L, Van den Bergh R, Nguon C, Debackere M, Abello Peiri C, et al. Closing in on the Reservoir: Proactive Case Detection in High-Risk Groups as a Strategy to Detect *Plasmodium falciparum* Asymptomatic Carriers in Cambodia. *Clin Infect Dis*. 2018;66:1610–7.

165. Sanann N, Peto TJ, Tripura R, Callery JJ, Nguon C, Bui TM, et al. Forest work and its implications for malaria elimination: a qualitative study. *Malaria Journal*. 2019;18:376.
166. Taffon P, Rossi G, Kindermans J-M, Van den Bergh R, Nguon C, Debackere M, et al. “I could not join because I had to work for pay.”: A qualitative evaluation of falciparum malaria pro-active case detection in three rural Cambodian villages. *PLoS ONE*. 2018;13:e0195809.
167. Canier L, Khim N, Kim S, Sluydts V, Heng S, Dourng D, et al. An innovative tool for moving malaria PCR detection of parasite reservoir into the field. *Malaria Journal*. 2013;12:405.
168. Moody A. Rapid diagnostic tests for malaria parasites. *Clin Microbiol Rev*. 2002;15:66–78.
169. Yeung S, McGregor D, James N, Kheang ST, Kim S, Khim N, et al. Performance of Ultrasensitive Rapid Diagnostic Tests for Detecting Asymptomatic Plasmodium falciparum. *Am J Trop Med Hyg*. 2020;102:307–9.
170. Stratil A-S, Vernaev L, Lopes S, Bourny Y, Mannion K, Hamade P, et al. Eliminating Plasmodium falciparum malaria: results from tailoring active case detection approaches to remote populations in forested border areas in north-eastern Cambodia. *Malaria Journal*. 2021;20:108.
171. Kheang ST, Sovannaroth S, Barat LM, Dysoley L, Kapella BK, Po L, et al. Malaria elimination using the 1-3-7 approach: lessons from Sampov Loun, Cambodia. *BMC Public Health*. 2020;20:544.
172. Kunkel A, Nguon C, Iv S, Chhim S, Peov D, Kong P, et al. Choosing interventions to eliminate forest malaria: preliminary results of two operational research studies inside Cambodian forests. *Malar J*. 2021;20:51.
173. WHO’s Mekong Malaria Elimination Programme. Countries of the Greater Mekong ready for the “last mile” of malaria elimination. 2020.
174. Ministry of Health, Kingdom of Cambodia. Cambodia Malaria Elimination Action Framework 2016-2020. Phnom Penh; 2016.
175. Bancone G, Menard D, Khim N, Kim S, Canier L, Nguong C, et al. Molecular characterization and mapping of glucose-6-phosphate dehydrogenase (G6PD) mutations in the Greater Mekong Subregion. *Malaria Journal*. 2019;18:20.
176. Cui L, Yan G, Sattabongkot J, Cao Y, Chen B, Chen X, et al. Malaria in the Greater Mekong Subregion: heterogeneity and complexity. *Acta Trop*. 2012;121:227–39.
177. Gryseels C, Durnez L, Gerrets R, Uk S, Suon S, Set S, et al. Re-imagining malaria: heterogeneity of human and mosquito behaviour in relation to residual malaria transmission in Cambodia. *Malar J*. 2015;14:165.

178. Wangdi K, Clements AC. Ending Malaria Transmission in the Asia Pacific Malaria Elimination Network (APMEN) Countries: Challenges and the Way Forward. In: Manguin S, Dev V, editors. *Towards Malaria Elimination - A Leap Forward*. InTech; 2018.
179. Killeen GF. Characterizing, controlling and eliminating residual malaria transmission. *Malar J*. 2014;13:330.
180. Durnez L, Mao S, Denis L, Roelants P, Sochantha T, Coosemans M. Outdoor malaria transmission in forested villages of Cambodia. *Malaria Journal*. 2013;12:329.
181. Vythilingam I, Sidavong B, Chan ST, Phonemixay T, Vanisaveth V, Sisoulad P, et al. Epidemiology of malaria in Attapeu Province, Lao PDR in relation to entomological parameters. *Trans R Soc Trop Med Hyg*. 2005;99:833–9.
182. Ismail I a. H, Notananda V, Schepens J, Organization WH. Studies on malaria and responses of *Anopheles balabacensis balabacensis* and *Anopheles minimus* to DDT residual spraying in Thailand. 1973.
183. Nustsathapana S, Sawasdiwongphorn P, Chitprarop U, Cullen J. The behaviour of *Anopheles minimus* Theobald (Diptera: Culicidae) subjected to differing levels of DDT selection pressure in northern Thailand. *Bulletin of entomological research*. 1986;76:303–12.
184. Stothard JR, Sousa-Figueiredo JC, Betson M, Seto EYW, Kabatereine NB. Investigating the spatial micro-epidemiology of diseases within a point-prevalence sample: a field applicable method for rapid mapping of households using low-cost GPS-dataloggers. *Trans R Soc Trop Med Hyg*. 2011;105:500–6.
185. Vazquez-Prokopec GM, Bisanzio D, Stoddard ST, Paz-Soldan V, Morrison AC, Elder JP, et al. Using GPS technology to quantify human mobility, dynamic contacts and infectious disease dynamics in a resource-poor urban environment. *PLoS ONE*. 2013;8:e58802.
186. Vazquez-Prokopec GM, Stoddard ST, Paz-Soldan V, Morrison AC, Elder JP, Kochel TJ, et al. Usefulness of commercially available GPS data-loggers for tracking human movement and exposure to dengue virus. *Int J Health Geogr*. 2009;8:68.
187. Paz-Soldan VA, Reiner RC, Morrison AC, Stoddard ST, Kitron U, Scott TW, et al. Strengths and weaknesses of Global Positioning System (GPS) data-loggers and semi-structured interviews for capturing fine-scale human mobility: findings from Iquitos, Peru. *PLoS Negl Trop Dis*. 2014;8:e2888.
188. McGranahan DA, Geaumont B, Spiess JW. Assessment of a livestock GPS collar based on an open-source datalogger informs best practices for logging intensity. *Ecology and Evolution*. 2018. <https://doi.org/10.1002/ece3.4094>.
189. Rainham D, Krewski D, McDowell I, Sawada M, Liekens B. Development of a wearable global positioning system for place and health research. *Int J Health Geogr*. 2008;7:59.

