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# Potential of the mosquito *Aedes malayensis* as an arbovirus vector in South East Asia

Elliott Miot

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# Sorbonne Université

École doctorale 515 : “Complexité du Vivant”

*Medical Entomology and Vector-Borne Disease Unit, Institut Pasteur du Laos*

*Unité Interactions Virus-Insectes, Institut Pasteur, CNRS UMR 2000, Paris*

## **Potential of the mosquito *Aedes malayensis* as an arbovirus vector in South East Asia**

Par

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Thèse de Doctorat en Entomologie Médicale

Dirigée par Louis Lambrechts & Paul Brey

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*“Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make,  
because they lead little by little to the truth.”*

Jules Verne, A Journey to the Center of the Earth

*“La science, mon garçon, est faite d’erreurs, mais d’erreurs qu’il est bon de commettre, car  
elles mènent peu à peu à la vérité.”*

Jules Verne, Voyage au Centre de la Terre



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## Abstract

Many emerging arthropod-borne viruses (arboviruses) of humans such as dengue, Zika, yellow fever and chikungunya viruses originated in sylvatic cycles, where they circulate between non-human animals and forest-dwelling mosquitoes. Over the last few centuries, these arboviruses have emerged into sustained transmission cycles among humans, causing substantial mortality and morbidity. Dengue virus (DENV) has even become endemic in the human population and is currently responsible for close to 400 million infections each year. The initial mechanism of arbovirus emergence typically involves spillover transmission by mosquito species that ‘bridge’ sylvatic and human transmission cycles. These bridge vectors can also mediate ‘spillback’ transmission of human arboviruses establishing novel sylvatic cycles, as exemplified by yellow fever virus (YFV) in South America. Understanding and preventing arbovirus emergence requires detailed entomological knowledge at the interface between human populations and the sylvatic environment.

This PhD work focused on *Aedes malayensis*, a sylvatic mosquito species widely distributed in South East Asia, which was recently detected in peridomestic habitats in Singapore. We used a combination of field surveys and laboratory experiments to assess the potential of *Ae. malayensis* as an arbovirus vector in South East Asia. The PhD thesis is divided in four chapters.

The first chapter reports on mosquito larval and adult surveys conducted in a forested area of the Nakai Nam Theun National Protected Area (NNT NPA), Khammuane Province, Laos. We identified 54 mosquito taxa belonging to 11 genera, of which 16 species were new records in Laos, including *Ae. malayensis*. The second chapter deals with the host-seeking behavior of sylvatic mosquitoes in the NNT NPA. We used human-baited traps to identify several sylvatic mosquito species, including *Ae. malayensis*, that may engage in human-biting behavior and therefore act as bridge vectors. The third chapter addresses the potential of *Ae. malayensis* to act as an arbovirus bridge vector in forested area of the NNT NPA. Using both vector competence assays and olfactometer behavioral experiments, we found a relatively modest vector competence of sylvatic *Ae. malayensis* for DENV and YFV and a lack of detectable attraction to human odor in laboratory conditions. The fourth chapter assessed the ability of a peridomestic *Ae. malayensis* population in Singapore to transmit YFV based on vector competence experiments and a human-baited trap survey. Not only was this peridomestic *Ae. malayensis* population able to experimentally acquire and transmit YFV, but it was also found to engage in contact with humans in a field situation.

Together, this work showed that whereas a sylvatic *Ae. malayensis* population in Laos did not display a high potential as a bridge vector, its peridomestic counterpart in Singapore is a capable arbovirus vector. The wide distribution of the species and its ability to colonize urban settings such as high-rise building areas in Singapore calls for increased vigilance. More generally, we conclude that ancillary vectors should not be overlooked, and that basic entomological knowledge is essential to fully assess the risk of arbovirus emergence.

### Résumé

De nombreux virus transmis par les arthropodes, ou arbovirus (“arthropod-borne virus”), émergeant chez l’Homme, tels que les virus de la dengue, de Zika, de la fièvre jaune et du chikungunya, circulaient à l’origine entre animaux non-humains et moustiques forestiers dans des cycles selvatiques. Au cours des derniers siècles, ces arbovirus ont émergé chez l’Homme dans des cycles de transmission continue, causant une mortalité et une morbidité importantes. Le virus de la dengue (DENV) est même devenu endémique dans la population humaine et il est actuellement responsable de près de 400 millions d’infections chaque année. Le mécanisme initial de l’émergence des arbovirus implique typiquement un transfert, dit « spillover », par des espèces de moustiques « bridge vectors » qui font le lien entre cycles de transmission selvatiques et humains. Ces bridge vectors peuvent aussi permettre un transfert inverse, dit « spillback », des arbovirus humains établissant des nouveaux cycles selvatiques, comme ce fût le cas du virus de la fièvre jaune (YFV) en Amérique du Sud. Comprendre et prévenir l’émergence des arbovirus nécessitent des connaissances entomologiques détaillées à l’interface entre les populations humaines et l’environnement forestier.

Ce travail de doctorat s’est focalisé sur *Aedes malayensis*, un moustique selvatique largement répandu en Asie du sud-est, qui a récemment été détecté dans des habitats péri-domestiques à Singapour. Nous avons utilisé une combinaison d’enquêtes de terrain et d’expériences en laboratoire pour évaluer le potentiel d’*Ae. malayensis* comme vecteur d’arbovirus en Asie du sud-est. La thèse doctorale se divise en quatre chapitres.

Le premier chapitre décrit les recensements de moustiques aux stades larvaires et adultes, conduits dans l’écosystème forestier de la réserve nationale protégée de Nakai Nam Theun (NNT NPA), dans la province de Khammuane au Laos. Nous avons identifié 54 taxons de moustiques appartenant à 11 genres, parmi lesquels 16 espèces n’ayant jamais été décrites au Laos, notamment *Ae. malayensis*. Le second chapitre porte sur le comportement de recherche d’hôte des moustiques selvatiques dans la NNT NPA. Nous avons utilisé des pièges à appâts humains pour identifier plusieurs espèces de moustiques selvatiques, dont *Ae. malayensis*, qui pourraient être amenées à piquer l’Homme et donc agir comme bridge vectors. Le troisième chapitre discute du potentiel d’*Ae. malayensis* à jouer le rôle de bridge vector dans la région forestière du district de Nakai au Laos. En se basant à la fois sur des tests de compétence vectorielle et des expériences comportementales d’olfactométrie, nous avons observé une compétence relativement modeste d’une population selvatique d’*Ae. malayensis* pour le DENV et le YFV, et une absence d’attraction détectable pour l’odeur

humaine en conditions expérimentales. Le quatrième chapitre évalue la capacité d'une population péri-domestique d'*Ae. mayalensis* singapourienne à transmettre le YFV, à partir d'expériences de compétence vectorielle et de l'évaluation de leur attraction pour l'Homme sur le terrain. Non seulement cette population péri-domestique a été capable d'acquérir et de transmettre le YFV expérimentalement, mais elle a aussi été retrouvée en contact avec les humains sur le terrain.

L'ensemble de ce travail indique que, bien qu'une population selvatique de *Ae. malayensis* au Laos ne possède pas un fort potentiel en tant que bridge vector, son équivalent péri-domestique à Singapour est un vecteur compétent d'arbovirus. La vaste distribution de l'espèce et sa capacité à coloniser des zones urbaines, tels que des zones de gratte-ciels à Singapour appelle à une vigilance accrue. Plus généralement, nous concluons que les vecteurs secondaires ne doivent pas être négligés, et que des connaissances entomologiques de base sont essentielles pour évaluer complètement le risque d'émergence des arbovirus.

## Table of contents

<b>ACKNOWLEDGMENTS / REMERCIEMENTS .....</b>	<b>V</b>
<b>ABSTRACT .....</b>	<b>XII</b>
<b>RESUME.....</b>	<b>XIV</b>
<b>LIST OF FIGURES.....</b>	<b>XVIII</b>
<b>LIST OF ABBREVIATIONS.....</b>	<b>XIX</b>
<b>PART 1: LITERATURE REVIEW .....</b>	<b>1</b>
GENERAL INTRODUCTION .....	3
ARBOVIRUSES.....	7
<i>Definition</i> .....	7
<i>Arbovirus taxonomy</i> .....	7
<i>Major emerging arboviruses</i> .....	7
<i>Origin and emergence of arboviruses</i> .....	10
Historical records of arbovirus emergence.....	10
Arbovirus transmission cycles .....	13
Role of vectors in arbovirus emergence .....	15
Bridge vectors .....	15
Main domestic vectors of arboviruses worldwide.....	15
Ancillary vectors.....	19
Factors associated with arbovirus emergence .....	19
MOSQUITO VECTORS.....	25
<i>Mosquito taxonomy</i> .....	25
<i>Basic mosquito morphology</i> .....	27
<i>Mosquito bionomics</i> .....	31
Mosquito life cycle.....	31
Host-seeking behavior and dispersal.....	35
<i>Arbovirus transmission by mosquitoes</i> .....	37
Vectorial capacity.....	37
Vector competence.....	39
Arbovirus propagation within the mosquito vector.....	41
<b>PART 2: EXPERIMENTAL WORK.....</b>	<b>43</b>
DESCRIPTION OF THE FIELD SITES .....	45
<i>Field sites in Laos</i> .....	45
<i>Field sites in Singapore</i> .....	49
CHAPTER 1: SURVEY OF THE SYLVATIC MOSQUITO FAUNA IN A FORESTED AREA OF THE NAKAI NAM THEUN NATIONAL PROTECTED AREA.....	51
<i>Summary</i> .....	53
<i>Article 1: New records and updated checklist of mosquitoes (Diptera: Culicidae) from Lao People's Democratic Republic, with special emphasis on adult and larval surveillance in Khammuane Province..</i>	55
<i>Article 2: First Record of Aedes (Stegomyia) malayensis Colless (Diptera: Culicidae) in the Lao People's Democratic Republic, Based on Morphological Diagnosis and Molecular Analysis</i> .....	69
CHAPTER 2: IDENTIFICATION OF SYLVATIC MOSQUITO SPECIES ATTRACTED TO HUMANS IN A FORESTED AREA OF THE NAKAI NAM THEUN NATIONAL PROTECTED AREA .....	77
<i>Summary</i> .....	79
<i>Article 3: Diversity, abundance and daily activity pattern of human-seeking mosquitoes in a forested area of the Nakai district, Laos</i> .....	81
CHAPTER 3: POTENTIAL OF THE SYLVATIC MOSQUITO <i>Aedes malayensis</i> TO ACT AS AN ARBOVIRUS BRIDGE VECTOR IN FORESTED AREA OF THE NAKAI NAM THEUN NATIONAL PROTECTED AREA.....	101
<i>Summary</i> .....	103
<i>Article 4: Potential of the sylvatic mosquito Aedes malayensis to act as an arbovirus bridge vector in forested area of the Nakai district, Laos</i> .....	105
CHAPTER 4: POTENTIAL OF PERIDOMESTIC <i>Aedes malayensis</i> MOSQUITOES TO TRANSMIT YELLOW FEVER VIRUS IN SINGAPORE.....	131

<i>Summary</i> .....	133
<i>Article 5: A peridomestic Aedes malayensis population in Singapore can transmit yellow fever virus</i> .....	135
GENERAL DISCUSSION AND PERSPECTIVES .....	147
CONCLUSIONS .....	154
REFERENCES .....	155
APPENDIX 1 .....	170
OTHER SCIENTIFIC WORK NOT INCLUDED IN THE THESIS .....	171

## List of figures

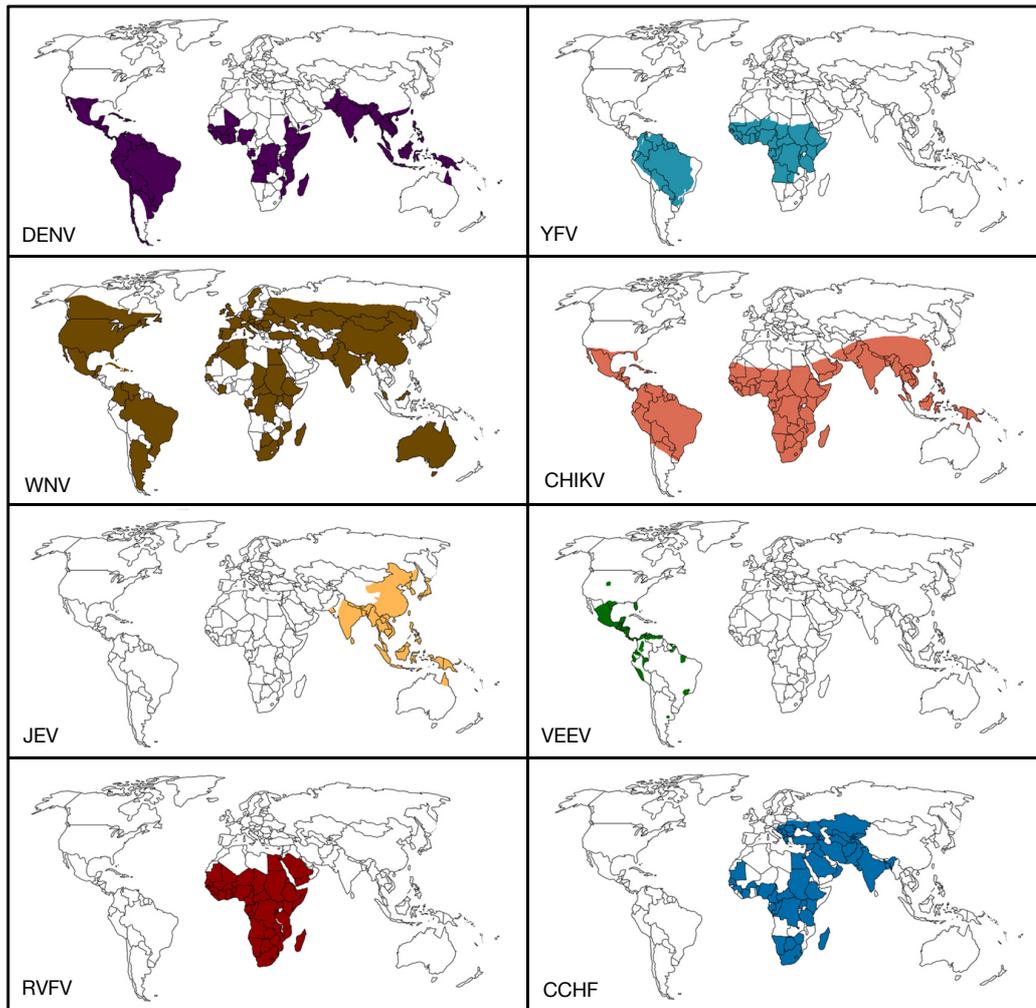
<b>FIGURE 1: GLOBAL DISTRIBUTION OF SOME OF THE MOST IMPORTANT ARBOVIRUSES OF HUMAN MEDICAL IMPORTANCE IN 2015</b> .....	2
<b>FIGURE 2: GLOBAL DISTRIBUTION OF RELATIVE RISK OF EMERGING INFECTIOUS DISEASES CAUSED BY VECTOR-BORNE PATHOGENS</b> .....	2
<b>FIGURE 3: MAJOR ARTHROPOD VECTOR GROUPS WITH EXAMPLES OF ARBOVIRUSES TRANSMITTED</b> .....	6
<b>FIGURE 4: ARBOVIRUS TRANSMISSION CYCLES</b> .....	12
<b>FIGURE 5: WORLDWIDE DISTRIBUTION OF DOCUMENTED, CONTEMPORARY FOCI OF CIRCULATION OF SYLVATIC DENGUE VIRUS AND SYLVATIC YELLOW FEVER VIRUS AND HISTORIC FOCI OF SYLVATIC YELLOW FEVER VIRUS</b> .....	12
<b>FIGURE 6: COMPARISON OF THE MAJOR MOSQUITO VECTORS AND NON-HUMAN PRIMATE HOSTS INVOLVED IN SYLVATIC TRANSMISSION, SPILLOVER AND URBAN TRANSMISSION OF YELLOW FEVER VIRUS AND DENGUE VIRUS</b> .....	14
<b>FIGURE 7: GLOBAL GEOGRAPHICAL DISTRIBUTION OF <i>Aedes aegypti</i></b> .....	14
<b>FIGURE 8: GEOGRAPHICAL DISTRIBUTION OF <i>Aedes aegypti formosus</i> AND <i>Aedes aegypti aegypti</i> WITH RECENT EVENTS OF INTRODUCTION OR CHANGE OF BEHAVIOR</b> .....	16
<b>FIGURE 9: GLOBAL GEOGRAPHICAL DISTRIBUTION OF <i>Aedes albopictus</i></b> .....	16
<b>FIGURE 10: OBSERVED ANNUAL AVERAGE <i>Aedes</i> HOUSEHOLD INDEX AND ANNUAL CLINICAL INCIDENCE OF DENGUE FEVER IN SINGAPORE</b> .....	18
<b>FIGURE 11: URBAN GROWTH IN ASIAN AND AMERICAN CITIES, 1950 – 2010</b> .....	18
<b>FIGURE 12: DIAGRAM SHOWING HOW URBANIZATION SHAPES IMMATURE <i>Aedes</i> MOSQUITO BREEDING SITES AND SPECIES IN SOUTH-EASTERN CÔTE D’IVOIRE</b> .....	20
<b>FIGURE 13: INTERNATIONAL TOURIST ARRIVALS BY REGION</b> .....	20
<b>FIGURE 14: REINFESTATION OF TROPICAL AMERICA BY <i>Aedes aegypti</i>, 1930–2011</b> .....	20
<b>FIGURE 15: THE FREQUENCY OF RESISTANCE TO DELTAMETHRIN IN <i>Aedes aegypti</i>, 2006–2015</b> .....	22
<b>FIGURE 16: MORPHOLOGY OF A CULICINAE FOURTH-INSTAR LARVA</b> .....	26
<b>FIGURE 17: MORPHOLOGY OF A MOSQUITO PUPA</b> .....	26
<b>FIGURE 18: MORPHOLOGY OF AN ADULT FEMALE MOSQUITO</b> .....	26
<b>FIGURE 19: EXAMPLE OF A DICHOTOMOUS MORPHOLOGICAL KEY TO IDENTIFY MOSQUITO SPECIES</b> .....	29
<b>FIGURE 20: MOSQUITO LIFE CYCLE</b> .....	30
<b>FIGURE 21: EXAMPLES OF MOSQUITO LARVAL BREEDING SITES</b> .....	30
<b>FIGURE 22: EXAMPLES OF MOSQUITO EGG-LAYING STRATEGIES</b> .....	32
<b>FIGURE 23: DIAGRAMMATIC REPRESENTATION OF THE GONOTROPHIC CYCLE OF A FEMALE MOSQUITO</b> .....	32
<b>FIGURE 24: SCHEMATIC OF THE MOSQUITO PERIPHERAL OLFACTORY SYSTEM</b> .....	34
<b>FIGURE 25: GENOTYPE-BY-GENOTYPE INTERACTIONS BETWEEN DENGUE VIRUS AND <i>Aedes aegypti</i></b> .....	38
<b>FIGURE 26: EFFECT OF MOSQUITO POPULATION, VIRUS STRAIN AND TEMPERATURE ON THE OVERALL PROPORTION OF FEMALES THAT HAD INFECTIOUS VIRUS IN THEIR SALIVA 6 DAYS AFTER ORAL EXPOSURE OF <i>Aedes albopictus</i> FEMALES TO CHIKUNGUNYA VIRUS</b> .....	38
<b>FIGURE 27: STEPS OF ARBOVIRUS PROPAGATION IN A MOSQUITO FOLLOWING AN INFECTIOUS BLOOD MEAL</b> .....	40
<b>FIGURE 28: MAP OF THE LAO FIELD SITES</b> .....	44
<b>FIGURE 29: PHOTOS OF THE FIELD STATION IN OUDOMSOUK AND THE WAY TO THE STUDY AREAS</b> .....	44
<b>FIGURE 30: PHOTOS OF THE NAM NOY FIELD SITE</b> .....	46
<b>FIGURE 31: PHOTOS OF THE NAM ON FIELD SITE</b> .....	46
<b>FIGURE 32: MAP OF THE FIELD SITES IN SINGAPORE</b> .....	48
<b>FIGURE 33: PHOTOS OF SINGAPORE FIELD SITES</b> .....	48
<b>FIGURE 34: INFLUENCE OF BLOOD SOURCE ON DENGUE VIRUS INFECTION RATE IN <i>Aedes aegypti</i></b> .....	102
<b>FIGURE 35: DISTRIBUTION OF <i>Aedes albopictus</i> AND <i>Aedes malayensis</i> IN URBAN PARKS OF SINGAPORE</b> .....	132
<b>FIGURE 36: DISTRIBUTION OF <i>Aedes malayensis</i> IN HIGH-RISE APARTMENT BLOCKS IN SINGAPORE</b> .....	134

## List of abbreviations

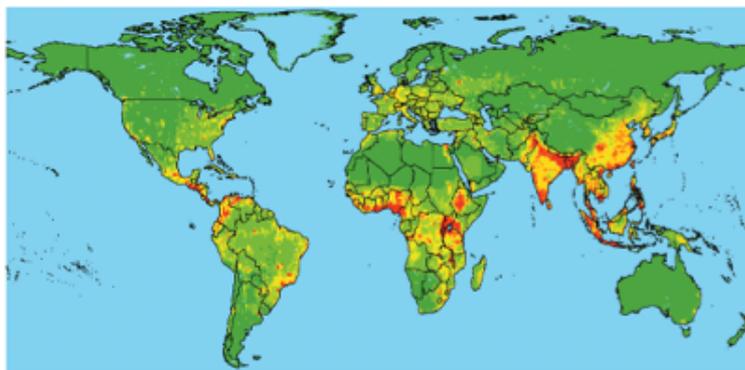
Ant	antennae
BG	Biogents
CCHF	Crimean–Congo hemorrhagic fever
CDC	Centers for Disease Control and prevention
CE	composite eyes
CHIKV	chikungunya virus
CO <sub>2</sub>	carbon dioxide
DENV	dengue virus
EIP	extrinsic incubation period
GR	gustatory receptor
HI	halters
HLC	human-landing catch
IATA	international air transportation authority
ICTV	international committee on taxonomy of viruses
IR	ionotropic receptor
JEV	Japanese encephalitis virus
MEB	midgut escape barrier
MIB	midgut infection barrier
MP1p	maxillary palpus
NEA	National Environment Agency
NNT	Nakai Nam Theun
NPA	national protected area
OBP	odorant-binding protein
OR	odorant receptor
P	proboscis
PFU	plaque-forming unit
RVFV	Rift valley fever virus
SGEB	salivary gland escape barrier
SGIB	salivary gland infection barrier
TBEV	tick-borne encephalitis virus
UNWTO	world tourism organization
VEEV	Venezuelan equine encephalitis virus
VT	vertical transmission
WHO	world health organization
WMPA	watershed management and protection authority
WNV	West Nile virus
YFV	yellow fever virus
ZIKV	Zika virus



# Part 1: literature review



**Figure 1: Global distribution of some of the most important arboviruses of medical importance in 2015.** Dengue virus (DENV); yellow fever virus (YFV); West Nile virus (WNV); chikungunya virus (CHIKV); Japanese encephalitis virus (JEV); Venezuelan equine encephalitis virus (VEEV); Rift valley fever virus (RVFV); Crimean–Congo hemorrhagic fever (CCHF) virus. Reproduced from (Iranpour et al. 2016).



**Figure 2: Global distribution of relative risk of emerging infectious diseases caused by vector-borne pathogens.** The relative risk is calculated from regression coefficients and variable values, categorized by standard deviations from the mean and mapped on a linear scale from green (lower values) to red (higher values). Reproduced from (Jones et al. 2008).

## General introduction

Arthropod-borne viruses (arboviruses) currently represent a major global health concern causing substantial mortality and morbidity around the world (Figure 1). They are a diverse group of viruses transmitted by hematophagous arthropods, usually referred to as vectors. For instance, dengue virus (DENV) is present in at least 128 countries and is estimated to cause around 390 million human infections per year, including 96 million symptomatic cases (Bhatt et al. 2013). Every year, yellow fever virus (YFV) is responsible for 200,000 cases and 30,000 deaths and it is endemic in 47 African and South American countries (World Health Organization (WHO) 2019c). The past few decades have seen a growing number of arbovirus-related outbreaks and epidemics with, for example, the introduction of West Nile virus (WNV) into North America in 1999 followed by large outbreaks, and the two global pandemics caused by chikungunya virus (CHIKV) and Zika virus (ZIKV) (Weaver and Reisen 2010; Gould et al. 2017).

Prevention and therapeutic strategies against most arboviruses are very limited. There are still no effective antiviral drugs available and most of the treatments are anti-symptomatic. Some vaccines have been developed and licensed, such as for YFV (Stamaril<sup>®</sup>), Japanese encephalitis virus (JEV; Ixiaro<sup>®</sup>), or more recently DENV (Dengvaxia<sup>®</sup>), with variable efficacy (Staples et al. 2015; Fischer et al. 2010; da Silveira et al. 2019). In addition to vaccination with only a limited arsenal of arboviral vaccines, controlling mosquito vector populations is still the primary prevention method against arboviruses (Achee et al. 2019; Flores and O'Neill 2018).

Many human-pathogenic arboviruses originated in tropical forests where they circulate between vertebrate animals and forest-dwelling mosquitoes in sylvatic transmission cycles. Over the last few centuries, some of these arboviruses have emerged into sustained transmission cycles among humans (Vasilakis et al. 2011). The transition between sylvatic and human transmission cycles typically involves “bridge vectors”, which are mosquito species feeding on both wild animals and humans that have the ability to transmit arboviruses.

South East Asia is one of the regions that are most at risk for the emergence of vector-borne disease based on socio-economic, environmental, and ecological correlations (Jones et al. 2008) (Figure 2). The mosquito fauna of many areas of South East Asia is still poorly characterized, especially in remote locations at the interface between human populations and potential sylvatic transmission cycles of arboviruses. This is the case of Laos where only recently the work of Rueda et al. (2015) established a first comprehensive checklist of

mosquito species present in the country based on the literature and limited field collections. Moreover, while the ability of some mosquito species to transmit pathogens has been extensively studied in some areas of South East Asia (e.g. Thailand, Vietnam, and Cambodia), it has not been investigated in other areas and for other species than the most common urban mosquito species (Souza-Neto et al. 2019). Overall, there is a lack of detailed entomological investigations in areas of South East Asia where the risk of arbovirus emergence is high.

In this context, this PhD work focused on the mosquito *Aedes malayensis* and its potential as an arbovirus vector in sylvatic and peridomestic settings of South East Asia. The mosquito *Ae. malayensis* is widely distributed in South East Asia with records in Thailand, Cambodia, Vietnam, Peninsular Malaysia, the Andaman and Nicobar Islands, and Taiwan (Huang 1972; Tewari et al. 1995; Rattanarithikul et al. 2010). A strain of *Ae. malayensis* from Bangkok, Thailand was found to be experimentally susceptible to DENV (Rosen et al. 1985). It was also recently identified in urban parks of Singapore as a putative vector of DENV and CHIKV (Mendenhall et al. 2017).

The PhD thesis is divided into two main parts. The first part is a brief literature review that presents the background of the work and is divided into two sections: (i) generalities on arboviruses, their transmission cycles, and emergence mechanisms, and (ii) mosquito vectors and vectorial transmission. The second part of the thesis describes the experimental work and is structured in four chapters.

The first chapter reports on field surveys that we initially conducted to better characterize the sylvatic mosquito fauna in a forested area of Laos. We performed several larval and adult collections in the Nakai Nam Theun National Protected Area (NNT NPA), Nakai district, Khammuane province. Following these initial surveys, we attempted to colonize some of the most relevant mosquito species in the laboratory, and successfully did so for *Ae. malayensis*. This work resulted in two research articles published in the *Journal of Vector Ecology* and the *US Army Medical Department Journal*.

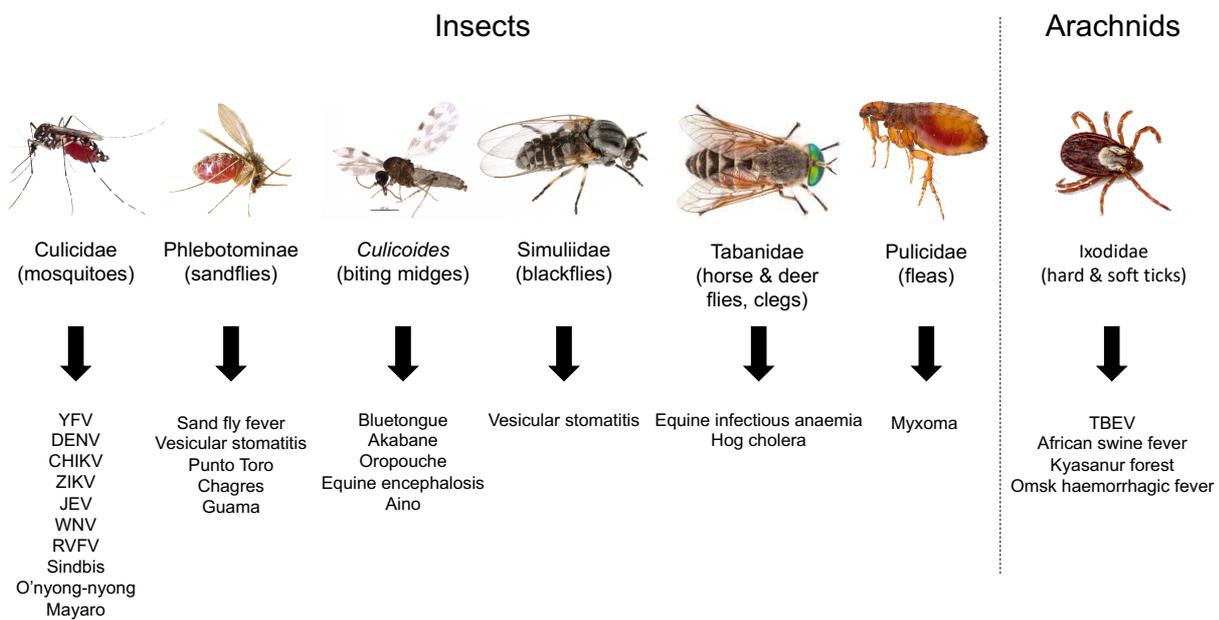
The second chapter describes investigations on the host-seeking behavior of sylvatic mosquitoes, including *Ae. malayensis*, in the NNT NPA. To identify potential bridge vectors, we used human-baited double-net traps (Tangena et al. 2015) to catch sylvatic mosquito species attracted to humans. The results from this work constitute a manuscript in preparation for publication.

The third chapter presents the laboratory assessment of *Ae. malayensis* potential to act as an arbovirus bridge vector in Laos. We experimentally evaluated the vector competence of

our sylvatic *Ae. malayensis* population for DENV and YFV. We also performed olfactometer behavioral bioassays to evaluate its attraction to human sent. A research article presenting the results of this work is under review for publication in *Parasites & Vectors*.

The fourth chapter describes a study conducted on a peridomestic population of *Ae. malayensis* in Singapore. We evaluated the vector competence of this peridomestic *Ae. malayensis* population for YFV and conducted field surveys in the urban parks of Singapore to assess the likelihood of host-vector contact. The results of this work were recently published as a research article in *PLoS Neglected Tropical Diseases*.

The thesis ends with a general discussion of the results and their perspectives for future research.



**Figure 3: Major arthropod vector groups with examples of arboviruses transmitted.** Yellow fever virus (YFV); dengue virus (DENV); chikungunya virus (CHIKV); Zika virus (ZIKV); Japanese encephalitis virus (JEV); West Nile virus (WNV); Rift valley fever virus (RVFV); tick-borne encephalitis virus (TBEV). Adapted from (Mellor 2000).

## Arboviruses

### Definition

Arboviruses consist of a polyphyletic group of viruses that share the ability of being transmitted between a vertebrate host and a hematophagous arthropod vector. A wide range of arthropods are involved in the transmission of arboviruses. They include insects such as mosquitoes, phlebotomine sandflies, and biting midges of the genus *Culicoides* but also arachnids such as ticks (Figure 3). Mosquitoes are the most frequent vectors of arboviruses.

### Arbovirus taxonomy

As of today, 537 arboviruses have been described (Centers for Disease Control and Prevention 2019) with at least 134 causing illness in humans (Gubler 2002). All arboviruses are RNA viruses with the exception of the DNA virus African swine fever virus, the only representative of the *Asfivirus* (Family: *Asfarviridae*) genus. Almost all arboviruses belong to eight viral families: *Flaviviridae*, *Togaviridae*, *Nairoviridae*, *Peribunyaviridae*, *Phenuiviridae*, *Orthomyxoviridae*, *Reoviridae*, and *Rhabdoviridae* (Weaver and Reisen 2010; International Committee on Taxonomy of Viruses (ICTV) 2019). The most important arboviruses for public health belong to the five first families, particularly viruses from the *Flavivirus* (e.g. YFV and DENV), *Alphavirus* (e.g. CHIKV), *Orthonairovirus* (e.g. Crimean-Congo hemorrhagic fever virus), *Orthobunyavirus* (e.g. Tahyna virus), and *Phlebovirus* (e.g. Rift Valley fever virus) genera (Weaver and Reisen 2010; ICTV 2019).

### Major emerging arboviruses

Some of the most important arboviruses infecting humans are *Aedes*-borne flaviviruses such as DENV, YFV, and ZIKV. DENV is the most prevalent human arbovirus worldwide and it is primarily transmitted by the mosquito *Aedes aegypti* (Lambrechts et al. 2010; Brady and Hay 2019). DENV comprises four genetically distinct clades (DENV-1, -2, -3 and -4) that have each emerged from a non-human primate reservoir in four sustained transmission chains in humans (Vasilakis et al. 2011). The four DENV types are often referred to as serotypes because infection with one serotype confers lifelong immunity to that serotype. Infection with a heterotypic DENV serotype is a major risk factor for developing severe dengue due to the mechanism of antibody-dependent enhancement (Katzelnick et al. 2017; Salje et al. 2018). However, it has been shown that within-serotype antigenic variation is almost as broad as between-serotype variation, suggesting a more complex genetic and immunological organization of DENV (Katzelnick et al. 2015). The emergence of dengue hyperendemicity

associated with periodic epidemics is largely due to the urbanization of tropical regions that enhanced the conditions for *Ae. aegypti* development and therefore the efficiency of interhuman transmission, associated with a failure in vector control (Gubler et al. 2014). This is especially true in poor areas where water is stored indoors and the lack of trash disposal results in the accumulation of containers and used tires filled with rainwater that act as breeding sites (Weaver and Reisen 2010).

YFV is a long-known arbovirus against which a very effective vaccine was developed more than 70 years ago (Monath and Vasconcelos 2015). However, several yellow fever outbreaks have recently occurred in Africa (Angola, Democratic Republic of the Congo, Uganda, and Nigeria) and in South America (Brazil) (Grobbelaar et al. 2016; WHO 2016c; WHO 2016b; WHO 2019a; WHO 2019b). The 2016-2017 YFV outbreak in Brazil resulted from infections acquired in forested areas, indicating virus spillover from susceptible wild primates, that expanded to previously YFV-free areas (Faria et al. 2018; Moreira-Soto et al. 2018). During these outbreaks, increasing numbers of unvaccinated travelers who become infected and return to non-endemic countries have raised the risk of YFV introduction to unprecedented levels, especially in the historically YFV-free Asia-Pacific region (Gubler 2018).

ZIKV was first isolated in 1947 in the Zika forest of Uganda (Dick et al. 1952) and did not represent a public concern for 60 years until the 2007 outbreak in Yap island, Federated States of Micronesia where more than 73% of the population were infected (Duffy et al. 2009). It was followed by several large-scale epidemics in French Polynesia (2013) and other Pacific islands (2014). This was a turning point for the disease with the appearance of previously unknown symptoms such as congenital ZIKV syndromes, Guillain-Barré syndromes, and non-vectorial transmission (e.g. sexual, vertical) (Musso and Gubler 2016). From then, it reached South America and rapidly spread across the continent with autochthonous circulation in 2016 in more than 20 countries in South, Central, and North America and the Caribbean (Musso and Gubler 2016). The scale of the pandemic prompted the World Health Organization (WHO) to consider ZIKV emergence a “Public Health Emergency of International Concern” (Gould et al. 2017).

Another *Aedes*-associated arbovirus that emerged globally in the last decades is the alphavirus CHIKV. The virus was first isolated in Tanzania in 1953 (Robinson 1955). For a long time, chikungunya was not regarded as an important arboviral disease and often misdiagnosed with dengue (Gould et al. 2017). CHIKV is now recognized as an important and dangerous arbovirus following two major epidemics. First, in 2004 in Kenya where it rapidly

spread to islands of the Indian Ocean such as La Réunion where 244,000 cases and an attack rate of 35% were reported in 2005-2006 (Renault et al. 2007). A second epidemic occurred in 2006 with the introduction of CHIKV to India, which resulted in millions of cases (Weaver and Reisen 2010). Both epidemics presented a higher death rate than previous ones and resulted in the introduction of the virus through travelers to other parts of the world such as Italy (Rezza et al. 2007) and France (Grandadam et al. 2011). Interestingly, the Indian Ocean epidemics were associated with adaptive evolution of the virus, through a single amino-acid substitution in the envelope protein, which increased its transmissibility by *Aedes albopictus* mosquitoes (Schuffenecker et al. 2006; Vazeille et al. 2007). However, it is difficult to determine if this adaptation was a cause or a consequence of these epidemics.

Besides *Aedes* mosquitoes, *Culex* mosquitoes have also been incriminated in the emergence of arboviruses such as WNV, which is only second to DENV in terms of geographical distribution (Gould et al. 2017). WNV was well known in the Old World but was not responsible for severe cases in humans, with no report of human or avian deaths (Gould et al. 2017). In 1999, WNV was introduced into New York, from where it spread rapidly and extensively across the United States of America and neighboring countries, resulting in a high number of deaths in birds, horses, and humans, together with the appearance of WNV-associated encephalitis (Lanciotti et al. 1999; Briese et al. 1999).

### Origin and emergence of arboviruses

#### *Historical records of arbovirus emergence*

Arboviruses have been affecting human populations for centuries. Evidence of their presence can be found as far as the Antiquity. For example, the death of Alexander the Great in 323 BC was suggested to be linked, based on records made by Plutarch, to a WNV encephalitis (Marr and Calisher 2003); or, a reference in a Chinese encyclopedia of the Jin dynasty (265 – 420 AD) describing a potential dengue-like illness called “water sickness”, which was thought to be linked to flying insects associated with water (Gubler et al. 2014).

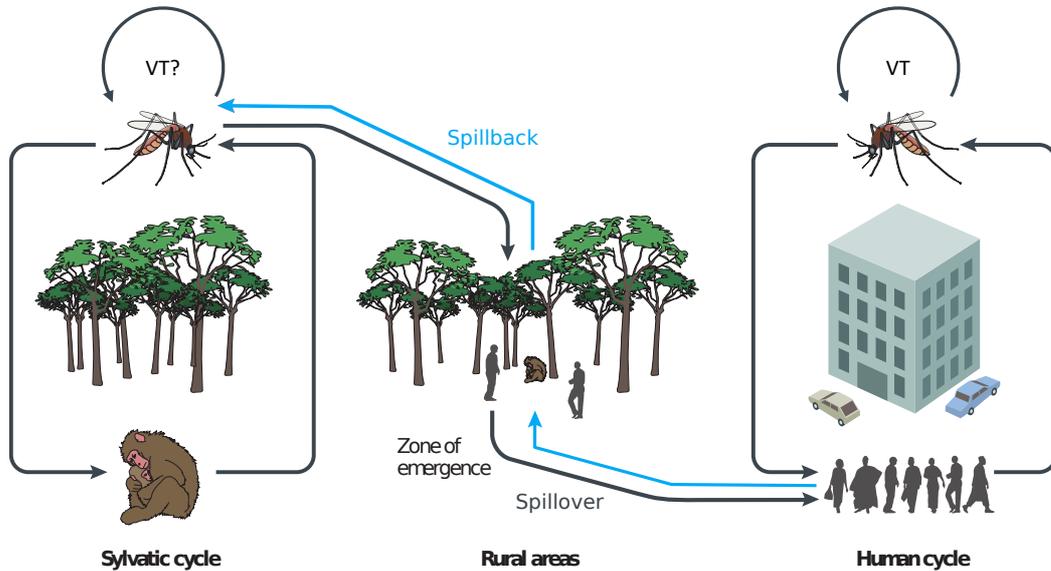
However, arboviruses began to be a major global health concern with YFV, which was introduced from West Africa into the Americas about 400 years ago and contributed to shape the expansion of settlements and colonial powers between the 15<sup>th</sup> and 19<sup>th</sup> century (Bryant et al. 2007). This started with the beginning of extensive overseas explorations by Europeans known as the age of discovery (15<sup>th</sup> – 17<sup>th</sup> centuries) with the first epidemics of yellow fever (Nogueira 2009), but also dengue (Gubler et al. 2014), in port cities of the New World, and especially in the islands of the West Indies. Then, during the colonial era (17<sup>th</sup> – 19<sup>th</sup> centuries), because of great military expeditions and the creation of more secure trade routes, intense epidemics of yellow fever and dengue started to break out. In South America, YFV was first introduced into Brazil before expanding to neighboring countries such as Paraguay, Uruguay and Argentina (Soper 1977). In North America, all of the Atlantic coast and the Gulf of Mexico were affected, making life in some of the ports practically impossible. For example, they were as many as seven major yellow fever epidemics in New York City between 1702 and 1800 (Nogueira 2009).

The emergence of YFV is thought to have been fueled by the geographical expansion of the highly anthropophilic mosquito species, *Ae. aegypti*. Originally a zoophilic forest species from Africa, *Ae. aegypti* invaded most of the tropical and sub-tropical world during the last few centuries. ‘Domestication’ through adaptation to the human environment allowed *Ae. aegypti* to travel within sailing ships to the New World and to establish in port cities harboring large populations of susceptible human hosts (Brown et al. 2014). *Aedes aegypti* is now known as ‘the yellow fever mosquito’.

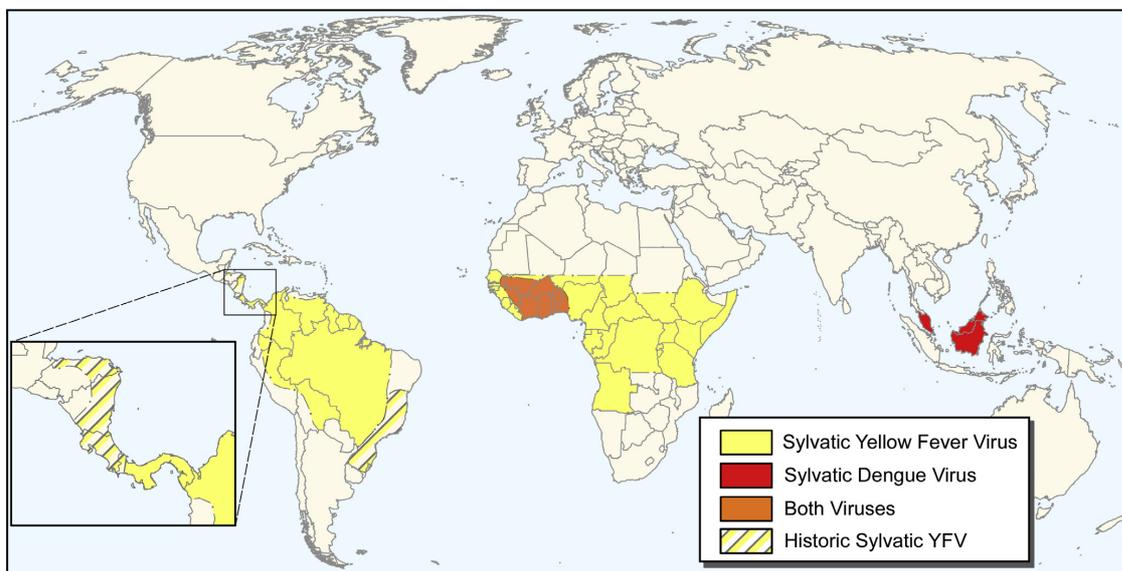
A turning point in the fight against yellow fever was the great epidemic of the Mississippi valley, United States of America in 1878 with a total toll of about 100,000 cases and 20,000 deaths (Woodworth et al. 1879). This epidemic created a public demand for an international investigation of the disease and indirectly became the starting point for the

discovery of its mechanism of transmission (Nogueira 2009; Soper 1977). At the end of the 19<sup>th</sup> century, the role of an arthropod vector in arbovirus transmission was hypothesized for the first time by Dr. Carlos J. Finlay (Finlay 1881). It was formally demonstrated almost 20 years later by Dr. Walter Reed and his team for the role of the mosquito *Ae. aegypti* in the transmission of YFV (Reed et al. 1900).

Interestingly, at the time, two hypotheses about the origin of YFV existed (Nogueira 2009). The first stated that the disease and its urban vector were imported into the Americas from the Guinean Coast of Africa, and this is what most researchers would agree with nowadays. The second hypothesis was that YFV originated in the Americas. This alternative view was mainly based on two types of historical observations: (i) the absence of any historical records of a disease with yellow fever-like symptoms prior the discovery of the New World; (ii) the study of the Mayan codices “Chumayel” and “Tizimin” made by the Bishop of Yucatan, Crescencio Carrillo Ancora, which described the fourth epidemic of “black vomit” in Yucatan in 1648 (Barrett and Higgs 2007). Yet, there was no other report of the disease until that year, and since the peninsula was only found in 1517, the three previous epidemics should have occurred before 1517 (Nogueira 2009). However, recent phylogenetic analyses, based on YFV sequences from 22 countries over a period of 76 years, supported an African origin around 1,500 years ago followed by an introduction into the Americas 300-400 years ago (Bryant et al. 2007).



**Figure 4: Arbovirus transmission cycles.** Vertical transmission (VT). Adapted from (Vasilakis et al. 2011).



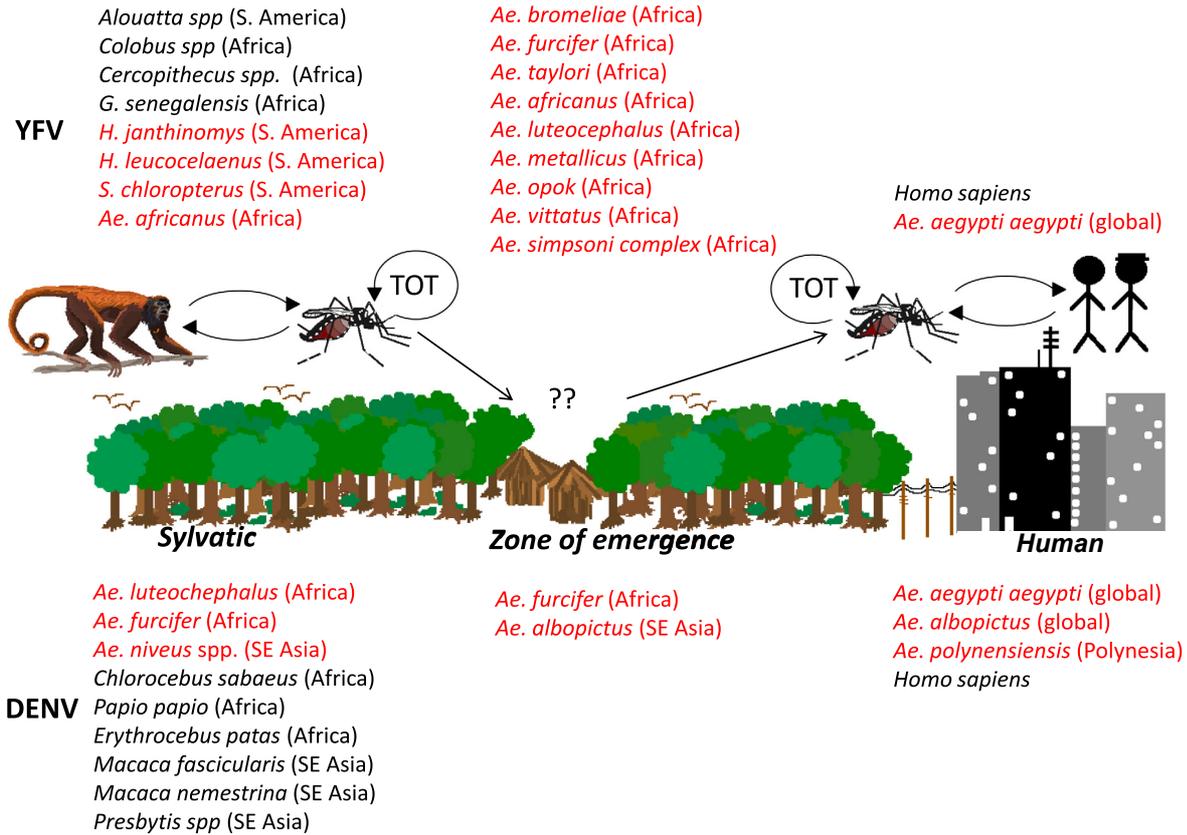
**Figure 5: Worldwide distribution of documented, contemporary foci of circulation of sylvatic dengue virus and sylvatic yellow fever virus and historic foci of sylvatic yellow fever virus.** Reproduced from (Hanley et al. 2013).