190. Duncan S, Stewart TI, Oliver M, Mavoa S, MacRae D, Badland HM, et al. Portable global positioning system receivers: static validity and environmental conditions. *Am J Prev Med.* 2013;44:e19-29.
191. Forin-Wiart M-A, Hubert P, Sirguy P, Poulle M-L. Performance and Accuracy of Lightweight and Low-Cost GPS Data Loggers According to Antenna Positions, Fix Intervals, Habitats and Animal Movements. *PLOS ONE.* 2015;10:e0129271.
192. Tobin C, Bailey DW, Trotter MG. Tracking and sensor-based detection of livestock water system failure: A case study simulation. *Rangeland Ecology & Management.* 2021;77:9–16.
193. Hast M, Searle KM, Chaponda M, Lupiya J, Lubinda J, Sikalima J, et al. The use of GPS data loggers to describe the impact of spatio-temporal movement patterns on malaria control in a high-transmission area of northern Zambia. *Int J Health Geogr.* 2019;18:19.
194. Seto EYW, Knapp F, Zhong B, Yang C. The use of a vest equipped with a global positioning system to assess water-contact patterns associated with schistosomiasis. *Geospat Health.* 2007;1:233–41.
195. Qi F, Du F. Tracking and visualization of space-time activities for a micro-scale flu transmission study. *International Journal of Health Geographics.* 2013;12:6.
196. Tessema S, Wesolowski A, Chen A, Murphy M, Wilhelm J, Mupiri A-R, et al. Using parasite genetic and human mobility data to infer local and cross-border malaria connectivity in Southern Africa. *eLife.* 2019;8:e43510.
197. Wesolowski A, Taylor AR, Chang H-H, Verity R, Tessema S, Bailey JA, et al. Mapping malaria by combining parasite genomic and epidemiologic data. *BMC Medicine.* 2018;16:190.
198. Wesolowski A, Buckee CO, Engø-Monsen K, Metcalf CJE. Connecting Mobility to Infectious Diseases: The Promise and Limits of Mobile Phone Data. *J Infect Dis.* 2016;214 suppl_4:S414–20.
199. Huber C, Watts A, Grills A, Yong JHE, Morrison S, Bowden S, et al. Modelling airport catchment areas to anticipate the spread of infectious diseases across land and air travel. *Spat Spatiotemporal Epidemiol.* 2021;36:100380.
200. Wirth FN, Johns M, Meurers T, Prasser F. Citizen-Centered Mobile Health Apps Collecting Individual-Level Spatial Data for Infectious Disease Management: Scoping Review. *JMIR Mhealth Uhealth.* 2020;8:e22594.
201. Sun S, Folarin AA, Ranjan Y, Rashid Z, Conde P, Stewart C, et al. Using Smartphones and Wearable Devices to Monitor Behavioral Changes During COVID-19. *J Med Internet Res.* 2020;22:e19992.
202. Kerr J, Duncan S, Schipperijn J, Schipperijn J. Using global positioning systems in health research: a practical approach to data collection and processing. *Am J Prev Med.* 2011;41:532–40.

203. Badland HM, Duncan MJ, Oliver M, Duncan JS, Mavoa S. Examining commute routes: applications of GIS and GPS technology. *Environ Health Prev Med.* 2010;15:327–30.
204. Oliver M, Badland H, Mavoa S, Duncan MJ, Duncan S. Combining GPS, GIS, and accelerometry: methodological issues in the assessment of location and intensity of travel behaviors. *J Phys Act Health.* 2010;7:102–8.
205. McGarigal K, Marks BJ. FRAGSTATS: spatial pattern analysis program for quantifying landscape structure. General technical report PNW (USA). 1995.
206. Peng J, Wang Y, Zhang Y, Wu J, Li W, Li Y. Evaluating the effectiveness of landscape metrics in quantifying spatial patterns. *Ecological Indicators.* 2010;10:217–23.
207. Wagman J, Grieco JP, Bautista K, Polanco J, Briceño I, King R, et al. A comparison of two commercial mosquito traps for the capture of malaria vectors in northern belize, central america. *J Am Mosq Control Assoc.* 2014;30:175–83.
208. Obenauer PJ, Abdel-Dayem MS, Stoops CA, Villinski JT, Tageldin R, Fahmy NT, et al. Field Responses of Anopheles gambiae Complex (Diptera: Culicidae) in Liberia using Yeast-Generated Carbon Dioxide and Synthetic Lure-Baited Light Traps. *Journal of Medical Entomology.* 2013;50:863–70.
209. Ponlawat A, Khongtak P, Jaichapor B, Pongsiri A, Evans BP. Field evaluation of two commercial mosquito traps baited with different attractants and colored lights for malaria vector surveillance in Thailand. *Parasit Vectors.* 2017;10:378.
210. Achee NL, Youngblood L, Bangs MJ, Lavery JV, James S. Considerations for the use of human participants in vector biology research: a tool for investigators and regulators. *Vector Borne Zoonotic Dis.* 2015;15:89–102.
211. Tangena J-AA, Thammavong P, Hiscox A, Lindsay SW, Brey PT. The Human-Baited Double Net Trap: An Alternative to Human Landing Catches for Collecting Outdoor Biting Mosquitoes in Lao PDR. *PLoS One.* 2015;10.
212. Service MW. *Mosquito Ecology: Field Sampling Methods.* Springer Netherlands; 1993.
213. Silver JB. *Mosquito Ecology: Field Sampling Methods.* 3rd edition. Springer Netherlands; 2008.
214. St Laurent B, Sukowati S, Burton TA, Bretz D, Zio M, Firman S, et al. Comparative evaluation of anopheline sampling methods in three localities in Indonesia. *Malar J.* 2018;17:13.
215. Le Goff G, P C, E F, V R, Comparaison R, Trois D. COMPARISON OF THREE SAMPLING METHODS OF MAN-BITING ANOPHELINES IN ORDER TO ESTIMATE THE MALARIA TRANSMISSION IN A VILLAGE OF SOUTH CAMEROON. 2016.
216. Service MW. A critical review of procedures for sampling populations of adult mosquitoes. *Bulletin of Entomological Research.* 1977;67:343–82.

217. Davis JR, Hall T, Chee EM, Majala A, Minjas J, Shiff CJ. Comparison of sampling anopheline mosquitoes by light-trap and human-bait collections indoors at Bagamoyo, Tanzania. *Med Vet Entomol.* 1995;9:249–55.
218. Hii JL, Smith T, Mai A, Ibam E, Alpers MP. Comparison between anopheline mosquitoes (Diptera: Culicidae) caught using different methods in a malaria endemic area of Papua New Guinea. *Bull Entomol Res.* 2000;90:211–9.
219. Le Goff G, Carnevale P, Robert V. [Comparison of catches by landings on humans and by CDC light traps for sampling of mosquitoes and evaluation of malaria transmission in South Cameroon]. *Ann Soc Belg Med Trop.* 1993;73:55–60.
220. Derek Charlwood J, Nenhep S, Sovannaroeth S, Morgan JC, Hemingway J, Chitnis N, et al. “Nature or nurture”: survival rate, oviposition interval, and possible gonotrophic discordance among South East Asian anophelines. *Malar J.* 2016;15:356.
221. Davidson JR, Sukowati S, Shinta, Asih PBS, Syafruddin D, Baskin RN, et al. Using barrier screens to characterize mosquito composition, flight activity, and abdominal status in South Lampung, Indonesia. *Parasit Vectors.* 2018;11.
222. Burkot TR, Russell TL, Reimer LJ, Bugoro H, Beebe NW, Cooper RD, et al. Barrier screens: a method to sample blood-fed and host-seeking exophilic mosquitoes. *Malaria Journal.* 2013;12:49.
223. Davidson JR, Wahid I, Sudirman R, Makuru V, Hasan H, Arfah AM, et al. Comparative field evaluation of kelambu traps, barrier screens and barrier screens with eaves for longitudinal surveillance of adult Anopheles mosquitoes in Sulawesi, Indonesia. *Parasites & Vectors.* 2019;12:399.
224. Keven JB, Katusele M, Vinit R, Koimbu G, Vincent N, Thomsen EK, et al. Species abundance, composition, and nocturnal activity of female Anopheles (Diptera: Culicidae) in malaria-endemic villages of Papua New Guinea: assessment with barrier screen sampling. *Malar J.* 2019;18.
225. Degefa T, Yewhalaw D, Zhou G, Lee M-C, Atieli H, Githeko AK, et al. Evaluation of the performance of new sticky pots for outdoor resting malaria vector surveillance in western Kenya. *Parasit Vectors.* 2019;12:278.
226. Brown R, Hing CT, Fornace K, Ferguson HM. Evaluation of resting traps to examine the behaviour and ecology of mosquito vectors in an area of rapidly changing land use in Sabah, Malaysian Borneo. *Parasites & Vectors.* 2018;11:346.
227. Abong’o B, Yu X, Donnelly MJ, Geier M, Gibson G, Gimnig J, et al. Host Decoy Trap (HDT) with cattle odour is highly effective for collection of exophagic malaria vectors. *Parasites & Vectors.* 2018;11:533.
228. Mathenge EM, Misiani GO, Oulo DO, Irungu LW, Ndegwa PN, Smith TA, et al. Comparative performance of the Mbita trap, CDC light trap and the human landing catch in

the sampling of *Anopheles arabiensis*, *An. funestus* and culicine species in a rice irrigation in western Kenya. *Malar J.* 2005;4:7.