*Arbovirus transmission cycles*

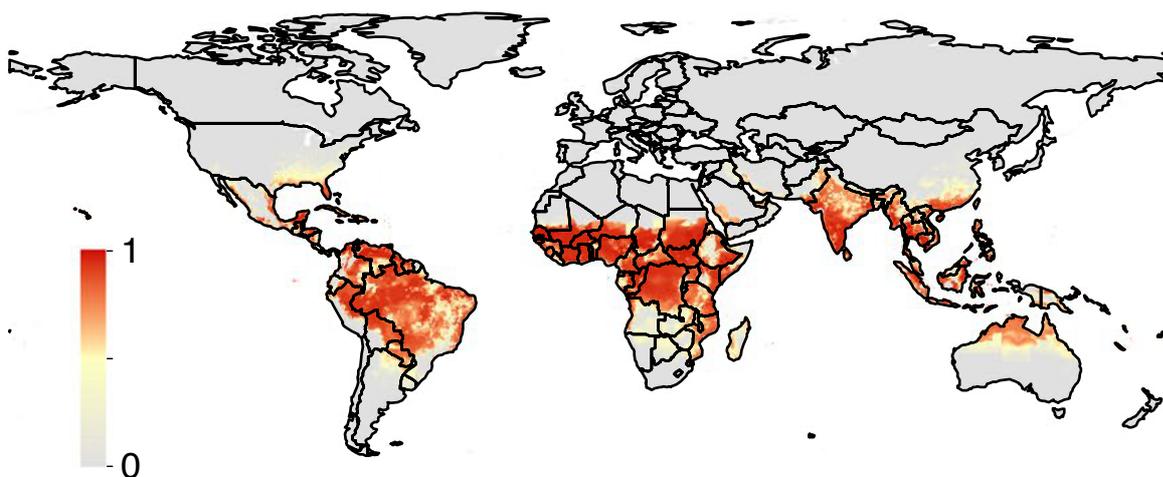
Arboviruses typically circulate in two main transmission cycles, the sylvatic (usually ancestral) cycle where they circulate between vertebrate animals and forest-dwelling mosquitoes, and the human/urban cycle where they circulate between humans and anthropophilic mosquitoes (Figure 4). These two canonical cycles are interconnected by “zones of emergence” at the interface between forest and rural areas where arboviruses may spillover to the human cycle or “spill back” to the sylvatic cycle. A third cycle called the savannah cycle may have represented an intermediate step of YFV spillover to urban cycles in Africa (Barrett and Higgs 2007). In the absence of vertebrate hosts or adverse conditions for horizontal transmission, mosquitoes can also transmit arboviruses to their progeny through vertical transmission (Lequime et al. 2016).

Nowadays, sylvatic transmission cycles of enzootic arboviruses still exist around the world. For example, sylvatic DENV cycles remain active in places such as jungles of peninsular Malaysia and West Africa, and so do sylvatic YFV cycles in tropical forests and surrounding savannah of Africa and South America (Figure 5) (Hanley et al. 2013).

The emergence or re-emergence of sylvatic arboviruses into the human population is an important public health concern (Cardosa et al. 2009) that calls for careful investigations at the interface between human populations and the sylvatic environment. Another concern is the risk of spillback of human arboviruses establishing new sylvatic transmission cycles in previously untouched regions (Hanley et al. 2013; Althouse et al. 2016; Lourenço-de-Oliveira and Failloux 2017). Not only are newly established sylvatic arbovirus cycles virtually impossible to eradicate, but such spillback events can have potentially serious consequences for wildlife. This was exemplified by the introduction of YFV into the Americas in the 15<sup>th</sup> century, establishing sylvatic transmission cycles within the Amazon, Araguaia, and Orinoco river basins (Bryant et al. 2007). Other spillback events have been recorded for DENV-1 in Malaysia (Teoh et al. 2010) and DENV-2 in Africa (Weaver 2013). Most of the spillover events lead to dead-end infections of a single host or vector (Hanley et al. 2013). Onward infection of a novel host usually begins in physical proximity to the sylvatic vectors. Arbovirus emergence implies a cross-species transmission event that may require the virus to overcome barriers to infection of the new host and/or vectors (Parrish et al. 2008).



**Figure 6: Comparison of the major mosquito vectors (red font) and non-human primate hosts (black font) involved in sylvatic transmission, spillover and urban transmission of yellow fever virus (YFV, top) and dengue virus (DENV, bottom). Adapted from (Hanley et al. 2013).**



**Figure 7: Global geographical distribution of *Aedes aegypti*. The color scale represents the probability of the *Ae. aegypti* occurrence with a spatial resolution of 5 by 5 km. Reproduced from (Kraemer et al. 2019).**

## *Role of vectors in arbovirus emergence*

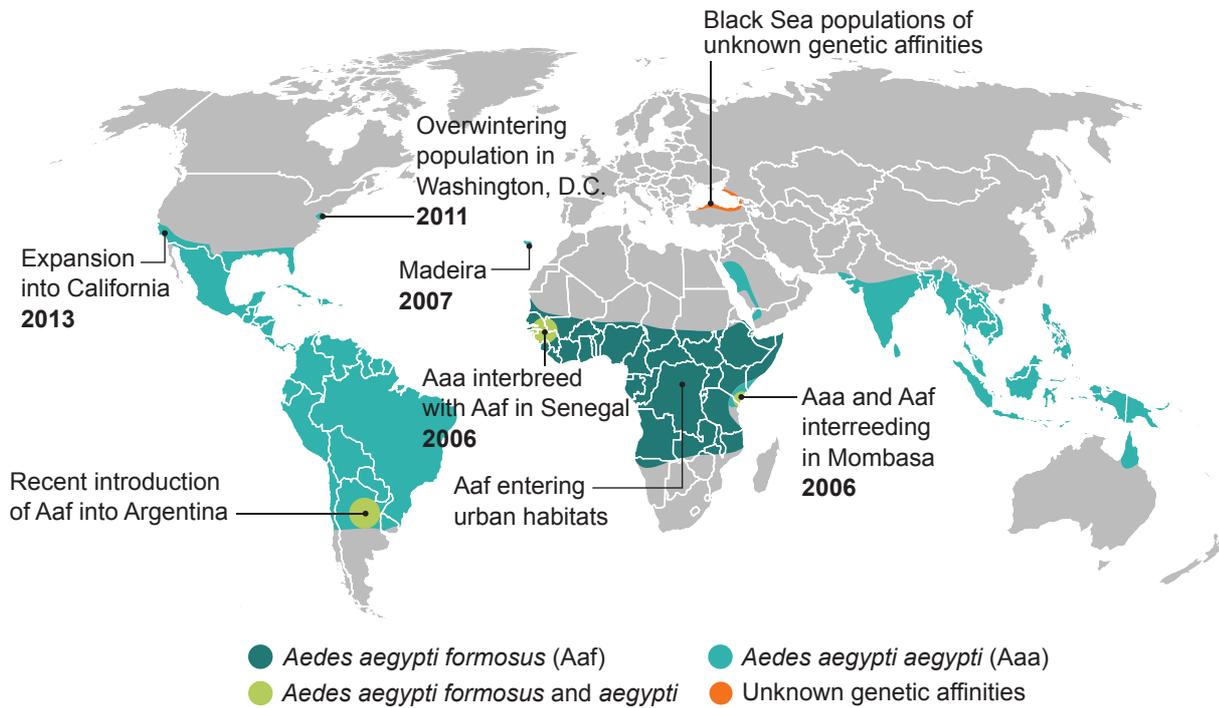
### Bridge vectors

The overlap of mosquito species and hosts involved in sylvatic vs. human arbovirus transmission cycles can result in spillover or spillback transmission (Figure 6). For example, mosquitoes from the *Aedes niveus* Complex are known primatophilic, canopy-dwelling species implicated in the DENV sylvatic cycle in Malaysia that can opportunistically descend to the ground level and feed on humans (Rudnick 1986). Vectors that feed on a broad range of mammalian hosts can facilitate cross-species viral exposures (Parrish et al. 2008). On the other hand, if vectors are highly host species-specific the probability of spillover is low (Hanley et al. 2013). Mosquito species that make the link between sylvatic and human transmission cycles are referred to as bridge vectors. Bridge vectors are competent for arbovirus transmission and display host-feeding behavior that include both humans and wild animals.

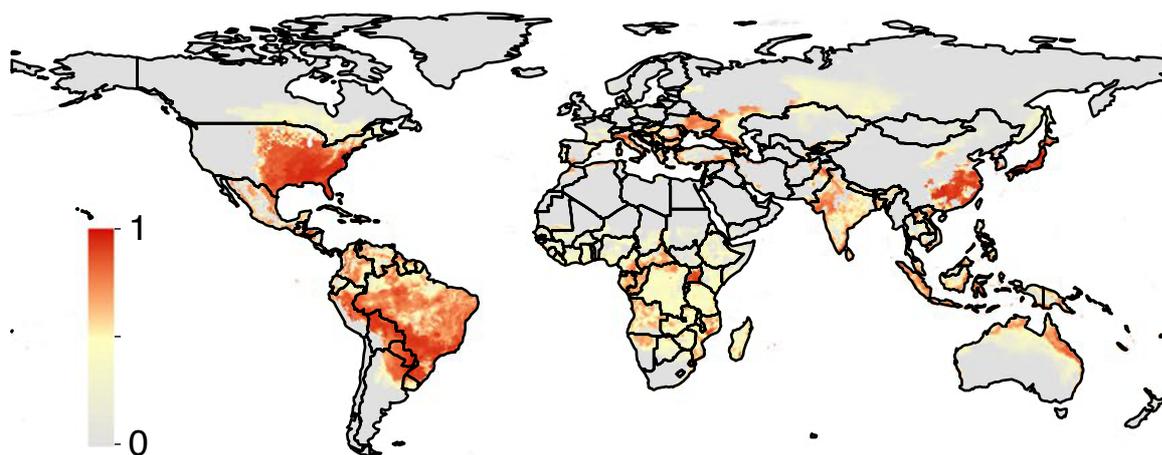
### Main domestic vectors of arboviruses worldwide

Arbovirus circulation in human transmission cycles is performed by domestic vectors. Some studies have suggested that arbovirus emergence in humans requires adaptation to peridomestic mosquito species, but additional work needs to be done to fully evaluate this hypothesis (Moncayo et al. 2004; M. Diallo et al. 2005).

*Aedes aegypti*, the “yellow fever mosquito”, is the most important domestic vector of arboviruses of human medical importance such as DENV, YFV, CHIKV, and ZIKV. This mosquito species originated in Africa where populations with ancestral features of the species can still be found (Powell and Tabachnick 2013). *Aedes aegypti* consists of two subspecies that were originally defined based on morphological (e.g. color, scales) and ecological (e.g. habitat, feeding behavior) characteristics. However, the relevance of morphological characters to distinguish the two subspecies was subsequently questioned because scaling patterns were shown vary between populations (McClelland 1974). Nevertheless, recent genetic studies supported the existence of two subspecies of *Ae. aegypti* based on genome-wide analyses (Gloria-Soria et al. 2016). The ancestral, sylvatic subspecies called *Ae. aegypti formosus* typically displays a dark scaling pattern and a preference for non-human blood and natural breeding sites, and is primarily found in sub-Saharan Africa. The cosmopolitan subspecies called *Ae. aegypti aegypti*, has a lighter scaling pattern and is highly adapted to human habitats where it feeds almost exclusively on humans and breeds in artificial containers (Powell and Tabachnick 2013). It is this cosmopolitan form *Ae. aegypti aegypti* that, during the last centuries, colonized the tropical and subtropical regions of the world (Figure 7), first



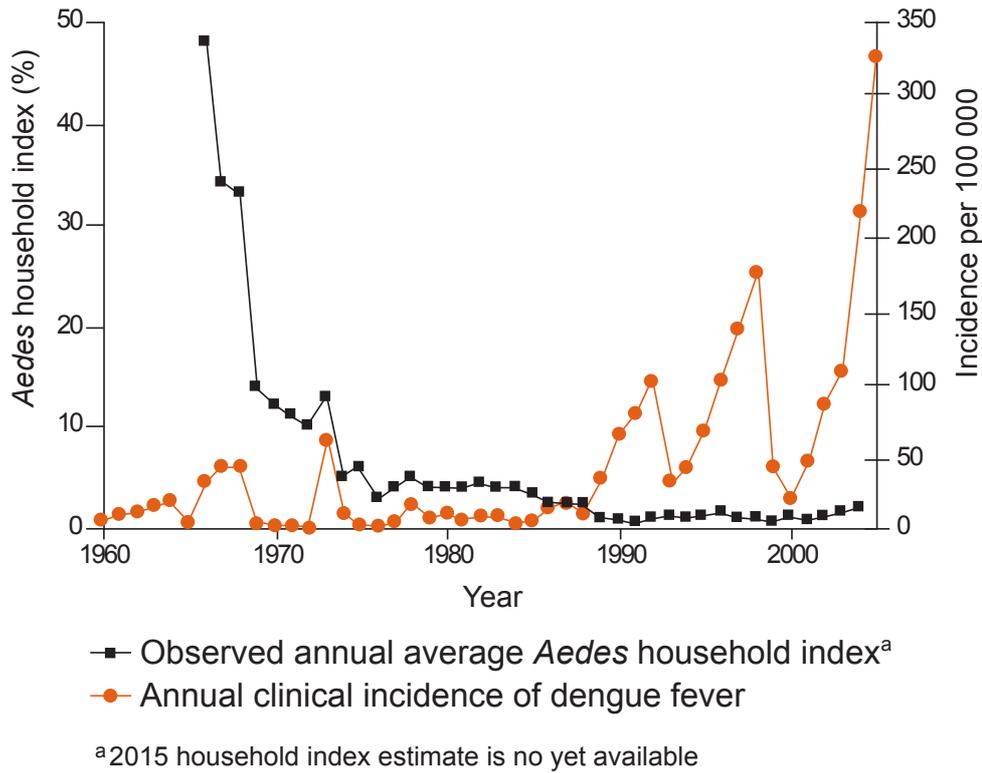
**Figure 8: Geographical distribution of *Aedes aegypti formosus* and *Aedes aegypti aegypti* with recent events of introduction or change of behavior.** Reproduced from (Powell 2016).



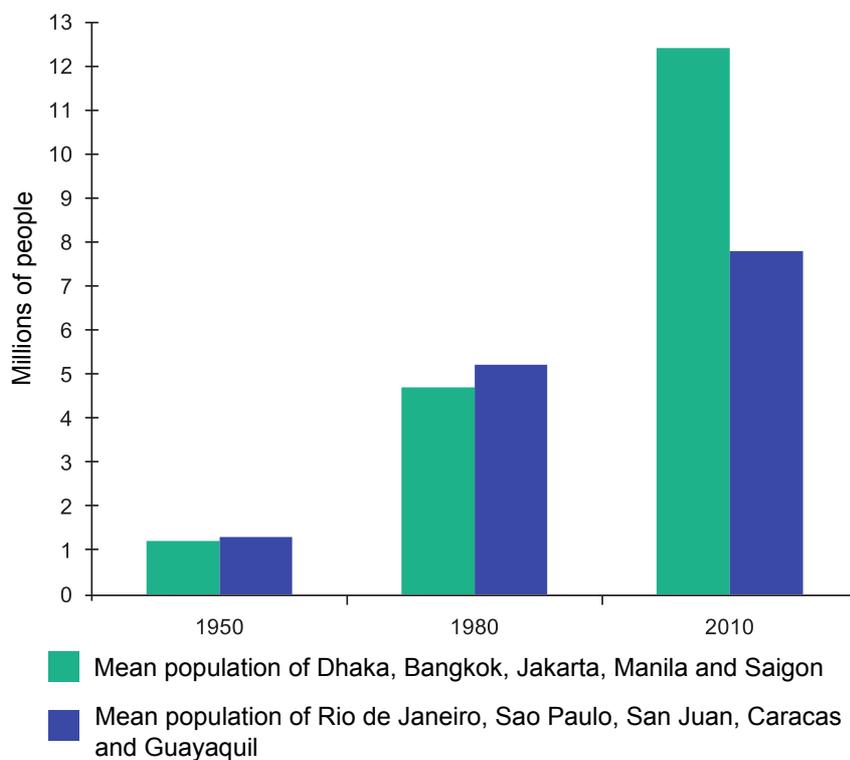
**Figure 9: Global geographical distribution of *Aedes albopictus*.** The color scale represents the probability of the *Ae. albopictus* occurrence with a spatial resolution of 5 by 5 km. Reproduced from (Kraemer et al. 2019).

by being brought to the New World and later to Europe, Asia, and the Pacific islands (Kotsakiozi et al. 2018). However, recent genetic investigations tend to breakdown this dichotomic classification of *Ae. aegypti formosus* vs. *Ae. aegypti aegypti* (Powell 2016). Their geographic distributions are overlapping with evidence of hybridization events. There is no longer a clear ecological separation between *Ae. aegypti aegypti* populations outside of Africa and *Ae. aegypti formosus* populations breeding in domestic habitats in Africa (Figure 8).

*Aedes albopictus*, the “Asian tiger mosquito”, is a generalist feeder that bites animals and humans opportunistically, although its preference for humans has been reported when a choice is present (Delatte et al. 2010). For a long time, *Ae. albopictus* was considered mostly as a pest and an arbovirus vector of secondary importance. However, like *Ae. aegypti*, it is experimentally able to transmit more than 20 arboviruses (Gratz 2004) and was incriminated in DENV and CHIKV epidemics in the absence of *Ae. aegypti* or in rural areas (Paupy et al. 2009). *Aedes albopictus* is originally a zoophilic, forest-dwelling species from tropical South East Asia (Mogi et al. 2015). In the last few decades, it has become one of the most invasive species in the world (Invasive Species Specialist Group 2019) and can now be found in both temperate and tropical areas of Asia, Europe, North and South America, Africa, and a number of islands in the Pacific and Indian Oceans from 40°N to 40°S (Paupy et al. 2009) (Figure 9). Its spread across the world was the third case of human-aided dispersal of a mosquito vector after *Ae. aegypti* and the *Cx. pipiens* Complex (Lounibos 2003) and was largely due to the ability of *Aedes* mosquito eggs to survive desiccation. One the main human activities that contributed to the dispersal of *Ae. albopictus* is the trade of used tires that serve as breeding sites (Reiter and Sprenger 1987). In addition, some populations of *Ae. albopictus* from temperate regions were able to adapt to colder climate such as in Europe, North America, and northern Asia (Hawley et al. 1987). In those populations, cold acclimation and shortening of the photoperiod during autumn as well as the ability to induce a facultative diapause increase eggs cold-hardiness and allow overwintering (Mori and Oda 1981; Hanson and Craig 1994; Lacour et al. 2015).



**Figure 10: Observed annual average *Aedes* household index and annual clinical incidence of dengue fever in Singapore.** Reproduced from (Egger et al. 2008).



**Figure 11: Urban growth in Asian and American cities, 1950 – 2010.** Reproduced from (Gubler 2011).

### Ancillary vectors

For a long time, secondary vectors of arboviruses have been poorly studied and have not been included in the management of mosquito vector populations. However, vector status is a dynamic process and *Ae. albopictus* exemplifies how a previously ancillary vector can become one of the most prominent arbovirus vectors worldwide.

Ancillary vectors also can be responsible for “cryptic” arbovirus transmission in the absence of primary vectors. For example, Singapore has implemented strict and sustained vector control measures associated with household structural improvements that achieve very low densities of *Aedes* mosquitoes, with an *Aedes* household index (i.e. percentage of all habitations with *Aedes* mosquitoes breeding sites) reduced from 50% to 1% (Egger et al. 2008). Paradoxically, despite a successful vector control program, the last two decades have seen the re-emergence of DENV, CHIKV, and ZIKV in Singapore (Hapuarachchi et al. 2016; Koh et al. 2008; Leo et al. 2009; Maurer-Stroh et al. 2016) (Figure 10). It has been suggested that ancillary vectors untargeted by vector control may contribute to arbovirus transmission. For instance, the peridomestic mosquito *Ae. malayensis* breeds in urban parks of Singapore and was shown to be experimentally competent for DENV and CHIKV (Mendenhall et al. 2017).

### Factors associated with arbovirus emergence

Many factors have been associated with arbovirus emergence in the last century, among which the three most prominent are unplanned urbanization, globalization, and failure in vector control (Gubler 2011). Urbanization and the development of tropical megacities has undoubtedly promoted the emergence of arboviruses. After the 1950s, a growing number of people were driven to cities followed by unprecedented and poorly planned urban growth, especially in Asia and in the Americas (Figure 11).

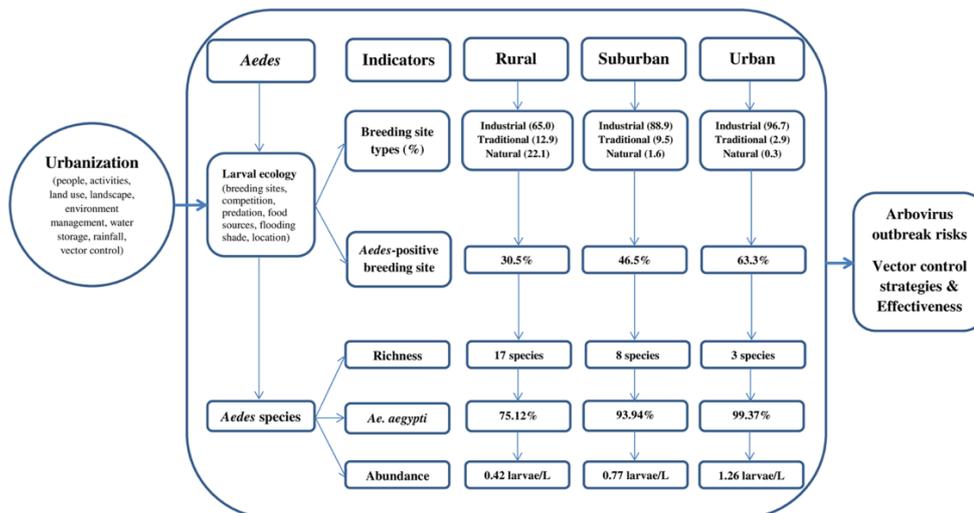


Figure 12: Diagram showing how urbanization shapes immature *Aedes* mosquito breeding sites and species in south-eastern Ivory Coast. Reproduced from (Zahouli et al. 2017).

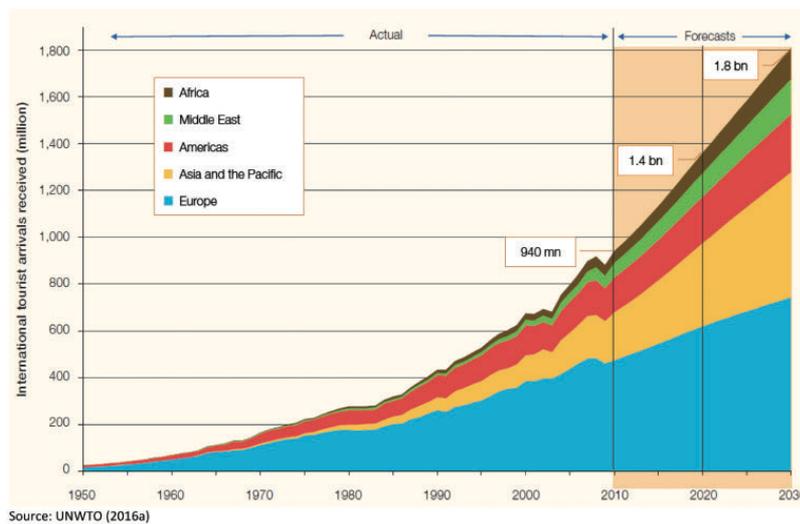


Figure 13: International tourist arrivals by region. Reproduced from (Glaesser et al. 2017).

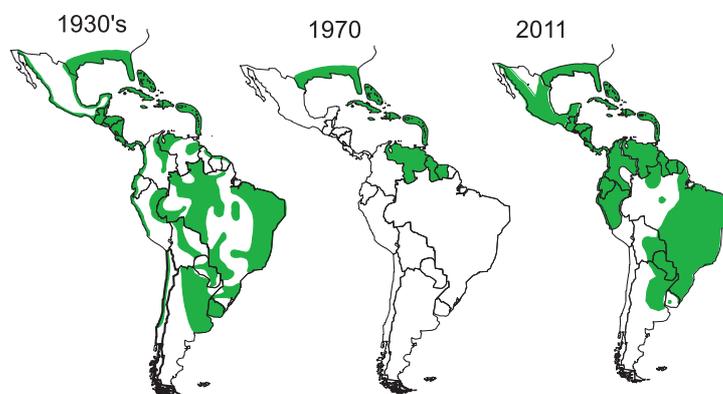
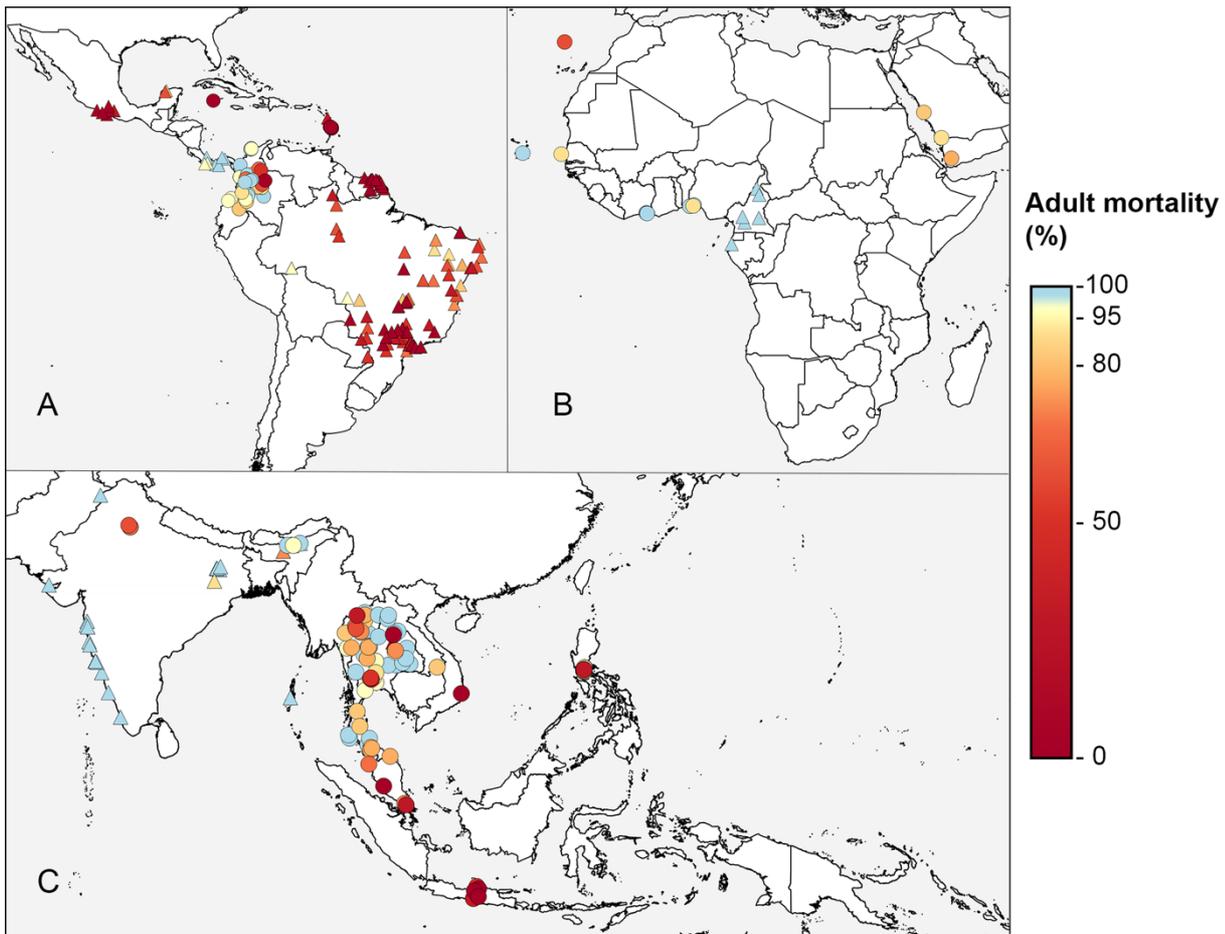


Figure 14: Reinfestation of tropical America by *Aedes aegypti*, 1930–2011. Reproduced from (Gubler 2011).

The degree of urbanization is positively associated with the densities of anthropophilic *Aedes* species. Local outbreaks of DENV, YFV, CHIKV, or ZIKV typically arise 5-15 years after infestation by *Ae. aegypti* and *Ae. albopictus*, showing that their presence is an essential risk factor of arbovirus emergence (Kraemer et al. 2019). Urbanization especially promotes infestation by *Ae. aegypti* because it produces highly suitable breeding sites for this species (Zahouli et al. 2017) (Figure 12). The high density of humans living in close proximity with equally large vector populations in urban areas greatly increases the opportunity of blood feeding and therefore of disease outbreaks.

Globalization is a main factor underlying arbovirus emergence through human activities such as tourism, trade and transport. During the last decades, airplanes have become the main mode of long-distance transportation for humans, animals, goods, and pathogens (Gubler 2018). In 2018, the international air transportation authority (IATA) reported 4.3 billion passengers travelling by plane. The world tourism organization (UNWTO) reported around 1.2 billion international tourist arrivals (i.e. overnight visitors) in 2015 and predicted an increase in the next decades, especially in the Asia-Pacific region and in the Americas (Glaesser et al. 2017) (Figure 13). This leads to the rapid dispersal of arboviruses and vectors across the world and significantly increases the risk of arbovirus emergence in places previously untouched.

Finally, the failure of vector control has been a key factor in arbovirus emergence in the last century (Morrison et al. 2008). The lack of implementation or sustained mosquito control programs and the lack of knowledge on mosquito ecology resulting in unsuitable strategies have been a major problem worldwide in the fight against vector-borne diseases (Paupy et al. 2009). The eradication program of *Ae. aegypti* in the Americas in the 1950s until the 1970s was very successful (Soper 1963), but as soon as the program was stopped *Ae. aegypti* started to re-infest tropical countries in the region (Gubler 2011) (Figure 14). The international tire trade has facilitated the spread of *Ae. albopictus*, yet no regulations concerning storage and transportation of used tires have been implemented to limit the spread of the species (Urbanelli et al. 2000). In the past decades, numerous insecticides have been restricted because of health risk issues, insecticide resistance, or non-registration, leaving fewer options in some regions (Zaim and Guillet 2002).



**Figure 15: The frequency of resistance to deltamethrin in *Aedes aegypti*, 2006–2015.** Results from adult bioassays using 0.05% insecticide for 1 hour are denoted as circles. Results from non-standard adult bioassays (including different diagnostic doses and exposure periods) are denoted as triangles. The map is zoomed to the three regions with data. (A) Americas. (B) Africa/Arabian Peninsula. (C) Asia. Reproduced from (Moyes et al. 2017).

Evolution of insecticide resistance in mosquitoes is a growing problem in Asia and the Americas for the most widely used classes of insecticides (pyrethroids and organophosphates) targeting *Ae. aegypti* and *Ae. albopictus* (Moyes et al. 2017) (Figure 15). The use of a new insecticides is generally rapidly followed by the appearance of resistant vector populations (Epelboin et al. 2018). Temephos resistance in *Ae. aegypti* larvae is a major concern because the lack of available, safe, and cost-effective larvicides that are compatible with drinking water because water storage containers are commonly used as breeding sites (Zaim and Guillet 2002). Targeting larval breeding sites can be difficult because of their numbers, variety, and lack of access (Epelboin et al. 2018). When not all breeding sites can be reached, eradication becomes almost impossible (Scholte and Schaffner 2015). The large amounts of insecticides, trained personnel, and financial resources needed for vector control can be difficult to implement and to maintain in developing countries (Paupy et al. 2009). This results in unchecked vector populations that can spread arboviruses.

Other factors than urbanization, globalization and vector control failure have been implicated in arbovirus emergence, such as climate and climate change (Githeko et al. 2000). Climate anomalies such as the El Niño Southern Oscillation have been implicated in the emergence of DENV in the South Pacific (Hales et al. 1999) and suggested to contribute to the dispersion of ZIKV in the Americas (Paz and Semenza 2016). Climate change could increase the distribution of vector species northward, expand the transmission season, or even influence vectorial capacity (Kraemer et al. 2019; Gould and Higgs 2009). It could also accelerate the decline of sylvatic habitats, resulting in more frequent contact between sylvatic and urban cycles and by that, raising the probability of spillover events (Tabachnick 2016). However, the effect of climate change on vector-borne pathogen transmission is nonlinear and may result in increased transmission potential in some areas and decreased in others (Mordecai et al. 2019; Ryan et al. 2019). For example, climate warming may release populations from cold stress near lower thermal margins (e.g. warmer winter temperatures), resulting in expansion in latitudes and altitudes, but populations near upper thermal margins would be subjected to increased heat stress that could restrict geographic expansion (Mordecai et al. 2019). For example, transmission of DENV, CHIKV, and ZIKV occurs between 18°C and 34°C (Mordecai et al. 2017).

Land use changes can also increase the probability of arbovirus emergence. Deforestation, resulting from logging and mining operations or clear-cutting for agricultural development, can increase the risk of arbovirus transmission (Walsh et al. 1993; Norris 2004;

Ali et al. 2017). Forest disturbance is associated with a shift in mosquito breeding site types that favors colonist species; these colonist species are usually arbovirus vectors and are more abundant than in undisturbed forests (Melrose 2001; Loaiza et al. 2017). In the Brazilian Amazon, deforestation was suggested as a factor implicated in the emergence of Mayaro and Oropouche viruses (Vasconcelos et al. 2001).

Agriculture can also be implicated in arbovirus emergence, for example in South East Asia, where the rapid expansion and development of rubber plantations have been associated with increased risk of exposure to arboviruses such as DENV (Tangena et al. 2017). In South East Asia, vast areas covered in paddy fields for rice cultivation have provided ideal breeding sites for mosquito species that transmit JEV (Chunsuttiwat 1989).

Finally, other human activities such as incursions in forested area (e.g. ecotourism, hunting) and can also increase the risk of arbovirus emergence (Terzian et al. 2015; Figueiredo 2007).

## Mosquito vectors

### Mosquito taxonomy

Mosquitoes are arthropods belonging to the class of insects, the order of Diptera, and the family of Culicidae. In June 2019, the Culicidae comprised 3,565 valid species (Harbach 2019). This list is constantly evolving with new species being continuously described, the extinction and appearance of new species, and the merging of species that are reclassified as synonyms. The Culicidae family is divided in two subfamilies, the Anophelinae and the Culicinae. The Anophelinae include three genera and encompass all the *Anopheles* mosquitoes, mainly known as vectors of the malaria-causing parasites of the *Plasmodium* genus but also of some arboviruses such as o'nyong-nyong virus. The Culicinae include most of the major arbovirus vectors.

According to the classification found in the “Systematic Catalog of Culicidae” (Gaffigan et al., n.d.), in June 2019 the subfamily Culicinae comprised 3,077 species, further divided into 38 genera belonging to 11 tribes. However, following the work of Reinert, Harbach & Kitching (Reinert et al. 2004; Reinert et al. 2006; Reinert et al. 2008; Reinert et al. 2009) a new classification was proposed based on comprehensive morphological data from all life stages of the tribe (Harbach 2019). This new classification has the same tribes but they are grouped in 110 genera and two groups with uncertain placement. Major changes occurred in the tribe Aedini, with for example the well-known vector *Ae. aegypti* becoming *Stegomyia aegypti*. However, this new classification is still controversial. Wilkerson et al. (2015) argued that the new classification is unstable and overcomplicated by questioning seven of the eleven taxonomic naming criteria proposed by Vences et al. (2013). One important aspect is that it could lead to confusion for operational community in charge of mosquito control or with information retrieval from the literature (Wilkerson et al. 2015).

In this thesis I chose to follow the historical classification of Aedini found in the “Systematic Catalog of Culicidae” (Gaffigan et al., n.d.) for consistency with the literature used in this work.

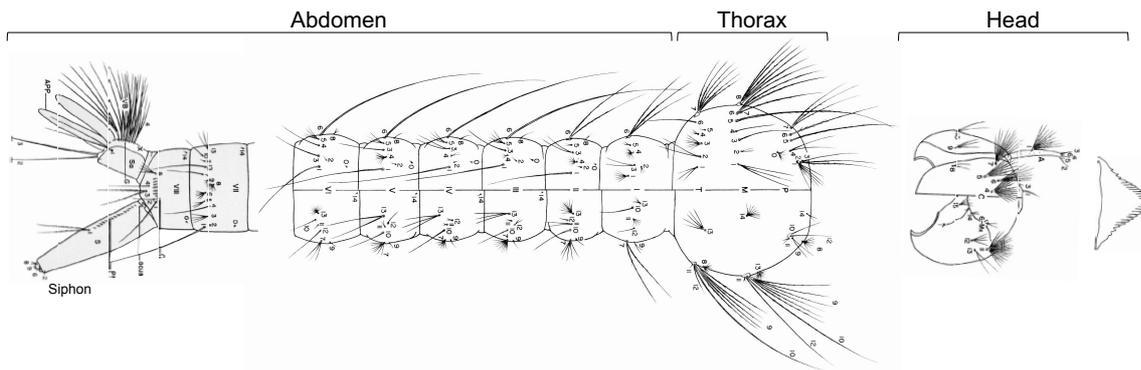


Figure 16: Morphology of a Culicinae fourth-instar larva. Adapted from (Rattanarithikul et al. 2010).

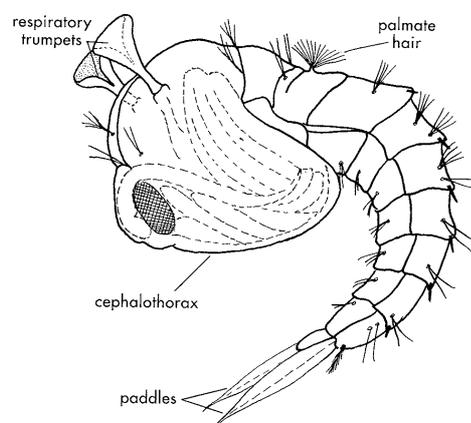


Figure 17: Morphology of a mosquito pupa. Reproduced from (Service 2012).

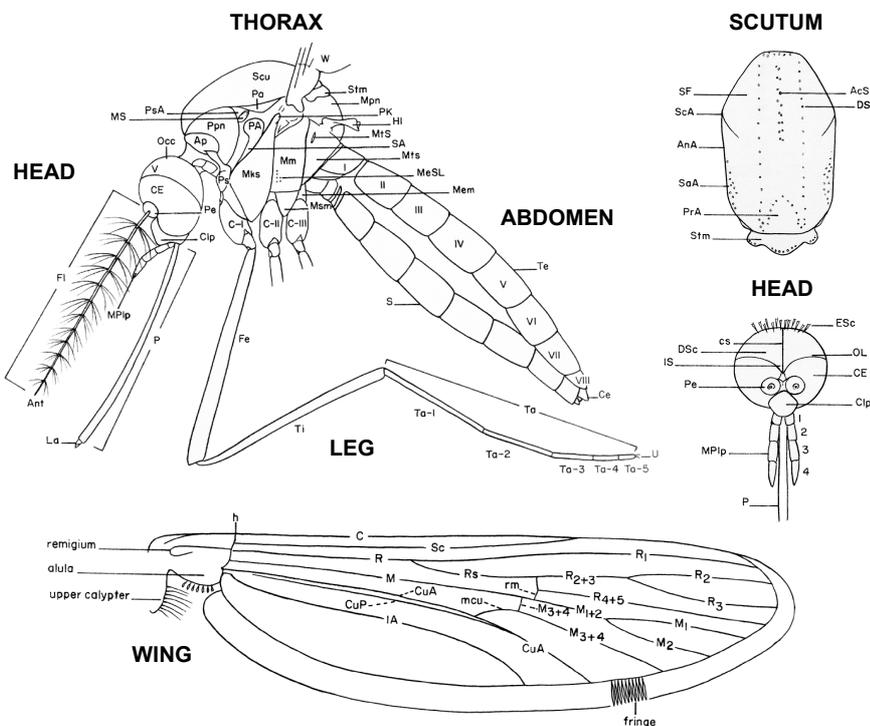


Figure 18: Morphology of an adult female mosquito. Reproduced from (Rattanarithikul et al. 2010).

### Basic mosquito morphology

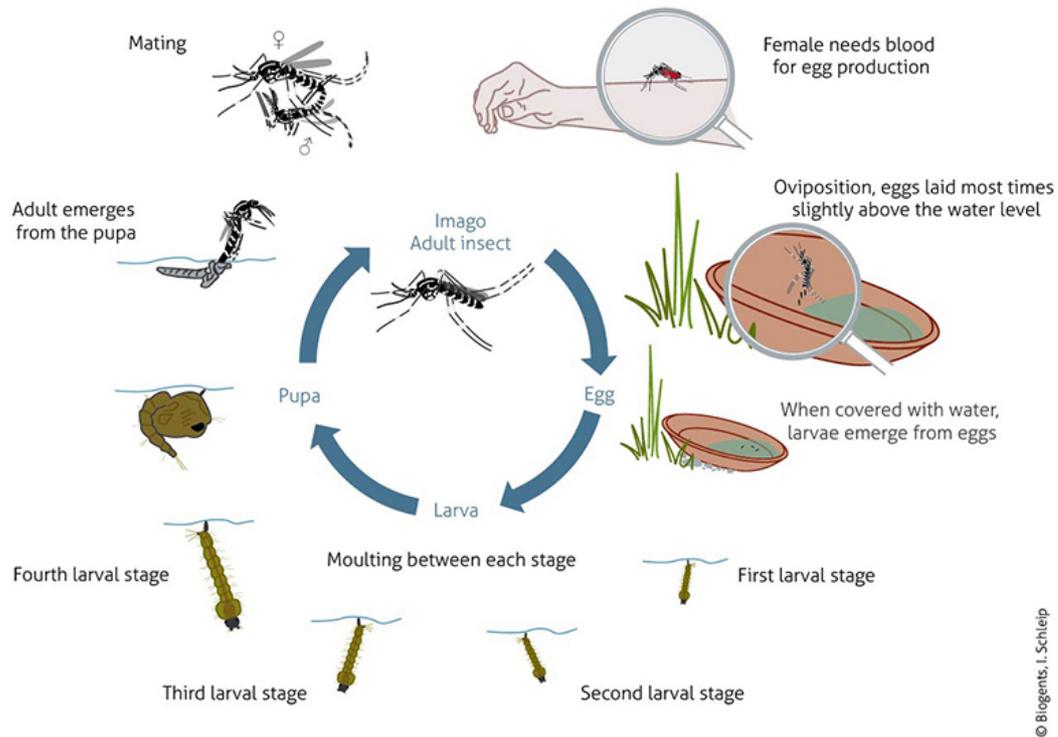
Mosquito larvae are legless with a well-formed head where the mouthparts are located, a thorax, and an abdomen (Figure 16). The only functional respiratory openings are spiracles located at the end of the abdomen, on the siphon for Culicinae or on the dorsal surface of the last abdomen segment for Anophelinae (Clements 2000).

The pupa has a comma-like shape (Figure 17). Head and thorax are fused and form a cephalothorax where the respiratory apparatus (i.e. respiratory trumpets) are located. The abdomen is terminated by two large paddles for propulsion (Clements 2000).

Adult mosquitoes have an elongated body, measuring 2-20 mm depending on the species. The adult mosquito body is divided into three main parts: head, thorax, and abdomen (Figure 18). The head contains the sensory parts, composite eyes (CE), antennae (Ant), and maxillary palpus (MPlp), as well as the mouthparts, i.e. the composite proboscis (P). Usually, an easy way to sex-sort mosquitoes is to examine the antennae, which harbor many long hairs with a feathery appearance in males and whorls of short hairs in females (Service 2012). The thorax is where the legs and the wings are attached. Only the forewings are functional, the hindwings have regressed into small oscillating sensory organs called halteres (HI) that increase flight stability (Clements 2000). The abdomen is divided into 10 segments with the last one being the genital apparatus.







**Figure 20: Mosquito life cycle.**



**Figure 21: Examples of mosquito larval breeding sites.** (1) decaying fruit husks, (2) fresh fruit husks, (3) tree holes, (4) puddles, (5) tires, (6) rock holes, (7) storage containers, (8) discarded containers and (9) bamboo holes. Reproduced from (D. Diallo et al. 2012).

## Mosquito bionomics

### *Mosquito life cycle*

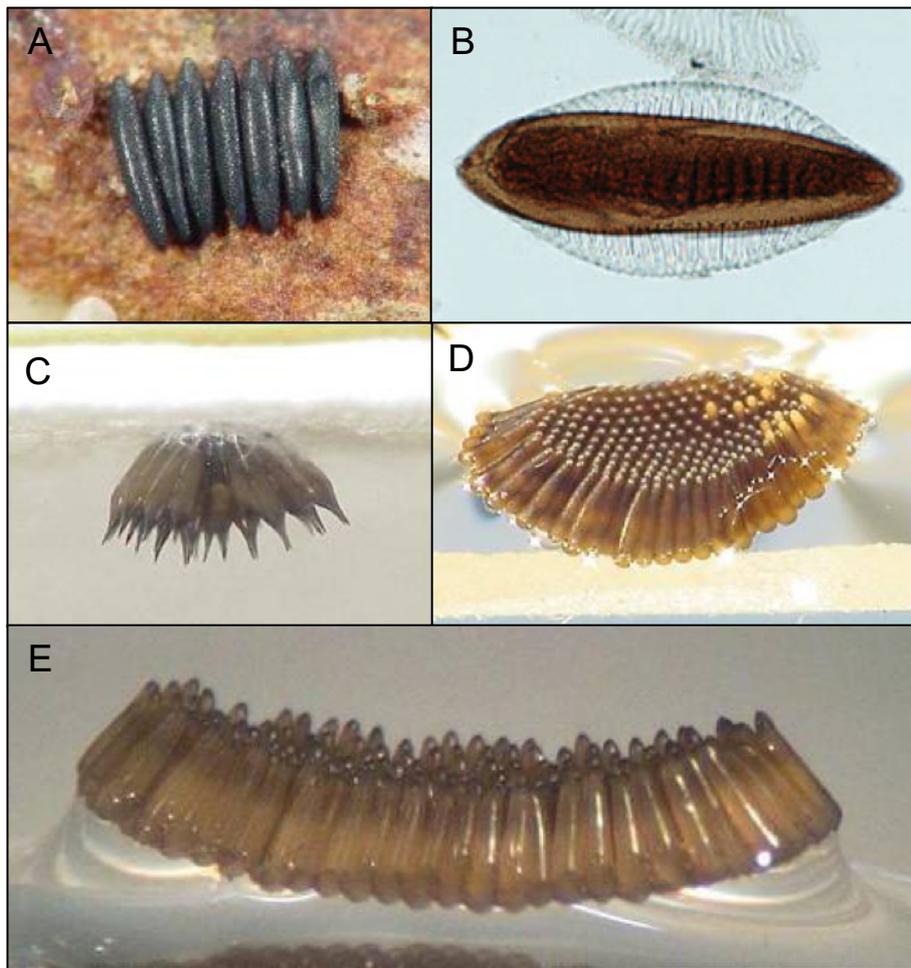
The mosquito life cycle proceeds in two phases, an aquatic phase called *preimaginal* with several immature stages (i.e. egg, larva, pupa) and an aerial phase called *imaginal* (i.e. adults) (Figure 20).