229. Laganier R, Randimby FM, Rajaonarivelo V, Robert V. Is the Mbita trap a reliable tool for evaluating the density of anopheline vectors in the highlands of Madagascar? *Malar J.* 2003;2:42.

230. Sikaala CH, Killeen GF, Chanda J, Chinula D, Miller JM, Russell TL, et al. Evaluation of alternative mosquito sampling methods for malaria vectors in Lowland South - East Zambia. *Parasit Vectors.* 2013;6:91.

231. Sikulu M, Govella NJ, Ogoma SB, Mpangile J, Kambi SH, Kannady K, et al. Comparative evaluation of the Ifakara tent trap-B, the standardized resting boxes and the human landing catch for sampling malaria vectors and other mosquitoes in urban Dar es Salaam, Tanzania. *Malaria Journal.* 2009;8:197.

232. Williams CR, Long SA, Russell RC, Ritchie SA. Field efficacy of the BG-Sentinel compared with CDC Backpack Aspirators and CO₂-baited EVS traps for collection of adult *Aedes aegypti* in Cairns, Queensland, Australia. *J Am Mosq Control Assoc.* 2006;22:296–300.

233. Mburu MM, Zembere K, Hiscox A, Banda J, Phiri KS, van den Berg H, et al. Assessment of the Suna trap for sampling mosquitoes indoors and outdoors. *Malar J.* 2019;18:51.

234. Lee HI, Seo BY, Shin E-H, Burkett DA, Lee J-K, Shin YH. Efficiency Evaluation of Nozawa-Style Black Light Trap for Control of Anopheline Mosquitoes. *Korean J Parasitol.* 2009;47:159–65.

235. Hutchinson RA, West PA, Lindsay SW. Suitability of two carbon dioxide-baited traps for mosquito surveillance in the United Kingdom. *Bulletin of Entomological Research.* 2007;97:591–7.

236. Andrews ES, Schoeler GB, Gozalo AS, Carbajal F, Lopez-Sifuentes V, Turell MJ. Species Diversity, Seasonal, and Spatial Distribution of Mosquitoes (Diptera: Culicidae) Captured in Aotus Monkey-Baited Traps in a Forested Site Near Iquitos, Peru. *J Med Entomol.* 2014;51:1127–35.

237. Robert V, Le Goff G, Ariey F, Duchemin J-B. A possible alternative method for collecting mosquito larvae in rice fields. *Malaria journal.* 2002;1:4.

238. Mwingira VS, Mayala BK, Senkoro KP, Rumisha SF, Shayo EH, Mlozi MRS, et al. Mosquito larval productivity in rice-fields infested with *Azolla* in Mvomero District, Tanzania. *Tanzania Journal of Health Research.* 2009;11.

239. Kongmee M, Achee NL, Lerdthusnee K, Bangs MJ, Chowpongpan S, Prabaripai A, et al. Seasonal abundance and distribution of *Anopheles* larvae in a riparian malaria endemic area of western Thailand. *Southeast Asian J Trop Med Public Health.* 2012;43:601–13.

240. Debrah I, Afrane YA, Amoah LE, Ochwedo KO, Mukabana WR, Zhong D, et al. Larval ecology and bionomics of *Anopheles funestus* in highland and lowland sites in western Kenya. *PLoS One*. 2021;16:e0255321.
241. Mehravaran A, Vatandoost H, Oshaghi MA, Abai MR, Edalat H, Javadian E, et al. Ecology of *Anopheles stephensi* in a malarious area, southeast of Iran. *Acta Med Iran*. 2012;50:61–5.
242. Nambunga IH, Ngowo HS, Mapua SA, Hape EE, Msugupakulya BJ, Msaky DS, et al. Aquatic habitats of the malaria vector *Anopheles funestus* in rural south-eastern Tanzania. *Malar J*. 2020;19:219.
243. Prussing C, Saavedra MP, Bickersmith SA, Alava F, Guzmán M, Manrique E, et al. Malaria vector species in Amazonian Peru co-occur in larval habitats but have distinct larval microbial communities. *PLoS Negl Trop Dis*. 2019;13:e0007412.
244. Alam MS, Al-Amin HM, Elahi R, Chakma S, Kafi MAH, Khan WA, et al. Abundance and Dynamics of *Anopheles* (Diptera: Culicidae) Larvae in a Malaria Endemic Area of Bangladesh. *J Med Entomol*. 2018;55:382–91.
245. Gimonneau G, Pombi M, Choisy M, Morand S, Dabiré RK, Simard F. Larval habitat segregation between the molecular forms of the mosquito *Anopheles gambiae* in a rice field area of Burkina Faso, West Africa. *Medical and Veterinary Entomology*. 2012. <https://doi.org/10.1111/j.1365-2915.2011.00957.x>.
246. Vittor AY, Pan W, Gilman RH, Tielsch J, Glass G, Shields T, et al. Linking deforestation to malaria in the Amazon: characterization of the breeding habitat of the principal malaria vector, *Anopheles darlingi*. *Am J Trop Med Hyg*. 2009;81:5–12.
247. Minakawa N, Munga S, Atieli F, Mushinzimana E, Zhou G, Githeko AK, et al. Spatial distribution of anopheline larval habitats in Western Kenyan highlands: effects of land cover types and topography. *Am J Trop Med Hyg*. 2005;73:157–65.
248. Reisen WK, Meyer RP, Shields J, Arbolante C. Population ecology of preimaginal *Culex tarsalis* (Diptera: Culicidae) in Kern County, California. *J Med Entomol*. 1989;26:10–22.
249. Balkew M, Mumba P, Dengela D, Yohannes G, Getachew D, Yared S, et al. Geographical distribution of *Anopheles stephensi* in eastern Ethiopia. *Parasit Vectors*. 2020;13:35.
250. Ya-Umphun P, Cerqueira D, Parker DM, Cottrell G, Poinsignon A, Remoue F, et al. Use of an *Anopheles* Salivary Biomarker to Assess Malaria Transmission Risk Along the Thailand-Myanmar Border. *J Infect Dis*. 2017;215:396–404.
251. Remoue F, Cisse B, Ba F, Sokhna C, Herve J-P, Boulanger D, et al. Evaluation of the antibody response to *Anopheles* salivary antigens as a potential marker of risk of malaria. *Trans R Soc Trop Med Hyg*. 2006;100:363–70.
252. Drame PM, Machault V, Diallo A, Cornélie S, Poinsignon A, Lalou R, et al. IgG responses to the gSG6-P1 salivary peptide for evaluating human exposure to *Anopheles* bites in urban areas of Dakar region, Sénégal. *Malar J*. 2012;11:72.

253. Drame PM, Poinsignon A, Dechavanne C, Cottrell G, Farce M, Ladekpo R, et al. Specific antibodies to Anopheles gSG6-P1 salivary peptide to assess early childhood exposure to malaria vector bites. *Malar J.* 2015;14.
254. Londono-Renteria B, Drame PM, Weitzel T, Rosas R, Gripping C, Cardenas JC, et al. An. gambiae gSG6-P1 evaluation as a proxy for human-vector contact in the Americas: a pilot study. *Parasites & Vectors.* 2015;8:533.
255. Pollard EJM, Patterson C, Russell TL, Apairamo A, Oscar J, Arcà B, et al. Human exposure to Anopheles farauti bites in the Solomon Islands is not associated with IgG antibody response to the gSG6 salivary protein of Anopheles gambiae. *Malar J.* 2019;18:334.
256. Kearney EA, Agius PA, Chaumeau V, Cutts JC, Simpson JA, Fowkes FJ. Anopheles salivary antigens as serological biomarkers of vector exposure and malaria transmission: A systematic review with multilevel modelling. *eLife.* 2021;10:e73080.
257. Sagna AB, Sarr JB, Gaayeb L, Drame PM, Ndiath MO, Senghor S, et al. gSG6-P1 salivary biomarker discriminates micro-geographical heterogeneity of human exposure to Anopheles bites in low and seasonal malaria areas. *Parasit Vectors.* 2013;6:68.
258. Dayananda KK, Achur RN, Gowda DC. Epidemiology, drug resistance, and pathophysiology of Plasmodium vivax malaria. *J Vector Borne Dis.* 2018;55:1–8.
259. Rosado J, White MT, Longley RJ, Lacerda M, Monteiro W, Brewster J, et al. Heterogeneity in response to serological exposure markers of recent Plasmodium vivax infections in contrasting epidemiological contexts. *PLOS Neglected Tropical Diseases.* 2021;15:e0009165.
260. Orjuela-Sánchez P, Brandi MC, Ferreira MU. Microsatellite analysis of malaria parasites. *Methods Mol Biol.* 2013;1006:247–58.
261. Auburn S, Barry AE. Dissecting malaria biology and epidemiology using population genetics and genomics. *International Journal for Parasitology.* 2017;47:77–85.
262. Fola AA, Kattenberg E, Razook Z, Lautu-Gumal D, Lee S, Mehra S, et al. SNP barcodes provide higher resolution than microsatellite markers to measure Plasmodium vivax population genetics. *Malar J.* 2020;19:375.
263. Diez Benavente E, Campos M, Phelan J, Nolder D, Dombrowski JG, Marinho CRF, et al. A molecular barcode to inform the geographical origin and transmission dynamics of Plasmodium vivax malaria. *PLoS Genet.* 2020;16:e1008576.
264. Noisang C, Prosser C, Meyer W, Chemoh W, Ellis J, Sawangjaroen N, et al. Molecular detection of drug resistant malaria in Southern Thailand. *Malar J.* 2019;18:275.
265. Fulakeza J, McNitt S, Vareta J, Saidi A, Mvula G, Taylor T, et al. Comparison of msp genotyping and a 24 SNP molecular assay for differentiating Plasmodium falciparum recrudescence from reinfection. *Malar J.* 2019;18:84.