Oviposition sites can be very different depending on the species (Figure 21). Females lay their eggs in natural sites, either ground water habitats such as puddles, rice fields, mangroves, marsh or even crab holes, or container habitats such as tree holes, rock pools, fruit shells or bamboos (Rattanaarithikul, Harrison, et al. 2005). Some mosquito species adapted to man-made breeding sites such as freshwater containers, plant saucers and used tires (Rattanaarithikul, Harrison, et al. 2005).

After egg laying, the embryogenesis proceeds until hatching (e.g. *Anopheles*, *Culex*) or is interrupted and enters a state of dormancy. Indeed, the eggs of some genera (e.g. *Aedes*) can sustain desiccation more or less efficiently, allowing eggs to survive until more favorable environmental conditions (Clements 2000). For example, *Ae. aegypti* eggs acquire desiccation resistance during embryogenesis between 11 and 13 hours after egg-laying (Rezende et al. 2008). This resistance can be extremely long with records of *Ae. aegypti* eggs surviving up to five years in laboratory conditions when left undisturbed and protected from predation (Mayilsamy 2019).

After 1-2 days submerged, eggs hatch and release larvae in the water. Larvae develop through four larval stages (L1, L2, L3, and L4) with molting between each of them, allowing larvae to grow in size. Most mosquito larvae are microphagous, feeding on tiny particles and microorganisms, but some species are predators (e.g. *Toxorhynchites* and *Lutzia*), feeding on other mosquito larvae. Larval development typically takes 4-8 days but this duration depends on the species and is greatly influenced by several factors such as water temperature, food availability, and larval density (Courret et al. 2014). Another factor influencing mosquito development that has gained interest recently is the microbiota. For example, the composition of bacterial communities in *Ae. aegypti* breeding sites can affect the duration of larval development (Dickson et al. 2017).

At the end of larval development, L4 instars molt into pupae. During this pupal phase, mosquitoes undergo a complete metamorphosis that leads to the aerial adult stage (i.e. *imago*). Pupae are behaviorally active (e.g. evade predation) but do not feed. Pupation can last 24-72h depending on the species and environmental factors.



**Figure 22: Examples of mosquito egg-laying strategies.** (A) *Aedes* sp. eggs on dry surface, (B) *Anopheles* sp. egg with floats on both sides of the egg, (C) *Mansonia* sp. eggs raft attached under debris, (D) *Coquillettidia* sp. egg raft, (E) *Culex* sp. egg raft. Images from © Southern Monitoring Services Ltd.



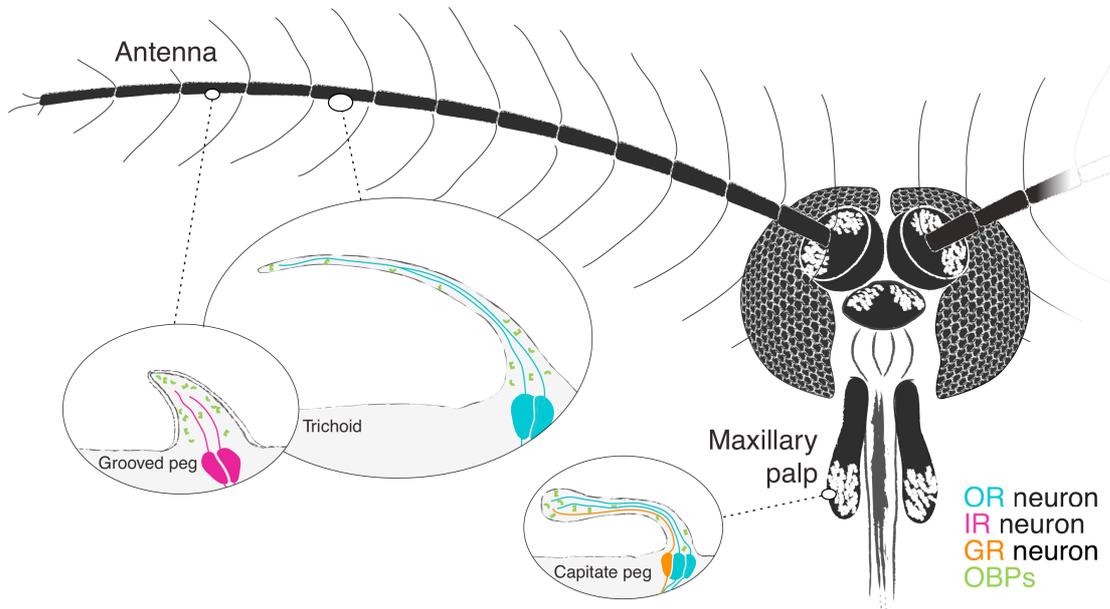
**Figure 23: Diagrammatic representation of the gonotrophic cycle of a female mosquito.** Adapted from (Service 2012).

Generally, mosquito males emerge earlier, have a shorter lifespan and are smaller than females. They feed exclusively on plant and fruit sugars as well as honeydew, which provide energy for flight activity, survival, and may promote reproduction (Spencer et al. 2005; Clements 2000). Newly emerged males undergo morphological changes (e.g. inversion of terminalia, maturation of male accessory glands) during the first days as *imago* before being sexually active (Clements 2000).

Contrary to males, mosquito females are typically hematophagous and use the proteins of the blood meal to produce their eggs, except for some genera such as *Toxorhynchites* that have accumulated resources during larval development (Clements 2000). Males also rely on sugar source for their energy but some species such as *Ae. aegypti* usually forgo frequent sugar feeding, preferring blood as an energy source (Edman et al. 1992). The influence of seasonality on sugar feeding has been reported, most likely due to the presence of preferred flowering plants, where *Ae. aegypti* females increased their sugar consumption during the dry season (Spencer et al. 2005).

Females can be sexually active as soon as their emergence for *Deinocerites cancer* (Provost and Haeger 1967) but it generally takes 24-48 hours between emergence and mating. Females can mate with several males during their lifetime but usually they are inseminated only once. They store sperm in specialized organs called spermatheca and use them for the rest of their life. After oogenesis for around 3 days, females lay up to 500 eggs using different strategies depending on the species (Figure 22), either in a unique breeding site (e.g. egg raft of *Culex* mosquitoes) or split in different breeding sites (e.g. *Ae. aegypti*).

The gonotrophic cycle refers to the sequence of host-seeking, blood-feeding (which may include several blood meals for some species such as *Ae. aegypti* (Scott et al. 1993)), blood digestion, egg maturation and egg laying. Female mosquitoes can undergo several gonotrophic cycles during their lifetime (Duvallet et al. 2018) (Figure 23).



**Figure 24: Schematic of the mosquito peripheral olfactory system.** Schematic of peripheral olfactory system. The mosquito antenna, maxillary palp, and tip of proboscis (not shown) are covered with sensory hairs called sensilla (insets) that house olfactory sensory neurons. The tuning of individual neurons is determined by the binding specificity of several different types of proteins. Odorant-binding proteins (OBPs) carry odorants through the aqueous lymph within each hair to the neuronal membrane, where odorant receptors (ORs), ionotropic receptors (IRs), or gustatory receptors (GRs) recognize them and cause the neuron to fire. Individual receptors and OBPs recognize only a subset of odorants and therefore confer specificity to the neurons or sensory hairs in which they are found. Other accessory proteins may also contribute. There are three major morphological types of olfactory sensilla (insets). Capitate pegs on the maxillary palp house one neuron expressing carbon-dioxide-sensitive GRs and two neurons expressing select ORs. Trichoid and grooved-peg sensilla on the antenna house neurons that likely express ORs and IRs, respectively, based on odor-response profiling. Evolutionary changes in the sequence or expression of peripheral olfactory genes may contribute to host preference by making the mosquito more or less sensitive to specific human and animal odorants. Reproduced from (McBride 2016).

*Host-seeking behavior and dispersal*

In order to locate their host, female mosquitoes use different sensory cues such as chemical (e.g. carbon dioxide, lactic acid, ammonia), visual, thermal or even acoustic signals. This detection process is highly integrated. Indeed, any of these sensory cues alone represents an incomplete host signal not reliably indicative of a host being present in the vicinity. A mosquito female thus integrates multimodal sensory information before choosing to pursue a potential host (McMeniman et al. 2014). Mosquitoes possess two main organs for their sense of smell. The maxillary palps measure the levels of carbon dioxide (CO<sub>2</sub>) and the antennae detect other host-released odors (Dekker et al. 2005) (Figure 24). In most of the cases, CO<sub>2</sub> is the primary activator for mosquito host-seeking behavior (McBride 2016). For example, *Ae. aegypti* females poorly respond to human odor alone but, in the presence of CO<sub>2</sub>, they are immediately sensitized and increase their responsiveness to skin odor (Dekker et al. 2005). Similarly, the attraction to a heat signature needs to be associated with CO<sub>2</sub> detection but not for a visual response (Liu and Vosshall 2019). The presence of CO<sub>2</sub> modulates the response to discrete visual stimuli that facilitate host tracking (Vinauger et al. 2019). Depending on the ecology of some mosquito species, other cues can be more important for host seeking. For some species of the genus *Uranotaenia* that blood feed on amphibians (i.e. ectotherms), sound (e.g. frog calls) is the primary stimulus to guide them when looking for a host (Toma et al. 2005).

Two types of mosquito dispersal behaviors exist, the first is referred to as appetential flight (i.e. active dispersal) and is usually associated with a short-distance flight that stops after the goal is achieved (e.g. oviposition, host-seeking) (Service 1997). There are five primary reasons in mosquito dispersal, which are to find resting sites, sugar sources, mates, blood sources, and oviposition sites (Service 1997). Little dispersal is therefore needed if these are close (Service 1997). The flight range of mosquitoes is highly species-specific with an average distance ranging from 25 m to 6 km (Verdonschot and Besse-Lototskaya 2014). The other type of dispersal behavior is known as non-appetential flight (i.e. passive dispersal), which can be long distance and is either windborne or human-related (e.g. planes, boats) (Service 1997). Some mosquito species are adapted to disperse during strong winds, suggesting that long-distance dispersal is not necessarily a passive process (Verdonschot and Besse-Lototskaya 2014). This windborne migratory process was recently demonstrated for malaria vectors in the Sahel (Huestis et al. 2019).



## Arbovirus transmission by mosquitoes

*Vectorial capacity*

Several intrinsic and extrinsic factors can influence the dynamics of arbovirus transmission by mosquitoes (Kramer and Ebel 2003). Vectorial capacity is a multifactorial metric that represents the overall efficacy of an arthropod population to transmit a pathogen among humans. The concept of vectorial capacity was first developed for malaria transmission in the work of Ronald Ross and then George MacDonald (MacDonald 1957). It was subsequently refined by the contributions of various mathematicians and scientists over 70 years (Smith et al. 2012). The vectorial capacity equation proposed by Garrett-Jones (Garrett-Jones 1964) based on the Ross-MacDonald model is mathematically described as follows:

$$V = \frac{ma^2p^n b}{-\ln(p)}$$

Where:

$V$  = vectorial capacity,

$m$  = vector density in relation to the vertebrate host,

$a$  = probability a vector feeds on a host in one day (i.e. daily biting rate),

$p$  = probability of vector daily survival,

$n$  = duration of extrinsic incubation period or EIP (in days)

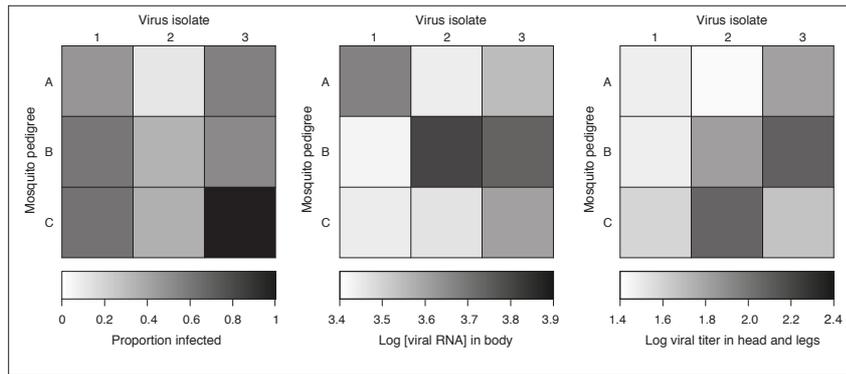
(i.e. time between initial exposure of the vector to the pathogen and the moment it becomes infective to another host),

$b$  = vector competence

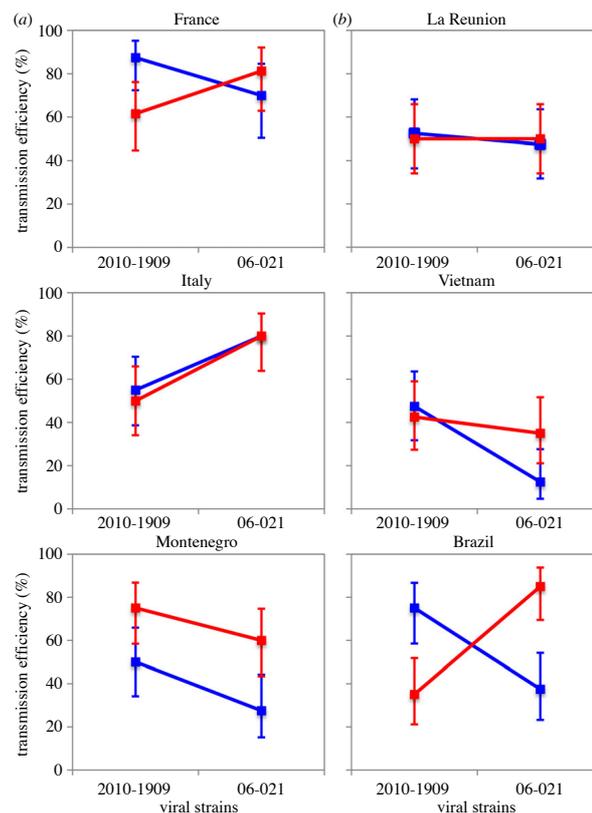
(i.e. proportion of vectors ingesting an infective meal that successfully become infectious),

and  $1/-\ln(p)$  = lifespan in days after the EIP (Kramer and Ebel 2003).

This metric is still widely used in the fight against arboviruses and their vectors because it is useful to understand the outcome of interventions and guide policy making (Brady et al. 2016).



**Figure 25: Genotype-by-genotype interactions between dengue virus and *Aedes aegypti*.** The matrices show experimental measurements of three different vector competence indices for all pairwise combinations of three DENV isolates and three mosquito pedigrees. Phenotypic values are converted to shades of grey according to the scale bar indicated below. Each vector competence index depends significantly on the specific combination of vector genotype and virus isolate. Reproduced from (Lambrechts 2011).



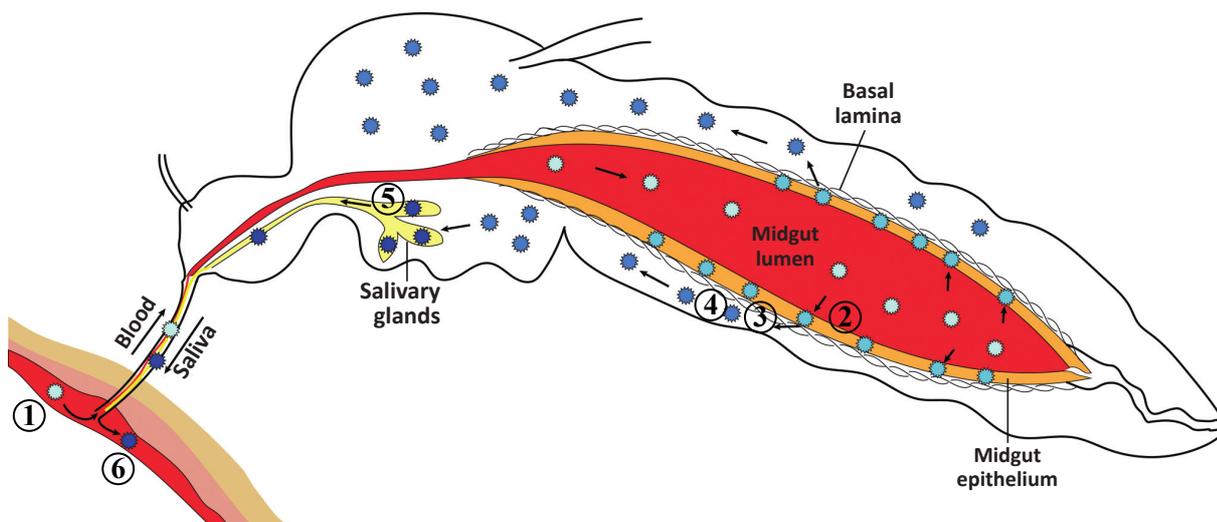
**Figure 26: Effect of mosquito population, virus strain and temperature on the overall proportion of females that had infectious virus in their saliva 6 days after oral exposure of *Aedes albopictus* females to chikungunya virus.** Different panels represent different mosquito populations: (a) temperate regions; (b) tropical regions. In each panel, the average percentage of female with CHIKV-positive saliva across two experiments is shown for two virus strains (indicated on the x-axis) under two temperature regimes (blue line: 20°C; red line: 28°C). Vertical bars are the confidence intervals of the percentages. Reproduced from (Zouache et al. 2014).

*Vector competence*

Vector competence is one component of vectorial capacity. It is defined as the intrinsic ability of a mosquito to acquire a pathogen, to allow its replication, and to transmit it to a new susceptible host (Kramer and Ebel 2003). Vector competence of a mosquito species for an arbovirus generally depends on the specific combination of the mosquito and the virus genotypes (Lambrechts et al. 2009; Lambrechts 2011). Thus, the level of vector competence reflects the effects of the vector genotype, the virus genotype, and their interaction (Figure 25). Genotype-by-genotype interactions between mosquitoes and arboviruses can be complicated by environmental factors (e.g. temperature), resulting in genotype-by-genotype-by-environment interactions (Zouache et al. 2014) (Figure 26).

Vector competence is usually evaluated in the laboratory by experimental infection. A population of mosquito reared under standardized conditions (i.e. synchronized hatching, control of larval density, nutrition, temperature, humidity, and photoperiod) is challenged with an artificial blood meal spiked with a viral solution. Engorged mosquitoes are incubated during a single time period, or several time intervals to assess the dynamics of virus transmission (EIP). Four parameters are conventionally estimated to characterize vector competence:

- **Infection rate** = proportion of infected females. It relates to the ability of the virus to establish an infection of the midgut epithelium.
- **Dissemination rate** = proportion of infected females with a systemic infection (usually determined by detecting virus in head tissues or wings/legs). It relates to the ability of the virus to escape from the midgut epithelium and invade the rest of the mosquito body.
- **Transmission rate** = proportion of females with a disseminated infection that have virus-positive saliva. It relates to the ability of the virus to invade the salivary glands and be released in saliva.
- **Transmission efficiency** = summary metric estimated as the overall proportion of infectious females, i.e. with virus-positive saliva among all females tested.



**Figure 27: Steps of arbovirus propagation in a mosquito following an infectious blood meal.** (1) ingestion of the infectious blood meal, (2) virus infects and replicates in the midgut epithelial cells, (3) release of virions in the general cavity, (4) secondary amplification: virus infects and replicates in secondary tissues (e.g. fat body cells, hemocytes), (5) virus infects and replicates in salivary gland epithelial cells as well as in other tissues, (6) virus released along the saliva during a subsequent blood meal (Kramer and Ebel 2003). Adapted from (Rückert and Ebel 2018).

Vector competence is one of the frequently measured parameters when evaluating the ability of a vector to transmit a pathogen (Souza-Neto et al. 2019). Yet, vector competence is a linear component of the vectorial capacity equation and therefore a relatively weaker contributor compared to EIP or daily survival, for example. This is explained by the greater difficulty to obtain data on other parameters of vectorial capacity. It is important to keep in mind that a low vector competence does not necessarily imply low transmission. Even a poorly competent vector can mediate an arbovirus epidemics if other vectorial capacity parameters (e.g. vector density, biting rate) are high enough (Miller et al. 1989).

#### *Arbovirus propagation within the mosquito vector*

Infection of a competent mosquito with an arbovirus proceeds in several steps (Figure 27). Following an infectious blood meal, the virus is ingested with the blood and infects the epithelial cells of the midgut before the formation of the peritrophic matrix (12-30 hours post blood meal, depending on the species), which physically separates the blood and the epithelium during digestion (Franz et al. 2015). After a phase of virus replication in the midgut epithelium, newly formed virions are released in the general cavity of the mosquito followed by a secondary amplification phase in other tissues (e.g. fat body cells, hemocytes, or nerve tissue). Dissemination of the virus occurs via the hemolymph (Franz et al. 2015). Then, virions infect other organs including the epithelial cells of the salivary glands and sometimes the reproductive tissue. Eventually, virions are released in the saliva during a subsequent blood meal, allowing infection of a new vertebrate host.

During their propagation within the mosquito, viruses encounter a series of anatomical barriers that can limit infection, dissemination and/or transmission of the virus and therefore determine vector competence (Kramer and Ebel 2003). The first barrier prevents infection of epithelial cells of the midgut and is referred to as the midgut infection barrier (MIB) (see 2 in

Figure 27). The second barrier prevents virus dissemination from the infected midgut epithelium and is referred to as the midgut escape barrier (MEB) (see 3 in

Figure 27). Finally, the salivary gland infection and escape barriers (SGIB and SGEB, respectively) prevent virus infection of the salivary glands and virus release in the saliva, respectively (see 5 in

Figure 27) (Franz et al. 2015).

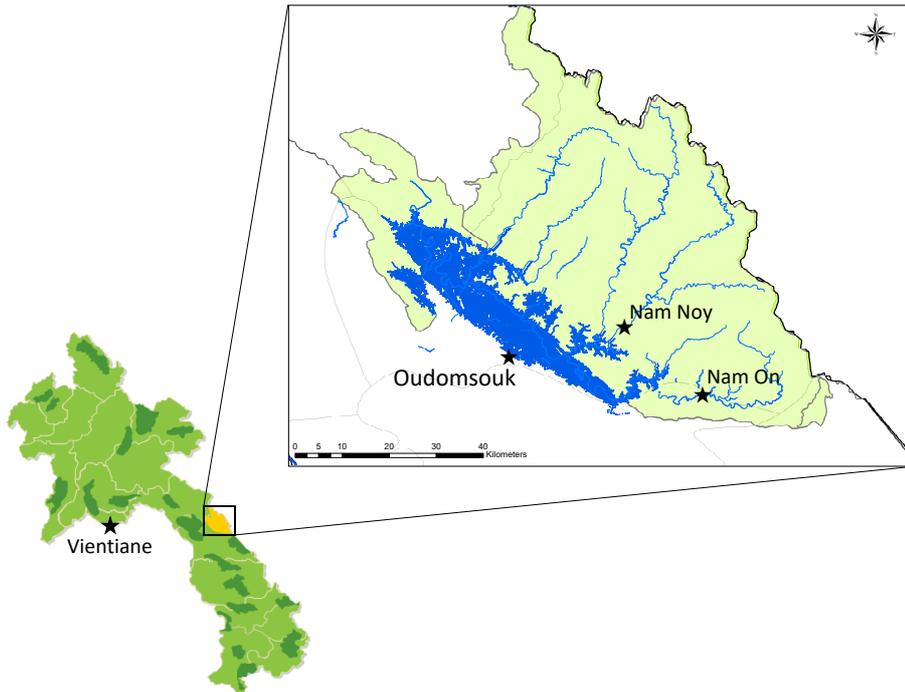
The time interval between the ingestion of the infectious blood meal and when the mosquito becomes able to transmit the virus is called the EIP and is an influential parameter of vectorial capacity. The EIP is influenced by several intrinsic factors such as the virus and

## Literature review – Mosquito vectors

vector genotypes, as well as by environmental factors such as temperature (Hardy et al. 1983). For example, the EIP of DENV at 30°C is 2-15 days (Chan and Johansson 2012) but can be as short as 2-3 days for CHIKV (Vazeille et al. 2007; Johansson et al. 2014). Arboviral infections in the mosquito, unlike in the vertebrate host, are generally persistent and infected females can transmit the virus after each bite. Thus, once infected, a mosquito can potentially transmit the virus for the rest of its life.

# Part 2: Experimental work

## Description of the field sites



**Figure 28: Map of the Lao field sites.** The map of Laos is segmented by province with the location of all the NPAs in dark green, including the NNT NPA in yellow. The blow-up shows the NNT NPA (light green) with the different tributaries descending the Annamite range and ending in the Nam Theun 2 Dam reservoir (blue). Black stars represent the location of the Institut Pasteur du Laos field station in Oudomsouk and the two sampling sites, Nam Noy and Nam On.



**Figure 29: Photos of the field station in Oudomsouk and the way to the study area.**

## Description of the field sites

An essential component of this PhD work was performed in the field in Laos and in Singapore. The section below describes the study areas.

### Field sites in Laos

In Laos, our fieldwork was conducted in the central part of the country, in the Nakai district, Khammuane province. This province is bordered by Thailand to the West, Borikhamxay province to the North, Vietnam to the East, and Savannakhet province to the South. We worked within the primary forest of the Annamite Range in the NNT NPA, also known as the Watershed Management and Protection Authority (WMPA) (Figure 28). The NNT NPA has a tropical monsoon climate (“Am” on the Köppen climate classification) with an average annual precipitation of 2,500-3,000 mm, mean monthly temperatures and relative humidity ranging from 20°C to 29°C and from 75% to 95%, respectively. This area was ideal for our investigation because it consists of dry evergreen forests, cloud forests and mountainous riverbeds that are the biotopes of various mosquito and mammal species including at least nine monkey species with increasing incursions of humans. About 20,000 humans reside in and around this area (NT2 WMPA 2019). To travel from our field laboratory located in Oudomsouk village to the study area in the NNT NPA, we used a boat to cross the reservoir of the Nam Theun 2 dam and go up the different tributaries (Figure 29).

Description of the field sites



**Figure 30: Photos of the Nam Noy field site.**



**Figure 31: Photos of the Nam On field site.**

We mainly sampled along, and in the forest surrounding, the Nam Noy river (17°46'07.07" N, 105°22'54.8" E) for a total of 8 one-week missions in January, February, March, May, August, November-December 2017, and twice in March 2018. The altitude of the study area ranges from 566 m to 784 m above sea level with rocky riverbeds at the bottom of densely forested slopes on both sides (Figure 30).

We also assessed the potential of another field site further south along the Nam On river (17°38'11.97" N, 105°32'19.32" E; altitude 540 m) during two days in May 2017. The area consists of a large rocky clearing in the forest where the river widens and reaches a waterfall before continuing downhill (Figure 31). However, this area was deemed too far and difficult to access and was therefore not further investigated.

Description of the field sites

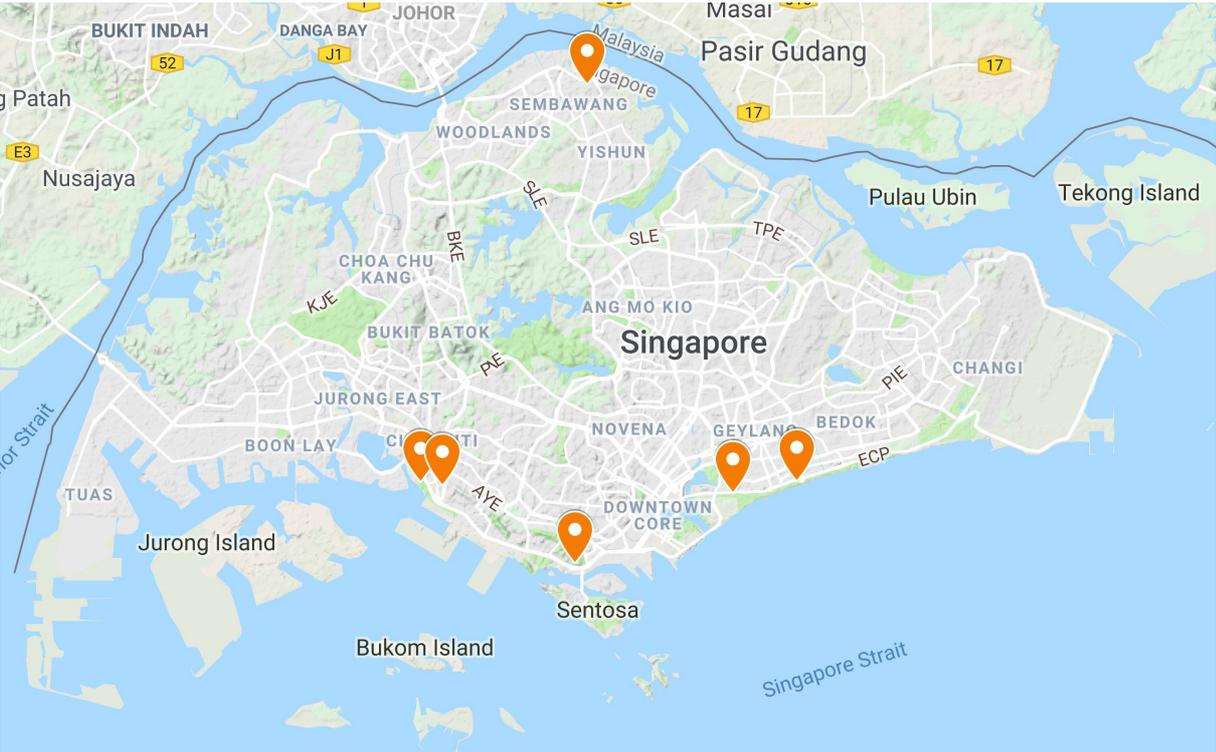


Figure 32: Map of the field sites in Singapore. Orange pins mark the location of the field sites. Generated using Google Maps.



Figure 33: Photos of Singapore field sites.

### Field sites in Singapore

In Singapore, I worked in urban parks located within the city for 2 weeks in March 2019 from 7 a.m. to 7 p.m. I sampled mosquitoes in 5 different national parks, East Coast Park – Zone A (01°17'42.39" N, 103°52'59.64" E) and C (01°17'58.92" N, 103°54'29.47" E), Mount Faber Park (01°16'01.97" N, 103°49'15.89" E), Clementi Woods Park (01°17'52.92" N, 103°46'08.83" E), West Coast Park (01°17'58" N, 103°45'38.02" E) and a forest on private land in Sembawang (01°27'23.37" N, 103°49'33.54" E) (Figure 32). During the fieldwork, temperature ranged from 26°C to 34°C and relative humidity ranged from 32% to 87%. It rained approximately 55 mm. The collection sites consisted of patches of dense secondary forest often surrounded by open spaces and/or crossed by hiking trails (Figure 33).



# Chapter 1

## Survey of the sylvatic mosquito fauna in a forested area of the Nakai Nam Theun National Protected Area



**“This chapter reports on mosquito larval and adult surveys conducted in a forested area of the NNT NPA.”**



## Summary

The Lao mosquito fauna is still not well characterized, especially in remote forested areas such as our study area. The work of Rueda et al. (2015) began to establish a comprehensive checklist of mosquito species in the country, but it was mainly based on a literature review and only few field collections.

In order to update the checklist of mosquitoes in our study area, we performed larval and adult mosquito collections in the NNT NPA along several rivers: Nam Theun (January 2012), Nam Mon (March 2012), Nam Noy (December 2011, March-April 2012, February, March, May, August, November, December 2017, and February-March 2018), and Nam On (May 2017). Mosquito larvae were collected in various types of breeding sites such as rock pools along the riverbanks, tree holes, temporary pools of water, fruit husks, and bamboo shoots. We also constructed bamboo traps, i.e. large bamboos cut in small pieces and filled with fresh water, that we deployed across the Nam Noy field site to create semi-natural breeding sites. Adult mosquitoes were captured using a combination of commercial traps (e.g. CDC light traps, BG sentinel traps) and active collections using vacuum-powered and mouth aspirators, and butterfly nets. Larvae were reared until adulthood and sorted morphologically back in the laboratory.

During our field surveys, we identified 54 mosquito taxa belonging to 11 genera, of which 15 species were new records in Laos. We also described *Ae. malayensis*, a species found throughout South East Asia (Huang 1972; Tewari et al. 1995; Rattananarithikul et al. 2010), for the first time in Laos based on morphological characters and molecular analyses. We produced photos of the morphological characters allowing proper identification of *Ae. malayensis* and distinction from closely related species that are found in sympatry, such as *Ae. albopictus*. We also provided information on the bionomics of *Ae. malayensis*.

Next, I attempted to colonize the four most abundant mosquito species in our collections: *Aedes macfarlanei*, *Aedes elsiae*, *Ae. albopictus*, and *Ae. malayensis*. They are at least five major steps that should be overcome to successfully rear wild mosquitoes in laboratory conditions: larval development (water type, diet), adult survival (sugar source, physico-chemical conditions), mating (specific behavior), blood feeding (blood source), and oviposition. I did not succeed to colonize *Ae. macfarlanei* and *Ae. elsiae*, as no eggs were obtained despite successful rearing and blood feeding. It is likely that the laboratory setup did not meet the conditions required for mating (copulation, fertilization) and/or oviposition steps.

## Chapter 1: Survey of the sylvatic mosquito fauna in a forested area of the Nakai Nam Theun National Protected Area

However, I successfully colonized *Ae. albopictus* and *Ae. malayensis*, which allowed subsequent laboratory studies on these mosquito populations.

Personal contributions to the chapter:

I was part of the team that performed all the field collections in 2017 and 2018 along the Nam Noy and Nam On rivers. I morphologically identified the mosquitoes from both field sites alongside Dr. Maysa T. Motoki, who also performed the subsequent molecular identifications. I established the sylvatic *Ae. albopictus* and *Ae. malayensis* colonies and was in charge of their maintenance in the insectary.

Article 1: New records and updated checklist of mosquitoes (Diptera: Culicidae) from Lao  
People's Democratic Republic, with special emphasis on adult and larval surveillance in  
Khammuane Province

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Maysa T. Motoki, Khamsing Vongphayloth, Leopoldo M. Rueda, Elliott F. Miot, Alexandra  
Hiscox, Jeffrey C. Hertz, and Paul T. Brey.

*Journal of Vector Ecology* 44 (1): 76-88. 2019.

## New records and updated checklist of mosquitoes (Diptera: Culicidae) from Lao People's Democratic Republic, with special emphasis on adult and larval surveillance in Khammuane Province

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**ABSTRACT:** A list of mosquitoes from the Nakai Nam Theun National Protected Area along the Nam Theun, Nam Mon, Nam Noy, and Nam On rivers, Nakai District, Khammuane Province, Lao People's Democratic Republic (Lao PDR) is presented. Fifty-four mosquito taxa were identified, including 15 new records in the Lao PDR. A fragment of the mtDNA cytochrome c oxidase subunit I (COI) gene, barcode region, was generated for 34 specimens, and together with four specimens already published, it represented 23 species in eight genera. In addition, an updated checklist of 170 mosquito taxa from Lao PDR is provided based on field collections from Khammuane Province, the literature, and specimens deposited in the Smithsonian Institution, National Museum of Natural History (SI-NMNH), Washington, DC, U.S.A. This paper provides additional information about the biodiversity of mosquito fauna in Lao PDR. *Journal of Vector Ecology* 44 (1): 76-88. 2019

**Keyword Index:** Culicidae, mosquito surveillance, DNA barcode, Lao PDR, Khammuane Province, mosquito species list.

### INTRODUCTION

Cambodia, China, Myanmar, Thailand, Vietnam, and Lao People's Democratic Republic (Lao PDR) constitute the Greater Mekong Sub-region (GMS), where diseases caused by mosquito-borne pathogens remain a significant source of morbidity (Jones et al. 2008, Hii and Rueda 2011). Malaria and dengue are the most important public health challenges in the GMS region (Hewitt et al. 2013, Suwonkerd et al. 2013, Undurraga et al. 2013, WHO 2016), while other mosquito-borne infections like chikungunya, Zika, and Japanese encephalitis remain a persistent threat (Hotez et al. 2015). Furthermore, the human population movement between Africa and China has resulted in an increased risk of yellow fever cases in China (Ling et al. 2016).

In Lao PDR, the major diseases of the region are transmitted by known species, which act as a primary vector in the surrounding southeast countries. *Aedes aegypti* (Linnaeus), the primary vector of dengue, chikungunya, and Zika viruses (WHO 2009, Lao et al. 2014) is the main vector for all four serotypes of dengue flaviviruses (DENV1-4) in Lao PDR (Khampapongpane et al. 2014, Somlor et al. 2017). *Anopheles dirus* Peyton and Harrison, *An. maculatus* Theobald and *An. minimus* Theobald are the most important vectors of malaria (Pholsena 1992, Meek 1995, Toma et al.

2002, Vythilingam et al. 2005, Jorgensen et al. 2010, Hii and Rueda 2013, Suwonkerd et al. 2013). Despite some studies that identified *Plasmodium* in the mosquito salivary glands (Toma et al. 2002), little is known about the vector status, distribution, and ecology of not only the anopheline, but of most species of culicidae in Lao PDR.

Nakai Nam Theun National Protected Area (NNT NPA), known as the Watershed Management and Protection Authority (WMPA), is Lao PDR's second largest national protected area in Nakai District, Khammuane Province. It is recognized as an important biodiversity site in Southeast Asia and globally, containing mammals, birds, reptiles, amphibians (WCS 2018), and insects, including mosquitoes. It is also the home of rare or newly discovered species of animals (Musser et al. 2005, Luu et al. 2013, Luu et al. 2016, Luu et al. 2017). There is an abundance of suitable mosquito habitats, making this region suitable for their development. Epidemiological and entomological studies previously have been carried out in Khammuane Province (Kobayashi et al. 1997, 2000; Toma et al. 2002), including field work in Ban Natan Region (Rueda et al. 2015). There is a lack of information about species vectors, and the major work for primary vectors still has not been accomplished in the Lao PDR.

In order to build on the knowledge regarding emerging and re-emerging vector-borne diseases, an improved

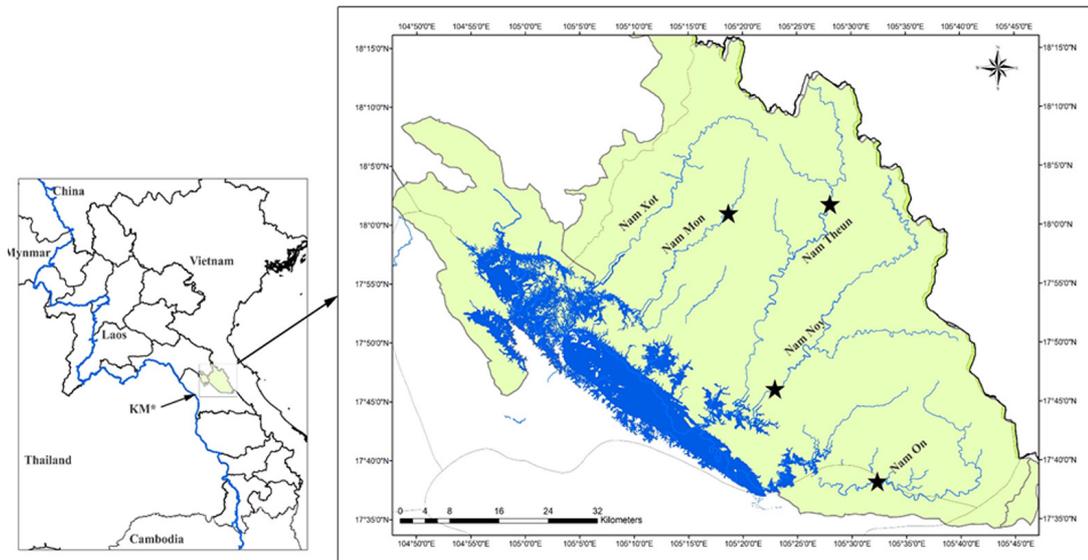


Figure 1. Map showing sampling locations of mosquitoes in Lao PDR. KM = Khammuane Province; Black stars = sampling locations; Blue line = Mekong River and its tributaries.

understanding of species composition, vector status of arthropod insects, together with increased understanding of their bionomics and ecology, we chose locations along the rivers in NNT NPA to support the mapping of health risk and vector-control interventions in the Lao PDR. We report here an extensive surveillance of mosquitoes conducted in Khammuane Province in remote areas along the rivers of Nam Theun, Nam Mon, Nam Noy, and Nam On. This study provides information about mosquito fauna of the Khammuane Province, in addition to the surveillance records of Rueda et al. (2015). Molecular identifications for some specimens of 23 mosquito species were conducted using the cytochrome *c* oxidase subunit I (*COI*), barcode region. Species recorded were combined with other published reports and an updated checklist of mosquitoes from Lao PDR is presented.

## MATERIALS AND METHODS

### Study area and specimen collection

Mosquito adult and larval collections were carried out in the Nakai Nam Theun National Protected Area (NNT NPA), known as the Watershed Management and Protection Authority (WMPA), located in Nakai District, Khammuane Province, Lao PDR. Khammuane Province, located in the south-central part of the country, is bordered by Borlikhamxay Province to the north and northwest, Vietnam to the east, Savannakhet Province to the south, and Thailand to the west. It is located about 350 km south of the capital, Vientiane. It has a tropical climate, with a temperature range of 30-34° C, and average humidity of 60-80%.

Immature and adult mosquitoes were sampled along the Nam Theun River (18.03333°N, 105.466667°E) in January, 2012; Nam Noy River (17.768548°N, 105.381989°E) in December, 2011, March-April, 2012, February, March, May,

August, November, December, 2017, and February-March, 2018; Nam Mon River (17.99416°N, 105.276664°E) in March, 2012; and Nam On River (17.636658°N, 105.538699°E) in May, 2017 (Figure 1).

Adult mosquitoes were collected using mouth aspirators, insect nets, and human-baited double net (HDN) traps (Tangena et al. 2015). Larval and pupal collections were carried out in temporary water habitats including rock pools, tree holes, and bamboo traps. They were collected using a standard larval dipper (350 ml, 13 cm diameter; BioQuip, Rancho Dominguez, CA, U.S.A.) and carefully transferred into a WhirlPak® plastic bag (BioQuip, CA, U.S.A.) using pipettes to transport back to the laboratory. Some specimens were individually link-reared to adults, as morphological voucher specimens for this work.

### Morphological identification

Emerged adults were pinned on paper points, each given a unique collection number, properly labeled, and identified using diagnostic morphological characters (Rattananarithkul et al. 2005, 2006a, 2006b, 2007, 2010). All samples were separated by species, sex, and site. Whole mosquitoes or their legs were stored for molecular identifications. Voucher specimens were deposited at the Entomology Collection, Institut Pasteur du Laos (IPL), Vientiane, Lao PDR and at the SI-NMNH. The surveillance data records will be incorporated into the MosquitoMap section of VectorMap (<http://vectormap.si.edu/>).

### Molecular identification

DNA from single legs plucked from pinned adults or whole ethanol preserved adults was extracted using the Macherey-Nagel NucleoSpin® Tissue (GmbH & Co. KG, Germany) according to manufacturer's instructions. The

fragment of mtDNA *COI* gene was amplified using the PCR Master Mix 2X (Promega Corporation, U.S.A.) utilizing LCO1490 and HCO2198 primers (Folmer et al. 1994). The PCR protocol consisted of a 1 min denaturation at 94°C and five cycles at 94°C for 40 s, 45°C for 40 s, and 72°C for 1 min, followed by 30 cycles at 94°C for 40 s, 49°C for 40 s, and 72°C for 1 min and 5-min extension at 72°C. PCR amplicons were electrophoresed in 1.5% TAE agarose gels stained with GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, U.S.A.), and then the PCR products were cleaned by adding ExosapIT<sup>™</sup> (USB Co, Cleveland, OH, U.S.A.). Samples were put back into the thermocycler and run at 37°C for 30 min, followed by 80°C for 15 min.

All sequencing reactions were carried out in both directions using the original primers and the Big Dye Terminator Kit v.3.1 (PE Applied Biosystems, Warrington, England, UK), analyzed on an ABI Prism 3500xL - Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). Sequences were edited in Sequencher<sup>™</sup> v.5.4.6 (Genes Codes Co, Ann Arbor, MI, U.S.A.), and then aligned using Geneious 9.1.6 (Kearse et al. 2012). A bootstrapped (Felsenstein 1985) Neighbor Joining tree (Saitou and Nei 1987) was employed based on 5,000 replicates. The evolutionary distances were calculated using the Kimura-2 parameters method (Kimura 1980) conducted in MEGA v.7 (Kumar et al. 2016).

## RESULTS

### Biodiversity survey

The mosquito larval and pupal habitats along the Nam Mon, Nam Theun, Nam On, and Nam Noy rivers included bamboo nodes (with stored rainwater), temporary pools, and water pockets along the edges of rivers. A total of 6,736 specimens of mosquitoes were collected (Table 1), of which 5,318 (79%) were identified to the species level. The identities of 9.9% (667 specimens) could only be determined to the genus or subgenus level and 11.1% (751 specimens) consist of a group or a complex of species.

The collected samples belonged to 54 mosquito taxa in 11 genera: *Aedes* Meigen, *Anopheles* Meigen, *Armigeres* Theobald, *Culex* Linnaeus, *Heizmannia* Ludlow, *Lutzia* Theobald, *Topomyia* Leicester, *Toxorhynchites* Theobald, *Tripteroides* Giles, *Udaya* Thurman, and *Uranotaenia* Lynch Arribalzaga (Table 1). *Aedes macfarlanei* (Edwards) (n = 1,845) and *Ae. elsiae* (Barraud) (n = 1,803) were the most dominant species, followed by *Ae. albopictus* (Skuse) (n = 1,123). DNA barcodes were generated for 34 specimens (Genbank numbers: MH427551-MH427584). Comparison of all these sequences in conjunction with morphological analysis allowed the verification of 23 species in eight genera, including *Aedes*, *Armigeres*, *Culex*, *Heizmannia*, *Lutzia*, *Udaya*, *Toxorhynchites*, and *Tripteroides*. *Aedes vittatus* (Bigot) (MG242527 and KU380451 - query cover 100%, identity 99%), *Ar. subalbatus* (Coquillett) (KJ768109 and KM502248 - query cover 100%, identity 100%), *Cx. bicornutus* (Theobald) (AB738196 and AB738259 - query cover 100%, identity 99%), *Cx. pallidothorax* Theobald (LC054474 and

AB738170 - query cover 100%, identity 100%), and *Lt. vorax* Edwards (LC054507 and LC054509 - query cover 100%, identity 99%) were compared to sequences already published in the Genbank. *Culex bicornutus* and *Cx. pallidothorax* were identified initially by morphological characters as *Cx. Mammilifer* Group and *Cx. Wilfredi* Group, and barcode sequences allowed us to correctly identify these two species. *Armigeres*, *Culex*, *Heizmannia*, and *Tripteroides* require further morphological and molecular analyses to clarify the species identities (Figures 2 and 3).

### Mosquito list from Lao PDR

An updated checklist of mosquitoes in Lao PDR includes a total of 170 species (Table 2). They consist of 19 genera, namely *Aedes* (42 species), *Anopheles* (42), *Armigeres* (25), *Coquillettia* (2), *Culex* (23), *Ficalbia* (1), *Heizmannia* (5), *Hodgesia* (1), *Lutzia* (2), *Mansonia* (4), *Mimomyia* (2), *Orthopodomyia* (1), *Topomyia* (1), *Toxorhynchites* (4), *Tripteroides* (3), *Udaya* (1), *Uranotaenia* (3), and *Verrallina* (3).

*Aedes* (*Christophersomyia*) sp., *Ae.* (*Hopkinsius*) *albocinctus* (Barraud), *Ae.* (*Collessius*) *elsiae*, *Ae.* (*Gilesius*) sp., *Cx.* (*Culex*) *Mimeticus* Complex, *Cx.* (*Culiciomyia*) *pallidothorax*, *Cx.* (*Culiciomyia*) Complex, *Cx.* (*Lophoceraomyia*) *bicornutus*, *Hs.* (*Mattinglyia*) *achaetae* (Leicester), *Hs.* (*Mattinglyia*) sp., *Lt.* (*Metalutzia*) *halifaxii* (Theobald), *Tx.* (*Toxorhynchites*) *gravelly* (Edwards), *Tx.* (*Toxorhynchites*) *sunthorni* Thurman, *Tp.* (*Tripteroides*) Complex, and *Ur.* (*Uranotaenia*) *macfarlanei* Edwards are new records for the Lao PDR (Tables 1 and 2).

### Voucher specimens examined

The vouchers are available at the entomology collections of the SI-NMNH and IPL. Morphological identifications are listed alphabetically by genus, subgenus, and species, including their collection numbers, as shown in supplementary data 1. Some mosquito specimens had been used for virus screening studies while other whole mosquitoes were used for DNA extraction; therefore, they are not available in the collections.

## DISCUSSION

### Previous mosquito surveys in Lao PDR

Lao PDR have been considered endemic for malaria (Pholsena 1992), however scientific information about its prevalence is unknown and the mosquito vectors have not yet been identified accurately (Toma et al. 2002). Study of epidemiology of malaria in Khammuane Province has been supported since 1995 by the Japan International Cooperation Agency (JICA) with the cooperation of the Institute of Malaria, Parasitology, and Entomology (IMPE), Vientiane, Lao PDR (Kobayashi et al. 1997).

Through JICA support in Khammuane Province, *Anopheles* species were collected from forest villages (Kobayashi et al. 1997) and in a location surrounded by rice fields (Kobayashi et al. 2000). In the same province, Toma et al. (2002) conducted an epidemiological study and collected 28 anopheline species in an endemic village area, where *An.*

Table 1. Summary of collected mosquito adults and larvae in Nam Mon, Nam Theun, Nam On, and Nam Noy Rivers, Nakai District, Khammuane Province, Lao PDR. RP = rock pool; BT = bamboo trap; AD = adult collection using mouth aspirators, insect nets and/or human-baited double net (HDN) trap. Species in bold are new records in Lao PDR.

Species	Nam Mon	RP	BT	AD	Nam Theun	RP	BT	AD	Nam On	RP	BT	AD	Nam Noy	RP	BT	AD
<i>Aedes (Bruceharrisonius) greeni</i> (Theobald)	-	-	-	-	-	-	-	-	-	-	-	-	1♀	-	-	X
<b><i>Aedes (Christophersomyia) sp.</i></b>	-	-	-	-	-	-	-	-	-	-	-	-	1♀	-	-	X
<i>Aedes (Collessius) elsiae</i> (Barraud)	16♀, 13♂	X	-	-	481♀, 464♂	X	-	-	3♀, 4♂	X	-	-	436♀, 386♂	X	X	-
<i>Aedes (Collessius) macfarlanei</i> (Edwards)	106♀, 89♂	X	-	-	161♀, 137♂	X	-	-	292♀, 228♂	X	-	-	439♀, 393♂	X	-	X
<i>Aedes (Downsiomyia) ganapathi</i> Colless	-	-	-	-	-	-	-	-	-	-	-	-	3♀	-	-	X
<i>Aedes (Downsiomyia) sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-	1♀	-	-	X
<i>Aedes (Fredwardsius) vittatus</i> (Bigot)	-	-	-	-	-	-	-	-	-	-	-	-	13♀, 9♂	X	-	-
<b><i>Aedes (Gilesius) sp.</i></b>	-	-	-	-	13♀, 14♂	X	-	-	-	-	-	-	-	-	-	-
<i>Aedes (Hopkinsius) albocinctus</i> (Barraud)	-	-	-	-	-	-	-	-	-	-	-	-	1♀	-	-	X
<i>Aedes (Hulecoeteomyia) chrysolineatus</i> (Theobald)	2♀, 3♂	X	-	-	11♀, 10♂	X	-	-	-	-	-	-	-	-	-	-
<i>Aedes (Hulecoeteomyia) saxicola</i> Edwards	1♀	X	-	-	7♀, 10♂	X	-	-	-	-	-	-	12♀	-	-	X
<i>Aedes (Phagomyia) prominens</i> (Barraud)	-	-	-	-	-	-	-	-	-	-	-	-	4♀	X	-	X
<i>Aedes (Phagomyia) sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-	2♀	-	-	X
<i>Aedes (Stegomyia) albopictus</i> (Skuse)	3♀	X	-	-	-	-	-	-	1♀	X	-	-	790♀, 329♂	X	X	X
<i>Aedes (Stegomyia) desmotes</i> Giles	-	-	-	-	-	-	-	-	-	-	-	-	34♀, 2♂	-	-	X
<i>Aedes (Stegomyia) gardnerii imitator</i> (Leicester)	-	-	-	-	-	-	-	-	-	-	-	-	1♀	-	-	X
<i>Aedes (Stegomyia) malayensis</i> (Colless)	-	-	-	-	-	-	-	-	-	-	-	-	60♀, 40♂	X	-	X
<i>Aedes sp.</i>	3♀, 1♂	X	-	-	-	-	-	-	-	-	-	-	71♀, 37♂	X	-	X
<i>Anopheles (Cellia) Maculatus Group</i>	1♀, 5♂	X	-	-	2♀, 6♂	X	-	-	1♀	X	-	-	2♂	X	-	-
<i>Anopheles sp.</i>	1♀, 2♂	X	-	-	-	-	-	-	-	-	-	-	8♀, 6♂	X	-	X
<i>Armigeres (Armigeres) subalatus</i> (Coquillett)	-	-	-	-	-	-	-	-	-	-	-	-	2♀	-	-	X
<i>Armigeres (Armigeres) sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-	50♀	-	-	X
<i>Armigeres (Leicesteria) longipalpis</i> (Leicester)	-	-	-	-	-	-	-	-	-	-	-	-	5♀, 5♂	X	X	X
<i>Armigeres (Leicesteria) sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-	8♀	X	-	X
<i>Armigeres sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-	5♀	-	-	X
<b><i>Culex (Culex) Mimetico Complex</i></b>	1♀	X	-	-	1♂	X	-	-	-	-	-	-	-	-	-	-
<i>Culex (Culex) Mimulus Complex</i>	5♀, 3♂	X	-	-	19♀, 24♂	X	-	-	26♀, 18♂	X	-	-	198♀, 159♂	X	X	-
<i>Culex (Culex) vishnu</i> Theobald	-	-	-	-	-	-	-	-	-	-	-	-	7♀	-	-	X
<i>Culex (Culicomyia) nigropunctatus</i> Edwards	66♀, 32♂	X	-	-	-	-	-	-	-	-	-	-	17♀, 22♂	X	-	-
<b><i>Culex (Culicomyia) pallidothorax</i></b> Theobald	-	-	-	-	-	-	-	-	-	-	-	-	5♀	-	X	-
<b><i>Culex (Culicomyia) Complex</i></b>	-	-	-	-	-	-	-	-	-	-	-	-	143♀, 115♂	X	X	X
<i>Culex (Lophoceraomyia) bicornutus</i> (Theobald)	-	-	-	-	-	-	-	-	1♀	X	-	-	-	-	-	-
<i>Culex (Lophoceraomyia) Mammilifer and Wilfredi Groups</i>	11♀, 5♂	X	-	-	2♂	X	-	-	10♀, 2♂	X	-	-	35♀, 10♂	-	-	X
<i>Culex (Oculeomyia) sinensis</i> Theobald	-	-	-	-	1♂	X	-	-	-	-	-	-	-	-	-	-
<i>Culex sp.</i>	26♀, 9♂	X	-	-	8♀, 5♂	X	-	-	2♀, 6♂	X	-	-	34♀, 57♂	X	X	X
<i>Heizmannia (Heizmannia) sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-	157♀	X	-	X
<b><i>Heizmannia (Mattinglyia) achaetae</i></b> (Leicester)	-	-	-	-	-	-	-	-	-	-	-	-	55♀	-	-	X
<b><i>Heizmannia (Mattinglyia) sp.</i></b>	-	-	-	-	-	-	-	-	-	-	-	-	31♀	-	-	X
<i>Heizmannia sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-	9♀	X	-	X
<b><i>Lutzia (Metalutzia) halifaxii</i></b> (Theobald)	-	-	-	-	-	-	-	-	1♀	X	-	-	-	-	-	-
<i>Lutzia (Metalutzia) vorax</i> Edwards	7♀, 14♂	X	-	-	2♀	X	-	-	1♀	X	-	-	33♀, 31♂	X	X	-
<i>Lutzia sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-	1♀	-	-	X
<i>Topomyia sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-	1♀	-	-	X
<b><i>Toxorhynchites (Toxorhynchites) graveyi</i></b> (Edwards)	-	-	-	-	-	-	-	-	-	-	-	-	1♀, 1♂	-	X	-
<b><i>Toxorhynchites (Toxorhynchites) sunthorni</i></b> Thurman	-	-	-	-	-	-	-	-	-	-	-	-	5♂	X	X	-
<i>Toxorhynchites sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-	2♀	-	X	X
<i>Tripteroides (Rachionotomyia) sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-	1♂	-	X	-
<b><i>Tripteroides (Tripteroides) Complex</i></b>	-	-	-	-	-	-	-	-	-	-	-	-	22♀	-	X	X
<i>Tripteroides sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-	1♀	-	-	X
<i>Udaya argyrurus</i> (Edwards)	-	-	-	-	-	-	-	-	-	-	-	-	1♀	-	-	X
<i>Uranotaenia (Pseudoficalbia) novobscura</i> Barraud	-	-	-	-	-	-	-	-	-	-	-	-	4♂	X	-	-
<i>Uranotaenia (Pseudoficalbia) sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-	1♀	-	-	X
<b><i>Uranotaenia (Uranotaenia) macfarlanei</i></b> Edwards	-	-	-	-	-	-	-	-	-	-	-	-	3♀, 1♂	X	-	-
<i>Uranotaenia (Uranotaenia) sp.</i>	-	-	-	-	-	-	-	-	-	-	-	-	1♀	-	-	X

*dirus* and *An. minimus* were identified as vectors by detecting sporozoite in mosquito salivary glands. Subsequently, a preliminary malaria survey was conducted in Bolikhamxay, Champasak, Luang Prabang, Saravan, Savannakhet, Sayaboury, Sekong, and Vientiane Provinces (Vythilingam et al. 2001). Vythilingam et al. (2003) carried out a longitudinal study on the prevalence of *Anopheles* in three villages in Sekong Province. From 2002 to 2004, studies were conducted

to determine the malaria vectors in Attapeu Province (Sidavong et al. 2004, Vythilingam et al. 2005), and a list of all mosquito species found in that province was reported by Vythilingam et al. (2006). We collected only few specimens (34) of *Anopheles* species. Half were identified as *An. (Cel.) Maculatus Group*, and the remainder were not possible to identify to species, named as *An. sp.* (Table 1).

In a diversity mosquito survey, Rueda et al. (2015)

Table 2. Updated checklist of mosquito species from Lao PDR. # indicates observed in field collection.

	Species	References
1	<i>Aedes (Aedimorphus) alboscuteallatus</i> Theobald	Vythilingam et al. 2006; WRBU 2018; Rueda et al. 2015
2	<i>Aedes (Aedimorphus) caecus</i> Theobald	Tangena et al. 2017
3	<i>Aedes (Aedimorphus) orbitae</i> Edwards	Tangena et al. 2017
4	<i>Aedes (Aedimorphus) pipersalatus</i> (Giles)	Vythilingam et al. 2006; WRBU 2018
5	<i>Aedes (Aedimorphus) vexans</i> (Meigen)	Vythilingam et al. 2006; WRBU 2018; Rueda et al. 2015
6	<i>Aedes (Borichinda) cavernicola</i> Rattananarithikul and Harbach	Tangena et al. 2017
7	<i>Aedes (Bothaella) eldridgei</i> Reinert	Rueda et al. 2015
8	<i>Aedes (Bothaella) helenae</i> Reinert	Tangena et al. 2017
9	<i>Aedes (Bruceharrisonius) greenii</i> (Theobald)	Tangena et al. 2017, #
10	<i>Aedes (Collessius) elisiae</i> (Barraud)	<b>new record</b>
11	<i>Aedes (Collessius) macfarlanei</i> (Edwards)	WRBU 2018, #
12	<i>Aedes (Danielsia) alboataeniata</i> (Leicester)	Tangena et al. 2017
13	<i>Aedes (Diceromyia) iyengari</i> Edwards	Vythilingam et al. 2006; WRBU 2018
14	<i>Aedes (Downsiomyia) ganapathi</i> Colless	Rueda et al. 2015, #
15	<i>Aedes (Downsiomyia) harinasutai</i> Knight	Rueda et al. 2015
16	<i>Aedes (Downsiomyia) inermis</i> Colless	Tangena et al. 2017
17	<i>Aedes (Downsiomyia) niveus</i> (Ludlow)	WRBU 2018
18	<i>Aedes (Fredwardsius) vittatus</i> (Bigot)	Tsuda et al. 2002; WRBU 2018; Tangena et al. 2017; #
19	<i>Aedes (Hopkinsius) albocinctus</i> (Barraud)	<b>new record</b>
20	<i>Aedes (Hulecoeteomyia) chrysolineatus</i> (Theobald)	Vythilingam et al. 2006; WRBU 2018; Rueda et al. 2015; Tangena et al. 2017; #
21	<i>Aedes (Hulecoeteomyia) formosensis</i> Yamada	Rueda et al. 2015
22	<i>Aedes (Hulecoeteomyia) reinerti</i> Rattananarithikul and Harrison	Rueda et al. 2015; Tangena et al. 2017
23	<i>Aedes (Hulecoeteomyia) saxicola</i> Edwards	Tangena et al. 2017; #
24	<i>Aedes (Kenknightia) dissimilis</i> (Leicester)	Rueda et al. 2015; Tangena et al. 2017
25	<i>Aedes (Kenknightia) harbachi</i> Reinert	Tangena et al. 2017
26	<i>Aedes (Lorrainea) fumidus</i> Edwards	Tangena et al. 2017
27	<i>Aedes (Mucidus) quasiferinus</i> Mattingly	Tangena et al. 2017
28	<i>Aedes (Neomelaniconion) lineatopennis</i> (Ludlow)	Rattananarithikul et al. 2010
29	<i>Aedes (Paraedes) ostentatio</i> (Leicester)	Rattananarithikul et al. 2010
30	<i>Aedes (Phagomyia) khazani</i> Edwards	Apiwathnasorn 1986; Tangena et al. 2017
31	<i>Aedes (Phagomyia) prominens</i> (Barraud)	Apiwathnasorn 1986; Rueda et al. 2015; Tangena et al. 2017; #
32	<i>Aedes (Stegomyia) aegypti</i> (Linnaeus)	Apiwathnasorn 1986; Tsuda et al. 2002; Hiscox et al. 2013a,b; Rueda et al. 2015; WRBU 2018; Tangena et al. 2017
33	<i>Aedes (Stegomyia) albopictus</i> (Skuse)	Apiwathnasorn 1986; Vythilingam et al. 2006; Chen-Hussey 2012; Hiscox et al. 2013a,b; Rueda et al. 2015; Tangena et al. 2017; Motoki et al. 2018; #
34	<i>Aedes (Stegomyia) annandalei</i> Theobald	Tangena et al. 2017
35	<i>Aedes (Stegomyia) craggi</i> (Barraud)	Tangena et al. 2017
36	<i>Aedes (Stegomyia) desmotes</i> Giles	Tangena et al. 2017; #
37	<i>Aedes (Stegomyia) gardnerii imitator</i> (Leicester)	Vythilingam et al. 2006; Tangena et al. 2017; #
38	<i>Aedes (Stegomyia) malayensis</i> Colless	Motoki et al. 2018; #
39	<i>Aedes (Stegomyia) pseudalbopictus</i> (Borel)	Vythilingam et al. 2006; WRBU 2018; #
40	<i>Aedes (Stegomyia) pseudoscutellaris</i> Theobald	Rueda et al. 2015
41	<i>Aedes (Stegomyia) seatoi</i> Huang	Tangena et al. 2017
42	<i>Aedes (Tewarius) pseudonummatius</i> Reinert	Rueda et al. 2015
43	<i>Anopheles (Anopheles) alboataeniatus</i> (Theobald)	Vythilingam et al. 2001
44	<i>Anopheles (Anopheles) argyropus</i> (Swellengrebel)	Hii and Rueda 2013
45	<i>Anopheles (Anopheles) baezai</i> Gater	Chen-Hussey 2012; Tangena et al. 2017
46	<i>Anopheles (Anopheles) baileyi</i> Edwards	Apiwathnasorn 1986; WRBU 2018; Tangena et al. 2017
47	<i>Anopheles (Anopheles) barbirostris</i> Van der Wulp	Hii and Rueda 2013; Lefebvre 1938; Vythilingam et al. 2001, 2006; Sidavong et al. 2004; WRBU 2018
48	<i>Anopheles (Anopheles) barbumbrosus</i> Strickland and Chowdhury	Kobayashi et al. 1997; Toma et al. 2002; Tangena et al. 2017
49	<i>Anopheles (Anopheles) donaldi</i> Reid	Hii and Rueda 2013; Vythilingam et al. 2006; Sidavong et al. 2004; WRBU 2018
50	<i>Anopheles (Anopheles) hodgkini</i> Reid	Toma et al. 2002; Chen-Hussey 2012; Suwonkerd et al. 2013; Tangena et al. 2017

Table 2 continued...

collected 43 taxa in Ban Natan area, Khammuane Province and reported a total of 101 species from Laos, including ten newly recorded *Aedes* and *Orthopodomyia* species. In our survey, we identified 14% of the same taxa collected by Rueda et al (2015). Their survey was employed in the west of Khammuane Province, in the cave environment close to the villages, and our surveys were done in the eastern region

along the Nam Theun, Nam Mon, Nam Noy, and Nam On rivers.

Recently, Tangena et al. (2017) reported 51 additional new records to the list of Lao PDR mosquito fauna from Luang Prabang Province, including *Aedes*, *Anopheles*, *Armigeres*, *Culex*, *Heizmania*, *Lutzia*, and *Udaya* species (42 species), five species complexes and four specimens identified

Table 2. Continued.

50	<i>Anopheles (Anopheles) hodgkini</i> Reid	Toma et al. 2002; Chen-Hussey 2012; Suwonkerd et al. 2013; Tangena et al. 2017
51	<i>Anopheles (Anopheles) hyrcanus</i> (Pallas)	Sidavong et al. 2004
52	<i>Anopheles (Anopheles) insulaeflorum</i> (Swellegrebel and Swellegrebel de Graaf)	Tangena et al. 2017
53	<i>Anopheles (Anopheles) separatus</i> (Leicester)	Tangena et al. 2017
54	<i>Anopheles (Anopheles) sinensis</i> Wiedemann	Hewitt et al. 2013; Lefebvre 1938
55	<i>Anopheles (Anopheles) umbrosus</i> (Theobald)	Vythilingam et al. 2003; Marcombe et al. 2017; Tangena et al. 2017
56	<i>Anopheles (Anopheles) whartoni</i> Reid	Tangena et al. 2017
57	<i>Anopheles (Cellia) aconitus</i> Doenitz	Gaschen 1934; Hii and Rueda 2013; Apiwathnasorn 1986; Vythilingam et al. 2001; Sidavong et al. 2004; Toma et al. 2002; Vythilingam et al. 2003; Vythilingam et al. 2006; Hiscox et al. 2013a; WRBU 2018; Marcombe et al. 2017; Tangena et al. 2017
58	<i>Anopheles (Cellia) annularis</i> Van der Wulp	Hii and Rueda 2013
59	<i>Anopheles (Cellia) culicifacies</i> Giles	Hii and Rueda 2013; Gaschen 1934; WRBU 2018; Kobayashi et al. 1997; Toma et al. 2002; Chen-Hussey 2012; Tangena et al. 2017
60	<i>Anopheles (Cellia) dirus</i> Peyton and Harrison	Hii and Rueda 2013; Tsuda et al. 2002; Vythilingam et al. 2001, 2003, 2005, 2006; WRBU 2018; Kobayashi et al. 1997, 2000; Toma et al. 2002; Sidavong et al. 2004; Chen-Hussey 2012; Suwonkerd et al. 2013; Tangena et al. 2017
61	<i>Anopheles (Cellia) dravidicus</i> Christophers	Hii and Rueda 2013; Vythilingam et al. 2006; Sidavong et al. 2004; WRBU 2018; Marcombe et al. 2017
62	<i>Anopheles (Cellia) epiroticus</i> Linton and Harbach	Tangena et al. 2017
63	<i>Anopheles (Cellia) harrisoni</i> Harbarch and Manguin	WRBU 2018
64	<i>Anopheles (Cellia) indefinitus</i> (Ludlow)	Hii and Rueda 2013; WRBU 2018
65	<i>Anopheles (Cellia) jamesii</i> Theobald	Apiwathnasorn 1986; Kobayashi et al. 2000; Vythilingam et al. 2001; Toma et al. 2002; Chen-Hussey 2012; Hii and Rueda 2013; WRBU 2018; Tangena et al. 2017
66	<i>Anopheles (Cellia) jeyporiensis</i> James	Hii and Rueda 2013; Gaschen 1934; Lefebvre 1938; Vythilingam et al. 2003; WRBU 2018; Apiwathnasorn 1986; Kobayashi et al. 1997; Chen-Hussey 2012; Suwonkerd et al. 2013; Tangena et al. 2017
67	<i>Anopheles (Cellia) karwari</i> (James)	Hii and Rueda 2013; Vythilingam et al. 2003, 2005; Sidavong et al. 2004; WRBU 2018
68	<i>Anopheles (Cellia) kochi</i> Donitz	Hii and Rueda 2013; Lefebvre 1938; Apiwathnasorn 1986; Kobayashi et al. 1997, 2000; Vythilingam et al. 2001, 2003, 2006; Sidavong et al. 2004; WRBU 2018; Toma et al. 2002; Chen-Hussey; Marcombe et al. 2017; Tangena et al. 2017
69	<i>Anopheles (Cellia) maculatus</i> Theobald	Gaschen 1934; Lefebvre 1938; Apiwathnasorn 1986; Kobayashi et al. 1997; Vythilingam et al. 2001, 2003, 2005, 2006; Sidavong et al. 2004; Toma et al. 2002; Chen-Hussey 2012; Hii and Rueda 2013; Suwonkerd et al. 2013; WRBU 2018; Marcombe et al. 2017
70	<i>Anopheles (Cellia) minimus</i> Theobald	Lefebvre 1938; Apiwathnasorn 1986; Kobayashi et al. 1997, 2000; Vythilingam et al. 2001, 2003, 2005, 2006; Toma et al. 2002; Sidavong et al. 2004; Chen-Hussey 2012; Hii and Rueda 2013; Suwonkerd et al. 2013; WRBU 2018; Marcombe et al. 2017
71	<i>Anopheles (Cellia) nivipes</i> (Theobald)	Vythilingam et al. 2001, 2003, 2006; Sidavong et al. 2004; WRBU 2018; Marcombe et al. 2017
72	<i>Anopheles (Cellia) notanandai</i> Rattanarithikul and Green	Hii and Rueda 2013; Vythilingam et al. 2006; Sidavong et al. 2004; WRBU 2018
73	<i>Anopheles (Cellia) pallidus</i> Theobald	Vythilingam et al. 2001, 2003; Sidavong et al. 2004
74	<i>Anopheles (Cellia) pampanai</i> Buttiker and Beales	Vythilingam et al. 2001, 2006; Toma et al. 2002; Sidavong et al. 2004; Chen-Hussey 2012; WRBU 2018; Marcombe et al. 2017; Tangena et al. 2017
75	<i>Anopheles (Cellia) philippinensis</i> Ludlow	Lefebvre 1938; Apiwathnasorn 1986; Kobayashi et al. 1997, 2000; Vythilingam et al. 2001, 2003, 2006; Sidavong et al. 2004; Toma et al. 2002; Chen-Hussey 2012; Hii and Rueda 2013; Hiscox et al. 2013a; Suwonkerd et al. 2013; WRBU 2018; Marcombe et al. 2017; Tangena et al. 2017
76	<i>Anopheles (Cellia) pseudowillmori</i> (Theobald)	Hii and Rueda 2013; Vythilingam et al. 2006; Sidavong et al. 2004; WRBU 2018; Rueda et al. 2015
77	<i>Anopheles (Cellia) rampae</i> Harbach and Somboon	Marcombe et al. 2017
78	<i>Anopheles (Cellia) sawadwongporni</i> Rattanarithikul and Green	Vythilingam et al. 2006; Sidavong et al. 2004; WRBU 2018; Marcombe et al. 2017
79	<i>Anopheles (Cellia) splendidus</i> Koidzumi	Vythilingam et al. 2001, 2003, 2006; Sidavong et al. 2004; WRBU 2018
80	<i>Anopheles (Cellia) subpictus</i> Grassi	Hii and Rueda 2013; Vythilingam et al. 2001
81	<i>Anopheles (Cellia) sundalcus</i> (Rodenwaldt)	Hii and Rueda 2013
82	<i>Anopheles (Cellia) tessellatus</i> Theobald	Apiwathnasorn 1986; Kobayashi et al. 1997; Vythilingam et al. 2001, 2006; Toma et al. 2002; Sidavong et al. 2004; Chen-Hussey 2012; Hii and Rueda 2013; Hiscox et al. 2013a; WRBU 2018; Marcombe et al. 2017; Tangena et al. 2017
83	<i>Anopheles (Cellia) vagus</i> Donitz	Hii and Rueda 2013; Gaschen 1934; Lefebvre 1938; Vythilingam et al. 2001, 2003, 2006; Marcombe et al. 2017
84	<i>Anopheles (Cellia) varuna</i> Iyengar	Vythilingam et al. 2001, 2003, 2006; Toma et al. 2002; Sidavong et al. 2004; WRBU 2018; Hii and Rueda 2013; Tangena et al. 2017
85	<i>Armigeres (Armigeres) aureolineatus</i> (Leicester)	WRBU 2018
86	<i>Armigeres (Armigeres) confusus</i> Edwards	Tangena et al. 2017
87	<i>Armigeres (Armigeres) durhami</i> (Edwards)	WRBU 2018
88	<i>Armigeres (Armigeres) foliatus</i> Brug	Tangena et al. 2017
89	<i>Armigeres (Armigeres) jugraensis</i> (Leicester)	Tangena et al. 2017
90	<i>Armigeres (Armigeres) kesseli</i> Ramalingam	Tangena et al. 2017

Table 2. Continued.

90	<i>Armigeres (Armigeres) kesseli</i> Ramalingam	Tangena et al. 2017
91	<i>Armigeres (Armigeres) kuchingensis</i> Edwards	Apiwathnasorn 1986; Vythilingam et al. 2006; WRBU 2018; Tangena et al. 2017
92	<i>Armigeres (Armigeres) laoensis</i> Toma and Miyagi	Toma and Miyagi 2003; WRBU 2018
93	<i>Armigeres (Armigeres) malayi</i> (Theobald)	Tangena et al. 2017
94	<i>Armigeres (Armigeres) moultoni</i> Edwards	Apiwathnasorn 1986; WRBU 2018; Tangena et al. 2017
95	<i>Armigeres (Armigeres) setifer</i> Delfinado	Vythilingam et al. 2006; WRBU 2018
96	<i>Armigeres (Armigeres) subalbatus</i> (Coquillett)	Apiwathnasorn 1986; Vythilingam et al. 2006; Hiscox 2011; Hiscox et al. 2016; WRBU 2018; Tangena et al. 2017; #
97	<i>Armigeres (Armigeres) theobaldi</i> Barraud	Vythilingam et al. 2006; WRBU 2018; Tangena et al. 2017
98	<i>Armigeres (Leicesteria) annulipalpis</i> (Theobald)	Tangena et al. 2017
99	<i>Armigeres (Leicesteria) annulitarsis</i> (Leicester)	Apiwathnasorn 1986; WRBU 2018; Tangena et al. 2017
100	<i>Armigeres (Leicesteria) balteatus</i> Macdonald	Tangena et al. 2017
101	<i>Armigeres (Leicesteria) digitatus</i> (Edwards)	Tangena et al. 2017
102	<i>Armigeres (Leicesteria) dolichocephalus</i> (Leicester)	Apiwathnasorn 1986; WRBU 2018; Tangena et al. 2017
103	<i>Armigeres (Leicesteria) flavus</i> (Leicester)	Apiwathnasorn 1986; Rueda et al. 2015; WRBU 2018; Tangena et al. 2017
104	<i>Armigeres (Leicesteria) inchoatus</i> Barraud	Tangena et al. 2017
105	<i>Armigeres (Leicesteria) longipalpis</i> (Leicester)	Apiwathnasorn 1986; Rueda et al. 2015; WRBU 2018; Tangena et al. 2017; #
106	<i>Armigeres (Leicesteria) magnus</i> (Theobald)	Apiwathnasorn 1986; Rueda et al. 2015; WRBU 2018; Tangena et al. 2017
107	<i>Armigeres (Leicesteria) omissus</i> (Edwards)	Tangena et al. 2017
108	<i>Armigeres (Leicesteria) pectinatus</i> (Edwards)	WRBU 2018
109	<i>Armigeres (Leicesteria) traubi</i> Macdonald	Tangena et al. 2017
110	<i>Coquillettidia (Coquillettidia) crassipes</i> (Van der Wulp)	Vythilingam et al. 2006; WRBU 2018
111	<i>Coquillettidia (Coquillettidia) ochracea</i> (Theobald)	WRBU 2018; Rueda et al. 2015
112	<i>Culex (Culex) alis</i> Theobald	Chen-Hussey 2012; Tangena et al. 2017
113	<i>Culex (Culex) edwardsi</i> Barraudi	Tangena et al. 2017
114	<i>Culex (Culex) fuscocephala</i> Theobald	Apiwathnasorn 1986; Vythilingam et al. 2006; Chen-Hussey 2012; Hiscox et al. 2013a; WRBU 2018; Tangena et al. 2017
115	<i>Culex (Culex) gelidus</i> Theobald	Apiwathnasorn 1986; Toma et al. 2002; Vythilingam et al. 2006; Hiscox 2011; Chen-Hussey 2012; WRBU 2018; Tangena et al. 2017
116	<i>Culex (Culex) hutchinsoni</i> Barraud	Vythilingam et al. 2006; Chen-Hussey 2012; WRBU 2018; Tangena et al. 2017
117	<i>Culex (Culex) Mimeticus</i> Complex	<b>new record</b>
118	<i>Culex (Culex) Mimulus</i> Complex	Tangena et al. 2017; #
119	<i>Culex (Culex) perplexus</i> Leicester	Chen-Hussey 2012; Tangena et al. 2017
120	<i>Culex (Culex) pseudovishnui</i> Colless	Vythilingam et al. 2006; WRBU 2018
121	<i>Culex (Culex) quinquefasciatus</i> Say	Apiwathnasorn 1986; Vythilingam et al. 2006; Chen-Hussey 2012; Hiscox et al. 2013a; Rueda et al. 2015; WRBU 2018; Tangena et al. 2017
122	<i>Culex (Culex) sitiens</i> Wiedemann	Apiwathnasorn 1986; Chen-Hussey 2012; Tangena et al. 2017
123	<i>Culex (Culex) tritaeniorhynchus</i> Giles	Vythilingam et al. 2006; WRBU 2018; Rueda et al. 2015
124	<i>Culex (Culex) vishnui</i> Theobald	Vythilingam et al. 2006; WRBU 2018; Toma et al. 2002; Chen-Hussey 2012; Hiscox et al. 2013a; Tangena et al. 2017; #
125	<i>Culex (Culex) whitei</i> Barraud	Chen-Hussey 2012; Tangena et al. 2017
126	<i>Culex (Culex) whitmorei</i> (Giles)	Apiwathnasorn 1986; Toma et al. 2002; Vythilingam et al. 2006; Hiscox 2013a; Chen-Hussey 2012; WRBU 2018; Tangena et al. 2017
127	<i>Culex (Culicomyia) Complex</i>	<b>new record</b>
128	<i>Culex (Culicomyia) dispectus</i> Bram	Tangena et al. 2017;
129	<i>Culex (Culicomyia) nigropunctatus</i> Edwards	Vythilingam et al. 2006; Chen-Hussey 2012; Rueda et al. 2015; WRBU 2018; Tangena et al. 2017; #
130	<i>Culex (Culicomyia) pallidothorax</i> Theobald	<b>new record</b>
131	<i>Culex (Culicomyia) papuensis</i> (Taylor)	Tangena et al. 2017
132	<i>Culex (Culicomyia) termi</i> Thurman	Tangena et al. 2017
133	<i>Culex (Eumelanomyia) foliatus</i> Brug	Chen-Hussey 2012; Tangena et al. 2017
134	<i>Culex (Lophoceraomyia) binocornutus</i> (Theobald)	<b>new record</b>
135	<i>Culex (Lophoceraomyia) Mammilifer and Wilfredi</i> Groups	Tangena et al. 2017

Table 2. Continued.

136	<i>Culex (Oculeomyia) bitaeniorhynchus</i> Giles	Apiwathnasorn 1986; Vythilingam et al. 2006; Chen-Hussey 2012; Hiscox et al. 2013a; WRBU 2018; Tangena et al. 2017
137	<i>Culex (Oculeomyia) longicornis</i> Sirivanakarn	Tangena et al. 2017
138	<i>Culex (Oculeomyia) pseudosinensis</i> Colless	Vythilingam et al. 2006; WRBU 2018; Tangena et al. 2017
139	<i>Culex (Oculeomyia) sinensis</i> Theobald	Vythilingam et al. 2006; WRBU 2018; #
140	<i>Ficalbia minima</i> (Theobald)	WRBU 2018
141	<i>Heizmannia (Heizmannia) chengi</i> Lien	Tangena et al. 2017
142	<i>Heizmannia (Heizmannia) complex</i> (Theobald)	Apitwathnasorn 1986; WRBU 2018; Tangena et al. 2017
143	<i>Heizmannia (Heizmannia) demeyloni</i> Mattingly	Tangena et al. 2017
144	<i>Heizmannia (Heizmannia) mattinglyi</i> Thurman	Tangena et al. 2017
145	<i>Heizmannia (Mattinglyia) achaetae</i> (Leicester)	<b>new record</b>
146	<i>Hodgesia malay</i> Leicester	WRBU 2018
147	<i>Lutzia (Metalutzia) halifaxii</i> (Theobald)	<b>new record</b>
148	<i>Lutzia (Metalutzia) vorax</i> Edwards	Tangena et al. 2017; #
149	<i>Mansonia (Mansonioides) annulifera</i> (Theobald)	Vythilingam et al. 2006; WRBU 2018
150	<i>Mansonia (Mansonioides) dives</i> (Schiner)	Vythilingam et al. 2006
151	<i>Mansonia (Mansonioides) indiana</i> Edwards	Vythilingam et al. 2006; WRBU 2018
152	<i>Mansonia (Mansonioides) uniformes</i> (Theobald)	Vythilingam et al. 2006; WRBU 2018; Rueda et al. 2015
153	<i>Mimomyia (Mimomyia) chamberlaini</i> Ludlow	WRBU 2018
154	<i>Mimomyia (Mimomyia) hybrida</i> (Leicester)	WRBU 2018
155	<i>Orthopodomyia albipes</i> Leicester	Rueda et al. 2015
156	<i>Topomyia (Topomyia) gracilis</i> Leicester	WRBU 2018
157	<i>Toxorhynchites (Toxorhynchites) albipes</i> (Edwards)	WRBU 2018
158	<i>Toxorhynchites (Toxorhynchites) gravelyi</i> (Edwards)	<b>new record</b>
159	<i>Toxorhynchites (Toxorhynchites) kempfi</i> (Edwards)	WRBU 2018
160	<i>Toxorhynchites (Toxorhynchites) sunthorni</i> Thurman	<b>new record</b>
161	<i>Tripteroides (Rachionotomyia) aranoides</i> (Theobald)	WRBU 2018
162	<i>Tripteroides (Rachionotomyia) ponmeki</i> Miyagi and Toma	Miyagi and Toma 2001; WRBU 2018
163	<i>Tripteroides (Tripteroides) Complex</i>	<b>new record</b>
164	<i>Udaya argyrurus</i> (Edwards)	Tangena et al. 2017; #
165	<i>Uranotaenia (Pseudoficalbia) nivipleura</i> Leicester	WRBU 2018
166	<i>Uranotaenia (Pseudoficalbia) novobscura</i> Barraud	WRBU 2018; #
167	<i>Uranotaenia (Uranotaenia) macfarlanei</i> Edwards	<b>new record</b>
168	<i>Verrallina (Verrallina) dux</i> (Dyar and Shannon)	WRBU 2018
169	<i>Verrallina (Harbachius) yusafi</i> (Barraud)	Tangena et al. 2017
170	<i>Verrallina (Verrallina) lugubris</i> (Barraud)	Tangena et al. 2017

to genus level. All voucher specimens in the report of Tangena et al. (2017), however, were unfortunately damaged or lost, and we were not able to conduct any further morphological examinations or DNA analysis of those specimens from this study. Attempts should be made to recollect specimens reported by Tangena et al. (2017) to further confirm the true identity of the species, particularly by using molecular analysis. None of the reports mentioned above aid molecular analysis, except Marcombe et al. (2017), which identified 13 *Anopheles* species/groups by molecular methods.

Lao PDR has an extensive network of rivers, such as the Mekong river and its tributaries, which facilitate population movement within the country and between the country and its neighbors and can also be a source of vector-disease spread. The Mekong River and its tributaries, with the presence of a tropical climate, can be a good resource for development of larvae and their spread throughout the country. For these reasons, many provinces in Lao PDR remain to be sampled, particularly those border areas, due to the presence of efficient vectors and extensive population movement between Lao PDR and its neighboring countries.

### Current biodiversity survey in Khammuane Province

#### *Aedes*

Most *Aedes* species were found in sympatry, in rock pools, and a few specimens in bamboo traps (Table 1). *Aedes macfarlanei* and *Ae. elsiae* were the most abundant species collected (Table 1). The biology and vector status of these two species remain unknown. Several species of *Aedes*, especially of subgenus *Stegomyia*, are major vectors of various organisms that cause human infectious diseases such as dengue, yellow fever, chikungunya, Zika viruses, and filariasis (Huang 1979, 2004, Peters 1992).

A bootstrap tree was made by adding two specimens of *Ae. malayensis* Colless (MG921175-MG921176) and two specimens of *Ae. albopictus* (MG921177-MG921178) collected in this survey already published in Motoki et al. (2018) (Figure 2). Morphological characters were corroborated with DNA barcodes to confirm *Ae. malayensis*, *Ae. albopictus* (identified in Motoki et al. 2018), *Ae. elsiae*, *Ae. saxicola*, *Ae. ganapathi*, *Ae. vittatus*, and *Ae. prominens* (Figure 2).

The subgenus *Gilesius* comprises *Ae. alius* Lien and

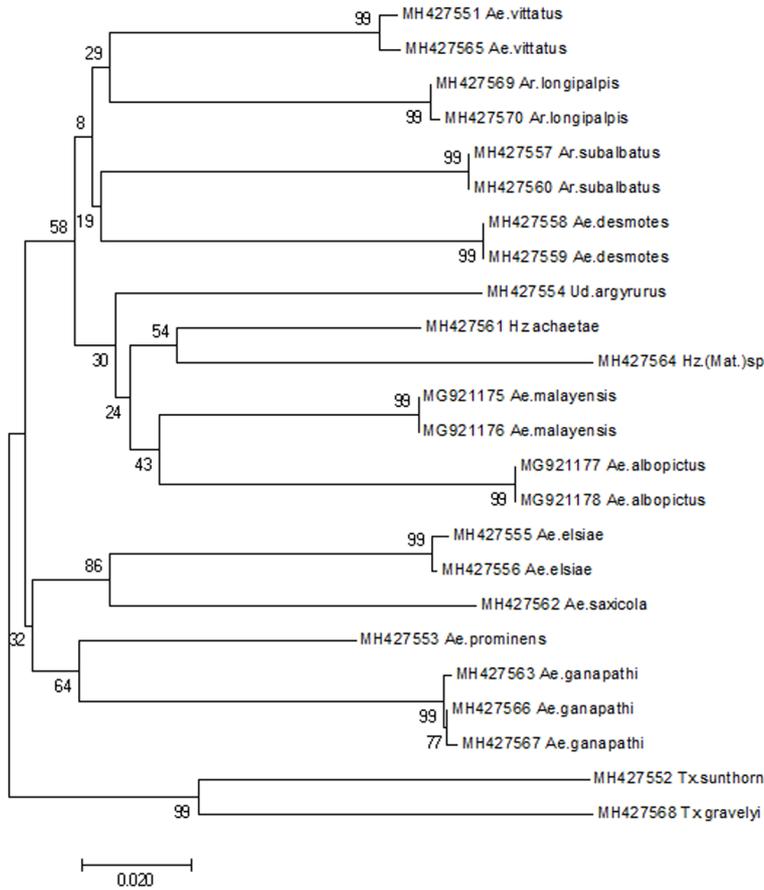
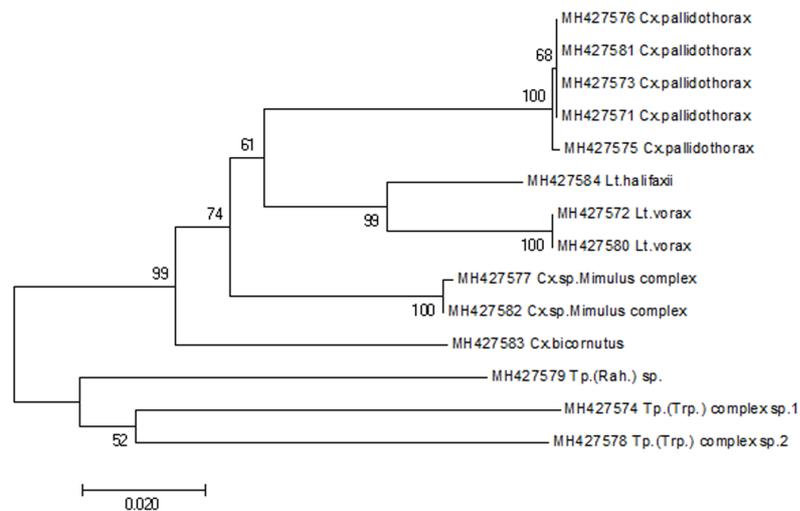


Figure 2. Bootstrapped tree of *COI* gene of *Aedes*, *Armigeres*, *Heizmannia*, *Udaya* and *Toxorhynchites* species (MH427551-MH427570) inferred using the neighbor-joining method based on 5,000 replicates of Tamura-Nei algorithm. Bootstrap values less than 50% are not shown. Scale bar represents sequence (%) divergence between samples. *Aedes malayensis* (MG921175-MG921176) and *Ae. albopictus* (MG921177-MG921178) were added from Motoki et al. (2018).

Figure 3. Bootstrapped tree of *COI* gene of *Culex*, *Lutzia* and *Tripteroides* species (MH427571-MH427584) inferred using the neighbor-joining method based on 5,000 replicates of Tamura-Nei algorithm. Bootstrap values less than 50% are not shown. Scale bar represents sequence (%) divergence between samples.



*Ae. pulchriverter* (Giles) (WRBU 2018). We identified 27 specimens as *Ae. (Gilesius)* sp. in rock pools along the Nam Theun River; however, more analyses are needed to accurately identify these specimens.

#### **Armigeres and Heizmannia**

All species of these two genera were found in the Nam Noy River area and most specimens were collected using manual aspirators and HDN traps, while a small number were collected from rock pools. At least four species of subgenus *Armigeres* and one species of subgenus *Leicesteria* in genus *Armigeres*, and at least five species of subgenus *Heizmannia* in genus *Heizmannia* are found in Lao PDR. *Armigeres (Armigeres) laoensis* Toma and Miyagi was described in 2003 based on samples from Khammuane Province (Toma and Miyagi 2003). Morphological characters of specimens from our collections were corroborated with DNA barcodes to confirm *Ar. subalbatus*, *Ar. longipalpis*, *Hs. Achaetae*, and *Hs. (Mat.)* sp (Figure 2). DNA barcodes were also generated for an additional number of samples of *Heizmannia* and *Armigeres* species. We assumed that *Hs. (Mat.)* sp. should be *Hs. catesi* (Lien); however, more samples should be checked molecularly and morphologically to determine if those specimens comprise only one species or a complex of species.

*Armigeres subalbatus* (Figure 2) has been known to be a vector of Japanese encephalitis virus (Liu et al. 2013), human filariasis in India (Das et al. 1983), and zoonotic filariasis in Malaysia (Cheong et al. 1981, Muslim et al. 2013). Furthermore, Hiscox et al. (2016) isolated a dengue 3 virus from *Ar. subalbatus* but did not conduct competency studies to confirm its status as a vector. The vector status of other species of *Armigeres* and all the species of *Heizmannia* remains unknown.

#### **Udaya and Topomyia**

Only one specimen was found of each genus, namely *Udaya argyrurus* (Edwards) and *Topomyia* sp. These two species were collected using manual aspirators and HDN traps (Table 1). No species of these genera appear to be of medical or economic importance to humans.

#### **Anopheles**

Numerous specimens of *Anopheles* were collected from Khammuane Province in rock pools using manual aspirators. Marcombe et al. (2017) tested insecticide resistance of *Anopheles* species in Lao PDR and identified 13 *Anopheles* species/groups by molecular methods. We collected 17 specimens of *An. maculatus* Group but could not identify the specimens to species using morphological characters. Some *Anopheles* species (*An. dirus*, *An. maculatus*, *An. minimus*) have been incriminated as vectors of malaria in Lao PDR, but the medical importance of many other anopheline species remains unknown.

#### **Culex and Lutzia**

Samples were collected from bamboo and rock pools using HDN traps. Various species of *Culex* are primary vectors of Japanese encephalitis virus in Thailand, Vietnam, Malaysia,

and India (Gould et al. 1974, Thoa et al. 1974, Gingrich et al. 1992, Samuel et al. 1998) and vectors of filariasis in India, Sri Lanka, and Thailand (Iyengar 1938, Carter 1948, Sucharit et al. 1988, Jitpakdi et al. 1998). Larvae of *Lutzia* species are predaceous, particularly on larvae of other mosquitoes (Rattanaarithikul et al. 2005). The COI barcode sequences from this report identified *Cx. pallidothorax* in *Cx. (Culiciomyia)* Complex and *Cx. bicornutus* in subgenus *Lophoceraomyia* of genus *Culex* (Figure 3). About 258 specimens of *Cx. (Culiciomyia)* Complex and 75 specimens of *Cx. (Lophoceraomyia)* spp. were identified (Table 1). Furthermore, *Lt. vorax* and *Lt. halifaxii* were identified both morphologically and molecularly (Figure 3). Additional DNA barcodes need to be generated to examine the diversity of the *Cx.* complex in Lao PDR.

#### **Tripteroides**

Little is known about the medical importance of this genus, except for a few specimens that attack and bite humans (Rattanaarithikul et al. 2007). The habitats of larvae of *Tp. ponmeki* Miyagi and Toma seem to be similar to species of the genus *Armigeres* (Miyagi and Toma 2001). Larvae were found in bamboo and one adult was captured by HDN traps in our collections. COI barcodes identified one species of *Tp. (Rachionotomyia)* sp. and two species into the *Tp. (Tripteroides)* Complex (Figure 3).

#### **Toxorhynchites**

The species of *Toxorhynchites* from our samples were collected in rock pools and bamboo (Figures 2 and 3). *Toxorhynchites gravely* (Edwards), *Tx. sunthorni* Thurman, and other species of the subgenus *Toxorhynchites* have been reported in Thailand. Because of their predatory behavior, usually a few larvae of other mosquitoes are found in association with the larvae of this genus in the same habitat. Larvae of *Toxorhynchites* species feed principally on the larvae of other mosquito species (Rattanaarithikul et al. 2007).