266. Lin JT, Hathaway NJ, Saunders DL, Lon C, Balasubramanian S, Kharabora O, et al. Using Amplicon Deep Sequencing to Detect Genetic Signatures of Plasmodium vivax Relapse. *J Infect Dis.* 2015;212:999–1008.
267. Rao PN, Uplekar S, Kayal S, Mallick PK, Bandyopadhyay N, Kale S, et al. A Method for Amplicon Deep Sequencing of Drug Resistance Genes in Plasmodium falciparum Clinical Isolates from India. *J Clin Microbiol.* 2016;54:1500–11.
268. Lerch A, Koepfli C, Hofmann NE, Kattenberg JH, Rosanas-Urgell A, Betuela I, et al. Longitudinal tracking and quantification of individual Plasmodium falciparum clones in complex infections. *Scientific Reports.* 2019;9:3333.
269. Lerch A, Koepfli C, Hofmann NE, Messerli C, Wilcox S, Kattenberg JH, et al. Development of amplicon deep sequencing markers and data analysis pipeline for genotyping multi-clonal malaria infections. *BMC Genomics.* 2017;18:864.
270. Potapov P, Tyukavina A, Turubanova S, Talero Y, Hernandez-Serna A, Hansen MC, et al. Annual continuous fields of woody vegetation structure in the Lower Mekong region from 2000-2017 Landsat time-series. *Remote Sensing of Environment.* 2019;232:111278.
271. Saah D, Tenneson K, Matin M, Uddin K, Cutter P, Poortinga A, et al. Land Cover Mapping in Data Scarce Environments: Challenges and Opportunities. *Front Environ Sci.* 2019;7.
272. Bhumiratana A, Sorosjinda-Nunthawarasilp P, Kaewwaen W, Maneekan P, Pimnon S. Malaria-associated rubber plantations in Thailand. *Travel Medicine and Infectious Disease.* 2013;11:37–50.
273. Kobayashi J, Somboon P, Keomanila H, Inthavongsa S, Nambanya S, Inthakone S, et al. Malaria prevalence and a brief entomological survey in a village surrounded by rice fields in Khammouan province, Lao PDR. *Trop Med Int Health.* 2000;5:17–21.
274. Messina JP, Pan WK. Different ontologies: land change science and health research. *Curr Opin Environ Sustain.* 2013;5:515–21.
275. FAO. FRA 2000 | Global Forest Resources Assessments | Food and Agriculture Organization of the United Nations. 2000. <http://www.fao.org/forest-resources-assessment/past-assessments/fra-2000/en/>. Accessed 9 Jul 2021.
276. Guerra CA, Snow RW, Hay SI. A global assessment of closed forests, deforestation and malaria risk. *Ann Trop Med Parasitol.* 2006;100:189–204.
277. Prothero RM. Malaria, Forests and People in Southeast Asia. *Singapore Journal of Tropical Geography.* 2002;20:76–85.
278. Diaz A, Drakeley C, Fornace K. Land use and land cover changes-implications for malaria elimination. WHO Strategic Advisory Group on Malaria Eradication. Zenodo; 2020.

279. Chua TH, Manin BO, Vythilingam I, Fornace K, Drakeley CJ. Effect of different habitat types on abundance and biting times of *Anopheles balabacensis* Baisas (Diptera: Culicidae) in Kudat district of Sabah, Malaysia. *Parasit Vectors*. 2019;12.
280. Sonko ST, Jaiteh M, Jafali J, Jarju LB, D'Alessandro U, Camara A, et al. Does socio-economic status explain the differentials in malaria parasite prevalence? Evidence from The Gambia. *Malaria Journal*. 2014;13:449.
281. Opeskin B. Malaria in Pacific populations: seen but not heard? *J Pop Research*. 2009;26:175–99.
282. Somi MF, Butler JR, Vahid F, Njau JD, Kachur SP, Abdulla S. Use of proxy measures in estimating socioeconomic inequalities in malaria prevalence. *Tropical Medicine & International Health*. 2008;13:354–64.
283. Ingstad B, Munthali AC, Braathen SH, Grut L. The evil circle of poverty: a qualitative study of malaria and disability. *Malaria Journal*. 2012;11:15.
284. Fornace KM, Alexander N, Abidin TR, Brock PM, Chua TH, Vythilingam I, et al. Local human movement patterns and land use impact exposure to zoonotic malaria in Malaysian Borneo. *eLife*. 2019;8:e47602.
285. Uspensky I, Braun S. Cannibalism in mosquito larvae during microbial larvicide potency tests. *Folia Parasitologica*. 2020. <https://doi.org/10.14411/fp.2020.005>.
286. Focks DA. Toxorhynchites as biocontrol agents. *J Am Mosq Control Assoc*. 2007;23 2 Suppl:118–27.
287. Marcombe S, Maithaviphet S, Bobichon J, Phommavan N, Nambanya S, Corbel V, et al. New insights into malaria vector bionomics in Lao PDR: a nationwide entomology survey. *Malaria Journal*. 2020;19:396.
288. Nguyen TQ, Nguyen MD, Pham VX, Ro HM, Edstein MD, Chow WK, et al. Entomological survey in two communes with residual malaria transmission in Gia Lai Province in the central highlands of Vietnam. *Malaria Journal*. 2021;20:403.
289. Sumarnrote A, Corbel V, Overgaard HJ, Celhay O, Marasri N, Fustec B, et al. Plasmodium Infections in Anopheles Mosquitoes in Ubon Ratchathani Province, Northeastern Thailand During a Malaria Outbreak. *J Am Mosq Control Assoc*. 2018;34:11–7.
290. Jacob CG, Thuy-Nhien N, Mayxay M, Maude RJ, Quang HH, Hongvanthong B, et al. Genetic surveillance in the Greater Mekong subregion and South Asia to support malaria control and elimination. *eLife*. 2021;10:e62997.
291. Hamilton WL, Amato R, Pluijm RW van der, Jacob CG, Quang HH, Thuy-Nhien NT, et al. Evolution and expansion of multidrug-resistant malaria in southeast Asia: a genomic epidemiology study. *The Lancet Infectious Diseases*. 2019;19:943–51.