#### **Uranotaenia**

*Uranotaenia (Pseudoficalbia) novobscura* Barraud and *Ur. (Uranotaenia) macfarlanei* Edwards were collected in rock pools or using HDN traps. One specimen of *Ur. (Pse.)* and one specimen of *Ur. (Ura.)* were not possible to identify morphologically, and they may need molecular analysis to confirm their species status. The species of genus *Uranotaenia* have not been incriminated as vectors of human pathogens; however, they have been found to be positive for certain viruses (Rattanaarithikul et al. 2007).

In summary, we generated 34 sequences of COI barcode region of Lao PDR samples, which could aid in molecular identification of 23 species (Figures 2 and 3). A list of mosquitoes from Lao PDR was updated containing 170 species, including 15 new taxa records (Table 2). *Aedes elsiae*, one of the new records in Lao PDR, and its abundance with *Ae. macfarlanei* (Table 1) in all locations collected, require more attention in relation to their vector status. The taxonomic status of *Armigeres* and *Heizmannia* and the diversity of *Culex* species still need to be clarified. Further analysis

including morphology plus molecular methods are required. We are in the process of elucidating the taxonomic status of *Armigeres* species in Lao PDR. This survey may help health personnel map out some risk areas for infectious diseases as well as vector control interventions. Nevertheless, additional studies, such as ecological surveys, are warranted on vector surveillance to improve the knowledge of the entomofauna in Lao PDR.

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Article 2: First Record of *Aedes (Stegomyia) malayensis* Colless (Diptera: Culicidae) in the  
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# First Record of *Aedes (Stegomyia) malayensis* Colless (Diptera: Culicidae) in the Lao People's Democratic Republic, Based on Morphological Diagnosis and Molecular Analysis

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## ABSTRACT

This is the first confirmed record of *Aedes (Stegomyia) malayensis* Colless from the Lao People's Democratic Republic. Its larvae were collected from rock pools and rock holes along the Nam Noy River in the Nakai Nam Theun National Protected Area, Khammuane Province. Larvae were reared in the laboratory and emerged adults were identified based on morphological characters and mitochondrial DNA analysis, using data from the mitochondrial cytochrome c oxidase subunit I. Detailed photographs of the morphological diagnostic characters and information on the bionomics of *Ae. malayensis* are included.

The Nakai Nam Theun National Protected Area (NNT NPA), known as the Watershed Management and Protection Authority area bordering Vietnam, is located in Nakai District, Khammuane Province, Lao People's Democratic Republic (PDR). It is an important Southeast Asian biodiversity area, containing mammals, birds, reptiles, amphibians,<sup>1</sup> and insects, including mosquitoes. It is also the home of a number of rare or newly discovered species of animals.<sup>2-5</sup>

Rueda et al<sup>6</sup> reported a total of 101 species from Laos, including newly recorded *Aedes (Stegomyia)* species collected from Khammuane province, ie *Aedes (Stegomyia) albopictus* (Skuse) and *Ae. (Stg.) pseudoscutellaris* (Theobald), and those reported in the literature, ie, *Ae. (Stg.) aegypti* (Linnaeus), *Ae. gardnerii imitator* (Leicester), *Ae. (Stg.) seatoi* Huang, *Ae. (Stg.) annandalei* (Theobald), *Ae. (Stg.) craggi* (Barraud), *Ae. (Stg.) malikuli* Huang, *Ae. (Stg.) perplexus* (Leicester), *Ae. (Stg.) desmotes* (Giles), *Ae. (Stg.) pseudalbopictus* (Borel).<sup>7-9</sup> All voucher specimens of these newly recorded species, as reported by Rueda et al,<sup>6</sup> were deposited in the Smithsonian Institution, National Museum of Natural History, Washington, DC, and in the Entomology Laboratory, Institut Pasteur Laos. Tangena et al<sup>10</sup> added an additional 51 species to the list of Lao mosquito fauna. However, all voucher specimens from that report were, unfortunately, damaged or lost, and we were not able to conduct any further morphological examinations or DNA analysis of those specimens from this study.

The subgenus *Stegomyia* of genus *Aedes* has 128 valid species worldwide,<sup>11</sup> with 24 species found in the Greater Mekong subregion of Asia (Cambodia, China, Laos, Myanmar, Thailand, and Vietnam), including 12 species from Lao PDR. Several species of subgenus *Stegomyia* are major vectors of various organisms that cause human infectious diseases such as dengue, yellow fever, chikungunya, Zika viruses, and filariasis.<sup>12-14</sup>

*Aedes aegypti* is the primary vector of dengue throughout the tropical and subtropical regions of the world.<sup>15</sup> *Aedes albopictus* is also an important vector in dengue epidemics.<sup>16-18</sup> *Aedes malayensis* Colless is widely distributed in many parts of Asia,<sup>19</sup> particularly Cambodia, India, Malaysia, Singapore, Taiwan, Thailand, and Vietnam.<sup>11</sup> A recent study showed a high susceptibility of *Ae. malayensis* and its vectorial capacity for both dengue serotype 2 and chikungunya.<sup>20</sup> This species is recorded for the first time in the Lao PDR. In this study, we confirmed the identification of *Ae. malayensis* from Laos based on the cytochrome c oxidase subunit I (*COI*) mitochondrial gene and the morphological diagnostic characteristics of adults, and compared them with *Ae. albopictus*.

## MATERIALS AND METHODS

### Specimen Collection

Larvae of mosquitoes were collected from aquatic habitats along the Nam Noy River (17.768548°N, 105.381989°E), Lao PDR (Figure 1) in March 2017, using standard larval dippers (350 ml, 13 cm diam ( BioQuip,

**FIRST RECORD OF *Aedes (Stegomyia) malayensis* Colless (Diptera: Culicidae) in the Lao People's Democratic Republic, based on morphological diagnosis and molecular analysis**

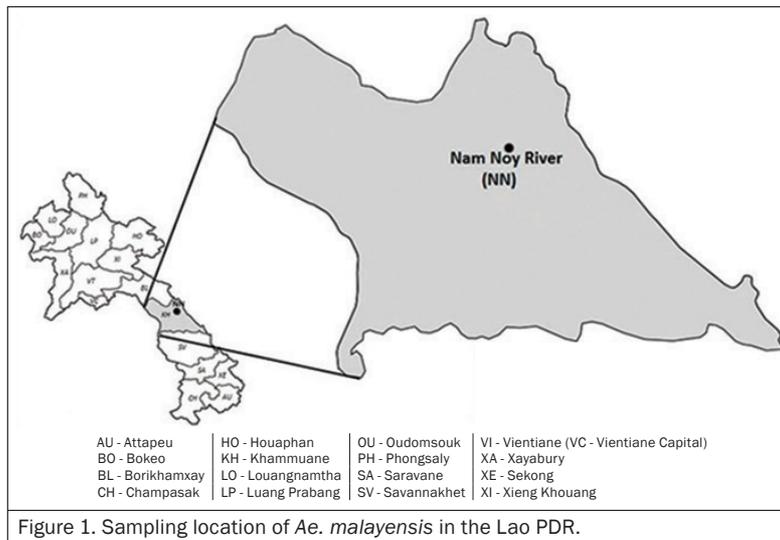


Figure 1. Sampling location of *Ae. malayensis* in the Lao PDR.

Rancho Dominguez, CA, USA). They were carefully transferred into a WhirlPak plastic bag (BioQuip) using pipettes, and transported to the laboratory of Institut Pasteur du Laos.

**Morphological Identification**

Emerged adults were pinned on paper points, each given a unique collection number, properly labeled, and identified using a stereomicroscope (Olympus SZX7, Tokyo, Japan) following the morphological keys of Rattanarithikul et al.<sup>21</sup> Voucher specimens were deposited in the Entomology Collection, Institut Pasteur du Laos, Vientiane, Lao PDR, and the National Mosquito Collections of the Smithsonian Institution, National Museum of Natural History, Washington, DC. Diagnostic characters of *Ae. malayensis* adults were photographed.

**DNA Extraction and Sequencing**

Total genomic DNA was extracted from a single whole mosquito using Macherey-Nagel NucleoSpin Tissue (GmbH & Co KG, Duren, Germany) according to manufacturer's instructions. The fragment of mitochondrial cytochrome c oxidase subunit I (mtDNA *COI*) gene was amplified using the polymerase chain reaction (PCR) Master Mix 2X (Promega Corporation, Madison, WI, USA) utilizing LCO1490 and HCO2198 primers.<sup>22</sup> The PCR protocol consisted of a one minute denaturation at 94°C and 5 cycles at 94°C for 40 seconds, 45°C for 40 seconds and 72°C for one minute, followed by 30 cycles at 94°C for 40 seconds, 49°C for 40 seconds and 72°C for one minute, and a 5-minute extension at 72°C. The PCR amplicons were electrophoresed in 1.5% TAE agarose gels stained with GelRed Nucleic Acid Gel Stain (Biotium Inc, Hayward, CA, USA), and the PCR

products were then cleaned by adding ExosapIT (USB Co, Cleveland, OH, USA). Samples were placed in the thermocycler and ran at 37°C for 30 minutes, followed by 80°C for 15 minutes.

All sequencing reactions were conducted in both directions using the original primers and the Big Dye Terminator Kit v.3.1 (PE Applied Biosystems, Warrington, UK), analyzed on an ABI Prism 3500xL - Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were edited in Sequencher v.5.4.6 (Genes Codes Co, Ann Arbor, MI, USA), and aligned using Geneious 9.1.6.<sup>23</sup> A bootstrapped<sup>24</sup> Neighbor Joining tree<sup>25</sup> was used based on

1,000 replicates. The evolutionary distances were calculated using the Kimura-2 parameters method<sup>26</sup> conducted in MEGA v.7.<sup>27</sup> All 658 base pair (bp) of the barcode fragment were included in pairwise comparisons. Two sequences of *Ae. albopictus* were used as an outgroup.

**RESULTS**

**Morphological Diagnosis**

The adult *Ae. malayensis* is very similar to *Ae. albopictus*, except for the diagnostic characters given below<sup>21</sup>:

1. A supraalar area of thorax with a patch of pale scales extended toward the scutellum in *Ae. malayensis*, and a supraalar area of thorax with spot of pale scales not extended toward the scutellum in *Ae. albopictus* (Figure 2);
2. Abdominal terga IV-VI with dorsal white bands connected to lateral pale patches in *Ae. malayensis*, and abdominal terga IV-VI with dorsal white bands separated from lateral spots in *Ae. albopictus* (Figure 3).

Furthermore, the adults of *Ae. malayensis* are very similar to adults of *Ae. alcasidi* Huang, *Ae. riversi* Bohart and Ingram, and *Ae. scutellaris* (Walker) and share the following diagnostic characters<sup>19</sup>:

1. Midfemur without median white line on anterior surface;
2. Wing with minute basal spot of white scales on costa; and
3. Hindtarsomere 5 entirely white.<sup>19</sup> Unfortunately, we did not have samples of *Ae. alcasidi*, *Ae. riversi*,

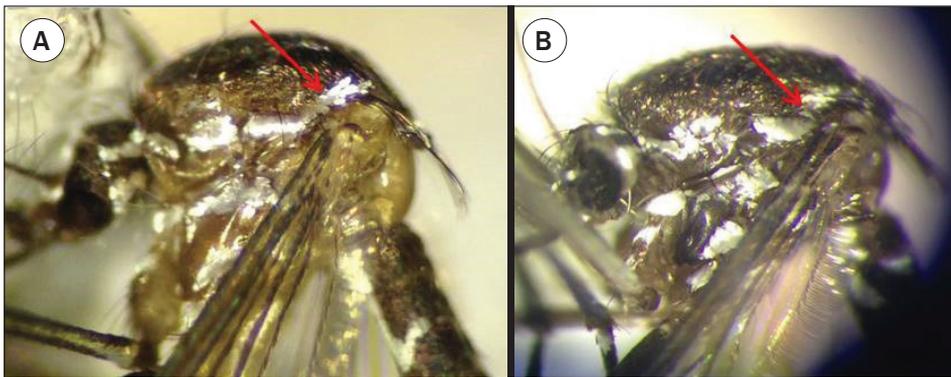


Figure 2. Morphological comparison of the thorax.  
 (A) *Ae. malayensis* - a supraalar area of thorax with a patch of pale scales extended upward toward the scutellum.  
 (B) *Ae. albopictus* - a supraalar area of thorax with spot of pale scales not extended toward the scutellum.

or *Ae. scutellaris* to compare, but we present the photographs of those diagnostic characters of *Ae. malayensis* from the Lao PDR (Figure 4).

#### Bionomics

Larvae (n=31) of *Ae. malayensis* were collected in the NNT NPA from rock holes and rock pools along the edges of the Nam Noy River, in an open area between the river and the forest. The habitats were not completely shaded, but some of them were naturally shaded by the rocks with fresh, clean, and cool water, and without vegetation (Figure 5). Larvae of *Ae. albopictus* were also collected from the same habitats in association with *Ae. malayensis*. Adults (n=17) of *Ae. malayensis* were collected using sweep nets near larval habitats. Adult females were attracted to humans and fed on their blood.

#### Molecular Characterization

The fragment of *COI* was sequenced for 4 specimens of *Ae. malayensis*, and 2 specimens of *Ae. albopictus* were used as an outgroup (GenBank numbers: MG921172-MG921178). We compared them with the DNA barcode sequences of our samples with those published previously (KY420809, KY420810, KY420811; KR349280, KR349282).<sup>20</sup> The amplicon length of the barcode sequence of *Ae. malayensis* was consistent at 658 bp (without primers). The base compositions were similar for all specimens, 14.31% G, 15.98% C, 29.22% A, and 40.49% T. The bootstrap consensus tree confirmed the differences observed in the morphology of the *Ae. malayensis* female adult compared with *Ae. albopictus* (Figure 6).

#### COMMENT

Larvae of *Ae. malayensis* have been found in tree holes in Singapore and Taiwan, and in coconut shells in Vietnam. In Thailand, they were found in rock holes, rock pools, water jars, and bamboo cups, while in Malaysia they were collected from rock pools, bamboo stumps, a coconut shell, and artificial containers.<sup>19</sup> In the Lao PDR, *Ae. malayensis* was found in rock pools (Figure 5) and rock holes together with *Ae. albopictus*. This sympatry

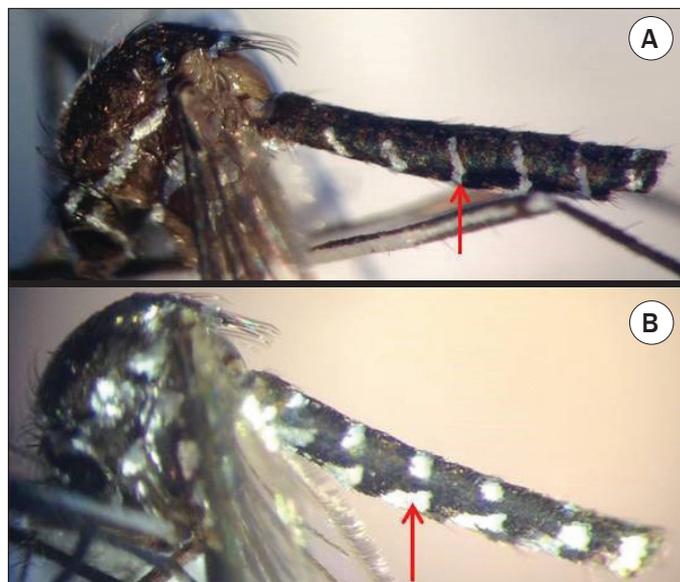


Figure 3. Morphological comparison of the abdominal terga IV-VI.  
 (A) *Ae. malayensis* - dorsal white bands connected to lateral pale patches.  
 (B) *Ae. albopictus* - dorsal white bands separated from lateral spots.

FIRST RECORD OF *Aedes (Stegomyia) malayensis* Colless (Diptera: Culicidae) in the Lao People's Democratic Republic, based on morphological diagnosis and molecular analysis

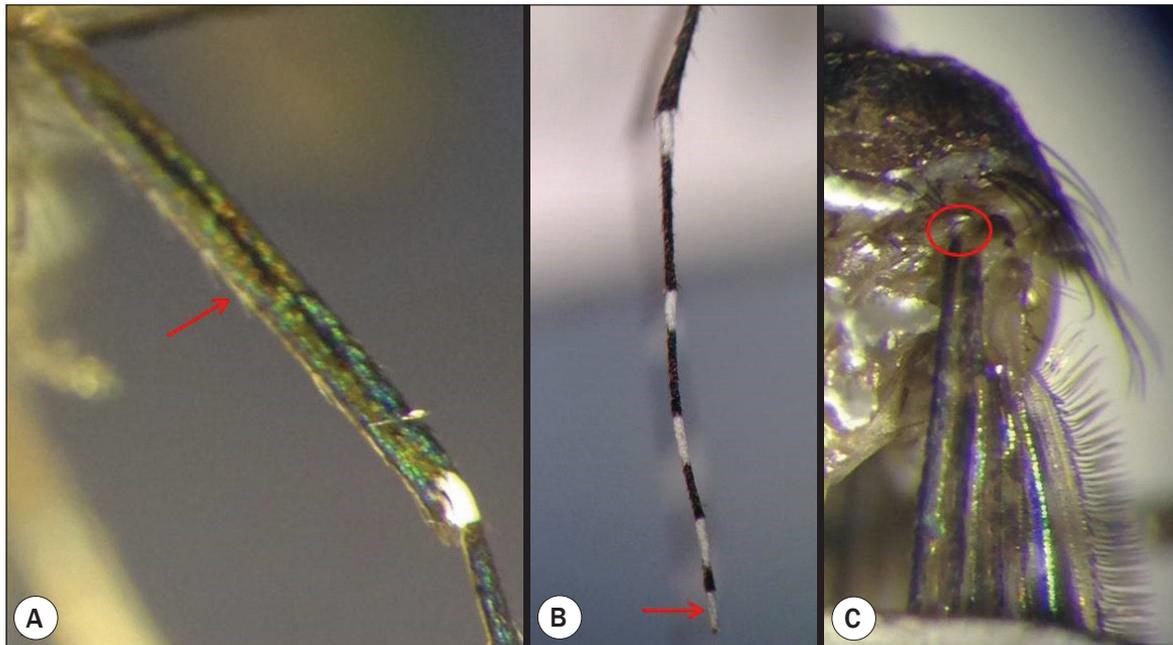


Figure 4. Morphological characters of *Ae. malayensis* that are similar to *Ae. alcasidi*, *Ae. riversi*, and *Ae. scutellaris*:  
(A) Midfemur without median white line on anterior surface.  
(B) Hindtarsomere 5 entirely white.  
(C) Wing with minute basal spot of white scales on costa.

was also observed in Singapore, Malaysia, Thailand, and Taiwan.<sup>19,28</sup> The larval habitat along the Nam Noy River is approximately 40 km from the nearest urban center of Oudomsouk (17.710971°N, 105.15086°E), and could only be accessed by a 3-hour boat trip.

Larvae of *Ae. malayensis* are morphologically similar to *Ae. albopictus* and are very difficult to separate from each other.<sup>28</sup> The body ornamentations of the adults and larvae are highly variable.<sup>28</sup> Despite the fact that *Ae. albopictus* is very similar to *Ae. malayensis*, the combination of some distinct morphological characters differentiate these 2 species<sup>21</sup> (Figures 2 and 3). Likewise, the adults of *Ae. malayensis* are very similar to adults of *Ae. alcasidi*, *Ae. riversi*, and *Ae. scutellaris*,<sup>19</sup> particularly the wings, midfemur, and hindtarsomere 5 (Figure 4). Overall, *Ae. malayensis* can be especially recognized by its abdominal ornamentation (Figure 3).

DNA barcoding of the mitochondrial *COI* has been an efficient and useful marker for mosquito identification and confirmation of new species.<sup>29-31</sup> Herein, the observed morphological differences are corroborated with the *COI* sequences. The *COI* sequences of *Ae. malayensis* from Lao PDR were clustered with the *COI* sequences of the Singapore samples, and distinguished from the

*Ae. albopictus*, thereby confirming the existence of *Ae. malayensis* in the Lao PDR (Figure 6).

Dengue, chikungunya, Zika, and yellow fever viruses are the most important pathogens associated with the species of subgenus *Stegomyia*.<sup>14,32</sup> Because of the important role of *Aedes (Stegomyia)* species in arbovirus transmission, Huang<sup>28</sup> described for the first time the larvae and pupae, and redescribed both adult sexes of *Ae. malayensis* from samples collected from the type locality in Pulau Hantu, Singapore.

Even though the medical importance of *Ae. malayensis* was not well known at the time, it was found to have strong anthropophilic behavior in Bo-Pia, Prachuap Khiri Khan, Thailand,<sup>19</sup> and India,<sup>33</sup> as well as our recent observations in Nakai, along the Nam Noy River. In addition, Rosen et al<sup>34</sup> reported that *Ae. malayensis* was susceptible to all 4 serotypes of dengue virus after oral infection, but the presence of virus in the mosquito saliva was not determined. Moreover, Mendenhall et al<sup>20</sup> compared the vector competence of *Ae. albopictus* and *Ae. malayensis* with *Ae. aegypti* from Singapore after oral infection with dengue serotype 2 and chikungunya viruses and observed the high susceptibility of *Ae. malayensis* and *Ae. albopictus* to both arboviruses.

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Importantly, the saliva of infected *Ae. malayensis* contained infectious particles for both viruses. This provided the evidence that *Ae. malayensis* and *Ae. albopictus* from Singapore possess all the necessary traits to transmit these arboviruses.

*Aedes malayensis* remains an understudied species of *Stegomyia*, although it is found in Singapore, Malaysia, Thailand, Cambodia, Vietnam, Taiwan, China (Hainan),<sup>19,29,35</sup> India,<sup>33</sup> and now in the Lao PDR. Because of the wide distribution of *Ae. malayensis* in many parts of Asia,<sup>19</sup> its high vector competence,<sup>20,34</sup> and its anthropophilic behavior,<sup>19,33</sup> more studies are warranted on the significance of this *Stegomyia* species as an arbovirus vector in the Lao PDR.



Figure 5. Larval habitats (rock holes) of *Ae. malayensis* in the Nakai District.

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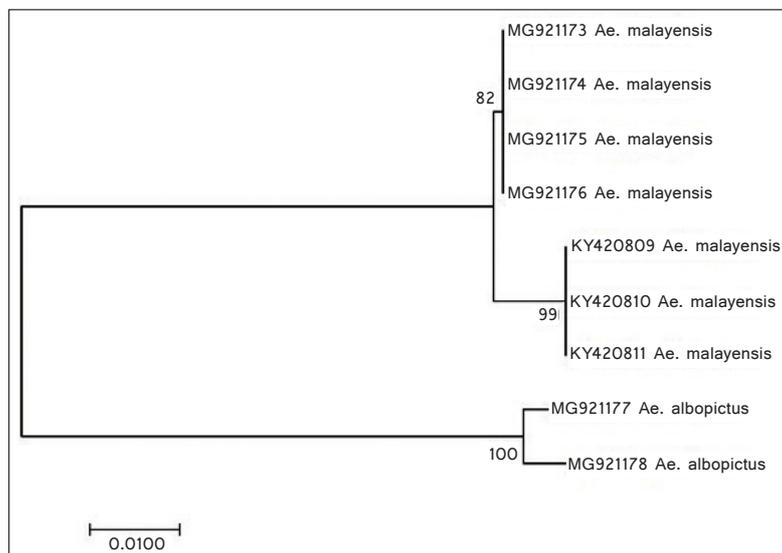


Figure 6. Bootstrapped NJ tree using *COI* sequences of *Ae. malayensis* (MG921172-MG921176 = Lao PDR; KY420809-KY420811 = Singapore<sup>20</sup>) based on 1,000 replicates of Tamura-Nei algorithm. Bootstrap values less than 50% are not shown. Scale bar represents sequence (%) divergence between samples. *Ae. albopictus* (MG921177-MG921178) was used as an outgroup.

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**FIRST RECORD OF Aedes (Stegomyia) Malayensis Colless (Diptera: Culicidae) IN THE LAO PEOPLE'S DEMOCRATIC REPUBLIC, BASED ON MORPHOLOGICAL DIAGNOSIS AND MOLECULAR ANALYSIS**

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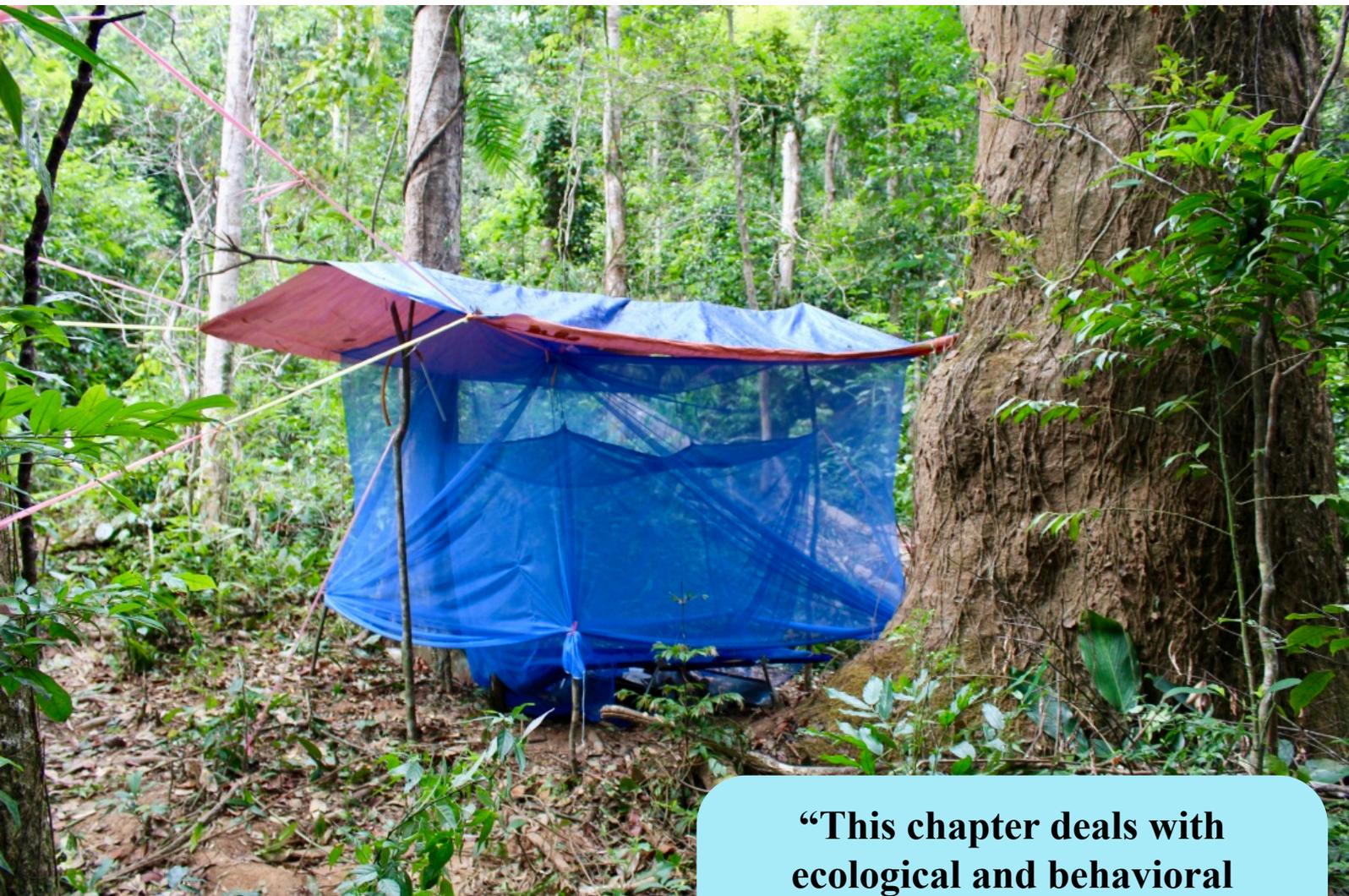
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# Chapter 2

## Identification of sylvatic mosquito species attracted to humans in a forested area of the Nakai Nam Theun National Protected Area



**“This chapter deals with ecological and behavioral aspects of sylvatic mosquitoes attracted to humans in the NNT NPA.”**



## Summary

Little is known about the vector status, ecology, and behavior of mosquito species in Laos. In addition to vector competence, vectorial capacity strongly depends on mosquito ecology and feeding behavior. Host preference, host availability, vector abundance, feeding frequency, encounter rate, and the temporal pattern of blood-feeding activity are some of the main ecological parameters that determine the effective contact between vectors and hosts (Kramer and Ebel 2003). Thus, characterizing the abundance, richness, diversity, and biting behaviors of mosquito species is an essential step in order to assess the risk of spillover of zoonotic, vector-borne pathogens to the human population (Vasilakis et al. 2011). The same is true for assessing the risk of spillback of human vector-borne pathogens establishing novel sylvatic transmission cycles in regions where they were previously absent (Hanley et al. 2013; Bryant et al. 2007).

We conducted a total of four 1-week missions in August (rainy season), November-December (dry season) 2017 and twice in March 2018 (intermediate season) in the NNT NPA. Mosquito species attracted to humans were specifically targeted using human-baited double-net traps (Tangena et al. 2015) deployed in three habitats (riverside, low-cover forest, high-cover forest) along a trail going from the Nam Noy riverbank to deeper in the forest. Mosquitoes were captured around the clock, sacrificed, grouped per 1-hour interval, and identified morphologically back in the laboratory. Based on the captures, we evaluated the abundance, richness, diversity, and daily activity pattern of human-attracted mosquito species and compared these indices between habitats and seasons.

We collected a total of 1,018 females that were morphologically assigned to 9 genera and 26 mosquito taxa, of which 87.6% represented only 8 mosquito species. Some of them are known or putative arbovirus vectors such as *Ae. albopictus*, *Cx. vishnui*, *Ar. subalbus*, and *Ae. malayensis*. Others have been poorly studied and little is known about their potential as pathogen vectors such as *Heizmannia* species, *Ae. desmotes*, and *Ar. jugraensis*. Analyses of species richness and diversity revealed that they were significantly higher during the rainy season, especially in the low-cover forest habitat. Daily activity patterns showed that all human-attracted mosquito species collected were only active during daytime regardless of seasons or habitats. Host-seeking activity differed between the low-cover forest habitat, where mosquitoes were mostly active during the afternoon, and the high-cover forest habitat, where they were more active in the morning, regardless of the season.

## Chapter 2: Identification of sylvatic mosquito species attracted to humans in forested area of the Nakai Nam Theun National Protected Area

The results of this study improve our understanding of mosquito-human interactions in the NNT NPA by providing new information on the species abundance, richness, diversity, and daily activity pattern of mosquitoes caught in human-baited traps. We characterized several human-attracted mosquito species, including *Ae. malayensis*, in a primary forest of Laos, which paves to way for identification of potential bridge vectors in this area.

Personal contributions to the chapter:

I was part of the team that performed all the field collections. I morphologically identified the specimens collected with the help of Dr. Maysa T. Motoki. I performed all the data analyses.

Article 3: Diversity, abundance and daily activity pattern of human-seeking mosquitoes in a forested area of the Nakai district, Laos

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*Manuscript in preparation.*

**Diversity, abundance and daily activity pattern of human-seeking mosquitoes in a forested area of the Nakai district, Laos**

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**Abstract**

Spillover of enzootic, vector-borne pathogens into human populations is often facilitated by mosquito species that ‘bridge’ sylvatic and human transmission cycles. Identifying such bridge vectors requires detailed entomological knowledge at the interface between human populations and the sylvatic habitats. Here, we used human-baited traps to evaluate the abundance, richness, diversity, and daily activity pattern of human-seeking mosquitoes across three habitats (riverside, low-cover forest, high-cover forest) and three seasons (dry, rainy, and intermediate) in a remote area of the Nakai Nam Theun National Protected Area, Laos. We captured a total of 1,018 females that were morphologically assigned to 9 genera and 26 mosquito taxa, of which 87.6% represented only 8 mosquito species. Species richness and diversity were significantly higher during the rainy season, especially for the low-cover forest habitat. All human-attracted mosquito species collected were only active during daytime regardless of seasons or habitats. This study characterized several human-attracted mosquito species in a primary forest of Laos, which paves the way for identification of potential bridge vectors in this area.

**Article Summary Line**

Several mosquito species attracted to humans were identified in a forested area of the Nakai district, Laos.

**Running Title**

Human-attracted mosquitoes in sylvatic Laos.

**Keywords**

Human-vector contact; Bridge vector; Double-net trap.

## Introduction

More than 80% of the global population is at risk of a vector-borne disease, with mosquito-borne diseases being the largest contributor to the human vector-borne disease burden [1]. Mosquito-borne diseases include the deadliest vector-borne disease, human malaria, which caused around 219 million cases and 435,000 deaths in 2017 [2]. Mosquito-borne diseases are also overrepresented among emerging infectious diseases [3,4]. In particular, the past few decades have seen an unprecedented emergence of epidemic mosquito-borne viruses [5]. Dengue, for instance, occurs in at least 128 countries, causing around 390 million human infections and resulting in 96 million symptomatic cases every year [6].

Many emerging mosquito-borne viruses of humans such as dengue virus (DENV), Zika virus (ZIKV), yellow fever virus (YFV) and chikungunya virus (CHIKV) originated in sylvatic transmission cycles where they circulate between vertebrate animals and forest-dwelling mosquitoes [7]. Zoonotic, vector-borne pathogens are generally brought into the human population by mosquito species referred to as bridge vectors, which make the link between sylvatic and human transmission cycles. Bridge vectors display host-feeding behavior towards both humans and non-human animals.

The Nakai Nam Theun National Protected Area (NNT NPA) is a forested area of the Nakai district, Khammuane province in Laos. It consists of dry evergreen forests, cloud forests and mountainous riverbeds that are the biotopes of various mosquito species with increasing incursions of humans. About 20,000 humans reside in and around this area bordering and within the primary forest of the Annamite Range [8]. The inventory of the mosquito species of the region has recently been updated [9] but little is known about their attraction to humans and therefore their potential to act as bridge vectors.

Density, blood-feeding frequency, and daily activity patterns are some of the main ecological parameters that allow effective contact between vectors and hosts [10]. Thus, characterizing the abundance, richness, diversity, and host-seeking behavior of mosquito species is an essential step to assess the risk of spillover of zoonotic mosquito-borne pathogens in the human population [7].

Here, we attempted to identify mosquito species that are attracted to humans using human-baited double-net traps in the NNT NPA. We investigated their abundance, richness, diversity, and human-seeking activity in different habitat types and seasons. Our results provide important baseline information to assess the risk of mosquito-borne disease emergence in this area.

## Materials & Methods

### Ethics statement

This study was approved by the Lao National Ethic Committee for Health Research (approval number 052/NECHR, issued 25-04-2017). Prior to the study, volunteers gave written informed consent for their participation.

### Study area and period

Adult mosquito sampling was conducted in a forested area of the Annamite Range, along the Nam Noy river (17.768548°N, 105.381989°E). The study area is located inside the NNT NPA, also known as the Nakai Nam Theun 2 Watershed Management and Protection Authority (WMPA) area, Nakai district, Khammuane province, Lao PDR. The NNT NPA has a tropical monsoon climate (“Am” on the Köppen climate classification) with an average annual precipitation of 2,500-3,000 mm, mean monthly temperatures and relative humidity ranging from 19°C to 29°C and from 75% to 95%, respectively. The altitude of the collection sites ranged from 566 m to 600 m above sea level with rocky riverbeds at the bottom of densely forested slopes on both sides. We conducted a total of four one-week missions, one in August 2017 (rainy season), one in November-December 2017 (dry season) and two in March 2018 (intermediate season). The recorded climatic data are presented in Table 1.

**Table 1: Climatic data of the study area.**

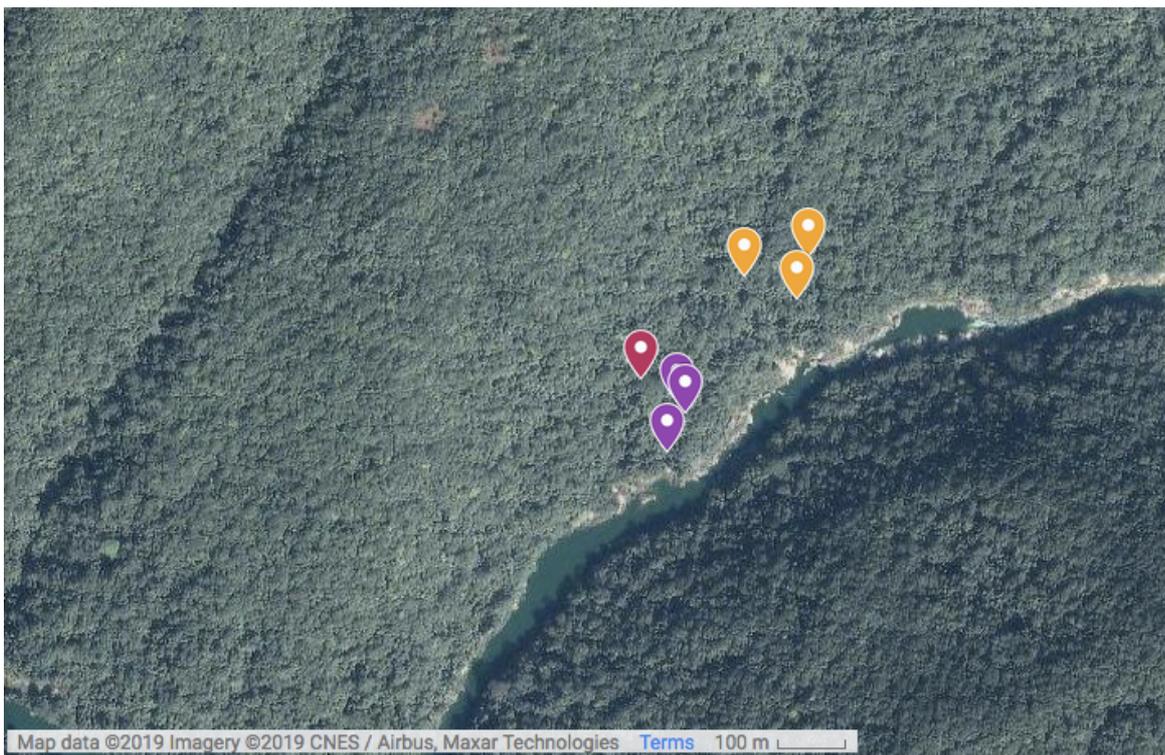
	Temperature (°C)	Relative humidity (%)	Average days with precipitation per month*
<b>August 2017</b>	24.9	94	23
<b>November- December 2017</b>	19.2	88.8	0
<b>March 2018</b>	24.9	80	4

\* data from the Norwegian Meteorological Institute

The mosquito collections were conducted in three distinct ecological habitats connected by a trail going from the riverbank into the forest. The three habitats were separated from one another by at least 100 m. The first habitat was located next to the Nam Noy river and is referred to as riverside (RS) habitat hereafter. The RS habitat is characterized by wide openings in the vegetation, low ground vegetation and high sunshine exposure. The second habitat was further away in the forest and was characterized by a thicker ground vegetation

## Chapter 2: Identification of sylvatic mosquito species attracted to humans in forested area of the Nakai Nam Theun National Protected Area

and denser tree cover with opening. It is referred to as low-cover forest (LCF) habitat hereafter. The third habitat was further away with thick vegetation, dense tree cover, and low sunlight exposure, and is referred to as high-cover forest (HCF) habitat hereafter. We collected in seven locations within those habitats, three in the RS habitat, one in the LCF, and three in the HCF (Figure 1). Collection sites within habitats were deployed in a radius of no more than 100 m. During each four missions, mosquitoes were collected during 6 consecutive days and nights with at least 1 site per habitat covered each time.



**Figure 1: Map of the Nam Noy field site with the location of the collection sites.** Generated using © Google Maps. Purple, red and yellow marks show the collection locations in the riverside habitat, low-cover forest habitat, and high-cover forest habitat, respectively.

### Mosquito collections

Mosquito species attracted to humans were specifically targeted using human-baited double-net traps [11] (Figure 2). Briefly, one human volunteer was resting on a long chair inside a small, untreated bed-net protected from mosquitoes for periods of 6 hours around the clock. A larger bed-net covered the smaller one with a 30-cm opening at the bottom allowing mosquitoes to enter the 20-cm space between bed-nets. A large plastic sheet was placed above bed-nets to protect from the weather. Every 10 min, mosquitoes trapped between bed-nets were collected by the volunteer using a mouth aspirator and if necessary, a head torch.

Aspirated mosquitoes were transferred into holding cups, killed and grouped per 1-hour interval into tubes containing silica gel.



**Figure 2: Human-baited double-net traps *in situ*.**

### **Morphological identification**

Adult mosquitoes were pinned on paper points and assigned a unique identification number. Identification was performed using a stereomicroscope (Olympus SZX7, Tokyo, Japan) following morphological keys from Thailand [12-17] and Vietnam [18]. Voucher specimens were stored in the Entomology Collection, Institut Pasteur du Laos, Vientiane, Lao PDR, and in the National Mosquito Collections of the Smithsonian Institution, National Museum of Natural History, Washington DC.

### **Statistical analyses**

Shannon diversity ( $H'$ ) indexes were calculated using the R package *vegan* [19]. The impact of season, habitat, and their interaction on richness and  $H'$  index was tested using two-way analysis of variance (ANOVA). All statistical analyses were performed using the R software version 3.5.2 [20] and graphical representations were generated with the R packages *ggplot2* [21].

## **Results**

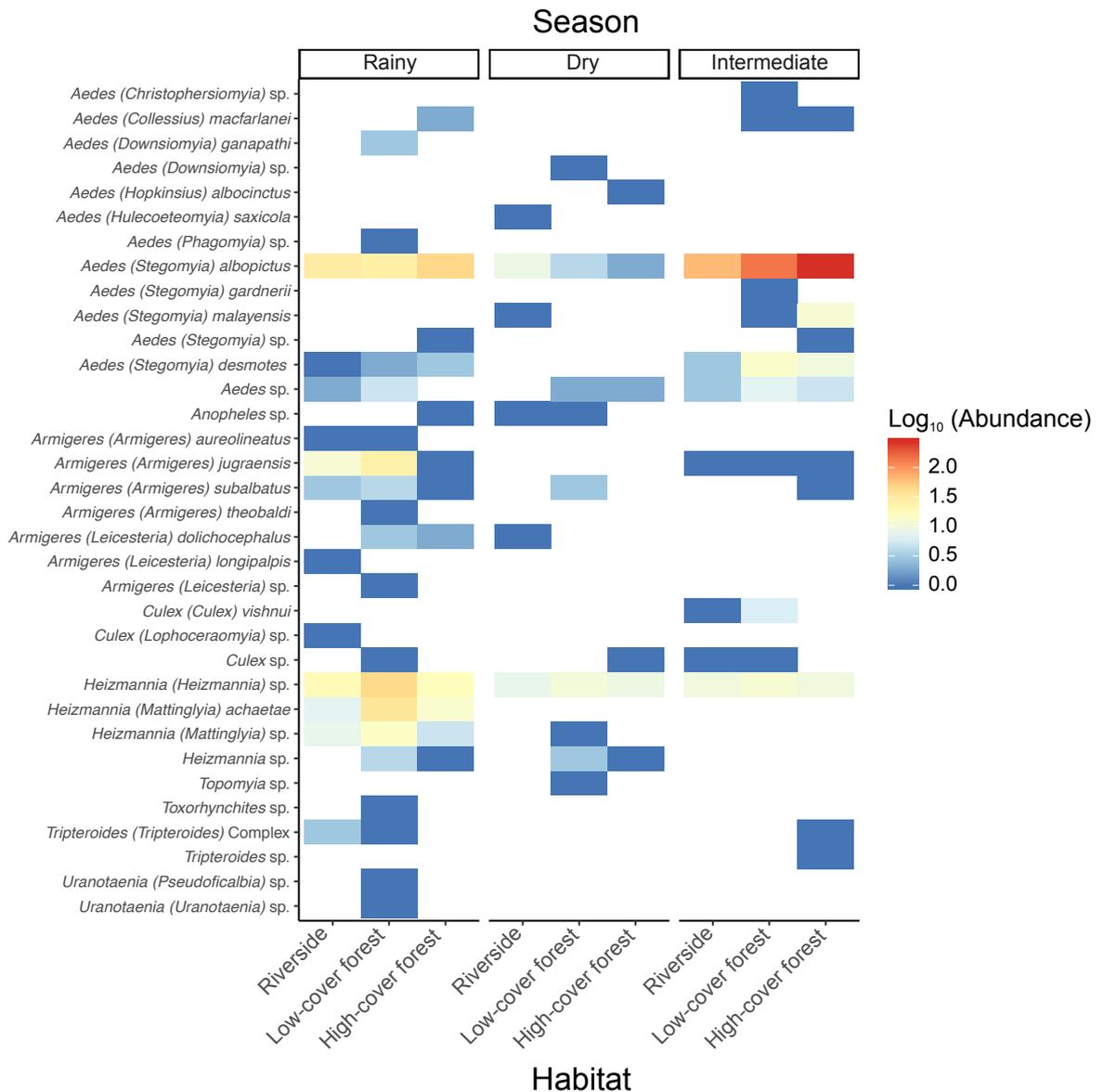
### **Species composition, abundance and richness**

Mosquito collections took place during four field trips of one week each between August 2017 and March 2018. Over the course of the four field trips, a total of 1,018 female mosquitoes were collected in the human-baited double-net traps. We identified 776 specimens (76.2%) to the species level, 224 specimens (22%) could only be determined to the genus or

## Chapter 2: Identification of sylvatic mosquito species attracted to humans in forested area of the Nakai Nam Theun National Protected Area

subgenus level, and 18 specimens were too damaged to be identified. The collected samples belonged to at least 26 different mosquito taxa in 9 genera: *Aedes* Meigen, *Anopheles* Meigen, *Armigeres* Theobald, *Culex* Linnaeus, *Heizmannia* Ludlow, *Topomyia* Leicester, *Toxorhynchites* Theobald, *Tripterooides* Giles, and *Uranotaenia* Lynch Arribalzaga. The most abundant mosquito species were *Ae. (Stegomyia) albopictus* (Skuse), *Hs. (Heizmannia) sp.*, *Hs. (Mattinglyia) achaetae* (Leicester), *Ar. (Armigeres) jugraensis* (Leicester), *Ae. (St.) desmotes* Giles, *Ae. (St.) malayensis* Colless, *Ar. (Arm.) subalbatus* (Coquillett), and *Cx. (Culex) vishnui* Theobald (Figure 3). *Hs. (Hsz.) sp.* specimens were initially identified as *Hs. (Hsz.) chengi* but preliminary molecular confirmation suggested that a complex of species might exist, and we decided to revert to the subgenus only.

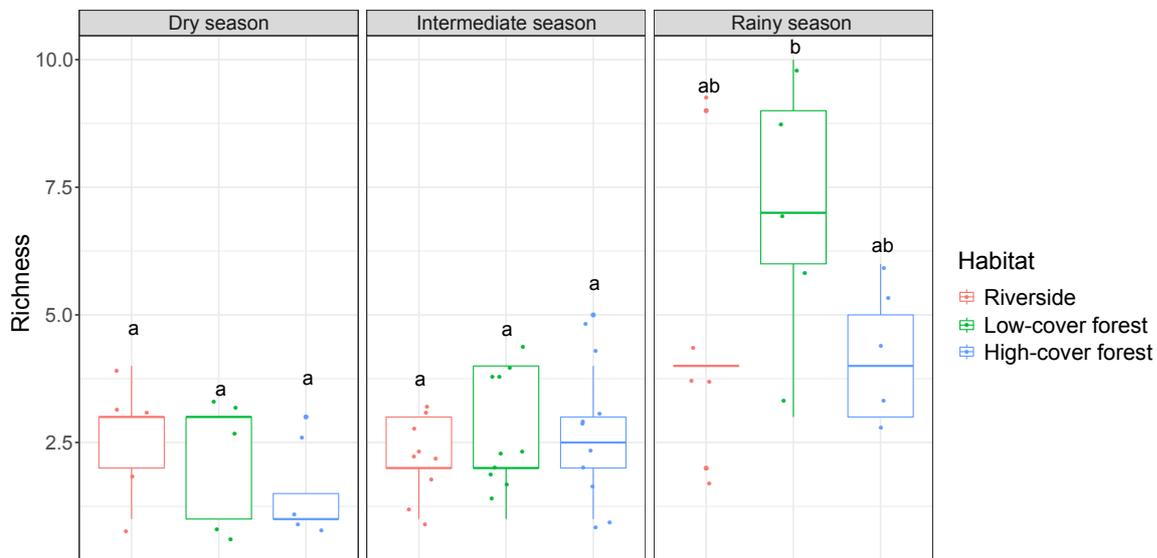
The species abundance was highly variable between seasons and to a lesser extent between habitats (Figure 3). *Aedes albopictus* was present all-year-long in the three habitats with a maximum abundance during the intermediate season and a minimum abundance during the dry season. *Heizmannia* species were predominant during the rainy season with *Hs. (Hsz.) sp.* being present all-year-long with a peak of abundance during the rainy season, and *Hs. (Mat.) achaetae* being present only during the rainy season. *Aedes desmotes*, *Ar. jugraensis*, and *Ar. subalbatus* were present only during the intermediate and rainy seasons with a peak of abundance during the intermediate season for the former one and an opposite pattern for the two latter ones. *Aedes malayensis* was not found during the rainy season and was most abundant in the HCF habitat during the intermediate season. *Culex vishnui* was only collected during the intermediate season and mainly in the LCF habitat.



**Figure 3: Species abundance of human-attracted mosquitoes stratified by season and habitat.** Heat map of the  $\log_{10}$ -transformed total number of mosquitoes caught in the human-baited traps shown by season (rainy, dry, intermediate) and habitat (riverside, low-cover forest, high-cover forest) for each species. Abundance ranges from dark blue (low) to red (high).

## Chapter 2: Identification of sylvatic mosquito species attracted to humans in forested area of the Nakai Nam Theun National Protected Area

Species richness was calculated as the number of unique mosquito taxa captured daily during the three seasons (rainy, dry, and intermediate) across the three habitats (RS, LCF, HCF). Season was the only statistically significant ( $p < 0.0001$ ) predictor of richness (Table 2). The highest richness values were observed during the rainy season with 4.6, 7.0, and 4.2 in RS, LCF, and HCF habitats, respectively and ranged from 1.5 (HCF – dry season) to 2.7 (LCF – intermediate season) in the other conditions (Figure 4). There were no statistically significant differences between the three habitats during dry and intermediate seasons or between RS and HCF habitats during the rainy season. There were no statistically significant differences between habitats during the rainy season. However, species richness was significantly higher ( $p < 0.001$ ) in the LCF habitat during the rainy season compared to the three habitats during the two other seasons.

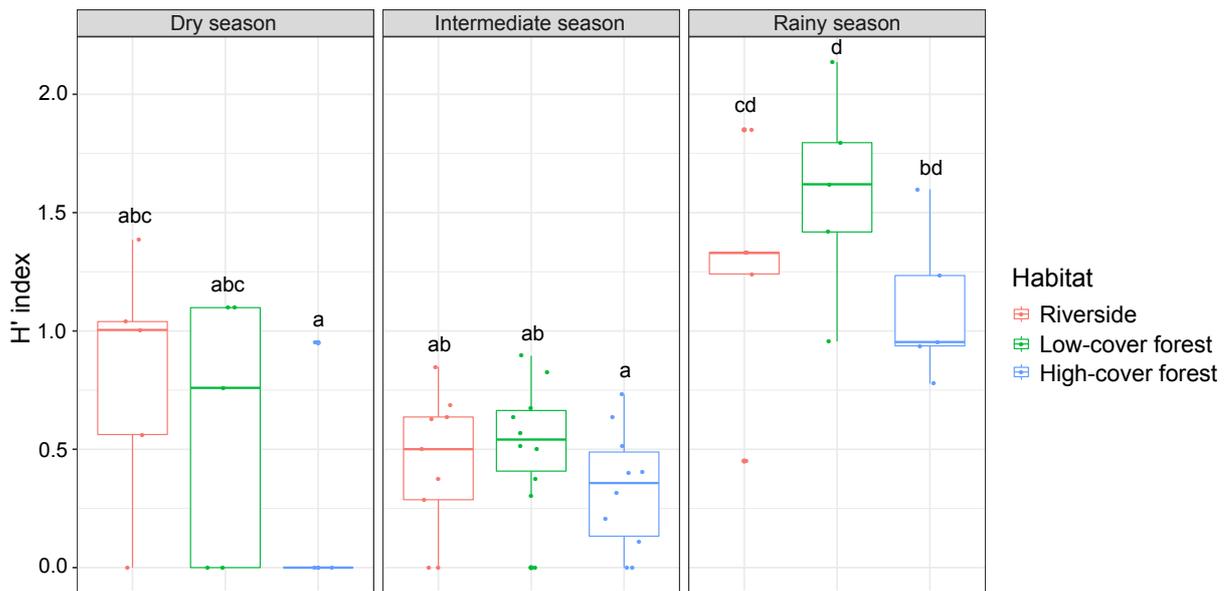


**Figure 4: Species richness of human-attracted mosquitoes stratified by season and habitat.** Boxplots of the daily number of mosquitoes caught in the human-baited traps shown by season (rainy, dry, intermediate) and habitat (riverside, low-cover forest, high-cover forest). Each point in the boxplots represents the species richness of a 24-hour mosquito collection period. Letters above the boxplots indicate statistical significance of the differences. Conditions sharing a letter are not significantly different.

### Species diversity

The Shannon diversity index ( $H'$ ) was used to compare species diversity between seasons and habitats. Season was the main statistically significant predictor of diversity ( $p < 0.0001$ ) followed by habitat ( $p = 0.0275$ ) (Table 2). The highest diversity indices were observed during the rainy season with  $H' = 1.24, 1.58,$  and  $1.1$  in the RS, LCF, and HCF

habitats, respectively and ranged from 0.19 (HCF – dry season) to 0.8 (RS – dry season) in the other conditions (Figure 5). Diversity indices did not differ significantly between dry and intermediate seasons across all habitats. There were no statistically significant differences between habitats during the rainy season. The low number of human-attracted mosquitoes collected in the three habitats during the dry season (only 70 specimens collected in total) resulted in a high variability of the  $H'$  index between each collection-day (e.g. it varied from 0 to 1.39 for the RS habitat).



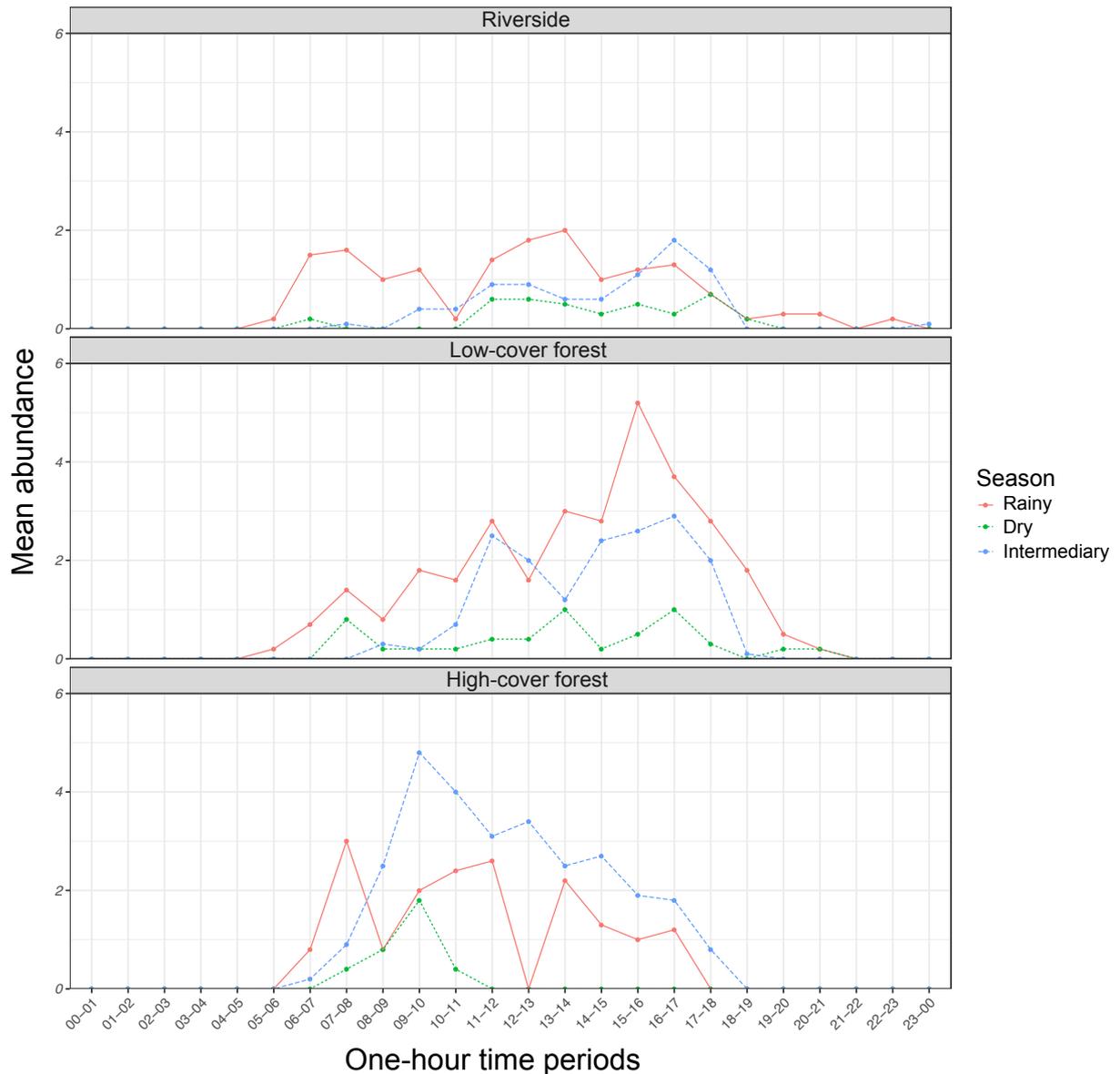
**Figure 5: Diversity of human-attracted mosquitoes stratified by season and habitat.** Boxplots of the daily Shannon diversity ( $H'$ ) index of mosquitoes caught in the human-baited traps by season (rainy, dry, intermediate) and habitat (riverside, low-cover forest, high-cover forest). Each point in the boxplots represents the  $H'$  index of a 24-hour mosquito collection period. Letters above the boxplots indicate statistical significance of the differences. Conditions sharing a letter are not significantly different.

**Table 2. Test statistics of species richness and Shannon diversity (H') index.** The impact of season, habitat, and their interaction on species richness and H' index was tested using two-way ANOVAs. Sum Sq = sum of squares; Df = degrees of freedom.

<b>Species richness</b>					
	<b>Df</b>	<b>Sum Sq</b>	<b>Mean Sq</b>	<b>F value</b>	<b>p value</b>
Season	2	94.27	47.13	21.308	2.2 x 10 <sup>-7</sup>
Habitat	2	9.59	4.80	2.169	0.125
Season x Habitat	4	17.91	4.48	2.024	0.106
Residuals	49	108.39	2.21		
<b>Shannon diversity index (H')</b>					
	<b>Df</b>	<b>Sum Sq</b>	<b>Mean Sq</b>	<b>F value</b>	<b>p value</b>
Season	2	8.040	4.020	27.827	7.54 x 10 <sup>-9</sup>
Habitat	2	1.117	0.558	3.866	0.0275
Season x Habitat	4	0.659	0.165	1.140	0.3486
Residuals	50	7.223	0.144		

### Human-seeking activity

Human-seeking activity was estimated for each habitat and season as the mean hourly number of female mosquitoes caught in the human-baited traps by 24-hour periods (Figure 6). In the RS habitat, human-seeking activity was relatively even from 07:00 to 18:00 during all seasons. In the LCF habitat, mosquitoes were mostly active during the afternoon. In the HCF habitat, in contrast, mosquitoes were mostly active in the morning. For all three habitats and seasons, almost no human-seeking activity was recorded at nighttime (from 19:00 to 6:00) with an abrupt cessation of activity at nightfall (from 18:00 to 19:00). Overall, there was lower activity during the dry season with a mean of around 0.3 mosquito caught per hour during the active period, compared to a mean of around 1.5 mosquitoes caught per hour during the two other seasons. Analyses of human-seeking activity at the species level showed different patterns of activity depending on the species (figure S1). *Aedes albopictus* and *Heizmannia* species were active from sunrise to sundown. *Aedes malayensis* and *Ae. desmotes* were mostly active during the afternoon. *Armigeres jugraensis* and *Ar. subalbatus* were mostly active during the late afternoon. *Culex vishnui* was mostly caught during the late afternoon and early night.



**Figure 6: Daily pattern of human-seeking mosquito activity stratified by habitat and season.** The mean hourly number of mosquitoes caught in the human-baited traps is plotted over time by habitat (riverside, low-cover forest, high-cover forest) and season (rainy, dry, intermediate). Hourly data were collected for 24-hour periods (rainy season: 6 consecutive days and nights; dry season: 6 consecutive days and nights; intermediate season: 2 x 6 consecutive days and nights).

## Discussion

In this study, we characterized mosquito-human interactions in the NNT NPA, Laos. We provided new information on species abundance and richness, diversity, and human-seeking activity patterns of sylvatic mosquito species using human-baited double-net traps. Our findings represent an important first step towards identification of potential bridge vectors in this area.

## Chapter 2: Identification of sylvatic mosquito species attracted to humans in forested area of the Nakai Nam Theun National Protected Area

Four mosquito genera accounted for 97% (987/1,018) of all human-seeking mosquitoes captured in the human-baited traps. The most common genus was *Aedes* (66.6%; 678/1,018) with three species accounting for 94% (637/678) of all *Aedes* captured. The most abundant species overall was *Ae. albopictus*, a well-known arbovirus vector of DENV, CHIKV, and ZIKV, which was present all-year-long in the area. This species is originally a zoophilic forest species from tropical South East Asia [22], which rapidly spread across the world in the last half century [23,24]. The second most abundant species, *Ae. desmotes*, has no record of medical significance but belongs to the same *Stegomyia* subgenus that includes the most important arbovirus vectors (e.g. *Ae. aegypti*) and was present most of the year. Finally, *Ae. malayensis*, another member of the *Stegomyia* subgenus, was found mostly during the intermediate season. This species is widely distributed across South East Asia with documented occurrence in Vietnam, Cambodia, Peninsular Malaysia and Singapore, Taiwan, the Andaman and Nicobar Islands, Laos, and Thailand [17,25-28]. Recently, it was found to be a competent vector of DENV, CHIKV, and YFV in Singapore [27,29]. Moreover, reports from various places in South East Asia showed that it would readily engage in human-biting behavior [25,26,29]. The second most common genus was *Heizmannia* (22.9%; 233/1,018) with *Hs. (Hez.)* sp. and *Hs. (Mat.) achaetae* representing 83.7% (195/233) of them. *Heizmannia* species have never been implicated as vectors of human pathogens and little is known about their bionomics, but they have been reported to bite humans in forests of South East Asia at daytime [17]. *Armigeres* mosquitoes were the third most abundant genus (6.3%; 64/1,018) with *Ar. jugraensis* and *Ar. subalbatus* representing 82.8% (53/64) of them. The latter has been implicated in the transmission of parasites causing human and zoonotic filariasis [30,31] as well as Japanese encephalitis virus (JEV) [32]. *Armigeres jugraensis* has no record of medical significance but belongs to the same subgenus as *Ar. subalbatus*. Finally, *Culex* represented 1.2% (12/1,018) of our captures, with only a single identification to the species level, *Cx. vishnui*, which is a known vector of JEV in many areas of South East Asia [33-35]. Other mosquito taxa were too rare to draw any conclusion regarding their attraction to humans and will need additional fieldwork to elucidate their status.

Our estimates of species richness and diversity were similar during the dry and intermediate seasons in all three habitats. They were higher during the rainy season especially in the LCF habitat, which is consistent with the expectation that rainfall promotes mosquito development. It is interesting to notice that species richness and diversity were higher in the LCF habitat than in the RS and HCF habitats. However, we would need to sample in other locations within the same LCF habitat because we did not have replicate collection sites for

this habitat type in our study. A more in-depth study of the habitat features could help to understand the factors underlying this observation (e.g. availability of breeding sites, protection from abiotic factors). For example, the RS habitat was more subject to the wind and the HCF habitat was less exposed to sunlight compared to the two other habitats. The absence of rain, lower temperatures and humidity, and presence of strong winds, all known environmental factors affecting mosquito survival [10], could explain the lower mosquito abundance observed during the dry season (only 70 mosquitoes collected in one week). The intermediate season was characterized by the overwhelming presence of *Ae. albopictus* in all habitats, which contributed to lower the overall diversity indices.

A possible confounding factor that could have influenced our results is that we did not fully randomize the human volunteers under the human-baited traps between each habitat. The collections involved a total of six different human volunteers, of which three, four and six conducted the collections in the RS habitat, LCF habitat, and HCF habitat, respectively, but not all volunteers-habitat combinations were represented (Table S1). Human attractiveness for mosquitoes varies significantly between individuals [36-38] and this could have influenced the captures in each habitat. Another limitation of our study is that because we could not identify every specimen to the species level, due to the lack of available sequences and the difficulty to discriminate morphologically some of the species, we might have underestimated the richness and diversity of the area.

One striking observation was that all human-attracted mosquito species collected, regardless of season or habitat, were only active during daytime. This indicates that the risk of human-vector contact in this area is exclusively diurnal. In a previous study, specimens of the *An. maculatus* Group, a species complex including nine different species and implicated in malaria transmission in Laos [39], was collected in the same area [9] but was not found in our collections. We only captured three *Anopheles* specimens, indicating the lack of human-attracted *Anopheles* species and a low risk of malaria transmission in the area.

Our field collections of human-seeking mosquitoes in the NNT NPA were dominated by eight mosquito species that represented 87.6% of all specimens. In the absence of vector competence assessment of the local populations, it is unknown to which extent these mosquito species could act as bridge vectors and therefore pose a risk of introducing novel pathogens into the human population. Some of the species, such as *Ae. albopictus* and *Cx. vishnui*, are already known as arbovirus vectors [33-35]. Other species, such as *Ae. malayensis* and *Ar. subalbatus*, are known to be experimentally competent for pathogen transmission [27,29-32].

## Chapter 2: Identification of sylvatic mosquito species attracted to humans in forested area of the Nakai Nam Theun National Protected Area

However, other species, such as *Heizmannia* species, *Ae. desmotes*, *Ar. jugraensis*, have been poorly studied and little is known about their potential as vectors of disease agents. We emphasize the need to expand studies of the sylvatic mosquito fauna, their bionomics and biting behavior, in order to identify candidate bridge vectors. Indeed, most of the major human arboviruses such as DENV, ZIKV, YFV and CHIKV originated in sylvatic transmission cycles [7]. Identifying potential bridge vectors is particularly important in the NNT NPA because of increasing incursions of humans. Most of the people living in rural Laos still depend on resources from the forest to sustain themselves. Additionally, the NNT NPA possesses a rich biodiversity [40] that attracts not only wildlife traffickers but also tourists. Indeed, one of the goals of WMPA is to develop ecotourism in the NNT NPA. Similar projects exist in the rest of country or are in development. Increased human movement promotes contact between forest-dwelling mosquitoes and humans and creates opportunities for pathogen emergence that should be monitored.

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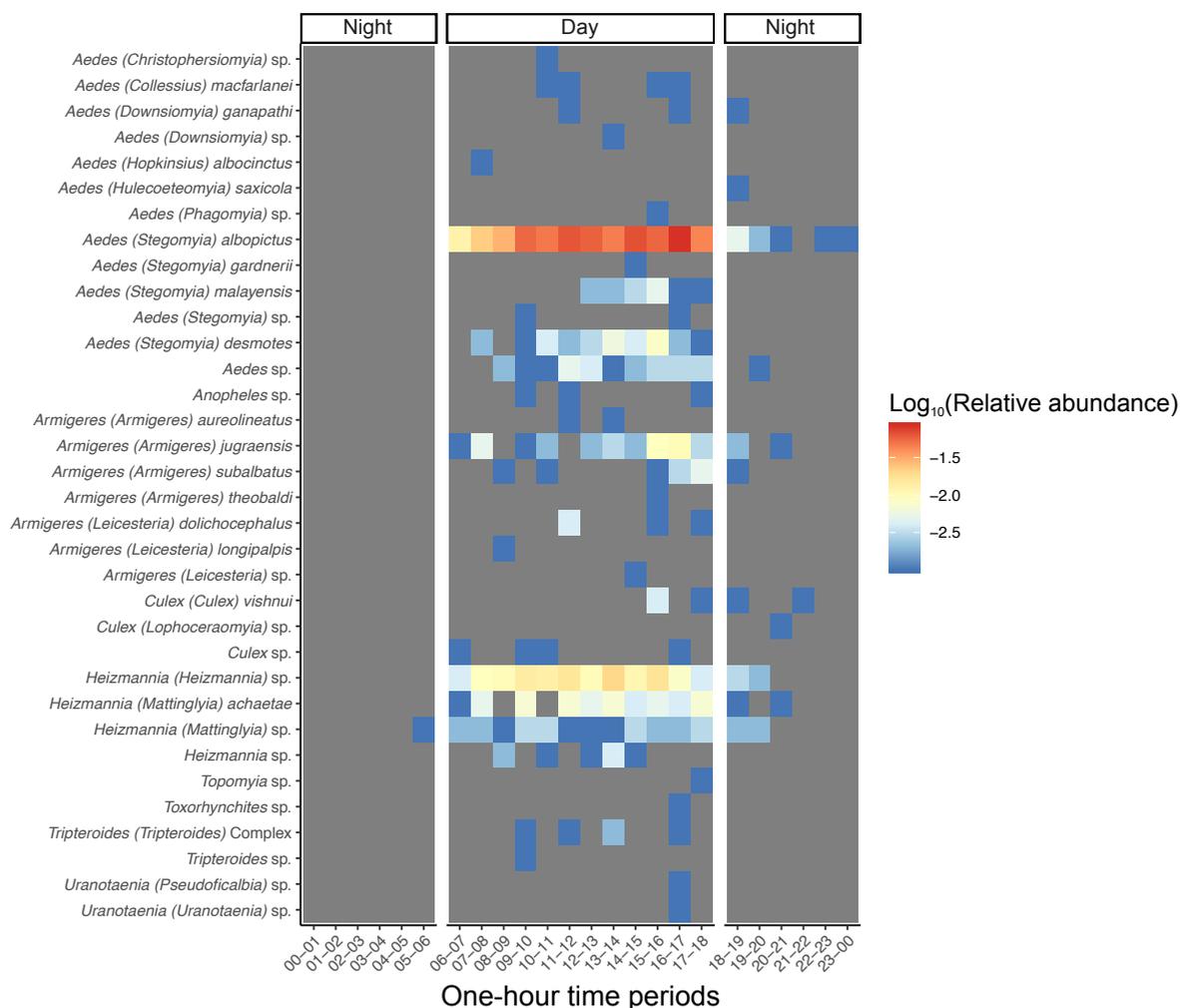
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Supplemental information



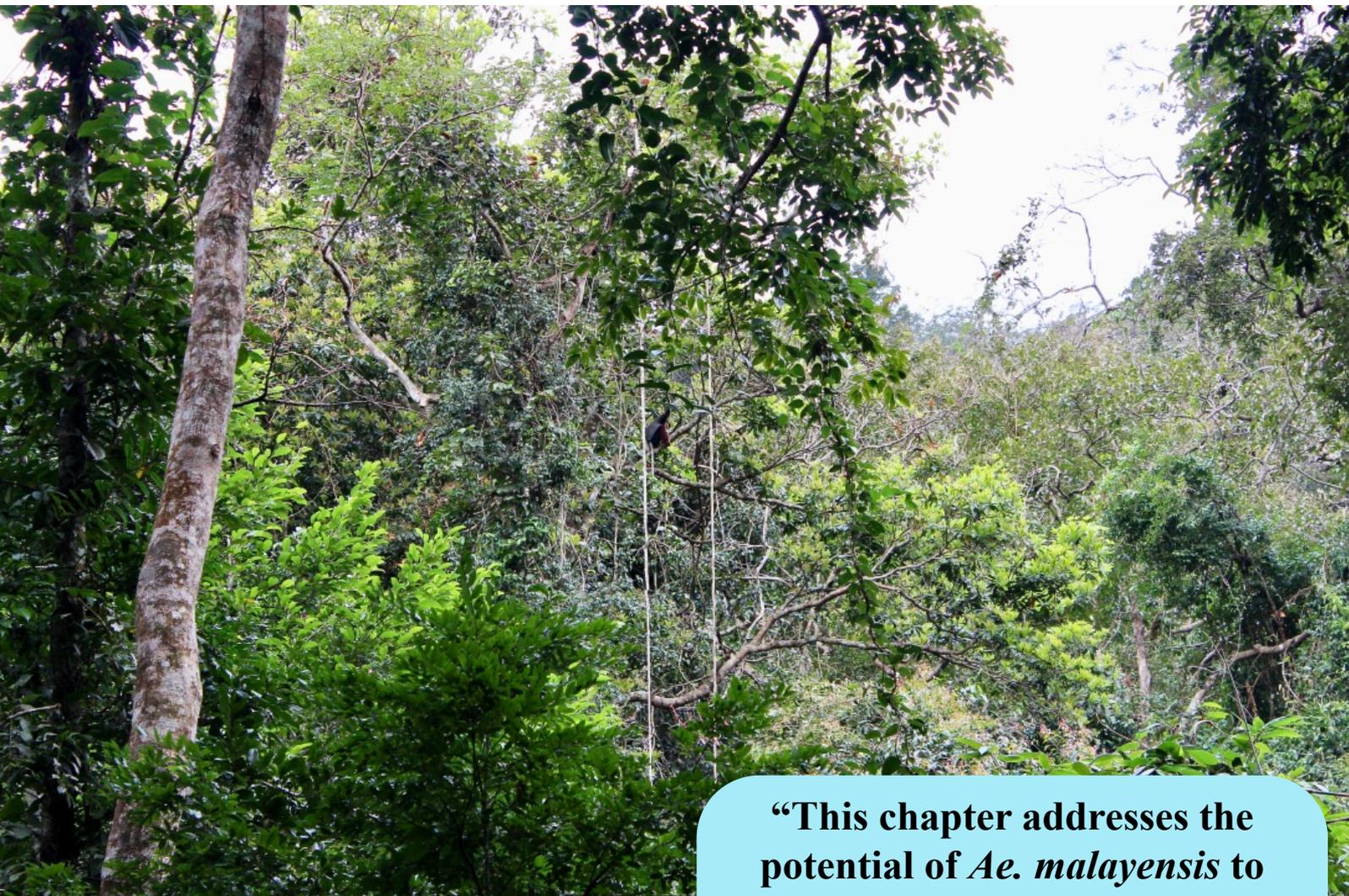
**Figure S1: Daily pattern of human-seeking activity for each mosquito taxon.** Heat map of the  $\text{log}_{10}$ -transformed hourly relative abundance of mosquitoes caught in the human-baited traps for each taxon. Data from each habitat and season were pooled.

**Table S1: Habitat types sampled by each human volunteer**

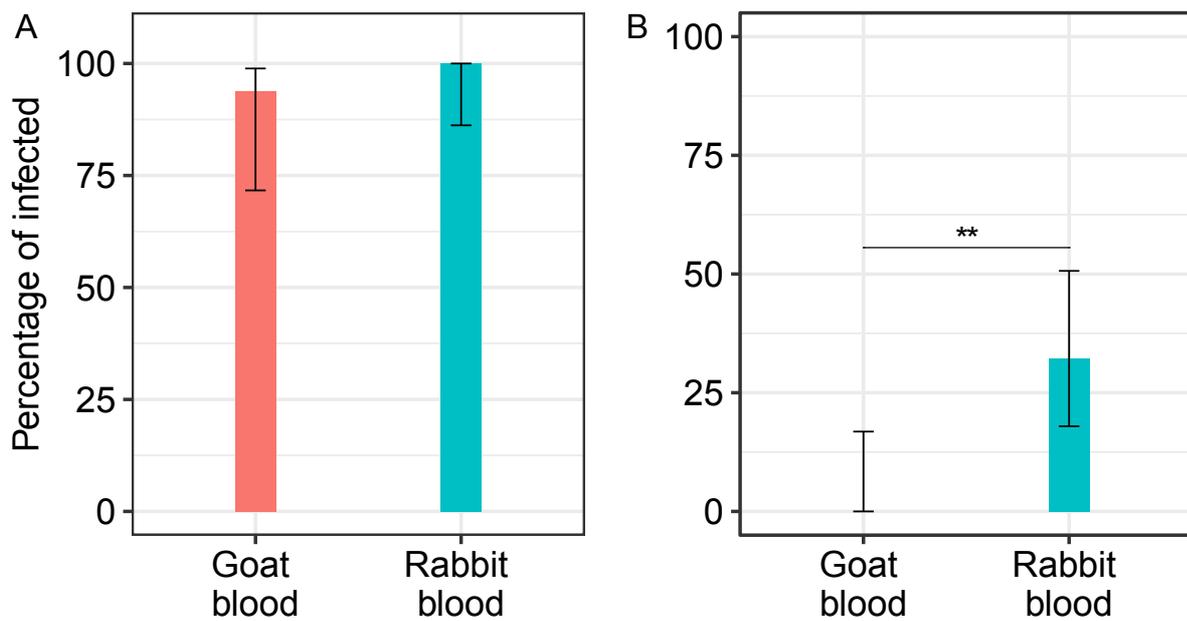
	Riverside	Low-cover forest	High-cover forest
<b>Volunteer 1</b>	X		X
<b>Volunteer 2</b>	X		X
<b>Volunteer 3</b>		X	X
<b>Volunteer 4</b>	X	X	X
<b>Volunteer 5</b>		X	X
<b>Volunteer 6</b>		X	X

# Chapter 3

## Potential of the sylvatic mosquito *Aedes malayensis* to act as an arbovirus bridge vector in forested area of the Nakai Nam Theun National Protected Area



“This chapter addresses the potential of *Ae. malayensis* to act as an arbovirus bridge vector in forested area of the NNT NPA based on vector competence and behavioral experiments.”



**Figure 34: Influence of blood source on dengue virus infection rate in *Aedes aegypti*.** Mosquitoes were orally challenged with a DENV-1 isolate mixed with goat or rabbit blood at a final titer of  $8.3 \times 10^6$  plaque-forming units (PFUs)/mL (A) and  $6.7 \times 10^5$  PFUs/mL (B) in two separate experiments. Bars represent the percentage of virus-positive females 14 days post infectious blood meal (i.e. infection rate) and the error bars are the 95% confidence intervals of the percentages. For each dose, a Fisher exact test was performed to test the influence of the blood source on infection rate. \*\*  $p < 0.01$ .

## Summary

Following our initial field surveys, we chose to focus on the mosquito *Ae. malayensis* to further characterize its potential to act as an arbovirus bridge vector in the NNT NPA. This was done by evaluating its vector competence and its attraction to human scent in laboratory conditions.

We measured the vector competence of our laboratory colony of *Ae. malayensis* recently derived from a sylvatic population (see Chapter 1) for DENV and YFV, using an urban *Ae. aegypti* population from Laos as a positive control. Mosquito were orally challenged with a low-passage DENV-1 isolate from Laos and a low-passage YFV isolate belonging to the West African lineage. Additionally, we performed behavioral experiments using a dual-port olfactometer to assess the specific attraction of this *Ae. malayensis* population to human odor in the presence of CO<sub>2</sub>.

In order to perform vector competence assays in Laos, I had to implement the protocol in the laboratory. One of the main issues was to find a reliable blood source. I initially used goat blood provided by a local research team, but we rapidly found out that infection rate was significantly lower when using goat blood instead of rabbit blood (Figure 34). Therefore, I had to set up a small facility for rabbit husbandry in the laboratory, in order to perform fresh blood draws prior to the artificial blood meals.

We found that our sylvatic *Ae. malayensis* population was competent for DENV-1, but to a lesser extent than our *Ae. aegypti* control. Due to low YFV infection rates overall, we were not able to conclusively demonstrate that this *Ae. malayensis* population was competent for YFV, but it was likely less competent than our *Ae. aegypti* control.

The olfactometer bioassays allowed us to measure both flight activity (i.e. percentage of females that spontaneously left the release chamber) and specific attraction to human odor (i.e. percentage of trapped mosquitoes that chose the trap with human odor). We observed that in the presence of human odor combined with CO<sub>2</sub>, a higher proportion of *Ae. malayensis* females were activated and started flying compared to CO<sub>2</sub> alone. However, there was no specific attraction to human odor, with equal proportions of mosquitoes caught in the trap with human odor combined with CO<sub>2</sub> and in the trap with CO<sub>2</sub> alone.

The relatively modest vector competence for DENV and YFV, combined with a lack of detectable attraction to human odor in laboratory conditions, indicate a low potential for this sylvatic population of *Ae. malayensis* to act as an arbovirus bridge vector in our study area. Nevertheless, we caution that opportunistic feeding on humans (see Chapter 2) may

### Chapter 3: Potential of the mosquito *Aedes malayensis* to act as an arbovirus bridge vector in forested area of the Nakai Nam Theun National Protected Area

occasionally contribute to bridge sylvatic and human transmission cycles. Further assessment of the risk of DENV and/or YFV spillback associated with *Ae. malayensis* should account for additional risk factors such as the presence of susceptible non-human primates and the probability of human-mediated introduction of arboviruses into the area.

Personal contributions to the chapter:

I performed all the vector competence experiments with assistance from Dr. Elodie Calvez at the Institut Pasteur du Laos for DENV and assistance from Dr. Fabien Aubry at the Institut Pasteur de Paris for YFV. I carried out the behavioral experiments at the London School of Hygiene and Tropical Medicine with the support of Ms. Catherine Oke.

Chapter 3: Potential of the mosquito *Aedes malayensis* to act as an arbovirus bridge vector in forested area of the Nakai Nam Theun National Protected Area

Article 4: Potential of the sylvatic mosquito *Aedes malayensis* to act as an arbovirus bridge vector in forested area of the Nakai district, Laos

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1 **Potential of the sylvatic mosquito *Aedes malayensis* to act as an arbovirus**  
2 **bridge vector in a forested area of the Nakai district, Laos**

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20 **Abstract**

21

22 **Background:** Many emerging arthropod-borne viruses (arboviruses) of global public health  
23 importance, such as dengue virus (DENV) and yellow fever virus (YFV), originated in  
24 sylvatic transmission cycles, where they circulate between wild animals and forest-dwelling  
25 mosquitoes. Arbovirus emergence in the human population typically results from spillover  
26 transmission via bridge vectors, which are competent mosquitoes feeding on both humans and  
27 wild animals. Another related, but less studied concern, is the risk of ‘spillback’ transmission  
28 from humans into novel sylvatic cycles, exemplified by the establishment of YFV in South  
29 America.

30 **Methods:** We colonized a sylvatic population of the mosquito *Aedes malayensis* from a  
31 forested area of the Nakai district, Laos in order to evaluate its potential to act as an arbovirus  
32 bridge vector. We measured its relative vector competence for DENV and YFV using an  
33 urban population of *Aedes aegypti* as a positive control. Vector competence was estimated 14  
34 days after oral exposure to an artificial infectious blood meal by detecting virus in the body,  
35 head and saliva of individual females. In addition, we performed olfactometer experiments to  
36 measure specific attraction to human odor of this *Ae. malayensis* population.

37 **Results:** Overall, the vector competence of *Ae. malayensis* for both arboviruses was lower  
38 than that of the *Ae. aegypti* controls, although we obtained unequivocal evidence of *Ae.*  
39 *malayensis* transmission potential for DENV. Olfactometer experiments showed that our *Ae.*  
40 *malayensis* colony did not display any detectable attraction to human scent in laboratory  
41 conditions.

42 **Conclusions:** The relatively modest vector competence for DENV and YFV, combined with  
43 a lack of detectable attraction to human odor, indicate a low potential for this sylvatic *Ae.*  
44 *malayensis* population to act as an arbovirus bridge vector. However, we caution that

### Chapter 3: Potential of the mosquito *Aedes malayensis* to act as an arbovirus bridge vector in forested area of the Nakai Nam Theun National Protected Area

45 opportunistic blood feeding on humans by sylvatic *Ae. malayensis* may occasionally  
46 contribute to bridge sylvatic and human transmission cycles.

47

#### 48 **Keywords**

49 *Aedes malayensis*; Dengue; Yellow fever; Arbovirus; Vector competence; Bridge vector;  
50 Host-seeking behavior.

51

#### 52 **List of Abbreviations**

53 BSA = bovine serum albumin

54 DENV = dengue virus

55 DENV-1 = dengue virus type 1

56 DR = dissemination rate

57 FBS= fetal bovine serum

58 FFA = focus-forming assay

59 FFU = focus-forming unit

60 IR = infection rate

61 NHP = non-human primate

62 NNT NPA = Nakai Nam Theun National Protected Area

63 PBS = phosphate-buffered saline

64 TE = transmission efficiency

65 TR = transmission rate

66 WMPA = Watershed Management and Protection Authority

67 YFV = yellow fever virus

68 **Declarations**

69 **Ethics approval and consent to participate**

70 This study used human blood samples to prepare mosquito artificial infectious blood meals.  
71 Healthy donor recruitment was organized by the local investigator assessment using medical  
72 history, laboratory results and clinical examinations. Biological samples were supplied  
73 through participation of healthy volunteers at the ICAReB biobanking platform (BB-0033-  
74 00062/ICAReB platform/Institut Pasteur, Paris/BBMRI AO203/[BIORESOURCE]) of the  
75 Institut Pasteur to the CoSImmGen and Diagmicoll protocols, which have been approved by  
76 the French Ethical Committee Ile-de-France I. The Diagmicoll protocol was declared to the  
77 French Research Ministry under reference DC 2008-68 COL 1. All adult subjects provided  
78 written informed consent.

79 **Consent for publication**

80 Not applicable.

81 **Availability of data and materials**

82 The datasets analyzed in the current study are available from the corresponding author on  
83 reasonable request.

84 **Competing interests**

85 The authors declare that they have no competing interests.

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### Chapter 3: Potential of the mosquito *Aedes malayensis* to act as an arbovirus bridge vector in forested area of the Nakai Nam Theun National Protected Area

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94 genetic epidemiology of malaria and other parasitic diseases in the Lao PDR for containing  
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96 interpretation, or the decision to submit the work for publication.

#### 97 **Authors’ contributions**

98 EFM, PTB and LL designed the study. EFM, EC, FA and SD performed vector competence  
99 experiments. MG provided the dengue virus isolate and various reagents in support of the  
100 virological assays. SM provided the *Ae. aegypti* colony. CO and JGL helped to design and  
101 perform the olfactometer experiments. EFM and LL analyzed the data. EFM and LL wrote the  
102 manuscript. All authors read and approved the final manuscript.

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108 their permission to collect in the NNT NPA and for providing technical support during the  
109 fieldwork. We are thankful to the staff of the Public Health Office, Nakai District,  
110 Khammuane province for their cooperation and field assistance.

111 **Introduction**

112           Many emerging arthropod-borne viruses (arboviruses) of humans such as dengue, Zika,  
113 yellow fever and chikungunya viruses originated in sylvatic transmission cycles where they  
114 circulate between vertebrate animals and forest-dwelling mosquitoes [1]. Over the last few  
115 centuries, these arboviruses have emerged into sustained transmission cycles among humans,  
116 causing substantial mortality and morbidity. Zoonotic arboviruses are typically brought into  
117 the human population by mosquitoes referred to as bridge vectors, which make the link  
118 between sylvatic and human transmission cycles. Bridge vectors are competent for arbovirus  
119 transmission and display host-feeding behavior towards both humans and non-human animals.  
120 The emergence or re-emergence of sylvatic arboviruses into the human population is an  
121 important public health concern [2] that calls for careful investigations at the interface  
122 between human populations and the sylvatic environment.

123           Another related, but less studied concern, is the risk of ‘spillback’ of arboviruses from  
124 the human population into novel sylvatic cycles in regions where they were previously absent  
125 [3]. Not only are newly established sylvatic arbovirus cycles virtually impossible to eradicate,  
126 but such spillback events can have potentially serious consequences for wildlife. This was  
127 exemplified by yellow fever virus (YFV), which was introduced into the Americas from West  
128 Africa about 400 years ago during the slave trade and contributed to shape the expansion of  
129 settlements and colonial powers [4]. YFV also established sylvatic transmission cycles within  
130 the Amazon, Araguaia, and Orinoco river basins where it has been responsible for deadly  
131 outbreaks among non-human primates of the New World. The incriminated bridge vectors  
132 were mosquitoes in the genera *Haemagogus* and *Sabethes* [5]. Recent reports from Kenya  
133 suggest that human dengue virus (DENV) infections may be a source of ‘spillback’  
134 transmission to baboon populations [6].

### Chapter 3: Potential of the mosquito *Aedes malayensis* to act as an arbovirus bridge vector in forested area of the Nakai Nam Theun National Protected Area

135           The Nakai National Biodiversity Conservation Area, Nakai district, Khammouane  
136 province consists of dry evergreen forests, cloud forests and mountainous riverbeds that are  
137 the biotopes of various mosquito species and monkeys with increasing incursions of humans.  
138 About 10,000 humans (1.95 persons/km<sup>2</sup>) reside in and around this area bordering and within  
139 the primary forest of the Annamite Range [7]. The mosquito *Aedes malayensis* in the  
140 *Stegomyia* sub-genus is widely distributed in South-East Asia with records in Thailand,  
141 Cambodia, Vietnam, Peninsular Malaysia, the Andaman and Nicobar Islands, and Taiwan [8-  
142 10]. It was also identified in peridomestic habitats of Singapore as a putative vector of YFV,  
143 DENV and chikungunya virus [10,11]. Recently, our mosquito surveys in the Nakai National  
144 Biodiversity Conservation Area indicated the presence of *Ae. malayensis* in this area [11,12].

145           Here, we evaluated the potential of *Ae. malayensis* to act as an arbovirus bridge vector  
146 in the Nakai National Biodiversity Conservation Area, using a combination of vector  
147 competence assays and behavioral experiments. Relative to *Ae. aegypti* controls, we found  
148 that our field-derived *Ae. malayensis* colony had similar vector competence indices for DENV  
149 type 1, but a lower susceptibility to YFV. In addition, olfactometer bioassay measurements  
150 showed that this *Ae. malayensis* population was not significantly attracted to human odor in  
151 laboratory conditions. We conclude that although this sylvatic *Ae. malayensis* population does  
152 not display a strong attraction to human odor, its vector competence for arboviruses such as  
153 DENV may contribute to bridge sylvatic and human transmission cycles when it engages in  
154 opportunistic blood feeding on humans.

155

#### 156 **Methods**

##### 157 1) Mosquitoes

158 Experiments were carried out with a laboratory colony derived from a sylvatic population of  
159 *Ae. malayensis* (7<sup>th</sup> generation) collected in March 2017 and subsequently maintained at the

### Chapter 3: Potential of the mosquito *Aedes malayensis* to act as an arbovirus bridge vector in forested area of the Nakai Nam Theun National Protected Area

160 Institut Pasteur du Laos. The *Ae. malayensis* colony was initiated with mosquito larvae  
161 collected along the Nam Noy River (17.768548°N, 105.381989°E) in the Nakai Nam Theun  
162 National Protected Area (NNT NPA), known as the Watershed Management and Protection  
163 Authority (WMPA), located in the Nakai district, Khammuane province, Laos [11]. A  
164 laboratory colony of *Ae. aegypti* (7<sup>th</sup> generation) maintained at the Institut Pasteur in Laos was  
165 used as a control. The *Ae. aegypti* colony originated in the town of Paksan (18.37134°N,  
166 103.66586°E), Paksan district, Bolikhamxay province, Laos. Mosquitoes were reared under  
167 controlled insectary conditions (28°C, 70% relative humidity, 12:12 hour light cycle). Eggs  
168 were hatched synchronously in a vacuum chamber for 1 hour. Larvae were reared in 24 x 34 x  
169 9 cm plastic trays containing 1.5 L of dechlorinated tap water and fed with Tetramin (Tetra)  
170 fish food at a density of 400 larvae per tray. Eight hundred adults were kept in 30 x 30 x 30  
171 cm Bugdorm-1 insect cages with permanent access to 10% sucrose solution. For olfactometer  
172 studies at the London School of Hygiene and Tropical Medicine, eggs from the *Ae.*  
173 *malayensis* and *Ae. aegypti* colonies were shipped to London and reared under similar  
174 insectary conditions.

#### 175 2) Viruses

176 Vector competence experiments were carried out with low-passage DENV type 1 (DENV-1)  
177 and YFV isolates. The DENV-1 isolate (H15-3000) was originally isolated in 2015 from the  
178 plasma of a patient hospitalized in one of the hospitals belonging to the Institut Pasteur du  
179 Laos DENV surveillance network in Vientiane capital, and was passaged three times in *Aedes*  
180 *albopictus* C6/36 cells before it was used in this study. Virus stocks were produced during the  
181 third passage following as previously described [13]. The YFV isolate (YFV-S79 strain)  
182 belongs to the West African lineage and was originally obtained in 1979 from the serum of a  
183 patient returning to France from Senegal [14]. Prior to its use in the experiments, the YFV  
184 isolate was passaged twice in newborn mouse brains and three times in C6/36 cells. Virus

### Chapter 3: Potential of the mosquito *Aedes malayensis* to act as an arbovirus bridge vector in forested area of the Nakai Nam Theun National Protected Area

185 stocks were produced during the last passage, as previously described for DENV [13]. Virus  
186 titration was performed by standard focus-forming assay (FFA) as previously described for  
187 DENV [13]. A mouse anti-dengue virus complex monoclonal antibody (MAB8705; Merck  
188 Millipore) diluted 1:200 in phosphate-buffered saline (PBS) supplemented with 1% bovine  
189 serum albumin (BSA; Interchim) was used as primary antibody for DENV. A mouse anti-  
190 flavivirus group antigen monoclonal antibody (MAB10216; Merck Millipore) diluted 1:1,000  
191 in PBS supplemented with 1% BSA (Interchim) was used as primary antibody for YFV.

#### 192 3) Oral Challenge

193 Mosquitoes were orally challenged with DENV-1 in two separate experiments and with YFV  
194 in a third one as previously described for DENV [13]. Briefly, 8-day-old females starved for  
195 24 hours were offered an artificial infectious blood meal in 3 rounds of 15 minutes using a  
196 Hemotek membrane-feeding apparatus with porcine intestine as membrane. Blood meals  
197 consisted of a 2:1 mix of washed erythrocytes (rabbit erythrocytes for DENV experiments and  
198 human erythrocytes for the YFV experiment) and virus suspension. Adenosine triphosphate  
199 (Merck) was added as a phagostimulant [15] to the blood meal at a final concentration of 10  
200 mM. In DENV experiments, mosquitoes were exposed to  $1.38 \times 10^7$  focus-forming units  
201 (FFUs)/mL of DENV-1. In the YFV experiment, they were exposed to  $1.84 \times 10^6$  FFUs/mL  
202 of YFV. Fully engorged females were sorted on wet ice, transferred into 1-pint cardboard  
203 containers and maintained in a climatic chamber (28°C, 70% relative humidity, 12:12 hour  
204 light cycle) with permanent access to 10% sucrose solution. Fourteen days after the blood  
205 meal, mosquitoes were cold-anesthetized to remove wings and legs for DENV experiments or  
206 paralyzed with triethylamine for the YFV experiment to collect saliva samples *in vitro* [16].  
207 The proboscis of each female was inserted into a 20- $\mu$ L pipet tip containing 5  $\mu$ L of fetal  
208 bovine serum (FBS). After 30 minutes of salivation, head and body were separated and stored  
209 individually at -80°C. The saliva-containing FBS was mixed with 45  $\mu$ L of Leibovitz's L-15

### Chapter 3: Potential of the mosquito *Aedes malayensis* to act as an arbovirus bridge vector in forested area of the Nakai Nam Theun National Protected Area

210 medium and immediately inoculated onto sub-confluent C6/36 cells for titration by FFA, as  
211 described above, without subsequent dilution.

#### 212 4) Virus Detection

213 In DENV-1 experiments, bodies were homogenized individually in 300  $\mu$ L of PBS. Body  
214 homogenates were centrifuged and viral RNA was extracted using the NucleoSpin RNA  
215 Virus kit (Macherey-Nagel) according to the manufacturer's instructions. Detection of  
216 DENV-1 RNA was performed with the EXPRESS one-step Superscript qRT-PCR kit  
217 (ThermoFisher Scientific) using the following program: 30 min at 45°C, 2 min at 95°C, 55  
218 cycles of 15 sec at 95°C and 30 sec at 60°C with a final step of 2 min at 25°C. The 25- $\mu$ L  
219 reaction volume contained 1x of reaction mix, 1.4  $\mu$ M of primers, 5  $\mu$ M of probe (forward:  
220 5'- AAGGACTAGAGGTTAKAGGAGACCC-3'; reverse: 5'-  
221 CGWTCTGTGCCTGGAWTGATG-3'; probe: 5'-  
222 TCTGGTCTTTCCCAGCGTCAATATGCTGTT-3'; [17]) and 5  $\mu$ L of RNA extract. In the  
223 YFV experiment, viral RNA was extracted by grinding bodies individually in 300  $\mu$ L of  
224 squash buffer (Tris 10 mM, NaCl 50 mM, EDTA 1.27 mM with final pH adjusted to 8.2)  
225 supplemented with proteinase K (1  $\mu$ L for 55.5  $\mu$ L of squash buffer) and by incubating 100  
226  $\mu$ L of body homogenates for 5 min at 56°C followed by 10 min at 98°C. Detection of YFV  
227 RNA was performed using a two-step RT-PCR reaction to generate a 192-bp amplicon  
228 located in a conserved region of the NS3 gene of YFV. Total RNA was reverse transcribed  
229 into cDNA with random hexamers using M-MLV reverse transcriptase (ThermoFisher  
230 Scientific) using the following program: 10 min at 25°C, 50 min at 37°C and 15 min at 70°C.  
231 The cDNA was subsequently amplified using DreamTaq DNA polymerase (ThermoFisher  
232 Scientific). The 20- $\mu$ L reaction volume contained 1x of reaction mix and 10  $\mu$ M of primers  
233 (forward: 5'-GCGTAAGGCTGGAAAGAGTG-3'; reverse: 5'-  
234 CTTCTCCCTTCATCCACAA-3'; [18]). The thermocycling program was 2 min at 95°C, 35

### Chapter 3: Potential of the mosquito *Aedes malayensis* to act as an arbovirus bridge vector in forested area of the Nakai Nam Theun National Protected Area

235 cycles of 30 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C with a final extension step of 7  
236 min at 72°C. Amplicons were visualized by electrophoresis on a 2% agarose gel. In all  
237 experiments, mosquito heads were homogenized in 300 µL of Leibovitz's L-15 medium  
238 supplemented with 2% FBS, centrifuged, and titrated by FFA as described above.

#### 239 5) Olfactometer Bioassays

240 Specific attraction of *Ae. malayensis* to human odor was evaluated using a dual-port  
241 olfactometer (160 x 60 x 43 cm) in a controlled environment room at the London School of  
242 Hygiene and Tropical Medicine. The experiments included *Ae. aegypti* as an anthropophilic  
243 control. To mimic human scent, sheer polyamide stocking washed in 70% ethanol was worn  
244 by the experimenter for 12 hours and stored at -20°C until use. An unworn stocking, which  
245 had been cleaned in the same manner, was used as control. Prior to the experiment, 5- to 7-  
246 day-old mosquitoes were deprived of sucrose for 24 hours. Batches of 20 to 30 females were  
247 transferred into a release chamber and allowed 1 hour to acclimatize. Stockings were thawed  
248 during 1 hour and placed inside the traps according to two distinct experimental designs. The  
249 first design, denoted "CO<sub>2</sub> + Human odor vs. CO<sub>2</sub> only" hereafter, consisted of one trap with  
250 human odor and one trap with no odor. The second design, denoted "CO<sub>2</sub> only vs. CO<sub>2</sub> only"  
251 hereafter, consisted of both traps without human odor and was used as a negative control. The  
252 air stream was heated and humidified, then subsequently directed to traverse either trap until  
253 it reached the release chamber. The air stream was supplemented with CO<sub>2</sub> (5%) released at  
254 the entrance of each trap at a rate of 175 mL/min. The air speed at the exit of the traps was set  
255 at 0.2 m/sec with a variation of no more than 0.01 m/sec between each trap. The sliding door  
256 of the release chamber was opened, and mosquitoes were allowed to enter the flight chamber  
257 for 20 min. During each run, mosquitoes that flew upwind towards the odor were caught  
258 inside the traps. A total of 9 replicates were performed for each mosquito species to test their  
259 attraction to human odor (CO<sub>2</sub> + Human odor vs. CO<sub>2</sub> only), in addition to 6 replicate runs for

### Chapter 3: Potential of the mosquito *Aedes malayensis* to act as an arbovirus bridge vector in forested area of the Nakai Nam Theun National Protected Area

260 each species to control for any bias in the olfactometer (CO<sub>2</sub> only vs. CO<sub>2</sub> only). We used a  
261 Latin square design to randomize the position of each trap between replicate runs. Flight  
262 activity was measured as the percentage of mosquitoes that exited the release chamber after  
263 20 min. Attraction to human odor was estimated as the percentage of trapped mosquitoes that  
264 chose the trap with human odor over the trap with no odor in the CO<sub>2</sub> + Human odor vs. CO<sub>2</sub>  
265 only design. Our measure of attraction to human odor ranged from 0% (full attraction to CO<sub>2</sub>  
266 without human odor) to 100% (full attraction to CO<sub>2</sub> with human odor) with 50% indicating a  
267 lack of either attraction.

#### 268 6) Statistical Analyses

269 All statistical analyses were performed using the R software version 3.5.2 [19] and graphical  
270 representations were generated with the R packages *ggplot2* [20]. Vector competence was  
271 evaluated using four conventional indices. Infection rate (IR) was estimated as the proportion  
272 of blood-fed females that became infected. Dissemination rate (DR) was estimated as the  
273 proportion of infected females that developed a systemic infection (i.e., with an infected head).  
274 Transmission rate (TR) was estimated as the proportion of females with a disseminated  
275 infection that released virus in their saliva. Transmission efficiency (TE) is a summary metric  
276 estimated as the overall proportion of blood-fed females with virus-positive saliva. Vector  
277 competence indices (IR, DR, TR and TE) were analyzed with a logistic regression model in  
278 which each individual mosquito was associated with a binary variable (infected = 1,  
279 uninfected = 0), followed by an analysis of deviance with the R package *car* [21]. For DENV  
280 experiments, the initial statistical model included the species, the experiment and their  
281 interaction as explanatory variables. The experiment effect between the two DENV  
282 experiments was statistically insignificant overall and subsequently removed from the model.  
283 In the olfactometer bioassay, flight activity and attraction to human odor were analyzed using  
284 a logistic regression model where each individual mosquito was associated with a binary

285 variable (flight activity: active = 1, inactive = 0; attraction: trap 1 = 0, trap 2 = 1), followed by  
286 an analysis of deviance with the R package *car* [21]. Both analyses accounted for the replicate  
287 run effect, which was statistically non-significant overall. Multiple comparison between  
288 conditions was performed using the estimated marginal means followed by Tukey's post-hoc  
289 test using the R package *emmeans* [22].

290

## 291 **Results**

### 292 **Lower DENV-1 vector competence of *Ae. malayensis* relative to *Ae. aegypti***

293 We measured the DENV-1 vector competence of 39 *Ae. malayensis* females and 53 *Ae.*  
294 *aegypti* females in two separate experiments. The data from both experiments were combined  
295 because initial analyses showed that none of the vector competence indices differed  
296 significantly between them. In each experiment, mosquitoes were exposed to an infectious  
297 dose of  $1.38 \times 10^7$  FFU/ml of DENV-1. Vector competence was analyzed 14 days post  
298 infectious blood meal. The proportion of blood-fed females that became infected (i.e., the  
299 infection rate; IR) was 69.2% (27/39) and 100% (53/53) for *Ae. malayensis* and *Ae. aegypti*,  
300 respectively (Figure 1). The difference in IR between the two species was statistically  
301 significant ( $p < 0.0001$ ). The proportion of infected females that developed a disseminated  
302 infection (i.e., the dissemination rate; DR) was 66.7% (18/27) for *Ae. malayensis* and 93.3%  
303 (42/45) for *Ae. aegypti* (Figure 1). The difference in DR between the two species was  
304 statistically significant ( $p = 0.004$ ). The proportion of females with a disseminated infection  
305 that also released virus in their saliva (i.e., the transmission rate; TR) was 33.3% (6/18) for *Ae.*  
306 *malayensis* and 54.8% (23/42) for *Ae. aegypti* (Figure 1). The difference in TR between the  
307 two species was not statistically significant ( $p = 0.125$ ). The overall proportion of blood-fed  
308 females with virus-positive saliva (i.e., the transmission efficiency; TE) was 15.4% (6/39) and  
309 51.1% (23/45) for *Ae. malayensis* and *Ae. aegypti*, respectively (Figure 1). The difference in

310 TE between the two species, which summarizes their relative vector competence, was  
311 statistically significant ( $p = 0.0004$ ). Together, these data indicate that *Ae. malayensis* is a  
312 competent DENV-1 vector, but to a lesser extent than the *Ae. aegypti* control population.

313

#### 314 **Lower YFV vector competence of *Ae. malayensis* relative to *Ae. aegypti***

315 We analyzed the YFV vector competence of 22 *Ae. malayensis* and 31 *Ae. aegypti* 14  
316 days after oral exposure to an infectious blood meal containing  $1.84 \times 10^6$  FFU/ml of YFV.  
317 The IR was 45.5% (10/22) and 96.8% (30/31) for *Ae. malayensis* and *Ae. aegypti*,  
318 respectively (Figure 2) and the difference between the two species was statistically significant  
319 ( $p < 0.001$ ). The DR was 10% (1/10) and 36.7% (11/30) for *Ae. malayensis* and *Ae. aegypti*,  
320 respectively (Figure 2), and the difference between the two species was not statistically  
321 significant ( $p = 0.087$ ). The TR was 0% (0/1) and 9.1% (1/11) for *Ae. malayensis* and *Ae.*  
322 *aegypti*, respectively (Figure 2), and the difference between the two species could not be  
323 statistically tested because there was only 1 *Ae. malayensis* female and thus no replication.  
324 Overall, there was no evidence for a statistically significant difference ( $p = 0.297$ ) in vector  
325 competence between the two species, as TE was 0% (0/22) and 3.2% (1/31) for *Ae.*  
326 *malayensis* and *Ae. aegypti*, respectively (Figure 2). However, the very small proportion of  
327 positive saliva samples overall (a single *Ae. aegypti* saliva sample was found positive) limited  
328 our ability to detect differences. Together, these data do not conclusively demonstrate the  
329 YFV vector competence of *Ae. malayensis*, but suggest that it is likely lower than that of the  
330 *Ae. aegypti* control population.

331

#### 332 **Lack of evidence for *Ae. malayensis* attraction to human odor**

333 In addition to vector competence, the ability of *Ae. malayensis* to act as an arbovirus  
334 bridge vector depends on its human-biting behavior. A typical bridge vector is expected to

### Chapter 3: Potential of the mosquito *Aedes malayensis* to act as an arbovirus bridge vector in forested area of the Nakai Nam Theun National Protected Area

335 display an intermediate level of attraction to humans, so that it bites humans and non-human  
336 animals in alternation. To assess its probability of human biting, we measured the attraction of  
337 *Ae. malayensis* to human odor using a dual-port olfactometer (Figure 3A). Flight activity in  
338 the presence of human scent (i.e., in the CO<sub>2</sub> + Human odor vs. CO<sub>2</sub> only design) was  
339 significantly lower ( $p = 0.001$ ) for *Ae. malayensis* than for *Ae. aegypti* with 70.7% (111/157)  
340 and 86.2% (213/247) of females exiting the release chamber after 20 min, respectively  
341 (Figure 3B). However, the flight activity of *Ae. aegypti* was not significantly influenced ( $p =$   
342 0.997) by human odor as 86.9% (146/168) of females exited the release chamber in the CO<sub>2</sub>  
343 only vs. CO<sub>2</sub> only design (Figure 3B), whereas the flight activity of *Ae. malayensis* was  
344 significantly enhanced ( $p = 0.002$ ) in the presence of human odor with 49.1% (57/116) exiting  
345 the release chamber in the CO<sub>2</sub> only vs. CO<sub>2</sub> only design (Figure 3B).

346 Attraction towards human odor of each species was estimated as a percentage of  
347 trapped females that were caught in the trap with human odor. This index of attraction varies  
348 from 0% (full attraction to CO<sub>2</sub> without human odor) to 100% (full attraction to CO<sub>2</sub> with  
349 human odor), with 50% indicating a neutral behavior (no effect of the human odor). To rule  
350 out any bias in the intrinsic attractiveness of the traps, we also performed control CO<sub>2</sub> only vs.  
351 CO<sub>2</sub> only experiments for each species. In the control experiments, 51% (95% CI: 36.8 –  
352 65.2) of *Ae. aegypti* females and 56.2% (95% CI: 28.9 – 83.6) of *Ae. malayensis* females  
353 chose one trap over the other. For both species, the percentage of mosquitoes in each trap did  
354 not significantly differ from 50%, which confirmed the lack of bias between the traps. As  
355 expected, 87.5% (95% CI: 81.9 – 93.1) of trapped *Ae. aegypti* females chose the trap with  
356 human odor (Figure 3C), indicating significant attraction to human odor. In contrast, 33.3%  
357 (95% CI: 14.3 – 52.3) of trapped *Ae. malayensis* chose the trap with human odor (Figure 3C),  
358 and this percentage was not statistically different from 50% indicating a lack of response to  
359 human odor. Accordingly, relative attraction towards human odor was significantly lower for

360 *Ae. malayensis* than for *Ae. aegypti* ( $p < 0.0001$ ; Figure 3C). Overall, these experiments  
361 revealed that *Ae. malayensis* was not attracted to human odor, but was activated by human  
362 odor as more females responded to the combination of CO<sub>2</sub> and human odor than to CO<sub>2</sub>  
363 alone.

364

### 365 **Discussion**

366 In this study, we demonstrated that a sylvatic *Ae. malayensis* population from a  
367 forested area of the Nakai district, Laos was competent for DENV-1 transmission, but to a  
368 lower extent than the *Ae. aegypti* control population. We also showed that this *Ae. malayensis*  
369 population had lower YFV vector competence indices than the *Ae. aegypti* control population,  
370 although TR was low for both species in our experimental conditions. Finally, whilst our  
371 olfactometer experiment did find greater flight activation of *Ae. malayensis* to CO<sub>2</sub> to human  
372 odor than CO<sub>2</sub> only, they did not display attraction to human odor under these laboratory  
373 conditions. Overall, our assessment indicates a low potential for this sylvatic *Ae. malayensis*  
374 population to act as an arbovirus bridge vector in rural Laos.

375 Mosquito vector competence for arboviruses depends on several tissue barriers  
376 associated with the midgut and the salivary glands [23]. The physiological and molecular  
377 nature of these barriers is poorly understood, but they are used as conventional phenotypes to  
378 characterize vector competence. IR informs on the midgut infection barrier (i.e., preventing  
379 infection of midgut epithelium), DR represents the midgut escape barrier (i.e., preventing  
380 virus exit from the midgut cells into the haemocoel), and TR relates to both salivary glands  
381 infection and escape barriers [24]. Our sylvatic colony of *Ae. malayensis* was experimentally  
382 competent for DENV-1, but its overall TE (= IR x DR x TR) was lower than that of the *Ae.*  
383 *aegypti* control. This difference resulted from significantly lower IR and lower DR, but not  
384 lower TR (Figure 1). This pattern suggests that the lower vector competence of this *Ae.*

### Chapter 3: Potential of the mosquito *Aedes malayensis* to act as an arbovirus bridge vector in forested area of the Nakai Nam Theun National Protected Area

385 *malayensis* population compared to the *Ae. aegypti* control is not primarily due to salivary  
386 glands barriers, but rather to midgut-related mechanisms hindering virus infection and  
387 dissemination. The pattern was different for YFV, for which all vector competence  
388 phenotypes were similar between the two species with the exception of IR (Figure 2). In our  
389 experiment, the same oral infectious dose of YFV infected about twice less *Ae. malayensis*  
390 than *Ae. aegypti*, whereas subsequently DR and TR did not differ significantly. Nevertheless,  
391 the low TR overall limited our ability to detect a difference between the two species. Further  
392 studies will be necessary to elucidate the mechanisms underlying differences in vector  
393 competence between *Ae. malayensis* and *Ae. aegypti*.

394 The results obtained with this sylvatic population of *Ae. malayensis* in Laos should not be  
395 extrapolated to other populations of the species. The mosquito *Ae. malayensis* is widely  
396 distributed across South-East Asia with documented occurrence in Vietnam, Cambodia,  
397 Peninsular Malaysia and Singapore, Taiwan, the Andaman and Nicobar Islands, Laos, and  
398 Thailand [8-11,25]. Recently, a peridomestic population of *Ae. malayensis* in Singapore was  
399 shown to be competent for diverse arboviruses such as DENV, YFV and chikungunya virus  
400 [10,11]. A human-baited trap survey demonstrated that this peridomestic *Ae. malayensis* in  
401 Singapore displayed an anthropophilic behavior [26], as was also documented for other field  
402 populations of *Ae. malayensis* in South-East Asia [8,9]. The discrepancy between the present  
403 and the earlier studies could be due to several, non-mutually exclusive explanations. First,  
404 there could be biological differences in human-biting behavior between different populations  
405 of the same species, as was observed for *Ae. aegypti* [27,28]. Second, behavioral assays in the  
406 laboratory may lack critical cues underlying host-seeking behavior in the field. Indeed,  
407 multiple sensory modalities such as CO<sub>2</sub>, heat and visual cues may contribute to attract *Ae.*  
408 *malayensis* to feed on humans in a natural situation [29,30]. Moreover, volatiles were  
409 collected from the foot only and *Ae. malayensis* might not respond to foot odor. Although our

### Chapter 3: Potential of the mosquito *Aedes malayensis* to act as an arbovirus bridge vector in forested area of the Nakai Nam Theun National Protected Area

410 behavioral bioassay successfully detected a strong attraction of *Ae. aegypti* to human scent, it  
411 is possible that for *Ae. malayensis* the experimental setup did not faithfully represent a natural  
412 situation. Additionally, the fact that more *Ae. malayensis* responded to the combination of  
413 CO<sub>2</sub> and human odor than CO<sub>2</sub> only indicated that they were activated by human odor,  
414 implying that human odor was enough to initiate flight and stimulate the beginning of a host  
415 location response, but perhaps not follow through to closer-range behavior.

416         Arbovirus bridge vectors represent the most likely mechanism of arbovirus spillover  
417 transmission to humans. Whereas sylvatic YFV transmission is only known to occur in Africa  
418 and South America, sylvatic DENV transmission cycles have been documented in the forests  
419 of South-East Asia. In particular, sylvatic DENV transmission was reported in peninsular  
420 Malaysia where sylvatic DENV strains were isolated from sentinel monkeys such as the crab-  
421 eating macaque (*Macaca fascicularis*) in remote forest reserves [1,2]. Crab-eating macaques  
422 are distributed across South-East Asia and can also be found in the forests of southern Laos  
423 [31]. Whether sylvatic DENV strains circulate in forests of Laos is unknown, but their  
424 emergence in the human population is a concern in those areas [1] because they can  
425 potentially result in severe disease [2]. The NNT NPA harbors abundant biodiversity with the  
426 presence of at least nine non-human primate (NHP) species such as rhesus macaques (*Macaca*  
427 *mulatta*), gibbons, langurs, and red-shanked doucs [32]. Rhesus macaques have been  
428 commonly used as laboratory models for arbovirus research [33] and as sentinel monkeys for  
429 arbovirus surveillance in the field [34]. Further investigations at the human-forest interface in  
430 Laos are necessary to evaluate the risk of spillover DENV transmission to humans.

431         Dengue is endemic in Laos with the circulation of at least three serotypes (i.e., DENV-  
432 1, -2 and -4) all year long and yearly epidemics during the rainy season [35,36]. Dengue  
433 epidemics occur not only in urban areas, but also in more rural settings. For instance, an  
434 outbreak of DENV-1 was detected in 2008 in a remote village of northwestern Laos [37].

435 DENV-3 was isolated from *Armigeres subalbatus* collected on the Nakai plateau [38]. DENV  
436 circulation in rural Laos increases the risk of spillback transmission to NHPs because most of  
437 the people living in rural Laos still depend on the forest to sustain themselves. Moreover, one  
438 of the goals of WMPA is to develop ecotourism in the NNT NPA. Similar projects exist in the  
439 country or are in development. Therefore, infected travelers represent another possible source  
440 of DENV introduction or re-introduction into the sylvatic environment. This risk is likely  
441 under-estimated as humans can transmit DENV to mosquitoes in the absence of clinical  
442 symptoms [39].

443

#### 444 **Conclusions**

445 The relatively modest vector competence for DENV and YFV, combined with a lack  
446 of detectable attraction to human odor, indicate a low potential for this sylvatic *Ae.*  
447 *malayensis* population to act as an arbovirus bridge vector. However, we caution that  
448 opportunistic blood feeding on humans by sylvatic *Ae. malayensis* may occasionally  
449 contribute to bridge sylvatic and human transmission cycles. The presence of susceptible  
450 NHPs combined with possibility of a human-mediated introduction of DENV into the sylvatic  
451 environment are additional risk factors that should be taken into account. Once entrenched, a  
452 newly established sylvatic cycle would undermine long-term DENV control in Laos.

453

454

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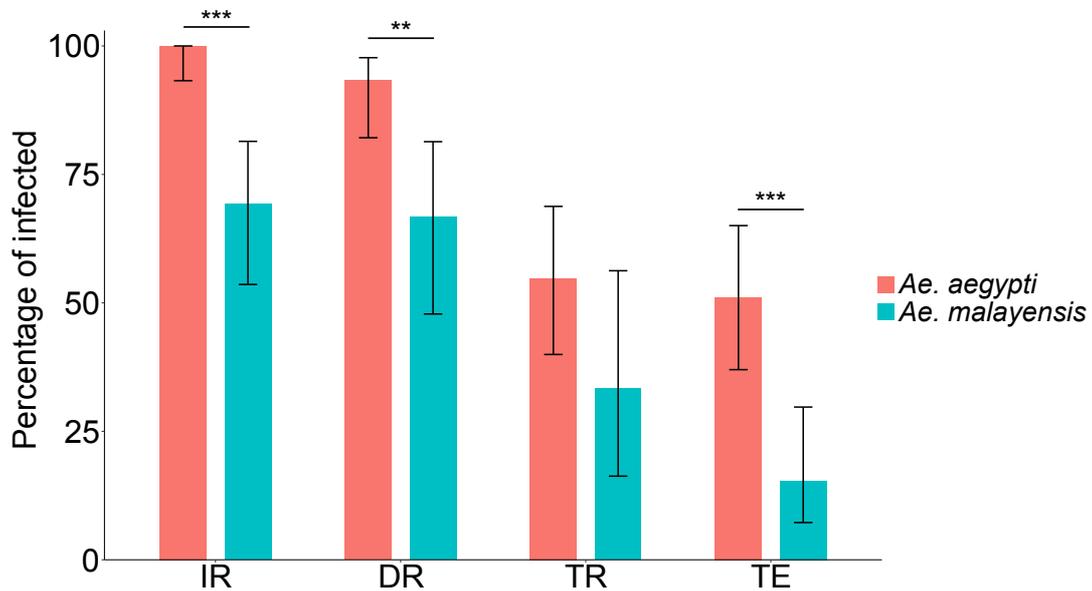
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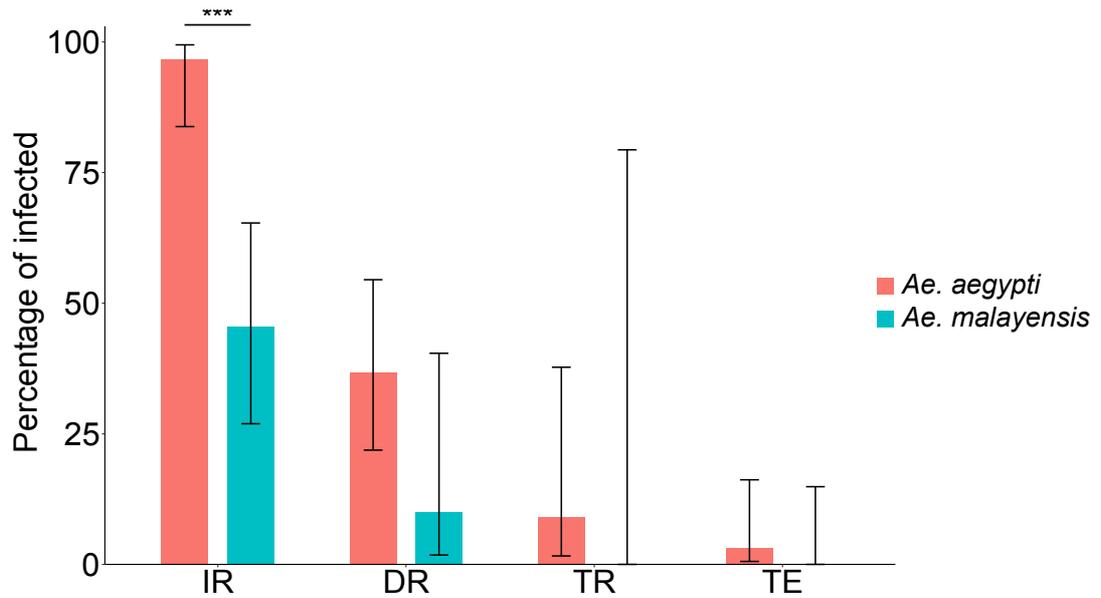
554 **Figures**

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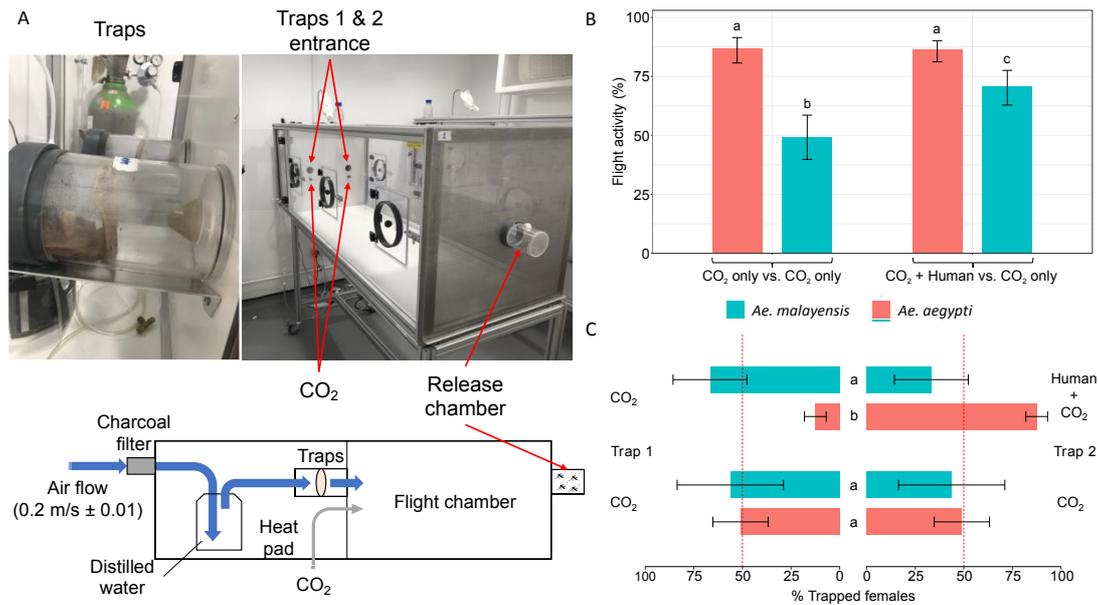
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557 **Figure 1: Vector competence of sylvatic *Ae. malayensis* and *Ae. aegypti* controls after**  
558 **exposure to  $1.38 \times 10^7$  FFUs/ml of DENV-1.** Bars represent the percentage of virus-positive  
559 mosquitoes 14 days post infectious blood meal and the error bars are the 95% confidence  
560 intervals of the percentages. Infection rate (IR) is the proportion of blood-fed females with an  
561 infected body. Dissemination rate (DR) is the proportion of infected females with virus  
562 disseminated to the head tissues. Transmission rate (TR) is the proportion of females with a  
563 disseminated infection that shed virus in their saliva. Transmission efficiency (TE) is the  
564 overall proportion of blood-fed females that shed virus in their saliva. The *Ae. aegypti*  
565 population was included as a positive control. The figure compiles data from two independent  
566 experiments that did not differ significantly. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .



567

568 **Figure 2: Vector competence of sylvatic *Ae. malayensis* and *Ae. aegypti* controls after**  
569 **exposure to  $1.84 \times 10^6$  FFUs/ml of YFV.** Bars represent the percentage of virus-positive  
570 mosquitoes 14 days post infectious blood meal and the error bars are the 95% confidence  
571 intervals of the percentages. Infection rate (IR) is the proportion of blood-fed females with an  
572 infected body. Dissemination rate (DR) is the proportion of infected females with virus  
573 disseminated to the head tissues. Transmission rate (TR) is the proportion of females with a  
574 disseminated infection that shed virus in their saliva. Transmission efficiency (TE) is the  
575 overall proportion of blood-fed females that shed virus in their saliva. The *Ae. aegypti*  
576 population was included as a positive control. \*\*\* $p < 0.001$ .



577

578 **Figure 3: Lack of laboratory evidence for *Ae. malayensis* attraction to human odor.** (A)

579 Schematic and pictures of the experimental setup to measure attraction to human odor in a

580 dual-port olfactometer. (B) Flight activity is the percentage of female mosquitoes that exited

581 the release chamber after 20 min. (C) Attraction to human odor was estimated as the

582 percentage of trapped mosquitoes that chose the trap with human odor, which ranges from 0%

583 (full attraction to CO<sub>2</sub> without human odor) to 100% (full attraction to CO<sub>2</sub> with human odor).

584 The red, vertical dashed lines indicate the expected percentage of trapped mosquitoes when

585 there is no preference for either trap (50%). Error bars represent 95% confidence intervals of

586 the percentages. Letters next to or above the bars indicate statistical significance. Conditions

587 with a letter in common are not significantly different from each other. The data shown in (B)

588 and (C) are pooled from 6 and 9 separate replicate trials for CO<sub>2</sub> only vs. CO<sub>2</sub> only and CO<sub>2</sub> +

589 Human odor vs. CO<sub>2</sub> only designs, respectively, which did not differ significantly. The *Ae.*

590 *aegypti* population was included as a positive control.

# Chapter 4

## Potential of peridomestic *Aedes malayensis* mosquitoes to transmit yellow fever virus in Singapore



“This chapter assessed the ability of a peridomestic *Ae. malayensis* population in Singapore to transmit YFV based on vector competence experiments and a human-baited trap survey.”

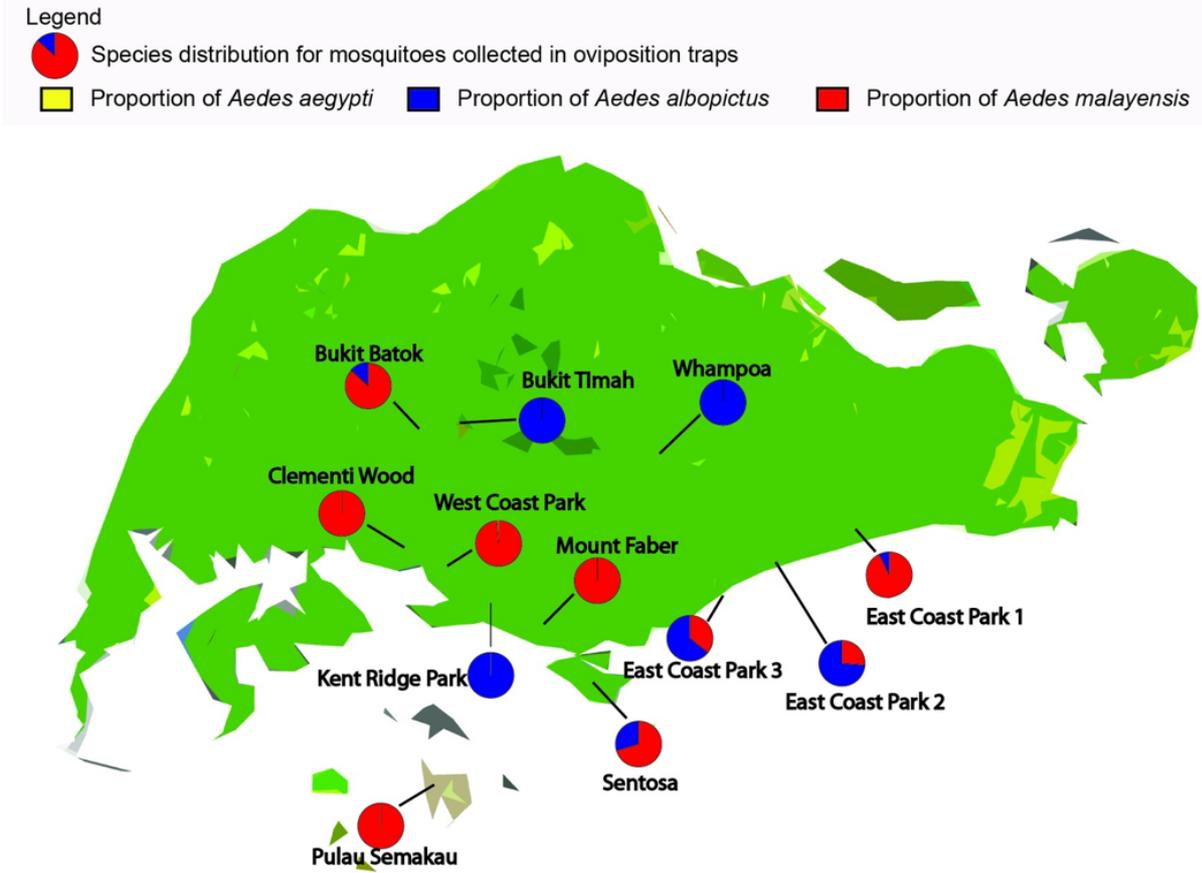


Figure 35: Distribution of *Aedes albopictus* and *Aedes malayensis* in urban parks of Singapore. Reproduced from (Mendenhall et al. 2017).

## Summary

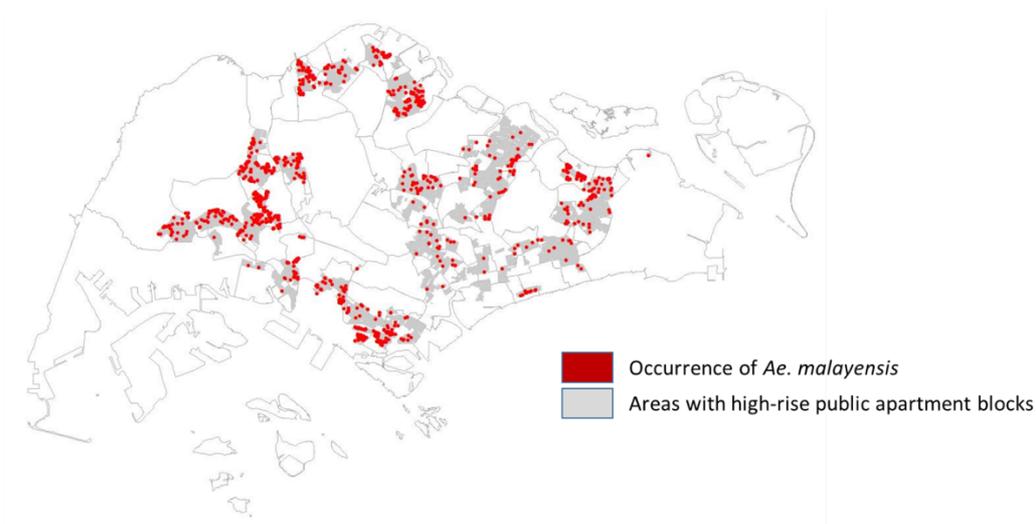
Following our evaluation of the vectorial capacity of a sylvatic population of *Ae. malayensis* in Laos (see Chapter 3), we extended our investigation to a peridomestic *Ae. malayensis* population found in urban parks of Singapore (Figure 35). This *Ae. malayensis* population was previously colonized by our collaborators at Duke-NUS Medical School who suspected that it could contribute to “cryptic” arbovirus transmission. Although vector control measures in Singapore achieve very low densities of *Aedes* mosquitoes (Egger et al. 2008), arboviruses such as DENV, CHIKV, and ZIKV have re-emerged in Singapore in the last two decades (Hapuarachchi et al. 2016; Koh et al. 2008; Leo et al. 2009; Maurer-Stroh et al. 2016). Indeed, this peridomestic *Ae. malayensis* population was recently shown to be experimentally competent for DENV and CHIKV (Mendenhall et al. 2017). However, its vector competence for YFV had not been evaluated, and its propensity to bite humans was unknown.

Until now, the Asia-Pacific region has remained YFV-free. However, the increasing influx of travelers coming from endemic regions of Africa and South America where recent YFV outbreaks have occurred has significantly increased the risk of YFV introduction in places like Singapore, a major hub for trade and tourism (Gubler 2018; Butler 2016). YFV introduction into the Asia-Pacific region was confirmed in 2016 when 11 workers infected with YFV in Angola returned to China, although fortunately it did not subsequently result in local transmission of the virus (WHO 2016a).

To evaluate the ability of *Ae. malayensis* to contribute to YFV transmission in Singapore, we used the colony established in 2014 by our collaborators in Singapore. First, we measured the vector competence of this *Ae. malayensis* colony for YFV, using *Ae. aegypti* as a positive control. Mosquito were orally challenged with a low-passage YFV isolate belonging to the West African lineage. Second, I performed a small-scale field survey in several parks and a forested area of Singapore in March 2019 to evaluate the probability of host-vector contact between humans and *Ae. malayensis*. I sampled mosquitoes at 6 locations from 7 a.m. to 7 p.m. using a human-baited double-net trap: East Coast Park zone A (2 days), East Coast Park zone C (1 day), Mount Faber park (1 day), Clementi woods park (1 day), West Coast park (1 day), and a forested area in the northern part of the city in Sembawang (3 days). We also obtained data from the island-wide Gravitrap surveillance network implemented by Singapore National Environment Agency (NEA) to monitor the spatial and temporal variability of adult *Aedes* mosquito populations.

## Chapter 4: Potential of peridomestic *Aedes malayensis* mosquitoes to transmit yellow fever virus in Singapore

Overall, the peridomestic *Ae. malayensis* population from Singapore was experimentally competent for YFV to a similar level as our *Ae. aegypti* control. During the small-scale field survey, *Ae. malayensis* was caught in the human-baited trap in one of the urban parks (Sembawang). The most abundant species collected was *Ae. albopictus* (89%) followed by *Ae. malayensis* (9%). Moreover, out of the 504,771 mosquitoes collected in Gravitrap, the predominant species was *Ae. aegypti* (80%), but a total of 1,931 *Ae. malayensis* were caught in 1,741 traps (Figure 36).



**Figure 36: Distribution of *Aedes malayensis* in high-rise apartment blocks in Singapore.** Map based on data collected from the island-wide Gravitrap surveillance network in 2018.

Together, this study provided evidence that peridomestic *Ae. malayensis* mosquitoes in Singapore are competent vectors for YFV, which may not only engage in contact with humans in urban parks but also breed in domestic settings such as housing blocks. Therefore, we conclude that *Ae. malayensis* could contribute to YFV transmission in Singapore if the virus were to be introduced.

Personal contributions to the chapter:

I performed the vector competence experiments with assistance from Dr. Fabien Aubry at Institut Pasteur in Paris. I performed all the fieldwork and mosquito identification in Singapore. The Gravitrap data was generated and analyzed by our collaborators from Singapore NEA.

Article 5: A peridomestic *Aedes malayensis* population in Singapore can transmit yellow fever virus

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RESEARCH ARTICLE

## A peridomestic *Aedes malayensis* population in Singapore can transmit yellow fever virus

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### Abstract

The case-fatality rate of yellow fever virus (YFV) is one of the highest among arthropod-borne viruses (arboviruses). Although historically, the Asia-Pacific region has remained free of YFV, the risk of introduction has never been higher due to the increasing influx of people from endemic regions and the recent outbreaks in Africa and South America. Singapore is a global hub for trade and tourism and therefore at high risk for YFV introduction. Effective control of the main domestic mosquito vector *Aedes aegypti* in Singapore has failed to prevent re-emergence of dengue, chikungunya and Zika viruses in the last two decades, raising suspicions that peridomestic mosquito species untargeted by domestic vector control measures may contribute to arbovirus transmission. Here, we provide empirical evidence that the peridomestic mosquito *Aedes malayensis* found in Singapore can transmit YFV. Our laboratory mosquito colony recently derived from wild *Ae. malayensis* in Singapore was experimentally competent for YFV to a similar level as *Ae. aegypti* controls. In addition, we captured *Ae. malayensis* females in one human-baited trap during three days of collection, providing preliminary evidence that host-vector contact may occur in field conditions. Finally, we detected *Ae. malayensis* eggs in traps deployed in high-rise building areas of Singapore. We conclude that *Ae. malayensis* is a competent vector of YFV and re-emphasize that vector control methods should be extended to target peridomestic vector species.

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### Author summary

Yellow fever is a dreadful disease caused by a mosquito-borne virus circulating in Africa and South America. Historically, the Asia-Pacific region has remained free of yellow fever but the ever increasing influx of travelers puts places such as Singapore at unprecedented risk of yellow fever virus introduction. The present study characterized the potential contribution of a mosquito species called *Aedes malayensis* to yellow fever virus transmission

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in Singapore. *Aedes malayensis* breeds in urban parks of Singapore and is suspected to have participated in the resurgence of other mosquito-borne diseases such as dengue because it is not targeted by current mosquito control measures. Not only was *Ae. malayensis* able to experimentally acquire and transmit yellow fever virus, but it was also found to engage in contact with humans in a field situation. This empirical evidence indicates that *Ae. malayensis* is a competent vector of yellow fever virus and should be targeted by mosquito control programs.

## Introduction

The case-fatality rate of yellow fever virus (YFV) ranges from 15% to 50% [1] and is one of the highest among arthropod-borne viruses (arboviruses). YFV is endemic in 47 countries in Africa and South America, with an estimated annual incidence of around 200,000 cases and 30,000 deaths [2]. Despite an efficient vaccine against YFV, the past few years have seen a growing number of YFV outbreaks (Democratic Republic of the Congo, Angola, Uganda, Brazil and most recently Nigeria) [3–5]. During such outbreaks, increasing numbers of unvaccinated travelers who become infected and return to non-endemic countries have raised the risk of YFV introduction to unprecedented levels [6]. The Asia-Pacific region has remained free of YFV until now but the risk of introduction has never been higher [6–8]. In 2016, eleven Chinese workers infected with YFV in Angola who returned to China were the first cases of YFV diagnosed in travelers to Asia [7]. Over two billion immunologically naïve people live in Asia and the current vaccine production capacity would be insufficient to prevent a massive YFV epidemic, while mosquito control programs would be overwhelmed [8].

With 18.5 million visitors in 2018, Singapore is a global hub for tourism, trade and transport that includes one of the busiest ports in the world and a major international airport. These features put Singapore at high risk for introduction of arboviruses, including YFV. Strict and sustained vector control measures together with household structural improvements, currently achieve very low densities of the domestic arbovirus vector *Aedes aegypti* [9]. Re-emergence of dengue virus (DENV), chikungunya virus (CHIKV) and Zika virus in Singapore in the last two decades [10–13] supports the hypothesis that peridomestic mosquito species untargeted by vector control measures may contribute to 'cryptic' arbovirus transmission [14]. In particular, the peridomestic mosquito *Aedes malayensis* (a member of the *Stegomyia* subgenus) breeds in urban parks of Singapore and is experimentally competent for DENV and CHIKV [14].

Here, we evaluated the potential contribution of the peridomestic mosquito *Ae. malayensis* to YFV transmission in Singapore. Vector competence assays in the laboratory and a small-scale field survey provided evidence that indeed *Ae. malayensis* could contribute to YFV transmission in Singapore.

## Methods

### Ethics statement

This study used human blood samples to prepare mosquito artificial infectious blood meals. Healthy donor recruitment was organized by the local investigator assessment using medical history, laboratory results and clinical examinations. Biological samples were supplied through participation of healthy volunteers at the ICAREB biobanking platform (BB-0033-00062/ICAREB platform/Institut Pasteur, Paris/BBMRI AO203/[BIORESOURCE]) of the Institut

Pasteur to the CoSIImmGen and Diagmicoll protocols, which have been approved by the French Ethical Committee Ile-de-France I. The Diagmicoll protocol was declared to the French Research Ministry under reference DC 2008–68 COL 1. The use of human-baited double net trap was approved by the National Environment Agency (NEA) of Singapore (NEA/PH/CLB/19-00004). All adult subjects provided written informed consent.

### Mosquitoes

Experiments were carried out with a laboratory colony derived in 2014 from a wild population of *Ae. malayensis* in Singapore and subsequently maintained at the Duke-NUS Medical School for >50 generations [14]. The 8<sup>th</sup> generation of a laboratory colony of *Ae. aegypti* maintained at the Institut Pasteur in Laos was used as a control. The *Ae. malayensis* colony was initiated with mosquito eggs collected from two forested areas located in the Northern (Sembawang) and the Southern (East Coast Park) regions of Singapore [14]. The *Ae. aegypti* colony originated in the town of Paksan, Paksan district, Bolikhamsai province, Laos. Mosquitoes were reared under controlled insectary conditions (28°C, 70% relative humidity, 12:12 hour light cycle). Eggs were hatched synchronously in a vacuum chamber for 1 hour. Larvae were reared in 24 x 34 x 9 cm plastic trays containing 1.5 L of dechlorinated tap water and supplemented with Tetramin (Tetra) fish food at a density of 400 larvae per tray. Six hundred adults were kept in 30 x 30 x 30 cm Bugdorm-1 insect cages with permanent access to 10% sucrose solution.

### Virus

Vector competence experiments were carried out with a low-passage YFV isolate (YFV-S79 strain) belonging to the West African lineage, which was originally obtained in 1979 from the serum of a patient returning to France from Senegal [15]. Prior to its use in the experiments, the YFV isolate was passaged twice in newborn mouse brains and three times in *Aedes albopictus* C6/36 cells. Virus stock was produced during the last passage, as previously described for DENV [16]. Virus titration was performed by standard focus-forming assay (FFA), as previously described for DENV [16] with the exception of the primary antibody. A mouse anti-flavivirus group antigen monoclonal antibody MAB10216 (Merck Millipore) diluted 1:1,000 in phosphate-buffered saline supplemented with 1% bovine serum albumin (Interchim) was used as primary antibody.

### Virus challenge

In two separate experiments (referred to as experiments 1 and 2 hereafter), mosquitoes were orally challenged with YFV as previously described for DENV [16]. Briefly, eight-day-old females deprived of sucrose for 24 hours were offered an artificial infectious blood meal in 3 rounds of 15 min using a Hemotek membrane feeding apparatus with porcine intestine as the membrane. Blood meals consisted of a 2:1 mix of washed human erythrocytes and virus suspension. Adenosine triphosphate (Merck) was added as a phagostimulant [17] to the blood meal at a final concentration of 10 mM. To obtain a dose-response curve, three separate blood meals with increasing YFV concentrations were prepared. In experiment 1, mosquitoes were exposed to  $2.9 \times 10^6$  focus-forming units (FFUs)/mL,  $2.1 \times 10^5$  FFUs/mL, or  $1.8 \times 10^4$  FFUs/mL of YFV. In experiment 2, they were exposed to  $8.1 \times 10^5$  FFUs/mL,  $5.4 \times 10^4$  FFUs/mL, or  $3.6 \times 10^3$  FFUs/mL of YFV. Fully engorged females were sorted on wet ice, transferred into 0.5-L cardboard containers and maintained in a climatic chamber (28°C, 70% relative humidity, 12:12 hour light cycle) with permanent access to 10% sucrose solution. At 10 and 14 days post blood meal, mosquitoes were cold anesthetized (experiment 1) or paralyzed with

triethylamine (experiment 2) to perform *in vitro* salivation [18]. In experiment 1, the wings and legs were removed and stored at  $-80^{\circ}\text{C}$  prior to the salivation assay. In experiment 2, the heads were removed and stored at  $-80^{\circ}\text{C}$  after the salivation assay. The proboscis of each female was inserted into a 20- $\mu\text{L}$  pipet tip containing 5  $\mu\text{L}$  of fetal bovine serum (FBS). After 30 min of salivation, the body remainders were stored dry at  $-80^{\circ}\text{C}$ . The saliva-containing FBS was mixed with 45  $\mu\text{L}$  of Leibovitz's L-15 medium and immediately inoculated onto C6/36 cells for titration by FFA, as described above, without subsequent dilution.

### Virus detection

Bodies were homogenized individually in 300  $\mu\text{L}$  of lysis buffer (Tris 10 mM, NaCl 50 mM, EDTA 1.27 mM with a final pH adjusted to 8.2) supplemented with proteinase K (1  $\mu\text{L}$  for 55.5  $\mu\text{L}$  of buffer). Body homogenates were centrifuged and 100  $\mu\text{L}$  of supernatant was incubated for 5 min at  $56^{\circ}\text{C}$  then 10 min at  $98^{\circ}\text{C}$  to extract viral RNA. Detection of YFV RNA was performed using a two-step RT-PCR reaction to generate a 192-bp amplicon located in a conserved region of the NS3 gene of YFV. Total RNA was reverse transcribed into cDNA with random hexamers using M-MLV reverse transcriptase (ThermoFisher Scientific) using the following program: 10 min at  $25^{\circ}\text{C}$ , 50 min at  $37^{\circ}\text{C}$  and 15 min at  $70^{\circ}\text{C}$ . The cDNA was subsequently amplified using DreamTaq DNA polymerase (ThermoFisher Scientific). The 20- $\mu\text{L}$  reaction volume contained 1x of reaction mix and 10  $\mu\text{M}$  of primers (forward: 5'-GCGTAAG GCTGAAAGAGTG-3'; reverse: 5'-CTTCCTCCCTTCATCCACAA-3'; [19]). The thermocycling program was 2 min at  $95^{\circ}\text{C}$ , 35 cycles of 30 sec at  $95^{\circ}\text{C}$ , 30 sec at  $60^{\circ}\text{C}$ , and 30 sec at  $72^{\circ}\text{C}$  with a final extension step of 7 min at  $72^{\circ}\text{C}$ . Amplicons were visualized by electrophoresis on a 2% agarose gel. Mosquito wings/legs and heads were homogenized in 300  $\mu\text{L}$  of Leibovitz's L-15 medium supplemented with 2% FBS, centrifuged, and titrated by FFA as described above.

### Host-vector contact

Adult mosquito sampling was conducted in a forested area in the northern part of Singapore (Sembawang; 1.456493, 103.825983) in March 2019. Host-vector contact was evaluated using a human-baited double net trap as previously described [20]. Briefly, a human volunteer was resting on a long chair inside a small, untreated bed-net protected from mosquitoes during daylight hours (7 a.m. to 7 p.m.). A larger bed-net covered the smaller one with a 30-cm opening at the bottom allowing mosquitoes to enter the 20-cm space between bed-nets. A large plastic sheet was placed above the bed-nets as a protection. Every 10 min, the human volunteer collected mosquitoes trapped between bed-nets using a mouth aspirator. Aspirated mosquitoes were transferred into holding cups, killed and grouped per 1-hour interval. Back in the laboratory, mosquitoes were morphologically identified and stored at  $-80^{\circ}\text{C}$ .

### Gravitrapp survey

The presence of *Ae. malayensis* in Singapore's high-rise public housing was assessed using surveillance data obtained in 2018 from the Singapore NEA. The island-wide Gravitrapp surveillance network was implemented by NEA to monitor the spatial and temporal variability of adult *Aedes* mosquito populations and to evaluate the effectiveness of vector control measures. In 2018, approximately 53,000 Gravitrapps were deployed in about 9,200 public apartment blocks across the island. The Gravitrapp is a simple, hay infusion-filled cylindrical trap with a sticky inner surface to catch ovipositing gravid female *Aedes* mosquitoes [21].

### Statistical analyses

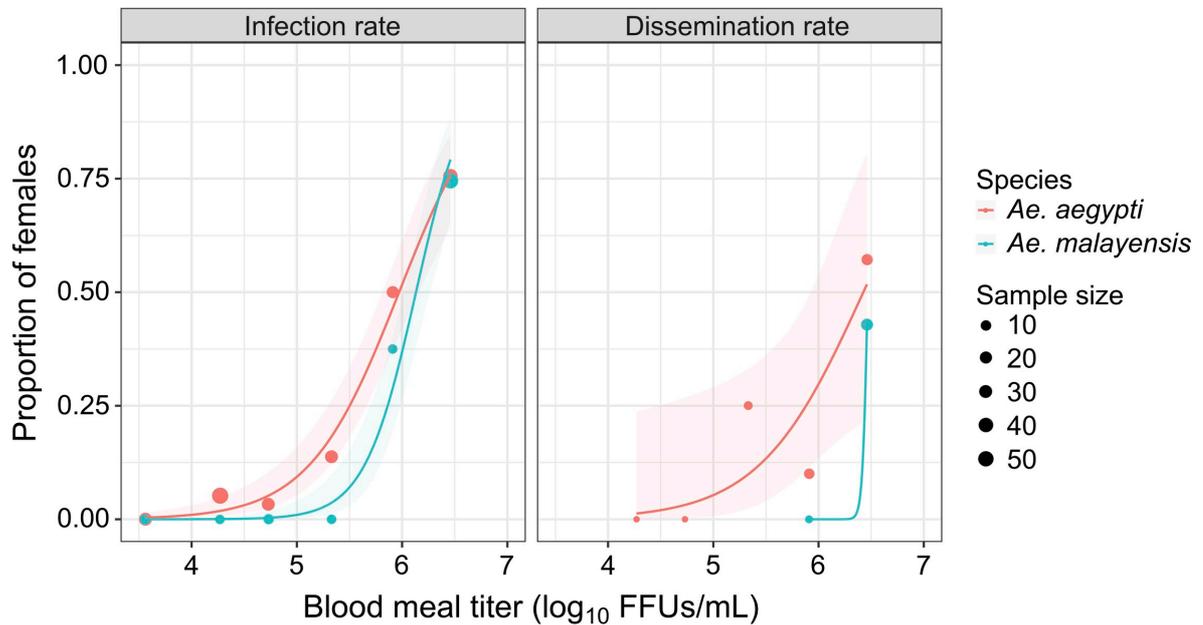
Vector competence was estimated by measuring virus infection, dissemination and transmission rates. Infection rate was estimated as the proportion of YFV-positive mosquitoes among all mosquitoes tested. Dissemination rate was estimated as the proportion of infected mosquitoes with YFV-positive wings and legs (experiment 1) or head (experiment 2). Transmission rate was estimated as the proportion of mosquitoes with a disseminated infection that had YFV-positive saliva. In addition, transmission efficiency was calculated as the proportion of mosquitoes with infectious saliva among all mosquitoes tested. Vector competence indices were analyzed with a logistic regression model in which each individual mosquito was associated with a binary variable (1 = YFV-positive or 0 = YFV-negative), followed by an analysis of deviance with the R package *car* [22]. An initial analysis showed that infection rates were not statistically significantly different between 10 and 14 days post blood meal and both time points were subsequently combined. The analysis of dissemination and transmission rates was restricted to 14 days post blood meal because the two time points differed and there was too little data on day 10 for a meaningful analysis. The experiment effect was statistically non-significant overall and removed from the model. For infection and dissemination rates, the final regression model included the infectious dose ( $\log_{10}$ -transformed blood meal titer), the mosquito species (*Ae. aegypti* and *Ae. malayensis*), and their interaction. The 50% oral infectious dose (OID<sub>50</sub>) and 10% oral infectious dose (OID<sub>10</sub>) values and their respective 95% confidence intervals were derived from the logistic fits of infection rates using the R package *MASS* [23]. The analysis of transmission rate was restricted to the highest infectious dose and the final regression model only included the mosquito species. All statistical analyses were performed using the R software version 3.5.2 [24] and graphical representations were generated with the R packages *ggplot2* [25] and *ggpubr* [26].

### Results

We measured the vector competence of 68 and 31 *Ae. malayensis* females and 132 and 76 *Ae. aegypti* females in experiments 1 and 2, respectively. The data from both experiments were combined because initial analyses showed that none of the vector competence indices differed significantly between them. In each experiment, mosquitoes were exposed to three different YFV infectious doses to establish a dose-response curve. Vector competence was analyzed 14 days post blood meal, with the exception of infection rates that also included mosquitoes sampled at 10 days post blood meal.

The proportion of blood-fed females that became infected (i.e., the infection rate) increased with the infectious dose (Fig 1). The highest infectious dose ( $2.9 \times 10^6$  FFUs/mL) resulted in 74.5% and 75.6% infected females for *Ae. malayensis* and *Ae. aegypti*, respectively. Infectious dose was the only statistically significant ( $p < 0.0001$ ) predictor of infection rate (Table 1), although there was a slight difference in the shape of the dose-response curve between the two species (Fig 1). Although the OID<sub>50</sub> was similar between the two species, the OID<sub>10</sub> was lower for *Ae. aegypti*. The OID<sub>50</sub> was  $5.97 \log_{10}$  FFUs/mL (95% CI =  $5.81$ – $6.14 \log_{10}$  FFUs/mL) for *Ae. aegypti* and  $6.13 \log_{10}$  FFUs/mL (95% CI =  $6.02$ – $6.25 \log_{10}$  FFUs/mL) for *Ae. malayensis*. The OID<sub>10</sub> was  $5.03 \log_{10}$  FFUs/mL (95% CI =  $4.78$ – $5.28 \log_{10}$  FFUs/mL) for *Ae. aegypti* and  $5.59 \log_{10}$  FFUs/mL (95% CI =  $5.41$ – $5.78 \log_{10}$  FFUs/mL) for *Ae. malayensis*.

The proportion of infected females that developed a disseminated infection (i.e., the dissemination rate) also increased with infectious dose (Fig 1). The highest infectious dose resulted in 42.9% and 57.1% dissemination in *Ae. malayensis* and *Ae. aegypti*, respectively. Infectious dose was the only statistically significant ( $p < 0.0001$ ) predictor of dissemination



**Fig 1. Peridomestic *Ae. malayensis* in Singapore are orally susceptible to YFV.** Dose-response curves are shown for the *Ae. malayensis* population from Singapore under study and a control *Ae. aegypti* population. Infection rate is the proportion of blood-fed females testing YFV-positive 10–14 days post blood meal. Dissemination rate is the proportion of YFV-infected females with YFV-positive wings/legs or head 14 days post blood meal. The line represents the logistic regression of the data and the shaded area is the 95% confidence interval of the fit. The data shown are combined from two separate experiments.

<https://doi.org/10.1371/journal.pntd.0007783.g001>

rate (Table 1). Together, these results indicated that although *Ae. aegypti* was slightly more susceptible than *Ae. malayensis* at low infectious doses, the two species had a similar YFV susceptibility overall.

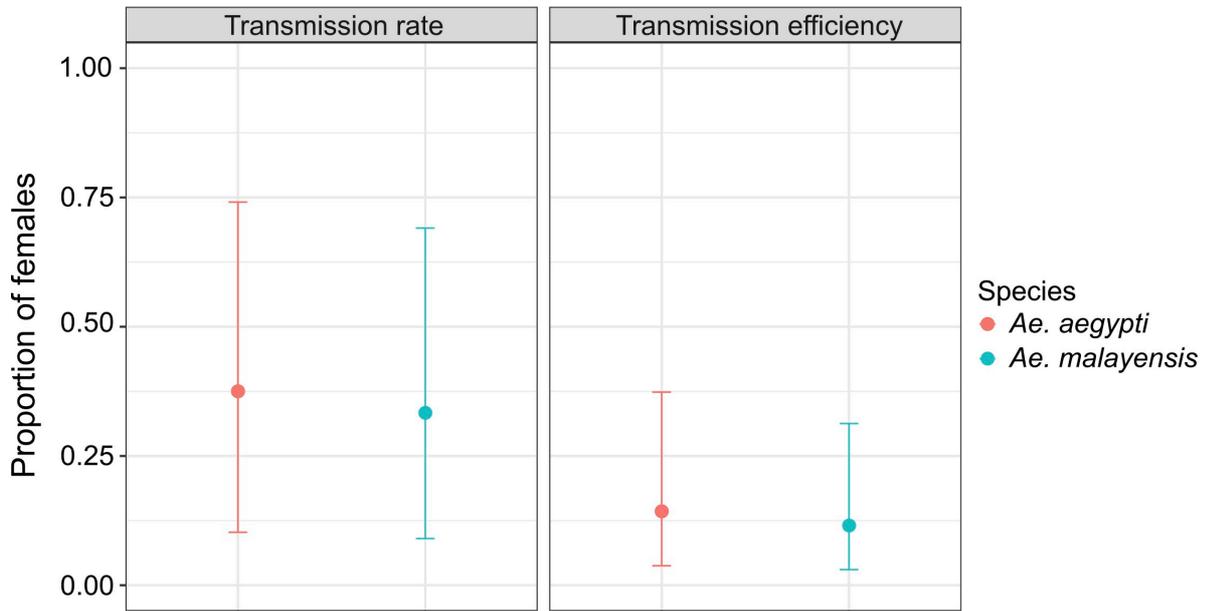
The proportion of females with a disseminated infection that had YFV-positive saliva (i.e., the transmission rate) was measured at 14 days post blood meal only for the highest infectious dose (Fig 2). YFV was detected in the saliva of 33.3% (3/9) of *Ae. malayensis* females and 37.5% (3/8) of *Ae. aegypti* females, and the difference between the two species was not statistically significant ( $p = 0.8576$ ). Vector competence can be summarized by the overall proportion of blood-fed females that had YFV-positive saliva at the highest infectious dose (i.e., the transmission efficiency). Transmission efficiency was 11.5% (3/26) for *Ae. malayensis* and 14.3% (3/21) for *Ae. aegypti* (Fig 2), and the difference between the two species was not statistically significant ( $p = 0.7795$ ).

**Table 1. Test statistics of YFV infection and dissemination rates.** Infection and dissemination rates were analyzed by logistic regression. The model included the effect of the YFV oral infectious dose ( $\log_{10}$ -transformed blood meal titer), the mosquito species (*Ae. aegypti* or *Ae. malayensis*) and their interaction.

	Infection rate			Dissemination rate		
	LR $\chi^2$	Df	<i>p</i> value	LR $\chi^2$	Df	<i>p</i> value
Dose	97.42	1	<0.0001	5.504	1	0.0190
Species	3.325	1	0.0682	1.040	1	0.3079
Dose x Species	3.099	1	0.0783	1.001	1	0.3171

Df: degrees of freedom; LR: likelihood ratio.

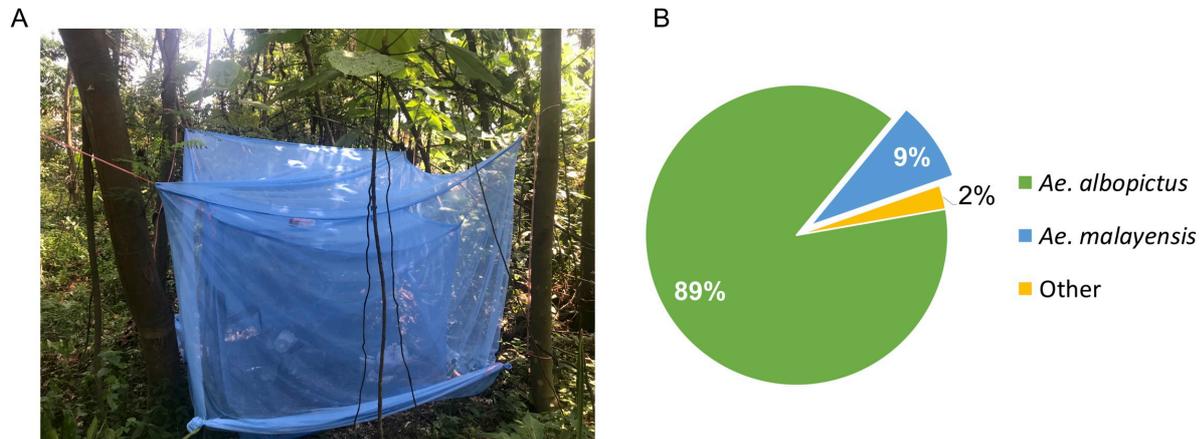
<https://doi.org/10.1371/journal.pntd.0007783.t001>



**Fig 2. Peridomestic *Ae. malayensis* in Singapore are competent for YFV transmission.** Transmission potential is shown for the *Ae. malayensis* population from Singapore under study and a control *Ae. aegypti* population 14 days after oral exposure to  $2.9 \times 10^6$  FFU/mL of YFV. Transmission rate is the proportion of females with a disseminated infection that had infectious YFV in their saliva 14 days post blood meal. Transmission efficiency is the proportion of all blood-fed females that had infectious YFV in their saliva 14 days post blood meal. Vertical bars represent 95% confidence intervals of the proportions. The data shown are from two separate experiments.

<https://doi.org/10.1371/journal.pntd.0007783.g002>

In addition to vector competence, the ability of *Ae. malayensis* to transmit YFV depends on its propensity to bite humans. To assess host-vector contact of peridomestic *Ae. malayensis* in Singapore, we surveyed day-biting mosquitoes in a forested area of Sembawang, Singapore in



**Fig 3. Peridomestic *Ae. malayensis* in Singapore are attracted to humans.** (A) Human-baited double net trap setup in the forested area of Sembawang, Singapore. (B) Distribution of mosquito species among 115 female specimens collected at daytime in the human-baited double net trap.

<https://doi.org/10.1371/journal.pntd.0007783.g003>

March 2019 using a human-baited double net trap (Fig 3A). A total of 115 female mosquitoes were captured during three days of collection. The most abundant species was *Ae. albopictus* (89%) followed by *Ae. malayensis* (9%) (Fig 3B).

Out of 504,771 mosquitoes collected in Gravitrap traps deployed by NEA in Singapore's high-rise public apartment blocks in 2018, the predominant mosquito species was *Ae. aegypti* (80% of all captures). However, a total of 1,931 *Ae. malayensis* (0.38%) were caught in 1,741 traps. The average capture rate was 0.0013 *Ae. malayensis* per trap and per week, with an average of 1.1 specimen per *Ae. malayensis*-positive trap.

## Discussion

Our study indicates that peridomestic *Ae. malayensis* mosquitoes can contribute to YFV transmission in Singapore based on two lines of evidence. First, our laboratory mosquito colony recently derived from a wild *Ae. malayensis* population in Singapore was experimentally competent for YFV, to a level that was not statistically different from that of the *Ae. aegypti* controls. Adding to earlier data demonstrating vector competence of this colony for DENV and CHIKV [14], our results are the first experimental evidence, to our knowledge, that peridomestic *Ae. malayensis* from Singapore can potentially transmit YFV acquired from an infectious blood meal. Second, we captured *Ae. malayensis* females in a human-baited trap. Although our host-seeking experiment lacked a non-baited control trap, it provided preliminary evidence that this peridomestic *Ae. malayensis* population in Singapore may engage in human-biting behavior, as was already observed for other populations of the species in South-East Asia [27,28].

Despite its wide distribution in South-East Asia with records in Laos, Thailand, Cambodia, Vietnam, Peninsular Malaysia, the Andaman and Nicobar Islands, and Taiwan [27–30], the mosquito *Ae. malayensis* has been relatively poorly studied. Recently, however, *Ae. malayensis* was found prevalent in numerous urban parks of Singapore [14]. Not only were *Ae. malayensis* eggs collected in the forested areas of the parks but they were also found in some open-air areas frequented by humans [14]. The current study reports that in 2018, the National Vector Surveillance program in Singapore detected the presence of *Ae. malayensis* in Gravitrap traps [21] outside the urban parks of the island. Although the majority of *Aedes* mosquitoes found in public apartment blocks were *Ae. aegypti*, *Ae. malayensis* was detected in Gravitrap traps deployed in high-rise building areas, underlining the potential of this mosquito species to colonize urban habitats. It was recently hypothesized that peridomestic mosquito species, such as *Ae. malayensis*, which escape vector control measures targeting domestic vector species, may have contributed to the (re-)emergence of arboviruses in Singapore in the last two decades [14]. The well-known peridomestic mosquito *Ae. albopictus*, which was highly abundant in our human-baited trap survey, significantly increases the risk of arbovirus transmission in Singapore [14]. Control of peridomestic mosquitoes is a challenge because in addition to artificial breeding sites, less accessible natural sites such as tree holes need to be targeted as well [31].

Although we did not measure other parameters that influence the vectorial capacity of a mosquito species, such as adult density and survival, and the duration of the virus extrinsic incubation period [32], vector competence and attraction to humans make *Ae. malayensis* a potential vector of YFV and other arboviruses that deserves increased attention from public health authorities. The level of *Ae. malayensis* vector competence for YFV that we observed was in the same range as our *Ae. aegypti* controls from Laos, and as Brazilian *Ae. aegypti* that were orally challenged with the same virus isolate in a previous study [33]. It is worth noting, however, that vector competence results cannot be readily compared if they did not use the same oral infectious dose, or if other factors such as virus strain and detection assays were different. The dose-response curves that we generated in the present study will be useful for

future comparisons because they provide absolute estimates of vector competence indices, such as the  $OID_{50}$ . Moreover, two vector species or populations may have a similar level of vector competence at a given infectious dose, but vary at a different dose. For example, the DENV type 2 susceptibility of two field-derived populations of *Ae. aegypti* in Thailand were similar at a medium dose, but different at a low dose [34]. This example indicates that the minimal threshold for infection may differ between mosquito populations. Likewise, in the present study, we observed similar YFV  $OID_{50}$  values for *Ae. aegypti* and *Ae. malayensis* but *Ae. aegypti* had a lower YFV  $OID_{10}$  value. In natural conditions, the oral infectious dose is the primary driver of successful human-to-mosquito transmission of arboviruses [35,36].

Despite an efficient vaccine, YFV still remains an important public health concern in Africa and South America. YFV is maintained in sylvatic cycles between non-human primates and arboreal mosquitoes, and human outbreaks occur when the virus is introduced into an urban cycle mediated by *Ae. aegypti* [37]. Although there is no evidence of a sylvatic cycle of YFV in the Asia-Pacific region, the risk of spillover transmission exists. The lack of YFV outbreaks in the Asia-Pacific region to date could be due to cross-protective immunity conferred by other widespread flaviviruses such as DENV and Japanese encephalitis virus, but it could also reflect relatively fewer opportunities for YFV introduction until now. Rampant globalization now puts the Asia-Pacific region at an unprecedented risk of YFV introduction [6–8]. Billions of immunologically naïve people, together with competent *Ae. aegypti* populations [38,39], create a dangerous set of conditions that are theoretically favorable to a massive YFV epidemic. On the other hand, a scenario whereby YFV would establish a novel sylvatic cycle [40] seems unlikely in Singapore given the small size of resident non-human primate populations. Clearly, places with high densities of the domestic vector *Ae. aegypti* are most at risk of spillover transmission, but we emphasize that in the absence of *Ae. aegypti*, ancillary vectors should not be overlooked. Competent mosquito species such as *Ae. malayensis* could facilitate introduction of YFV into the Asia-Pacific region. In Singapore, strict and sustained vector control measures have achieved low densities of *Ae. aegypti* [9] but this has failed to prevent arbovirus outbreaks in the last two decades [10]. Following a previous study on the ability of *Ae. malayensis* from Singapore to transmit DENV and CHIKV [14], we reiterate the need to account for peridomestic species in the management of mosquito vector populations.

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## General discussion and perspectives

South East Asia is one of the hotspots for vector-borne disease emergence (Jones et al. 2008). However, some regions of South East Asia, such as Laos, are still understudied. In these regions, there is little knowledge of the mosquito fauna and of their vector status, distribution, and ecology. This lack of basic entomological data undermines proper evaluation of the risk of vector-borne disease emergence and adequate implementation of vector control measures. Laos has a network of 20 NPAs covering 14% of the country (Namintha 2019). The NNT NPA is the largest and most diverse natural forest area of Laos with more than 20,000 people living in and around the protected area (NT2 WMPA 2019). We initially conducted field surveys to provide an up-to-date inventory of the mosquito species of the NNT NPA and more generally of the country. During these surveys, one of the predominant mosquito species that we found was *Ae. malayensis*, a member of the *Stegomyia* subgenus widely distributed in South East Asia (Huang 1972; Tewari et al. 1995; Rattanaarithikul et al. 2010) that had not been previously recorded in Laos. We also noticed that *Ae. malayensis* females were flying around us and biting us. At about the same time, a study in Singapore identified the same species as a potential vector of DENV-2 and CHIKV based on vector competence experiments, but the study did not provide evidence of human-vector contact (Mendenhall et al. 2017). Due to this coincidence, we decided to investigate if *Ae. malayensis* was attracted to humans in field conditions, as was reported in other field-based studies (Huang 1972; Tewari et al. 1995). The presence of *Ae. malayensis* specimens caught in our human-baited traps subsequently confirmed this hypothesis.

Following our field-based surveys and successful colonization of *Ae. malayensis* in the laboratory, we tested the vector competence of our sylvatic colony for DENV-1 and YFV and found a relatively modest vector competence for both viruses. Next, we evaluated the degree of attraction to human odor of this *Ae. malayensis* population, but we did not detect any significant attraction toward human scent combined with CO<sub>2</sub> relative to CO<sub>2</sub> alone in our experimental conditions. Lastly, we obtained the peridomestic *Ae. malayensis* colony from Singapore that was identified as a potential vector of DENV-2 and CHIKV (Mendenhall et al. 2017) to evaluate its potential for YFV transmission. We found that the peridomestic *Ae. malayensis* population in Singapore was as competent for YFV as our *Ae. aegypti* control. Moreover, a small-scale field experiment using a human-baited trap provided preliminary evidence of human-vector contact in an urban park of Singapore. This peridomestic *Ae.*

*malayensis* population in Singapore putative potential to transmit arboviruses is exacerbated by its ability to colonize more urban settings such as high-rise building areas in the city.

One of the limitations of our larval surveys of the NNT NPA was that an unknown proportion of breeding sites were presumably too difficult to access or even to locate due to the topography of the area and the thickness of the vegetation. This may have resulted in an overrepresentation of some of the breeding sites that were easier to access such as rock pools along the riverbanks and tree holes close to the ground. The bias that was consequently introduced in our mosquito diversity surveys cannot be readily quantified, mosquito species preferentially breeding close to the ground were likely overrepresented. To maximize our collection success, we artificially increased the number of breeding sites in the area by deploying bamboo traps. However, many of the bamboo traps were also colonized by other animal species, in particular frogs that ate most of the mosquito larvae. Thus, we modified the design of the bamboo traps to allow shallower water levels and minimize the presence of undesired residents. Several species that were not collected in the natural breeding sites were found in the bamboo traps. Despite that, we also caught mosquito adults belonging to species that were never found in our larval collections. This could be due, in part, to the vertical stratification of mosquito breeding preferences ranging from ground level to high in the canopy (Mitchell and Rockett 1979; Lira-Vieira et al. 2013). This stratification is influenced by various abiotic factors, such as temperature, relative humidity, light gradient, and wind speed, but also specific mosquito behaviors such as swarming and mating activities, preference of oviposition sites (e.g. epiphytes or tree holes), or habitat of their hosts (Mitchell and Rockett 1979). For example, many mosquito species involved in arbovirus sylvatic cycles reside in the canopy where they feed on non-human primates (Vasilakis et al. 2011; Hanley et al. 2013). Moreover, some mosquito species change their vertical preference (ground vs. canopy) depending on the season (Lira-Vieira et al. 2013), which could explain why we only collected some species during specific months. Shifts in breeding site preferences might reflect variation in the availability of larval breeding sites, which is lowest during the dry season and highest during the rainy season. It would be interesting to conduct a more in-depth study of vertical stratification. We attempted to deploy CDC light traps and BG sentinel traps in the canopy but were not able to collect any mosquitoes. A proper characterization of the mosquito fauna in the canopy will require more specialized equipment and techniques. For example, we could place traps on elevated platforms or climb on trees with high epiphyte covering to look for potential breeding sites, following earlier work in Malaysia (Rudnick

1986). Another possibility would be to borrow the approach of the canopy biodiversity inventory project “Radeau des cimes”, which sampled biodiversity in the canopy from the top, using balloons and raft structures placed on top of the trees (Blog du radeau des cimes 2019).

One of the difficulties we encountered during the field surveys was the presence of *Toxorhynchites* and *Lutzia* species in our larval samples. These mosquito genera engage in predatory behavior and even cannibalism in absence of other preys and they ate the other larvae sharing the same container (Rattanaarithikul et al. 2007; Rattanaarithikul, Harbach, et al. 2005). We subsequently changed our storage protocol in the field and separated known predatory species as soon as possible, but it was sometimes difficult to detect them within the pool of larvae, especially at early stages of development. Another difficulty was the presence of numerous ants that killed the adult mosquitoes that we captured or that emerged from the collected larvae.

Due to the remote location of the Nam Noy field site and the absence of information on the arboviruses that might circulate in the area, we could not implement the human-landing catch (HLC) technique to collect mosquito species attracted to humans. We opted for an alternative, safer method using human-baited double-net traps, which minimize the risk of bite for the experimenter (Tangena et al. 2015). Human-baited double-net traps were shown to capture a similar number of mosquitoes as HLC, although they may underestimate the proportion of *Aedes* species, presumably because of failed entry or limited time spent inside the trap (Tangena et al. 2015). To address this potential bias, we increased the frequency of collection by collecting every 10-15 minutes within each one-hour interval to minimize losses.

A caveat of our study design was that we did not include any trap without a human bait (i.e. negative control) to account for mosquitoes seeking shelter or mosquitoes entering the bed net accidentally without any specific attraction to humans. This phenomenon was reported by Tangena et al. (2015) who observed a slight increase in mosquito diversity in human-baited traps compared to HLC. As a result, we could not subtract a background level of non-specific captures to determine the threshold above which a mosquito species can be considered to be specifically attracted to humans. This is especially problematic for species with a low number of specimens captured because it could reflect rare species attracted to humans or more abundant species not attracted to humans. To address this issue, we used previous evidence of human-biting behavior to make conclusions about species with a low

number of specimens captured. In any case, using a control trap without a human bait would have been difficult in the field. Mosquitoes are not expected to stay within the trap for a long time, even more so in the absence of bait in the trap, and it would require an experimenter to come every hour to collect the trapped specimens. The intermittent presence of the experimenter in the trap or its vicinity would necessarily introduce a bias because it would inevitably leave cues (e.g. volatiles) that would attract human-seeking mosquitoes even when the experimenter is away.

Overall, HLC and human-baited double-net traps only provide proxy estimates of human exposure to mosquitoes. Our initial goal was to identify human-biting mosquito species present in the NNT NPA forest and to determine the host range of these species by blood meal analysis (Crabtree et al. 2013). We initially envisioned collecting blood-engorged females in the field to determine the blood-meal source by DNA genotyping. Unfortunately, we were unable to find any blood-fed females. This was surprising because other studies in the forests of Brazil (Mucci et al. 2015), Senegal (D. Diallo et al. 2013), or the USA (Mehus and Vaughan 2013) successfully did so, with up to 30% of their collections based on active methods (e.g. vacuum aspiration) for the latter study. Our lack of success could be explained by the difference in species composition present in Laos, where sylvatic mosquitoes may have different preferences in habitats or resting sites. For example, if their primary hosts (e.g. non-human primates) mainly reside in the canopy, they might rest in the vicinity or in places that we could not reach. The overall mosquito density could also be lower in our study area.

The attraction to humans displayed by *Ae. malayensis* during our fieldwork in Laos and Singapore was not confirmed by olfactometer bioassays in the laboratory. This discrepancy could be explained by several, non-mutually exclusive hypotheses. First, there could be intrinsic biological differences in human-seeking behavior among different populations of *Ae. malayensis*, as was described for *Ae. aegypti* (McBride et al. 2014; McBride 2016). Because sylvatic *Ae. malayensis* was caught in human-baited traps in Laos, this explanation is unlikely because we used the colony derived from this sylvatic population in the olfactometer bioassays. Nevertheless, we cannot exclude that human-seeking behavior may have evolved during the seven generations of colonization and maintenance on live quail blood, prior to the behavioral experiments. It would be interesting in the future to compare *Ae. malayensis* populations from a range of habitats (sylvatic, peridomestic, and urban) to test the hypothesis that increased exposure to humans translates into increased attraction. Second, our behavioral assay was an oversimplification of a natural situation. In particular, we only tested attraction

of human odor combined with CO<sub>2</sub> relative to CO<sub>2</sub> alone. Our experimental setup may have lacked critical cues that are essential for host-seeking behavior such as thermal stimuli, visual cues, or chemical cues and their integrations (McMeniman et al. 2014; Liu and Vosshall 2019). Moreover, the scent of only one person was used and it was only collected from the foot. Inter-individual variation in the attractiveness of human odor in mosquitoes due to difference in body odor is well documented (Qiu et al. 2006; Logan et al. 2008; Logan et al. 2010). Composition of the skin microbiota has also been implicated in human attractiveness to mosquitoes (Verhulst et al. 2011). Yet, the human skin microbiota can be very different depending on the body part and therefore produce different metabolites (Byrd et al. 2018). It is possible that *Ae. malayensis* females do not respond to foot odor. Interestingly, we observed increased flight activity of *Ae. malayensis* when human odor was added to CO<sub>2</sub>, indicating that human scent combined with CO<sub>2</sub> was sufficient to initiate a host-seeking response. Flight activation is the first response to host cues in the sequence of behaviors that lead from long-range orientation to short-range approach and final contact with the host (Clements 1999). The lack of response to human odor that we observed based on the trapped mosquitoes indicates that we may have missed some stimuli to follow through to close-range behavior. To test this hypothesis, we could use a live-host olfactometer assay such as the one used by McBride et al. (2014), in which more cues are represented (visual, chemical, and thermal) than when only using volatiles collected on human skin.

The two *Ae. malayensis* populations that we tested, i.e. sylvatic *Ae. malayensis* from Laos and peridomestic *Ae. malayensis* from Singapore, displayed different levels of vector competence. The population from Singapore was highly competent for DENV-2, CHIKV (Mendenhall et al. 2017), and YFV. On the other hand, the sylvatic population from Laos had a relatively modest competence for DENV-1 and YFV. Intra-specific differences in vector competence are well known and depend on the combination of vector and virus genotypes (Lambrechts et al. 2009; Lambrechts 2011). The difference that we observed between the two *Ae. malayensis* populations in their vector competence for the same YFV isolate reemphasizes the fact that vector competence is not a universal property of the species as a whole. It would be interesting to study the vector competence of other *Ae. malayensis* populations across South East Asia to determine the entire range of vector competence variation at the species level.

Vector competence assays are obviously an abstraction of reality. For instance, exposing mosquitoes to a single viral dose does not represent a natural situation where

mosquitoes may bite people at different stages of the viremic period. In natural conditions, the oral infectious dose is the main driver of successful human-to-mosquito arbovirus transmission (Duong et al. 2015; Nguyet et al. 2013). In order to address this aspect, we generated dose-response curves by exposing mosquitoes to different concentrations of virus in the blood meal. Not only dose-response curves provide absolute estimates of vector competence indices (i.e. relative to the dose), but they also allow assessment of mosquito susceptibility at lower doses than are typically tested (Pongsiri et al. 2014). This approach will benefit future comparisons between research teams, which can prove to be difficult otherwise (Souza-Neto et al. 2019). A challenge of dose-response experiments is that they require significantly larger numbers of mosquitoes. We failed to generate dose-response curves for our sylvatic *Ae. malayensis* colony because it suffered high mortality in the biosafety level-3 laboratory, which resulted in insufficient sample sizes for a meaningful analysis. It is noteworthy that our data do not allow us to make any conclusion on the relationship between the sylvatic or peridomestic origin of the *Ae. malayensis* populations and their vector competence. This is because this comparison lacks replication and would require multiple replicates of sylvatic and peridomestic populations to be tested.

Taxonomical identification of mosquito species is a crucial, and often neglected, part in the fight against vector-borne diseases. Information on mosquito species distribution in a given region can help local authorities to implement proper and efficient vector control programs and to evaluate the risk of arbovirus emergence. Morphological identification and proper descriptions of discriminatory morphological characters are essential. Yet, it takes a considerable amount of time, effort, and money to catalog and systematically describe the entomological fauna of a region. In particular, it is often difficult to find funding for this type of descriptive, ‘naturalist’ science. In many places, morphological characters are the only option to identify mosquito species, due to lack of laboratory equipment and/or funds to perform molecular identification. Paradoxically, there is a shortage of qualified personnel with an expertise on mosquito morphology in developing countries where mosquito diversity is the highest. This skill is progressively disappearing worldwide with people increasingly opting for recent genetic or proteomic approaches. These new approaches are really powerful and have been instrumental to disentangle species complexes (Collins et al. 1987; Dumas et al. 2016). Moreover, they are convenient because they can be used at any mosquito life stage. However, most of the available databases of sequences or mass spectrometry profiles are

incomplete or contain mis assigned sequences. This makes them very difficult to use in high-diversity regions where understudied or even undescribed species are present.

This lack of available data was one of the main difficulties during this PhD project. There was often no molecular identification possible and specimens were hardly identifiable morphologically. There was no proper mosquito description in Laos, and we relied on dichotomous keys from neighboring countries (i.e. Thailand and Vietnam). Dichotomous keys are useful but are not error-proof because undescribed species or local variations of known species can lead to misidentifications. For most of our specimen identifications, there were no sequence data available and morphological identification was unsure beyond the genus level.

## Conclusions

### Conclusions

Together, this PhD work showed that whereas a sylvatic *Ae. malayensis* population in Laos did not display a high potential as a bridge vector, its peridomestic counterpart in Singapore is a capable arbovirus vector. The wide distribution of the species in South East Asia and its ability to colonize urban settings such as high-rise building areas in Singapore calls for increased vigilance. Indeed, the current features of *Ae. malayensis* are reminiscent of those of *Ae. albopictus* prior to its spread across the globe. The two closely related species (both from the *Stegomyia* subgenus) share many ecological similarities and are often found in sympatry. It is possible that the two species compete for the same ecological niche and that for now *Ae. albopictus* prevails. However, should vector control campaigns eliminate or diminish the ecological pressure from *Ae. albopictus*, *Ae. malayensis* might take over. Therefore, I believe it is important to monitor this species more closely, to evaluate its vector competence for arboviruses in other locations, and include it in vector surveillance networks to monitor its spread in urban areas. Importantly, some preliminary work on insecticide resistance that I performed in Laos revealed that our sylvatic *Ae. malayensis* colony from the NNT NPA was resistant to DDT, malathion, permethrin, and was suspected to be resistant to deltamethrin and temephos when exposed to the doses recommended by WHO for *Ae. aegypti* (Appendix 1). These data indicate that chemical control of this species could prove to be difficult should it become necessary. *Aedes malayensis* and *Ae. albopictus* close morphological similarity (i.e. identical to an untrained eye) could represent an additional problem because of probable misidentifications. Thus, training operational staff in charge of vector surveillance in morphological identification of mosquitoes is a crucial skill that needs to be maintained, especially in zones at risk of emergence. More generally, I conclude that ancillary vectors should not be overlooked, and that basic entomological knowledge is essential to fully assess the risk of arbovirus emergence.

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## Appendix

### Appendix 1

**Susceptibility status of *Ae. albopictus* and *Ae. malayensis* laboratory colonies derived from sylvatic populations (see Chapter 1) to different insecticides following WHO protocols.**

<b>Insecticide</b>	<b>Species</b>	<b>Number of replicates</b>	<b>Percentage mortality (Status)</b>
Dieldrin 4%	<i>Ae. albopictus</i>	2	98.95 (S)
Malathion 0.8%	<i>Ae. albopictus</i>	3	13 (R)
Permethrin 0.25%	<i>Ae. albopictus</i>	3	100 (S)
Deltamethrin 0.05%	<i>Ae. albopictus</i>	3	100 (S)
DDT 4%	<i>Ae. albopictus</i>	3	92 (RS)
Malathion 0.8%	<i>Ae. malayensis</i>	2	58 (R)
Permethrin 0.25%	<i>Ae. malayensis</i>	3	52.3 (R)
Permethrin 0.25% + piperonyl butoxide	<i>Ae. malayensis</i>	2	58.5 (R)
Deltamethrin 0.05%	<i>Ae. malayensis</i>	2	95.5 (RS)
DDT 4%	<i>Ae. malayensis</i>	2	5 (R)
Temephos (0.0132 mg/L)	<i>Ae. malayensis</i>	1	92 (RS)

S = susceptible (mortality > 98%); RS = resistance suspected (90% < mortality < 98%); R = resistant (mortality < 90%).

Other scientific work not included in the thesis

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## Abstract

Many emerging arthropod-borne viruses (arboviruses) of humans such as dengue, Zika, yellow fever and chikungunya viruses originated in sylvatic cycles, where they circulate between non-human animals and forest-dwelling mosquitoes. Over the last few centuries, these arboviruses have emerged into sustained transmission cycles among humans, causing substantial mortality and morbidity. Dengue virus (DENV) has even become endemic in the human population and is currently responsible for close to 400 million infections each year. The initial mechanism of arbovirus emergence typically involves spillover transmission by mosquito species that 'bridge' sylvatic and human transmission cycles. These bridge vectors can also mediate 'spillback' transmission of human arboviruses establishing novel sylvatic cycles, as exemplified by yellow fever virus (YFV) in South America. Understanding and preventing arbovirus emergence requires detailed entomological knowledge at the interface between human populations and the sylvatic environment.

This PhD work focused on *Aedes malayensis*, a sylvatic mosquito species widely distributed in South East Asia, which was recently detected in peridomestic habitats in Singapore. We used a combination of field surveys and laboratory experiments to assess the potential of *Ae. malayensis* as an arbovirus vector in South East Asia. The PhD thesis is divided in four chapters. The first chapter reports on mosquito larval and adult surveys conducted in a forested area of the Nakai Nam Theun National Protected Area (NNT NPA), Khammuane Province, Laos. We identified 54 mosquito taxa belonging to 11 genera, of which 16 species were new records in Laos, including *Ae. malayensis*. The second chapter deals with the host-seeking behavior of sylvatic mosquitoes in the NNT NPA. We used human-baited traps to identify several sylvatic mosquito species, including *Ae. malayensis*, that may engage in human-biting behavior and therefore act as bridge vectors. The third chapter addresses the potential of *Ae. malayensis* to act as an arbovirus bridge vector in forested area of the NNT NPA. Using both vector competence assays and olfactometer behavioral experiments, we found a relatively modest vector competence of sylvatic *Ae. malayensis* for DENV and YFV and a lack of detectable attraction to human odor in laboratory conditions. The fourth chapter assessed the ability of a peridomestic *Ae. malayensis* population in Singapore to transmit YFV based on vector competence experiments and a human-baited trap survey. Not only was this peridomestic *Ae. malayensis* population able to experimentally acquire and transmit YFV, but it was also found to engage in contact with humans in a field situation.

Together, this work showed that whereas a sylvatic *Ae. malayensis* population in Laos did not display a high potential as a bridge vector, its peridomestic counterpart in Singapore is a capable arbovirus vector. The wide distribution of the species and its ability to colonize urban settings such as high-rise building areas in Singapore calls for increased vigilance. More generally, we conclude that ancillary vectors should not be overlooked, and that basic entomological knowledge is essential to fully assess the risk of arbovirus emergence.

**Keywords:** *Aedes malayensis*; Culicidae; yellow fever; dengue; arbovirus; vector competence; bridge vector; host-seeking behavior; host-vector contact; South East Asia.

## Résumé

De nombreux virus transmis par les arthropodes, ou arbovirus ("arthropod-borne virus"), émergent chez l'Homme, tels que les virus de la dengue, de Zika, de la fièvre jaune et du chikungunya, circulaient à l'origine entre animaux non-humains et moustiques forestiers dans des cycles selvatiques. Au cours des derniers siècles, ces arbovirus ont émergé chez l'Homme dans des cycles de transmission continue, causant une mortalité et une morbidité importantes. Le virus de la dengue (DENV) est même devenu endémique dans la population humaine et il est actuellement responsable de près de 400 millions d'infections chaque année. Le mécanisme initial de l'émergence des arbovirus implique typiquement un transfert, dit « spillover », par des espèces de moustiques « bridge vectors » qui font le lien entre cycles de transmission selvatiques et humains. Ces bridge vectors peuvent aussi permettre un transfert inverse, dit « spillback », des arbovirus humains établissant des nouveaux cycles selvatiques, comme ce fut le cas du virus de la fièvre jaune (YFV) en Amérique du Sud. Comprendre et prévenir l'émergence des arbovirus nécessitent des connaissances entomologiques détaillées à l'interface entre les populations humaines et l'environnement forestier.

Ce travail de doctorat s'est focalisé sur *Aedes malayensis*, un moustique selvatique largement répandu en Asie du sud-est, qui a récemment été détecté dans des habitats péri-domestiques à Singapour. Nous avons utilisé une combinaison d'enquêtes de terrain et d'expériences en laboratoire pour évaluer le potentiel d'*Ae. malayensis* comme vecteur d'arbovirus en Asie du sud-est. La thèse doctorale se divise en quatre chapitres. Le premier chapitre décrit les recensements de moustiques aux stades larvaires et adultes, conduits dans l'écosystème forestier de la réserve nationale protégée de Nakai Nam Theun (NNT NPA), dans la province de Khammuane au Laos. Nous avons identifié 54 taxons de moustiques appartenant à 11 genres, parmi lesquels 16 espèces n'ayant jamais été décrites au Laos, notamment *Ae. malayensis*. Le second chapitre porte sur le comportement de recherche d'hôte des moustiques selvatiques dans la NNT NPA. Nous avons utilisé des pièges à appâts humains pour identifier plusieurs espèces de moustiques selvatiques, dont *Ae. malayensis*, qui pourraient être amenées à piquer l'Homme et donc agir comme bridge vectors. Le troisième chapitre discute du potentiel d'*Ae. malayensis* à jouer le rôle de bridge vector dans la région forestière du district de Nakai au Laos. En se basant à la fois sur des tests de compétence vectorielle et des expériences comportementales d'olfactométrie, nous avons observé une compétence relativement modeste d'une population selvatique d'*Ae. malayensis* pour le DENV et le YFV, et une absence d'attraction détectable pour l'odeur humaine en conditions expérimentales. Le quatrième chapitre évalue la capacité d'une population péri-domestique d'*Ae. mayalensis* singapourienne à transmettre le YFV, à partir d'expériences de compétence vectorielle et de l'évaluation de leur attraction pour l'Homme sur le terrain. Non seulement cette population péri-domestique a été capable d'acquérir et de transmettre le YFV expérimentalement, mais elle a aussi été retrouvée en contact avec les humains sur le terrain.

L'ensemble de ce travail indique que, bien qu'une population selvatique de *Ae. malayensis* au Laos ne possède pas un fort potentiel en tant que bridge vector, son équivalent péri-domestique à Singapour est un vecteur compétent d'arbovirus. La vaste distribution de l'espèce et sa capacité à coloniser des zones urbaines, tels que des zones de gratte-ciels à Singapour appelle à une vigilance accrue. Plus généralement, nous concluons que les vecteurs secondaires ne doivent pas être négligés, et que des connaissances entomologiques de base sont essentielles pour évaluer complètement le risque d'émergence des arbovirus.

**Mots clés :** *Aedes malayensis* ; Culicidae ; fièvre jaune ; dengue ; arbovirus ; compétence vectorielle ; bridge vector ; recherche d'hôte ; contact hôte-vecteur ; Asie du Sud Est.