292. Li Y, Shetty AC, Lon C, Spring M, Saunders DL, Fukuda MM, et al. Detecting geospatial patterns of Plasmodium falciparum parasite migration in Cambodia using optimized estimated effective migration surfaces. *International Journal of Health Geographics*. 2020;19:13.
293. Imwong M, Dhorda M, Myo Tun K, Thu AM, Phyo AP, Proux S, et al. Molecular epidemiology of resistance to antimalarial drugs in the Greater Mekong subregion: an observational study. *The Lancet Infectious Diseases*. 2020. [https://doi.org/10.1016/S1473-3099\(20\)30228-0](https://doi.org/10.1016/S1473-3099(20)30228-0).
294. Gruenberg M, Lerch A, Beck H-P, Felger I. Amplicon deep sequencing improves Plasmodium falciparum genotyping in clinical trials of antimalarial drugs. *Sci Rep*. 2019;9:17790.
295. Barry AE, Waltmann A, Koepfli C, Barnadas C, Mueller I. Uncovering the transmission dynamics of Plasmodium vivax using population genetics. *Pathog Glob Health*. 2015;109:142–52.
296. Messerli C, Hofmann NE, Beck H-P, Felger I. Critical Evaluation of Molecular Monitoring in Malaria Drug Efficacy Trials and Pitfalls of Length-Polymorphic Markers. *Antimicrob Agents Chemother*. 2017;61:e01500-16.
297. Jones S, Kay K, Hodel EM, Gruenberg M, Lerch A, Felger I, et al. Should Deep-Sequenced Amplicons Become the New Gold Standard for Analyzing Malaria Drug Clinical Trials? *Antimicrobial Agents and Chemotherapy*. 2021;65:e00437-21.
298. Miller RH, Hathaway NJ, Kharabora O, Mwandagalirwa K, Tshetu A, Meshnick SR, et al. A deep sequencing approach to estimate Plasmodium falciparum complexity of infection (COI) and explore apical membrane antigen 1 diversity. *Malaria Journal*. 2017;16:490.
299. Talundzic E, Ravishankar S, Kelley J, Patel D, Plucinski M, Schmedes S, et al. Next-Generation Sequencing and Bioinformatics Protocol for Malaria Drug Resistance Marker Surveillance. *Antimicrobial Agents and Chemotherapy*. 2018;62:e02474-17.
300. Neafsey DE, Juraska M, Bedford T, Benkeser D, Valim C, Griggs A, et al. Genetic Diversity and Protective Efficacy of the RTS,S/AS01 Malaria Vaccine. *N Engl J Med*. 2015;373:2025–37.
301. Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, et al. Global trends in emerging infectious diseases. *Nature*. 2008;451:990–3.
302. Pallen MJ. Reply to Updating benchtop sequencing performance comparison. *Nat Biotechnol*. 2013;31:296.
303. R Core Team. R: A language and environment for statistical computing. www.R-project.org. 2022.
304. Hartig F. DHARMA: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models. 2021.

305. Rosado J, Ruybal-Pesántez, S, Han J, Munro J, Bourke C, White M, et al. A novel amplicon deep sequencing tool for studying *Plasmodium vivax* infections at the clonal level. manuscript in preparation.
306. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods*. 2016;13:581–3.
307. Fisher RG, Smith DM, Murrell B, Slabbert R, Kirby BM, Edson C, et al. Next generation sequencing improves detection of drug resistance mutations in infants after PMTCT failure. *Journal of Clinical Virology*. 2015;62:48–53.
308. Kwon S, Park S, Lee B, Yoon S. In-depth analysis of interrelation between quality scores and real errors in Illumina reads. *Annu Int Conf IEEE Eng Med Biol Soc*. 2013;2013:635–8.
309. McElhoe JA, Holland MM, Makova KD, Su MS-W, Paul IM, Baker CH, et al. Development and assessment of an optimized next-generation DNA sequencing approach for the mtgenome using the Illumina MiSeq. *Forensic Science International: Genetics*. 2014;13:20–9.
310. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3--new capabilities and interfaces. *Nucleic Acids Res*. 2012;40:e115.
311. Tobler WR. A Computer Movie Simulating Urban Growth in the Detroit Region. *Econ Geogr*. 1970;46:234–40.
312. Piantadosi S, Byar DP, Green SB. The ecological fallacy. *Am J Epidemiol*. 1988;127:893–904.
313. Hahn MB, Gangnon RE, Barcellos C, Asner GP, Patz JA. Influence of Deforestation, Logging, and Fire on Malaria in the Brazilian Amazon. *PLOS ONE*. 2014;9:e85725.
314. Olson SH, Gangnon R, Silveira GA, Patz JA. Deforestation and malaria in Mâncio Lima County, Brazil. *Emerging Infect Dis*. 2010;16:1108–15.

Appendices

Appendix 1: REDCap form used to describe breeding sites. For every round of collection, we created new entries for each breeding sites rather than doing a follow-up. Indeed, breeding sites would change over time and not be recognisable, or they would dry up and disappear. In addition, many new breeding sites had appeared between the first round of collection (October-November 2019) and third round (July-August 2020).

Breeding Sites Description

Breeding site ID: _____

This record must be completed for every breeding site during larvae collection.

IDENTIFICATION

1) Latitude: _____

2) Longitude: _____

3) Date and time: _____

4) Can you describe the breeding site? No Yes Not attainable

5) Name of the person collecting this data:

- Anais
- Bros
- Chantrea
- Koeun
- Sokchan
- Amelie
- Other

5) If other, please name them: _____

6) Collectors names:

- Anais
- Bros
- Chantrea
- Koeun
- Sokchan
- Amelie
- Other

6) If other, please name them: _____

CONDITIONS

7) Water temperature (°C): _____

(If NA, leave blank)

8) Air temperature (°C): _____

(If NA, leave blank)

9) Relative humidity (%): _____

(If NA, leave blank)

10) Wind: None Little Medium
 High

11) Weather: Sunny Cloudy Rainy

HABITAT

12) Dimensions of the breeding site in cm:

_____ (Example: 50*30)

13) Habitat class: Stagnant temporary water bodies
 Running water
 (Semi)-permanent natural water bodies
 Natural containers
 Artificial containers
 Other

14) a) Habitat description: Puddles and ditches
 Road tracks
 Ponds
 Marshes
 Rice paddies
 Other

14) a) If other, please describe: _____

14) b) Habitat description: Rivers
 Streams
 Ditches and trenches
 Drains and canals
 Other

14) b) If other, please describe: _____

14) b) Was there current where you collected the larvae? No Yes

14) c) Habitat description: Ponds
 Marshes
 Other

14) c) If other, please describe: _____

14) d) Habitat description: Rock pools
 Tree holes
 Bamboo holes
 Coconut shells
 Leaves dropped on the floor
 Plants' axils and leaves
 Other

14) d) If other, please describe: _____

14) e) Habitat description:

- Pots
 Basins and pits
 Water reservoir
 Cans
 Bottle
 Plastic or rubbish
 Other

14) e) If it is a water reservoir, please precise which kind:

- Clay Plastic Metal
 Other

14) e) If other kind of reservoir, please describe:

14) e) If it is a bottle, please describe which material:

- Plastic
 Glass
 Other

14) e) If other, please describe:

14) e) If other, please describe:

14) f) If other habitat class, please describe:

15) Is there a (partial) layer of organic or mineral matter on habitat's bottom?

- No Yes

15) Which kind?

- Leaves
 Mud
 Gravel or rocks
 Other

15) If other, please describe:

16) Depth:

- < = 10 cm > 10 cm

17) Edges:

- Shallow Abrupt

18) Water turbidity:

- None Little Medium
 High

19) Pollution:

- No Yes

19) If yes, please describe:

- Plastic or other rubbish
 Debris
 Oil
 Animal faeces
 Other

19) If other, please describe:

20) Water colour:

21) Shading:

None Partial Complete

22) Dominant land use class(es) around the site:

- Village
 Fields
 Plantations
 Forest

22) Fields of which kind?

- Cassava
 Rice
 Vacant or fallow
 Other

22) If other kind of fields, please precise:

22) Plantations of which kind?

- Cashew nut trees
 Rubber trees
 Banana trees
 Other

22) If other kind of plantations, please precise:

FLORA

23) Presence of vegetation in the breeding site:

No Yes

23) Quantity of vegetation in the breeding site (% of surface):

- < 5%
 5-25%
 25-50%
 50-75%
 75-100%

23) Vegetation type within the site:

- Submerged
 Emergent
 Floating
 Algae
 Other

23) If other, please describe:

FAUNA

24) Presence of mosquito larvae: No Yes

24) Encountered mosquito larvae:

- Anopheles
- Aedes
- Culex
- Not identified
- Other

24) If other, please describe:

25) Presence of predators: No Yes Not determined

25) Encountered predators:

- Coleoptera
- Hemiptera
- Odonata
- Crustacean
- Mosquito larvae
- Fish
- Other

25) a) Which Coleoptera?

- Dysticidae
- Hydrophilidae
- Not identified
- Other

25) a) If other Coleoptera, please describe:

25) b) Which Hemiptera?

- Notonectae
- Corixidae
- Nepidae
- Not identified
- Other

25) b) If other Hemiptera, please describe:

25) c) Which Odonata?

- Dragonflies
- Damselflies
- Not identified
- Other

25) c) If other Odonata, please describe:

25) d) Which Crustacean?

- Cyclopoids
- Not identified
- Other

25) d) If other Crustacean, please describe:

25) e) Which mosquito larvae?

- Toxorynchites
- Not identified
- Other

25) e) If other mosquito larvae, please describe:

25) f) If other type of predators, please describe:

26) Presence of other animals:

No Yes

26) Which other animals?

Amphibian
 Domestic animals
 Other

26) If other, please describe:

27) Is there enough water for taking at least one dip?

No Yes

27) Number of dips:

_____ (Up to 20)

28) Presence of visible people:

No Yes

28) How many of them?

< = 10 people > 10 people

29) Remarks

PICTURES

30) Photo 1:

(Breeding site and surroundings)

31) Photo 2:

(Breeding site and surroundings)

32) Photo 3:

(Breeding site only)

33) In case of a very wide breeding site, photo 4:

(Where the larvae were sampled within the breeding site)

VERIFICATION

All boxes, containers and samples are correctly identified?

No Yes

Nothing to describe then!

Potential remarks: