The risk of vector-borne disease exposure in rubber plantations of northern Lao PDR

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‘The risk of vector-borne disease exposure in rubber plantations of northern Lao PDR’

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Thesis submitted to Durham University in fulfilment of the requirements of the degree of Doctor in Philosophy

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Unprecedented economic growth in South-East Asia has encouraged the expansion of rubber plantations. Outbreaks of vector-borne diseases occur in these plantations, yet data on the vector dynamics is limited. In this thesis I describe the mosquito ecology in rubber plantations compared to neighbouring habitats in northern Lao PDR, to assess the risk of vector-borne diseases for rubber workers and villagers, and to identify how to mitigate these risks.

I carried out a study to identify an ethically sound alternatives to human landing catches (HLC). The human-baited Double Net trap (HDN) collected similar numbers of *Anopheles* and *Culex* as HLC, but under-estimated the number of *Aedes albopictus*. As both HLC and HDN are crude ways of identifying the human-biting rate, the HDN is a representative method to estimate the human-biting rate outdoors without exposing collectors to mosquito bites.

Using the HDN, I compared the adult mosquito dynamics in the secondary forests, immature rubber plantations, mature rubber plantations and villages. A total of 113 species were identified, including 61 species not documented in Lao PDR before The highest number of mosquitoes were collected in the secondary forests. Three of the four most common species found were vector species; the dengue and chikungunya vector *Ae. albopictus*, the lymphatic filariasis vector *Ar. kesseli* and the JE vector *Cx. vishnui*. Additionally, in all habitats a daily exposure to malaria vectors was found.

To assess the risk of exposure to vector-borne diseases I explored the local human behaviour using sociological methods. Compared to staying in the village, dengue exposure risk increased when working in the plantations, which was exasperated when also living in these man-made forests. By contrast, malaria vector exposure risk decreased when living in the plantations.

I identified the characteristics of mosquito breeding sites in rubber plantations and villages. *Aedes albopictus* immature stages were most frequently collected from tyres and latex collection cups in the mature rubber plantations and from tyres and water containers (< and > 10 L) in the villages. A majority of the *Cx. quinquefasciatus* were collected from water containers (< and > 10 L) in the mature rubber plantations and villages. *Anopheles dirus s.l.* were mostly collected from puddles in the immature rubber plantations and villages.

This thesis emphasizes the importance of implementing mosquito control in the rubber plantations for the control of dengue disease. Larval control and personal protection methods are possible vector control methods for our study area. The successful implementation of vector control requires an inter-sectoral approach, with strong collaboration between the health sector, rubber industry and local communities.

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List of abbreviations

ACTs	Artemisinin-based Combination Therapy
AFD	Agence Française de Développement
ASEAN	Association of South-East Asian Nations
BG	Biogents
Bti	*Bacillus thuringiensis israelensis*
CDC	Centers for Disease Control and Prevention
CHIK	Chikungunya virus
CMPE	Centre for Malaria Parasitology and Entomology
CI	Confidence Interval
CO_2	Carbon dioxide
DALYs	Disability-Adjusted Life Years
DEET	N,N-Diethyl-m-toluamide
DENV	Dengue virus
EPI	Expanded Programme on Immunization
FAO	Food and Agriculture Organization
GDP	Gross Domestic Product
GEE	Generalized Estimating Equation
GLM	Generalized Linear Modelling
GMR	Greater Mekong Region
HDN	Human-baited Double Net
HLC	Human Landing Catches
IPL	Institut Pasteur du Laos
IRS	Indoor residual spraying
IVM	Integrated Vector Management

ITN	Insecticide-treated net
LLIN	Long-lasting insecticidal net
LSM	Larval source management
JE	Japanese Encephalitis
MEA	Millennium Ecosystem Assessment
MOU	Memorandum Of Understanding
OR	Odds Ratio
PDR	People's Democratic Republic
RDT	Rapid diagnostic test
RH	Relative Humidity
RR	Rate Ratio
SD	Standard Deviation
SEA	South-East Asia
SEARO	World Health Organization Regional Office for South-East Asia
s.l.	*sensu lato*
s.s	*sensu stricto*
USD	United States Dollar
VBD	Vector borne disease
WHO	World Health Organization
WPRO	World Health Organization Regional Office for the Western Pacific Region

Declaration by candidate

I, Julie-Anne Akiko Tangena, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. All individuals pictured in this thesis gave prior consent to the publication of their image. This thesis is submitted in accordance with the requirements of the Durham University for the Degree of Doctor of Philosophy.

Signed: 

Date: 05-08-2016

Acknowledgements

Since arriving in Laos for the first time three years ago, this country has captured my heart with its friendly smiles, delicious food, 'bor penyang' and beerlao. I would therefore like to start by thanking the people of Lao PDR, without whom this project would not have been possible. I will always feel a special connection with you, your language and your culture. Women never look more beautiful than when they are wearing a traditional 'sin' and the nature in the country is unparalleled.

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I could not have done this work without the help of the very talented young lady Phoutmany. Your calm presence, preciseness and hard work have been invaluable. Thank you especially for being my doorway into the Lao culture and Lao perspectives. The same also applies to Hong. Thank you for being a great driver, cook and most importantly a great friend. I am forever thankful. Hopefully I will be able to repay your friendship in the Netherlands one day.

My gratitude goes out to the health and agriculture departments of Nane and Xieng Ngeun district, and the provincial head of Luang Prabang for their assistance. I would like to thank the Lao National Agriculture and Forestry Research Institute (NAFRI) and the Center for Malaria and Parasite Epidemiology (CMPE) of Lao PDR for their contribution both in the field and during the different meetings. I would like to thank the ECOMORE project, funded by the L'Agence Française de Développement (AFD), for making this project possible.

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I would like to dedicate this thesis to my late friend Machteld Klok, who passed away in a tragic accident at the start of my PhD. Your enthusiasm for life has shown me to be thankful for every experience we have.

Julie-Anne Tangena, 05-08-2016

Transliteration from Lao to English

There are many tones and intonations in the Lao language that cannot be transliterated using roman letters. The Lao government has therefore had difficulty keeping to one standard spelling for villages, districts and even provinces. Throughout the years slightly different spelling have been used in official documents, on road signs and by people. Although the government is in the process of standardizing all names, this document is currently not available. In this thesis I therefore used the transliteration most commonly used in Luang Prabang province.

Authors' contributions

The original project idea was conceived by Dr. Paul Brey (PB). All study protocols were written and designed by myself (JT), Prof. Steve Lindsay (SL) and PB. The project was managed by JT. Translation of materials into Lao language, such as the information sheets and consent forms, were done by Phoutmany Thammavong (PT) Meetings with village heads, district heads, provincial heads and national government were organized by PT under supervision of JT. Data were collected and species were identified by JT and PT throughout the three year study. Members of staff from the National Agriculture and Forestry Research Instititute (NAFRI) helped JT and PT with the environmental measurements fieldwork. For the Rapid Rural Appraisals protocol I cooperated with Mr. Sophoan from 'Agronomes et Vétérinaires Sans Frontières' Cambodia. The behavioural survey was designed by JT and Julia Ledien (JL) from Institut Pasteur Cambodia and conducted by JT, PT and JL. During the survival study JT and PT received assistance in the field from Somsanith Chonephetsarath (SC). All *Ae. albopictus* samples were molecularly tested for viral presence by the French virology laboratory of IPL under supervision of Dr. Marc Grandadam. Molecular identification of *Anopheles* species was conducted at Kasetsart University partially by JT and completed by Mrs. Naritsara under the supervision of Pr. Theeraphap. All data were entered by JT, PT and SC. Data cleaning was conducted by JT. Data analysis was performed by JT under supervision of SL. This thesis was written by JT under supervision of SL and PB. The mosquito trap images were made by Cas Eggerding. JT received important input from Anne Wilson for chapter 2 and from Dr. Alexandra Hiscox for chapter 3. Financial management and administrative support was provided by members of IPL.

1 Introduction

The project team in front of the field laboratory, from left to right; me, Hong and Phoutmany

1.1 Environmental changes – A Global Perspective

People have made unparalleled changes to the environment, improving the lives of billions of people. Since 1990 more than 2.6 billion people have gained access to improved drinking water due to the establishment of water systems [1]. Globally in the last 30 years, despite the total population in poor countries increasing by 2 billion, the total number of people living in extreme poverty has decreased by 0.7 billion [2]. The improvement of human livelihood has often been achieved by misuse of the natural ecosystems. An estimated 60 % of ecosystem services examined by the Millennium Ecosystem Assessment (MEA) are used unsustainably [3] with negative trade-offs including biodiversity loss, decreased natural purification of air and water, decreased protection from disasters and increased climate change [4]. An estimated 1/3 of the ice and desert free land surface has been transformed into cropland or grazing land for food production [5]. From 1990 to 2006 agricultural land increased by 34 million ha [6]. The transformation of the land is not expected to slow down, with the global population expected to reach between 9.0 and 13.2 billion people in 2100 with 95 % probability [7]. To keep up with this growth, the MEA estimated that from 2005 to 2055 demand for food will grow by 70 % to 80 %, for which an extra 10 % to 20 % of grassland and forestland will need to be converted to cropland by 2050 [3, 8]. More worryingly, the human population is polluting water faster than nature can recycle and purify it [9]. By 2050 an estimated 40 % of the world's population will be living in areas where water use exceeds availability [10].

Climate change is impacting the environment, resulting in changes of the local ecology and the land-use. This is leading to both increased disease spread and a population that is more susceptible to diseases [11-13]. The average temperature in the world is expected to increase more than 1.5 ^{o}C by the end of the 21st century [14]. It has been estimated that due to the warmer climate from 1981 to 2002 the yields of maize, wheat and other major crops have dropped significantly, with an estimated 40 megatons of harvest lost every year [9]. Furthermore, climate change has increased rainfall in some areas, and localised variation in extreme events including floods, droughts and tropical cyclones [15, 16]. It has been estimated that, if the current trajectory continues, between 2030 and 2050 climate change related impacts could cause an extra 250.000 deaths per year [4]. It remains a real challenge to find and maintain a balance between protecting the environment and its resources, whilst increasing the economy and living standards of poor communities.

1.2 Relation between environmental changes and vector-borne diseases

Currently, more than 80 % of the global population lives in regions at risk of at least one vector-borne disease. More than 50 % of the global population is at risk of at least two vector-borne diseases [17]. An increasing number of studies are showing changes in the environment as a major driver of vector-borne diseases [18-23]. These include environmental changes such as changes in wildlife habitat, surface water availability, agricultural land, urbanization accompanied by human migration and climate change (Figure 1.1). These changes can increase the number of vector breeding sites, increase host and vector distribution, increase interaction of hosts with vectors and change biodiversity [18-22, 24-26]. The environmental changes can also impact the vector-borne disease dynamics indirectly. For example, insecticides and herbicides are often used on agricultural land. The use of these chemicals increases the selective pressure on the local mosquito populations. This results in a higher rate of resistance development. Once resistance to insecticides is established in the vector population, it is more difficult to control the vectors using these insecticides. This is especially problematic, as some resistance mechanisms result in cross resistance to insecticides not used before. There are many examples of how environmental changes have resulted in an increase of vector-borne diseases. Nevertheless, environmental changes do not necessarily cause increase in vector-borne disease incidence. There is a complex balance dependent on the ecosystems affected, type of land-use change, disease-specific transmission dynamics, sociocultural changes, the specific vector ecology and the susceptibility of human populations [11, 27].

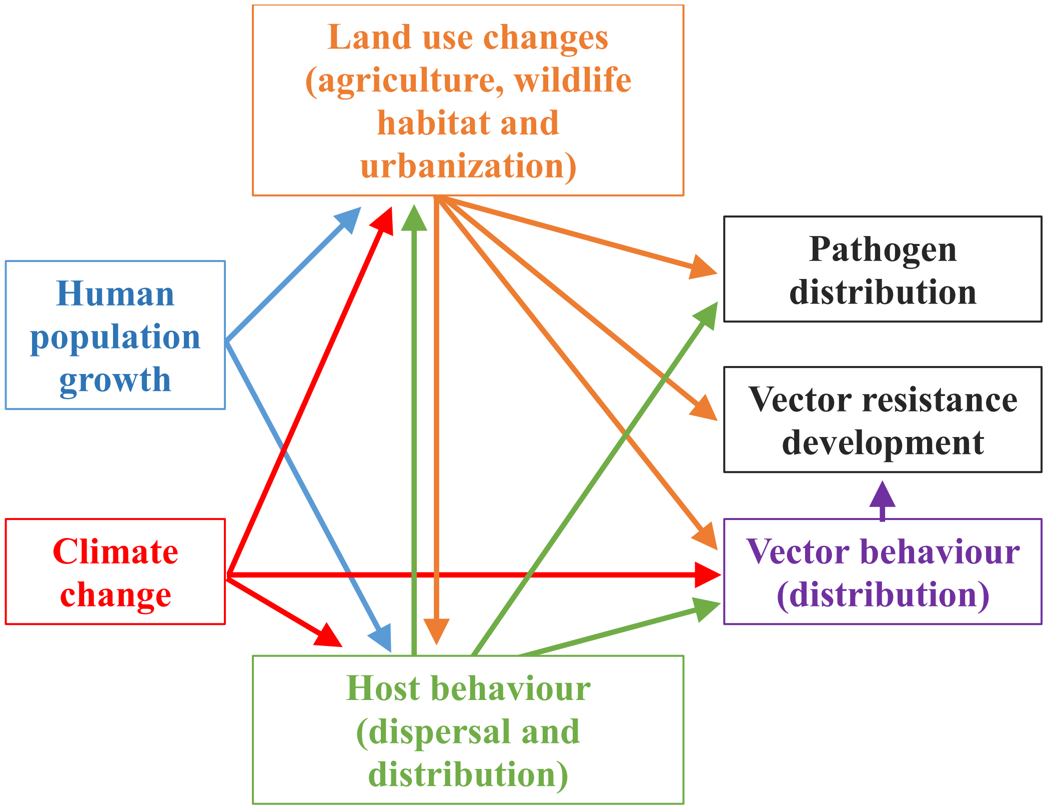

Figure 1.1 Direct and indirect impact of human population growth, climate change and land use changes on vector-borne disease dynamics

It is challenging to identify the exact impact of environmental changes on the vector-borne diseases. An example of how environmental modifications can alter vector-borne disease incidence in a variety of ways, is the building of irrigation systems [28, 29]. These systems are essential for food production, with an estimated 40 % of global food crops dependent on irrigation. Irrigation systems increase the surface water availability in the area. These water areas are often good mosquito breeding sites. The establishment of irrigations can therefore lead to an increase in the number of vector mosquitoes [30]. In India, after the development of an irrigation project, the annual malaria parasite index increased from 0.01 to 37.9. Another irrigation project in India resulted in a nine fold increase of malaria cases [31, 32]. A more recent study from western Ethiopia showed a 4.6 to 5.7 fold increase in the annual exposure rate to malaria infectious mosquitoes in irrigated sugarcane areas compared to traditional and non-irrigated agro-ecosystems [33]. By contrast, a review on the impact of irrigation on malaria incidence showed that, in 11 studies where malaria was stable, no difference was found between irrigated villages and non-irrigated villages. Interestingly, in some studies a lower malaria incidence was found in irrigated villages due to the replacement of the vector by a less efficient vector. For example, the change from *Anopheles funestus* by the less anthropophilic *Anopheles arabiensis* [29]. Environmental modifications can either increase or decrease risk of vector-borne diseases dependent on the local environment, human behaviour and local vector population.

Climate change is expected to impact the vector-borne disease incidence considerably [14, 19, 34]. However it remains challenging to predict the exact impact of climate change on vector-borne disease dynamics [35]. Warmer temperatures are suggested to increase the distribution and development rate of both mosquitoes and the pathogens [36-39]. Furthermore, extreme weather events related to climate change will results in unstable water levels, changing vegetation structures and altering aquatic predator dynamics [40]. Additionally, the extreme weather events increase the exposure and vulnerability of the human population to vector-borne diseases.

Due to climate change both malaria and dengue are expected to expand in range. For malaria, the conservative estimate is that due to climate change an additional 360 million people will be at risk of *Plasmodium falciparum* and *Plasmodium vivax* malaria in 2080 [41]. This increased distribution of malaria to previously non-endemic areas can cause a considerable reduction in healthy years for the local population [39]. By 2080, due to climate change, an additional 2.5 billion people will be at risk of dengue disease [36]. Moreover, there is an increased risk of emerging infectious diseases related to climate change [42]. According to the Lancet Commission on Health and Climate Change [4], climate change could partly reverse the health gains achieved in recent decades. However, it also provides the opportunity for governments and non-governmental organizations to work toward a climate resilient health system which simultaneously improves the health of the people and slows down climate change [34].

1.3 Relations between deforestation and vector-borne diseases in South-East Asia

In recent decades, deforestation has mostly been associated with tropical regions [43]. Trees are being cut on a vast scale to provide income from the sale of tropical hardwood, to make way for infrastructure, urbanization and, most importantly, to provide more agricultural land for crops. The main crops for which forests are being cut are rubber, cashew and sugar cane. In the last two centuries, the global forest area has been reduced by approximately 30 % [44]. South-East Asia (SEA) currently has one of the highest rates of tropical deforestation. The most heavily deforested areas are the North-East and southern part of Lao PDR and the North-East of Cambodia. Forests are shrinking, with a total of 33 million hectare (ha) of tropical rainforest lost between 1990 and 2010, resulting in 203 million ha of rainforest left in 2010. An additional 16 million ha are expected to be lost from 2010 to 2020, decreasing the area of SEA covered in forest from 49 to 46 % [45].

Changes in mosquito ecology and disease risk after ecological disruption is difficult to predict and even more difficult to relate to vector-borne disease incidence. Deforestation can have a varied impact on mosquito-borne disease risk. This is dependent on the complicated dynamics of the environmental factors (light intensity, temperature, air movement, humidity, microclimates, soil, water condition, ecology of local flora and fauna), the ecology of the local mosquito population, the human activities and the human-wildlife contact [27, 46-49]. For example, there is lower risk of malaria in the intact Brazilian Amazon forests compared to the fragmented forests. This is a result of the intact forests containing fewer malaria vector breeding sites, a larger vector predator population and more mammals (which result in the dilution of the disease) [50]. On the contrary, in SEA deforestation often results in lower malaria incidence. This is related to the primary malaria vector of SEA, *An. dirus sensu lato*, which mainly resides in the forested areas [51, 52]. However, if crops are planted in these cleared grassy areas, this can further change the vector ecology. The choice of crops for the cleared area together with the local vector ecology, are two important factors influencing the risk of vector-borne diseases after deforestation [27, 51]. In areas where *An. dirus s.l.* are the main vector, forest replacement by rice cultivation led to a reduction in malaria. This was due to the absence of canopy cover. Yet when tree-crop plantations were established in these areas, the canopy cover was re-established and led to the establishment of the same forest malaria species [27]. In areas where non-forest malaria vectors are the main vector, the establishment of rice fields led to an increase in malaria incidence. It is important to note that the increase or decrease in number of vector mosquitoes does not necessarily correspond to vector-borne disease incidence change. More mosquitoes can result in more competition for resources or changes in human behaviour [53].

Wildlife in SEA carry many different viruses that are transmitted by arthropods. These arboviral diseases, like dengue and chikungunya, can be transmitted from forest mammals to people with sometimes little or no genetic changes necessary to adapt to the human hosts [54]. Globally, about 60 % of emerging diseases are zoonotic viruses, with 72 % of these originating from wildlife [55]. For arboviral diseases to emerge and spread, the proximity and interaction of humans with forested areas are important. The deforestation process can increase human-wildlife contact, increasing the risk of zoonotic diseases spreading from the forest habitat to the human population. This increased interaction occasionally leads to disease outbreaks [56]. Deforestation activity is thought to be one of the most important factors contributing to emerging and re-emerging vector-borne diseases [48, 54, 57-62]. Once deforestation has occurred, the natural environment of the reservoir hosts and mosquitoes is reduced, thereby limiting the human-wildlife contact. However, due to increasing populations, people may start living closer to the border of the forest. This can increase human-wildlife interaction again and increase the risk of zoonotic virus transmission (Figure 1.2). Deforestation changes the interaction between human and wildlife, sometimes resulting in the establishment of zoonotic diseases in the human populations.

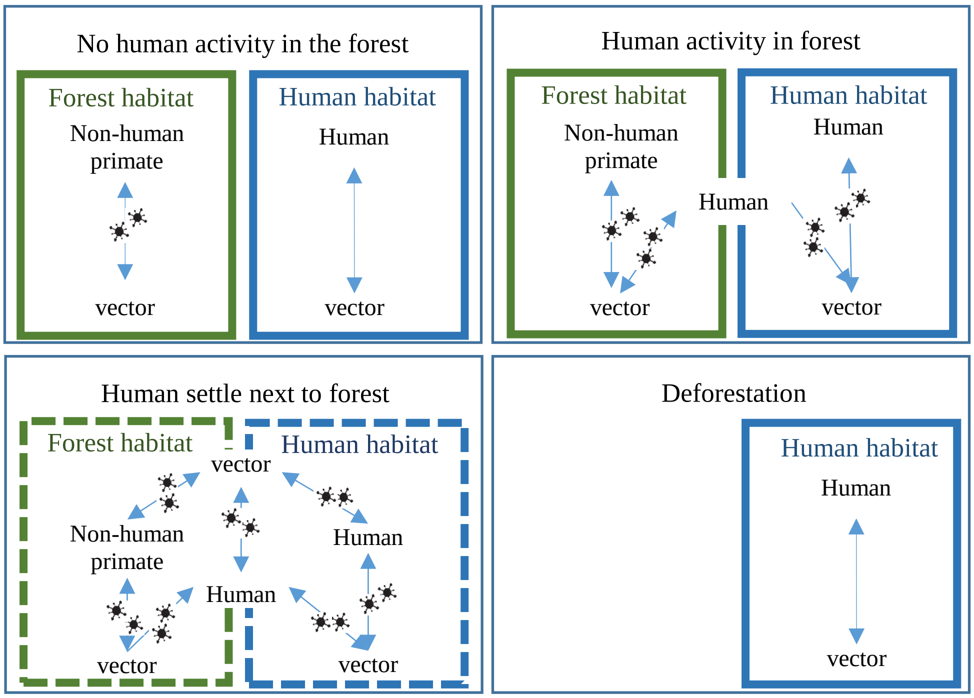

Figure 1.2 Dynamics of forest vector-borne diseases. ✱✱ denotes the disease pathogens

1.4 Lao PDR

1.4.1 Geography and economy

Lao PDR is a lower-middle income country landlocked by Myanmar, China, Viet Nam, Cambodia and Thailand (Figure 1.3). It is a country roughly the size of the United Kingdom, with a total land area of 236,800 km^2. The Mekong is the largest and most important river in Lao PDR, spanning over 1,898 km within the country [63]. It provides irrigation, energy, food and infrastructure to the country. It also functions as the border with parts of Thailand and Myanmar. The country has a diverse land cover, with mountainous forest areas in the north and lowland planes around the Mekong in the middle and South. According to the Lao statistics bureau only 5 % of the land is arable throughout the year [63]. The main crops cultivated in the country are rice, maize, soybean, peanut and tobacco. Unfortunately, due to the fast economic growth, the natural resources are under pressure. According to the Food and Agriculture Organization (FAO) Lao PDR, together with Cambodia, has the fastest deforestation rate in the region [45].

Figure 1.3 Location of Lao PDR and its provinces in South-East Asia [64]

Lao PDR is exceptionally diverse in its ethnicities. In 2014, Lao PDR consisted of 6.7 million inhabitants with more than 57 recognized ethnic groups [65]. More than half of the Lao population lives in rural areas (63 %), with low population densities throughout the country. Population density in Lao PDR is 24 people/km^2 compared to 232 people/km^2 in Viet Nam and 127 people/km^2 in Thailand [66]. In 2005, Lao PDR consisted of 31,210 km of road, significantly less than the 210,000 km of road in Viet Nam (with a land area of 332.698 km^2). Due to the high spread of the population and the low density of roads, accessibility of health centres for people living in some areas of Lao PDR is still problematic (Figure 1.4 A) [66]. Poverty in Lao PDR is very heterogeneous with difficult to access areas in the south of Lao PDR and the mountainous areas in the north lagging behind (Figure 1.4 B) [66]. According to the 2005 household census, 35 % of the total population had access to drinking water with 58 % of households having access to electricity.

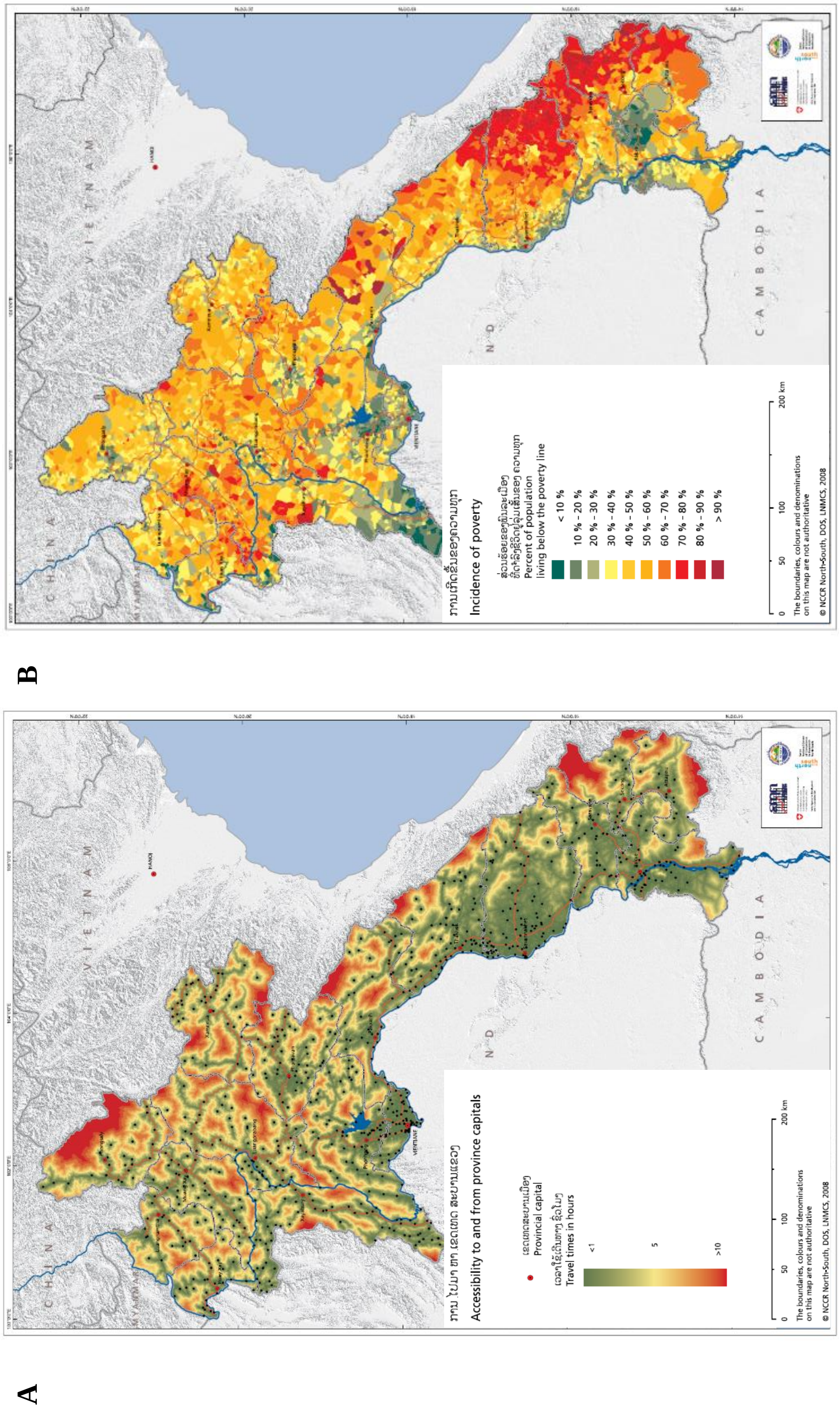

Figure 1.4 Socio-economic maps of Lao PDR with data from 2005 census (A) Accessibility to and from health centres in Lao PDR (B) Incidence of poverty in Lao PDR

Since the economic reforms in the late 1980s, Lao PDR has become one of the fastest growing economies in Asia. In 2014 the Gross Domestic Products (GDP) grew with 7.5 %. More than half of the country's wealth is comprised of natural resources, including timber and minerals [65]. An estimated 78.5 % of the Lao population is working in agriculture. As a member of the Association of South-East Asian Nations (ASEAN), Lao PDR is slowly becoming a land-linked country more than a land-locked country, with increased integration in the regional and global economy. An estimated 5 % of the population is migrating to neighbouring provinces or countries every year to find land for agricultural production, opportunities for education, employment, and access to social services [66]. Due to the increase of travel between countries for trade and workforce, infectious diseases are more easily spread. Examples include the spread of artemisinin-resistant malaria parasites and the identification of chikungunya in Lao PDR originating from neighbouring countries [67, 68]. These issues are currently being addressed by the government in a new five year Strategic Plan [69]. The economic reforms in Lao PDR are changing the dynamics of the country at an unprecedented speed, presenting new challenges for the health of the population.

1.4.2 Rubber plantations

Rubber tree cultivation is a new kind of mass farming not seen in Lao PDR before. Lao PDR has seen a rapid increase in rubber plantation area, with 900 ha of mature plantations in 2010, increasing 163 fold to 147,000 ha in 2015 [70]**.** The hectarage for rubber plantations is still rather low compared to neighbouring countries [71, 72] (Table 1.1). In the future the rubber plantation area is likely to further expand to 342,400 ha [70]. The impact of its expansion on the local ecosystems remain poorly understood.

Table 1.1 Latex production in 2010, adapted from [70, 73]

Country	Production ('000 tonnes)
Thailand	3300
Indonesia	2592
Malaysia	1000
Lao PDR	1.3

Rubber cultivation is seen as a potential sustainable alternative for poor farmers in SEA. A large area of SEA is suitable for rubber plantations and these plantations provide a relatively high income [74, 75]. The rubber trees are planted for the production of latex, which can be tapped until the tree is 30 years old. These trees can then be sold for wood and replaced by new rubber trees. The economic benefits of rubber plantation cultivation for the population are dependent on the local situation. The rubber plantations have improved the livelihood in villages of Luang Namtha province (Lao PDR) and South-West China. The livelihood improved especially amongst ethnic minorities [76-78]. Farmers earned approximately 6,000 to 8,000 USD per hectare per year from growing rubber, which is considerably more than from rice farming, non-timber forest products and eco-tourism [79]. In 2006, profit for rubber plantation areas in Lao PDR was 880 USD/ha compared to 146 USD for rice and 903 USD for opium [70]. Although the cultivation has improved the livelihood of small-scale local rubber plantation owners, in areas with large foreign owned rubber plantations economic benefits for the local populations are small. Rubber plantations are often established on agricultural land and surrounding forests, excluding the local population from resources that provided them with food and security [80, 81]. Furthermore, the tappers on these big plantations are often migrants, resulting in little or no employment for the local inhabitants. Rubber plantations therefore offer both opportunities and hardship depending on how and by whom they are established.

The establishment of rubber plantations has a trade-off with environmental resources. Even though rubber cultivation helps reduce carbon dioxide (CO_2) in the atmosphere, these areas show a 19 % lower soil carbon stock then the originally present natural forests [82]. Additionally, although soil erosion is less than for other crop plantations, rubber plantations still cause significant soil erosion compared to the natural forests [83, 84]. One of the biggest environmental impacts of the rubber cultivation is the local underground water depletion due to the latex extraction [77, 84-86]. In southern China, the water depletion was so severe rubber planting was banned in 2006 [86]. The establishment of large rubber plantations can lead to significant changes in the environmental resources and need to be closely monitored.

Establishment of rubber plantations in Lao PDR is one of the most important factors contributing to forest loss, together with cashew and sugarcane production [45]. Huge areas of primary and secondary forests are being cut to make space for rubber plantations. This rapid and uncontrolled expansion has been conducted without proper surveys and have resulted in the loss of many primary rain forests [45, 71, 87]. The Lao forests have been described as one of the most diverse habitats in the world [88-91]. Cutting these forests for the establishment of monoculture rubber plantations results in a large decrease of the local biodiversity. The rubber plantations are maintained regularly by cutting the undergrowth between trees, further decreasing the biodiversity in the area.

The rubber plantations do not provide the key biodiversity assets which are essential for many flora and fauna species to survive and remain. This results in a collapse of the forest ecosystem, which was present before deforestation. For example, the low density and diversity of plants in the rubber plantations results in a lack of food and shelter for organisms, such as pollinators. The decrease in pollinators will impact the supporting ecosystem services, which are necessary for certain plants and crops to reproduce. The loss of biodiversity also leads to a loss in some essential regulatory ecosystem services, such as the loss of predators of mosquito larvae and adults. This loss in regulatory services could increase the number of mosquitoes and subsequently increase the risk of disease for the local population. Additionally fungicides are regularly sprayed in the rubber plantations to decrease risk of tree blight [70]. These fungicides persist in the soil and migrate to waterways, impacting both the terrestrial and aquatic ecosystems in an area beyond the plantations. Due to the establishment of rubber plantations, the local population is also deprived of the non-wood forest products present in the forest areas (provisioning ecosystem services). An estimated 80 % of people in developing countries are dependent on the forests for their primary health and nutritional value [45]. In Lao PDR wild foods are consumed by 80 % of the population on a daily basis. These wild foods include insects, small mammals, fruits, edible leaves, nuts, roots and mushroom [92]. The self-sufficiency of the local population is further decreased by the competition of rubber plantations with other food crops such as rice, coffee, corn and sweet potatoes [71]. The Lao government is currently encouraging intercropping of rubber trees with upland rice, pineapple, corn, maize, sesame and other crops to increase crop diversity. This intercropping is making people more self-sufficient and provides extra income for farmers. The loss of forests and their biodiversity, for the establishment of rubber plantations, is leading to the loss of many ecosystem services essential for the environment and the local population. There is a clear need to identify the direct and indirect impact of the rubber plantation establishment, both short term and long term, on the ecosystem services present. This is essential, as the impact of this loss in services is likely to impact an area far greater than only the rubber plantation area.

1.5 Vector-borne diseases in Lao PDR

1.5.1 Malaria

Malaria is endemic in Lao PDR with a highly heterogeneous distribution (Figure 1.5). There is low focal malaria transmission in the mountainous areas (>1200 m) in the North, low transmission in the lowland plains of the Mekong in the centre of the country and high transmission in the forested areas in the South [93-95]. The peak transmission period is during the rainy season from May to October. *Plasmodium falciparum* is the most common malaria parasite in the country. The second most common malaria parasite is *P. vivax*. Occasionally, *P. malariae* and *P. ovale* are also reported [96, 97]. At present, Lao PDR is the only country in the region where *P. knowlesi* has not been detected. In 2013, about 73 % of all malaria cases were *P. falciparum* and 27 % *P. vivax* compared to only 3 % *P. vivax* cases in 2010 [98, 99]. The proportion of *P. vivax* cases has increased. This is likely related to the dormant stages of this parasite in the liver, which are more difficult to eliminate. Furthermore, the proportion change is likely related to the improved surveillance and the high number of seasonal workers that introduce *P. vivax* from other malaria-endemic areas. About 90 % of all malaria cases are from the five southernmost provinces of Lao PDR. Consequently, about 36 % of the population in Lao PDR live in high transmission areas with 60 % of the population living at risk [95, 98]. According to the Ministry of Health, the population most at risk of malaria are the ethnic minority groups, forest fringe inhabitants, new forest settlers, children under 15 years and seasonal workers [100-103]. The seasonal workers in the Greater Mekong Region (GMR) consists of at least 4 million workers, emphasizing the need to develop specific control measures to protect these vulnerable groups [104]. The difficulty of protecting these vulnerable populations is exacerbated by the cultural differences, language barriers, their remoteness from good infrastructure (Figure 1.4 A) and weak healthcare services. Malaria in Lao PDR is highly heterogeneously distributed, with a large number of vulnerable groups that are difficult to protect.

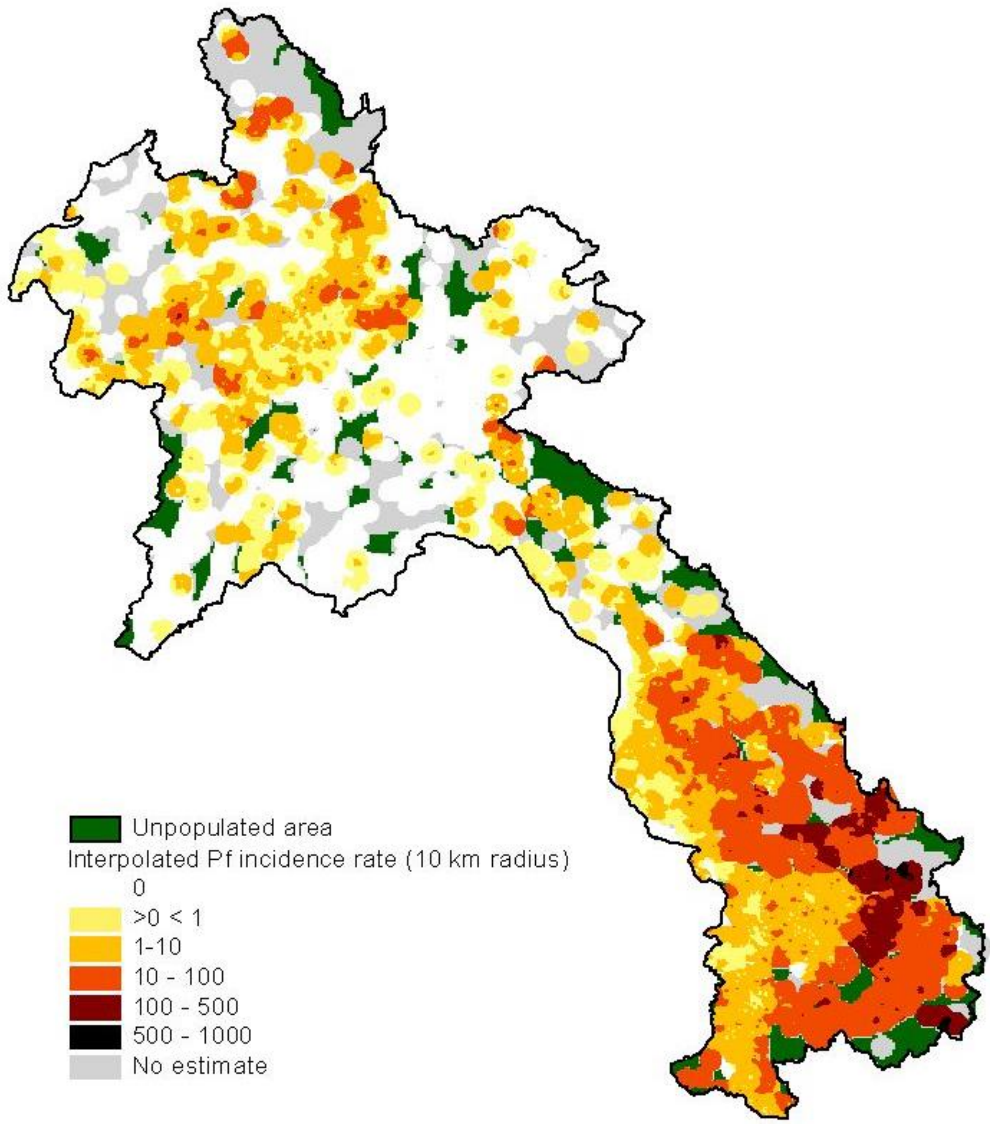

Figure 1.5 Malaria incidence distribution in Lao PDR in 2010 [98, 105]

In Lao PDR malaria transmission is most intense in forests and on fringes of forests, where the important anthropophilic malaria vector *An. dirus s.l.* resides [106-108]. In southern Lao PDR, the major vector *An. dirus s.l.* is looking for a blood meal from 19.00-06.00 h with a peak in host-seeking activity at 22.00 h [97, 108]. The mosquito species has a variable biting preference with indoor biting preference in Attapeu province and no preference for either indoor or outdoor biting in Sekong province [97, 108]. *Anopheles dirus s.l.* prefers to lay eggs in slow moving or stagnant water in forested areas, with larvae found in the shaded areas of the water [109]. Apart from *An. dirus s.l.*, important vectors of malaria in Lao PDR include *Anopheles minimus s.l., Anopheles maculatus s.l.* and *Anopheles jeyporiensis* [96, 97, 108]. Currently, more than 41 anopheline species have been identified in Lao PDR. Possible secondary malaria vectors include *Anopheles harrisoni*, *An. aconitus, Anopheles nivipes, Anopheles philippinensis, Anopheles sinensis*, *Anopheles sawadwongporni,* and *Anopheles vagus* [52, 94, 96, 97, 102, 108, 110, 111]. In Lao PDR, apart from the primary vectors and *An. philippinensis*, no malaria positive mosquitoes have been identified. However, in neighbouring Viet Nam and Thailand, using circumsporozoite protein (csp) ELISA methods, these possible secondary malaria vectors have been implicated as vectors [53, 94, 112, 113]. It should be noted that csp ELISA methods alone may give false positives [114]. It is therefore necessary to confirm the csp ELISA results with PCR techniques to confirm infection of mosquitoes with malaria parasites. This has not been done yet for any of the secondary vectors.

In 1998, Lao PDR had the highest malaria incidence (7.9 cases per 1,000 population) and mortality rate (8.6 deaths per 100,000 population) in the GMR [18]. Malaria incidence has since decreased from 9.1 cases per 1,000 people in 2002 to 3.5 cases per 1,000 people in 2010. This decrease is due to the increased malaria control measures and economic development of the country. However malaria incidence in Lao PDR is not under control yet. In 2012 the World Health Organization (WHO) reported a malaria epidemic in the southern provinces. A threefold increase in malaria cases was reported in 2012 compared to 2011. Malaria cases have since fallen, yet outbreaks continue in Saravan and Champasack provinces [115]. The outbreaks of malaria in the southern provinces of Lao PDR have been linked to the rapid environmental changes occurring in the provinces. The changes include mining, rubber plantation establishment, hydro-dam constructions and deforestation. There is, furthermore, great concern that the movement of seasonal workers is hastening the spread of diseases to other regions in SEA and beyond [67, 116]. An example is the artemisinin-resistant strains, which is already present in most of mainland SEA [67, 117]. Disturbingly, there is evidence that during the epidemic in 2012 foreign migrant workers from provinces in Viet Nam, where the artemisinin-resistant malaria parasites are established, have spread the resistant strains to Champasack and Attapeu provinces [115, 118]. The Lao Ministry of Health is intensifying malaria control efforts and targeting remaining endemic communities and key risk groups [100].

The control of malaria disease in Lao PDR is achieved by the free distribution of long-lasting insecticidal nets (LLINs), by the early diagnosis with rapid diagnostic tests and by providing free treatment with antimalarials [100]. A great proportion of the malaria control efforts is funded by 'The Global Fund to Fight AIDS, Tuberculosis and Malaria'. As the distribution of LLINs in Lao PDR was associated with reduced *An. dirus s.l.* density and decrease in malaria slide positivity rates [119, 120], since 2003 LLINs have been distributed to the Lao population free of charge. In 2008, 82 % of the population at risk of malaria had access to free LLINs. Studies have shown that the available bed nets in Lao PDR are not always fully utilized and need to be properly maintained for continuous protective effect [120, 121]. Therefore, education on the use and maintenance of LLINs should be facilitated. Additionally, to control vector mosquitoes since 2010 Indoor Residual Spraying (IRS) and larvicides are recommended in high risk areas and during outbreaks [100]. With the current high dependence on insecticides for vector control in Lao PDR, development of insecticide resistance in malaria vectors should be closely monitored. Several malaria vectors have already been identified to be less sensitive to insecticides in the Mekong region which could threaten the malaria control programmes [122]. There is a study ongoing in Lao PDR to identify the presence of insecticide-resistant malaria vectors and their distribution throughout eight provinces (project MALVEC, Institut Pasteur du Laos).

In 2004, Quinine and Doxycycline were used for *P. falciparum* treatment, and Artemesinin-based combination therapy (ACT) (Artemether + Lumefantrine, Coartem®) was used for *P. vivax* treatment [98, 100]. Since 2005, the most commonly used treatment for both malaria parasites in Lao PDR is Coartem ® [123]. One of the main problems in treating malaria cases in Lao PDR is the high number of counterfeit and substandard malaria medicine available. These suboptimal malaria medicine contain no or too low amount of the active ingredients necessary to kill the malaria parasites. Worryingly, some antimalarials tested contained the ineffective medicine Chloroquine and banned pharmaceuticals [124, 125]. The use of these substandard medicine has led to deaths and has contributed to the spread of artemisinin resistance [124]. A study from 2001 showed three of the eight Artesunate formulations (38 %) bought in Lao pharmacies were counterfeit. Similarly two studies from 2003 showed 54 % (25/46) and 90 % (27/30) of Artesunate antimalarials bought in Lao pharmacies were counterfeit [126-128]. Since then, the government has taken steps to improve the quality of the medicine. A recent study in 2012 showed none of the 158 Artesunate antimalarials bought in Lao pharmacies were counterfeit, although 25 % were still substandard [125]. The antimalarials used for the treatment of malaria in Lao PDR should be closely monitored for quality to ensure the Lao population has access to effective and safe antimalarials.

1.5.2 Dengue and Chikungunya

In Lao PDR, both the dengue virus (DENV) and chikungunya virus (CHIK) have been reported. They have similar symptoms of fever, rash and joint pains, causing misdiagnosis in areas where both diseases occur [129]. Even though chikungunya is rarely fatal, DENV infection can lead to haemorrhagic fever with plasma leakage, severe bleeding and impairment of organs. The global annual costs of dengue have been estimated at 8.9 billion USD [130]. There is no specific treatment for either disease [131]. Typically, the peak transmission season for dengue and chikungunya in Lao PDR is during the rainy season, from May to October. Both pathogens are present in the natural forests, with similar mosquitoes transmitting the viruses amongst the macaques (*Macaca)*. This transmission cycle between wild animals and vectors is called the sylvatic cycle. Furthermore the disease is present in the human population.

Both diseases are endemic in SEA with spatial distribution of dengue and chikungunya varying, even in small areas [54, 132-134]. The vectors known to transmit dengue and chikungunya are *Aedes (Stegomyia) albopictus* and *Aedes (Stegomyia) aegypti*. Both vectors easily adapt to new environments with *Ae. albopictus* predominating in rural and *Ae. aegypti* in urban areas [135]. The immature stages are mostly found in indoor artificial containers closely associated with human dwellings [136]. Typically, these mosquitoes do not fly far, remaining within 100 m of where they emerged. They feed almost entirely on humans, mainly during daylight hours, both indoors and outdoors. These mosquito species easily adapt to new surroundings, increasing their distribution throughout the world. This ever increasing distribution of *Ae. albopictus* and *Ae. aegypti* is resulting in the spread of dengue and chikungunya disease.

The DENV consists of at least four different serotypes, which have all caused outbreaks in Lao PDR [137]. During different surveys conducted from 1991 to 1994 between 58 % and 100 % of surveyed population in Lao PDR were found positive for DENV antibodies [138-140]. Highest prevalence of dengue infections was found in adults in the plain areas along the Mekong [140, 141]. The latest outbreak dates from March 2013 when a four-fold increase in DENV infections occurred. A total of 1,070 cases were reported from January to March 2013, mostly DENV3, compared to 269 cases in the same months a year before [137, 142]. Little is known about chikungunya distribution and incidence in Lao PDR. Chikungunya was first recorded in 1994 in Vientiane capital and rural Khammouane province by detection of antibodies [138]. In 2012 the virus itself was identified in Khammouane province, with data currently being analysed [68]. Dengue and chikungunya case reporting in Lao PDR has been incomplete and inconsistent in the past. Since 2013 the reporting has improved. Currently samples of all suspected dengue and chikungunya cases from the provincial hospitals are tested in a central laboratory using PCR techniques for confirmation of the diseases. Information on the prevalence of dengue and chikungunya is increasing, with indication both diseases will continue to be important causes of disease in the region.

1.5.3 Japanese encephalitis

Japanese encephalitis (JE) is a vaccine preventable disease for which no alternative treatment is available. The JE virus originates from Indonesia/Malaysia, from where it has evolved and spread throughout Asia [143]. The peak in cases occurs in the rainy season and early dry season from June to November. The disease typically starts with flu-like symptoms of fever, headache and disorientation. One out of every 250 cases leads to more severe symptoms with seizures, spastic paralysis and comas. In about 30 % of the severe cases the disease leads to death. About 20-30 % of severe cases that do not lead to death, lead to permanent intellectual, behavioural or neurological problems [144]. In Asia, around 3 billion people are at risk of the disease. The human host is considered a dead-end host, with a short period when a low density of viruses is present in the blood. This short viremia makes it almost impossible for mosquitoes to become infected with JE when feeding on a human host. The risk of disease is generally associated with the presence of their natural reservoir *Ardeidae* water birds, like herons and egrets. Furthermore the risk of disease is also considered to be associated with the domestic pigs, their amplifying host. In neighbouring countries both *Culex tritaeniorhynchus s.l.* and *Culex quinquefasciatus* have been identified as the main JE vectors [145]. It is likely that these species also play an important role in Lao PDR. The disease has been found to be related to the lowland rice agriculture where both the zoophilic primary vectors of JE, *Culex tritaeniorhynchus s.l.* and *Culex quinquefasciatus*, reside [145, 146]. The primary mosquito vectors breed in rice fields and fly to the peri-domestic areas for their blood meal. The vectors can fly up to 1.5 km from their breeding site to find a host [147]. Other (possible) vectors of JE in Lao PDR include *Culex pipiens, Culex vishnui, Ae. albopictus, Aedes togoi, Culex annulus, Culex quinquefasciatus* and *Armigeres subalbatus* [147, 148]. Entomological and molecular studies are needed to identify the important vectors of JE in Lao PDR.

According to the WHO, there are globally more than 68,000 confirmed JE cases every year, with an estimated 20,400 deaths [149]. In Lao PDR, JE is endemic with 78 % of the Lao population at risk, particularly children [139, 141, 150, 151]. In Lao PDR there are limited data on the number of JE cases. Until recently, Lao PDR did not have countrywide coverage of JE vaccines. Control of JE relied mostly on the malaria vector control (LLINs, IRS and larvicide distribution), which also decreased densities of JE vectors. Studies in China have shown that LLINs distributed for malaria control also resulted in a 20 % decrease of the primary JE vector *Cx. tritaeniorhynchus* and in some areas a 48 % decrease in disease incidence [152, 153]. However, the use of insecticides to control JE vectors is of temporary nature and not always effective [147]. In 2014 JE immunization campaigns were started throughout the country. To complete the coverage throughout the country, in April 2015 a final campaign was commenced. This campaign, funded by the vaccine alliance, has the goal to vaccinate an additional 1.5 million children between 1-15 years with the JE vaccine. From 2016 the country will include the JE vaccine in the national Expanded Programme on Immunization (EPI), a routine vaccination programme for vaccinating new-born babies, to ensure continued countrywide coverage. With help of the mass vaccination campaigns JE is likely to become less prevalent in Lao PDR.

1.5.4 Lymphatic filariasis

Worldwide, lymphatic filariasis is an important source of disability, with the disease causing an estimated 40 million disfigured and incapacitated people [154, 155]. Lymphatic filariasis is generally caused by the nematode *Wuchereria bancrofti*, with other causes in Asia including *Brugia malayi* and *Brugia timori* infection. The nematodes reside in the lymph system of people, where it decreases its function. In some cases this can results in lymphedema; accumulation of fluids which leads to abnormal enlargements of body parts.

Lymphatic filariasis is transmitted by a wide variety of mosquitoes including *Aedes*, *Anopheles*, *Culex* and *Mansonia* species [156]. In Africa, important vectors of lymphatic filariasis include *Culex pipiens*, *Culex quinquefasciatus*, *Anopheles gambiae* and *Anopheles funestus* [157-161]. In South America, the main vector is *Cx. quinquefasciatus* [162]. Generally in Asia, *W. bancrofti* is transmitted by *Mansonia* and *Anopheles* species. In addition, *B. malayi* and *B. timori* are transmitted by *Culex quinquefasciatus* [163]. In India and Indonesia, *Cx. pipiens* has also been identified as an important vector [164]. The disease is endemic in Lao PDR, with currently no data on the vector species. In Thailand important vector species include *Anopheles barbirostris*, *Mansonia annulifera*, *Cx. pipiens* and *Cx. quinquefasciatus* [165-167]. Vector dynamics of lymphatic filariasis is expected to be similar to neighbouring Thailand. However, this needs to be confirmed with the identification of lymphatic filariasis infected mosquitoes in Lao PDR.

The disease can be contained with diethylcarbamazine (DEC), albendazole or ivermectin, which kills the nematodes circulating in the blood. The global mass drug administration of albendazole and ivermectin from 2000 to 2012 has prevented an estimated 96.71 million cases. However, an estimated 67.88 million cases still remain [166]. Many countries in Asia are working towards elimination of the disease using Mass Drug Administration [163]. Although progress has been made, it will be very challenging to eliminate the disease throughout the region without proper understanding of the vector dynamics. Lymphatic filariasis is an important cause of disease in Lao PDR with limited data on its prevalence and dynamics in the country.

1.5.5 Other emerging infectious diseases

Emerging infectious diseases are of great importance in global health with 177 emerging and re-emerging human-pathogens identified by a recent survey [168]. These diseases are newly recognized, newly evolved or diseases increased in incidence and/or expanded in geographical area. About 75 % of emerging and re-emerging pathogens are zoonotic. South-East Asia is a hotspot for emerging vector-borne diseases with cases of dengue variants, evolved JE and zika [169-171]. As a recent study on causes of non-malarial fever in Lao PDR highlighted, vector-borne diseases are an important source of febrile disease [172]. Research on potential animal reservoirs and the vectors associated with these pathogens is becoming increasingly important. This is especially important with a large area of thick forests and wildlife still present in Lao PDR [168, 173, 174]. An example of an emerging vector-borne disease in Lao PDR is rickettsial disease, which represents the second most important cause of non-malarial disease in SEA [175, 176]. Although recognized as an important cause of disease in the region, detailed epidemiology and understanding is limited [177]. Another example of an emerging infectious disease in Lao PDR is the zika virus which, after large worldwide attention, was also found to be locally transmitted in Lao PDR [171]. Detailed information on the dynamics and causal mechanisms of environmental changes is only available for a small number of vector-borne diseases. The challenge for the health systems, both in Lao PDR and in the region, will be to further develop the ability to identify and manage these poorly studied vector-borne diseases [172, 176]. .

Study rationale

The area of land cultivated for rubber is expanding rapidly in Lao PDR. I anticipated that the changes in ecology from primary and secondary rainforest, to cleared land cultivated for rubber and the maturation of these rubber trees will likely result in an altered risk from vector-borne diseases; predominantly malaria and dengue. This study will examine the vector ecology in rubber plantations compared to village and forest habitats, identify risk factors for vector-borne diseases, and make recommendations on how best to reduce the incidence of vector-borne disease for public health workers, governments, and those working in the rubber industries of Lao PDR and other countries in SEA.

Goal of the thesis

The goal of this study was to assess the potential risk of vector-borne diseases in rubber plantations in northern Lao PDR.

Study objectives

The study objectives were:

- Determine the most efficient, reliable and ethically sound method for sampling human-biting mosquitoes in dengue-endemic areas (chapter 3).
- Compare the adult mosquito diversity and abundance in the four major rural habitats: secondary forests, immature rubber plantations (<5 years), mature rubber plantation (>8 years) and rural villages situated close to rubber plantations (chapter 4).
- Describe the behaviour of villagers and rubber workers in the study area that are likely to increase the risk of vector-borne diseases (chapter 5).
- Investigate where mosquitoes are breeding in rural villages, immature and mature rubber plantations (chapter 6).

Using these objectives I made clear recommendations to public health workers, governments and those working in the rubber industries of Lao PDR on how to decrease the risk of vector-borne diseases arising in rubber plantations.

Hypotheses

Malaria: The immature rubber plantations, compared to the secondary forests, were expected to have a lower density and diversity of the important forest malaria vectors *An. dirus s.l.* and *An. minimus s.l.* The lower density of forest malaria vectors was expected, as the canopy cover is not fully developed in the immature plantations. This lack of canopy was predicted to result in lower humidity and higher temperatures compared to the secondary forests, leading to a less preferable habitat for forest mosquitoes. The vector *An. maculatus s.l.* was expected to be collected in the immature plantations, as these mosquitoes prefer sunlit pools for their larval habitats. The density of *An. dirus s.l.* and *An. minimus s.l.* was expected to increase as the canopy developed in the rubber plantations. The mature rubber plantations were therefore expected to contain a similar abundance of forest malaria vectors as the secondary forest habitats. Furthermore, more human activity was predicted to be present in mature rubber plantations compared to the secondary forests. Mature rubber plantations were therefore expected to be the areas where risk of exposure to malaria vectors was highest.

Dengue: Dengue in Lao PDR is transmitted by *Ae. aegypti* and *Ae. albopictus*. *Aedes aegypti* mosquitoes were expected to be collected in the rural villages. The establishment of mature rubber plantations was expected to result in a high number of bowls used for collecting rubber. These bowls were predicted to become good breeding sites for *Ae. albopictus*, resulting in a large number of these dengue vectors in the mature rubber plantations. The *Ae. albopictus* mosquitoes were also expected to be present in immature rubber plantations. However numbers were likely to be lower, due to the lack of latex collection cups, lower humidity and higher temperatures. *Aedes albopictus* mosquitoes were also anticipated to be present in the secondary forest habitats, with larvae surviving in the cut bamboo. Human activity was expected to be high in the mature rubber plantations, resulting in a high risk of dengue vector exposure in mature rubber plantations. Furthermore, villagers that visit the secondary forests during the day were predicted to be at high risk of dengue.

2 Risk and control of mosquito-borne diseases in South-East Asian rubber plantations

A latex collection cup filled with coagulated latex, attached to a mature rubber tree in village Silalek

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2.1 Abstract

Unprecedented economic growth in South-East Asia (SEA) has encouraged the expansion of rubber plantations. This land-use transformation is changing the risk of mosquito-borne diseases in the region. Mature plantations provide ideal habitats for forest mosquitoes, including *Anopheles minimus s.l.*, *Anopheles dirus s.l.*, both malaria vectors, and *Aedes albopictus*, a dengue and chikungunya vector. Migrant workers may introduce vector-borne disease pathogens into plantation areas, most worryingly artemisinin-resistant malaria parasites. Additionally, the close proximity of rubber plantations to natural forest increases the threat from zoonosis, where new vector-borne pathogens spill over from wild animals into the human population. There is therefore an urgent need to scale up vector control and access to health care for rubber workers. This requires an inter-sectoral approach with strong collaboration between the health sector, rubber industry and local communities.

2.2 Mosquito-borne diseases in South-East Asia

In South-East Asia (SEA) the most important vector-borne diseases are malaria and dengue. Great progress has been made in malaria control in the SEA region. The WHO estimates that from 2000-2014 there was a reduction in global malaria cases from 2.9 million to 1.6 million, with malaria mortality rates falling by 60 % [98]. In SEA malaria mortality rate has declined by 85 % and in the western Pacific region by 65 % (Figure 2.1 A). This remarkable drop has been achieved by the massive deployment of long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS), improved access to diagnosis, and effective treatment with artemisinin combination therapies (ACTs) [98]. Consequently, many countries in SEA are now planning for malaria elimination. By contrast, dengue cases have increased in many parts of SEA and the disease is endemic in many places (Figure 2.1 B). Recent dengue epidemics have been recorded in Cambodia, Indonesia, Malaysia, the Philippines and Thailand [131]. Malaria and dengue are the most important mosquito-borne diseases in SEA, with a necessity to mitigate their risk.

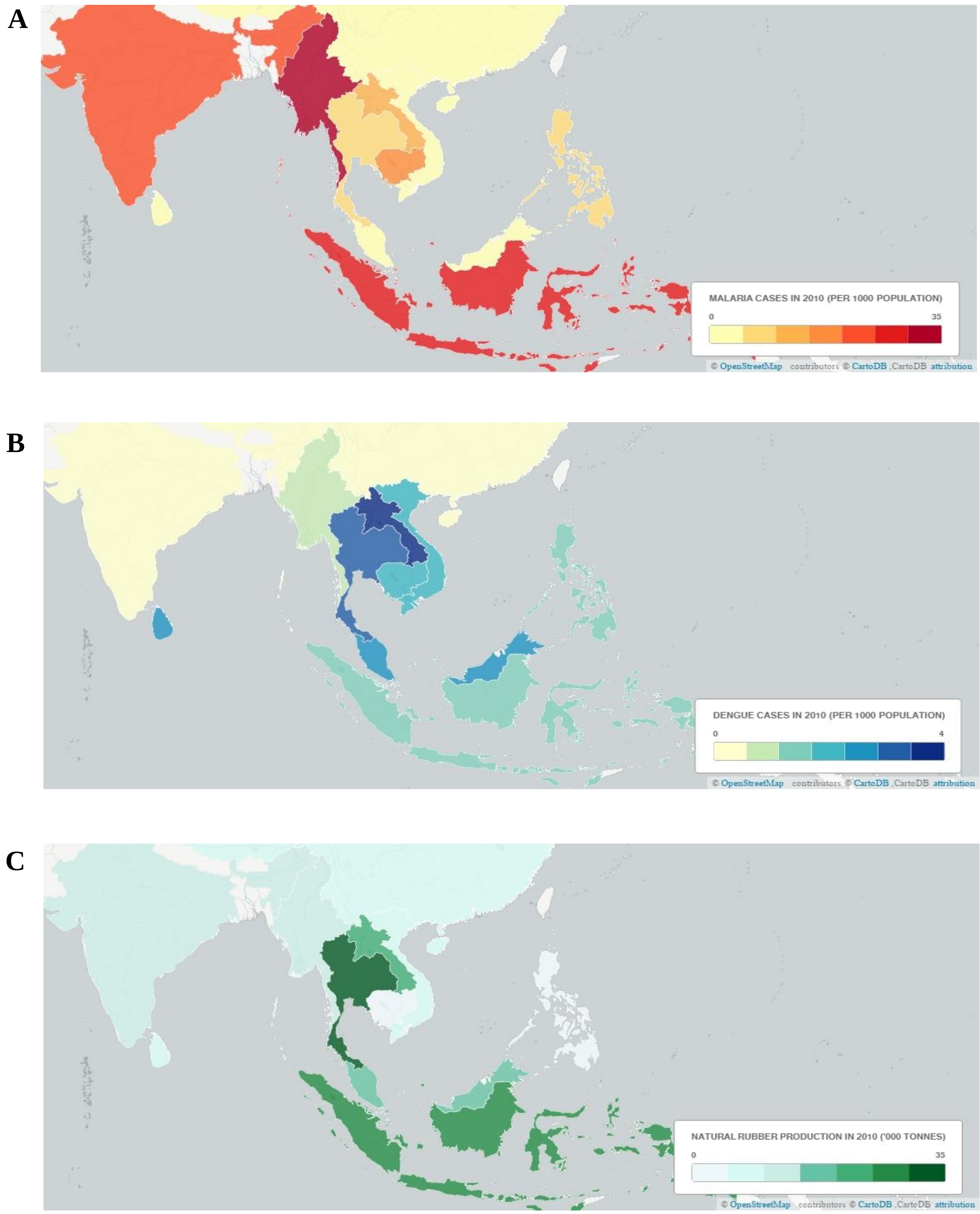

Figure 2.1 Distribution maps of the two main vector-borne disease and rubber plantation area in South-East Asia. (A) Malaria cases in 2010 (B) Dengue cases in 2010 (C) Natural rubber production in 2010, adapted from [73, 98, 178, 179]. Images made using © CartoDB

The risk of both malaria and dengue disease depends intimately on the environment, with major land-use changes often increasing the risk of transmission [20]. As a consequence of the economic development in the region over the past 30 years, there has been an unprecedented increase in rubber plantation area in SEA. Here I examine the potential threat posed by the expansion of rubber plantation area and suggest ways of protecting plantation workers from mosquito-borne diseases, focusing on vector control.

2.3 Rubber tree cultivation in South-East Asia

Hevea brasiliensis is a tropical softwood tree that produces nearly all of the world's natural rubber. Since the tree is of economic importance, many clones have been developed that vary in latex production, wood productivity, disease resistance and soil-nutrient adaptation. Rubber trees are usually grown in a nursery and planted in the plantation when they are between 1-2 m high. Fungicides, herbicides and fertilisers are used to increase their development rate and protect them from tree blight. To my knowledge, insecticides are not used in the plantations. Typically, when the rubber trees are seven years old or when 70 % of the trees have a circumference of >50 cm, tapping is commenced (Figure 2.2 A) [70]. Tapping is generally conducted during the rainy season from June to November when rubber trees are physiologically active. Latex, the milky suspension of rubber particles, is present outside the phloem in the latex vessels of the bark. These vessels are curved at a 30° angle up the tree in a right-handed spiral. This spiral makes tapping latex difficult and requires skill from the rubber tappers. A series of thin slices of bark are cut in half of a spiral around the trunk without damaging the growing layer (Figure 2.2 B). If tapping has been done carefully the same area of bark can be tapped again after a few years. The latex seeps out of the cut into a gutter and is collected in a collection cup (Figure 2.2 C). The latex slowly coagulates within three hours of tapping and the flow stops. Tapping is done typically from 21.00 to 05.00 h when phloem flow is highest [70]. On average every worker taps 750 trees per night, equivalent to 1.5 ha of rubber plantation. Tapping occurs every two days. Latex can be tapped for up to 30 years, after which the trees are felled and sold as tropical softwood. The latex from the cups is collected in large buckets. Depending on the facilities, the latex is left liquid by adding ammonium or coagulated by adding 94 % formic acid. Liquid latex is filtered and processed into sheets. Solid latex sheets can be processed in many ways with the quality of the rubber depending on the method. Rubber plantations are labour intensive cultivations that provide income for up to 30 years.

A **B** **C**

Figure 2.2 Rubber plantations in Lao PDR (A) Mature rubber plantation (B) Rubber worker tapping latex (C) Rubber tree with collection cup filled with latex

2.4 Expansion of rubber plantations in South-East Asia

The economy and population of SEA has been growing exponentially, which has stimulated the establishment of rubber plantations. The population has increased two fold from 1950 to 2000, with currently an estimated 610 million people living in SEA. It is expected that the population will continue growing at a rapid pace, with projections of a 50 % increase from 2012 to 2050 [44]. Since 1978, the gross domestic product (GDP) per capita has increased from 1,339 USD in 1978 to 10,540 USD in 2010 [180]. Although economic growth has resulted in improvement of the standard of living for many, others including ethnic minorities and lower educated people are lagging behind. The government of Lao PDR and Viet Nam, among other SEA countries, have been stimulating the establishment of rubber plantations as a poverty alleviating crop in the last decade.

Monocultures of the rubber tree *H. brasiliensis* are hugely important commercial crops with plantations in SEA supplying more than 90 % of the global demand for natural rubber [181]. The growth of the Chinese economy resulted in a high demand for rubber, with record high rubber prices. This has led to an expansion of rubber plantations. In 2010, SEA had 9.2 million ha of rubber plantations, with the largest plantations in Indonesia (2.9 million ha), Thailand (2.6 million ha), and Malaysia (1.1 million ha) (Figure 2.1C) [45]. Although rubber prices have dropped since the onset of the 2008 global financial crisis, when world industrial production contracted [182, 183], it is anticipated that large hectares of rubber will continue to be cultivated across SEA in the future.

The deforestation of natural forest and the subsequent cultivation of rubber represents a change in habitats and a shift in vectors as the rubber trees mature (Table 2.1). Rubber plantations are essentially man-made forests with generally higher humidity and lower temperatures under the canopy than non-tree crop. This environment makes the plantations ideal for forest vectors, including the important malaria vectors *Anopheles dirus s.l.* and the dengue and chikungunya vector *Aedes albopictus* [184, 185]. Furthermore, rubber plantations provide a wide range of mosquito larval habitats that support a diverse vector fauna such as latex-collecting cups, water-storage containers, slow-running streams, water pools and puddles, [185, 186].

The expansion of rubber plantations has created a high demand for labour which is changing the dynamics of the diseases in the region. I estimated that in the next decade 4.5 to 6 million people will work on rubber plantations in SEA (assuming 13.5-17.7 million ha of rubber plantations by 2024 [187], with one person tapping 3 ha [70]). In many plantations workers are largely poor itinerant workers. This mobile, migrant and sometimes illegal population may be non-immune and working in disease-endemic countries, or they may be carrying pathogens into disease-free areas; leading to increased cases in the migrant workforce or in local communities, respectively [116, 188]. The risk from vector-borne diseases is increased further for plantation workers as they often do not interact with official health-care providers due to the difficult accessibility of health services, the economic factors, the lack of local language skills, the lack of knowledge on mosquito-borne diseases, for fear of deportation or a combination of these [189, 190]. Both the local population and migrant population are at increased risk of vector-borne diseases due to the establishment of rubber plantations.

Table 2.1 Land-use development with the resulting change in dominant vectors and disease risk

	Secondary forest	**Bare land**	**Immature rubber Plantation**	**Mature rubber Plantation**
Change in land use				
Dominant vectors	*An. dirus s.l.* *An. minimus s.l.* *Ae. albopictus*	*An. maculatus s.l.*	*An. maculatus s.l.* *Ae. albopictus*	*An. dirus s.l.* *An. minimus s.l.* *Ae. albopictus*
Malaria risk	High	Low	Low	Medium
Dengue risk	Medium	Low	Low	High
Emerging disease risk	High	Low	Low	Medium

2.5 Malaria in rubber plantations

Malaria outbreaks have been reported in rubber plantations of SEA. The first account of malaria in rubber plantations dates from 1907 in Malaysia, when a malaria epidemic swept through rubber plantations with non-immune immigrant workers [191]. Since then malaria outbreaks have been regularly reported in rubber plantations throughout SEA, most frequently in Thailand [98, 192-195]. The relative importance of malaria vector species in rubber plantations varies according to the site and time of year. For example, in Malaysia *Anopheles umbrosus s.l.* was the primary malaria vector in lowland rubber plantations whilst *Anopheles maculatus s.l.* was dominant in highland rubber plantations [196]. In Thailand *An. dirus s.l.* and *Anopheles minimus s.l.* were the most important vectors in rubber plantations during the dry season [197], while in the rainy season *An. dirus s.l., An. minimus s.l.*, *An. maculatus s.l.* and *Anopheles aconitus* were the main vectors [198]. Mature rubber plantations also support other malaria vectors, including *Anopheles barbirostris s.l.* and *Anopheles latens* [199, 200].

While many species of malaria vectors have been collected from rubber plantations, it is unclear which of these actually breed in the plantations. *Anopheles baimaii* larvae (from the *An. dirus* complex) have been collected from rubber plantations in Thailand [200], while *An. aconitus* and *Anopheles annularis* larvae were found in Indonesian plantations [201]. In Borneo and Thailand *An. maculatus s.l.*, *An. barbirostris s.l.*, *An. dirus s.l.* and *An. umbrosus s.l.* were recorded breeding on the edges of plantations, but not within [202, 203]. Although evidence for anopheline larvae in rubber plantations is limited, potential breeding sites for malaria vectors are abound; particularly partially shaded slow-running streams, pools and puddles next to the unpaved roads used for transporting latex. Rubber plantations can provide suitable breeding sites for malaria vectors, although research is limited on the occurrence of this.

In general, *An. minimus s.l.* and *An. dirus s.l.* are considered the principal vectors in rubber plantations because both prefer breeding in shaded forests [27, 109, 197, 199, 200].

Anopheles dirus s.l. is a highly anthropophilic forest mosquito that is present mostly in the rainy season. They breeds in shaded, temporary bodies of fresh, stagnant water in hilly or mountainous zones, including ground pools, puddles and wells found in natural forests and rubber plantations [199, 204]. *Anopheles minimus s.l*. is a more zoophilic mosquito that is common in the drier season (Table 2.2). They commonly breed in partially-shaded margins of slow-running streams in low hill forests [205]. In areas where *An. dirus s.l.* is the main vector, the replacement of deforested bare areas with rubber, leads to increased malaria incidence [27]. Although not yet investigated, a similar trend is expected for *An. minimus s.l*.

Table 2.2 Summary of the major malaria and dengue vectors of South-East Asia and their typical breeding and biting behaviours

Vector-borne diseases	Vector	Habitat preference	Preferred breeding sites	Biting peak	Behaviour	Average flight distance (m)	Seasonality	Sources
Malaria	*An. minimus s.l.*	ranging from dense forests to agricultural fields	clear unpolluted water along shaded grassy edges of slow moving streams	throughout the night from 22.00 h to 5.00/6.00 h	anthropophilic/zoophilic and endophagic/exophagic	1500 to 3000	dry season	[94, 204-208]
	An. dirus s.l.	forest with high canopy coverage and forest fringe	temporary waters and stagnant/slow moving shaded water in forests	early morning and from 18.00 h to 1.00 h	anthropophilic and endophagic/exophagic	1500 to 3000	rainy season	[94, 109, 199, 204, 206, 209]
	An. maculatus s.l.	low canopy coverage	sunlit streams and ground pools	early morning and from 20.00 h to 23.00 h	anthropophilic/zoophilic and exophagic	1500 to 3000	rainy season and dry season	[109, 199, 204, 206, 210-212]

(Continued from previous page)

Vector-borne diseases	Vector	Habitat preference	Preferred breeding sites	Biting peak	Behaviour	Average flight distance (m)	Seasonality	Sources
Dengue	*Ae. albopictus*	urban and rural habitats	artificial or natural containers outdoors	06.00 to 11.00 h and 16.00 to 19.00 h	anthropophilic and exophagic	100 to 1000	rainy season	[213-215]
	Ae. aegypti	urban habitats, becoming more common in rural areas	artificial containers with no reliable water supply indoors	all day	anthropophilic and endophagic/exophagic	100 to 1000	rainy season	[136, 214, 215]

The risk of malaria transmission in rubber plantations depends critically on the daily activities of the rubber workers. Rubber plantation workers in SEA, unlike those in Africa, tap latex at night, when latex yields are highest, exposing them to malaria vectors. For example, Thai tappers work from 21.00 to 05.00 h, which coincides with peak malaria vector biting times [198]. Additionally, whole families may live and work in the rubber plantations, also exposing them to evening-biting mosquitoes when resting in their poorly-constructed houses [216, 217]. Moreover, as rubber tapping is seasonal work, disease incidence can increase markedly due to the influx of workers during the tapping season [188]. In southern Laos, an influx of malaria-infected workers from neighbouring countries, some of whom worked in rubber plantations, increased the number of malaria cases from 17,529 in 2011 to 46,140 in 2012 [115].

With limited data available on the fine-scale distribution of *Plasmodium* species in SEA, it is difficult to understand how rubber plantations affect the population dynamics of malaria parasites. The most common malaria parasites in SEA are *Plasmodium falciparum* and *Plasmodium vivax*, with fewer cases of *Plasmodium malariae*, *Plasmodium knowlesi* and *Plasmodium ovale* [98]. Currently, there is great interest in the artemisinin-tolerant *P. falciparum* strains that originally developed in Cambodia and are now present in most of mainland SEA [67]. Recent studies on the Thailand-Myanmar and Thailand-Cambodia borders have shown the important role of migrant rubber workers in spreading malaria, especially *P. falciparum* and *P. vivax* multidrug resistance [192, 195]. *Plasmodium knowlesi* cases have been reported in rubber workers on the Thai-Myanmar border [218]. Unlike other malaria species, *P. knowlesi* is naturally infective to macaques including *Macaca fascicularis*, the long-tailed macaque, which is found in rubber plantations [219]. *Plasmodium knowlesi* has been reported in all SEA countries, except Lao PDR [220, 221]. The presence of primate reservoir hosts, *Anopheles* mosquitoes and plantation workers in rubber plantations makes rubber tapping a high-risk practice for *knowlesi* malaria. It is likely that rubber plantations are potential residual malaria transmission zones and could support the spread of artemisinin-resistant *P. falciparum*, as well as *P. knowlesi*.

2.6 Dengue in rubber plantations

The key reason for the rapid spread of the dengue virus (DENV) is its adaptation to the highly anthropophagic day-biting mosquitoes *Aedes aegypti* and *Ae. albopictus* (Table 2.2). Dengue is principally an urban disease, where it is transmitted by *Ae. aegypti*. However, in rural areas *Ae. albopictus* thrives and is often responsible for outbreaks [213]. Recent rubber plantation epidemics include 16,367 cases in 2010 in a Malaysian rubber plantation [222] and 3,760 cases in 2012 in an Indian plantation [223]. Although there are few data on dengue epidemics in rubber plantations, since rubber plantations make ideal habitats for *Ae. albopictus* [224] the threat from dengue must be taken seriously.

Aedes albopictus thrives in rubber plantations since they provide a plethora of potential breeding sites including latex-collection cups, tree holes and water-storage containers around the homes of rubber workers [225, 226]. As rubber workers and their families live within or close to the rubber plantations, they are exposed to these day-biting mosquitoes. According to one study in Thailand, rubber plantation houses have 18.3 times higher odds of having at least one container with *Aedes* larvae (not identified to species) than town houses [227]. Importantly, *Ae. albopictus* frequently lay their eggs in latex-collection cups that fill with rain water and can produce adult mosquitoes during the long tapping break outside the main rainy season, or due to interruptions in tapping during the rainy season. *Aedes* mosquitoes thrive in these collecting cups as they contain latex residues and decaying leaves for nutrients. In one Malaysian study 96 % of the adult and larvae mosquitoes collected in rubber plantations were *Ae. albopictus* [184]. Similarly, *Ae. albopictus* was dominant in an Indian plantation, where mosquito larvae were found in 80 % of collection cups outside the tapping season, with 98 % of these cups containing *Ae. albopictus* larvae [228]. Other *Aedes* mosquitoes collected in rubber plantations include *Ae. aegypti*, *Ae. chrysolineatus*, *Ae. niveus*, *Ae. vexans* and *Ae. vittatus* [185].

The risk of dengue is increased further by the close proximity of rubber plantations to the natural forest where the sylvatic cycle of dengue is present [54]. In Viet Nam 79 % of the rubber plantations in the Central highlands were planted in partly deforested forests [229]. In such situations the risk of dengue infection is likely to be enhanced as dengue-infected non-human primates, like *Presbytis* and *Macaca* species, enter the rubber plantations to feed. This exposes the dengue vectors in the rubber plantations to the forest arbovirus. Additionally, rubber workers who visit the natural forest in search of food can be exposed to dengue vectors from the forest [219]. Although data on dengue cases in rubber plantations is limited, the presence of the vector, the proximity of the sylvatic cycle and the high exposure risk of rubber workers suggest a substantial risk of dengue in rubber plantations.

2.7 Chikungunya - an emerging disease in rubber plantations

Since many new and emerging infectious diseases are vector borne [230] it is possible that rubber plantations, with their close proximity to natural forests, large work force and presence of anthropophilic vectors, could be a nidus for pathogens to spill over from forest animals into local human communities. Although information on new and emerging diseases in rubber plantations is limited, the rich diversity of mosquito species found in these environments highlights the potential risk of exposure to new pathogens [185, 186].

Chikungunya is one example of a virus with a sylvatic cycle that has spilled over to rubber-plantation workers. The chikungunya virus (CHIK) has spread across many parts of SEA where it has resulted in severe outbreaks, with 2 million cases reported in India [231]. Chikungunya in SEA has been mostly an urban disease, typically found in dengue-endemic areas. However, like dengue, chikungunya cases are becoming more common in rural areas [68]. In Kerala, India a province with large rubber plantations, a chikungunya epidemic occurred with 24,052 cases in 2006-2007. A post-epidemic survey found a 78 % seroprevalence among males, with 74 % of them involved in rubber plantation-related activities [232]. More recently, in 2012 there were 14,277 cases reported in India. Many of those infected were working in rubber plantations, where *Aedes* mosquitoes were breeding in coconut shells used for latex collection [223]. Although knowledge on the diseases and vectors circulating in the rubber plantations remains limited, there is a clear indication that these habitats can become significant areas for chikungunya transmission.

2.8 Vector control in rubber plantations

There is a need to identify vector control methods to reduce the risk of vector-borne diseases in rubber plantations. Investing in the health of rubber plantation workers will be financially beneficial to the rubber industry and economies of SEA because vector-borne disease outbreaks result in high vector control costs, medical costs, absenteeism and lower productivity [233, 234]. A historical analysis suggested that a malaria outbreak could increase costs of rubber plantation cultivation by 20 %, due to sick workers forcing the employment of expensive skilled labour to keep production stable [191]. In India the economic burden of malaria is estimated at 1,940 million USD of which 75 % was due to loss of earnings for patients and supporting family [235]. In Viet Nam a country-wide dengue outbreak cost the economy 12 million USD for vector control, surveillance, information, education, communication and other direct and indirect costs [236]. In general, proper implementation of vector control in rubber plantations will be beneficial for all parties involved.

Vector control in rubber plantations should involve a combination of interventions; targeting both indoor- and outdoor-biting mosquitoes, providing protection against day-time and night-time biting, and using both insecticide-based and non-insecticide-based vector control methods. Vector control should furthermore draw on vector control measures both from within and outside the health sector. In the present example, this would entail collaboration between the health sector, rubber industry, and local communities of plantation workers. Vector control should be supported by strong entomological and epidemiological surveillance to determine the most appropriate tools and implementation strategies. These strategies should be closely monitored and regularly evaluated. This is essentially integrated vector management (IVM), a WHO-recommended adaptive management approach to vector control [237]. Complementary activities that should be implemented alongside vector control in rubber plantations include the training of migrant community volunteers, facilitating health communication, promoting interaction with health workers, and improving access to basic health services for prompt and effective diagnosis, and treatment of vector-borne diseases [117]. Currently, many different community protection and personal protection strategies are suggested for preventing mosquito-borne diseases, with the choice of vector control interventions urgently needing further research in a variety of settings [238, 239]. Here I provide some guidance on possible interventions in rubber plantations.

2.8.1 Protection against outdoor biting

Protecting people against outdoor-biting mosquitoes is one of the biggest challenges facing vector control today with the current tools representing, at best, partial protection [238]. The topical application of mosquito repellent is perhaps the most common method used for protection outdoors (Figure 2.3 A). Examples of topical repellents include citronella, para-menthane-3,8-diol, lemon eucalyptus (*Eucalyptus maculata citriodon*), picaridin and the best known, N,N-diethyl-m-toluamide (DEET) [240, 241]. While they protect individuals from mosquitoes for several hours [242, 243] a recent systematic review and meta-analysis concluded that topical repellents are not protective against falciparum or vivax malaria [244]. This lack of efficacy against clinical disease may be because topical repellents do not protect for long enough and because it requires high user compliance [245]. Therefore, while topical repellents are useful for personal protection, they cannot be recommended as public-health interventions. New approaches are therefore needed that function in an automated fashion, for longer periods and require lower user compliance.

Figure 2.3 Personal protection methods. (A) Topically applied repellent (B) Permethrin-treated work clothing (C) Metofluthrin emanator worn on a belt (D) Pyrethroid mosquito coil worn on a belt

Although to my knowledge no scientific study has assessed the protective efficacy of long-sleeved clothing, organisations like the WHO recommend wearing long-sleeved clothing to protect from mosquito bites [246, 247]. Greater protection would be achieved by using insecticide-treated clothing, especially on large industrial plantations where it can be incorporated in workers' clothing for greater acceptability (Figure 2.3 B) [248]. Insecticide-treated clothing is protective against bites from *Anopheles* and *Aedes* mosquitoes [249, 250] and personal protection is enhanced when an insecticide and repellent are combined [251, 252]. However, there is only weak evidence that treated clothing is protective against clinical malaria [249]. Therefore before insecticide-treated clothing can be used routinely by rubber plantation workers, further research is needed to make insecticide-treated clothing more resistant to washing, to ultraviolet light exposure and to wear-and-tear [250].

Another method of outdoor protection is the use of spatial repellents fitted to the individual, such as a metofluthrin-emitting machine worn on a belt (Figure 2.3 C). Metofluthrin emanators can reduce exposure to *Ae. albopictus* by 70 % for about 3 h while the individual is mobile [253]. Similarly mosquito coils, although most commonly burnt indoors, can be inserted into a metal case and worn by a mobile person (Figure 2.3 D). More studies are needed to understand the true value of personal spatial repellents for both indoor and outdoor protection. If these interventions are effective for longer periods, they could be a convenient and cheap solution for protecting rubber workers.

Apart from the application of physical and chemical barriers against mosquitoes, vector control could include the alteration of the mosquito reproduction and immune response against pathogens, using the intracellular bacterium *Wolbachia*. This bacterium is a common mutualist for arthropods. It can rapidly spread in the mosquito population and could inhibit the development of the dengue virus [254]. *Wolbachia* is always present in *Ae. albopictus* mosquitoes while it is absent in *Ae. aegypti*. The introduction and establishment of this bacterium in two natural Australian *Ae. aegypti* populations has shown the potential of this bacterium in vector control [254]. Possibly the same strategy could be used for the control of zika [255]. Moreover, the bacterium could be used for malaria control, although research on infection of *Anopheles* species with *Wolbachia* is still in early phases [256]. Although several countries, including the United States of America, are looking into the possibilities of using *Wolbachia* to control dengue and zika disease, to date no studies have shown the decrease in dengue or zika incidence in humans after introduction of the *Wolbachia* bacterium [257].

2.8.2 Protection against indoor biting

Many vector control methods against indoor biting exist. The key indoor interventions used for malaria control, LLINs and IRS [98], can be effective even against vectors that are generally considered to be exophilic, such as *An. dirus s.l., An. minimus s.l.* and *Ae. albopictus*. However, both intervention methods are threatened by the rise of insecticide-resistant vectors [258]. A general recommendation is that LLINs should be distributed to plantation workers and their families, since even in the presence of pyrethroid-resistant vectors, nets provide a physical barrier against malaria.

Good housing is protective against indoor-biting mosquitoes [259, 260] with traditional houses made from bamboo having more gaps in walls and floors for mosquitoes to enter houses, compared with modern houses [217]. Houses raised on platforms with few entry points for mosquitoes are protective; as is keeping cattle away from houses [216, 217]. House screening or the use of insecticide-treated curtains should be considered for protecting against malaria and dengue in rubber plantations [260, 261]. Although rebuilding houses for rubber workers might be too costly, simple measures such as screening houses should be recommended.

Spatial repellents such as mosquito coils or metofluthrin impregnated plastic strips for use in the home may be effective at reducing the indoor density of mosquitoes [262, 263]. Although epidemiological data on the impact of spatial repellents on disease transmission is limited [264], several studies have shown the indoor protection of spatial repellents against malaria. In China transfluthrin coils provided 77 % protection against malaria, which increased to 94 % protection when combined with LLINs [262]. In Indonesia metofluthrin-treated coils showed 52 % protection against malaria [265]. These studies are encouraging but further research is needed, before they can be recommended as public-health tools for rubber plantations.

2.8.3 Larval Source Management

Larval source management (LSM) is an important complementary method for vector control in rubber plantations. Environmental management has been practised successfully for malaria control throughout SEA from the early 1900s [266], but is used less today. In situations where *Anopheles* breeds in streams, small dams could be constructed and water released periodically to flush the streams. In India flushing resulted in an 85 % reduction in positive dips of *Anopheles* larvae and pupae [267]. For dengue and chikungunya control, a simple intervention would be inverting the latex-collection cups and storing them in rain-proof shelters when not tapping for long periods. This activity could be made compulsory in large-scale plantations to decrease number of suitable mosquito breeding sites. Rain guards, that stop water from running into the latex cups, could also be used for larval control. These guards are already used on some plantations for higher latex quality and yield, as they stop rainwater and debris falling into the latex. Rain guards decreased water in latex cups five-fold compared with cups without a guard [268]. Additionally, around the home and peri-domestic environment of rubber workers, mosquito-breeding sites should be prevented by removing garbage and covering water containers [131, 136].

Apart from reducing the number of waterbodies, mosquito breeding can be decreased by making the waterbodies less suitable for breeding. Larvicides could be applied in rubber plantations to reduce vectors, but I know of no studies where this has been done. The disadvantage of larvicides is that in many cases, sites need retreatment every 7-14 days. Larvicides can therefore only be cost-effective where breeding sites are few, fixed and findable [269]. Biological control agents can also be used for vector control in rubber plantations. One of the best examples is the use of *Mesocyclops*, a copepod that feeds on mosquito larva. Community-based programmes introduced these copepods into large water-storage jars in Viet Nam so successfully, that they eliminated dengue from large parts of the country [270]. However, the success of these programmes was dependent on large water-storage jars being the dominant breeding sites for *Ae. aegypti*, which may not be the case in rubber plantations. Another example of a biological control agent that could potentially be released in the large water-storage jars close to tappers' houses is larvivorous fish, such as *Gambusia* spp. and *Poecilia reticulate*. A recent systematic review identified a lack of evidence that fish were effective control agents, so additional studies are necessary [271]. Other examples of natural predators include the nematode *Romanomermis iyengari* and the naturally occurring predatory mosquito *Toxorhynchites splendens* which have been effective at reducing *Ae. albopictus* larvae in rubber plantations [226, 272]. More well-conducted studies are needed on the impact of larval control on disease incidence, before recommendation can be made regarding the use of larvicide and biological agents in rubber plantations.

2.8.4 Genetic control

In the future, genetic control of mosquitoes may be an effective method of vector control in rubber plantations. Genetic control has been studied for several vector species, including the chikungunya and dengue vectors *Ae. aegypti* and *Ae. albopictus* [273, 274]. The development of the CRISPR-Cas9, a system that can change specific DNA sequences which is heritable, has opened many doors. Possible implementation of this system includes disease control, making drugs, de-extinction, vector control, better food production and disease models [275]. This genome editing technology is currently being tested for *Anopheles gambiae* and *Ae. aegypti* to produce high numbers of males, to decrease reproduction rate and develop resistance to pathogens such as malaria parasites, dengue viruses and nematodes [276-280]. Currently, apart from the release of sterile males and insects with a dominant lethal gene (RIDL), most genetic control methods remain at an early stage of development [273, 274, 281]. As genetic control is species specific, in rubber plantations there are opportunities for dengue vector control, with only the vector *Ae. albopictus* seemingly important in this habitat.

2.9 Concluding remarks

For the foreseeable future, large hectares of rubber plantations will continue to be cultivated for latex across SEA. There is a threat that these plantations may become malaria hot spots, making it difficult to eliminate this disease. The presence of high numbers of *Ae. albopictus* in rubber plantations suggests that dengue and chikungunya could be easily introduced in these environments. Moreover, there is concern that as yet unknown pathogens may spill over to the rubber-worker population from animals living in or close to the rubber plantations.

Future mosquito-borne disease control in rubber plantations should focus on developing IVM strategies alongside prompt and effective treatment of vector-borne diseases, and education about vector-borne disease transmission and prevention. For malaria vector control, large-scale deployment of LLINs, and in some sites stream flushing and larvicides, would be protective. For dengue and chikungunya control, inverting the latex-collection cups after latex collection and storing them in rain-proof shelters is essential. Although there is currently a lack of methods for personal outdoor protection, wash-proof insecticide-treated clothing or spatial repellent emanators may provide long-term protection for plantation workers. Understanding the migration patterns of plantation workers in SEA within countries and cross-border is a crucial challenge for effective disease control and is even more urgent with the rapid spread of ACT-tolerant malaria parasites across the region. National and international cooperation is imperative for successful control and management of vector-borne diseases, not only strengthening the capacity for mosquito control but also identifying vulnerable population groups and residual transmission areas. Importantly, this is an issue that threatens the growth and productivity of the rubber industry in the region, so control implementation should be a partnership between the health sector, local communities, and industry.

3 The human-baited double net trap: an alternative to human landing catches for collecting outdoor biting mosquitoes in Lao PDR

Adult mosquito collection in the secondary forests of Thinkeo village using the Human Landing Catches and Human baited Double Net trap

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3.1 Abstract

Estimating the exposure of people to mosquito-borne diseases is a key measure used to evaluate the success of vector control operations. The current best standard (gold standard) is to use human landing catches where mosquitoes are collected off the exposed limbs of human collectors. This is, however, an unsatisfactory method since it potentially exposes individuals to a range of mosquito-borne diseases. In this study, several sampling methods were compared to find a method that is representative of the human-biting rate outdoors, but which does not expose collectors to mosquito-borne infections.

The sampling efficiency of four odour-baited traps were compared outdoors in rural Lao PDR: the human-baited double net (HDN) trap, Centers for Disease Control and Prevention (CDC) light trap, Biogents (BG) sentinel trap and BG Suna trap. Subsequently the HDN, the best performing trap, was compared directly with human landing catches (HLC), the 'gold standard', for estimating human-biting rates.

HDNs collected 11 to 44 times more mosquitoes than the other traps, with the exception of the HLC. The HDN collected similar numbers of *Culex* (Rate Ratio (RR) 1.26, 95 % CI 0.74-2.17) and *Anopheles* mosquitoes (RR 1.16, 95 % confidence interval (CI) 0.61-2.20) as HLC, but under-estimated the numbers of *Aedes albopictus* (RR 0.45, 95 % CI 0.27-0.77). Simpson's index of diversity was 0.845 (95 % CI 0.836-0.854) for the HDN trap and 0.778 (95 % CI 0.769-0.787) for HLC, indicating that the HDN collected a greater diversity of mosquito species than HLC.

Both HLC and HDN can distinguish between low and high biting rates and are crude ways to measure the human-biting rate. The HDN is a simple and cheap method to estimate the human-biting rate outdoors without exposing collectors to mosquito bites.

3.2 Introduction

An important metric used for quantifying the risk of infection with mosquito-borne pathogens is the estimation of the human-biting rate (the number of mosquito bites per person per day or night). Developing methods for estimating human-biting rates that do not expose collectors to vector-borne pathogens has been a major challenge in vector ecology, especially for species biting outdoors [282, 283]. Despite the development of innovative trapping methods the traditional human landing catch (HLC) method, where mosquitoes are collected when landing on exposed limbs, is still considered the current best standard (gold standard) [282, 284-286]. The strength of the HLC method is also its weakness, as participants are exposed to potentially infective mosquito bites while performing catches. HLC can expose participants to diseases such as dengue for which no chemoprophylaxis or sterilising vaccine exists. Furthermore, whilst collectors can be protected from malaria using chemoprophylaxis [287], this method cannot be used where *Plasmodium* strains are less sensitive to antimalarials [288]. Alternative mosquito collection methods are therefore necessary.

Here I set out to determine the most efficient, reliable and ethically sound method for outdoor sampling of human-biting mosquitoes in dengue-endemic areas of Lao PDR. In the first experiment I compared the human-baited double net trap (HDN), Centers for Disease Control and Prevention (CDC) light trap, Biogents (BG) sentinel trap and Biogents (BG) Suna trap. The HDN trap [282, 289] consists of two box nets; one protecting the collector and a second larger net which is placed directly over the inner net. The outer net is raised off the ground so that mosquitoes attracted to the human-bait are collected between the two nets. The trapping method has been used in many different regions of the world with varying success [282, 290-292]. CDC light traps have been used for outdoor mosquito surveillance in Asia [293-297], though their primary use has been for estimating indoor-biting rates [284, 298, 299]. The BG-sentinel trap releases artificial host-odours and employs attractive visual cues to attract outdoor-biting *Aedes* mosquitoes and is routinely used for surveillance [300-305]. The newly developed odour-baited BG-Suna trap is effective at sampling *Anopheles gambiae* mosquitoes outdoors in Kenya [306]. In the second experiment the trap collecting the highest number of mosquitoes was compared directly against HLC to determine whether an alternative to the current 'gold standard' could be found. I hypothesized that no trapping method would collect similar numbers as the HLC. However I expected the HDN to collect the most comparable numbers, due to the use of a human participant as an attraction for the mosquitoes.

3.3 Materials and Methods

3.3.1 Study sites

The study was conducted in Luang Prabang province, Lao PDR during the middle of the rainy season in 2013 and at end of the rainy season in 2014. The area has a tropical monsoon climate with one hot, rainy season from May to October. During the study period temperatures ranged between 14.6℃ and 34.5℃ with a relative humidity of 21.8 % to 100 %. The 2013 annual rainfall in the study area was 1,746 mm. The annual rainfall in 2014 was 1,415 mm. For experiment one the teak plantation (19°41'09.19"N 102°07'13.84"E) and the primary school (19°41'08.27"N 102°07'12.99"E), both bordering Thinkeo village, were chosen for day and night collections, respectively. For experiment two, the secondary forest next to Silalek village (19°37'04.57"N 102°03'27.67"E) was chosen for day collections and the primary school in Thinkeo village for night collections.

3.3.2 Study participants

Before starting the study, verbal informed consent was provided by village leaders to conduct our studies in their villages. Participants who conducted HLCs and HDNs gave written, informed consent for their participation. A total of 36 healthy participants, males and females between 18 and 55 years old, were paid for their participation. Participants were given the opportunity to receive vaccination against Japanese encephalitis (JE) free of charge and were offered free medical treatment when they showed any symptoms suspected to be caused by mosquito-borne diseases. The study took place in an area without malaria. Human Landing Catches were conducted when dengue transmission was low according to information provided by the Ministry of Health of Lao PDR [307].

3.3.3 Mosquito collection methods

3.3.3.1 Human-baited double net trap

During catches performed with HDNs, one adult occupied one trap. According to the different experiments, mosquitoes were collected by participants for six or eight hours. Participants rested on a metal-framed bed with fabric inlay (20 cm high x 200 cm long x 70 cm wide) and were fully protected from mosquitoes by a small untreated blue polyester bed net (97 cm high x 200 cm long x 100 cm wide, mesh size 1.5 mm). The small bed net was hung over the bed to the ground. A larger untreated bed net (100 cm high x 250 cm long x 150 cm wide, mesh size 1.5 mm), which was also not treated with insecticide, was hung over the smaller net and was raised 30 cm above the ground [282, 286] (Figure 3.1 A). Mosquitoes were caught in the 20 cm gap between the two nets. Both nets were protected from rain by plastic-sheeting roofs. For 10 minutes of every hour participants raised the bottom of the inner net and aspirated mosquitoes caught between the nets into paper-cups. Mosquito catches for each hour were aspirated into different paper cups. When participants were not collecting mosquitoes, they rested inside the inner net. Participants had access to a stopwatch to monitor the time. A total of 36 participants collected mosquitoes using the HDN during this comparison study. Every collection day two field supervisors were present to verify the participants conducted the collections as instructed.

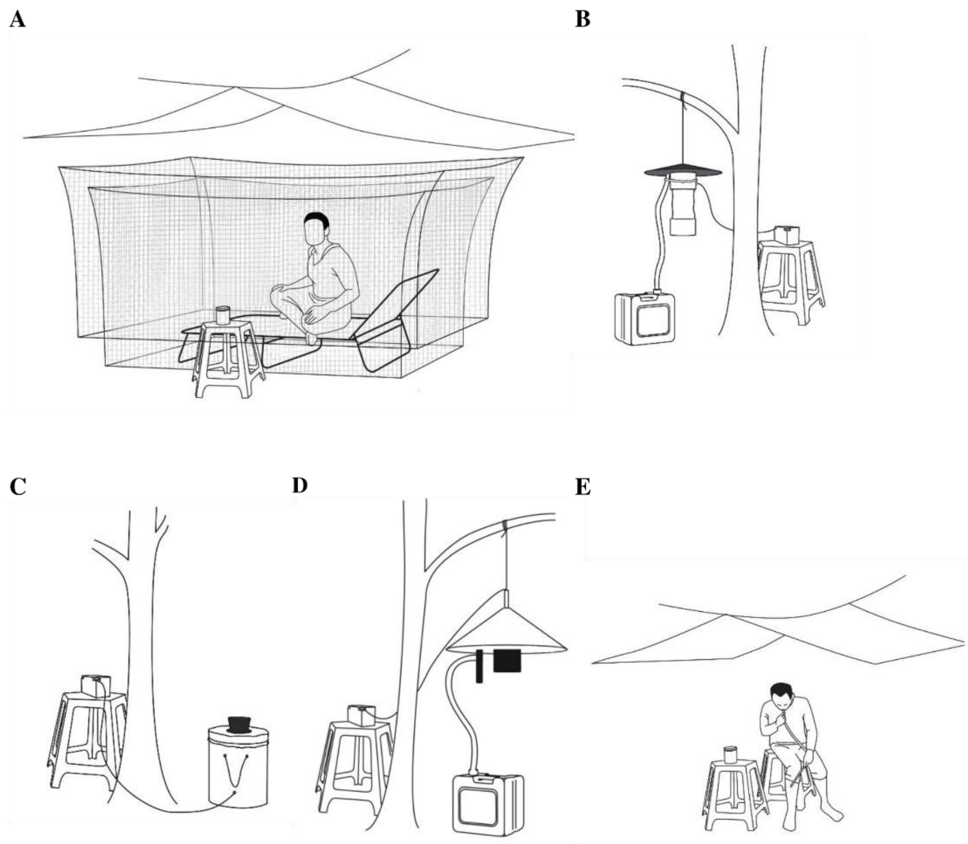

Figure 3.1 Mosquito sampling methods. (A) Human-baited double net trap with collecting cup (B) Odour-baited CDC light trap connected to a 6V battery with CO_2-produced by sugar fermentation in the attached jerry can (C) Odour-baited BG-sentinel trap connected to a 12V battery (D) Odour-baited BG-Suna trap connected to a 12V battery with CO_2-produced by sugar fermentation in the attached jerry can (E) Human landing catch method with collecting cup

3.3.3.2 CDC Light trap

CDC light traps (model 1912, John W. Hock Company, USA) with the supplied incandescent bulb were suspended from trees with the lightbulb 1.5 m above the ground (Figure 3.1 B). Mosquitoes attracted to the trap were sucked into the collection container by a 6V (6Ah) battery-powered fan. Carbon dioxide produced by fermentation of sugar with yeast was supplied to the trap and one human-scented sock acted as an attractant [308]. CO_2 was produced by mixing 250 g sugar (SPOON, Kasetphol sugar ltd, Thailand), 17.5 g yeast (Saf instant, Le saffre, Thailand) and 2.5 L water in a clean plastic jerry-can one hour before trapping [309]. The CO_2 produced, passed along tubing (two cm diameter) and was released at the trap entrance. Human-scented nylon socks, worn by one local volunteer for 24 hrs, were hung next to the trap entrance and replaced after eight days [310, 311]. When not in use the socks were stored in unused glass bottles at -20 °C in order to preserve the human odour [310, 311].

3.3.3.3 BG-sentinel trap

The BG-sentinel trap (Model 7.5, Biogents, Germany) is an odour-baited counter-flow trap. The trap was comprised of a collapsible tubular container (40 cm high x 36 cm diameter) placed on the ground with the trap mouth opening upwards. The trap mouth opening was positioned 40 cm above the ground. Air was drawn into the black funnel trap opening (10 cm diameter), which then passed across the solid BG-lure® inside the main body and was forced out of the trap-top through the gauze surrounding the funnel. The BG-lure® is a mixture of lactic acid, ammonium hydrogen carbonate and hexanoic acid. Air movement was created by a fan powered by a 12V battery (11Ah) (Figure 3.1 C). Mosquitoes attracted to the trap passed through the funnel and were collected in a gauze bag attached internally to the black funnel.

3.3.3.4 BG-Suna trap

The BG-Suna trap (Biogents, Germany) is an odour-baited counter-flow trap with the trap mouth opening downwards. The trap was suspended 1.5 m above the ground, with the funnel opening set 1.0 m above the ground. The trap was powered by a 12V battery (11 Ah). At the base of the conical trap (52 cm high x 39 cm diameter) air was drawn into the trap through a black funnel (10 cm diameter). A synthetic blend of attractants mimicking human skin odours, impregnated on to nylon strips hanging in the cone of the trap, were blown out of the trap base through a perforated plastic cover surrounding the funnel entrance [312]. A jerry-can producing CO_2, prepared as described above in section 3.3.3.2 CDC Light trap, was connected to the trap via a CO_2 release tube located near the trap entrance (Figure 3.1 D). Mosquitoes attracted to the trap passed through the trap funnel and were collected in a collection bag attached to the funnel inside the trap.

3.3.3.5 Human Landing Catches

HLC were conducted by 32 adults who collected mosquitoes landing on their exposed legs with an aspirator. During collection participants sat on a 40 cm high stool and were protected from the rain by a plastic-sheeting roof (Figure 3.1 E). Mosquitoes were aspirated from the exposed legs and collected in paper cups covered with netting. Collections were performed for 50 minutes every hour with a 10 minute break [284]. Each participant collected mosquitoes for eight hours. Participants were involved in the comparison study for four days, of which two days were spent conducting the HLCs.

3.3.4 Sample size considerations

The first experiment was designed to detect whether HDN collected > 50 % more mosquitoes than other type of traps. This figure was chosen as we wanted to identify the method which clearly collected more mosquitoes than the other traps. Small differences between traps were not of interest. The power analysis was done using preliminary HDN data from the study area (n = 12 days, mean no. mosquitoes/12 hrs 48, standard deviation 23). The sample size required to identify a minimum difference of 50 % in mosquito numbers between traps was 15 replicates of 12 hrs (power $\omega = 0.8$, alpha $\alpha = 0.05$) [313]. The power of the sample size calculation is calculated by 1- β, with β symbolizing the Type II error. The Type II error represents the possibility of failing to reject the null hypothesis, when the null hypothesis is wrong. The alpha symbolizes the Type I error, which represents the possibility of wrongly rejecting the null hypothesis. The sample size of 15 was increased to 20 catching occasions for a balanced design. For the second experiment, sample size was calculated to identify if at least 20% of variance was explained by the relation between the traps. Therefore a minimum correlation of 0.44 (square root of 0.2) between the best trap and HLC was chosen for the power analysis.

Sample size calculations suggested 30 replicates were needed to obtain this (ω 0.8, α 0.05) [313], which was increased to 32 for a balanced design.

3.3.5 Human-baited double net trap and outdoor traps comparison (Experiment one)

In July 2013, comparisons were made between the HDN, CDC light trap, BG-sentinel trap and BG-Suna trap in teak plantations for day collections and at a primary school for night collections. These habitats were chosen, as the preliminary study showed that the highest day collections were done in the teak plantations and highest night collections were done in the primary school. Day collections were made for 10 days from 07.00 to 19.00 h and night collections for 10 nights from 19.00 to 07.00 h (i.e. 20 day and 20 night comparisons). All collections were conducted outdoors. At each site there were two parallel transects, 30 m apart. On each transect, each of the four trap types were randomly allocated to one of the four locations (four traps/transect) using a Latin square design. The traps were positioned five meters apart to ensure the human participant could keep an eye on the battery-powered traps. There were reports of thieves in the area and we did not want the traps to get stolen. Four participants conducted the HDN method, rotated randomly between the four transects (two transects/day and night) and changed every six hours. Therefore all four participant collected mosquitoes for six hours during both the day and night collections, randomly located in the two different transects. If a single trap malfunctioned all collections from that transect were discarded and the experiment repeated.

3.3.6 Human landing catch and human-baited double net trap comparison (Experiment two)

Collections were made in September and October 2014 in the secondary forest from 10.00 h to 18.00 h and at the primary school from 17.00 h to 01.00 h. All collections were conducted outdoors. HLC and HDN collections were made five meters apart, and then duplicated 50 m away (i.e. four traps per occasion). The HLC and HDN comparisons were done in close proximity, as participants did not feel comfortable on their own at night. This set up was repeated 16 times for day collections and 16 times for night collections (total of 64 comparisons between HLC-HDN). A group of four participants were randomly assigned to one of the four locations for four days or nights, so that variation in attractiveness between collectors was compounded with location attractiveness. Although collection sites are located in the same habitat, small differences can exist between these sites in their attractiveness to mosquitoes. We therefore included the location as a possible variable, for which we compensated partly by ensuring both mosquito trapping methods collected mosquitoes equally often in every collection site. After four days/nights of collection the participant group was changed for a group of four new participants. While participants were linked to a location, traps were rotated between locations using a four by four Latin-square design. The Latin-square design was repeated eight times (32 comparison days) to ensure that the location variation, trapping ability of each participant and odour of each participant would not be associated with one trapping method. Thus, in total 32 participants took part in the comparison, collecting mosquitoes using both the HLC and HDN method.

3.3.7 Mosquito identification

Mosquitoes were morphologically identified to species complex using stereo-microscopes and recognized keys of the Indochinese region [314].

3.3.8 Data analysis

Analyses of sampling efficiency for both experiments were performed using generalized linear modelling (GLM) with a negative binomial model for count data and a log-link function (IBM SPSS statistics, ver. 20). Species diversity was compared for day and night collections using Simpson’s index of diversity (1-D) with results representing diversity from 0 (no diversity) to 1 (infinite diversity) [315, 316].

3.3.9 Ethics

The study was approved by the ethics committee of the Ministry of Health in Lao PDR (approval number 017/NECHR issued 21-04-2013, Appendix 1) and the School of Biological and Biomedical Sciences Ethics Committee, Durham University (issued 25-07-2013, Appendix 2). Additionally, the use of HLC was approved by the Comité de Recherche Clinique de l'Institut Pasteur (approval number 2014-19 issued 08-07-2014, Appendix 3). The information sheet and consent form were provided in Lao language (English version Appendix 4).

3.4 Results

3.4.1 Human-baited double net trap and outdoor traps comparison (Experiment one)

Overall 1,144 female mosquitoes (978 HDN, 35 CDC light trap, 102 BG-sentinel trap, 29 BG-Suna trap) belonging to 48 species (45 species HDN, 16 species CDC light trap, 20 species BG-sentinel trap, 13 species BG-Suna trap) were collected. Seven mosquitoes could not be identified. Of the total number of female mosquitoes collected 31.2 % were *Aedes* mosquitoes (357/1144), with *Aedes albopictus* most abundant (51.8 %, 185/357), 23.3 % were *Anopheles* mosquitoes (267/1144), with *Anopheles barbumbrosus* most abundant (73.4 %, 196/267) and 16.3 % were *Culex* mosquitoes (188/1144), with *Culex vishnui* most common (40.4 %, 76/188). A total of 12.9 % of collected mosquitoes were *Heizmania* species (147/1144), with *Heizmania mattinglyi* most common (95.2 %, 140/147). About 30 % of sampling occasions yielded no mosquitoes (0 % of HDN, 65 % of CDC light trap, 2.5 % of BG-sentinel trap, 52.5 % of BG-Suna trap).

Mosquito numbers varied significantly between traps, but not by location or collection date (Table 3.1). Overall HDN traps caught 34.5 (95 % confidence interval (CI) 18.9-66.7) times more mosquitoes than CDC light traps, 11.0 (95 % CI 6.5-18.5) times more than BG-sentinel traps and 43.5 (95 % CI 22.7-76.9) times more than BG-Suna traps (detailed information Table 3.2). More *Aedes* mosquitoes, including *Ae. albopictus*, *Anopheles* mosquitoes and *Culex* mosquitoes were collected by the HDN traps than the three other traps during both the day and the night.

Table 3.1 Generalized linear modelling of female mosquitoes collected by the human-baited double net trap and outdoor traps comparison (experiment one)

	Time of day	Catch size (95 % CI)	Location *P*	Date *P*	Trap type *P*
Total mosquitoes	**Day**	8.46 (5.47-11.45)	0.742	0.372	<0.001*
	Night	5.84 (3.54-8.13)	0.248	0.104	<0.001*
***Aedes* mosquitoes**	**Day**	3.91 (2.50-5.33)	0.902	0.540	<0.001*
	Night	0.55 (0.12-0.98)	0.508	0.070	0.002*
Aedes albopictus	**Day**	2.23 (1.37-3.08)	0.954	0.871	<0.001*
***Anopheles* mosquitoes**	**Night**	3.06 (1.71-4.41)	0.302	0.203	<0.001*
***Culex* mosquitoes**	**Night**	2.06 (1.12-3.00)	0.527	0.528	<0.001*

Results are shown for day (n = 10) and night (n = 10) collections, for all locations (n = 8), for all collection dates (n = 10) and for all trap types (n = 4). As the catch sizes were too low, no night values for Aedes albopictus *and no day values for* Anopheles *and* Culex *mosquitoes are shown. CI, confidence interval. *significantly different,* P*<0.05*

Table 3.2 Means and rate ratio of female mosquitoes collected by the human-baited double net trap and outdoor traps comparison, calculated using generalized linear modelling (experiment one)

		HDN		CDC light trap			BG-sentinel trap			BG-Suna trap		
		Mean catch size (95% CI)	**RR**	**Mean catch size (95% CI)**	**RR (95% CI)**	***P***	**Mean catch size (95% CI)**	**RR (95% CI)**	***P***	**Mean catch size (95% CI)**	**RR (95% CI)**	***P***
Total	**Day**	28.65 (22.71-34.59)	1	0.15 (-0.02-0.32)	0.005 (0.001-0.02)	<0.001*	4.25 (3.17-5.33)	0.14 (0.07-0.29)	<0.001*	0.80 (0.31-1.29)	0.02 (0.009-0.05)	<0.001*
	Night	20.25 (14.66-25.84)	1	1.60 (0.47-2.73)	0.06 (0.02-0.13)	<0.001*	0.85 (0.34-1.36)	0.03 (0.01-0.08)	<0.001*	0.65 (0.19-1.11)	0.03 (0.01-0.07)	<0.001*
Aedes	**Day**	13.15 (10.12-16.18)	1	0.10 (-0.04-0.24)	0.007 (0.001-0.03)	<0.001*	2.15 (1.29-3.01)	0.14 (0.07-0.30)	<0.001*	0.25 (-0.01-0.51)	0.02 (0.005-0.05)	<0.001*
	Night	1.75 (0.05-3.45)	1	0.05 (-0.05-0.15)	0.03 (0.003-0.29)	0.003*	0.30 (0.03-0.57)	0.09 (0.002-0.47)	0.004*	0.10 (-0.04-0.24)	0.04 (0.004-0.31)	0.002*

(Continued from previous page)

		HDN		CDC light trap			BG-sentinel trap			BG-Suna trap		
		Mean catch size (95 % CI)	**RR**	**Mean catch size (95 % CI)**	**RR (95 % CI)**	***P***	**Mean catch size (95 % CI)**	**RR (95 % CI)**	***P***	**Mean catch size (95 % CI)**	**RR (95 % CI)**	***P***
Aedes albopictus	**Day**	7.70 (5.75-9.65)	1	0.05 (-0.05-0.15)	0.006 (0.001-0.05)	<0.001*	1.0 (0.60-1.40)	0.12 (0.05-0.27)	<0.001*	0.15 (-0.02-0.32)	0.02 (0.004-0.06)	<0.001*
Anopheles	**Night**	11.00 (7.39-14.61)	1	1.15 (0.16-2.14)	0.06 (0.02-0.16)	<0.001*	0.10 (-0.11-0.31)	0.005 (0.001-0.03)	<0.001*		N/A	
Culex	**Night**	7.25 (4.48-10.02)	1	0.25 (0.04-0.46)	0.03 (0.01-0.09)	<0.001*	0.25 (-0.01-0.51)	0.03 (0.01-0.10)	<0.001*	0.50 (0.06-0.94)	0.05 (0.02-0.13)	<0.001*

Mosquito data are shown for day (n = 10) and night (n = 10) collections. As the catch sizes were too low, no night values for Aedes albopictus *mosquitoes and no day values for* Anopheles *and* Culex *mosquitoes are depicted. CI, confidence interval; N/A, Not Applicable; RR, rate ratio. *significantly different,* P*<0.05*

3.4.2 Human landing catch and human-baited double net trap comparison (Experiment two)

A total of 8,282 female mosquitoes (4,967 HLC, 3,315 HDN), belonging to 66 species (48 species HLC, 63 species HDN) were collected. From the total number of female mosquitoes collected, 39.9 % were *Heizmania* species (3,308/8,282), with *Heizmania mattinglyi* most common (91.6 %, 3,029/3,308), 35.4 % were *Aedes* species (2,934/8,282), with *Ae. albopictus* most frequent (58.4 %, 1,714/2,934), 7.4 % were *Culex* species (612/8,282), with *Cx. vishnui* most abundant (83.0 %, 508/612) and 2.5 % were *Anopheles* species (205/8,282), with *An. barbumbrosus* most common (77.1 %, 158/205) (Figure 3.2, Figure 3.3). Only 21 possible malaria vectors (*Anopheles minimus s.l., Anopheles maculatus s.l., Anopheles dirus s.l.* and *Anopheles barbirostris s.l.*) were collected. The HLC collected a total of 86 *Anopheles* mosquitoes of which 18.6 % (16/86) were putative malaria vectors. The HDN collected a total of 119 *Anopheles* mosquitoes of which 12.6 % (15/119) were putative malaria vectors. Nearly 20 % of sampling occasions yielded no mosquitoes (21 % HDN, 19 % HLC).

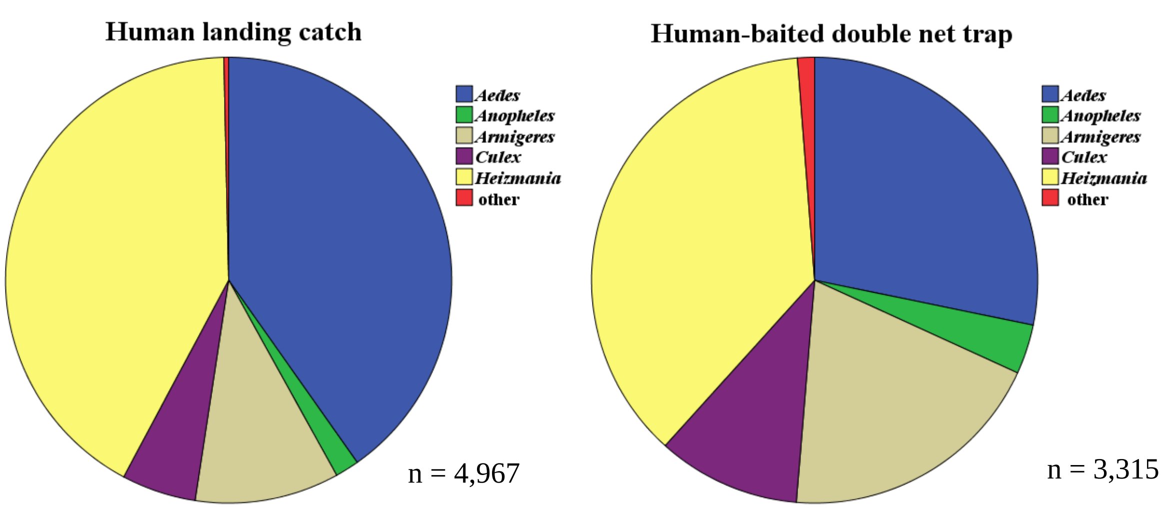

Figure 3.2 Species diversity of mosquitoes collected by the human landing catch method and the human-baited double net trap (experiment two)

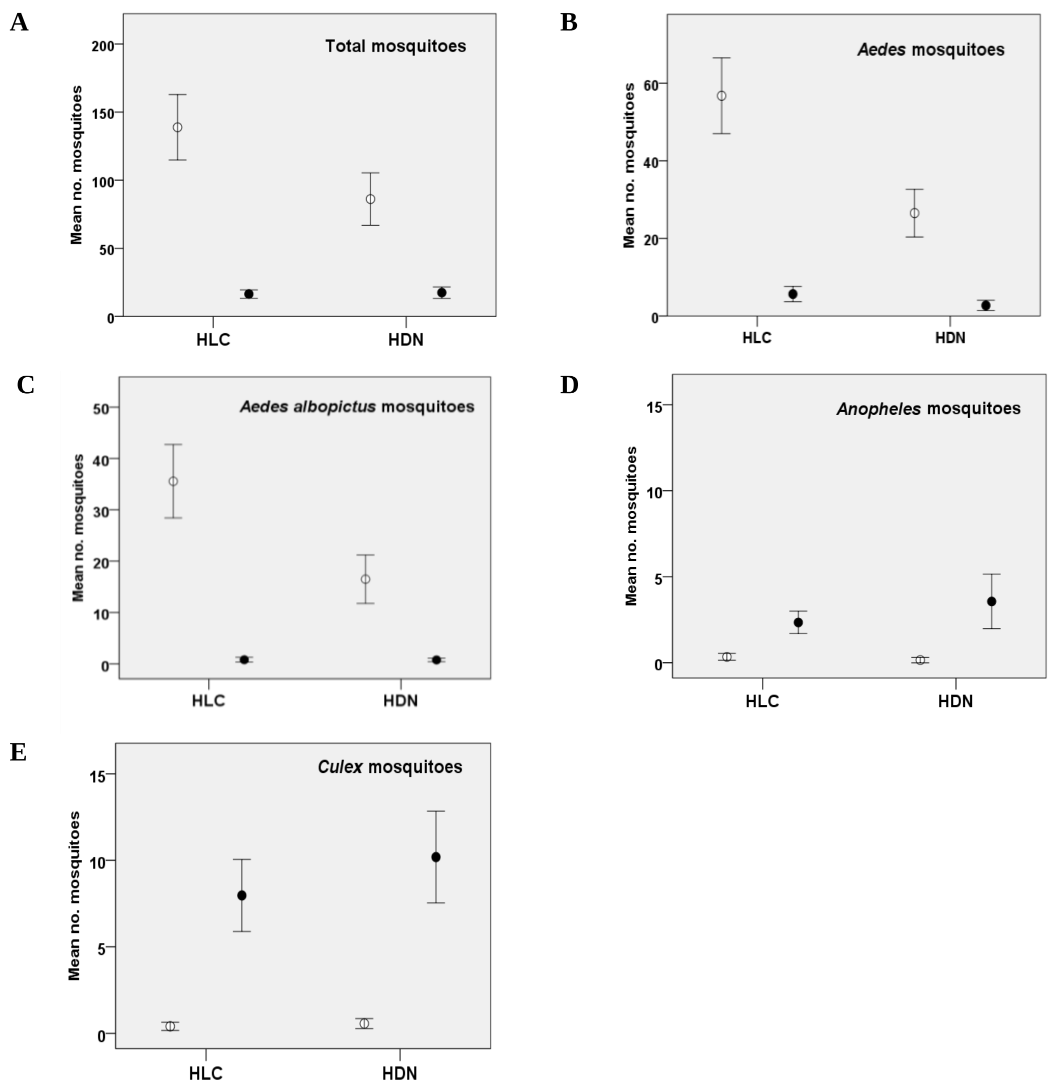

Figure 3.3 Means and 95% confidence interval of female mosquitoes collected by the human landing catch and the human-baited double net trap comparison both day ○ and night ● (experiment two). (A) Total female mosquitoes collected (B) Total female *Aedes* mosquitoes collected (C) Total female *Aedes albopictus* mosquitoes collected (D) Total female *Anopheles* mosquitoes collected (E) Total female *Culex* mosquitoes collected. HLC, human landing catch method; HDN, human-baited double net trap

The HDN trap collected similar number of total mosquitoes as HLC (Rate ratio, RR = 0.78, 95 % CI 0.55-1.13, P = 0.186). However, more detailed analysis showed that, whilst HDN collected a similar number of *Anopheles* and *Culex* mosquitoes when compared to HLC, the HDN method under-estimated the number of *Aedes* species, including the number of *Ae. albopictus,* by half (Table 3.3). Sampling was not affected by trap location nor date. The HDN and HLC collected similarly high number of mosquitoes during the day and similarly low number during the night (Figure 3.4). However, no correlation was evident within the day collections or the night collections. Species diversity calculated using Simpson's index of diversity was 0.845 (95 % CI 0.836-0.854) for the HDN collection method, which was higher than for the HLC method (1-D = 0.778 with 95 % CI 0.769-0.787).

Table 3.3 Generalized linear modelling of female mosquitoes collected by the human landing catch and the human-baited double net trap comparison (experiment two)

		Mean catch size					HDN
	Time of day	**HLC (95 % CI)**	**HDN (95 % CI)**	**Location *P***	**Date *P***	**Trap *P***	**RR (95 % CI)**
Total mosquitoes	**Day**	138.81 (114.74-162.89)	86.16 (66.91-105.40)	0.637	0.941	0.054	0.60 (0.36 - 1.01)
	Night	16.41 (13.34-19.47)	17.44 (13.24-21.64)	0.570	0.814	0.946	0.98 (0.58 - 1.66)
***Aedes* species mosquitoes**	**Day**	56.78 (47.01-66.55)	26.53 (20.38-32.76)	0.741	0.968	0.003*	0.46 (0.27 - 0.77)
***Aedes albopi*ctus**	**Day**	35.56 (28.42-42.71)	16.47 (11.74-21.20)	0.817	0.938	0.003*	0.45 (0.27 - 0.77)
***Anopheles* species mosquitoes**	**Night**	2.34 (1.76-3.00)	3.56 (1.98-5.15)	0.358	0.353	0.648	1.16 (0.61-2.20)
***Culex* species mosquitoes**	**Night**	7.97 (5.89-10.10)	10.18 (7.53-12.84)	0.236	0.434	0.397	1.26 (0.74 - 2.17)

Mosquito data are shown for total of day (n = 16) and night (n = 16) collections for all locations (n = 4), for all collection dates (n = 16) and for all trap types (n = 2). As the catch sizes were too low, no night values for Aedes *and* Aedes albopictus *mosquitoes and no day values for* Anopheles *and* Culex *mosquitoes are depicted. CI, confidence interval; HLC, Human Landing Catches; HDN, Human-baited Double Net; RR, rate ratio. *significantly different,* P*<0.05*

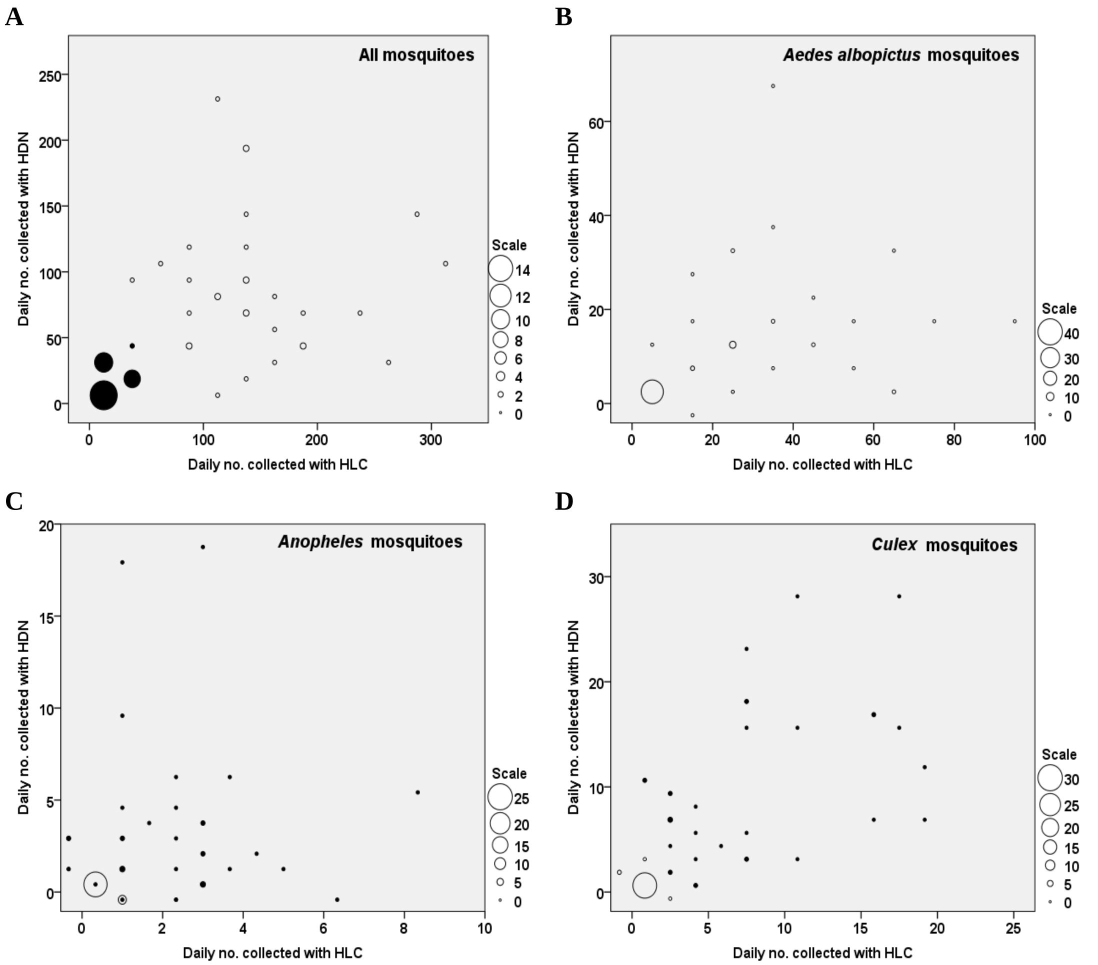

Figure 3.4 The female mosquitoes collected by the human landing catch (HLC) trap and the human-baited double net (HDN) trap (experiment two) for both day ○ and night ● (A) All mosquitoes collected by HLC (n = 4967) and HDN (n = 3315) (B) All *Aedes albopictus* collected by HLC (n = 1163) and HDN (n = 551) (C) All *Anopheles* collected by HLC (n = 86) and HDN (n = 119) (D) All *Culex* collected by HLC (n = 268) and HDN (n = 344)

3.5 Discussion

This study demonstrated that the HDN method is more efficient at collecting outdoor mosquitoes than CDC light traps, BG-sentinel traps and BG-Suna traps, and that it can be used as a more 'ethical' alternative to HLC.

The low mosquito numbers collected by the traps not using human subject was disappointing, but perhaps not surprising. Although indoors, CDC light traps can capture more vector mosquitoes than HLC [317-320], the suitability of outdoor CDC light traps for collecting mosquitoes is debatable [296, 297, 321]. The success of these traps outdoors is expected to be highly dependent on mosquito species present. Furthermore, this trap is likely more efficient at night due to the use of a light, with the trap in this study collecting a higher number of mosquitoes at night than during the day. The BG-sentinel trap is a tool used for routine mosquito surveillance of *Ae. albopictus* and *Aedes aegypti* in North America, Singapore and Australia [304, 305, 322]. The BG-sentinel trap collected a higher number of mosquitoes during the day than the night, with the visual cues of the trap most likely involved in attracting the mosquitoes. In this study the BG-sentinel trap caught more *Aedes* species than both the CDC light trap and the BG-Suna trap. Although no *Aedes* mosquito studies have been conducted for the BG-Suna traps, many studies have shown the BG-sentinel traps' superiority over CDC light traps for collecting *Aedes* mosquitoes [323-325]. Nevertheless, in our study the number of *Aedes* mosquitoes caught with the BG sentinel trap were seven times lower than for the HDN traps. The BG-Suna trap has only been tested in one study in Kenya and the blend of attractants was developed based on host-seeking behaviour of the two African mosquitoes *Anopheles coluzzii* and *Anopheles gambiae s.s.* [306]. It is probable that skin odours from adults in Asia differ from those in Africa and consequently the BG-Suna blend is less attractive to Asian mosquitoes. In conclusion the CDC light trap, BG-sentinel trap and BG-Suna trap cannot be recommended for estimating outdoor human-biting rates in this study area.

It is important to consider the many advantages of traps for which no human participant is necessary, compared to traps involving a human participant. Compared to the use of HLC or HDN methods, a high number of battery-powered traps can be deployed at one time at very low cost. These traps are less labour intensive, they are easy to install and need little preparation. Furthermore, there is no variation in catching efficiency between the battery-powered traps, which is impossible to achieve for traps involving a human participant. In addition, the use of battery-powered traps does not have ethical concerns. Therefore, even though battery-powered traps are currently not as efficient in collecting outdoor mosquitoes, their use is essential for many entomological studies. One of the main challenges in the future will be to develop a battery-powered trap and lure which attracts similar number and diversity of mosquitoes outdoors in Asia, as traps involving a human participant.

Gater (1935) appears to be the first to describe the use of the HDN method [326]. Since then HDN traps have been used in Africa, Asia and South America with varying success [282, 290-292]. This study showed that mosquito catches made with HDN and HLC are similar. Closer scrutiny of the data revealed this effect was due to sampling in two study areas, one with higher mosquito numbers than the other. Such an effect has been seen previously in comparisons between HLC and CDC light traps where an overall positive linear association was found between the two collection methods [327, 328]. In both cases when only low density data were considered no correlation was evident. Concluding both HLC and HDN can distinguish between low and high mosquito densities.

In this study, the HDN collected similar numbers of *Anopheles* and *Culex* mosquitoes as HLC, but under-estimated the number of *Aedes* mosquitoes. The *Anopheles* species composition and the proportion of putative malaria mosquitoes collected, were similar between the two trapping types. In earlier studies in Africa the HLC collected twice as many mosquitoes as human-baited single bed net in Uganda [329], almost four times as many in Nigeria [330] and 7.5 times as many as a double net trap in Cameroon [290]. Concerns have been raised about HDN collections underestimating the true mosquito abundance as mosquitoes would escape the double net trap when they cannot feed [282, 290, 331]. In the study this concern was reduced by conducting hourly collections. However, the HDN method still systematically underestimated the number of *Ae. albopictus* collected compared to the HLC method. This is presumably because mosquitoes either failed to enter the trap or did not persist for very long in the HDN. Nonetheless it should be recognised that both HDNs and HLCs are only proxy estimates of exposure. It is likely that HDNs slightly under-estimate biting rates, whilst HLCs over-estimate biting rates, since few people sit still all night exposing their limbs to mosquitoes. Human-biting rate estimates derived from both can be improved by increasing trap numbers to reduce variance. The conclusion is that whilst both HLC and HDN can distinguish between low and high mosquito densities, they are both crude ways of estimating biting rates.

A slightly greater diversity of mosquito species were collected with the HDN trap compared with the HLC method. This suggests the HDN trap collects anthropophilic mosquitoes coming to feed, mosquitoes seeking shelter and mosquitoes entering the bed net accidentally. It likely collects fewer, but nevertheless representative numbers of, anthropophilic mosquitoes compared to HLC. The HDN is therefore appropriate for identifying outdoor mosquito diversity in SEA.

The main limitation of this study was that it was only powered to explore whether there was a relationship between total mosquito numbers caught using HLC and HDN. In the future, there is need to increase the sample size to explore the relationship between the catching efficiency and parity rates of both methods when sampling *Aedes*, *Anopheles* and *Culex* species separately. Furthermore, the sporozoite rates of the malaria vectors collected in the different traps should be investigated. A study in Cameroon has shown that anopheline parity and sporozoite rates were similar between the HLC and HDN trapping methods [290]. Studies are needed in Asia to confirm this for Asian *Anopheles* mosquitoes. Additional studies will also need to be done to investigate if the HDN allows detection of seasonal variation and if the HDN method could be used as early warning for increases in disease transmission intensity. Moreover, I did not check whether any of the collected mosquitoes had fed on the participants under the nets, so I cannot rule out the possibility that some participants were bitten during these collections.

3.6 Conclusion

HDN can be used for sampling anthropophilic mosquitoes outdoors and is likely to work in similar settings in SEA. It is a simple and cheap method for estimating human-biting rates. Most importantly this procedure is an ethically acceptable alternative to HLC as it protects individuals from exposure to mosquito bites directly. This method could become the preferred method for collecting entomological data in areas where outdoor disease transmission occurs. Furthermore this method is of use in areas where dengue is endemic and where malaria parasites are less sensitive to antimalarials. Further studies are nevertheless needed to confirm the catching efficiency of HDNs against single vector species in other parts of Asia and the tropics.

4 Mosquito density and diversity in natural and man-made forest habitats in rural Lao PDR

Three Human-baited Double Net traps in an immature rubber plantation near Thinkeo village

4.1 Abstract

Vector-borne disease outbreaks, such as dengue and malaria, are still regularly occurring in Lao PDR. Worryingly, these outbreaks have been linked to changes in land-use, such as the expansion of cities, hydro-dams and rubber plantation areas. Unfortunately there have been very few studies that report the mosquito fauna in the country. The objective of this study was to identify the mosquito dynamics, specifically the vector species dynamics, in rural habitats common in northern Lao PDR.

I carried out a longitudinal study to compare the abundance and diversity of adult mosquitoes in four rural habitats common in northern Lao PDR: secondary forests, villages, immature rubber plantations and mature rubber plantations. Collections were made for nine months using Human-baited Double Net traps in three study areas, each consisting of the four different habitat. Generalized estimating equations were used to explore differences in mosquito abundance, and the Simpson's diversity index was used to explore differences in species diversity in the different habitats.

During 15,552 hours of collection 24,927 female mosquitoes were collected, including 61 species newly recorded in Lao PDR. All habitats showed high species diversity (Simpson indexes between 0.82-0.86). The highest number of mosquitoes were collected in the secondary forests during both the rainy season (4.4 female mosquitoes per person per hour, 95% confidence interval (CI) 4.2-4.6) and the dry season (1.9 female mosquitoes per person per hour, 95% CI 1.6-2.0). Three of the four most common species found in the study habitats were vector species; the dengue and chikungunya vector *Ae. albopictus*, the lymphatic filariasis vector *Ar. kesseli* and the JE vector *Cx. vishnui*. Additionally, in all habitats a daily exposure to malaria vectors, such as *An. maculatus s.l*, *An. dirus s.l.*, *An. minimus s.l.* and *An. barbirostris s.l.* was found.

In all habitats vector mosquitoes were collected, with the highest density identified in the secondary forests. Larval control and personal protection methods are possible vector control methods for our study area. However, human behavioural studies are necessary to understand the true mosquito exposure of the population in the different habitats. Additional entomological studies are also necessary to identify the breeding habitats and effectiveness of the personal protection methods.

4.2 Introduction

South-East Asia (SEA) is a region where the population is at high risk of vector-borne diseases [55, 205, 332]. This risk has been exacerbated by changes in the environment, such as surface water availability, urbanization, establishment of cash crops, large-scale movement of people and climate change [18-23]. Cash crops, such as rice, sugar cane and rubber, drastically change the environment and its suitability for vector mosquitoes [27]. For example, in Sri Lanka there have been several examples of where rice cultivations were an important habitat for malaria and Japanese encephalitis (JE) epidemics [333, 334]. Other examples of an increase in malaria and JE incidence after rice cultivation were also seen in China, India, Indonesia, Malaysia and Nepal [27, 335-339].

Vector-borne diseases are an important source of febrile disease in Lao PDR [172]. Malaria is endemic in Lao PDR with a highly heterogeneous distribution [93-95]. In 2012 a threefold increase in malaria cases was reported, compared to 2011. Malaria cases have since fallen, yet outbreaks continue in Saravan and Champasack provinces [115]. Dengue is also endemic in Lao PDR. The latest outbreak occurred in 2013, when a four-fold increase in dengue infections occurred compared to the year before, totalling 1,070 cases from January to March 2013 [137, 142]. Japanese encephalitis is also endemic in Lao PDR with 78 % of the population at risk [139, 141, 150, 151]. It is a vaccine preventable disease for which currently efforts are made for countrywide coverage. Another important vector-borne disease is lymphatic filariasis [154, 155]. The disease is endemic, but data on the disease dynamics are absent.

The outbreaks of vector-borne diseases in Lao PDR have been linked to the rapid environmental changes occurring. The changes include mining, rubber plantation establishment, hydro-dam constructions and deforestation. Lao PDR (People's Democratic Republic) has one of the fastest growing economies in SEA with a 6.4% increase in Gross Domestic Product in 2015 [65], helped partly by the 160 fold expansion of rubber plantations from 2010 to 2015. Rubber tree cultivation is a new kind of mass farming not seen in Lao PDR before and the impact of these changes on the vectors remain poorly understood [340]. The total rubber plantation area in SEA covers 9.2 million ha, about the size of Portugal [45]. These man-made forests provide environments for vector mosquitoes [341]. Outbreaks of dengue [223, 342], malaria [191-195, 343], and chikungunya [223, 232] have been recorded in rubber plantations of SEA, yet data on the abundance and diversity of mosquitoes in rubber plantations remains limited. Since rubber plantations are likely to expand in the country for at least the next decade [70], there is a need to understand if there is a high risk for exposure to vector mosquitoes in these habitats.

There is a general lack of information on mosquito species diversity and density in Lao PDR. There have only been a few entomological studies conducted [96, 97, 110, 205, 217, 340, 344-347]. These studies mostly focus on malaria vectors in the South of the country. To date, only 101 mosquito species have been recorded [96, 97, 110, 205, 217, 340, 344-347], compared to neighbouring Thailand and Viet Nam where more than 300 mosquito species have been identified [314, 348, 349]. For future vector control programmes more research on the mosquito abundance and species richness is necessary. This is especially vital information in a country like Lao PDR, where mosquito-borne diseases are a major public health threat.

The objective of this study was to identify the mosquito dynamics, specifically the vector species dynamics, in rural habitats common in Lao PDR. A longitudinal study was carried out in northern Lao PDR to determine the abundance and diversity of adult mosquitoes in four typical rural habitats: secondary forests, immature rubber plantations, mature rubber plantations and villages. I hypothesised that the highest density and diversity of vector mosquitoes would be found in the secondary forests. The mature rubber plantations, with high canopy cover, high humidity and stable temperatures, were also expected to provide an ideal habitat for forest mosquitoes such as the dengue vector *Aedes albopictus*. However due to the lack of canopy cover, immature rubber plantations and villages were not anticipated to contain a high density of vector mosquitoes.

4.3 Materials and Methods

4.3.1 Study sites

The study was conducted in northern Lao PDR in the three study sites Thinkeo (19°41’02.13”N 102°07’05.49”E), Silalek (19°37’02.80”N 102°03’05.70”E) and Houayhoy (19°33’03.22”N 101°59’42.42”E), in Xieng-Ngeun and Nane district, Luang Prabang province (Figure 4.1). These study sites were chosen for the accessibility of the different study habitats within 30 minutes from the main road. This is a hilly region with patches of secondary forests and rubber plantations. The most common cash crops in the area were maize, banana, rubber plantations, highland rice and lowland rice. On average, the elevation in Thinkeo study site was 468 m (range 328-596), in Silalek 671 m (range 520-786) and in Houayhoy 652 m (range 533-787).

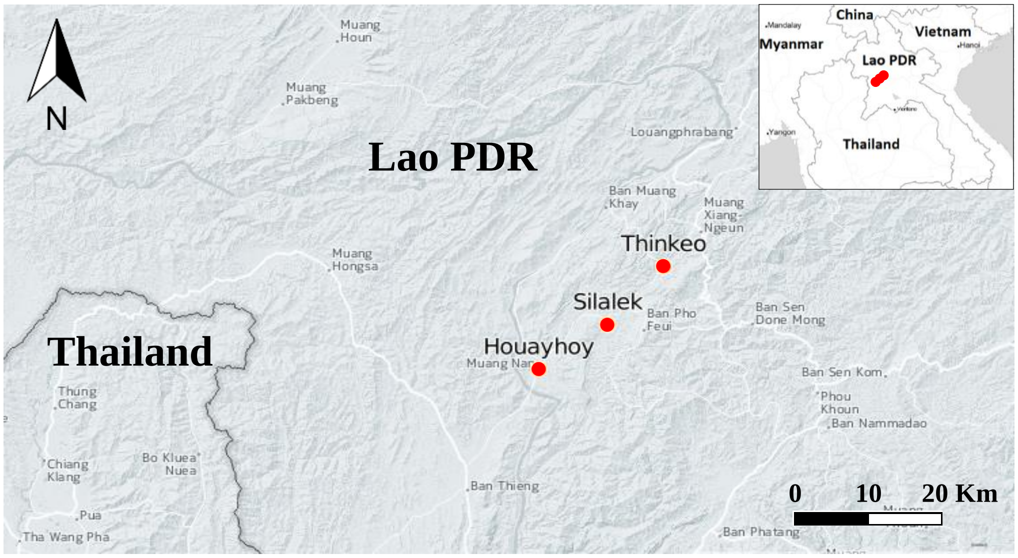

Figure 4.1 The three study sites in Luang Prabang province, northern Lao PDR (Carto DB © attribution)

In each study site, four habitats were surveyed (n_{total} = 12): a secondary forest, an immature rubber plantation, a mature rubber plantation and a local village (Figure 4.2). The secondary forests were defined as forests that had re-grown after the primary forests had been cut for timber. Thus, few mature trees were present and bamboo shrubs and small trees dominated. Immature plantations were classed as rubber trees less than five years old, which had not yet been tapped for latex. Mature rubber plantations were those where the trees were more than five years old and over 70% of the trees were tapped for latex for at least one year. The villages were linear rural settlements. According to the last census in 2013, the villages comprised of 700 to 1,000 inhabitants with on average 5.9 people per household. Generally, villagers lived in one storey bamboo houses with thatched roofs or brick houses with corrugated iron roofs.

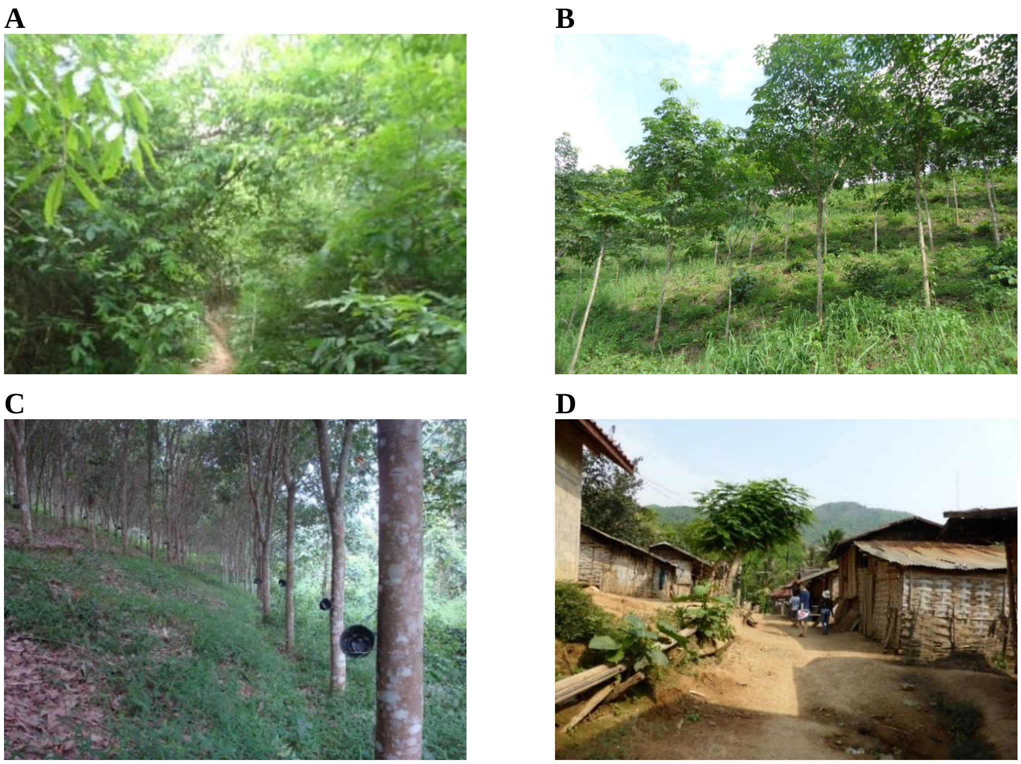

Figure 4.2 The four habitats investigated in this study. (A) secondary forest (B) immature rubber plantation (C) mature rubber plantation (D) rural village

In this study area, there is a single rainy season from May to October when vector-borne disease transmission is high [100]. The most common vector-borne disease in the study area is dengue, with the presence of Japanese encephalitis (JE) and lymphatic filariasis. According to the Lao Ministry of Health, during this study from July 2013 to June 2014 there were 199 reported dengue cases in Xieng-Ngeun district and 11 in Nane district. No information was available on the incidence rate of JE and lymphatic filariasis. Malaria was rare in the study area with two cases identified in 2013 and three cases in 2014. All malaria cases were thought to be imported from malaria endemic areas with no local transmission reported.

4.3.2 Study design

This longitudinal study was designed to identify the density and diversity of mosquito species present in the four habitats throughout the year. Routine entomological surveys were made monthly for nine months from July to November 2013 and in February, March, May and July of 2014. Surveillance was not conducted in December 2013, January 2014, April 2014 and June 2014 due to national holidays and local festivals. Temperature and humidity data were collected in all habitats throughout the study period, with measurements on the physical forest structure (undergrowth density, canopy cover, tree density, tree height and tree circumference) collected one time in June and July 2014.

4.3.3 Mosquito surveillance

Adult female mosquitoes were sampled using the Human-baited Double Net (HDN) trap [350], which is described in detail in chapter 3. Briefly, the HDN trap construction in this study consisted of one participant resting on a bamboo bed (30 cm high x 230 cm long x 100 cm wide) covered by two untreated bed nets (Figure 4.3). Every hour for 10 minutes the participant raised the bottom of the inner net and aspirated all mosquitoes caught between the two bed nets into labelled paper-cups. Three HDN traps were used in one habitat, placed five to 10 m apart. Three traps were set in one habitat to ensure the participants were not on their own during the night collections and a higher number of mosquitoes could be collected. Thus, 36 HDN traps were used in total, i.e. three study sites, each with four habitats and three traps in each habitat. A supervisor checked on the collecting participants periodically during the collecting period.

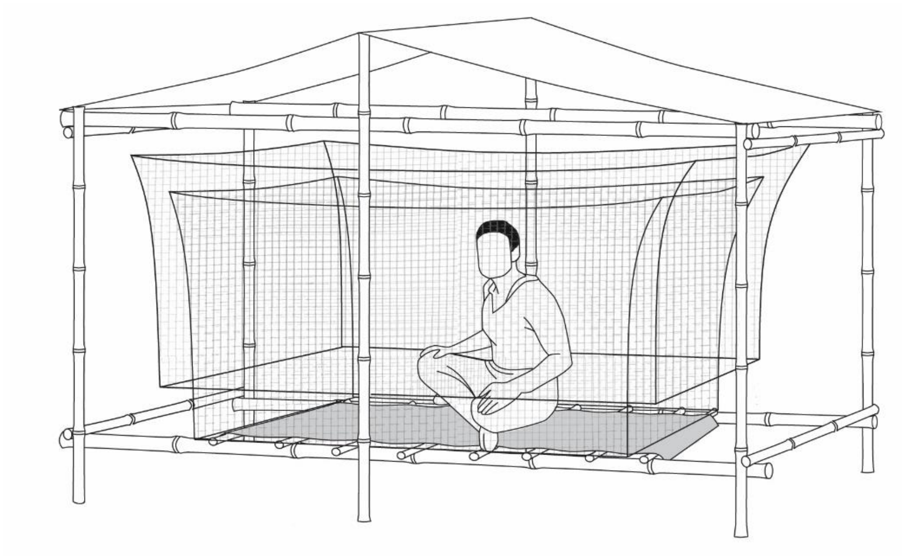

Figure 4.3 The Human-baited Double Net trap. Mosquito sampling method consisting of a bamboo construction with two bed nets

Verbal informed consent was provided by village leaders, after which participants were recruited during a village meeting. Participants gave written informed consent for their participation. A total of 78 healthy male and female participants (i.e. 24 participants and two supervisors per study site), between 18 and 55 years old were paid for their participation. Participants were provided with free medical treatment for infectious diseases throughout the study period and up to three weeks after participation.

During one month of collection in each study site four teams, each consisting of three participants, individually collected mosquitoes in an HDN trap in one of the four habitats for six hours. Thus, 12 collectors collected mosquitoes at one time, distributed over four different habitats at one site. The collection teams were distributed randomly between habitats using the research randomizer program [351]. After six hours, collectors were replaced by a second group of 12 collectors in four teams who continued collecting mosquitoes for a further six hours. This 12 hour collection period lasted from 06.00 h - 18.00 h or 18.00 h - 06.00 h and was repeated four times over several days until a total of two 24 hours collections were conducted in one study site (Figure 4.4). Similar collections were done in the other two study sites over a two weeks' time-period. Adult mosquito collections were therefore conducted for three weeks every month. At the start of the study, the study sites were randomly selected for collections in week one, two or three for the nine months using the randomizer program.

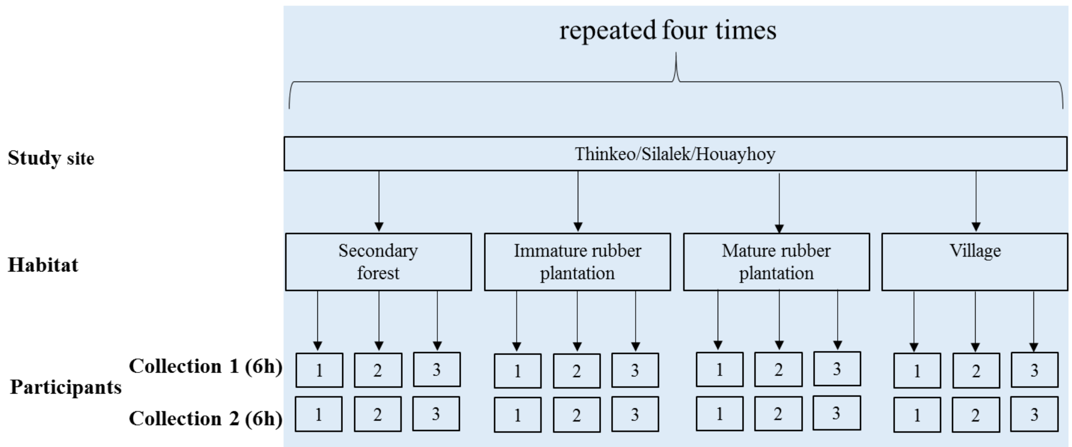

Figure 4.4 Monthly mosquito surveillance design. Adult mosquitoes were collected by 72 participants in three study sites, with each study site consisting of two collection groups that were collecting mosquitoes in four different habitats (secondary forests, immature rubber plantations, mature rubber plantations and villages). This design was repeated four times every month for a total of two 24 hour collection periods in each study site

The labelled paper-cups containing mosquitoes were frozen at -20℃ and mosquitoes were morphologically identified to species or species complex using stereo-microscopes and recognized keys of Thailand [314]. The *Anopheles minimus* group and *Anopheles maculatus* complex were identified molecularly to species using AS-PCR assays and species specific primers at Kasetsart University in Bangkok Thailand [352-357]. Due to problems with the PCR technique the *An. dirus* complex could not be molecularly identified.

4.3.4 Susceptibility test of adult **Aedes albopictus** *to DDT, bendiocarb, permethrin, deltamethrin and malathion*

The susceptibility of wild caught *Ae. albopictus* was assessed using the WHO bioassays [358]. Currently, no discriminating concentrations exists for *Ae. albopictus* susceptibility tests. As *Ae. aegypti* discriminating doses were unavailable during testing in the field, I used the doses for *Anopheles* mosquitoes with a regular check for knock down. Adult mosquitoes of unknown age were collected from June to August 2014, in October and in November 2014 in the secondary forests of Thinkeo study area (19°41'01.13"N 102°06'57.98"E) using two HDN traps and transported to the field laboratory in rearing cages (Bug dorm, Bioquip, 299 cm x 299 cm x 299 cm, mesh size 1.1 mm x 0.7 mm).

The adult mosquitoes were kept in the bug dorms in the field laboratory for at least 24 hrs with access to sugar water. Healthy adult mosquitoes were selected from the bug dorms by only selecting mosquitoes that were attracted to a human hand next to the cage. Approximately 20 adult female mosquitoes were placed in holding tubes for 60 minutes and damaged mosquitoes removed. Remaining mosquitoes were then exposed for 60 minutes to the insecticide-impregnated or appropriate control papers: 4 % DDT; 0.1 % bendiocarb; 0.75 % permethrin; 0.05% deltamethrin and 5% malathion (Vector Control Research Unit, University Sains Malaysia, Penang, Malaysia). Knockdown was checked every three minutes. Exposures were conducted at 27 °C ± 10 °C with 80 % ± 10 % relative humidity (RH). Mosquitoes were returned to the holding tube, given 10 % glucose solution and mortality recorded after 24 hours. Live mosquitoes were those able to fly and any knocked down mosquitoes that had lost legs or wings were considered dead. The analysis of the susceptibility tests followed WHO recommendations [358]. If more than 10% of the mosquitoes exposed to the control papers died, the insecticide exposures were adjusted for this mortality using the Abbott's formula. The Abbott's formula is a mathematical formula used to correct for control mortality [359].

4.3.5 Environmental measurements

Temperature and RH were measured in the four habitat types every 15 minutes during the mosquitoes collection period (two 24 hour periods per month) in each study site using four HOBO Pro data-loggers (Onset Computer Corporation model H08-031-08). This was repeated for the three study sites, resulting in six 24 hour climate data for each habitat type every month. Temperature and RH were measured for a total of nine months. The HOBO data loggers were attached to the tree nearest to the HDN traps at 1.80 m above the ground by a participant before mosquito collections were commenced. The same locations in each habitat were used throughout the study period.

In the three different habitats (secondary forests, immature and mature rubber plantations), measurements on the physical forest structure were made at the end of the study (June and July 2014). Using Google Earth© a 10 m x 10 m grid was fitted to each of the nine forest habitats (three forest habitats in three study sites). Ten squares were randomly chosen using the research randomizer program for each of the nine habitats [351], resulting in a total of 90 squares. In each square measurements were made to determine undergrowth density, canopy cover, tree density, tree height and tree circumference. Undergrowth density was measured at the four corners of each square by placing the centre of a two by two meters white sheet vertically on a corner with one side facing north and other facing south. The bottom of the sheet touched the ground and the sheet was held upright by bamboo. Pictures of the sheet were taken using a camera (Stylus TG-830 Tough, Olympus) on a tripod from four meters away and one meter off the ground [360]. Forty colour photographs were taken in each habitat (four corners of 10 squares) with 360 photographs taken in total. The pictures were analysed to measure proportion of vegetation in each picture using the threshold function of imag J software [361]. Canopy cover proportion was also measured in the four corners of each square [360]. Pictures of the sky were taken on the corners of each square, one meter above the ground and analysed similarly to the undergrowth density using imag J [360, 361]. Tree density was measured by counting the number of trees (defined as a perennial woody plant with the main trunk > 20 cm circumference) in each square. These tree counts included the rubber trees present. Tree height was measured for all trees using a clinometer (FIN-01510, Valimotie 7, Suunto, Finland) and tree circumference was measured at standard breast height, 1.37 m from the ground [362].

4.3.6 Data analysis

Generalized estimating equations (GEE) using a negative binomial model with log-link function were used to estimate the difference in mosquito density between habitats, study sites and months for both seasons (IBM SPSS statistics, version 20). The GEE was used to estimate the average response of the parameters, with collection days designated as the correlation variable between the different parameters. Species diversity was compared using Simpson's index of diversity with 95 % CI [316, 363]. The positively skewed daily mean temperature and the daily mean humidity were square root transformed and analysed with GEE using a linear model with odds ratio (OR) and 95 % CI. Undergrowth, canopy cover, tree height and tree circumference were averaged for each square before analysis. Undergrowth and height were positively skewed and transformed using the formula $\log_{10}(x+1)$ for undergrowth and $\log_{10}(x)$ for height. Undergrowth and height data were analysed with generalized linear modelling (GLM) using a linear model. Canopy cover and circumference were negatively skewed. Data was analysed with GLM using a gamma model with log-link function. Tree density data was analysed using GLM with Poisson log linear model.

4.3.7 Ethics

The study was approved by the Lao ethics committee (approval number 017/NECHR issued 21-04-2013) (Appendix 1) and the School of Biological and Biomedical Sciences Ethics

Committee, Durham University (issued 25-07-2013) (Appendix 2). Information sheet and consent form were provided in Lao language (English version Appendix 5).

4.4 Results

4.4.1 Mosquito surveillance

During 15,552 hours of collection 24,927 adult female mosquitoes were collected. One hundred and thirteen mosquito species were identified, including 61 species that have not been recorded previously in Lao PDR (Appendix 6) [96, 97, 102, 108, 110, 111, 205, 217, 340, 347]. Most mosquitoes were collected in secondary forests (55.3%), followed by immature rubber plantations (21.4%), mature rubber plantations (14.6%) and villages (8.7%). A total of 1,249 male mosquitoes were collected of which 71.2 % were *Ae. albopictus* (889/1,249). Thirteen female and nine male mosquitoes could not be identified to species. More than 60% (9,395/15,552) of the sampling occasions yielded no mosquitoes (37.8%, 1,470/3,888 in secondary forests; 64.7%, 2,300/3,888 in immature rubber plantations; 59.2%, 2,514/3,888 in mature rubber plantations; 80%, 3,111/3,888 in villages).

4.4.2 Mosquito density

The number of female mosquitoes collected varied by habitat (GEE, $P<0.001$), study site ($P<0.001$) and month ($P<0.001$). In both the rainy season and dry season more female mosquitoes were collected in the secondary forests than the other three habitats (all $P<0.0001$) (Table 4.1). Most mosquitoes were collected in Thinkeo study site and the least number of mosquitoes were collected in Silalek. The variability between collection months within one season was high, with collection numbers varying between 1.5 times higher and 1.5 times lower than June 2014 in the rainy season and March 2014 in the dry season.

Table 4.1 Multivariate analysis of variables associated with female adult mosquitoes collected using human-baited double net traps

Season	Explanatory variable	n	Mean number collected per person/h. (95% CI)	OR (95% CI)	*P*
Rainy (May - Oct)	**Habitat**				
	Immature rubber plantation	4118	1.59 (1.49-1.68)	0.33 (0.31-0.36)	<0.0001*
	Mature rubber plantation	3007	1.16 (1.08-1.24)	0.25 (0.23-0.27)	<0.0001*
	Village	1652	0.64 (0.55-0.72)	0.13 (0.12-0.14)	<0.0001*
	Secondary forest	11427	4.41 (4.19-4.62)	1	
	Study site				
	Thinkeo	8158	2.36 (2.22-2.50)	1.48 (1.39-1.57)	<0.0001*
	Silalek	5811	1.68 (1.57-1.80)	0.88 (0.83-0.94)	<0.0001*
	Houayhoy	6235	1.80 (1.69-1.92)	1	
	Month				
	July 2013	3442	1.99 (1.82-2.16)	1.05 (0.96-1.14)	0.311
	Aug. 2013	4852	2.81 (2.59-3.02)	1.50 (1.38-1.64)	<0.0001*
	Sept. 2013	3348	1.94 (1.75-2.12)	0.94 (0.86-1.02)	0.139
	Oct. 2013	2350	1.36 (1.23-1.49)	0.68 (0.62-0.74)	<0.0001*
	May 2014	2883	1.67 (1.51-1.83)	0.84 (0.77-0.92)	<0.0001*
	June 2014	3329	1.93 (1.76-2.10)	1	
Dry (Nov - April)	**Habitat**				
	Immature rubber plantation	1205	0.93 (0.77-1.09)	0.46 (0.41-0.51)	<0.0001*
	Mature rubber plantation	644	0.50 (0.42-0.57)	0.25 (0.22-0.28)	<0.0001*
	Village	512	0.40 (0.32-0.47)	0.20 (0.18-0.23)	<0.0001*
	Secondary forest	2362	1.82 (1.64-2.01)	1	
	Study site				
	Thinkeo	2492	1.44 (1.30-1.59)	2.07 (1.87-2.29)	<0.0001*
	Silalek	889	0.78 (0.67-0.89)	0.65 (0.58-0.73)	<0.0001*
	Houayhoy	1342	0.51 (0.43-0.60)	1	
	Month				
	Nov. 2013	1832	1.06 (0.94-1.18)	1.12 (1.01-1.25)	0.026*
	Feb. 2014	1205	0.70 (0.59-0.80)	0.74 (0.66-0.82)	<0.0001*
	Mar. 2014	1686	0.98 (0.84-1.11)	1	

*Results are shown for generalized estimating equations of factors affecting the collection of adult female mosquitoes with odds ratio (OR) and 95% confidence interval (CI) *significantly different,* P*<0.05*

In the secondary forests more female mosquitoes were collected in August and September 2013, when rainfall was highest, than in the other months combined (Figure 4.5). There was a similar monthly trend between the two rubber plantation habitats with generally lower numbers collected in mature rubber plantations than in immature rubber plantations. In both types of plantation during the August 2013 peak in rainfall between four and five times more mosquitoes were collected than in February 2014 when there was no rain. In the villages the numbers of female mosquito collected were low throughout the year, with generally less than one female mosquito per person per hour collected.

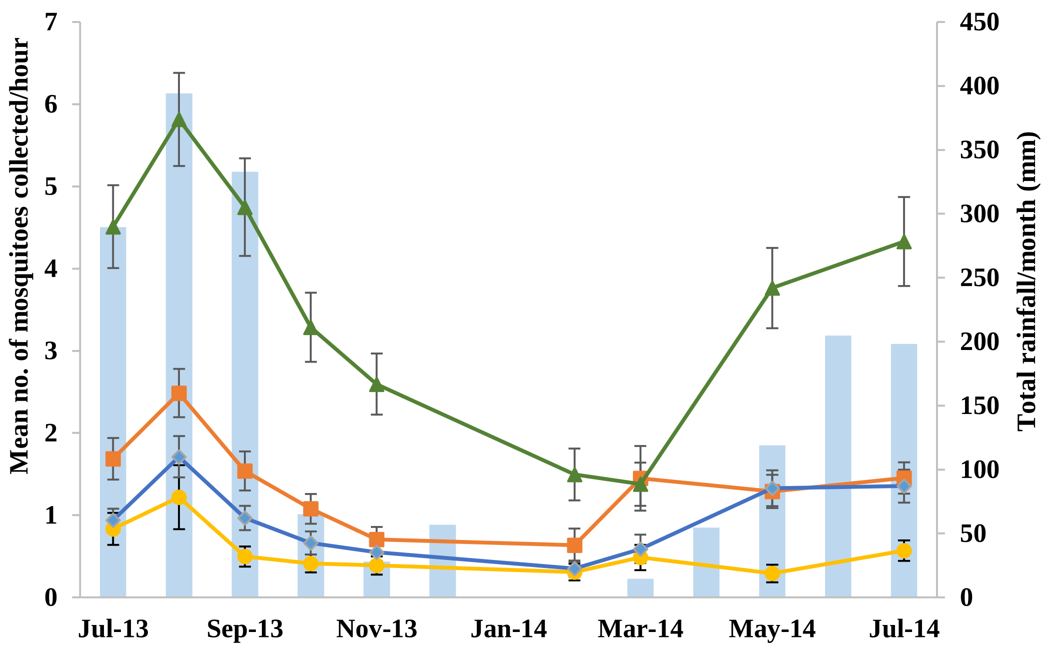

Figure 4.5 Seasonal variation of female mosquito numbers in different habitats. The average number of female mosquitoes collected per person per hour for each collection month in the four habitats (—▲— secondary forests, —■— immature plantations, —♦— mature plantations, —●— villages) with 95 % confidence intervals and total rainfall per month indicated with light blue bars ( )

4.4.3 Mosquito diversity

In the secondary forests 89 species were collected with a Simpson's index of 0.853 (95 % CI 0.850-0.856) which was slightly higher than for immature rubber plantations where 79 species were collected (0.843 with 95 % CI 0.838-848, t-test P<0.001) and mature rubber plantations where 72 species were collected (0.816 with 95 % CI 0.806-0.825, P<0.001). The diversity index in the secondary forests was lower than the diversity index found in the villages where 62 mosquito species were collected with an index of 0.864 (95 % CI 0.855-0.873, P=0.0182). The species distribution in the natural and man-made forest habitats showed similar trends with *Aedes* species dominating in the rainy season and *Culex* species in the dry season (Appendix 6). In the villages *Culex* species were more common in the rainy season with *Anopheles* mosquitoes the most abundant species in the dry season.

The dengue vectors *Ae. albopictus* (n = 6,302) and *Ae. aegypti* (n = 1), and the JE vectors *Culex vishnui* (n = 3,562), *Culex bitaeniorhynchus* (n = 75), *Culex fuscocephalus* (n = 70), *Culex quinquefasciatus* (n = 21) and *Culex gelidus* (n = 10) were collected during the study (Appendix 7). Many putative lymphatic filariasis vectors were also collected, comprising of *Armigeres kesseli* (n = 2,621), *Armigeres subalbatus* (n = 268) and *Culex quinquefasciatus*. Furthermore, the malaria vectors *An. maculatus* complex (n = 294), *Anopheles barbirostris* complex (n = 170), *An. minimus* group (n = 151), *Anopheles dirus* complex (n = 46), *Anopheles culcifacies* (n = 3), *Anopheles epiroticus* (n = 3) and *Anopheles philippinensis* (n = 1) were collected. Molecular identification of the *An. maculatus* complex resulted in the identification of *An. maculatus s.s.* (n = 180), *Anopheles pseudowillmori* (n = 36), *Anopheles dravidicus* (n = 10) and *Anopheles sawadwongporni* (n = 9) (Table 4.2). The remaining 59 mosquitoes could not be identified to species. For the *An. minimus* group, *An. minimus s.s.* (n = 85) and *Anopheles aconitus* (n = 63) were identified. Three samples from the *An. minimus* group could not be identified to species.

Table 4.2 Molecular identification of members of the *An. minimus* group and *An. maculatus* complex from different habitats

		Secondary forest	Immature rubber plantation	Mature rubber plantation	Village
***An. maculatus* complex**	***An. maculatus s.s.***	28	96	27	29
	An. pseudowillmori	3	9	10	14
	An. dravidicus	8	2	0	0
	An. sawadwongporni	3	2	1	3
***An. minimus* group**	***An. minimus s.s.***	8	17	16	44
	An. aconitus	7	12	8	36

More than half of the mosquitoes collected in the secondary forests were putative vector species (56.2 %, 7,746/13,786) (Figure 4.6 A). A majority of these putative vector mosquitoes were dengue vectors (26.4 %, 3,640/13,789). For the immature rubber plantations about half were putative vector species (49.7 %, 2,678/5,323) (Figure 4.6 B). Both dengue and JE vectors were most common (dengue 23.4 %, 1,248/5,323 and JE 20.4 %, 1,087/5,323). In the mature rubber plantations 56.4 % of the collected mosquitoes were putative vector species (2,060/3,651) (Figure 4.6 C). Similar to the secondary forests, a majority were dengue vectors (36.5 %, 1,331/3,651). In the villages 52.4 % of the collected mosquitoes were putative vector mosquitoes (1,134/2,164) with JE the most abundant vector species (30.2 %, 654/2,164) (Figure 4.6 D). In the secondary forests, compared to the other habitats, the highest number of dengue vectors (57.8 %, 3,640/6,303), JE vectors (41.0 %, 1,523/3,717) and lymphatic filariasis vectors (82.7 %, 2,406/2,910) were collected. Malaria vector numbers were similarly high in the secondary forests (27.0 %, 177/655), immature rubber plantations (31.8 %, 208/655) and villages (27.0 %, 177/655). In the following paragraphs I describe the most important and abundant vector species.

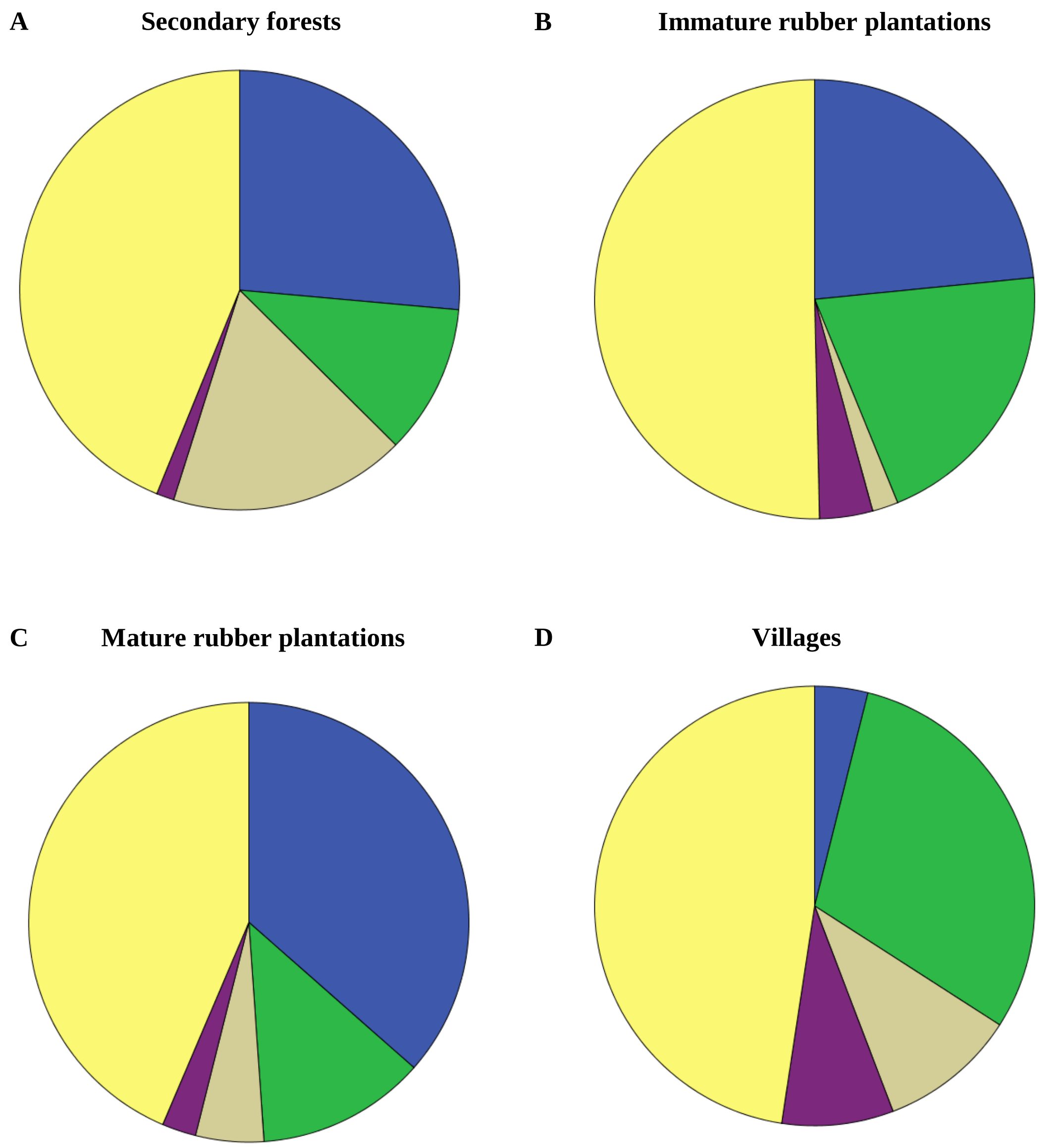

Figure 4.6 Proportion of putative vector species collected (A) Secondary forests (B) Immature rubber plantations (C) Mature rubber plantations (D) villages, with Putative dengue vectors (*Ae. albopictus* and *Ae. aegypti*) Putative Japanese encephalitis vectors (*Cx. vishnui, Cx. bitaeniorhynchus, Cx. fuscocephalus* and *Cx. gelidus*) Putative lymphatic filariasis vectors (*Ar. kesseli, Ar. subalbatus* and *Cx. quinquefasciatus*) Putative malaria vectors (*An. maculatus* complex, *An. barbirostris* complex, *An. minimus* group, *An. dirus* complex, *An. culcifacies*, *An. epiroticus* and *An. philippinensis* non-vectors

About 73 % of the collected *Aedes* mosquitoes were identified as *Ae. albopictus* (6,305/8,585). Most *Aedes* and *Ae. albopictus* were collected in the secondary forests during both the rainy season and dry season (all $P<0.001$) (Table 4.3, Table 4.4). A similar pattern was found for *Culex* mosquitoes where *Cx. vishnui* dominated (71%, 3,562/5,022) with largest numbers collected in the secondary forests during both seasons (all $P \leq 0.001$) (Table 4.3, Table 4.4). Few *Anopheles* mosquitoes were caught during the survey (n = 1,341) with 48% of samples collected in the village (648/1,341) (Table 4.3, Table 4.4). The putative malaria vectors *Anopheles maculatus s.l.* and *An. dirus s.l.* were most common in immature rubber plantations, *An. minimus s.l.* in villages and *An. barbirostris s.l.* in secondary forests.

Table 4.3 Multivariate analysis of habitat variability associated with female adult mosquito species collected using human-baited double net traps during the rainy season

Rainy season (May-Oct)	Habitat	n	Mean no. collected per person/hour (95 % CI)	OR (95 % CI)	*P*
***Aedes* mosquitoes**	**immature rubber plantation**	1729	0.67 (0.61-0.72)	0.41 (0.38-0.45)	<0.001*
	mature rubber plantation	1595	0.62 (0.56-0.67)	0.37 (0.35-0.41)	<0.001*
	village	185	0.07 (0.06-0.08)	0.04 (0.04-0.05)	<0.001*
	secondary forest	4361	1.68 (1.58-1.79)	1	
Ae. albopictus	**immature rubber plantation**	1185	0.46 (0.42-0.50)	0.38 (0.35-0.41)	<0.001*
	mature rubber plantation	1233	0.48 (0.43-0.52)	0.38 (0.35-0.42)	<0.001*
	village	77	0.03 (0.02-0.04)	0.02 (0.02-0.03)	<0.001*
	secondary forest	3281	1.27 (1.18-1.36)	1	
***Culex* mosquitoes**	**immature rubber plantation**	517	0.20 (0.17-0.23)	0.47 (0.41-0.53)	<0.001*
	mature rubber plantation	316	0.12 (0.10-0.14)	0.29 (0.25-0.33)	<0.001*
	village	909	0.35 (0.28-0.42)	0.75 (0.67-0.84)	<0.001*
	secondary forest	1090	0.42 (0.35-0.49)	1	
Cx. vishnui.	**immature rubber plantation**	273	0.11 (0.09-0.12)	0.47 (0.40-0.55)	<0.001*
	mature rubber plantation	142	0.05 (0.04-0.07)	0.24 (0.20-0.30)	<0.001*
	village	518	0.20 (0.14-0.26)	0.79 (0.68-0.91)	0.001*
	secondary forest	584	0.23 (0.18-0.27)	1	
***Anopheles* mosquitoes**	**immature rubber plantation**	163	0.06 (0.05-0.08)	1.03 (0.82-1.30)	0.790
	mature rubber plantation	73	0.03 (0.02-0.04)	0.46 (0.35-0.61)	<0.001*
	village	312	0.12 (0.10-0.14)	1.95 (1.60-2.39)	<0.001*
	secondary forest	158	0.06 (0.05-0.07)	1	
An. maculatus s.l.	**immature rubber plantation**	100	0.04 (0.03-0.05)	2.20 (1.54-3.14)	<0.001*
	mature rubber plantation	29	0.01 (0.01-0.02)	0.65 (0.40-1.03)	0.068
	village	42	0.02 (0.01-0.02)	0.93 (0.61-1.42)	0.722
	secondary forest	46	0.02 (0.01-0.02)	1	
An. minimus s.l.	**immature rubber plantation**	11	0.00 (0.00-0.01)	1.22 (0.50-2.95)	0.662
	mature rubber plantation	16	0.01 (0.00-0.01)	1.66 (0.72-3.80)	0.234
	village	50	0.02 (0.01-0.02)	4.99 (2.43-10.25)	<0.001*
	secondary forest	9	0.00 0.00-0.01)	1	
An. barbirostris s.l.	**immature rubber plantation**	9	0.00 (0.00-0.01)	0.18 (0.09-0.36)	<0.001*
	mature rubber plantation	8	0.00 (0.00-0.01)	0.16 (0.07-0.33)	<0.001*
	village	28	0.01 (0.01-0.02)	0.55 (0.35-0.88)	0.013*
	secondary forest	51	0.02 (0.01-0.03)	1	
An. dirus s.l.	**immature rubber plantation**	20	0.01 (0.00-0.01)	3.99 (1.49-10.67)	0.006*
	mature rubber plantation	5	0.00 (0.00-0.01)	0.99 (0.29-3.45)	0.994
	village	0			
	secondary forest	5	0.00 (0.00-0.00)	1	

*Results are shown using generalized estimating equations with odds ratio (OR) and 95 % confidence interval (CI). *significantly different,* P*<0.05*

Table 4.4 Multivariate analysis of habitat variability associated with female adult mosquito species collected using human-baited double net traps during the dry season

Dry season (Nov-Apr)	Habitat	n	Mean no. collected per person/hour (95 % CI)	OR (95 % CI)	*P*
***Aedes* mosquitoes**	**immature rubber plantation**	93	0.07 (0.05-0.09)	0.19 (0.15-0.24)	<0.001*
	mature rubber plantation	117	0.09 (0.07-0.11)	0.24 (0.19-0.30)	<0.001*
	village	18	0.01 (0.00-0.02)	0.04 (0.02-0.06)	<0.001*
	secondary forest	487	0.38 (0.32-0.44)	1	
Ae. albopictus	**immature rubber plantation**	63	0.05 (0.03-0.07)	0.17 (0.13-0.23)	<0.001*
	mature rubber plantation	98	0.08 (0.06-0.10)	0.27 (0.21-0.35)	<0.001*
	village	6	0.00 (0.00-0.01)	0.02 (0.01-0.04)	<0.001*
	secondary forest	359	0.28 (0.23-0.32)	1	
***Culex* mosquitoes**	**immature rubber plantation**	814	0.63 (0.48-0.77)	0.80 (0.70-0.91)	0.001*
	mature rubber plantation	322	0.25 (0.19-0.31)	0.32 (0.28-0.38)	<0.001*
	village	116	0.09 (0.07-0.11)	0.13 (0.10-0.16)	<0.001*
	secondary forest	938	0.72 (0.60-0.84)	1	
Cx. vishnui	**immature rubber plantation**	768	0.59 (0.45-0.74)	0.78 (0.68-0.90)	0.001*
	mature rubber plantation	298	0.23 (0.17-0.29)	0.31 (0.26-0.37)	<0.001*
	village	86	0.70 (0.05-0.09)	0.10 (0.08-0.12)	<0.001*
	secondary forest	893	0.69 (0.57-0.81)	1	
***Anopheles* mosquitoes**	**immature rubber plantation**	118	0.09 (0.06-0.12)	1.00 (0.76-1.31)	0.971
	mature rubber plantation	66	0.05 (0.04-0.07)	0.55 (0.40-0.76)	<0.001*
	village	336	0.26 (0.20-0.32)	2.76 (2.20-3.48)	<0.001*
	secondary forest	115	0.09 (0.07-0.11)	1	
An. maculatus s.l.	**immature rubber plantation**	37	0.03 (0.02-0.04)	4.13 (1.98-8.60)	<0.001*
	mature rubber plantation	20	0.02 (0.01-0.02)	2.20 (1.00-4.86)	0.051
	village	11	0.01 (0.00-0.01)	1.22 (0.50-2.96)	0.658
	secondary forest	9	0.01 (0.03-0.05)	1	
An. minimus s.l.	**immature rubber plantation**	17	0.01 (0.01-0.02)	2.43 (1.00-5.89)	0.050
	mature rubber plantation	8	0.01 (0.00-0.01)	1.14 (0.41-3.16)	0.803
	village	33	0.03 (0.02-0.04)	4.66 (2.05-10.60)	<0.001*
	secondary forest	7	0.01 (0.00-0.01)	1	
An. barbirostris s.l.	**immature rubber plantation**	3	0.00 (0.00-0.00)	0.06 (0.02-0.19)	<0.001*
	mature rubber plantation	3	0.00 (0.00-0.00)	0.06 (0.02-0.19)	<0.001*
	village	17	0.01 (0.01-0.02)	0.33 (0.19-0.57)	<0.001*
	secondary forest	51	0.04 (0.32-0.44)	1	
An. dirus s.l.	**immature rubber plantation**	11	0.01 (0.00-0.01)	-	
	mature rubber plantation	4	0.00 (0.00-0.01)		
	village	1	0.00 (0.00-0.00)		
	secondary forest	0			

*Results are shown using generalized estimating equations with odds ratio (OR) and 95 % confidence interval (CI). *significantly different,* P*<0.05*

4.4.4 Susceptibility test of adult **Aedes albopictus** *to DDT, bendiocarb, permethrin, deltamethrin and malathion*

A total of 133 adult *Ae. albopictus* mosquitoes were exposed to 4 % DDT, 166 mosquitoes to 0.1 % bendiocarb, 105 mosquitoes to 0.75 % permethrin, 108 mosquitoes to 0.05 % deltamethrin and 92 mosquitoes to 5 % malathion (Appendix 8). Furthermore, during the insecticide exposures, a total of 60 adult *Ae. albopictus* were exposed to each of the recommended control papers. As less than 10% of the control mosquitoes died during each exposure, the Abbott's formula was not used. Mortality after exposure to DDT was 99.1 % and for bendiocarb 98.8 %. For the remaining insecticides permethrin, deltamethrin and malathion mortality was 100 %. The wild caught female *Ae. albopictus* collected in the study were susceptible to all insecticides tested.

4.4.5 Environmental measurements

The mean collective temperature recorded in all habitats during the rainy season, for day and night combined, was 25.4℃ (range 15.3-39.9℃) with 84.2 % RH (range 19.0-100 %) and in the dry season was 23.2℃ (range 8.8-41.9℃) with 75.8 % RH (range 20.3-100 %). The temperature and humidity was similar between the four habitats investigated, with temperature only slightly lower in the secondary forests than the other habitats during the rainy season (Table 4.5).

Table 4.5 Mean temperature and relative humidity during the rainy and dry season in the four different habitats

		Temperature (°C)			Relative humidity (%)		
Season	**Habitat**	**Mean (95% CI)**	**OR (95% CI)**	***P***	**Mean (95% CI)**	**OR (95% CI)**	***P***
Rainy (May-Oct.)	**immature rubber plantation**	25.0 (24.4-25.7)	1.04 (1.02-1.06)	<0.001*	83.7 (80.0-87.4)	1.26 (0.92-1.72)	0.151
	mature rubber plantation	25.0 (24.2-25.7)	1.03 (1.00-1.06)	<0.001*	81.5 (74.2-88.8)	1.07 (0.70-1.64)	0.742
	village	26.1 (25.3-26.9)	1.15 (1.12-1.18)	<0.001*	82.7 (80.1-85.3)	1.20 (0.87-1.65)	0.264
	secondary forest	24.7 (24.0-25.4)	1		79.9 (73.4-86.5)	1	
Dry (Nov.-April)	**immature rubber plantation**	23.3 (21.5-25.1)	1.03 (0.98-1.07)	0.260	75.8 (68.0-83.5)	1.01 (0.92-1.12)	0.819
	mature rubber plantation	22.9 (21.3-24.5)	0.99 (0.95-1.03)	0.628	77.0 (69.3-84.7)	1.09 (0.97-1.21)	0.139
	village	23.5 (21.2-25.8)	1.04 (0.98-1.11)	0.208	74.0 (65.6-80.4)	0.92 (0.81-1.04)	0.166
	secondary forest	23.1 (20.9-25.2)	1		75.7 (66.3-85.1)	1	

*Results are shown using generalized estimating equations with odds ratio (OR) and 95 % confidence interval (CI). *significantly different,* P*<0.05*

The physical structure differed between the natural and man-made forests with undergrowth density and canopy cover higher in the secondary forests than in the immature rubber plantations and mature rubber plantations (Table 4.6). The tree density was similar for the secondary forests and immature rubber plantation and 1.24 times lower in the secondary forests than in the mature rubber plantations. Additionally, the tree height and tree circumference was lower in the secondary forest than in the immature rubber plantations and mature rubber plantations (Table 4.6).

Table 4.6 Difference in physical forest structure of the secondary forest compared to the immature rubber plantations and mature rubber plantations

Environmental factors	Habitat	Mean	(95% CI)	OR	(95% CI)	*P*
Undergrowth (% covered by undergrowth)	**immature rubber plantation**	12.1	(9.0-15.2)	0.63	(0.54-0.74)	<0.001*
	mature rubber plantation	4.5	(2.4-6.7)	0.40	(0.34-0.47)	<0.001*
	secondary forest	30.7	(25.5-35.9)	1		
Canopy (% covered by canopy)	**immature rubber plantation**	81.9	(76.3-87.5)	0.88	(0.83-0.94)	<0.001*
	mature rubber plantation	87.4	(85.2-89.5)	0.94	(0.88-1.00)	<0.001*
	secondary forest	93.0	(92.0-94.0)	1		
Tree density^ (no. of trees)	**immature rubber plantation**	6.1	(5.3-6.9)	1.17	(0.94-1.45)	0.158
	mature rubber plantation	6.4	(5.8-7.1)	1.24	(1.00-1.53)	0.048*
	secondary forest	5.2	(4.3-6.1)	1		
Height (m)	**immature rubber plantation**	11.6	(10.7-12.5)	1.05	(1.00-1.11)	0.033*
	mature rubber plantation	13.9	(13.3-14.6)	1.15	(1.10-1.21)	<0.001*
	secondary forest	10.8	(8.7-12.8)	1		
Circumference (cm)	**immature rubber plantation**	40.6	(37.3-44.0)	1.59	(1.33-1.89)	<0.001*
	mature rubber plantation	47.8	(45.7-49.9)	1.89	(1.58-2.26)	<0.001*
	secondary forest	25.8	(18.3-33.4)	1		

*Results are shown using generalized linear modelling with odds ratio (OR) and 95% confidence interval (CI). ^ all perennial trees, including rubber trees. *significantly different,* P*<0.05*

4.5 Discussion

This study described the abundance and diversity of adult mosquitoes, including vector species, in four typical rural habitats in northern Lao PDR. Species diversity was high in all habitats. Three of the four most common species found in the study habitats were vector species; the dengue and chikungunya vector *Ae. albopictus* [364, 365], the lymphatic filariasis vector *Ar. kesseli* [366] and the JE vector *Cx. vishnui* [148]. Additionally, in all habitats a daily exposure to malaria vectors, such as *An. maculatus s.l, An. dirus s.l., An. minimus s.l.* and *An. barbirostris s.l.* was found. Overall, the highest number of mosquitoes, including vector mosquitoes, were collected from the secondary forests.

This is the first study that documents the abundance, species richness and seasonality of mosquitoes in rubber plantations. In Lao PDR there have been few studies that report the mosquito fauna. To date 101 mosquito species have been recorded, including 41 *Anopheles* species [96, 97, 110, 205, 217, 340, 344-347]. This is in marked contrast with neighbouring Thailand where more than 300 different mosquito species have been identified, including at least 73 *Anopheles* species [314, 348]. The present study adds a further 61 species to the species list for mosquitoes in Lao PDR with the true mosquito diversity in the habitats investigated possibly greater than this study suggests, since the HDN may underestimate zoophilic mosquitoes [350]. The entomological studies that have been conducted in Lao PDR focus mainly on malaria vectors [96, 97, 102, 107, 108, 110, 111, 346] with few studies on dengue and JE vectors [135, 136, 217, 340, 367]. However, this emphasis is slowly shifting. A recent publication in Lao PDR underlined the importance of non-malarial vector-borne diseases present in Lao PDR including dengue and JE virus infections [172]. More research on the mosquito abundance and species richness in Lao PDR is necessary, with limited information on the mosquito dynamics in a country where mosquito-borne diseases are a major public health threat.

The important dengue, chikungunya and zika vector *Ae. albopictus* was the dominant mosquito species in the natural and man-made forests. It is not surprising to find high numbers of *Ae. albopictus* in the forests of northern Laos since it is a forest mosquito that originated from tropical forest areas in SEA [365, 368] and prefers shaded areas [214, 369, 370]. Similar studies in other parts of SEA also found *Ae. albopictus* to be the dominant species in forests and rubber plantations [184, 224, 228, 371]. For example, one study from Cambodia found that 98.2 % of mosquitoes collected in the forest were *Ae. albopictus* [371]. In a Malaysian study, 96 % of the adult and larvae mosquitoes found in a rubber plantation were *Ae. albopictus* [184]. This dominance of *Ae. albopictus* underlines the need to control exposure to dengue vectors in these habitat. Reducing exposure to *Ae. albopictus* in rubber plantations requires a combination of protection methods, including larval source reduction and personal protection methods. Detailed studies are needed in dengue endemic areas to identify the main breeding sites of *Ae. albopictus* in rubber plantations before larval source reduction can be successful [269]. Further research is also needed to identify the best outdoor personal protection method, with currently limited field studies on the use of permethrin treated clothing, transfluthrin emitting devices and mosquito coils in a portable cage [341].

In the villages a low number of *Ae. albopictus* were collected. Although not surprising that numbers were lower than in the forests, numbers were unexpectedly low. In a similar study in Thailand, a similar number of *Ae. albopictus* were collected in the villages as in the fragmented forests [348]. Other studies also show the preference of the mosquito species for peri-urban and village habitats compared to the forest habitats [106, 348]. This low number of *Ae. albopictus* collected in the village habitats could be related to the dengue outbreak in 2013-2014 in the study area. According to the Center of Malaria, Parasitology and Entomology (CMPE) of Lao PDR, the government operating procedures states that Indoor Residual Spraying (IRS) and larvicides are used in the villages where mosquito-borne disease outbreaks occur. Although official information on the use of vector control methods in the study area was unavailable, villagers were contradicting each other on the use of IRS and I did not find any residues of spraying on the wall nor large larvicide presence (chapter 6). The possible implementation of vector control in the villages during the dengue outbreak could have resulted in very low number of *Ae. albopictus* in the villages compared to the natural and man-made forest habitats. Additional adult mosquito surveys in the rural villages will give more insight into the dynamics of the important dengue vector in rural villages of northern Lao PDR.

In this study *Culex* mosquitoes, primarily the zoophilic *Cx. vishnui* mosquito, increased in numbers in the forest and rubber plantation habitats in the middle of the dry season after a period of little rain. This seems to be contradicting the general assumption that mosquito numbers increase after rain. This increase in numbers in the forest habitats is possibly related to their main breeding site rice fields [148, 372-374], which were closely situated to the villages, disappearing during the dry season. The *Culex* mosquitoes were forced to find other suitable breeding sites during the dry season. The mosquitoes might have moved to the neighbouring forest and rubber plantation habitats where waterbodies are present for a longer period of time, due to the canopy cover and undergrowth delaying desiccation. The broad waterbody preference of *Culex* mosquito, including large and sunlit waterbodies, increases the likelihood of finding suitable breeding sites. Furthermore, the number of predatory and competitive invertebrates possibly decreases during the dry season. This results in less competition for *Culex* mosquitoes and could lead to a proliferation of *Culex* mosquitoes in the dry season. Further larval investigation is needed to understand the true dynamics of the *Culex* mosquitoes in the study sites.

Few malaria vectors were collected in our study compared to other vector species, yet numbers did result in daily exposure. Exposure to vector mosquitoes does not directly relate to disease incidence, with a low density of *An. dirus s.l.* known to cause high malaria transmission [53, 197]. The most important malaria vectors collected in our study area were *An. maculatus s.s* and *An. minimus s.s.*, which is in accordance with the information provided by the CMPE of Lao PDR. In the rubber plantations the putative malaria vectors *An. maculatus s.s., An. minimus s.s, An dirus s.l., An barbirostris, An. umbrosus s.l.* and *An. jeyporiensis* were identified. All species except *An. jeyporiensis* have been identified in rubber plantations before [196-200]. The dynamics of malaria vectors was different between the habitats investigated, with the heliophobic mosquito *An. minimus s.s*. dominant in the villages, the more heliophilic *An. maculatus s.s.* dominant in the rubber plantations and the heliophilic swamp breeder *An. barbirostris s.l*. dominant in the secondary forests [199, 370]. Interestingly, hardly any *An. dirus s.l.* were collected in the secondary forests, even though these primary malaria vectors are often found in SEA forests [109, 375-377]. This contradiction between the behavioural preferences of mosquito species described in the literature and where they are found in practice emphasizes the heterogeneous behaviour of malaria vectors in SEA [378-380].

In the secondary forests the highest number of vector mosquitoes were collected, including vectors of dengue, JE and lymphatic filariasis. This result emphasizes the likely high risk of exposure to vector-borne diseases, when working and living in the secondary forest habitats. It is important to note, that in the collected mosquitoes the presence of pathogens were not tested for. Therefore the exact risk of exposure to vector-borne diseases is hard to identify. In some areas men have a higher risk of contracting malaria than women, due to their higher frequency of visiting the forests to collect wood and hunt at night [381-383]. This occupational hazard related to outdoor work is also identified for other vector-borne diseases, such as lyme disease, dengue and West Nile fever [384-387]. It is therefore essential to relate the entomological data we collected with the local sociological data. By identifying the groups that visit the forests most frequently, we can identify the people most at risk of dengue, JE and lymphatic filariasis in our study area. Control measures can then be focussed on these people most at risk.

This study has highlighted the rich and heterogeneous mosquito dynamics in SEA, as has been emphasized before [378, 379]. This richness of species is not unique for mosquitoes with the biodiversity of mammals, birds and plants in northern Lao PDR described as one of the richest in the world [88-91]. A higher species diversity was found in the secondary forests and villages compared to the rubber plantations, albeit this difference was very small. This variance found for species diversity could be related to the impact of habitat diversity on mosquito species diversity [348, 388, 389]. The rural village and fragmented forests have been described as ecotones in Thailand [348], which generally entails an elevated number of species as they include species from bordering ecological systems [390]. Furthermore, the secondary forests and villages could provide more varied breeding sites than the monoculture of rubber trees, resulting in sites suitable for a higher diversity of mosquito species.

The susceptibility tests that were conducted for adult *Ae. albopictus* were a first step towards understanding the insecticide resistance status in the study area. For this experiment, I used WHO discriminating concentrations recommended for *Anopheles* susceptibility tests. Future susceptibility tests for *Ae. albopictus* should be conducted using the appropriate concentrations for *Ae. albopictus* and *Ae. aegypti* exposures [391]. This would include the exposure to 4 % DDT for half an hour and exposure to a three times lower concentration of permethrin (0.25 %) for one hour. Furthermore, future susceptibility tests should be done in a room where temperatures are more stable and maximum temperatures do not exceed 30 degrees.

Evidence on the impact of land use change on mosquito dynamics and therefore impact on vector-borne disease risk is growing, yet knowledge on relations between the abundance and diversity of mosquito species, and the different habitats are limited [377, 389, 392, 393]. Finding a relationship between vector presence and habitat types is challenging, with often a mismatch between the human perceptions of structural habitat units, such as land cover types, and the functional habitat units for the vector mosquitoes [394]. The areas that are grouped as the same land cover type may have different functional resources for the mosquito. Therefore, more specific characterization of the different environments are necessary. In this study, the secondary forests were relatively young forests with high undergrowth and canopy cover, yet with a lower density and smaller trees than the rubber plantations. Compared to the secondary forests the immature rubber plantations had intermediate undergrowth with lower canopy cover and slightly bigger trees. The mature rubber plantations consisted of a high density of big trees with similar canopy cover as the immature rubber plantations and little undergrowth. The low undergrowth in the mature rubber plantations is expected, as rubber workers regularly clear the undergrowth in the tapped plantation area to enable access to the rubber trees. Higher canopy cover nor higher undergrowth was related to lower temperatures or higher humidity. Higher mosquito numbers were found in the secondary forests and immature rubber plantations compared to the mature rubber plantations and villages. High number of mosquitoes collected could be related to high undergrowth density, with both the secondary forests and immature rubber plantations showing a higher mosquito abundance and higher undergrowth density than the mature rubber plantations. The undergrowth may increase mosquito survival rate by providing shelter from predators and providing flowers for sugar. Furthermore, the leaves of the undergrowth might provide more suitable larval habitats for *Aedes* and *Culex* mosquitoes than areas with low percentage of undergrowth cover. Although more studies are necessary, high mosquito abundance may be linked to high undergrowth density and cutting undergrowth might be a potential vector control method.

4.6 Conclusion

There is risk of exposure to vectors of dengue, JE, lymphatic filariasis and malaria in all habitats investigated. Highest risk of vector exposure was in the secondary forests where dengue, JE and lymphatic filariasis vector mosquito numbers were highest. To protect the population from disease vector exposure, focus should be on the identification of the main vector larval breeding sites and on improving our knowledge on the protectiveness of personal protection methods. More importantly, social studies are essential. These human behaviour analyses will clarify in which habitats people spent time. This information related to the entomological data will show in which habitats risk of vector mosquito exposure is highest during the day and night. Additional studies on the mosquito dynamics in the forests, rubber plantations and villages in the south of the country, where malaria is endemic, might help to further understand the exact contribution of the different habitats to mosquito diseases and advance vector control. To identify relations between the abundance and diversity of mosquito species and the different habitats, it will be of importance to properly characterize the different habitats using functional habitat units related to mosquito presence.

5 Risk of vector-borne disease exposure for rubber workers compared to villagers in northern Lao PDR

Villagers and rubber workers participating in the Rapid Rural Appraisals in Silalek village

5.1 Abstract

The risk of vector-borne infections is dependent on the interaction between the susceptible human population and vector population. The objective was to explore how differences in human behaviour between rural villagers and rubber workers affected their risk of exposure to dengue, Japanese encephalitis (JE) and malaria vectors.

Mosquitoes were collected using human-baited double net (HDN) traps in three study sites, each consisting of four habitats (secondary forests, immature rubber plantations, mature rubber plantations and villages) in northern Lao PDR. Information on the daily activity of the local population was collected using rapid rural appraisals and surveys. Molecular identification of alphavirus and flavivirus presence in *Aedes albopictus* were conducted. Risk of mosquito-borne disease exposure was assessed by calculating the basic reproductive number (R_0) in the different habitats and comparing risk of exposure to vectors for local villagers and rubber workers.

The dengue vector *Ae. albopictus* (n = 6,302), the JE vector *Culex vishnui* (n = 3,562) and nine different malaria vector species (n = 655) including *Anopheles maculatus s.l.*, *Anopheles minimus s.l.* and *Anopheles dirus s.l.* were included in the risk analysis. *Aedes albopictus* collected in the natural and man-made forests contained pan-flavivirus sequences. The dengue basic reproductive number (R_0) was larger than 2.8 for all habitats except villages where $R_0 \leq 0.06$. For all habitats the main malaria vector in the rainy season was *An. maculatus s.l.* with $R_0 \geq 16.6$ and in the dry season *An. minimus s.l.* with $R_0 \geq 18.1$. Compared to villagers staying in the village, risk of dengue vector exposure was higher for villagers visiting the secondary forest during the day (odds ratio (OR) 35.99, 95 % confidence interval (CI) 24.61-52.62), rubber workers living in the villages (OR 3.22, 95 % CI 2.32-4.48) and rubber workers living in the plantations (OR 16.22, 95 % CI 11.51-22.86). Japanese encephalitis vector exposure and malaria vector exposure were also higher for villagers visiting the forest (OR_{JE}

1.38, 95 % CI 1.15-1.65; $OR_{malaria}$ 1.29, 95 % CI 1.15-1.44). Malaria vector exposure was lower for rubber workers that live in the rubber plantations (OR 0.63, 95 % CI 0.41-0.97) than for villagers staying in the village.

The present study suggests that visiting the forests during the day increases risk of dengue, JE and malaria vector exposure. Working in the rubber plantations also increases risk of dengue vector exposure, which is worsened when also living in these man-made forests. Working and living in the rubber plantations did reduce risk of exposure to malaria vectors. This study highlights the importance of implementing mosquito control in the secondary forests and rubber plantations with rubber workers at high risk of dengue vector exposure. Additionally, the population should be closely monitored for possible introduction of the malaria pathogen.

5.2 Introduction

Due to the rapid economic development in Asia, rubber has been in high demand. Natural rubber, obtained as latex from the rubber tree *Hevea brasiliensis*, provides 42 % of the global rubber demand [71, 395]. Outbreaks of mosquito-borne diseases, such as malaria, dengue and chikungunya are already occurring in rubber plantations of South-East Asia (SEA) [195, 198, 223, 232]. In the next decade an estimated 4.5 to six million workers will be necessary in the rubber plantations of SEA during the rainy season to tap the available mature rubber trees. This will result in a high demand for seasonal labour, which may aid the spread and increase the incidence of mosquito-borne disease in the region [341].

In Lao PDR the mature rubber plantation area increased from 900 ha in 2010 to 147,500 ha in 2015 [70]. Although rubber cultivation is expected to decrease with the slowdown in the Chinese economy, an estimated 342,400 ha of mature rubber plantations will still be tapped in Lao PDR in the next decade, employing over 100,000 people [70]. As the rubber workers are working and often living in the plantations, they will be exposed to the vector mosquitoes present in the rubber plantations. It has been suggested that rubber worker are at higher risk of malaria due to their nightly tapping activity [198, 341].

To achieve successful intervention of vector-borne diseases, an understanding of the transmission dynamics is essential. Identifying and comprehending the heterogeneous patterns of contact between pathogens, vectors and the susceptible population, results in more effective control [396-401]. Several studies have focussed on human behavioural elements that increase risk of vector-borne diseases. For example, in some areas it has been found that the number of hours spent working outdoors increases risk of lyme disease, West Nile fever and malaria [381-383, 385-387]. The identification of this risky behaviour allowed for the focus of disease prevention on groups within the population, such as certain occupations. Employers of these risky occupations can be actively involved in the protection of the workers [387]. Another example is the current focus of malaria prevention around the Thai borders on the migrant workers [117]. Human behaviour and the impact of this movement on the exposure to vector mosquitoes, and consequently exposure to the pathogens, is still poorly understood [397]. Fortunately, studies on the relations between human behaviour data, epidemiological data and entomological data are becoming more common. These interdisciplinary studies are essential to decrease disease incidence, especially in countries where small hotspots of disease transmission remains.

The objective of this study was to explore how differences in human behaviour between rural villagers and rubber workers affected their risk of exposure to dengue, Japanese encephalitis (JE) and malaria vectors in northern Lao PDR. The basic reproductive number (R_0) was calculated based on the Ross-Macdonald model [402]. The R_0 is an estimate of the number of new cases derived from one infective case before the patient dies or is cured [403, 404]. Values greater than one suggest that new infections could occur in an area if introduced, and values less than one indicate that new infections would not occur. Furthermore data on the behaviour of villagers and rubber workers was collected. This sociological data was related to mosquito behaviour using different human behaviour scenarios. The rubber workers tap latex at night and collect the coagulated latex during the day. I therefore hypothesized that rubber workers were at higher risk of exposure to dengue, JE and malaria vectors than the villagers.

5.3 Materials and Methods

5.3.1 Study sites

The study was conducted in the three study sites: Thinkeo (19°41’02.13”N 102°07’05.49”E), Silalek (19°37’02.80”N 102°03’05.70”E) and Houayhoy (19°33’03.22”N 101°59’42.42”E) in a hilly area of Xieng-Ngeun and Nane district, Luang Prabang province in northern Lao PDR. In each study site, four habitats were identified as adult mosquito collection sites: secondary forests, immature rubber plantations, mature rubber plantations and villages (description in chapter 4). The secondary forests were young forests consisting of young small trees with a high density of undergrowth. The immature rubber plantations were comprised of young rubber trees (<5 years) which had not been tapped for latex, and mature rubber plantations were those where >70 % of the trees were tapped for latex. The rural villages consisted of about 150 to 200 bamboo and brick houses. In the villages people from Lao loum, Khamou and Hmong ethnic groups were present. There is a single rainy season in the study area from May to October, when vector-borne disease cases are highest. The most common vector-borne disease in the area was dengue with no information available on the incidence of Japanese Encephalitis (JE). Malaria is not endemic in the area, but several vector species, including *Anopheles maculatus s.l.*, *Anopheles minimus s.l.* and *Anopheles dirus s.l.* are present (chapter 4).

5.3.2 Mosquito sampling method

Routine entomological measurements were made monthly for nine months from July to November 2013 and in February, March, May and July 2014 (study described in chapter 4).

The mosquito population was sampled using the human-baited double bed net (HDN) trap. This trap consists of a person surrounded by two nets: the internal net fully protects the occupant from mosquito bites, whilst the outer net is raised off the ground and traps mosquitoes coming to feed. Mosquitoes are collected from between the nets at hourly intervals during the day and night. A total of 36 HDN traps were used, 12 in each study site i.e. three HDN traps in each of the four different habitats. Mosquitoes were morphologically identified to species or species complex using stereo-microscopes and recognized keys of Thailand [314].

5.3.3 Molecular identification of presence or absence of virus in **Aedes albopictus**

The arboviral team of Institut Pasteur du Laos screened all *Aedes albopictus* collected during the entomological survey (chapter 4) for presence of alphaviruses and flaviviruses sequences. Alphaviruses are from the *Togaviridae* family and include diseases such as sindbis and chikungunya. Flaviviruses are from the *Flaviviridae* family and include diseases such as yellow fever, dengue, west Nile and JE. No malaria vectors were analysed for the presence of plasmodia parasites since the disease was not locally transmitted. The abdomen, wings and legs of all collected *Ae. albopictus* samples were pooled in tubes, with a maximum of 10 samples per tube. Pooling was done to keep costs of molecular analysis low. The head and thorax of the mosquitoes were stored individually for future reference. If pools were positive for viruses, the individual samples of head and thorax were tested for viral presence. The pools were separated for male/female, different habitats and month of collection. The blood fed female mosquitoes were analysed individually, as the blood containing abdomen could not be used for future analysis. RNA was extracted using the NucleoSpin^{R} 8 Virus (Ref: 740 643.5) extraction kit. The RNA samples were amplified using specific primers with RT-PCR and screened for the alphavirus (195 base pair) and flavivirus (143 base pair) genome sequence using agarose gel electrophoresis with the positive controls dengue, West Nile and JE virus for

Pan-flavi identification and chikungunya, Metri and Sindbis virus for Pan-alpha identification [405, 406].

5.3.4 Mosquito survival

Mosquito survival was assessed in July and August 2015 in the secondary forest, immature rubber plantation, mature rubber plantation and village of Thinkeo study site. Two HDN traps were deployed in each habitat from 17.00 h - 06.00 h. All possible *Anopheles* malaria vectors and *Ae. albopictus* were dissected using the Detinova method of parity [407]. The ovaries of the female mosquitoes were identified to be nulliparous, not laid eggs before, or parous, laid eggs before. Nulliparous mosquitoes are identified by the presence of coiled tracheole skeins in the ovary, while uncoiled skeins indicate a parous mosquito. The percentage of parous mosquitoes was used for mosquito survival. A high parity rate (>80 %) would indicate the mosquito population is long-lived, as a high percentage of the collected mosquitoes have laid eggs before (and therefore have blood fed before).

5.3.5 Basic reproductive number for mosquito-borne infections

The R_0 was calculated using several parameters to determine the R_0 of dengue and malaria in the secondary forests, immature rubber plantations, mature rubber plantations and villages during both the rainy season (May-September) and dry season (October-April).

5.3.5.1 Basic reproductive number for dengue

The R_0 of dengue was calculated for *Ae. albopictus,* the only dengue vector collected in the study area in numbers larger than n = 2. The following formula was used (1) with the description of parameters in Table 5.1 [408].

$$Dengue\ R_0 = \frac{a}{r} m a^2 e^{-\mu n} \frac{bd}{\mu} \qquad (1)$$

Table 5.1 Description of the parameters used for the dengue basic reproductive number model

	Description	**Formula/calculation**
a	Frequency of the vector mosquito feeding on a person/day	a = C/x
C	Proportion of mosquitoes fed on human blood instead of other animals	0.99 [409]
x	Gonotrophic cycle length, measured by the interval between blood meals taken	Conservative estimate of 4.5 days [410]. Multiple feeding is not taken into account
r	Rate of human recovery (1/number of days)	Four to five days [332, 408, 411, 412] So, 1/4.5
ma	Number of mosquito bites per person/day	Average number of mosquitoes collected per person/day
µ	Mortality rate of female mosquitoes	1- *p*
p	Daily survival probability of adult mosquitoes	$A^{1/X}$
A	Average proportion of parous mosquitoes	Proportion parous from the mosquito survival study
n	Development days of virus in mosquito	Using graph [413] with Average T_{dry} in study area= 23.2 Average T_{rain} in study area = 23.3
b	Proportion of female mosquitoes infective after taking infective blood meal	0.4 [408, 414]
d	Transmission from human to mosquito	0.4 [408, 414]

5.3.5.2 Basic reproductive number for malaria

The R_0 of malaria was calculated for both *Plasmodium falciparum* and *Plasmodium vivax* malaria infections. The R_0 for both malaria strains was calculated as 73 % of all confirmed malaria infections in Lao PDR are *P. falciparum* [98], yet the last malaria outbreak recorded close to the study area was caused by the parasite *P. vivax*. The R_0 was calculated for the malaria vectors *An. maculatus s.l., An. minimus s.l. and An. dirus s.l* separately, using their individual mosquito survival data and if possible using different parameters (Table 5.2). The following formulae was used (2) [415, 416].

$$Malaria\ R_0 = \frac{ma^2bp^n}{-\ln(p)r} \qquad (2)$$

Table 5.2 Description of the parameters used for the malaria basic reproductive number model

	Description	**Formula and calculation**
ma	Number of mosquito bites per person/day	Average number of mosquitoes collected per person/day
a	Frequency of the vector mosquito feeding on a person/day	a = C/x
C	Proportion of mosquitoes fed on human blood instead of other animals	1/3 proportion fed on human for *An. maculatus s.l. and An. minimus s.l.* [417] 2/3 proportion fed on human for *An. dirus s.l.*
x	Gonotrophic cycle length, measured by the interval between blood meals taken	2.35 days for *An. maculatus* [417, 418] Two days for rainy season and three days for dry season for *An. minimus s.l.*[419] Three days for *An. dirus s.l.* [108, 420]
b	Proportion of female mosquitoes developing parasites after taking infective blood meal	Dependent on genetic and non-genetic determinants [421, 422], conservative estimate of 0.5 for all [423]
p	Daily survival probability of adult mosquitoes	$A^{1/X}$
A	Average proportion of parous mosquitoes	Proportion parous from the mosquito survival study
n	Development days of parasite in mosquito (sporogonic cycle) using Moshkovsky's method	For *P. falciparum* the thermal sum required to complete parasite development is 111℃ above 16℃. For *P. vivax* the thermal sum required to complete parasite development is 105℃ above 14.5℃ [424] Average T_{dry} in study area= 23.2 Average T_{rain} in study area = 23.3
r	Rate of human recovery (1/number of days)	60 days, so 1/60 [425, 426]

5.3.6 Rapid Rural Appraisals

Daily and monthly activities of the rubber workers and villagers were described qualitatively using Rapid Rural Appraisals (RRA) in each of the three study sites in November 2013 [427]. Together with the village heads the setting of the meetings were chosen. All villagers and rubber workers from the study area were invited to participate in the RRA with a local translator present to facilitate the meeting. The villagers were asked to draw a map of their own village area with clear identification of the different habitats present. Using this map, among other details, the areas where mosquito nuisance is high, where mosquito larvae have been seen and where large water containers are present, were identified. This information was used for the larval surveys (chapter 6). The participants, according to their experience, were also asked to fill in two timetables together. The timetables recorded the monthly and/or hourly intensity from one to five (one: very low, five: very high) for: rainfall, temperature, mosquito numbers, villagers feeling unwell and the number of villagers migrating. Additionally, the intensity of the secondary forest visits, latex tapping, collecting latex and rice production (Seedling, Growth, Harvest phases) were recorded (method of RRA in Appendix 9). Although activities in many different habitats were described, including visits to farms, schools and cities, I simplified the behaviour to assume that when participants were not active in the secondary forests, rubber plantations or rice fields they were present in the villages.

5.3.7 Social survey on the daily activities of the local population

A survey was carried out in the three study villages in June 2015 to collect information on the daily activities of the local population the day before the survey. Furthermore, information was collected on their visits to the rubber plantations and the methods used to protect themselves from mosquito bites when outdoors (survey questionnaire in Appendix 9). The survey was anonymous with no sensitive information collected. The survey was conducted by a medical doctor fluent in the Lao. For realistic representation of the different villages, 54 people per village were surveyed (power $\omega = 0.8$, $\alpha=0.05$ and size effect of 0.5) [313]. This entails that 54 people per village need to be surveyed for an 80 % probability that the test correctly rejects the null hypothesis. The villages were visited twice, once in the morning and once in the afternoon for the data to be representative. Participants were selected in every third house from the beginning of the village. In each selected house the participant was chosen by myself using four sticks of different sizes. The shortest stick represented a small child, the second shortest a teenager, the third shortest a parent and the longest a grandparent. If no one fitted the age category the next house was selected until someone in the right age category was found. For small children that were not able to answer the survey, a parent or grandparent was involved.

Each participant had the right to refuse to answer questions. All participants were given a bar of soap after their participation in the survey.

5.3.8 Human behavioural patterns

The exposure risk to the dengue vector *Ae. albopictus*, JE vector *Culex vishnui* and malaria vectors was assessed using several behavioural scenarios. Three behavioural scenarios were selected, using the outcomes of the RRA's and social surveys, based on the behaviour of both villagers and rubber plantation workers in the rubber plantations and secondary forests. To identify risky behaviour, these scenarios were compared in their risk of vector exposure to the villagers staying in the village.

5.3.9 Data analysis

Mosquito sampling results from chapter 4 were averaged to describe the daily activity of dengue, JE and malaria vectors in the different habitats. R_0 were calculated and compared for the different habitats. The three RRA's were summarised by taking the mean intensity of activities from the three appraisals. All means were rounded up. The social survey results were described quantitatively, using the percentage of respondents. The behavioural patterns of rubber plantation workers and villagers were separated into four scenario's, for which risk of exposure to dengue, JE and malaria vectors was compared.

5.3.10 Ethics

The RRA's and surveys were approved by the Ministry of Health, Lao PDR and the provincial health department of Luang Prabang.

5.4 Results

5.4.1 Mosquito sampling

During the adult mosquito survey 24,927 female mosquitoes were collected. A total of 8,585 *Aedes* mosquitoes were collected in the different habitats of which 6,302 were *Ae. albopictus*.

Furthermore, a total of 5,022 *Culex* mosquitoes were collected of which 3,562 were *Cx. vishnui*. A total of 1,341 *Anopheles* mosquito species were collected, of which 655 were malaria vectors. The most common malaria vectors were *An. maculatus s.l.* (n = 294), *An. barbirostris s.l.* (n = 170), *An. minimus s.l.* (n =151 samples) and *An. dirus s.l.* (n = 46) (detailed description in chapter 4).

A high density of *Aedes albopictus* mosquitoes were collected from 06.00 h, which increased throughout the day with a peak at 18.00 h (Figure 5.1). Similar collection trends were found in the secondary forests, immature rubber plantations and mature rubber plantations. In these natural and man-made forest habitats *Ae. albopictus* mosquitoes were also collected in low numbers during the night. In the villages *Ae. albopictus* activity was low throughout the day and night. *Culex vishnui* showed very different behaviour compared to *Ae. albopictus* with peak activity in the evening from 18.00 h to 20.00 h for all habitats and low activity throughout the rest of the night until 06.00 h. After 06.00 h almost no *Cx. vishnui* were collected during the day until 18.00 h (Figure 5.1). Malaria vectors were collected in low numbers throughout the day and night. In the secondary forests the mosquito activity was highest from 06.00 h to 18.00 h with *An. barbirostris s.l.* mostly collected during the day and *An. maculatus s.l.* collected during the evening (Figure 5.1). In the immature rubber plantations malaria vectors were generally collected from 18.00 h to 05.00 h with a small increase from 18.00 to 20.00 h due to the increased activity of *An. maculatus s.l.* In the mature rubber plantations malaria vectors were collected most frequently between 18.00 to 21.00 h with high presence of *An. maculatus s.l.* In the villages malaria vectors were mostly collected from 18.00 to 20.00 h with a low number continued to be collected until 05.00 h. More than half of the 46 *An. dirus s.l.* collected during this study were caught in the immature rubber plantations (67 %, 31/46). About 20 % of *An. dirus s.l.* were collected in the immature rubber plantations (9/46). The remaining samples were collected in the forests (5/46). One sample of *An. dirus s.l.* was collected in the villages. The *An. dirus s.l.* mosquito samples collected in the different habitats showed similar behaviour. About 67% of total *An. dirus s.l.* were collected between 18.00 and 22.00 h. (30/46), with the remaining samples collected between 01.00 and 05.00 h.

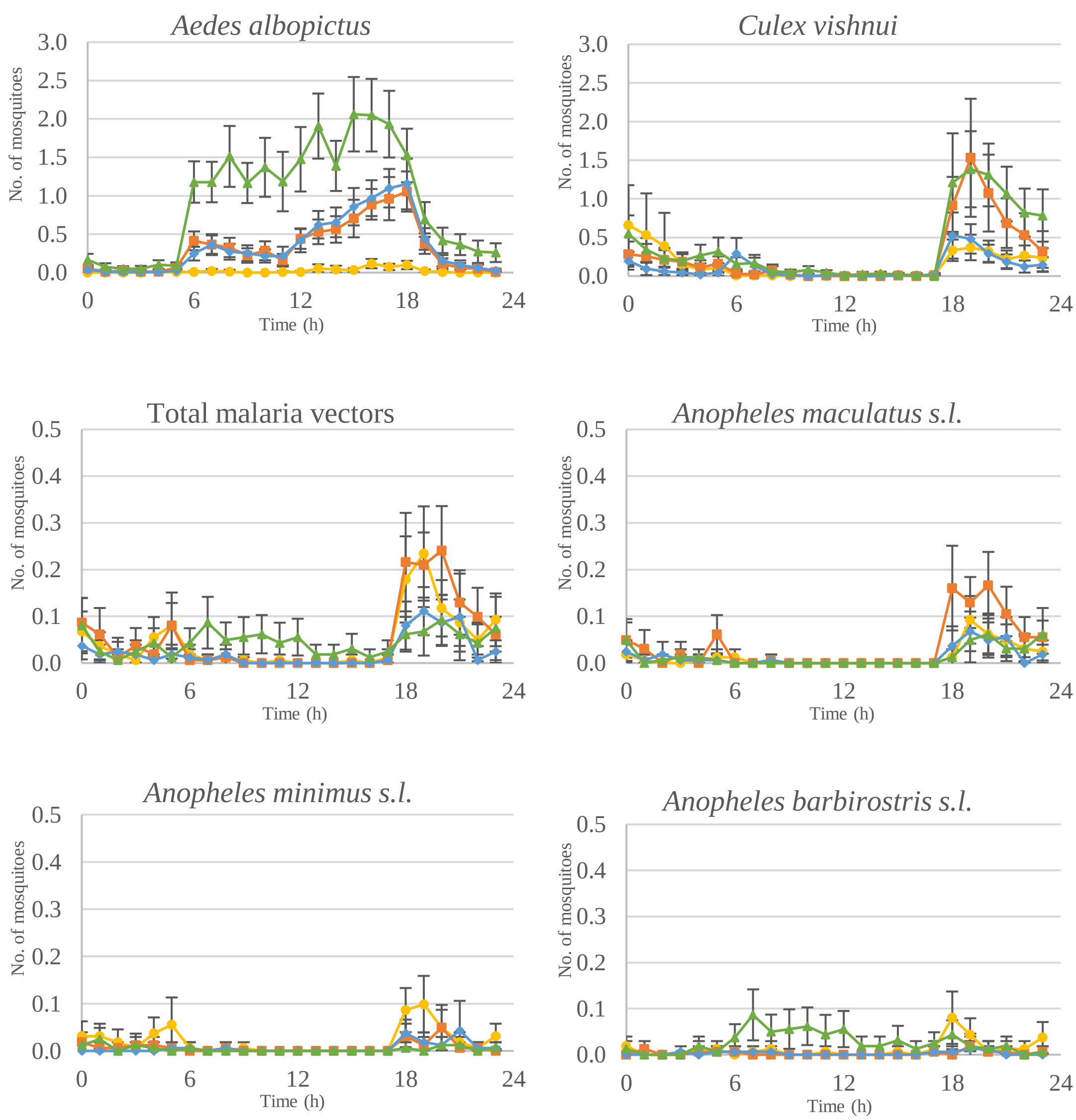

Figure 5.1 The average number of female mosquitoes collected per person/hour in the four different habitats (—▲— secondary forests, —■— immature plantations, —♦— mature plantations, —●— villages) for *Aedes albopictus*, *Culex vishnui*, *Anopheles* malaria vectors, *Anopheles maculatus s.l.*, *Anopheles minimus s.l.* and *Anopheles barbirostris s.l.* during 24 hrs. All including 95 % confidence interval

5.4.2 Molecular identification of presence or absence of virus in **Aedes albopictus**

A total of 7,191 *Ae. albopictus* mosquitoes (6,302 female, 889 male) were pooled in 1,252 tubes and tested for Pan-alphavirus and Pan-flavivirus sequences. None displayed amplicon of expected size for pan-alphaviruses. A total of 36 *Ae. albopictus* pools contained Pan-flaviviruses. Both male (6.8 %, 9/133) and female (2.4 %, 27/1,119) *Ae. albopictus* pools contained Pan-flaviviruses. No Pan-flaviviruses were found in the village pools (0/30), but 2.9 % of *Ae. albopictus* pools from the secondary forests had Pan-flaviviruses (20/690), 2.1 % from the immature rubber plantations (5/238) and 3.7 % from the mature rubber plantations (11/294).

5.4.3 Mosquito survival

A total of 1,197 dengue vector mosquitoes (*Ae. albopictus*) and 89 putative malaria vector mosquitoes (*Anopheles aitkenii* group, *An. dirus s.l., An. barbirostris s.l., Anopheles epiroticus, Anopheles hodgkini, An. maculatus s.l., An. minimus s.l., Anopheles tesselatus* and *Anopheles umbrosus s.l.*) were collected using the HDN trap. The ovaries were successfully dissected from 1,171 *Ae. albopictus* and 82 malaria vector mosquitoes for the identification of the parity status. A total of 26 *Ae. albopictus* and seven *Anopheles* malaria vectors were not successfully dissected. In general, the percentage of parous ovaries were high, with long living vector mosquitoes present in all habitats (Table 5.3). For the malaria R_0 calculations, parity rates of *An. maculatus s.l., An. minimus s.l.* and *An. dirus s.l.* were used separately. A total of 34 *An. maculatus s.l.* samples were collected, of which 31 were parous (91.2 %). A total of 18 *An. minimus s.l.* samples were collected, of which 17 were parous (94.4 %). A total of 14 *An. dirus s.l.* samples were collected, of which eight were parous (57.1 %).

Table 5.3 The mosquito survival, described as parity rate, for the dengue and putative malaria vector mosquitoes in the different habitats

Habitat	Vector species	Parity number	Parity rate (95 % CI)
Secondary forests	Dengue vectors	406/447	91 %
	Putative malaria vectors	13/14	93 %
Immature rubber plantations	Dengue vectors	234/269	87 %
	Putative malaria vectors	8/23	35 %
Mature rubber plantations	Dengue vectors	309/327	92 %
	Putative malaria vectors	5/10	50 %
Villages	Dengue vectors	3/5	60 %
	Putative malaria vectors	33/35	94 %
Total	Dengue vectors	953/1048	91 % (58 - 100)
	Putative malaria vectors	59/82	72 % (20 - 100)

Results are shown for the parity rate, which is the proportion of parous mosquitoes compared to the total number dissected. The dengue vectors were all Ae. albopictus *mosquitoes. The putative malaria vectors consisted of* An. aitkenii group, An. dirus s.l., An. barbirostris s.l., An. epiroticus, An. hodgkini, An. maculatus s.l., An. minimus s.l., An. tesselatus and An. umbrosus s.l. *mosquitoes. The 95 % confidence interval (CI) is given for the total number of parous mosquitoes.*

5.4.4 Basic reproductive number for mosquito-borne infections

5.4.4.1 Basic reproductive number for dengue

The R_0 for the dengue vector *Ae. albopictus* was calculated using the average number of *Ae. albopictus* bites per person per day in the different habitats (Appendix 10). The R_0 was considerably higher than one for all natural and man-made forest habitats during both the rainy season and dry season, and considerably lower than one for the villages (Table 5.4). The R_0 was highest in the secondary forests and second highest in the mature rubber plantations. Of the three forest habitats the R_0 was lowest in the immature rubber plantations.

Table 5.4 The basic reproductive number (R_0) for dengue vector *Ae. albopictus* in the secondary forest, immature rubber plantation, mature rubber plantation and village habitats during the rainy season and dry season

	Secondary forest	Immature rubber plantation	Mature rubber plantation	Village
Rainy season	42.0	9.5	18.8	0.06
Dry season	10.6	1.5	2.8	0.01

5.4.4.2 Basic reproductive number for malaria

The R_0 for malaria was calculated using the average number of bites per person per day (ma) for the different malaria vectors in each of the different habitats (Appendix 10). All habitats exhibited high malaria R_0 during both the rainy season and dry season, with similar outcomes for *P. falciparum* and *P. vivax* (Table 5.5). Both *An. maculatus s.l.* and *An. minimus s.l.* are important malaria vectors in the study sites whilst *An. dirus s.l.* is not.

Table 5.5 The basic reproductive number for *P. falciparum* and *P. vivax* malaria parasites calculated for the different vectors in the different habitats during the rainy season and dry season

	Malaria parasite	Malaria vector	secondary forest	immature rubber plantation	mature rubber plantation	village
Rainy season	***P. falciparum***	***An. maculatus s.l.***	28.6	64.0	16.6	28.6
		An. minimus s.l.	8.3	6.9	2.8	42.8
		An. dirus s.l.	0.2	0.5	0.1	0
	P. vivax	***An. maculatus s.l.***	31.2	69.8	18.1	31.2
		An. minimus s.l.	8.8	7.4	2.9	45.7
		A. dirus s.l.	0.3	0.7	0.2	0
Dry season	***P. falciparum***	***An. maculatus s.l.***	13.1	39.2	22.1	11.4
		An. minimus s.l.	18.1	41.6	36.1	84.9
		An. dirus s.l.	0.03	0.5	0.2	0.02
	P. vivax	***An. maculatus s.l.***	14.9	44.8	25.2	13.1
		An. minimus s.l.	19.3	44.3	38.5	90.6
		A. dirus s.l.	0.05	1.0	0.3	0.05

5.4.5 Rapid Rural Appraisals

Between 15 to 19 villagers participated in the two hour long RRA at each of the three study sites. Participants recognised that periods of high rainfall were associated with an increase in mosquito numbers (Table 5.6). Villagers felt unwell both during the rainy and dry season with a mention of dengue cases (Khai neung) when mosquito nuisance was high (Table 5.6). In the months December to February, when there were no farms to tend, some villagers travelled to other areas in Lao PDR and abroad to find work (Table 5.6).

From May to November Rice was cultivated and secondary forests were visited most frequently. Villagers generally visited the secondary forests from 05.00 h to 17.00 h to collect food and fire wood (Table 5.7). The forests are furthermore occasionally visited at night to hunt animals, like rodents and muntjacs. The high host-seeking activity of mosquitoes in the secondary forests overlapped with the human activity, resulting in high nuisance of mosquitoes. Rubber tapping occurred throughout the rainy season from May to October. Rubber trees are always tapped at night between 02.00 h to 07.00 h, when latex flow is highest. However, collecting of latex is more flexible and can occur at other times of the day and night.

Respondents indicated that there was a peak in mosquito activity in the villages from 18.00 h to 20.00 h which overlapped with the human activity in the villages (such as cooking, washing and relaxing), leading to nuisance of mosquitoes. No information was collected on mosquito and human activity in immature rubber plantations due to the low and irregular activity of villagers and rubber workers in these habitats.

In general the appraisals from the participants highlighted the importance of mosquito control both inside the villages and surrounding areas. Villagers experience vector-borne diseases and mentioned the nuisance from mosquitoes in the villages, natural and man-made forest habitats. As villagers travel to other areas in the region, introduction of new diseases to the area is possible and should be closely monitored. Villagers from the study areas had relatively good knowledge of mosquitoes and mosquito-borne diseases, with mosquito larvae easily identified in their surrounding habitats. However, these areas with mosquito larvae were not controlled as there seems to be a gap between knowledge and action.

Table 5.6 Summary of data obtained from the rapid rural appraisals on the monthly intensity of environmental variables and behavioural variables of villagers and rubber workers

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Rainfall					**	***	*****	*****	***	**	*	
Temperature			***	*****	*****	****	***	***	***	*		
Mosquito population		*	*	**	***	****	*****	*****	*****	****	***	**
Villagers feeling unwell	*						*	*	* (Dengue)	*	**	*
Migration	**	**										*
Villagers visit forests			***	***	***	****	*****	*****	****	****		
Rubber tapping								(End of April - Nov)				
Rice					Seedling			Growing		Harvest		

** Intensity symbol for the different variables according to the experience of the local villagers and rubber workers*

Table 5.7 Summary of data obtained from the rapid rural appraisals on the daily intensity of mosquito and human activity in different habitats

Time	Periods of mosquito activity			Periods of human activity				
	Secondary forests	Mature rubber plantations	villages	Secondary forests	Mature rubber plantations		Villages°	Rice fields
					Tapping	Latex collection		
07.00	****	***	*	High activity	High activity	High activity	Low activity	High activity
08.00	****	*	*	High activity		High activity	Low activity	High activity
09.00	****	*		High activity		High activity	Low activity	High activity
10.00	****	*		High activity		High activity	Low activity	High activity
11.00	****	*		High activity		Low activity	Low activity	High activity
12.00	****	*		High activity		Low activity	Low activity	High activity
13.00	****	*		High activity		Low activity	Low activity	High activity
14.00	****	*		High activity		Low activity	Low activity	High activity
15.00	****	*		High activity		Low activity	Low activity	High activity
16.00	****	*		High activity		Low activity	Low activity	High activity
17.00	****	**	*	High activity		Low activity	Low activity	High activity
18.00	*****	****	*****	Low activity		Low activity	High activity	
19.00	*****	***	*****	Low activity		Low activity	High activity	
20.00	*****	**	****	Low activity		Low activity	High activity	
21.00	**	**	***	Low activity		Low activity	High activity	
22.00	**	**	***	Low activity		Low activity	High activity	
23.00	**	**	**	Low activity		Low activity	High activity	
24.00	**	**	*	Low activity		Low activity	High activity	
01.00	**	**	*	Low activity		Low activity	High activity	
02.00	**	**	*	Low activity	High activity	Low activity	Low activity	
03.00	**	**	*	Low activity	High activity	Low activity	Low activity	
04.00	**	**	*	Low activity	High activity	Low activity	Low activity	
05.00	**	***	**	Low activity	High activity	Low activity	Low activity	
06.00	*****	****	****	Low activity	High activity	Low activity	Low activity	

*°The village behaviour is derived from when villagers are not in the secondary forests, rubber plantations and rice fields. * Intensity symbol for the different variables according to the experience of the local villagers and rubber workers from none to five*

5.4.6 Human activity survey

A total of 162 participants participated in the survey of which 8.6 % (14/162) were rubber workers. During the survey I found that 77 % (114/148) of the villagers of all ages visit the rubber plantation at least once every month (range in age one to 96 years). Most often villagers visit the rubber plantation to help with maintenance and cutting of grass, both in the mature and immature rubber plantations. Furthermore, they visit the rubber plantations when travelling to their farms, to collect food and to collect fire wood. About 91 % (147/162) of the villagers and rubber workers stayed in the village at night the day before the survey was conducted. The villagers generally slept from 20.00 h to 05.00 h. The remaining 6 % (10/16) slept on the farm and 3 % (5/162) worked in the rubber plantations. One person spent the whole night in the secondary forest. Usually the villagers between 14 and 55 years leave the village during the day from 07.00 h to 17.00 h with more than 40 % (65/162) of participants spending the day on the farm, more than 10 % (17/162) spending the day at school, 5 % (8/162) in rubber plantation, 3 % (5/162) in the forest and 3 % (4/162) in Luang Prabang city. About 39 % (63/162) of the villagers stayed in the village.

More than 90 % (148/162) of participants had insecticide-treated bed nets (ITNs) in their houses. However, the bed nets often contained holes and were too few to protect the whole family. A total of 34 % (55/162) of respondents used methods to protect themselves against mosquitoes when outdoors, with 60 % (33/55) of participants mentioning the use of mosquito coils and 35 % (19/55) mentioning the repellent N,N-Diethyl-meta-toluamide (DEET). About 7 % (4/55) of participants said they used long sleeves and 2 % (1/55) the use of lemongrass.

5.4.7 Human behavioural patterns

In the study areas there were two types of rubber plantation workers: local and migrant. Typically, local rubber workers owned a small two hectare plantation which they tap every two days during the night. They do not live in the rubber plantations, but sleep in the village. In contrast, migrant rubber workers work on large plantations (> 10ha) where they also sleep. I, therefore, used four behavioural scenarios to assess the risk from mosquito-borne diseases: (1) villager that stays in the village, (2) villager that visits the forest during the day from 05.00 h to 17.00 h (4) rubber worker that lives in the village, and (3) rubber worker that lives and works in the rubber plantations.

5.4.7.1 Villager that stays in the village

Villagers that stay in the village are only exposed to mosquitoes present in the village with highest vector exposure from 18.00 to 19.00 h (Figure 5.2). Exposure to *Ae. albopictus* mosquitoes in the village is low with only one mosquito exposure per person every two days (Table 5.8). The villager that stays in the village are exposed to more than three *Cx. vishnui* mosquitoes every 24 hrs (Table 5.8). When a bed net is used during the night from 20.00 to 05.00 h exposure to this JE vector can be decreased to less than one mosquito exposure every 24 hrs (Figure 5.2). Malaria vector exposure is approximately one mosquito exposure every 24 hrs in the village, with bed net usage probably halving this risk. Generally villagers that stay in the village are at risk of exposure to JE and malaria vectors, but not to dengue vectors.

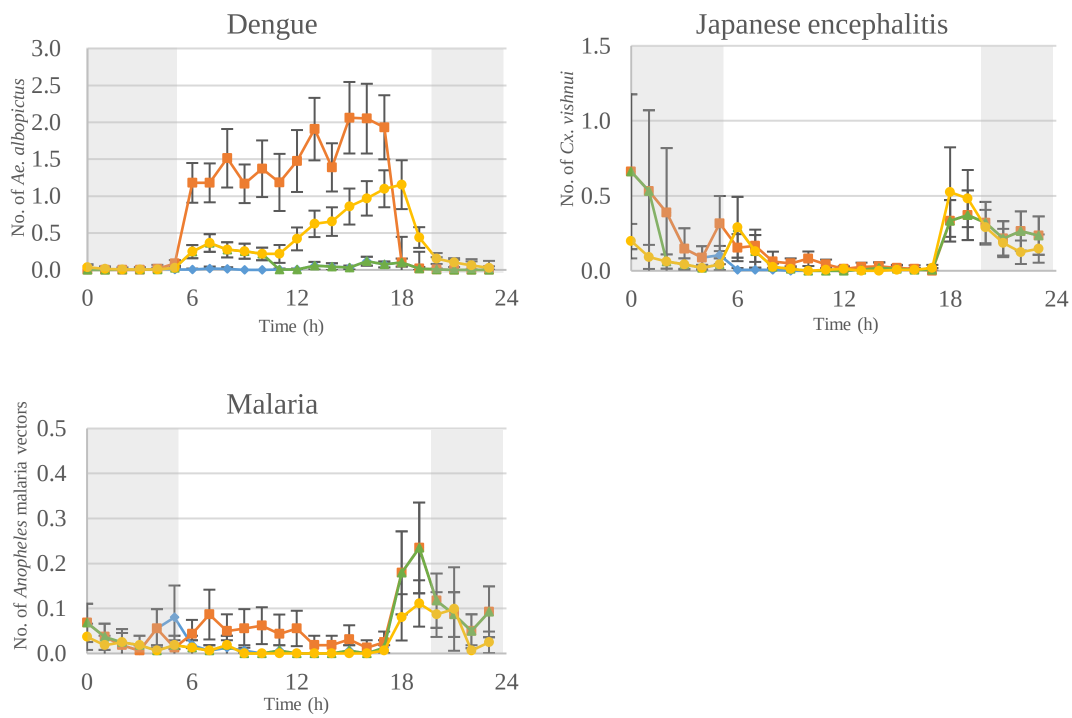

Figure 5.2 The average hourly exposure to female *Aedes albopictus* (dengue vector), *Culex vishnui* (Japanese encephalitis vector) and *Anopheles* malaria vectors for the different scenarios (villager that stays in village, villager that visits forest from 05.00 to 17.00 h, rubber worker that lives in the village and rubber worker that lives in the plantation) with the possible use of bed nets indicated from 20.00 h to 05.00 h with . All including 95 % confidence interval

Table 5.8 The daily risk of exposure to vectors for people in different scenarios

	Dengue risk			Japanese encephalitis risk			Malaria risk		
Exposure per 24 hrs	***Ae. albopictus*** **exposure (95% CI)**	**OR (95% CI)**	***P***	***Cx. vishnui*** **exposure (95% CI)**	**OR (95% CI)**	***P***	**Malaria vectors exposure (95% CI)**	**OR (95% CI)**	***P***
Villager that visits the secondary forest during the day	16.77 (14.12-19.42)	35.99 (24.61-52.62)	<0.001*	4.53 (2.85-6.22)	1.38 (1.15-1.65)	<0.001*	1.44 (1.06-1.83)	1.29 (1.15-1.44)	<0.001*
Rubber worker that lives in the village	1.64 (1.30-1.97)	3.22 (2.32-4.48)	<0.001*	3.60 2.25-4.96)	0.97 (0.92-1.03)	0.357	0.99 (0.73-1.25)	0.89 (0.79-1.01)	0.062
Rubber worker that lives in the plantations	8.22 (6.97-9.46)	16.22 (11.51-22.86)	<0.001*	2.72 (1.77-3.67)	0.82 (0.61-1.11)	0.195	0.57 (0.40-0.75)	0.63 (0.41-0.97)	0.037*
Villager that stays in the village	0.51 (0.35-0.68)	1		3.73 (2.06-5.40)	1		1.09 (0.79-1.39)	1	

*Results are shown using generalized estimating equations with odds ratio (OR) and 95 % confidence interval (CI). *significantly different,* P*<0.05*

5.4.7.2 Villager that visits the secondary forest during the day

In this scenario the villager visits the forest during the day from 05.00 h to 17.00 h and sleeps in the village at night. This activity occurs irregularly, most often in the rainy season, to collect food, wood and other commodities from the forest. Exposure to *Ae. albopictus* is highest in the secondary forests during the day from 06.00 h to 17.00 h (Figure 5.2). Therefore, if villagers visit the forest during the day, exposure risk to dengue vectors increases almost 36 times (Table 5.8). *Culex vishnui* exposure also increases when villagers visit the forest during the day. Villagers are exposed to JE vectors both in the village and in the rubber plantations. Exposure would be even higher if the villagers stayed in the forest until later, as *Cx. vishnui* activity in the secondary forests increases after 17.00 h (Figure 5.1). Exposure to malaria vectors occurs both in the forest and village with visiting the secondary forests during the day increasing the risk of malaria vector exposure 1.29 times. Risk of exposure to dengue, JE and malaria vectors is higher for villagers that visit the secondary forests than for villagers that stay in the village. Visiting the forest during the day is especially risky behaviour for dengue vector exposure.

5.4.7.3 Rubber worker that lives in the village

Rubber workers that live in the villages and work in the rubber plantations from 02.00 h to 10.00 h are exposed to both village and rubber plantation mosquitoes. Highest *Ae. albopictus* exposure occurs when working in the plantation, with peak exposure from 06.00 to 10.00 h (Figure 5.2, Table 5.8). Working in the plantations thus increases dengue vector exposure risk more than three times compared to when staying in the village. Risk of *Cx. vishnui* exposure is highest when rubber workers are resting in the village, resulting in similar JE vector exposure as villagers that stay in the village (Figure 5.2, Table 5.8). Similarly, malaria vector exposure is the same for rubber workers living in the village and villagers staying in the village with exposure to malaria vectors highest when present in the village (Figure 5.2). Risk of JE and malaria vector exposure does not increase due to latex tapping or collecting activity in the rubber plantations. However, for dengue vector exposure, working in the rubber plantations is risky behaviour.

5.4.7.4 Rubber worker that lives in the plantations

The rubber workers that live in the plantations are only exposed to mosquitoes present in the mature rubber plantations. When working and living in the rubber plantations risk of dengue vector exposure increases more than 16 fold compared to staying in the village (Figure 5.2, Table 5.8). However, rubber workers living in the plantations are exposed to similar number of *Cx. vishnui* mosquitoes as villagers staying in the village (Figure 5.2). Moreover, exposure to malaria vectors decreased 1.6 times when working and living in the plantations compared to villagers staying in the village. Living and working in the rubber plantations increased risk of dengue vector exposure and decreased risk of malaria vector exposure compared to villagers staying in the village while JE vector exposure remained the same.

5.5 Discussion

In an effort to assess the risk of exposure to mosquito-borne disease for villagers and those engaged in the rubber industry I investigated the overlap between mosquito behaviour and human behaviour in northern Lao PDR. I found that in all natural and man-made forest habitats dengue disease could establish itself, with malaria disease able to establish itself in all habitats investigated. Contrary to my hypothesis, rubber workers were not necessarily at higher risk of vector-borne diseases than villagers. This study suggests that visiting the forests during the day increases risk of dengue, JE and malaria vector exposure. Working in the rubber plantations also increases risk of dengue vector exposure, which is exasperated when also living in these man-made forests. However, contrary to expectation, working and living in the rubber plantations did not increase risk of exposure to JE vectors and even decreased risk of exposure to malaria vectors.

Dengue is a sylvatic disease that has been spread from the forest to rural and urban areas by the easily adjusting *Ae. albopictus* and *Ae. aegypti* [54]. As these *Aedes* mosquitoes generally have a high reproduction rate and can lay eggs in more than one land cover type, they cause outbreaks in a large variety of habitats including forested and urban areas [106, 365]. Risk of dengue was highest for villagers visiting the secondary forests and also high for rubber workers. This high risk was related to the high vector exposure, the flavivirus presence and the high R_0 calculated in both habitats. It should be noted that the flavivirus presence in *Ae. albopictus* does not necessarily mean that these mosquitoes are positive for human infective arboviruses. I found a clear higher risk for dengue infections and vector exposure in the natural and man-made forests than in the villages. According to the behavioural analysis both the natural and man-made forests are regularly visited by villagers, with rubber workers active in the rubber plantations. They are therefore important environments for dengue and possibly chikungunya transmission. The low risk of dengue infections in the rural villages surveyed, could be related to the possible implementation of vector control during the dengue outbreak in 2013-2014 (chapter 4, discussion) or more likely due to the preference of *Ae. albopictus* mosquitoes for the shaded forested habitats. Dengue vector control in Lao PDR is presently focussed on the village habitats. There is a clear need to broaden the control efforts to the surrounding forest and rubber plantation habitats using outdoor protection methods such as larval control and personal protection methods.

Almost 7 % of the male *Ae. albopictus* mosquitoes were positive for Pan-flaviviruses. As male mosquitoes do not feed on blood, the infection with flaviviruses is likely caused by vertical transmission, from parent to offspring. Vertical transmission is of importance for many vector-borne pathogens as this ensures that, even without pathogen transmission from vector to host, the viruses can persist. It is important to keep the presence of vertical transmission in mind when designing vector control programmes. Dengue vector control should not only focus on the human biting mosquitoes, but also include methods with which the male mosquitoes are targeted, such as larval control.

The rubber workers that tap latex in the rubber plantations at night and live in the village are exposed to similar number of JE and malaria vectors as villagers staying at home, with risk of malaria vector exposure decreasing when rubber workers also live in the rubber plantations. This is contrary to earlier suggestions that rubber tapping activity at night increases exposure to malaria vectors [198, 341]. Working in the rubber plantations from 02.00 to 10.00 h is not a risky behaviour for malaria vector exposure in this study area, due to the early evening host-seeking behaviour of the malaria vectors. However, the high R_0 of malaria calculated for all habitats does imply that if malaria is introduced in rubber plantations, it could easily establish itself. From the behavioural analysis, I identified two ways in which malaria could be introduced in the study area. Firstly, during the RRA I found clear indication that local villagers migrate yearly to find temporary work in other areas of SEA. The local population could unknowingly be exposed to malaria parasites when working in other regions and carry the parasites back to their own village. Secondly, many of the rubber plantations workers that live in the plantations are migrant workers that only live in the plantations during the rainy season to tap latex. These migrant workers could unknowingly introduce the malaria parasites from other areas in SEA to the rubber plantation areas. As these rubber plantations are also visited by the local population, the pathogen could then be spread to the villages. Although malaria is currently not an endemic disease in the study area, worryingly when the malaria pathogen is introduced, all necessary factors are present for malaria infections.

Resistance to pyrethroids in the mosquito population of SEA is increasing. Several malaria vectors have already been identified to be less sensitive to insecticides in the Mekong region [122]. This is threatening the effectiveness of the pyrethroid treated ITNs. The use of ITNs is also threatened by the change in mosquito behaviour [428, 429]. Mosquitoes in areas with a high density of ITNs can change their biting behaviour from night time biting towards early evening biting. Furthermore increased outdoor biting has occur [430]. Interestingly, more than 90 % of the population in our study area were in possession of ITNs. If these bed nets have been used consistently and correctly over a longer period of time, mosquitoes could have adjusted their behaviour to this. This could result in a peak in host-seeking behaviour before the villagers go to bed and high outdoor biting rate. It is difficult to relate my study data to the use of bed nets, as I only collected mosquito samples outdoors. Additionally, the numbers of *Anopheles* mosquitoes collected throughout the study were too low for clear behavioural descriptions. An additional study is necessary in the study area, in which mosquito samples are collected both indoors and ourdoors, to identify if behavioural changes have occurred due to the use of bed nets. It is always important to keep behavioural changes in mind in areas where ITNs are used. This is especially precedent in countries like Lao PDR, where vector control heavily depends on the use of ITNs.

The R_0 estimates have given a good insight into the vector-borne disease dynamics in our study area. A downside to using any calculation model is the lack of specificity. The R_0 calculations in this paper are no exception. Both models do not include landscape factors nor the vertical and sexual transmission of dengue viruses. Furthermore, they do not take into account the treatment of disease, heterogeneous exposure to vector mosquitoes nor the immunity of the population [431]. In addition the different parameters used for the calculations are all estimates. For example, often more than the estimated two-thirds of the *An. dirus s.l.* feed on humans. Moreover, *Anopheles* and especially *Aedes* mosquitoes feed multiple times during one gonotrophic cycle, which is not considered in the calculations. The high basic reproductive number calculated in this study is therefore expected to be an underestimation of the reality.

A further limitation of the R_0 calculations in this study has been the parity data used. The parity data was only collected at the beginning of the rainy season in July and August 2015. Parity can differ vastly between adjacent months [53, 432, 433]. Data should therefore have been collected throughout the 9 months of collection. In addition, the total number of mosquitoes dissected were too low and it is unclear if the collection method surveys different age classes equally. *Anopheles dirus s.l.* parity rate is generally high [97, 108], with these mosquitoes having a long survival rate [109]. In this study we identified a comparatively low *An. dirus s.l.* parity rate, which could be a misrepresentation of the reality, due to the low number of dissections conducted. For future R_0 calculations, survival rates of mosquitoes should be collected throughout the entire study, with at least 100 samples of each vector species successfully dissected.

There is a lack of suitable methods to measure human behaviour, especially on an individual scale, with limits to the predictability of human mobility [397, 434, 435]. In this study I used a combination of RRA's and surveys to collect human behaviour data, which is novel for vector-borne disease studies. I focussed on the destinations of villagers and not on the routes that villagers take. There are a number of techniques used to capture human movement, such as GPS tracking systems [436, 437], cellular phones [438] and photo voice [439]. In my study area in Lao PDR, GPS tracking was not possible, as villagers were hesitant of this method, possibly due to illegal activities and other personal affairs that occur. Furthermore, photo-voice was not comprehensive enough for the study. Instead in this study I used a combination of RRA's and surveys. The RRA's and surveys do not result in detailed quantitative information. Both methods are sensitive to memory decay, social desirability and other biases. Yet the methods combined did provide us with sufficient information to describe broad patterns of human behaviour and relate risk of vector-borne diseases to villagers and rubber workers behaviour.

From the behavioural analysis I identified that the biggest limitation of this study has been the exclusion of the rice fields and other farm habitats from the analysis. The behavioural summaries, especially the RRA's, highlighted that villagers spend considerable time on their farms. These habitats could therefore be an important site for disease transmission with one comparison study in Thailand showing higher mosquito abundance in rice fields, with a high proportion of the JE vector *Cx. vishnui*, compared to forests [348]. Furthermore, for more reliable behavioural data, it would be recommended to increase observational intervals, since human behaviour changes throughout the year. Conducting the survey every month will lead to a better understanding of the subtle changes in human behaviour throughout the year, which could be related to the monthly mosquito behaviour. For future vector-borne disease risk studies, I would recommend to perform RRA's and surveys before the start of the study, with the surveys repeated monthly for the duration of the study.

Currently, mosquito control in Lao PDR focusses on the distribution of Long-lasting Insecticide Treated nets (LLINS), Indoor Residual Spraying (IRS) and the distribution of larvicides in urban areas and rural villages. The current control strategies are not sufficient to control dengue, with dengue outbreaks still occurring regularly. There is therefore a need to better understand the dengue dynamics in Lao PDR. Identifying patterns of interaction between vectors and the population results in more effective and efficient interventions by targeting the key areas of transmission [396, 397]. This study, for example, has highlighted the importance of including the secondary forests and rubber plantations habitats in the mosquito-control strategies, especially for the control of dengue. Furthermore it has underlined that villagers visiting the secondary forests regularly and rubber plantation workers are at higher risk of exposure to vector-borne diseases. Vector control, education and disease surveillance should therefore be focussed on these vulnerable population groups. Furthermore, vector control in rubber plantations should focus on the rubber worker houses inside the plantations and on outdoor control. For control in rubber houses similar methods can be used as in the villages; use of LLINs, spatial repellents and screening of houses [341]. For outdoor control both personal protection and larval source management is necessary. Personal protection methods should include motivating rubber workers to wear long sleeved clothing and closed shoes when in the plantation. Additionally, insecticide-treated clothing, insecticide emanators and portable insecticide coils could be used for personal protection [341]. However, before recommendations on personal protection methods can be made, these methods need to be further investigated for their protection value against vector-borne diseases. Larval control in rubber plantations can be achieved by turning the latex collection cups upside down [341]. Furthermore, rubber workers should be encouraged to keep the plantations clean from garbage such as plastic bags, bottles and tyres. In forested areas mosquito control is more challenging than the rubber plantation areas. Particularly larval control is difficult to implement in the natural forests due to the vastness and diversity of breeding sites, and the high biodiversity of other insects present. Emphasis should therefore be on personal protection method, which are similar to the rubber workers. Additionally, LLINs should be used when staying in the forests overnight.

5.6 Conclusion

Mosquito-borne diseases are an important human health problem in Lao PDR yet the understanding of the disease dynamics, including the risk of certain human behaviour, is limited. The present study suggests that risk of dengue infection is higher for villagers who visit the forests and rubber workers, than for villagers that stay in the village. Furthermore, risk of JE and malaria is higher for villagers that visit the forest during the day. Currently, vector control in Lao PDR is focussed on control in villages. This study highlights the importance of broadening mosquito control to include the secondary forests and rubber plantations. Studies on personal protection methods are essential to advance the vector control in the area with a great need to understand the impact of insecticide treated clothing, portable coils and emitters on the disease dynamics. This study furthermore emphasizes the importance of including local human behaviour into the risk analysis and is a step towards better understanding of vector-borne disease dynamics in northern Lao PDR.

6 Surveillance of larval habitats in rubber plantations and villages: A baseline study in northern Lao PDR

Collecting mosquito larvae in the mature rubber plantation of Houayhoy village with help from local villagers

6.1 Abstract

Rubber plantations in South-East Asia (SEA) are important habitats for adult vectors of malaria, dengue, Japanese encephalitis (JE) and lymphatic filariasis. Understanding where the mosquito vectors breed in these plantations will enable the development of improved ways to control these diseases. I set out to identify the major breeding sites of mosquitoes in rubber plantations and rural villages.

Monthly larval surveys were carried out in three study sites in northern Lao PDR, each consisting of a mature rubber plantation, immature rubber plantation and village. Sampling sites were characterized based on biotic and abiotic information, and emerged adult mosquitoes were morphologically identified.

Between August and December 2014, 1,379 waterbodies were surveyed of which 53 % (724/1,379) contained immature mosquitoes. *Aedes* and *Culex* were most often found in waterbodies from mature rubber plantations (209/443; 108/200, respectively). *Anopheles* present waterbodies were most indentified in immature plantations (10/21). The highest number of *Aedes* immature stages were collected from cut bamboo (3,065/11,468). The highest number of *Culex* larvae were collected from tyres (2,265/7,916) and the highest number of *Anopheles* immature stages were collected from puddles (106/177). *Aedes albopictus* immature stages were most frequently collected from tyres and latex collection cups in the mature rubber plantations and from tyres and water containers (< and > 10 L) in the villages. A majority of the *Cx. quinquefasciatus* were collected from water containers (< and > 10 L) in the mature rubber plantations and villages. *Anopheles dirus s.l.* were mostly collected from puddles in the immature rubber plantations and villages.

The findings suggest that mature rubber plantations have similarly suitable breeding habitats for *Ae. albopictus*, *Cx. quinquefasciatus* and *An. dirus s.l.* species as the rural villages. As exposure to mosquitoes from the rubber plantations is high for both villagers and rubber

workers (chapter 5), current focus on village larval control in Lao PDR should broaden to include rubber plantation areas. Larval control can be implemented in rubber plantations and villages using environmental management in a community-based manner.

6.2 Introduction

In South-East Asia (SEA) the most important vector-borne diseases are dengue and malaria. Dengue disease incidence and spread has been increasing in the region, with epidemics recorded in almost all SEA countries in the past decade [142, 440, 441]. In Lao PDR the most recent dengue epidemic was in 2013 with more than 10,000 cases [137, 411]. In contrast, malaria has declined in SEA by 45% from 2.9 million cases in 2000 to 1.6 million cases in 2014 [98]. This decrease is mostly due to the utilization of long-lasting insecticidal nets (LLINs), effective treatment with artemisinin combination therapies (ACTs), indoor residual spraying (IRS) and improved access to diagnosis of malaria. In spite of this success Lao PDR is still experiencing malaria epidemics throughout the country, with the most recent outbreak in 2013 in the southern provinces [115].

Lao PDR currently has one of the fastest growing economies in Asia [65], which has resulted in huge changes in land use. One of the major land cover changes in Lao PDR is the establishment of rubber plantations, which has increased from 900ha in 2010 to 147,500ha in 2015 [70]. As a result agricultural land and natural forest areas have decreased. Since rubber plantations provide suitable habitats for dengue and malaria vectors [341] and vector-borne disease outbreaks have been recorded, there is a need to understand the vector dynamics in rubber plantations for control strategies.

Dengue and malaria vector control in Lao PDR has been dependent largely on the distribution of LLINs, the application of larvicides and, to a lesser extent, the use of IRS in the rural villages and urban areas [100]. In spite of the large deployment of these control methods, dengue and malaria remain important public health problems. Vector-borne disease control is furthermore threatened by the increasing tolerance of malaria parasite strains to artemisinin and increasing resistance of dengue and malaria vectors to pyrethroids and DDT [67, 116, 122, 442-444]. There is therefore a need to develop supplementary vector control methods to include in the Integrated Vector Management (IVM) programmes.

Control of mosquito breeding sites can be a complementary method to support the control of mosquito-borne diseases in Lao PDR. Larval control is an important vector control tool, as the water stages of mosquitoes are confined within the waterbodies and cannot readily escape [445]. Larval source management (LSM) is suggested to be especially important for areas where hotspots of malaria exist [396]. Large scale LSM has been successful in decreasing malaria cases [445-451]. Malaria transmission can be reduced by 70 to 90 % if the important waterbodies are treated with larvicides [446, 448, 449, 451, 452]. The application of larvicides decreased the risk of new malaria infection more than twice for children in Kenya [451]. This level of protection was shown independently from the use of insecticide treated nets (ITNs). The use of larval source reduction can, therefore, increase the protection rate against vector-borne disease in areas where ITNs are used as vector control [451, 453]. However, larvicides are largely ineffective if habitats are extensive and cannot be easily accessed. Furthermore, many mosquito control programs do not have the financial means to implement larval control effectively [454].

For the implementation of larval control to be successful in decreasing disease, detailed knowledge on the breeding preference of vector mosquitoes is necessary. These are labour intensive and dependent on the local environmental dynamics. Only a few larval surveys have been reported from Lao PDR [111, 137]. There is therefore a real need to better understand where the mosquito species breed and their waterbody characteristics. This is especially important in the North of Lao PDR, where malaria hotspots remain. I carried out a larval survey in rubber plantations and nearby rural villages to determine the major breeding sites of the vectors of *Aedes*, *Culex* and *Anopheles* mosquitoes.

6.3 Materials and Methods

6.3.1 Study site

Larval surveys were conducted in the three study sites Thinkeo (19°41’02.13”N 102°07’05.49”E), Silalek (19°37’02.80”N 102°03’05.70”E) and Houayhoy (19°33’03.22”N 101°59’42.42”E) in Xieng-Ngeun and Nane district, northern Lao PDR. The study site elevation ranged between 359 and 1428 m above sea level

6.3.2 Study design

In each study site, three habitats were surveyed monthly from August to December: a mature rubber plantation, an immature rubber plantation and a village (the same habitats as chapter 4). A total of nine study sites (three study sites in three habitats) were thus surveyed monthly. . During the survey period the mean daily temperature fluctuated between 8.8 °C and 35.2 °C and daily relative humidity (RH) ranged between 65 % and 100 %. The mean daily precipitation was between 0 and 141.2 mm with maximum rainfall in August 2014.

The mature rubber plantations were defined as plantations where more than 70% of the trees were tapped for latex for at least one year. The immature plantations were those with young untapped rubber trees (<7 years). Using Google earth® in each of the six rubber plantations a 1 km^2 area was selected for monthly surveillance. The 1 km^2 area was selected by placing the adult collection study site from chapter 4 at the centre of the 1 km^2 area. If the study site exceeded the habitat, the area was moved until the entire square was inside the habitat. Villages were roughly ±1 km^2, organized linearly with one paved road running through the centre of the village. I identified the borders of the village using the information received during the rapid rural appraisals (chapter 5). The entire village was surveyed for mosquito breeding sites.

6.3.3 Characterization of waterbodies

All potential breeding sites were surveyed in each 1 km^2 area. Waterbodies of the villages were surveyed both inside and outside the houses, provided the owners were present and they verbally consented. On a few occasions the owners of the houses refused access. In each of the nine study sites all waterbodies encountered were identified with a unique number. The position was recorded with a handheld Global Positioning System (Garmin GPS map 62sc, Garmin International Inc, Kansas, USA). All waterbodies were classified into one of the following categories: 1) cut bamboo, both still growing and used for construction of gates, pig stalls and chicken houses; 2) leaf axils of a banana tree; 3) discarded plastic, including broken shoes, plastic bottles and plastic bags; 4) water container < 10 L, generally containers used to transport water, such as buckets; 5) water container > 10 L, generally containers used to store water, including drums and cement tubs; 6) puddle, small (< 2 m diameter) and shallow (< 50 cm) standing water body with water originating from rainwater, usually drying out toward the end of the rainy season; 7) pool, generally medium sized (2-5 m diameter) standing water body with water originating from ground water and rainwater; 8) pond, permanent large (> 5 m) water body with water originating from both ground water and rain water; 9) stream fringe of year-round stream; 10) latex collection cup, both containing and not containing latex; 11) tree trunk; 12) ditch, a narrow channels (< 2 m diameter) dug at the side of a road or around a house; 13) leaf puddle, fallen leaves from different plants that provide small (< 20 cm diameter) temporary pockets of water; 14) tyre; 15) rice field, seasonally flooded areas used to grow lowland rice.

Detailed information of visual waterbody characteristics was also collected. The diameter (< 10 cm, 10-100 cm, >100 cm) and volume (< 5 mL, 5 mL - 50 mL, 1 L - 5 L, > 5 L) of each water body was estimated. Water depth was measured using a ruler. Water movement was noted by measuring the time a Post-it® (3M Company, 73 cm by 73 cm) moved five meters. Water movement was distinguished in no movement, slow moving water (>8 sec), moderate flow (4-8 sec) and fast flow (<4 sec). It was noted if the waterbody was in the shade during the day (sunlit, sometimes shade, always shade). The presence of small bags with Temephos was checked visually and noted. Furthermore, presence of submerged, emergent or floating aquatic plants was noted.

After the visual description of the waterbodies, the water chemistry was measured 10 cm below the water surface of every waterbody. Using a multi-probe meter (Multi 3420, Wissenschaftlich-Technische Werkstätten GmbH, Oberbayern, Germany) with an standard conductivity measuring cell (TetraCon® 925, WTW GmbH, Oberbayern, Germany), a pH electrodes with gel electrolyte (SenTix® 940-3, WTW GmbH, Oberbayern, Germany) and an optical dissolved oxygen sensor (FDO® 925, WTW GmbH, Oberbayern, Germany) the water temperature, pH, salinity and dissolved oxygen were measured. If a waterbody was too small, water was transferred into a small cup from where measurements were made. Furthermore, at least 20 mL of undisturbed water from every waterbody was transported back to the field laboratory for turbidity measurements using a turbidity measurement device (Turb® 355 IR, WTW GmbH, Oberbayern, Germany).

In the field laboratory, phosphate and nitrate concentrations were determined from water samples collected in the field. Due to the high costs, the number of tests were limited to 92 latex collection cups. Water samples were analysed using a photometer (pHotoFlex® STD, Wissenschaftlich-Technische Werkstätten (WTW) GmbH, Oberbayern, Germany) for phosphate (PO_4-3 TC, WTW GmbH, Oberbayern, Germany) and nitrate (NO_3-1 TC, WTW GmbH, Oberbayern, Germany) concentrations following standard programs of WTW (program number 314 and 316) [455].

6.3.4 Mosquito larvae and invertebrate sampling

The presence or absence of immature mosquitoes, other invertebrates, tadpoles and fish were determined by one to 10 dips (depending on the habitat size) with a standard 350 ml dipper (Bioquip, California, USA). Small water bodies were surveyed using a 5 ml pipette (Saysaath pharmacy, Sihome village, Lao PDR). In medium and large waterbodies dipping was focused on tufts of grass and other vegetation where larvae often aggregate. Collected water was emptied in a larval tray (34.3 cm x 25.4 cm x 3.8 cm, Bioquip, California, USA) and examined for mosquito larvae, pupae, other invertebrates, tadpoles and fish. If any vertebrate or invertebrate was found in a waterbody, these waterbodies were surveyed for an additional 10 minutes using a fine net and dipper, to collect more samples. All mosquito and other invertebrate specimens were transferred to separate see-through plastic bags (whirl-pack bags, Bioquip, California, USA) and transported back to the field laboratory for further identification.

6.3.5 Mosquito and invertebrate identification

In the field laboratory, mosquito larvae from each waterbody were transferred to larval trays. The mosquito larvae were separated into anophelini, aedini, culicini, toxorhynchitini, ficalbiini and sabethini. Mosquitoes were then identified to early stage larvae (1^{st} and 2^{nd}), late stage larvae (3^{rd} and 4^{th} stage) or pupae. After basic identification, mosquito larvae and pupae from each waterbody were transferred to a breeding cone (Bioquip, California, USA) or small 100 mL cups covered by netting. Mosquito larvae were fed ground dry cat food (Whiskas mackerel flavour, Chantuk, Thailand) every two days. All emerged adult mosquitoes were morphologically identified to species using recognized Thai identification keys [314].

Invertebrates collected in the field were identified within two days of the survey, using a stereomicroscope with 10 × magnification and a standard identification key for the British freshwater invertebrates, to the following taxonomic groups: beetle larvae (*Coleoptera*), beetle adults (*Coleoptera*), dragonfly and damselfly larvae (*Odonata*; suborder *Anisoptera* and *Zygoptera*), may fly larvae (*Ephemeroptera*), larvae of non-biting midges (*Chironomidae*), phantom midges (*Chaoboridae*), diptera larvae, waterflea (*Cladocera*), Ostracods and waterbugs (*Heteroptera*) [456].

6.3.6 Data analysis

The proportion of different mosquito taxa present in the different habitats was compared using Simpson's index of diversity with results representing diversity from 0 (no diversity) to 1 (infinite diversity) [315, 316]. Generalized estimating equations (GEE) with binary logistics were used to estimate the difference in waterbody characteristics (study site, habitat, diameter, volume, depth, shade, vegetation and waterbody type) between waterbodies where *Aedes*, *Culex* and *Anopheles* larvae and pupae were present or absent (IBM SPSS statistics, version 20). If numbers of waterbodies surveyed were fewer than 25, the waterbodies were combined. Forward stepwise binary logistic regressions were conducted to ensure the key values were stable. Generalized linear models (GLM) with binary logistics were used to identify the important water chemistry (Salinity, dissolved oxygen, temperature, pH, turbidity, nitrate and phosphate) that could influence larval density with odds ratio (OR) and 95 % confidence interval (CI). Waterbodies with less than 50 larvae collected were combined. Generalized estimating equations (GEE) were used to estimate the difference in immature mosquito density between waterbodies using Poisson log linear model for count data with OR and 95 % CI.

6.4 Results

6.4.1 Mosquito larval survey

Between August and December 2014, 1,379 waterbodies were surveyed of which 53 % (724/1,379) contained mosquito larvae and/or pupae. In 2.5 % of the waterbodies small bags of Temephos larvicide were found (35/1,379). No immature mosquitoes were identified in these larvicide treated waterbodies. In 32 % of surveyed waterbodies immature stages of *Aedes* were found (443/1,379) with a total of 11,468 immature *Aedes* mosquitoes collected (48.8 % early instars, 43.4 % late instars and 7.8 % pupae). In 14.5 % of surveyed waterbodies *Culex* mosquitoes were found (200/1379) with 7,916 immature *Culex* mosquitoes collected (52.6 % early instars, 42.2 % late instars and 5.2 % pupae). About 1.5 % of surveyed waterbodies contained *Anopheles* (21/1,379) with 177 *Anopheles* larvae and pupae collected (46.3 % early instar, 48.0 % late instar, 5.7 % pupae).

The greatest number of mosquito present waterbodies were found in September, when rainfall was highest, with the highest proportion of mosquito present waterbodies identified in the subsequent month October (Figure 6.1). Waterbodies surveyed were similar for the rainy season and the dry season with the exception of the rice fields, which dried out during the dry season. The three most abundant waterbodies were latex collection cups, Water containers < 10L and cut bamboo. These waterbodies accounted for 48 % of all waterbodies surveyed. The mosquito diversity, calculated using the Simpson's index, showed similar mosquito diversity in the waterbodies of the mature rubber plantations (0.697, 95% CI 0.625-0.768), immature rubber plantation waterbodies (0.769, 95% CI 0.706-0.832, t-test *P* = 0.259), and villages (0.671, 95 % CI 0.572-0.770, *P* = 0.222).

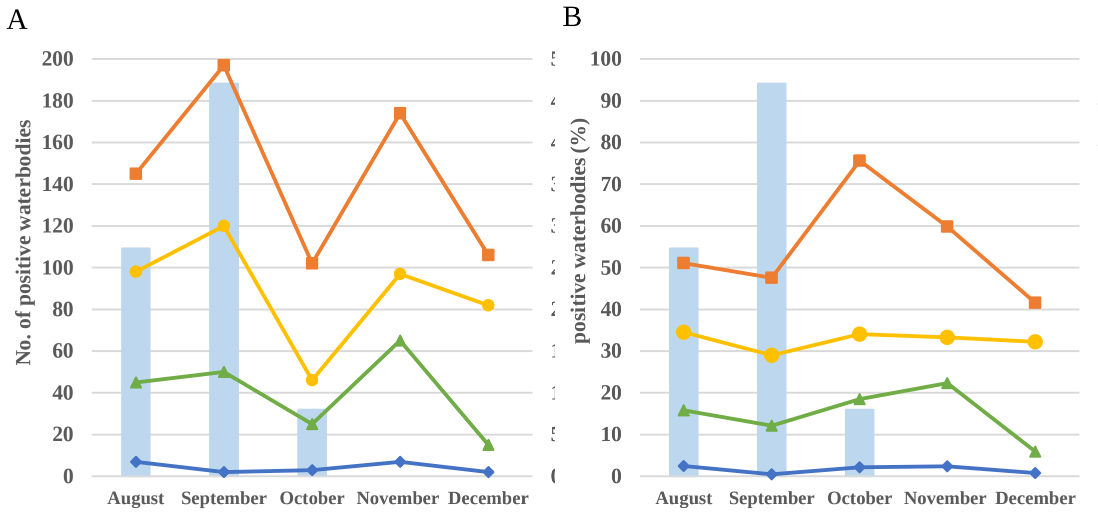

Figure 6.1 Waterbodies containing immature mosquitoes (—■— total of all species, —●— *Aedes* species, —▲— *Culex* species, —♦— *Anopheles* species) with total rainfall per month indicated with light blue bars. (A) the total number of waterbodies in which immature mosquito stages were present (B) the proportion of waterbodies in which immature mosquito stages were present.

Non-mosquito invertebrates were found in 26.8 % of surveyed waterbodies (370/1,379). In the mature rubber plantations lowest proportion of waterbodies contained non-mosquito invertebrates with highest proportion found in immature rubber plantations (Appendix 11). Tadpoles were found in nine waterbodies and fish in three. A total of 556 invertebrates were collected and identified. The most commonly identified invertebrates were *Chaoboridae* (n = 180) and *Chironomidae* (n = 108).

6.4.2 Presence and absence of larvae and pupae

More *Aedes* larvae were collected in Silalek and Thinkeo study sites, compared to Houayhoy. *Aedes* larvae were most often found in small waterbodies such as cut bamboo, tyres, tree trunks, leaf axils, latex collection cups, other natural waterbodies, other artificial waterbodies, containers for water and discarded garbage (Table 6.1). Lower salinity, lower nitrate and lower phosphate concentrations were also associated with *Aedes* larvae presence (Appendix 12).

Aedes pupae were associated with water volume, low level of shade and vegetation absence (Table 6.2). Water bodies smaller than 50 mL were four times more likely to contain *Aedes* pupae than water bodies larger than five litres. Higher turbidity was also associated with *Aedes* pupae presence (Appendix 12). Similar to *Aedes* larvae, the pupae were also found in small waterbodies like cut bamboo, tyres, container for water and tree trunks.

More *Culex* larvae were collected in Silalek and Thinkeo study sites than in Houayhoy. The presence of *Culex* larvae was associated with the habitat, waterbody diameter larger than 10 cm, low level of shade and other invertebrate presence (Table 6.3). Waterbodies in mature rubber plantations were three times more likely to contain *Culex* larvae than in the villages. The waterbodies with *Culex* larvae were also associated with lower salinity and higher turbidity than when *Culex* larvae were absent (Appendix 13). *Culex* larvae were most frequently found in tyres, water containers, puddles, tree trunks, latex collection cups, other natural waterbodies, other artificial waterbodies and discarded garbage. Similar to *Culex* larvae, more *Culex* pupae were collected in Silalek and Thinkeo study sites than in Houayhoy. The presence of *Culex* pupae were associated with diameter of waterbody larger than 100 cm and low level of shade (Table 6.4). No water characteristics measured during the study were associated with *Culex* pupae presence (Appendix 13). *Culex* pupae were most likely to be found in tyres, puddles, other natural waterbodies, other artificial waterbodies, containers for water >10 L, latex collection cups and tree trunks.

Presence of other invertebrates in the water increased the chance of *Anopheles* larvae presence more than three times from 0.7 % positivity rate to 2.2 % positivity rate. Water characteristics could not be compared for *Anopheles* larvae due to the low number of positive waterbodies. *Anopheles* larvae were most commonly found in tree trunks, puddles, cut bamboo, other artificial waterbodies, other natural waterbodies, water containers > 10 L, tyres and latex collection cups (Table 6.5). Number of waterbodies containing *Anopheles* pupae were too few for general analysis.

Table 6.1 Multivariate analysis of factors associated with *Aedes* larvae presence

		n waterbodies surveyed	% *Aedes* larvae present	OR	(95% CI)	*P*
Study site	**Thinkeo**	480	36.3	1.86	(1.49-2.32)	<0.001*
	Silalek	338	35.8	1.85	(1.37-2.50)	<0.001*
	Houayhoy	561	26.4	1		
Habitat	**Mature rubber plantation**	621	33.5	1.46	(0.93-2.27)	0.097
	Immature rubber plantation	179	33.0	1.39	(0.64-3.05)	0.407
	Village	579	30.4	1		
Diameter	**<10 cm**	566	41.5	2.31	(1.43-3.74)	0.001*
	10-100 cm	677	29.2	2.34	(1.61-3.41)	<0.001*
	>100cm	136	7.4	1		
Volume	**50 mL – 5 L**	464	28.2	0.77	(0.61-0.98)	0.035*
	> 5L	218	14.2	0.49	(0.36-0.66)	<0.001*
	< 50 mL	697	40.3	1		
Shade	**absent**	153	30.1	1.22	(0.77-1.94)	0.395
	Sometimes	814	31.2	1.20	(0.98-1.46)	0.072
	Always	412	34.7	1		
Vegetation	**absent**	1289	33.0	0.61	(0.28-1.30)	0.199
	present	90	20.0	1		
Other invertebrates˜	**Absent**	872	29.9	0.96	(0.67-1.40)	0.847
	Present	507	35.9	1		
Waterbody type	**Cut bamboo**	156	62.2	31.82	(14.67-69.05)	<0.001*
	Leaf axil	102	44.1	10.04	(4.64-21.73)	<0.001*
	Discarded garbage	140	23.6	5.06	(2.34-10.93)	<0.001*
	Container for water (< 10 L and > 10 L)	313	26.5	9.58	(4.40-20.90)	<0.001*
	Stream fringe	42	2.4	0.52	(0.11-2.52)	0.415
	Latex collection cup	321	33.0	7.06	(3.15-15.82)	<0.001*
	Tree trunk	50	52.0	15.86	(8.08-31.15)	<0.001*
	Tyre	65	49.2	19.45	(8.09-46.79)	<0.001*
	Other natural waterbodies°	30	36.7	8.61	(4.55-16.31)	<0.001*
	Other artificial waterbodiesˆ	30	10.0	4.16	(0.87-19.95)	0.074
	Puddle	130	4.6	1		

Generalized estimating equations were used to calculate the P*-value with odds ratio (OR) and 95% confidence interval (95% CI) ˜other invertebrates, including other mosquito larvae ° other natural waterbodies (pools and leaf puddles) ˆ other artificial waterbodies (ponds, ditches and rice fields) *significantly different,* P*<0.05*

Table 6.2 Multivariate analysis of factors associated with *Aedes* pupae presence

		n waterbodies surveyed	% *Aedes* pupae present	OR	(95% CI)	*P*
Study site	**Thinkeo**	480	8.1	1.07	(0.55-2.07)	0.843
	Silalek	338	13.6	1.53	(0.92-2.57)	0.103
	Houayhoy	561	9.3	1		
Habitat	**Mature rubber plantation**	621	8.2	1.28	(0.77-2.13)	0.334
	Immature rubber plantation	179	11.7	1.19	(0.51-2.80)	0.686
	Village	579	11.2	1		
Diameter	**<10 cm**	566	15.4	6.11	(0.60-61.75)	0.125
	10-100 cm	677	7.2	4.49	(0.73-27.64)	0.105
	>100cm	136	0.7	1		
Volume	**50 mL – 5 L**	464	9.1	0.62	(0.38-1.03)	0.065
	> 5L	218	2.3	0.26	(0.11-0.61)	0.002*
	< 50 mL	697	12.9	1		
Shade	**absent**	153	11.1	1.34	(0.92-1.95)	0.126
	Sometimes	814	9.1	1.42	(1.06-1.91)	0.020*
	Always	412	11.2	1		
Vegetation	**absent**	1289	10.4	1.49	(1.30-1.72)	<0.001*
	present	90	3.3	1		
Other invertebrates˜	**Absent**	872	10.0	1.23	(0.75-2.03)	0.417
	Present	507	9.9	1		
Waterbody type	**Cut bamboo**	156	31.4	14.49	(3.25-64.66)	<0.001*
	Leaf axil	102	7.8	2.29	(0.33-15.69)	0.398
	Discarded garbage	140	9.3	2.88	(0.77-10.73)	0.116
	Container for water (< 10 L and > 10 L)	313	8.0	6.34	(1.43-28.16)	0.015*
	Stream fringe	42	2.4	2.61	(0.31-21.90)	0.378
	Latex collection cup	321	3.7	1.11	(0.37-3.33)	0.855
	Tree trunk	50	16.0	5.49	(1.41-21.33)	0.014*
	Tyre	65	23.1	17.44	(3.19-95.46)	0.001*
	Other natural waterbodies°	30	13.3	4.72	(0.82-27.18)	0.082
	Other artificial waterbodiesˆ	30	0.0			
	Puddle	130	1.5	1		

Generalized estimating equations were used to calculate the P-*value with odds ratio (OR) and 95% confidence interval (95% CI) ˜other invertebrates, including other mosquito larvae ° other natural waterbodies (pools, and leaf puddles) ˆ other artificial waterbodies (ponds, ditches and rice fields) *significantly different,* P*<0.05*

Table 6.3 Multivariate analysis of factors associated with *Culex* larvae presence

		n waterbodies surveyed	% *Culex* larvae present	OR	(95% CI)	*P*
Study site	**Thinkeo**	480	18.1	2.21	(0.93-5.27)	0.073
	Silalek	338	20.4	3.15	(1.71-5.82)	<0.001*
	Houayhoy	561	8.4	1		
Habitat	**Mature rubber plantation**	621	17.6	3.18	(2.21-4.56)	<0.001*
	Immature rubber plantation	179	14.5	2.00	(0.78-5.17)	0.151
	Village	579	11.7	1		
Diameter	**<10 cm**	566	7.6	0.34	(0.15-0.77)	0.010*
	10-100 cm	677	18.9	0.79	(0.51-1.22)	0.286
	>100cm	136	23.5	1		
Volume	**50 mL – 5 L**	464	19.0	0.95	(0.53-1.71)	0.875
	> 5L	218	17.0	0.68	(0.40-1.17)	0.165
	< 50 mL	697	11.2	1		
Shade	**absent**	153	11.8	0.80	(0.39-1.66)	0.550
	Sometimes	814	16.7	1.59	(1.08-2.33)	0.019*
	Always	412	11.9	1		
Vegetation	**absent**	1289	14.3	0.78	(0.29-2.11)	0.625
	present	90	21.1	1		
Other invertebrates˜	**Absent**	654	6.9	0.23	(0.14-0.38)	<0.001*
	Present	725	21.8	1		
Waterbody type	**Cut bamboo**	156	7.7	0.37	(0.21-0.65)	0.001*
	Leaf axil	102	5.9	0.16	(0.03-0.87)	0.034*
	Discarded garbage	140	6.4	0.41	(0.14-1.18)	0.099
	Container for water <10 L	186	10.2	0.73	(0.34-1.55)	0.416
	Container for water >10 L	127	20.5	1.43	(0.61-3.34)	0.410
	Stream fringe	42	2.4	0.06	(0.01-0.24)	<0.001*
	Latex collection cup	321	11.8	0.29	(0.05-1.86)	0.192
	Tree trunk	50	24.0	0.94	(0.18-4.90)	0.939
	Tyre	65	46.2	2.97	(1.00-8.82)	0.051
	Other natural waterbodies°	30	23.3	0.43	(0.09-2.15)	0.304
	Other artificial waterbodiesˆ	30	36.7	2.37	(0.58-9.76)	0.231
	Puddle	130	24.6	1		

Generalized estimating equations were used to calculate the P*-value with odds ratio (OR) and 95% confidence interval (95% CI) ˜other invertebrates, including other mosquito larvae ° other natural waterbodies (pools and leaf puddles) ˆ other artificial waterbodies (ponds, ditches and rice fields) ˜other invertebrates, including other mosquito larvae *significantly different,* P*<0.05*

Table 6.4 Multivariate analysis of factors associated with *Culex* pupae presence

		n waterbodies surveyed	% *Culex* pupae present	OR	(95% CI)	*P*
Study site	**Thinkeo**	480	5.2	4.50	(2.18-9.31)	<0.001*
	Silalek	338	5.9	5.76	(3.53-9.40)	<0.001*
	Houayhoy	561	1.3	1		
Habitat	**Mature rubber plantation**	621	4.3	1.62	(0.76-3.46)	0.210
	Immature rubber plantation	179	4.5	1.74	(0.35-8.68)	0.502
	Village	579	2.9	1		
Diameter	**<10 cm**	566	1.6	0.28	(0.10-0.85)	0.024*
	10-100 cm	677	4.3	0.36	(0.20-0.67)	0.001*
	>100cm	136	10.3	1		
Volume	**50 mL – 5 L**	464	4.3	0.90	(0.52-1.54)	0.700
	> 5L	218	6.0	0.74	(0.47-1.18)	0.206
	< 50 mL	697	2.7	1		
Shade	**absent**	153	2.6	0.77	(0.37-1.63)	0.495
	Sometimes	814	4.9	2.44	(1.16-5.10)	0.018*
	Always	412	1.9	1		
Vegetation	**absent**	1289	3.8	2.76	(0.89-8.61)	0.080
	present	90	3.3	1		
Other invertebrates˜	**Absent**	654	2.9	0.76	(0.39-1.49)	0.423
	Present	725	4.6	1		
Waterbody type	**Cut bamboo**	156	1.3	0.18	(0.02-1.28)	0.087
	Leaf axil	102	1.0	0.11	(0.01-1.72)	0.114
	Discarded garbage	140	0.0			
	Container for water <10 L	186	0.5	0.11	(0.03-0.39)	0.001*
	Container for water >10 L	127	5.5	0.87	(0.36-2.11)	0.751
	Stream fringe	42	0.0			
	Latex collection cup	321	3.4	0.35	(0.08-1.59)	0.173
	Tree trunk	50	6.0	0.58	(0.08-3.97)	0.576
	Tyre	65	12.3	1.51	(0.59-3.89)	0.389
	Other natural waterbodies°	30	10.0	0.57	(0.08-4.40)	0.594
	Other artificial waterbodiesˆ	30	10.0	1.01	(0.19-5.25)	0.994
	Puddle	130	10.0	1		

Generalized estimating equations were used to calculate the P*-value with odds ratio (OR) and 95% confidence interval (95% CI) ˜other invertebrates, including other mosquito larvae ° other natural waterbodies (pools and leaf puddles) ˆ other artificial waterbodies (ponds, ditches and rice fields) *significantly different,* P*<0.05*

Table 6.5 Multivariate analysis of factors associated with *Anopheles* larvae presence

		n waterbodies surveyed	% *Anopheles* larvae present	OR	(95% CI)	*P*
Study site	**Thinkeo**	480	1.0	1.79	(0.85-3.79)	0.127
	Silalek	338	3.3	1.82	(0.90-3.68)	0.097
	Houayhoy	561	0.9	1		
Habitat	**Mature rubber plantation**	621	1.1	1.73	(0.40-7.45)	0.462
	Immature rubber plantation	179	5.6	4.50	(0.91-22.33)	0.066
	Village	579	0.7	1		
Diameter	**<10 cm**	566	0.2	0.03	(0.00-2.22)	0.107
	10-100 cm	677	1.5	0.29	(0.02-3.78)	0.347
	>100cm	136	7.4	1		
Volume	**50 mL – 5 L**	464	2.8	1.61	(0.14-18.44)	0.701
	> 5L	218	2.3	0.59	(0.08-4.58)	0.615
	< 50 mL	697	0.4	1		
Shade	**absent**	153	3.3	0.92	(0.13-6.68)	0.933
	Sometimes	814	1.4	0.85	(0.17-4.29)	0.842
	Always	412	1.2	1		
Vegetation	**absent**	1289	1.2	0.47	(0.22-1.02)	0.055
	present	90	5.6	1		
Other invertebrates˜	**Absent**	611	0.7	0.29	(0.09-0.88)	0.030*
	Present	768	2.2	1		
'Waterbody type	**Cut bamboo**	156	0.6	0.77	(0.02-35.51)	0.895
	Leaf axil	102	0.0			
	Discarded garbage	140	0.0			
	Container for water <10 L	186	0.0			
	Container for water >10 L	127	0.8	0.38	(0.02-9.56)	0.558
	Stream fringe	42	0.0			
	Latex collection cup	321	0.3	0.14	(0.02-1.36)	0.091
	Tree trunk	50	4.0	1.99	(0.37-10.61)	0.419
	Tyre	65	1.5	0.30	(0.04-2.49)	0.266
	Other natural waterbodies°	30	3.3	0.62	(0.07-5.56)	0.667
	Other artificial waterbodiesˆ	30	6.7	0.77	(0.23-2.59)	0.669
	Puddle	130	9.2	1		

Generalized estimating equations were used to calculate the P*-value with odds ratio (OR) and 95% confidence interval (95% CI) ˜other invertebrates, including other mosquito larvae ° other natural waterbodies (pools and leaf puddles) ˆ other artificial waterbodies (ponds, ditches and rice fields) ˜other invertebrates, including other mosquito larvae *significantly different,* P*<0.05*

6.4.3 Density of immature mosquito stages in waterbodies

The most immature *Aedes* mosquito productive waterbody was cut bamboo, with highest average density of *Aedes* immature stages found in tyres (Table 6.6). The most productive waterbody for immature *Culex* mosquitoes were tyres, with highest densities per waterbody found in water containers > 10 L, pools, ditches and tyres (Table 6.7). Highest total number and density of *Anopheles* immature stages were collected from puddles (Table 6.8).

Table 6.6 Density of *Aedes* immature stages collected per waterbody type in comparison to cut bamboo

	Total number of mosquitoes collected	Mean number of mosquitoes per waterbody				
	n_{total}	n_{mean}	(95% CI)	OR	(95% CI)	*P*
Leaf axil	461	10.2	(7.4-13.1)	0.20	(0.12-0.33)	<0.001*
Discarded garbage	567	16.7	(9.2-24.1)	0.26	(0.14-0.46)	<0.001*
Latex collection cup	1,523	14.2	(10.9-17.5)	0.35	0.22-0.56)	<0.001*
Tree trunk	625	24.0	(13.5-34.6)	0.73	(0.37-1.44)	0.358
Leaf puddle	123	9.5	(1.4-17.5)	0.26	(0.12-0.58)	0.001*
Tyre	2,480	75.2	(42.2-108.1)	2.12	(1.01-4.46)	0.048*
Other natural habitats°	54	6.8	(0-14.8)	0.03	(0.01-0.07)	<0.001*
Other artificial habitats ˆ	2,570	29.5	(18.11-41.0)	0.71	(0.32-1.57)	0.391
Cut bamboo	3,065	31.6	(25.1-38.1)	1		

Generalized estimating equation to calculate the P*-value with odds ratio (OR) and 95% confidence interval (95% CI) ˆother artificial waterbodies (water containers, ponds, ditches and rice fields) °other natural waterbodies (puddles, pools and stream fringes) *significantly different,* P*<0.05*

Table 6.7 Density of *Culex* immature stages collected per waterbody type in comparison to tyres

	Total number of mosquitoes collected	Mean number of mosquitoes per waterbody				
	n_{total}	n_{mean}	(95% CI)	OR	(95% CI)	*P*
Cut bamboo	121	10.1	(2.8-17.4)	0.04	(0.01-0.17)	<0.001*
Discarded garbage	214	23.8	(8.7-38.8)	0.10	(0.02-0.38)	0.001*
Containers for water <10 L	940	49.5	(24.3-74.6)	0.28	(0.09-0.81)	0.019*
Containers for water >10 L	1,724	66.3	(30.0-102.6)	0.84	(0.28-2.51)	0.756
Puddle	979	30.6	(19.5-41.7)	0.26	(0.09-0.71)	0.009*
Pools	191	95.5	(0-85.5)	0.66	(0.08-5.27)	0.696
Ponds Rice fields	73	10.4	(4.0-16.9)	0.19	(0.05-0.79)	0.022*
Latex collection cups	775	18.9	(12.4-25.4)	0.05	(0.02-0.13)	<0.001*
Tree trunk	205	17.1	(3.0-31.2)	0.14	(0.04-0.47)	0.001*
Ditch	308	77.0	(0-164.9)	0.88	(0.20-3.79)	0.858
Other natural waterbodies°	121	9.3	(1.9-16.7)	0.03	(0.01-0.09)	<0.001*
Tyre	2,265	75.5	(25.4-125.6)	1		

Generalized estimating equation to calculate the P-*value with odds ratio (OR) and 95% confidence interval (95% CI) °other natural waterbodies (leaf axils, stream fringes and leaf puddles) *significantly different,* P*<0.05*

Table 6.8 Density of *Anopheles* immature stages collected per waterbody type in comparison to puddles

	Total number of mosquitoes collected	Mean number of mosquitoes per waterbody				
***Anopheles* mosquitoes**	n_{total}	n_{mean}	(95% CI)	OR	(95% CI)	*P*
Natural waterbodies°	46	15.3	(0-58.1)	0.226	(0.132-0.389)	<0.001*
Artificial waterbodiesˆ	25	4.2	(0.5-8.0)	0.044	(0.024-0.082)	<0.001*
puddles	106	8.8	(0-18.5)	1		

Generalized estimating equation to calculate the P-*value with odds ratio (OR) and 95% confidence interval (95% CI) °Natural waterbodies (leaf axils, pools, stream fringes, tree trunk and leaf puddles) ˆArtificial waterbodies (cut bamboo, discarded garbage, containers for water <10 L, containers for water >10 L, ponds, latex collection cups, ditch, tyre and rice fields) *significantly different,* P*<0.05*

6.4.4 Emerged adult mosquitoes

More than 27 % of the 29,537 larvae and pupae collected during the larval survey emerged in the field laboratory (8,107/29,537). A total of 8,075 adult mosquitoes were identified to 64 species, including 16 *Aedes*, 15 *Culex* and eight *Anopheles* species. Thirty-two adult mosquitoes could not be identified after emergence. Approximately 76 % of emerged *Aedes* mosquitoes were identified as *Aedes albopictus* and 56 % of emerged *Culex* mosquitoes were identified as *Cx. brevipalpis* (Appendix 14). For *Anopheles* mosquitoes, 84 % of emerged mosquitoes were identified as *Anopheles dirus s.l.*

From discarded garbage, tree trunk, tyre, latex collection cup, water container <10 L, water container >10 L and leaf puddle most often the mosquito species *Ae. albopictus* was identified (Appendix 15). From cut bamboo both *Ae. albopictus* and *Ae. annandalei* were common while in leaf axils generally the species *tripteroides* and *mimomyia* were found. In puddles and ponds *Cx. brevipalpis* most frequently emerged, *Cx. fuscocephalus* from pools and *Cx. quinquefasciatus* from ditches. Only one mosquito emerged from the stream fringes which was identified to *Cx. sitiens.* From the rice fields only three mosquitoes emerged which were all *Cx. vishnui s.l.*

6.4.5 Waterbody preference of mosquitoes in the different habitats

6.4.5.1 Mature rubber plantations

The greatest number of waterbodies were found in the mature rubber plantations (621/1,379) (Figure 6.2). About 56 % of these waterbodies contained mosquito larvae and/or pupae (349/621). From the mature rubber plantations the highest number of mosquito positive waterbodies (48 %, 349/724) were identified, with the highest number of immature mosquitoes collected (46 %, 13,425/29,537) (Figure 6.2). A high density of these immature mosquitoes were *Aedes* and *Culex* larvae (Figure 6.3). Furthermore, a high proportion of the *Culex* pupae were collected in the mature rubber plantations (Figure 6.3). About 29 % of the collected immature mosquitoes emerged in the field laboratory (3,895/13,425). Nearly 40 % of these emerged mosquitoes were *Ae. albopictus* (1,533/3,895) and 12 % *Cx. brevipalpis* (476/3,895). Compared to other habitats, the number of emerged *Ae. albopictus* were highest in the mature rubber plantations (Appendix 16).

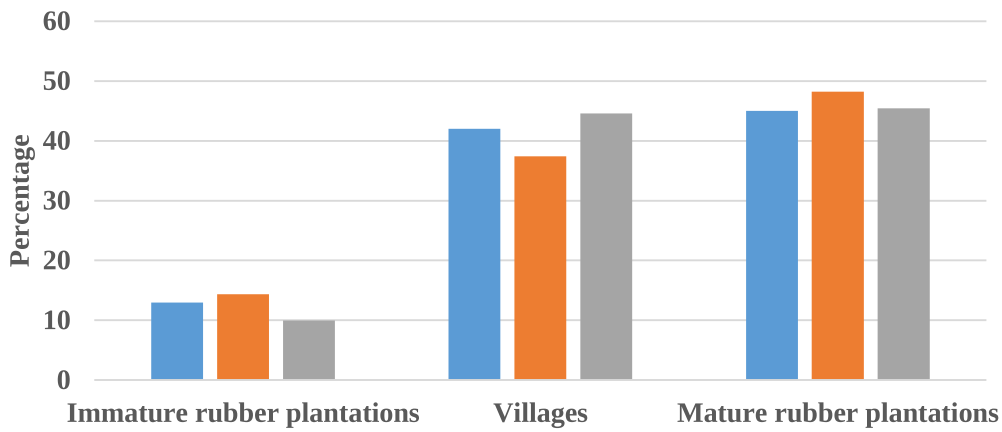

Figure 6.2 Relative importance of the different habitats in relation to the number of waterbodies identified and immature mosquitoes collected (■ % waterbodies found, ■ % waterbodies positive for immature mosquitoes, ■ % contribution to total immature mosquitoes collected)

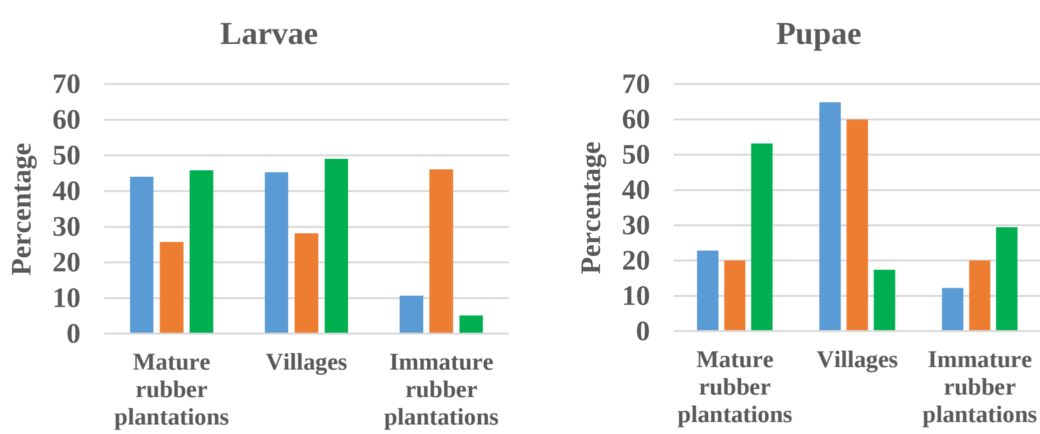

Figure 6.3 Relative importance of the different habitats in relation to the number of *Aedes*, *Anopheles* and *Culex* larvae and pupae collected (■ *Aedes*, ■ *Anopheles*, ■ *Culex*)

In the mature rubber plantations the tyres and latex collection cups contributed the most to the total number of larvae collected (Figure 6.4). The number of successfully reared *Ae. albopictus* were highest for the latex collection cups (Appendix 17). A total of 562 of the 1,533 *Ae. albopictus* emerged from the latex cups. From the water container > 10 L, a total of 21 of the 56 *Cx. quinquefasciatus* emerged (Appendix 17). Only one *An. dirus s.l.* was identified from the mosquito collections in the mature rubber plantations. This sample emerged from the puddle.

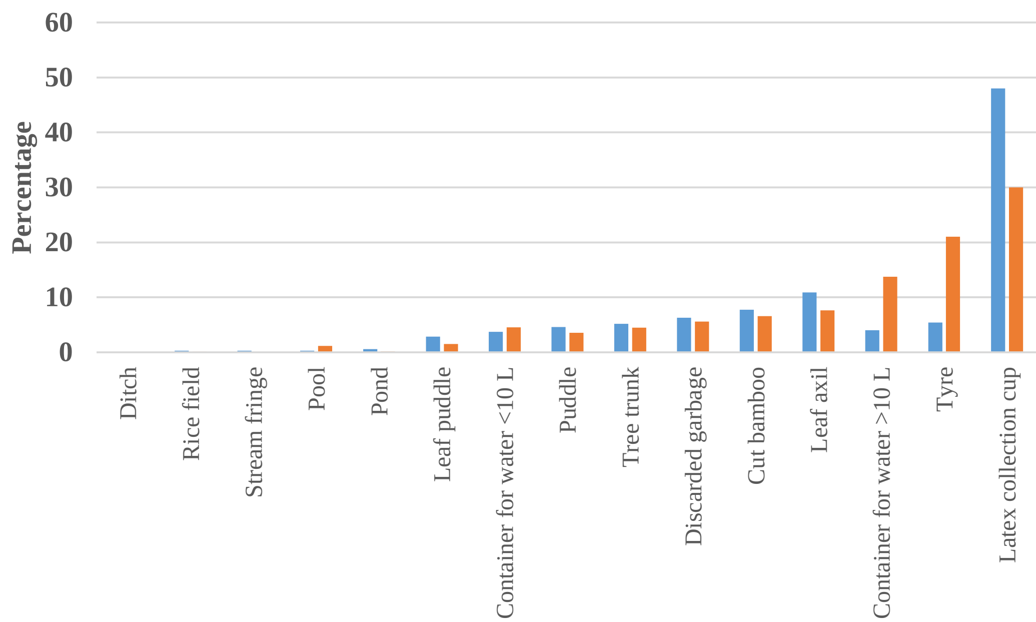

Figure 6.4 Relative importance of the mosquito positive waterbody types in mature rubber plantations and their relation to the total number of larvae collected (■ % positive for larvae ■ % contribution to total larvae population)

6.4.5.2 Immature rubber plantations

In the immature rubber plantations the lowest number of waterbodies were found (179/1,379) (Figure 6.2). About 58 % of these waterbodies were positive for mosquitoes (104/179). The total number of immature mosquitoes collected in the immature rubber plantations was 10 % of the total (2,949/29,537). Compared to the other habitats, *Anopheles* larvae were most common in the immature rubber plantations (Figure 6.3). About 37 % of the immature mosquitoes collected in the immature rubber plantations were successfully reared to adults (1,093/2,949). The most common mosquitoes were *Ae. albopictus* with 22 % (242/1,093) and *Cx. brevipalpis* with 14 % (148/1,093). Furthermore, a majority of the emerged *An. dirus s.l.* were collected from the immature rubber plantations (Appendix 16).

In the immature rubber plantations the leaf axils and cut bamboo contributed the most to the total number of larvae collected (Figure 6.5). The number of successfully reared *Ae. albopictus* were highest from the cut bamboo collections, where 121 of the 242 *Ae. albopictus* mosquitoes were identified (appendix 18). From the water container > 10 L, a majority of the *Cx. quinquefasciatus* emerged (54/74) (Appendix 18). A majority of the 26 *An. dirus s.l.* identified, were collected from the puddles (65 %, 17/26). Furthermore, 7 *An. dirus s.l.* were identified from tree trunks (Appendix 18).

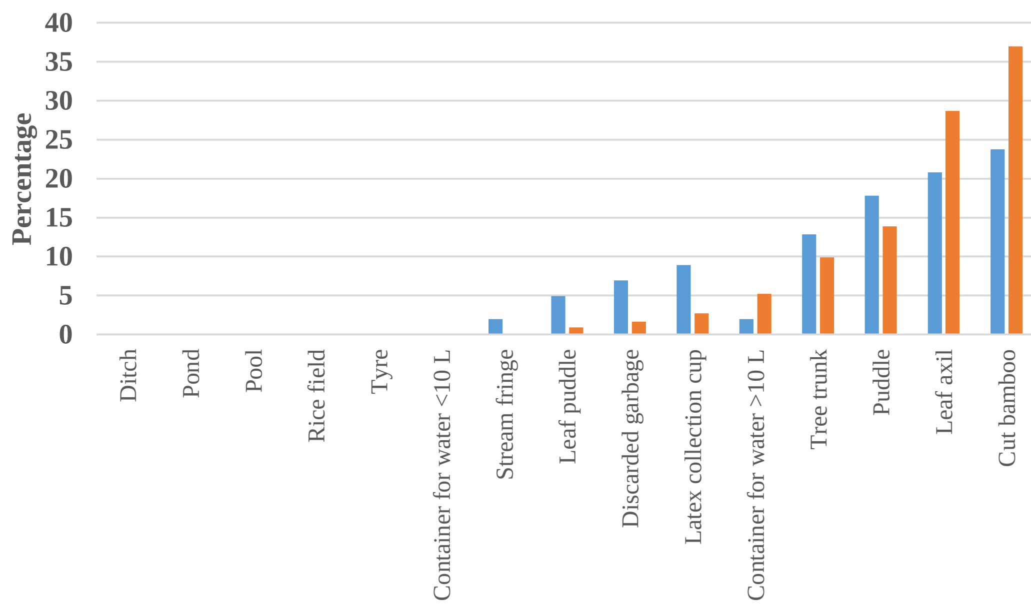

Figure 6.5 Relative importance of the mosquito larvae positive waterbody types in immature rubber plantations and their relation to the total number of larvae collected (■ % positive for larvae ■ % contribution to total larvae population)

6.4.5.3 Villages

In the villages the second greatest number of waterbodies were found (579/1379) (Figure 6.2). Slightly less than half of these waterbodies were positive for immature mosquitoes (47 %, 271/579). From these positive waterbodies 45% of all immature mosquitoes were collected (13,163/29,537), which was only slightly lower than the number collected in the mature rubber plantations (Figure 6.2). Similar to the mature rubber plantations, a high density of these immature mosquitoes were *Aedes* and *Culex* larvae (Figure 6.3). Additionally a high proportion of the *Aedes* and *Anopheles* pupae were collected in the villages (Figure 6.3). About 24 % of the collected immature mosquitoes were successfully reared to adults (3,119/13,163). Most common adult mosquitoes that emerged from the villages were again *Ae. albopictus* (34.4 %, 1,070/3,119) and *Cx. brevipalpis* (7.1 %, 223/3,119). Compared to the other habitats, the highest number of the vector *Cx. quinquefasciatus* emerged in the villages (Appendix 16).

In the villages the water containers > 10 L contributed the most to the number of larvae collected. The second most important contributor were cut bamboo (Figure 6.6). The number of *Ae. albopictus* collected were highest for waterbodies < 10 L (appendix 19), where 345 of the 1,070 *Ae. albopictus* were collected. The second and third most important waterbody for *Ae. albopictus* emergence were tyres (243/1,070) and water containers > 10 L (231/1,070) (Appendix 19). For *Cx. quinquefasciatus,* water containers < 10 L and > 10 L were the most important waterbodies (57/120 and 47/120, respectively) (Appendix 19). No *An. dirus s.l.* were reared from the larvae collected in the villages.

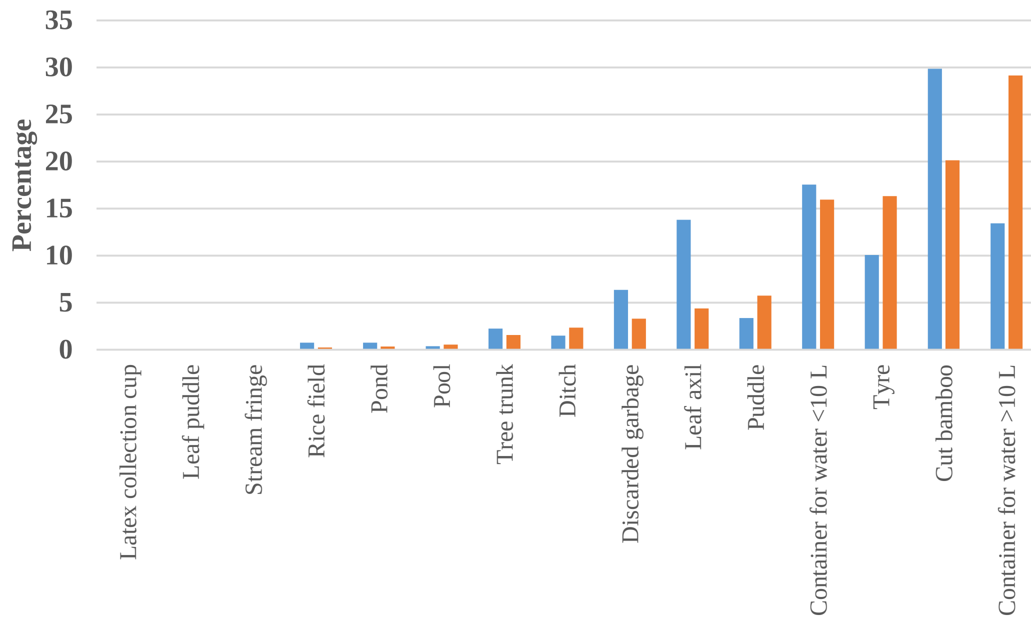

Figure 6.6 Relative importance of the mosquito larvae positive waterbody types in villages and their relation to the total number of larvae collected (% positive for larvae % contribution to total larvae population)

6.5 Discussion

In an effort to better understand where the important vector species breed and their waterbody characteristics, a survey of the mosquito breeding sites in rubber plantations and nearby villages were conducted. The data show that, although many breeding sites were found in villages, mature rubber plantations contained more suitable sites for *Aedes* and *Culex* larvae while immature rubber plantations had more suitable sites for *Anopheles* immature stages. *Aedes* immature stages, including the vector species *Ae. albopictus*, were most common in small temporary waterbodies such as cut bamboo, tyres and tree trunks. *Culex* immature stages were also most common in tyres, with also a high density per waterbody found in water containers (both < and > 10 L) and puddles. More than half of the emerged *Culex* were identified as the non-vector *Cx. brevipalpis* and a high number were identified as the Lymphatic filariasis and possible Japanese encephalitis (JE) vector *Cx. quinquefasciatus* [163, 165-167]. *Anopheles* larvae, mostly *An. dirus s.l.*, were most common in puddles. As there is risk of exposure to vector-borne diseases in rubber plantations, there is a need to control mosquitoes in these habitats. As some species have similar preference for breeding sites, focus can be on a few key waterbody types using environmental management in a community-based manner.

Aedes larvae and pupae were the most frequently collected genus during this study, with collections generally occurring in small waterbodies such as cut bamboo, tyres and tree trunks. A large majority of the emerged *Aedes* mosquitoes were identified to be the important dengue and chikungunya vector *Ae. albopictus*. The main waterbodies identified for *Ae. albopictus* mosquitoes in this study (cut bamboo, tyres, containers for water, latex collection cups, discarded garbage and tree trunks) were similar to larval studies in other SEA regions where the mosquitoes were generally found in temporary water of small volume and small diameter, and water containers [135, 457-462].

Culex were collected from a wider variety of waterbodies, with most common waterbodies identified as tyres, containers > 10 L, puddles, pools and ditches. Moreover, *Culex* larvae presence was associated with the presence of other invertebrates, including other mosquito species. This association with other invertebrates is dependent on the mosquito species and its interaction with the local habitat, with literature also showing high variation in tendencies of *Culex* mosquitoes preferring waterbodies with vegetation, turbidity and other biotic and abiotic factors [461, 463-470]. In this study *Culex brevipalpis* were most often collected from puddles, latex collection cups and ponds. As this mosquito species is not known to transmit diseases, few studies have been done on their waterbody preference. One study notes *Cx. brevipalpis* to be very diverse in breeding sites with both natural and artificial waterbodies, permanent and temporary waterbodies, shade and partially shaded waterbodies preferred [461]. We identified *Culex quinquefasciatus* in ditches, ponds, water container > 10

L and puddles. Similar to *Cx. brevipalpis* waterbody preference, the *Cx. quinquefasciatus* larval preferences is broad with no clear preference for specific characteristics, except the liking of turbid waters [461, 466-468].

Anopheles present waterbodies were found in low numbers throughout the survey. A majority of emerged *Anopheles* larvae were identified as *An. dirus s.l.*, an important vector of malaria. The *Anopheles* larvae were typically found in temporary waterbodies such as puddles, tree trunks, other natural waterbodies (pools and leaf puddles) and other artificial waterbodies (ponds, ditches and rice fields). Furthermore, *Anopheles* larvae presence was associated with the presence of other invertebrates, including other mosquito species. Several studies in SEA have similarly identified small, temporary and shaded puddles to be related to *An. dirus s.l.* abundance and distribution [109, 199, 370]. Even though *An. maculatus s.l*. and *An. minimus s.l.* are important possible malaria vectors collected from the study site (chapter 5), no *An. maculatus s.l.* and *An. minimus s.l.* larvae were collected during the survey. Bordering habitats should thus be surveyed in the future to understand if *Anopheles* mosquitoes possibly breed in these neighbouring habitats and enter the surveyed habitats for a blood meal.

The greatest number of immature mosquitoes were found in the mature rubber plantations, with tyres and latex collection cups identified as the most important *Aedes* (including *Ae. albopictus*) and *Culex* mosquito breeding sites. A majority of the *Cx. quinquefasciatus* were collected from water containers > 10 L and puddles. Furthermore, a large number of the *Anopheles* mosquitoes were collected from puddles. In the immature rubber plantations the number of immature mosquitoes were low. However the highest number of *Anopheles* immature stages were collected in this habitat, with a high number of *An. dirus s.l.* collected from puddles. There is a need to control mosquitoes in the rubber plantations, with the population at risk of exposure to mosquitoes when working in or traveling through the plantations and the possible risk of the mosquitoes moving to the surrounding habitats. In the mature rubber plantations the discarded garbage, including unused tyres, should be collected and disposed properly in covered containers to avoid exposure to rain. When latex tapping is not conducted for more than one week, all latex collection cups should be turned upside down to avoid *Ae. albopictus* and to a lesser degree *Cx. brevipalpis* breeding. This is also important when latex is not collected for more than one week as dengue vectors can breed in water that collects over the latex layer. After latex tapping is completed for the season, all latex collection cups should be collected in roofed sheds to diminish breeding sites. Mud roads in the plantation contain road puddles where *An. dirus s.l.* and *Cx. quinquefasciatus* mosquitoes breed. These puddles should be levelled in both the mature and immature rubber plantations by filling the cavities with gravel. Tarmac roads would reduce pooling more permanently, if good drainage is provided. All large water containers, generally located close to the rubber worker houses, should be covered with a lid, netting or treated with an insecticide such as temephos and *Bacillus thuringiensis israelensis* (bti) [471] to decrease *Cx. quinquefasciatus* and *Ae. albopictus* numbers. The use of larvicides are not necessary in the rubber plantations we investigated, as the main mosquito larvae waterbodies can easily be decreased by consistent use of the environmental measures mentioned.

In villages more than half of the cut bamboo waterbodies were found, mostly used for fences of vegetable gardens and in chicken houses. Additionally, water containers (both < and > 10 L) and tyres were found to be important mosquito breeding sites. In the villages unused bamboo-constructions should be broken down and cleared. Unused bamboo poles should be properly stored away from the rain and used bamboo should be cut at the joint, cut in length or filled to the rim with stones to ensure water cannot collect inside. To further decrease *Ae. albopictus*, and to simultaneously decrease *Cx. quinquefasciatus* mosquitoes, the small and large water containers surrounding the houses should be covered with a lid, netting or treated with *Bacillus thuringiensis israelensis* (bti) [471]. The use of temephos or other insecticides is not recommended, as *Aedes albopictus* larvae in the area have low levels of resistance to temephos, malathion and permethrin [444]. Some water containers >10 L cannot be covered. As these waterbodies are few, fixed and findable, the use of predaceous insects or fish can be considered. Although there is lack of evidence that fish can be effective control agents [271], the use of *Mesocyclops*, a copepod that feeds on mosquito larva, has been successful in Lao PDR and neighbouring Vietnam [270, 367].

To control vector-borne diseases in our study area overall community-based IVM campaigns are recommended alongside prompt and effective treatment of vector-borne diseases. To achieve sustainable larval control, the recommendations from larval surveys should be communicated and implemented during regular community-based mosquito source reduction activities. A community-based approach is considered essential for sustainable and effective vector control programs [239, 471-474], although a systematic review did not give confirmation [475]. Community-based control campaigns are focussed on the education of the local community on vector-borne diseases and the active involvement of the local population in the implementation of the vector control. This involvement increases success of IVM as it ensures the control methods are on par with the local epidemiological and cultural setting [239].

6.6 Conclusion

Rubber plantations have more suitable breeding habitats for *Aedes*, *Culex* and *Anopheles* species than villages. The findings furthermore suggest that mature rubber plantations have similarly suitable breeding habitats for *Ae. albopictus, Cx. quinquefasciatus* and *An. dirus s.l.* species as the rural villages. Current focus on village larval control in Lao PDR should thus broaden to include rubber plantations. The present study suggests that *Ae. albopictus* control should focus on the tyres and latex collection cups in the mature rubber plantations. Additionally, water containers should be covered to decrease *Ae. albopictus* and *Cx.*

quinquefasciatus. Moreover the road puddles in both the mature and immature rubber plantation areas should be cleared to decrease *Anopheles dirus s.l.* numbers. Larval control in villages should focus on decreasing waterbodies in cut bamboo, tyres and water containers, possibly with the additional use of *Mesocyclops* in permanent large water containers. Vector control intervention strategies should target these water bodies in a community-based manner, involving the rubber workers and villagers in the implementation of the environmental management strategies. This study has highlighted the importance of surveying habitats outside the villages for mosquito breeding sites, as these habitats can be important breeding sites for vector mosquitoes.

7 General discussion

The recent establishment of 147,000 ha of rubber plantations in Lao PDR has changed the natural landscape of the country. In this thesis I assessed how these changes may affect the risk from vector-borne diseases in rubber plantations in northern Laos.

The main objectives of this study were to determine:

- The most efficient method for surveying outdoor biting adult mosquitoes in rural Laos (chapter 3).
- Whether the number and variety of adult mosquitoes found in rubber plantations differed from that in secondary forest or local villages (chapter 4).
- How the risk of vector-borne diseases varied with human activities (chapter 5).
- The major mosquito breeding sites in rubber plantations and local villages (chapter 6).

7.1 Contributions to the field of medical entomology in Lao PDR

This study has increased our understanding of mosquito vectors in northern Lao PDR. I demonstrated that the Human-baited Double Net trap (HDN) is an efficient and ethically sound method for surveying outdoor-biting adult mosquitoes in rural Lao PDR. Using this method I have conducted the first study in Lao PDR assessing mosquito dynamics in rubber plantations. High species diversity was found in all habitats investigated (secondary forests, immature rubber plantations, mature rubber plantations, villages) including vectors of dengue, Japanese encephalitis (JE), lymphatic filariasis and malaria. Working in rubber plantations increased risk of dengue vector exposure compared to staying in the village with risk exacerbated when people also lived in these plantations. The highest risk of dengue was found in the secondary forests with negligible exposure risk in the village. Malaria vector exposure risk was highest for villagers that visited the forest during the day and lowest for rubber workers living in the plantations. It is the first study to stress the importance of rubber plantations in the dengue disease dynamics in Lao PDR.

I also identified the main breeding sites for mosquitoes in the rubber plantations. Larval control in the rubber plantations should focus on preventing water accumulation in latex collection cups, tree trunks, cut bamboo, removal of puddles and garbage to decrease number of *Aedes albopictus* and *Anopheles dirus s.l.* mosquitoes. Currently, deployment of long lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) in villages accounts for the majority of vector control methods used in Lao PDR. This study highlights the importance of implementing additional mosquito control interventions such as personal protection methods and larval control in the secondary forests and rubber plantations. The results from this study have been translated into clear recommendations which have been communicated to the public health workers, governments and those working in the rubber industries of Lao PDR.

The work described in this thesis is novel since it represents a comprehensive approach to assessing disease risk and control based on both the studies of adult and immature mosquito stages as well as the behaviour of people in different habitats in northern Lao PDR. To my knowledge no entomological studies have been published on the mosquito population in the north of the country with relatively few entomological studies from central and southern Lao PDR [96, 97, 101-103, 107, 108, 110, 111, 135, 136, 217, 340, 345, 346, 367]. The entomological studies that have been conducted in Lao PDR focus mostly on malaria vectors [96, 97, 102, 107, 108, 110, 111, 346] with few studies on dengue and JE vectors [135, 136, 217, 340, 367]. However, this emphasis is slowly shifting. A recent publication in Lao PDR underlined the importance of non-malarial vector-borne diseases present in Lao PDR including dengue, scrub typhus and JE virus infections [172]. Data on disease vectors in Lao PDR are still limited for many vector species with few descriptions of diseases transmitted by fleas, mites, ticks and sand-flies [175, 476-487]. The high diversity of mosquito species identified in this study, including a variety of vector species, have contributed to knowledge of the general mosquito fauna in Lao PDR and to knowledge of vector species dynamics and behaviour.

This study has been a first attempt at using both Rapid Rural Appraisals (RRA) and surveys in the identification of behaviours that increase the risk of exposure to dengue, JE and malaria vectors. These methods not only provided information on the human behaviour of the local population, it also gave the villagers a sense of ownership by involving them in the study from the start. The results emphasize the importance of including local human behaviour into the risk analysis, with risk to vector-borne diseases changing depending on the population movement. In Lao PDR behavioural analysis have rarely been included in vector-borne disease studies, even though this is especially important in a country with 57 recognized ethnic groups. Future medical entomology studies should include small behavioural studies like RRA's and surveys to relate entomological data to vector exposure risk for the local population.

From the beginning of this study I have worked closely with both the Ministry of Health and Ministry of Forestry and Agriculture of Lao PDR. This has been formally achieved by the signing of a Memorandum of Understanding (MOU) at the beginning of the project between the National Agriculture and Forestry Research Institute (NAFRI) and Institut Pasteur du Laos. Practically this cooperation entailed the organization of regular stakeholder meetings at country, provincial, district and village level which was attended by experts from neighbouring countries, representatives of both ministries and rubber stakeholders. During the different stakeholders meetings NAFRI regularly complemented my presentations with presentations on the rubber plantation dynamics from an agricultural perspective. Furthermore, members of the NAFRI staff supported us during the collection of environmental data and visited us in the field to see the fieldwork in action. This study is the first study in Lao PDR to have integrated the Ministry of Health and Ministry of Forestry and Agriculture in one project to mitigate vector-borne disease risk. This approach has been novel for the governmental officers involved and has created awareness of the overlap between the goals of different ministries. The study has resulted in inclusion of my recommendations in the five year Strategic plan for vector control, organized by the Ministry of Health, and has resulted in the contribution of one chapter on 'health risks for rubber workers' in an information book on rubber plantations from the Ministry of Forestry and Agriculture. This book will be published in 2016 and will be distributed to the rubber industry, provincial offices and district offices all over Lao PDR. The cooperation between the Ministry of Health and the Ministry of Agriculture from the beginning of this project has facilitated this study in achieving its study objectives and has improved the communication of my recommendations to the important stakeholders in Lao PDR and the region.

7.2 Key findings

Below I describe the key findings for each of the four research objectives.

7.2.1 Sampling outdoor-biting mosquitoes

Estimating the number of mosquito bites per person per day or night is a key metric used for quantifying the risk of infection with mosquito-borne pathogens. This human-biting rate is typically estimated using human landing catches (HLC), with which mosquitoes are collected off exposed limbs. However, this method potentially exposes individuals to infective mosquito bites. There has been no suitable alternative method identified for outdoor mosquito collections in South-East Asia (SEA). Hence I tested a range of sampling techniques which do not expose participants to mosquito bites. In the first experiment I compared the human-baited double net trap (HDN), CDC light trap, BG-Sentinel trap and BG-Suna trap. The HDN trap method collected 11 to 44 times more mosquitoes than the other traps. In the succeeding experiment the trap collecting the highest number of mosquitoes, the HDN trap, was compared directly against HLC to determine whether it could be an alternative for outdoor mosquito sampling. The HDN collected similar numbers of *Anopheles* (Rate Ratio (RR) 1.2, 95 % confidence interval (CI) 0.6-2.2) and *Culex* (RR 1.3, 95 % CI 0.7-2.2) mosquitoes as HLC, but under-estimated the numbers of *Ae. albopictus* by half (RR 0.45, 95 % CI 0.27-0.77). It should be recognised that both HDNs and HLCs are only proxy estimates of exposure. It is likely that HDNs slightly under-estimates biting rates, whilst HLCs over-estimates biting rates. The HDN is a simple and cheap method to estimate the human-biting rate outdoors without exposing collectors to mosquito bites. This trapping method will be an important tool in areas where no chemical prophylaxis can be used for the endemic vector-borne diseases.

The main limitation of the comparison study between HLC and HDN has been the focus of the comparison study on the total number of mosquitoes collected using the two methods. Before HDNs can be recommended as an alternative to HLC throughout SEA, additional studies are needed to confirm the catching efficiency of HDNs against individual vector species. Power calculations for individual species will result in more replicates for the comparison between HLC and HDNs. Furthermore, the sample size calculations should take into account the parity rates of the mosquitoes collected. Additionally, for *Anopheles* collections the malaria infection rates will be of interest. The comparisons should be conducted in a number of different sites throughout the world to understand its efficiency in different habitats with different mosquito populations.

Apart from finding a suitable alternative to HLC using a human participant, it is important to investigate alternatives that do not need a human participant. These methods are less labour intensive, easy to install and need little preparation. Furthermore, there is no variation in catching efficiency between the traps and, most importantly, there is no ethical concern. One of the main challenges for entomology in the future will be to develop a trap and lure, which attracts similar number and diversity of mosquitoes as traps involving a human participant. These traps should be developed in such a way that the mosquitoes remain intact, so that parity rates and sporozoite rates can be identified. These traps can be used for vector surveillance. A study in Kenya showed that the collection of a large numbers of the malaria vector mosquito *Anopheles funestus* using the BG Suna trap, decreased the disease prevalence in the local population [488]. The traps could therefore also become an important vector control method.

7.2.2 Adult mosquito diversity and abundance

In Lao PDR no study on the vector-borne dynamics in rubber plantations have been conducted, even though both malaria and dengue outbreaks occur regularly in SEA rubber plantations. I therefore carried out a study using HDN traps to compare the abundance and diversity of adult mosquitoes in four rural habitats common in northern Lao PDR: the secondary forests, the immature rubber plantations, the mature rubber plantations and the local villages. A total of 24,927 female mosquitoes were collected during the nine months of adult mosquito surveys, including 61 species not documented in the country before. This study showed that mosquito abundance was highest in the secondary forests, two to three times lower in the immature rubber plantations, four times lower in the mature rubber plantations and five to seven times lower in the villages. High species diversity was found in all habitats (Simpson's Indexes ranging from 0.82 to 0.86), including vectors of dengue, JE, lymphatic filariasis and malaria.

Aedes albopictus is the dominant mosquito in the secondary forests and rubber plantations, indicating that these natural and man-made forests could play an important role in the spread of dengue and chikungunya in the region. Furthermore, as this mosquito is an opportunistic feeder that can be a bridge vector between the sylvatic and human population [365, 489], there could be a substantial risk of emerging infectious diseases in rubber plantations. Overall there is a low risk of exposure to vectors of JE, lymphatic filariasis and malaria in all habitats investigated. However, risk of exposure to dengue vectors was considerable in both the natural and man-made forests.

An important limitation of the adult mosquito survey has been the exclusion of the rice fields and other farmland habitats. Villagers spend considerable time on their farm, possibly making these habitats important area for disease transmission. However, this information was not available during the writing of the protocol. Consequently for the designing of future entomological studies, behavioural surveys should be conducted before the writing of the protocol as to include important behavioural information of the local population. Furthermore, more detailed behavioural data can be obtained in future studies by conducting the surveys every month, instead of just at one time point. This regular collection of human behaviour data throughout the collection period is not very labour intensive and will result in more comprehensive information on the human behaviour changes throughout the year. These data could then be linked to the monthly mosquito behaviour which will give a better idea of the changes in vector-borne disease dynamics in the different habitats throughout the year. For future entomological studies I would recommend collecting basic behavioural information of the local human population before writing the protocol and regular behavioural surveys for the duration of the study to collect data on human behaviour variations.

The susceptibility tests that were conducted during this study gave a first indication of the resistance status of adult *Ae. albopictus* mosquitoes in northern Lao PDR. Currently, no WHO discriminating concentrations for *Ae. albopictus* exists. As this species is an important vector species for several diseases [365, 454, 457, 490], it is important to identify the discriminating concentrations. These concentrations can then be used as a reference for *Ae. albopictus* susceptibility tests throughout the world. The concentrations used in this study were discriminating doses for *Anopheles* mosquitoes. This preliminary study therefore needs to be repeated using the WHO discriminating concentrations for *Ae. aegypti* susceptibility tests [391]. Furthermore, future susceptibility tests should be done in a room where temperatures are more stable and maximum temperatures do not exceed 30 degrees.

7.2.3 Risky behaviours

In the next decade an estimated four and a half to six million seasonal workers will be working in rubber plantations of SEA. The rubber tappers work in the plantations during both the day and night, exposing them to different vectors compared to the local population. In this study I explored differences in human behaviour between rural villagers and rubber workers using RRA's and surveys, to assess the risk of exposure to mosquito vectors in northern Lao PDR. The human presence in the different habitats was related to the mosquito dynamics using four scenarios. In all natural and man-made forest habitats flavivirus present *Ae. albopictus* were identified. Furthermore, the dengue basic reproduction rate (R_0) was between 2.8 and 42.0, which indicates that the disease can established itself in these habitats. Risk of dengue vector exposure compared to staying in the village was 36 times higher for villagers that visited the secondary forests during the day, more than three times higher for rubber worker activity and 16 times higher when working and living in the plantations. Furthermore, when visiting the secondary forests during the day risk of exposure to JE vectors was 1.38 times higher and exposure to malaria vectors 1.3 times higher compared to staying in the village. However, contrary to my hypothesis, working and living in the plantations decreased risk of malaria exposure by 1.6 times compared to staying in the village. The malaria R_0 did indicate that once the malaria parasites are introduced to rubber plantations, malaria could establish itself and result in outbreaks with *Anopheles maculatus s.l.* the main vector in the rainy season (R_0 16.6-64.0) and *Anopheles minimus s.l.* the main vector in the dry season (R_0 319.3-44.3). This study highlights the importance of implementing mosquito control in the secondary forests and rubber plantations for the control of dengue disease and emphasizes the importance of including local human behaviour in the risk analysis.

The R_0 calculations have been a great way to compare the vector-borne disease dynamics between the different habitats in our study area. It is, however, important to keep in mind that these R_0 calculations are calculated by modelling the dynamics between certain variables using different assumption and estimates. An example is the parity data, which was only collection for two months, resulting in a very low number of results. Furthermore, many variables were not included in the model, such as landscape factors, vertical transmission of disease, sexual transmission disease, treatment of disease, heterogeneous exposure to vector mosquitoes, multiple feedings in one gonotrophic cycle and the immunity of the population [431]. The R_0 results are therefore an over-simplification of the reality, using many untested assumptions. Therefore, these results should be used with caution, keeping in mind the variables that were not included and the estimates and assumptions used.

In this chapter, I combined entomological data with human behaviour data to identify risky behaviour. There are only a limited number of ways with which human behaviour can be measured, such as GPS tracking systems [436, 437], cellular phones [438] and photo voice [439]. GPS tracking is currently seen as the best method of collecting data on human behaviour. However this method is expensive and is often perceived as intrusive by the participants. Another useful method would be the use of cellular phone data. However, this information could not be accessed for our study area. I therefore decided to focus on rapid rural appraisals and surveys to collect the data necessary. It is important to note that both methods are sensitive to memory decay and social desirability. When conducting a social study to collect data on the human behaviour, it is important to consider the different methods carefully by identifying the data necessary, the finances available and the local population dynamics and culture.

7.2.4 Mosquito breeding sites

According to the adult mosquito survey, rubber plantations are important habitats for adult vectors of dengue, JE, lymphatic filariasis and malaria. Vector control in Lao PDR has been dependent largely on the distribution of LLINs, the application of temephos in village water containers and, to a lesser extent, the use of IRS. However, these methods are not sufficient to control outdoor vectors nor breeding of mosquitoes in other waterbodies. Here I identified the major breeding sites of vector mosquitoes and their waterbody characteristics in rubber plantations and in nearby villages. During five months of survey 1,379 waterbodies were found, of which 53 % contained immature mosquito stages. Mature rubber plantations provided the highest number of mosquito breeding sites of the three habitats surveyed. In the mature rubber plantations the highest number of immature mosquitoes were collected. Rubber plantations had more suitable breeding habitats for *Aedes, Culex* and *Anopheles* species than rural villages. The main *Ae. albopictus* breeding sites were tyres and latex collection cups. The *Culex quinquefasciatus* were collected from water containers larger than 10 L and *Anopheles* mosquitoes from puddles. In the immature rubber plantations only a low number of larvae were collected. However the number of *An. dirus s.l.* collected were higher than for the other two habitats, with puddles identified as their main breeding site. In the villages tyres and water containers were important water bodies for *Ae. albopictus*, with the water containers also identified as important habitats for *Culex quinquefasciatus*.

During the adult survey *Culex vishnui*, *An. maculatus s.l.* and *An. minimus s.l.* were identified to be important vectors in the study area. However, breeding sites of these JE and malaria vectors were not identified during the larval surveys, except for three *Cx. vishnui* larvae collected from the rice fields. *Culex vishnui* generally breed in streams, pools, rice fields and ditches, *An. maculatus s.l.* in clear flowing waters exposed to the sun and *An. minimus s.l.* in clear flowing waters in the shade [370]. Although streams, pool and ditches were present in the study area, I did not identify the breeding sites of these vectors. This difficulty to identify the breeding sites could be related to the large dispersal range of these vector species. *Culex* and *Anopheles* mosquitoes are known to fly several kilometres from where they emerge in search of a blood host [370]. Vector species might therefore be breeding in bordering habitats and travelling to rubber plantations and villages in search of a blood meal. For this reason bordering habitats need to be surveyed to identify the main breeding sites of the JE and malaria vectors. If the main breeding sites of *An. maculatus s.l.* and *An. minimus s.l.* are clear slow running streams/rivers, as has been identified in neighbouring countries [206], these mosquito species can be easily controlled by canalizing the rivers close to the human settlements. *Culex vishnui* breeding in the pools and rice fields can be controlled by releasing their natural predators into the rice fields and pools.

Larval surveys are labour intensive and challenging to conduct properly. It is possible certain water bodies were not found during our larval survey. This is especially likely in the immature rubber plantations where the high and dense undergrowth made it challenging to find water containers. Moreover, it is likely that in the streams not all immature mosquitoes were identified. The streams were long, winding and often difficult to reach. Even if not all waterbodies were surveyed, this study has given a first insight into the larval breeding habitats of many different mosquito species in northern Lao PDR. More specifically, this larval survey has given highlighted the many different mosquito species that breed in the latex collection cups. The information on breeding sites of vector mosquitoes will help design evidence based vector control programs in the area, which is currently lacking.

The findings suggest that current focus on village larval control in Lao PDR should broaden to include rubber plantations. In the mature rubber plantations, the latex collection cups should be turned upside down after emptying latex, rubbish such as unused tyres should be cleared, puddles should be filled up and tree trunks removed. Additionally, all water containers should be covered. In the immature rubber plantations the puddles should also be removed. In the villages all bamboo should be filled to decrease waterbodies. Furthermore tyres should be properly stored and water containers covered. Possibly, in large water containers *Mesocyclops* can be introduced. This larval source management should be implemented as an addition to the already existing methods of controlling adult mosquitoes.

Implementation of larval control should be planned in a community-based manner every three to six months, focussing on education and participation of the population.

7.3 Study limitations

During this study focus has been on identifying risk of vector-borne diseases in the different habitats for rubber workers and local population. From this risk analysis and larval survey, recommendations were made. Regrettably these recommendations have not been implemented in this study. As no other control studies have been reported in rubber plantations, empirical evidence is needed to confirm my recommendations will decreases exposure to mosquito bites and will lead to a sustainable decrease in vector-borne disease incidence. Furthermore, vector control studies in other rubber plantation areas in the regions are necessary to identify if the recommendations can be generalized for other rubber plantation areas. The relation between vector presence and disease presence is especially important to consider, with decrease in vector numbers not necessarily resulting in a decrease in disease incidence. Throughout the world there are areas where low vector densities still result in a high number of cases [53, 197, 491, 492]. For example, in Thailand a low density of *An. dirus s.l.* is known to cause high malaria transmission [53, 197]. In the Gambia, less exposure to the vector mosquitoes lead to more clinical cases [492]. In Mali, a high density of anopheline mosquitoes lead to mosquitoes with lower vectorial capacity [491]. There are, therefore, many aspects and dynamics to consider when implementing vector control. In addition, the implementation of control measures are not linearly linked to decrease in vector-borne disease incidence due to resilience and resistance of mosquitoes to control methods [431].

On a broader scale, a limitation of this study has been the gap in knowledge on the major vector-borne diseases carried by rubber workers, with the suggestion that rubber plantations could be a nidus for pathogens spill-over from wild animals into the human populations due to their close proximity to natural forests, large work force and presence of anthropophilic vectors [54, 185, 186, 224, 232, 341]. Furthermore, limited data are available on the vector-borne diseases present in the local population with inadequate facilities for proper disease diagnostics in the district and provincial hospitals. Analysing the blood of rubber workers and villagers for vector-borne disease infections, including malaria resistant strains, will be of interest. Furthermore, the pathogen strains can be genetically related to neighbouring strains to gain information on the dispersal of disease [493] as currently little is known about the migration patterns of rubber workers and the local villagers. This is of key importance for understanding the vector-borne disease dynamics in the country. Recommendations can be made using this information to mitigate vector-borne diseases throughout the region, including the spread of drug resistant strains of malaria.

7.4 Recommendations for the Lao health officials and rubber industry

This study was conducted to understand the dynamics of vector mosquitoes in northern Lao PDR which could be translated into recommendations for the local Lao health officials and the rubber industry stakeholders. The most important recommendation from this study is the necessity to include rubber plantations in vector control programmes, which are currently mainly focussed on the villages. In the rubber plantations the following larval control can be implemented. If latex tapping is not conducted for more than one week, all latex collection cups should be turned upside down to avoid *Aedes* and to a lesser degree *Culex* breeding. This is also important when latex is not collected for more than one week as dengue vectors can breed in water that collects over the latex layer. After latex tapping is completed for the season, all latex collection cups should be collected in roofed sheds to diminish breeding sites. To further decrease *Aedes* and *Culex* mosquitoes the water containers and other waterbodies surrounding the rubber and village houses should be covered with a lid or netting, or treated with an insecticide or an microbial larvicide, *Bacillus thuringiensis israelensis* (Bti). The rotation of the used larvicides is of paramount importance to decrease the selection for resistant larvae. As there is already decreased susceptibility of mosquito larvae to temephos in the area, this insecticide is not recommended. Although there have been suggestions that the selection of resistance against larvicides can benefit the control efforts, if the resistance leads to a shorted life-span or reduced biting behaviour [494]. Moreover, all rubbish in plantations and villages should be properly disposed of in closed containers, including unused tyres. When using bamboo for construction, the open end of bamboo poles should be filled with gravel, cut at the joint or in the length to further decrease *Aedes* mosquitoes breeding. Tree trunks in the villages and rubber plantations should be removed as they are good breeding sites for *Aedes* and *Anopheles* mosquitoes. Mud roads in the plantation and villages contain road puddles where *Anopheles* mosquitoes breed. These puddles should therefore be levelled by filling the cavities with gravel. This needs to be done regularly during the rainy season when road use is intensive. Tarmac roads would reduce pooling more permanently, providing there was good drainage on either side of the road. To achieve sustainable larval control, the recommendations should be communicated and implemented during regular community-based mosquito source reduction activities.

Rubber workers should be encouraged to live in villages instead of inside the plantations, as this decreases exposure to dengue (which is a bigger risk in the study area than JE or malaria is). Additionally, my study has shown the necessity of outdoor vector protection methods to complement the larval control in rubber plantations and for protection in secondary forests. There is a need to compare personal protection methods such as insecticide-treated clothing and insecticide emitters for their efficacy outdoors in field settings to identify the methods which are most protective against mosquito bites. More importantly, it is essential to understand the protection of these methods at the community level. When this information is available, the mandatory use of personal protection methods by plantation workers can be discussed, as has been done in Bolivia [495]. For the implementation of personal protection methods to be protective for a community, the compliance is of utmost importance. A study from Cambodia showed that mass use of a topical repellent did not result in community protection against malaria as compliance remained suboptimal [496]. Until such information is available the local population should be encouraged to use methods that are known to decrease mosquito bites, including wearing thick long-sleeved clothing and applying topical repellents when visiting the natural and man-made forests, as exposure to vector mosquitoes is highest in these habitats. Although to my knowledge no scientific study has been done on the protectiveness of long-sleeved clothing compared to short-sleeved clothing, important organizations like the World Health Organization (WHO), and Center for Disease Control and Prevention (CDC) advise wearing long-sleeved clothing for protection from mosquito bites [246, 247]. Moreover, it should be safeguarded that all rubber worker families and villagers have access to LLINs, as JE and malaria vectors are seeking blood meals in the evening and at night when people (including the families of the rubber worker) are asleep.

Additional recommendations that are not directly related to the study results, but which are important for mitigating vector-borne disease risk is the education of the population and the swift treatment of the diseases. Of key importance is to improve the education of the local population and rubber workers on the vector-borne diseases and how to decrease their risk of exposure. Additionally, villagers should be taught why it is important to go to health centres when febrile, especially if they have just travelled from outside their district. This is important as vectors of most mosquito-borne diseases are present in the study area and introduction of pathogens could lead to an outbreak. Moreover, education on the importance of bed nets, the proper use of bed nets and their maintenance is important. This communication can be achieved by the organization of meetings by the health departments when distributing bed nets or when visiting villages for distribution of larvicides. These meeting should focus on teaching villagers about the risks of mosquitoes, where they breed and how they can protect themselves against this. Furthermore, this meeting should be used as an opportunity for villagers to share their problems related to mosquitoes which can help the health officers identify risk areas and focus-topics. After the meeting, the health worker from the district should walk around the village with the village representatives and teachers of the local schools to identify breeding sites. This information should then be communicated by the village representatives and teachers to the children and families in the village. Costs of this meeting and training should be paid by the dengue and malaria control funds from the government and will not be more than the per diem for the village workers and some simple materials such as posters and flyers. For a more detailed planning of the communication of mosquito control, social studies are necessary to identify the most suitable method for communication and motivation to act by the local population. In some villages it might have more impact to communicate recommendations using a play or the use of a puppet theatre (as seen for the ‘bird flu prevention’ by UNICEF [497]) as many villagers are illiterate and do not understand the Lao language well. Apart from the education of the population, the local health services should be prepared for outbreaks of dengue and malaria. Rapid diagnostic tests should be available in the local health hospitals to identify dengue and malaria. Similarly, malaria medicine should be available in the hospitals, even in non-endemic areas, so that malaria cases can be swiftly dealt with. The health workers should be provided sufficient training to conduct the tests, analyse the results and recommend treatment. Appropriate preparation for outbreaks also include regular surveys of disease incidence, which should be followed closely by the provincial health offices for early warning of outbreaks. These early warnings should result in the implementation of a clear action plan which focusses on the treatment of patients, communication to the affected areas and mosquito control.

7.5 Global perspective

The rubber plantations have been described as mosquito box amplification habitats by Sumodan *et al*. in SEA, with increased risk of dengue, malaria and chikungunya diseases [224]. Although other studies in SEA have shown the increased risk of malaria vector exposure for rubber workers [188, 198, 203, 498, 499], I did not recognise rubber tapping as a risky behaviour for malaria vector exposure in the study sites. I did however identify increased risk of dengue when working and living in the rubber plantations, which corresponds with results from other studies in the region [184, 225, 228]. It is of great importance to conduct further entomological surveys in other parts of SEA to understand the local dynamics of vector-borne diseases, especially in malaria endemic areas. Moreover, as rubber plantations are established in Africa and South America [500], where vector-borne diseases such as malaria, yellow fever and dengue result in morbidity and mortality [98, 501], the dynamics of vector-borne diseases should be investigated on a more global scale. As far as I am aware, no studies have been conducted on the risk of vector-borne diseases in rubber plantations outside SEA, except for an onchocerciasis risk study among residents of a rubber plantation in Liberia [502, 503]. With growing evidence of increased vector-borne disease risk in SEA rubber plantations, it is of interest to identify the vector-borne disease dynamics in rubber plantation areas of Africa and South America.

Our study has shown a high density of *Ae. albopictus* mosquitoes in the forests and rubber plantations. The areas I investigated are possibly areas where *Ae. albopictus* was originally present. However, data on mosquito dynamics in the past is limited. It could be possible that the mosquito species was recently introduced into this area, where the species has rapidly established. Our study area would not be an exception, with *Ae. albopictus* expanding globally at a rapid pace [457, 490, 504, 505]. With the expansion of *Ae. albopictus*, diseases transmitted by this mosquito are also expanding. Recent outbreaks of zika in South America grabbed the world's attention [171]. Yellow fever is still an important disease in Africa [506]. Furthermore, dengue and chikungunya outbreaks are becoming more frequent and wide spread [490, 506, 507]. As *Ae. albopictus* is an important vector mosquito throughout the world, it is important that WHO discriminating doses are identified for *Ae. albopictus* susceptibility tests. Furthermore, vector control programmes should not be limited to the housed areas, but include agricultural and forested areas too. This is especially important for the day biting mosquitoes, as exposure often occurs outside of the villages. Additionally, more studies are necessary to identify suitable personal protection methods that are protective for the community and identify other ways in which people can be protected from day biting mosquitoes.

Vector-borne disease studies are becoming more and more advanced, with modelling of data providing an important step towards predicting disease outbreaks [134, 508-512]. However, gaps still exist in many vector-borne disease studies on how to communicate results to the local governments and population. In this study, I have involved the important stakeholders from the beginning of the project. By organizing regular stakeholder meetings throughout the three year project, I have ensured that the results obtained from the study are of interest for the different stakeholders. The stakeholder meetings were a platform for me to present my preliminary data and receive ideas and comments from the participants. This has resulted in the inclusion of my work in documents from both the Ministry of Health and Ministry of Agriculture and Forestry. For any study conducted in the field, it is of upmost importance to involve the different stakeholders from the beginning. This also includes the input of the villagers where the study is conducted.

All vector-borne disease studies should be approached in a more interdisciplinary fashion for bigger impact, including participation of governmental organizations, entomologists, doctors, social scientists, climatologists and epidemiologists. In this study I combined the expertise of social scientists with entomologists. By combining data from both, a clearer image of the vector-borne disease dynamics could be described. This has led to clearer recommendations. Although my study lacked the involved of people from the medical field and epidemiologists, it already shows the value of working interdisciplinary. The future of vector-borne disease studies is a close collaboration between different scientists and the regional administrations, for the development of strategic vector control plans which engages the local population in the control of vector-borne diseases.

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Appendix 1 Ethical approval from the Lao government

Lao People's Democratic Republic
Peace Independence Democracy Unity Prosperity
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Ministry of Health
Council of Medical Science
National Ethics Committee
For Health Research (NECHR) No 017 /NECHR

Approval Notice

Paul BREY
Email: pbrey@pasteur.fr
Phone: +856 21 285321

RE: "Risk of vector-borne diseases in relation to rubber plantations in Lao PD Human landing catches ethical approval "

Paul BREY

Members of the Ethics Committee of the Lao People's Democratic Republic (PDR) have reviewed and approved your research.

Please note the following information about your approved research protocol:

Approval period: 2013-2016
Approved Subject Enrollment: 80
Sponsor: Agence Française de Développement
Implementing Panel/Project Investigator: Paul BREY

Please note that the Ethics Committee reserves the right to ask for further questions, seek additional or monitor the conduct of your research and consent process.

Vientiane Capital 4 / 21 /2013
President, National Ethics Committee
For Health Research

Prof.Dr Bounhong SOUTHAVONG

ສາທາລະນະລັດ ປະຊາທິປະໄຕ ປະຊາຊົນ ລາວ
ສັນຕິພາບ ເອກະລາດ ປະຊາທິປະໄຕ ເອກະພາບ ວັດທະນາຖາວອນ

ກະຊວງສາທາລະນະສຸກ
ສະຖາບັນ ປັດສະເຕີ ລາວ
ຕູ້ປ.ນ 3560 ຖະໜົນ ສາມແສນໄທ
ບ້ານ ເກົ້າຍອດ, ເມືອງ ສີສັດຕະນາກ
ນະຄອນຫຼວງວຽງຈັນ
ໂທ: 021 285321, ແຟັກ: 021 285326

ເລກທີ 239/ສປລ
ນະຄອນຫຼວງວຽງຈັນ, ວັນທີ 03 JUN 2014

ໃບສະເໜີ

ຮຽນ: ທ່ານປະທານຄະນະກຳມະການຈັນຍາທຳແຫ່ງຊາດ ຂອງ ສປປ ລາວ

ເລື່ອງ: ຂໍເພີ້ມເອກະສານຊ້ອນທ້າຍໃນການຄົ້ນຄ້ວາ ກ່ຽວກັບ "ເຕັກນິກຈັບຍຸງເວລາຕອມຄົນ ເພື່ອສຶກສາຄວາມສ່ຽງຂອງການເກີດພະຍາດ ທີ່ຕິດຕໍ່ຈາກແມງໄມ້ ທີ່ເປັນພາຫະນຳເຊື້ອ ຢູ່ສ່ວນຢາງພາລາໃນສປປ ລາວ"

- ອີງຕາມໃບອະນຸຍາດ ຂອງຄະນະກຳມະການຈັນຍາທຳແຫ່ງຊາດ ສະບັບເລກທີ 017 /NECHR ລົງວັນທີ 21/04/2013 ກ່ຽວກັບ "ເຕັກນິກຈັບຍຸງເວລາຕອມຄົນ ເພື່ອສຶກສາຄວາມສ່ຽງຂອງການເກີດພະຍາດ ທີ່ຕິດຕໍ່ຈາກແມງໄມ້ ທີ່ເປັນພາຫະນຳເຊື້ອ ຢູ່ສ່ວນຢາງພາລາໃນສປປ ລາວ"

ສະຖາບັນ ປັດສະເຕີ ລາວ ມີຈຸດປະສົງຂໍຮຽນສະເໜີມາຍັງທ່ານເພື່ອຂໍເພີ້ມ ເອກະສານຊ້ອນທ້າຍຂໍ້ທີ່ 11 ກ່ຽວກັບການສັກຢາວັກແຊງປ້ອງກັນພະຍາດເຫຍື້ອຫຸ້ມສະໝອງອັກເສບຍີ່ປຸ່ນ (JE) ໃຫ້ກັບຜູ້ເຂົ້າຮ່ວມການສຶກສາ, ເຊິ່ງຫຼັງຈາກໄດ້ພິຈາລະນາສະຖານທີ່ເຮັດການສຶກສາແລ້ວ ເຫັນວ່າຜູ້ເຂົ້າຮ່ວມການສຶກສາມີຄວາມສ່ຽງໃນການຕິດເຊື້ອພະຍາດເຫຍື້ອຫຸ້ມສະໝອງອັກເສບຍີ່ປຸ່ນ, ເພື່ອໃຫ້ເຂົາເຈົ້າຫຼຸດຜ່ອນຄວາມສ່ຽງການຕິດເຊື້ອພະຍາດດັ່ງກ່າວໃນເວລາເຮັດວຽກ ການຊັກຢາວັກແຊງຈຶ່ງມີຄວາມຈຳເປັນ.

ດັ່ງນັ້ນ ສະຖາບັນ ປັດສະເຕີ ລາວ ຈຶ່ງຮຽນສະເໜີມາຍັງທ່ານ ເພື່ອພິຈາລະນາ ແລະ ອານຸມັດຕາມທາງຄວນດ້ວຍ.

ດ້ວຍຄວາມນັບຖືຢ່າງສູງ

ຄຳເຫັນຂອງຫົວໜ້າກອງເລຂາ
ຄະນະກຳມະການຈັນຍາທຳແຫ່ງຊາດ
[illegible]

ດຣ ບຸບຜາ ທອງມະໄລວົງ
...HA THONGMALAYVONG

ຜູ້ອຳນວຍການສະຖາບັນ ປັດສະເຕີ ລາວ

ດຣ. ໂປນ ເຢູ

Appendix 2 Ethical approval from Durham University

Durham University

Biological and Biological Sciences
Devolved Ethics Sub-Committee

Application Form

(REF)	**ECOMORE, Lao PDR**
Chief Investigator:	**S.W. Lindsay and P. Brey**

SECTION 1: Basic information

For office use only	
Application received:	
Minutes referring: Comments were requested by e-mail circulation. Receive following comment: 'Rubber plantation workers who participate in the Focus Group Discussion are not having to have an open discussion in front of their superiors at work. I worry that any concern workers may express about their working conditions/risks in relation to exposer to vector-borne diseases could be used against them. So if possible, could workers be interviews with peers rather than 'line-managers' or superiors' Clarification was obtained from CI Steve Lindsay that superiors/plantation owners would not be present and confirmed that the discussions would be held with peer groups only.	
Signature to confirm approval of application	Date: 9.4.2013
Prof C J Hutchison, Chair SBBS Ethics Committee	25.7.2013
	Enclosed?
Consent Form(s)	Received
Participant Information Sheet (s)	N/A
Risk Assessment	Received
Documentation of adequate insurance (if applicable)	Approved
Documentation of statistical advice (if applicable)	

Full title of the study:	**" ECOnomic development, ecosystem MOdifications, and emerging infectious diseases Risk Evaluation"** **ECOMORE project** **Study Title: Risk Of Vector-Borne Diseases In Relation To Rubber Plantations In Lao PDR**

1.1 PRINCIPAL APPLICANT

Fist name:	Steve	Last Name:	Lindsay
Email:	S.W.Lindsay@durham.ac.uk	Phone:	41291

Appendix 3 Ethical approval from the CoRC, Paris

Institut Pasteur

Paris,
September 1st, 2014

File followed by :
Pôle Intégré de Recherche Clinique
Tel : 01 44 38 91 01
Email : secr-CORC@pasteur.fr

Project title : *«Risk of vector-borne diseases in relation to rubber plantations in Lao PDR»*

Dear Mr Brey,

Re : Committee's number 2014-19

Further to the notice from the Clinical Research Committee of July, 8th 2014, I hereby confirm:

The favorable notice for the conduct of the research subject to the provision of the documents requested in the Committee's examination statement on your project : ***«Risk of vector-borne diseases in relation to rubber plantations in Lao PDR»***

Attention :

After regulatory and ethical clearance and for a good recordkeeping, we would appreciate if you could send us not only all the finalized documents in the frame of the research, but also study progress statements, amendments and publications of the results related to the study.
We remind you that any significant modification of the protocol must be notified to us.

Yours sincerely,

Christian Bréchot
President

Encl : Statement of the Clinical Research Committee n° 2014-19 of July 8th, 2014

25-28, Rue du Docteur Roux
75724 Paris Cedex 15

Appendix 4 Information sheet and consent form comparison study

Information sheet for collection of outdoor mosquitoes using Human Landing Catches and the Double bed net method
Study name: Risk of vector-borne diseases in relation to rubber plantations in Lao PDR
Responsible scientist:
Dr Paul Brey and Julie-Anne Tangena of Institut Pasteur Laos

Introduction:
You are invited to take part in a research study. Before you decide it is important for you to understand why the research is being done. Allow me to explain what the study is all about and what it will involve. Please take time to listen to the information carefully. Ask us if there is anything that is not clear or understood.

Purpose:
Malaria and dengue cause a lot of illness in Lao PDR and we want to understand the risk for people in and surrounding rubber plantations and how we can help them. People get sick when bitten by a certain type of mosquito. The mosquito spits the germ into you, making you sick. To understand which control method is best to use, we need to understand the behaviour and species composition of the mosquitoes. To collect information on mosquitoes, generally a method is used which exposes people to mosquito bites. In this study we compare this commonly used method with a new method which protects people from mosquito bites, to understand if the new method can be just as good at collecting mosquitoes without exposing people to mosquito bites

What will happen if you agree to participate?
- Depending on the experiment you will be asked to sit under a bed net or collect mosquitoes off your legs using a sucking tube
- You will be exposed to mosquito bites which can make you sick
- You will collect mosquitoes during the day and night. We will need you to help us collect mosquitoes in the months of May till August 2014
- You will collect mosquitoes in and around your village, including the secondary rainforest and the rubber plantation
- We will expect you to participate for 8h with a varying starting time of 10:00 or 17:00
- You will have a 10 minute break each hour
- You must not drink alcohol before or during the catching
- You are not allowed to smoke in close proximity to the mosquito collection area.
- You will collect mosquitoes and put them into collection cups

You will receive medical treatment if you get sick during the study period. If you are feverish, please call **Dr. Phoutmany Thammavong at Tel: 020-77843543.**

Benefits: You will receive payment for this work of 35.000 kip for day collections and 50.000 for night collections. You will receive vaccination for protection against Japanese encephalitis. If you are unwell during this work or three weeks after you will receive prompt medical treatment free of charge.

Risks: Mosquitoes you are collecting in the different habitats may carry diseases. You will possibly be exposed to dengue, for which no medicine exists.

Participation: You have the right not to join the study and to leave the study at any time without penalty or loss of benefits to which you are otherwise entitled.

Confidentiality: Your individual information will be kept private.

Payment: There is payment for participation in this study of 35.000 kip for day collections and 50.000 for night collections.

Research sponsor: This research is sponsored by the development agency of France (AFD)

Ethical Approval: This study has been approved by the Lao ethics committee and Durham University

Please feel free to ask any questions if you have any, Dr. Phoutmany Thammavong at Tel: 020-77843543.

Informed consent form for collection of outdoor mosquitoes using human landing catches and the double bed net method

ECOMORE PROJECT: Risk of vector-borne diseases in relation to rubber plantations in Lao PDR

Village Name ………………………………………………………………..
Identification number participant:..........

Name of participant
……………………………………………………………… consents to participate in the above research study.

Participant Statement
I, the participant named above, have witnessed an ECOMORE staff member explaining the nature of the study as described on the information sheet. The purpose of the research study has been explained and opportunity has been given to ask questions concerning this study. Any such questions have been answered in full. Should any further questions arise concerning this study I know to contact **Dr. Phoutmany Thammavong at Tel: 020-77843543**
I understand that I may withdraw from this study at any time without penalty.

Name of Participant
…………………………………………………………signature……………………………

Date……………………………………………………………

Person obtaining consent
Name……………………………………………………………………signature

Date……………………………………………………………

Witness consent, if applicable
Witness Statement
I have witnessed the IPL staff member explaining the nature of the study as described on the information sheet to the person named above. The purpose of the research study has been explained opportunity has been given to ask questions concerning this study. Any such questions have been answered in full. Should any further questions arise concerning this study the participant knows to contact **Ms Phoutmany Thammavong at Tel: 020-77843543**

The participant understands that he/she may withdraw from this study at any time without penalty.

Name of witness ………………………………………signature……………………………

Date……………………………………………………………

Person obtaining consent ……………………………signature……………………………

Date……………………………………………………………

Appendix 5 Information sheet and consent form longitudinal study

Information sheet for collection of outdoor mosquitoes using the double bed net method
Study name: Risk of vector-borne diseases in relation to rubber plantations in Lao PDR
Responsible scientist:
Dr Paul Brey and Julie-Anne Tangena of Institut Pasteur Laos

Introduction:
You are invited to take part in a research study. Before you decide it is important for you to understand why the research is being done. Allow me to explain what the study is all about and what it will involve. Please take time to listen to the information carefully. Ask us if there is anything that is not clear or understood.

Purpose:
Malaria and dengue cause a lot of illness in Lao PDR and we want to understand the risk for people in and surrounding rubber plantations and how we can help them. People get sick when bitten by a certain type of mosquito. The mosquito spits the germ into you, making you sick. In this study we want to find out which and how many mosquitoes carry malaria and dengue, when people are most at risk and what time of year most people get bitten. This information is important for us to find ways of stopping the diseases where you live.

What will happen if you agree to participate?

- You will collect mosquitoes that are caught between two bed nets. You will be protected by a bed net for a majority of the time. During 10 minutes every hour you will be asked to lift up the bed net and collect mosquitoes between the two bed nets
- We will need your participation for four consecutive days every month from July 2013 to July 2014 and perhaps exceptionally at other times
- You will collect mosquitoes in groups of three in three double bed net construction in the secondary forests, mature rubber plantations, immature rubber plantations and villages. We will provide transport to the different locations
- We will expect you to participate for 6h both during the day and night
- You must not drink alcohol before or during the catching
- You will collect mosquitoes and put them into collection cups
- You will be exposed to mosquito bites which can make you sick
- You are not allowed to smoke in close proximity to the mosquito collection area

You will receive medical treatment if you get sick during the study period. If you are feverish, please call **Dr. Phoutmany Thammavong at Tel: 020-77843543.**

Benefits: You will receive payment for this work of 45.000 kip for 6 h during the day and 60.000 kip for 6 h during the night from 24.00 to 6.00 am. You will receive vaccination for protection against Japanese encephalitis. The results of the study will help us learn how best malaria and dengue can be controlled in the area where you live and work.
Risks: Mosquitoes you are collecting in the different habitats may carry diseases. You will possibly be exposed to dengue, for which no medicine exists. If you are unwell during this work and up to three weeks after, you will receive prompt medical treatment free of charge.
Participation: You have the right not to join the study and to leave the study at any time without penalty or loss of benefits to which you are otherwise entitled.
Confidentiality: Your individual information will be kept private. Personal information will be stored in a computer file without your name, but a number. The data showing the name associated with the number will be kept together with your personal information in a locked cupboard.
Payment: You will receive payment for this work of 45.000 kip during the day and 60.000 kip during the night.
Research sponsor: This research is sponsored by the development agency of France (AFD)
Ethical Approval: This study has been approved by the Lao ethics committee and Durham University

Please feel free to ask any questions if you have any, Dr. Phoutmany Thammavong at Tel: 020-77843543.

Informed consent form for collection of outdoor mosquitoes using the double bed net method

ECOMORE PROJECT: Risk of vector-borne diseases in relation to rubber plantations in Lao PDR

Village Name ………………………………………………………..
Identification number participant:..........

Name of participant
…………………………………………………………… consents to participate in the above research study.

Participant Statement
I, the participant named above, have witnessed an ECOMORE staff member explaining the nature of the study as described on the information sheet. The purpose of the research study has been explained and opportunity has been given to ask questions concerning this study. Any such questions have been answered in full. Should any further questions arise concerning this study I know to contact **Dr. Phoutmany Thammavong at Tel: 020-77843543**
I understand that I may withdraw from this study at any time without penalty.

Name of Participant
………………………………………………………signature……………………………

Date……………………………………………………………

Person obtaining consent
Name………………………………………………………………………signature

Date……………………………………………………………

Witness consent, if applicable

Witness Statement
I have witnessed the IPL staff member explaining the nature of the study as described on the information sheet to the person named above. The purpose of the research study has been explained opportunity has been given to ask questions concerning this study. Any such questions have been answered in full. Should any further questions arise concerning this study the participant knows to contact **Ms Phoutmany Thammavong at Tel: 020-77843543**

The participant understands that he/she may withdraw from this study at any time without penalty.

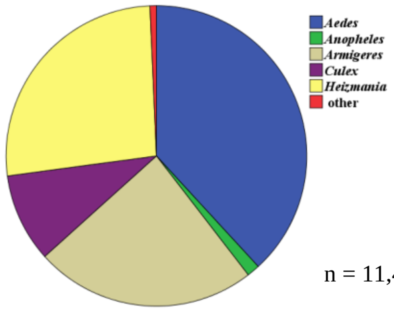
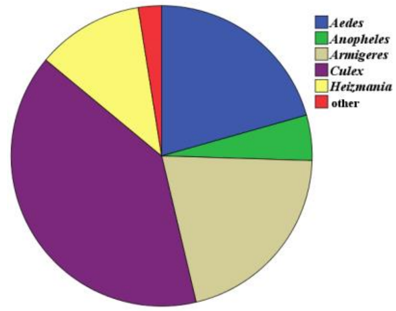
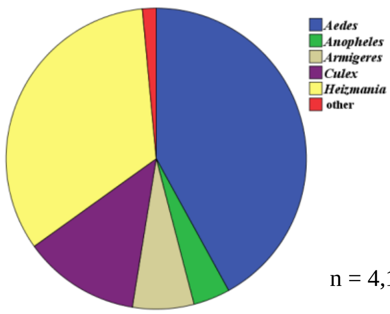
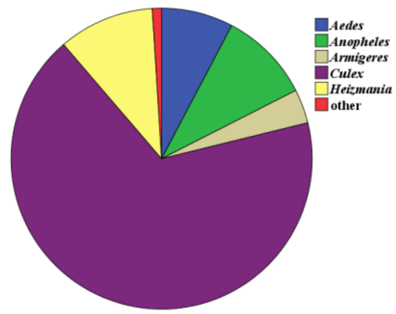
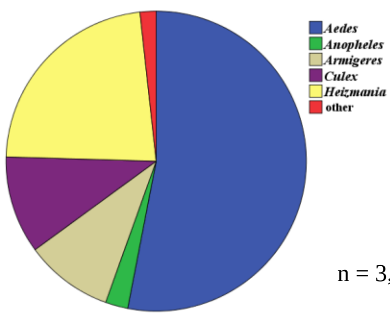
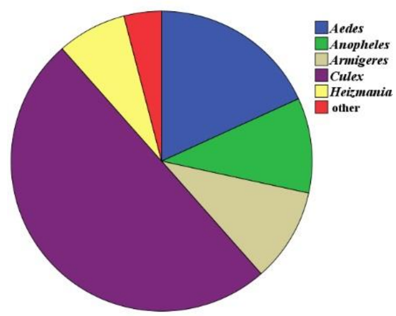
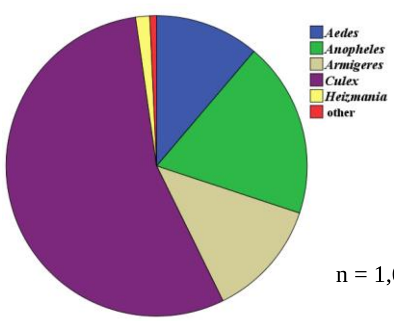
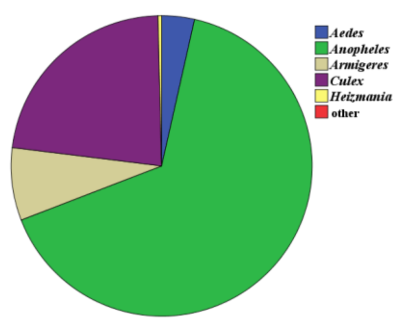
Name of witness ………………………………………signature……………………………

Date……………………………………………………………

Person obtaining consent ……………………………signature……………………………

Date……………………………………………………………

Appendix 6 Pie-chart of mosquito species distribution

Rainy season (May-Oct)	Dry season (Nov-Apr)
secondary forests	**secondary forests**
 n = 11,427	 n = 2,362
immature rubber plantations	**immature rubber plantations**
 n = 4,118	 n = 1,205
mature rubber plantations	**mature rubber plantations**
 n = 3,007	 n = 644
villages	**villages**
 n = 1,652	 n = 512

Appendix 7 Female adult mosquito species collected

List of all female adult mosquito species identified in the different habitats during the nine months of collection using the illustrated keys of the mosquitoes of Thailand [314]

	Village	Immature rubber plantation	Mature rubber plantation	Secondary forest	Total
*Ayurakitia sp**	0	1	1	4	6
*Aedes (Aedimorphus) caecus**	0	2	0	3	5
*Aedes (Aedimorphus) orbitae**	0	0	1	0	1
*Aedes (Borichinda) cavernicola**	0	0	0	1	1
Aedes (Bothaella) eldridgei	2	8	11	13	34
*Aedes (Bothaella) helenae**	0	6	4	5	15
*Aedes (Bruceharrisonius) greenii**	1	6	3	17	27
*Aedes (Danielsia) albotaeniata**	0	10	7	46	63
*Aedes (Downsiomyia) inermis**	0	0	1	0	1
Aedes (Downsiomyia) novonivea and *Aedes (Downsiomyia) litorea**	103	344	183	274	904
Aedes (Fredwardsius) vittatus	4	15	14	2	35
Aedes (HuIecoeteomyia) chrysolineata	0	1	1	0	2
Aedes (HuIecoeteomyia) formosensis and *Aedes (HuIecoeteomyia) pallirostris*	0	10	5	36	51
Aedes (HuIecoeteomyia) reinerti	1	18	5	47	71
*Aedes (HuIecoeteomyia) saxicola**	0	0	1	0	1
Aedes (Kenknightia) dissimilis	0	0	2	16	18
*Aedes (Kenknightia) harbachi**	0	0	1	5	6
*Aedes (Lorrainea) fumida**	0	0	0	2	2
*Aedes (Mucidus) quasiferinus**	0	0	1	0	1
*Aedes (Phagomyia) khazani**	0	8	5	23	36
Aedes (Phagomyia) prominens	0	0	0	1	1
Aedes (Stegomyia) aegypti	1	0	0	0	1
Aedes (Stegomyia) albopicta	83	1248	1331	3640	6302
Aedes (Stegomyia) gardnerii imitator	2	48	33	104	187
*Aedes (Stegomyia) seatoi**	0	0	2	1	3
*Aedes (Stegomyia Heteraspidion) annandalei**	4	37	40	367	448
*Aedes (Stegomyia Heteraspidion) craggi**	0	1	0	0	1
Aedes (Stegomyia Huangmyia) malikuli and *Aedes (Stegomyia Huangmyia) perplexa**	0	36	25	211	272
*Aedes (Stegomyia Xyele) desmotes**	0	4	12	18	34
*Aedes (Verrallina Harbachius) yusafi**	0	0	0	6	6
*Aedes (Verrallina Verrallina) lugubris**	0	1	0	0	1
*Anopheles (Anopheles) sp (Aitkenii group)**	0	3	1	22	26
*Anopheles (Anopheles) baezai**	15	0	0	3	18
Anopheles (Anopheles) baileyi	1	0	0	0	1

* Species not recorded in Lao PDR before

(Continued from previous page)

	Village	Immature rubber plantation	Mature rubber plantation	Secondary forest	Total
Anopheles (Anopheles) barbirostris s.s.	45	12	11	102	170
Anopheles (Anopheles) barbumbrosus	388	49	35	48	520
*An. sp. near Anopheles (Anopheles) gigas**	2	0	0	5	7
Anopheles (Anopheles) hodgkini	3	0	0	4	7
*Anopheles (Anopheles) insulaeflorum**	1	0	0	0	1
*Anopheles (Anopheles) separatus**	6	4	2	0	12
Anopheles (Anopheles) umbrosus	16	1	1	0	18
*Anopheles (Anopheles) whartoni**	1	0	0	0	1
Anopheles (Cellia) dirus s.l.	1	31	9	5	46
Anopheles (Cellia) culcifacies	2	1	0	0	3
*Anopheles (Cellia) epiroticus**	1	1	0	1	3
Anopheles (Cellia) jamesii	3	2	1	0	6
Anopheles (Cellia) jeyporiensis	0	1	1	0	2
Anopheles (Cellia) kochi	14	7	4	6	31
Anopheles (Cellia) maculatus s.l.	53	137	49	55	294
Anopheles (Cellia) minimus s.l.	83	28	24	16	151
Anopheles (Cellia) pampanai	6	1	1	3	11
Anopheles (Cellia) phillipinensis	0	0	0	1	1
Anopheles (Cellia) tessellatus	7	2	0	2	11
Anopheles (Cellia) varuna	0	1	0	0	1
*Armigeres (Armigeres) confusus**	0	0	0	1	1
Armigeres (Armigeres) foliatus and *Armigeres (Armigeres) kuchingensis**	5	4	9	95	113
*Armigeres (Armigeres) jugraensis**	4	0	2	4	10
*Armigeres (Armigeres) kesseli**	204	76	129	2212	2621
Armigeres (Armigeres) kuchingensis	1	0	0	2	3
*Armigeres (Armigeres) malayi**	0	2	2	12	16
Armigeres (Armigeres) moultoni	7	21	16	42	86
Armigeres (Armigeres) subalbatus	8	23	51	186	268
Armigeres (Armigeres) theobaldi	7	4	7	33	51
*Armigeres (Leicesteria) annulipalpis**	0	1	0	1	2
Armigeres (Leicesteria) annulitarsis	0	2	1	5	8
*Armigeres (Leicesteria) balteatus**	0	0	1	3	4
*Armigeres (Leicesteria) digitatus**	0	3	2	5	10
Armigeres (Leicesteria) dolichocephalus	1	57	36	101	195
Armigeres (Leicesteria) flavus	11	109	81	480	681

* Species not recorded in Lao PDR before

(Continued from previous page)

	Village	immature rubber plantation	mature rubber plantation	Secondary forest	Total
*Armigeres (Leicesteria) inchoatus**	0	1	0	2	3
Armigeres (Leicesteria) longipalpis	0	10	7	15	32
Armigeres (Leicesteria) magnus	1	1	5	7	14
*Armigeres (Leicesteria) omissus**	0	1	0	0	1
Armigeres (Leicesteria) pectinatus and *Armigeres (Leicesteria) vimoli*	0	0	0	4	4
*Armigeres (Leicesteria) traubi**	0	1	0	0	1
Coquillettidia sp	0	25	27	47	99
*Culex (Culex) alis**	85	55	52	133	325
*Culex (Culex) edwardsi**	0	0	0	1	1
Culex (Culex) fuscocephala	41	17	2	10	70
Culex (Culex) gelidus	2	3	0	5	10
Culex (Culex) hutchinsoni	7	9	2	1	19
Culex (Culex) mimulus and *Culex (Culex) murrelli**	1	0	1	0	2
Culex (Culex) perplexus and *Culex (Culex) whitei**	0	1	1	0	2
Culex (Culex) quinquefasciatus	7	3	3	8	21
*Culex (Culex) sitiens**	56	31	34	91	212
Culex (Culex) vishnui	604	1041	440	1477	3562
*Culex (Culex) whitei**	194	125	67	244	630
Culex (Culex) whitmorei	2	0	0	2	4
*Culex (Culiciomyia) dispectus**	0	0	0	1	1
Culex (Culiciomyia) fragilis and *Culex (Culex) spathifurca**	1	2	1	3	7
Culex (Culiciomyia) nigropunctatus	12	11	12	5	40
*Culex (Culiciomyia) papuensis**	5	1	1	2	9
*Culex (Culiciomyia) termi**	1	0	0	0	1
Culex (Eumelanomyia) brevipalpis and *Culex (Eumelanomyia) phangngae**	0	4	11	8	23
*Culex (Eumelanomyia) foliatus**	0	1	0	0	1
Culex (Lophoceraomyia) infantulus and *Culex (Lophoceraomyia) minutissimus**	0	0	0	2	2
Culex (Lophoceraomyia) sp (Mamilifer subgroup and *Wilfredi group)**	0	0	0	2	2
Culex (Oculeomyia) bitaeniorhynchus	7	26	11	31	75
*Culex (Oculeomyia) longicornis**	0	1	0	1	2
Culex (Oculeomyia) pseudosinensis	0	0	0	1	1
*Heizmannia (Heizmannia) chengi**	5	255	99	793	1152
Heizmannia (Heizmannia) complex	0	1	0	0	1

* Species not recorded in Lao PDR before

(Continued from previous page)

	Village	immature rubber plantation	mature rubber plantation	Secondary forest	Total
*Heizmannia (Heizmannia) demeilloni**	0	0	0	2	2
*Heizmannia (Heizmannia) mattinglyi**	22	1244	635	2497	4398
*Lutzia (Metalutzia) vorax**	2	0	0	1	3
*Malaya sp**	3	4	3	9	19
Mansonia sp	2	19	25	10	56
Mimomyia sp	1	1	0	1	3
Topomyia sp	0	3	2	4	9
*Toxorhynchites splendens and Toxorhynchites amboinensis**	0	0	1	2	3
Tripteroides sp	5	29	44	62	140
*Udaya argyrurus**	0	1	0	1	2
Uranotaenia sp	1	3	0	0	4

* Species not recorded in Lao PDR before

Appendix 8 Susceptibility test results

Results of the WHO susceptibility tube tests for wild-caught adult *Aedes albopictus* to DDT, bendiocarb, permethrin, deltamethrin and malathion

	Mosquito species	**Total no. adult exposed**	**Knock down after 16 min.**	**Knock down after 30 min.**	**Knock down after 46 min.**	**Knock down after 60 min.**	**Dead after 24hrs.**	**Suscep-tibility**
DDT (4 %)	*Ae. albopictus*	113	3	61	96	113	112	99.1 %
Bendiocarb (0.1 %)	*Ae. albopictus*	166	113	166	166	166	164	98.8 %
Permethrin (0.75 %)	*Ae. albopictus*	105	66	105	105	105	105	100 %
Deltamethrin (0.05 %)	*Ae. albopictus*	108	2	99	108	108	108	100 %
Malathion (0.08 %)	*Ae. albopictus*	92	21	92	92	92	92	100 %

Appendix 9 Rapid rural appraisal and survey questionnaire

Rapid rural appraisal

START with introducing all our staff and explaining why, how, and how long we will be doing this PRA today.

MAPPING:
Step 1: Ask the villagers to draw the village including all 4 of our collection sites.
Step 2: Where is the north, west, south, east?
Step 3: When villagers feel like everything is drawn, ask them what all the empty areas represent
Step 4: Confirm:

- Garbage
- Water areas
- Pig stalls
- Rice fields (high or low?)
- Forests
- Rubber/teak/banana plantation
- Mountains
- What is in the north of the village (which village?) In the south, west, east?
- Distance estimation

Step 5: Where are many mosquitoes? (village + rubber)
Step 6: Do you know where many larvae are?

CALENDER:
Step1: Ask to fill in the following in the table from 1 (Jan) to 12 (Dec) with the intensity of when a certain activity happens

- Rainfall*
- T*
- Rice production [Seedling, Growth phase, Harvest]
- When you go to forest*
- Rubber tapping[]
- Outbreak disease*
- mosquito population*
- migration*

Step 2: Why are there many mosquitoes in those months?
Step 3:Which diseases are common?
Step 4: Why seedling then?
Step 5: Why migrate in those months?
Step 6: When people come back from migration, problem of health?

CALENDER: TIME 0:00-0:00
Step 1: Show intensity of mosquitoes during the day *
Step 2: When tap rubber []
Step 3: When collect rubber[]
Step 4: What time are you most often in the forest?
Step 5: what time are you in the rice fields?

Questionnaire ECOMORE project

With this questionnaire we would like to collect information on the movement of villagers in our study area to understand how often they visit the different habitats in which we collected mosquitoes in 2013 and 2014; secondary forest, mature rubber plantation, immature rubber plantation and village. Using this information we hope to identify the risk of mosquito diseases in each habitat.

Date: - - 2015 **Code Questionnaire**:…………

I. Socio-demographic data

Village name:	☐Male / ☐ female	Age:.............

Expositions

1) Do you use method to protect yourself from mosquitoes (DEET, coil etc.)?

☐Yes ☐No

a. If yes, can you give some examples?

…………………………………………………………………………

2) Are you a rubber worker? ☐Yes ☐No

a. If no, do you ever visit the rubber plantations? ☐Yes ☐No

b. If yes, when do you visit and why?

....

....

See back of this document for question 3

3) **Please describe your movement yesterday**

	village	Farm	Immature rubber plantation	Mature rubber plantation	Secondary forest	……………………………………
1.00						
2.00						
3.00						
4.00						
5.00						
6.00						
7.00						
8.00						
9.00						
10.00						
11.00						
12.00						
13.00						
14.00						
15.00						
16.00						
17.00						
18.00						
19.00						
20.00						
21.00						
22.00						
23.00						
24.00						

Appendix 10 Mosquito bites per person per day

The average number of mosquito bites per person per day for the important vector species *Ae. albopictus*, *An maculatus s.l.*, *An minimus s.l.* and *An. dirus s.l.* in the secondary forest, immature rubber plantation, mature rubber plantation and village habitats during the rainy season and dry season.

	Rainy season				Dry season			
Vector species	**Secondary forest**	**Immature rubber plantation**	**Mature rubber plantation**	**Village**	**Secondary forest**	**Immature rubber plantation**	**Mature rubber plantation**	**Village**
Ae. albopictus	33.3	12.2	13.1	0.8	8.9	2.1	2.1	0.2
An. maculatus s.l.	0.4	0.7	0.3	0.4	0.2	0.7	0.4	0.2
An. minimus s.l.	0.07	0.06	0.02	0.3	0.1	0.3	0.3	0.7

Appendix 11 Presence of invertebrates in waterbodies

Number and proportion of waterbodies which contained immature mosquito stages and non-mosquito invertebrates in the different habitats

	Mature rubber plantation		Immature rubber plantation		Village	
Mosquito species	**n waterbodies positive**	**% contribution to total**	**n waterbodies positive**	**% contribution to total**	**n waterbodies positive**	**% contribution to total**
***Aedes* (Aedini)**	209	33.7	59	33.0	175	30.2
Culex	108	17.4	25	14.0	67	11.6
Anopheles	7	1.1	10	5.6	4	0.7
***Armigeres* (Aedini)**	67	10.8	16	8.9	42	7.3
***Mimomyia* (ficalbiini)**	38	6.1	19	10.6	32	5.5
***Tripetroides* (sabethini)**	6	1.0	8	4.5	6	1.0
Toxorhynchitus	8	1.3	6	3.4	8	1.4
Non-mosquito invertebrates	35	5.1	26	14.5	50	8.6

Appendix 12 Water characteristics associated with *Aedes*

Multivariate analysis of water characteristics associated with *Aedes* presence

		N_{mean} ***Aedes* absent** **(95% CI)**	**N_{mean}** ***Aedes* present** **(95% CI)**	***P***
larvae	**Salinity (µS/cm)**	343.5 (320.4-366.7)	275.5 (238.7-312.3)	0.002*
	Oxygen (mg/L)	7.2 (4.3-10.2)	38.0 (-7.5-83.4)	0.230
	Temperature (℃)	25.4 (24.7-26.0)	24.7 (24.3-25.1)	0.108
	pH	8.3 (6.0-10.6)	7.0 (6.9-7.1)	0.077
	Turbidity (NTU)	173.2 (152.7-193.8)	140.1 (115.5-164.6)	0.062
	Nitrate (mg/L NO_3)	1.0 (0.5-1.5)	0.04 (-0.02-0.10)	0.007*
	Phosphate (mg/L PO_4)	1.2 (0.8-1.5)	0.6 (0.5-0.8)	0.005*
pupae	**Salinity (µS/cm)**	320.0 (299.8-340.2)	345.6 (259.3-431.9)	0.492
	Oxygen (mg/L)	17.6 (2.5-32.6)	4.7 (4.3-5.2)	0.123
	Temperature (℃)	25.1 (24.6-25.6)	25.8 (25.1-26.4)	0.484
	pH	8.0 (6.3-9.7)	7.3 (7.1-7.4)	0.835
	Turbidity (NTU)	156.2 (139.7-172.7)	242.0 (175.4-308.5)	0.006*
	Nitrate^ (mg/L NO_3)	0.5 (0.2-0.8)	-	
	Phosphate (mg/L PO_4)	0.9 (0.7-1.0)	0.5 (-0.1-1.1)	0.387

Generalized linear modelling was used to calculate the P*-value with odds ratio (OR) and 95% confidence interval (CI) *significantly different,* $P<0.05$

Appendix 13 Water characteristics associated with *Culex*

Multivariate analysis of water characteristics associated with *Culex* presence

		N$_{mean}$ *Culex* absent (95% CI)	**N$_{mean}$ *Culex* present (95% CI)**	***P***
larvae	**Salinity (µS/cm)**	332.2 (310.5-354.0)	264.4 (218.0-310.7)	0.016*
	Oxygen (mg/L)	18.4 (2.0-34.8)	6.2 (4.0-8.3)	0.701
	Temperature (℃)	25.2 (24.7-25.7)	25.1 (24.5-25.6)	0.894
	pH	8.1 (6.2-9.9)	7.0 (6.9-7.1)	0.087
	Turbidity (NTU)	155.7 (138.8-172.6)	204.2 (155.1-253.3)	0.037*
	Nitrate (mg/L NO_3)	0.6 (0.3-0.8)	0.1 (-0.1-0.4)	0.284
	Phosphate (mg/L PO_4)	0.9 (0.7-1.1)	0.7 (0.5-0.9)	0.325
pupae	**Salinity (µS/cm)**	325.8 (305.4-346.1)	229.1 (156.1-302.0)	0.064
	Oxygen (mg/L O_2)	17.0 (2.5-31.5)	5.2 (4.6-5.7)	0.811
	Temperature (°C)	25.1 (24.6-25.6)	26.7 (25.6-27.9)	0.271
	pH	7.9 (6.3-9.6)	6.9 (6.5-7.2)	0.102
	Turbidity (NTU)	163.5 (147.0-180.0)	148.1 (73.0-223.1)	0.720
	Nitrate^ (mg/L NO_3)	0.5 (0.2-0.8)	-	
	Phosphate^ (mg/L PO_4)	0.9 (0.7-1.0)	0.8 (0.2-1.4)	0.875

Generalized linear modelling was used to calculate the P*-value with odds ratio (OR) and 95% confidence interval (CI) ^ data too few for statistical analysis *significantly different,* P*<0.05*

Appendix 14 Emergence of mosquitoes

Proportion of immature mosquitoes identified as adult mosquitoes for the three most important genera with the most frequently identified mosquito species

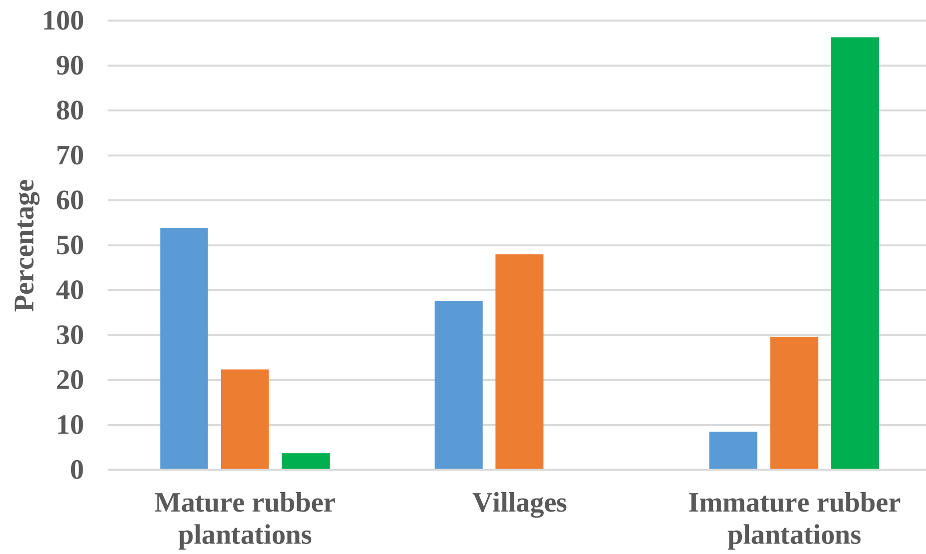
	Immature mosquitoes collected	% identified to species	Most common species	
Aedes	11,468	32.8	*Ae. albopictus*	2,845
			Ae. annandalei	557
Culex	7,916	19.0	*Cx. brevipalpis*	847
			Cx. quinquefasciatus	250
Anopheles	177	21.5	*An. dirus s.s.*	27
			An. barbumbrosus	3

Appendix 15 Mosquito species composition for every waterbody type

Total proportion of mosquito species in each waterbody type which have been collected in their immature stages, reared to adults and identified as adult mosquitoes

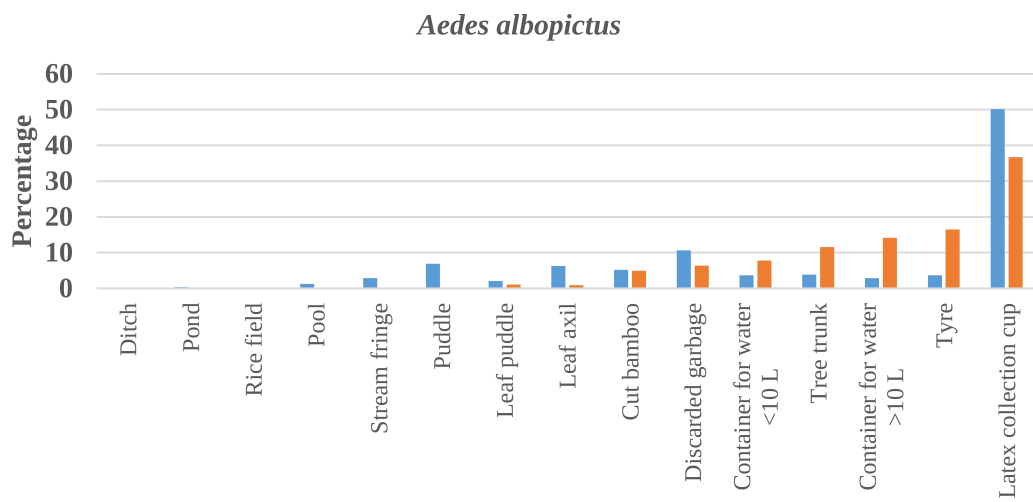
			Aedes species			*Culex* species			*Anopheles* species			
Habitat type	**Total immature mosquito**	**% identified to species**	**% *Ae. albo-pictus***	**% *Ae. annandalei***	**% Other *Aedes***	**% *Cx. brevipalpis***	**% *Cx. quinque-fasciatus***	**% Other *Culex***	**% *An. dirus s.l.***	**% *An. barbumbrosus***	**% Other *Anopheles***	**% Other species**
Tyre	4,974	21.4	46.5	0.4	0.9	13.3	0.7	1.8				36.3
Cut bamboo	4,623	30.9	20	33.7	4.6	4.8	0.9	0.8	0.1	0.1	0.1	34.8
Container for water >10 L	4,595	22.7	33.9	1.1	1.9	12.3	10.0	9.9			0.1	29.5
Latex collection cup	4,104	30.3	45.2	0.4	5.7	16.1	0.6	1.8				30.1
Container for water <10 L	3,942	38.5	37.0	1.3	2.2	10.5	5.1	1.7			0.1	39.5
Leaf axil	2,447	20.1	9.9	1.6	22.7	1.6	0.2	0.6				62.7
Puddle	1,642	20.2	3.0	0.3	0.6	25.9	7.8	23.8	5.4		1.5	14.5
Discarded garbage	1,234	30.1	62.1	4.8	2.2	1.9		0.3				28.8
Tree trunk	1,101	41.1	57.8	1.5	1.3	6.6	0.4	1.5	1.5			29.1
Ditch	308	5.8		11.1			50.0	38.9				
Leaf puddle	231	43.3	33		25	11		3				28
Pool	228	7.9	5.6				5.6	55.6				33.3
Pond	61	29.5			5.6	33.3	11.1	33.3		16.7		
Rice field	41	7.3						100				
Stream fringe	6	16.7						100	0			

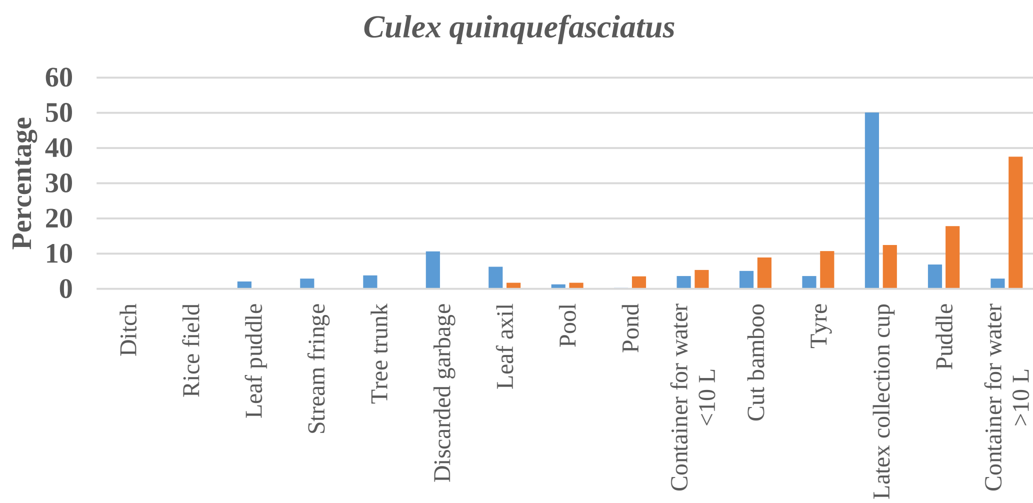
Appendix 16 Relative importance of the different habitats in relation to vector species



Relative importance of the different habitats (mature rubber plantations, villages and immature rubber plantations) in relation to the three most important vector species identified during the larval survey (■ *Aedes albopictus*, ■ *Culex quinquefasciatus*, ■ *Anopheles dirus s.l.*)

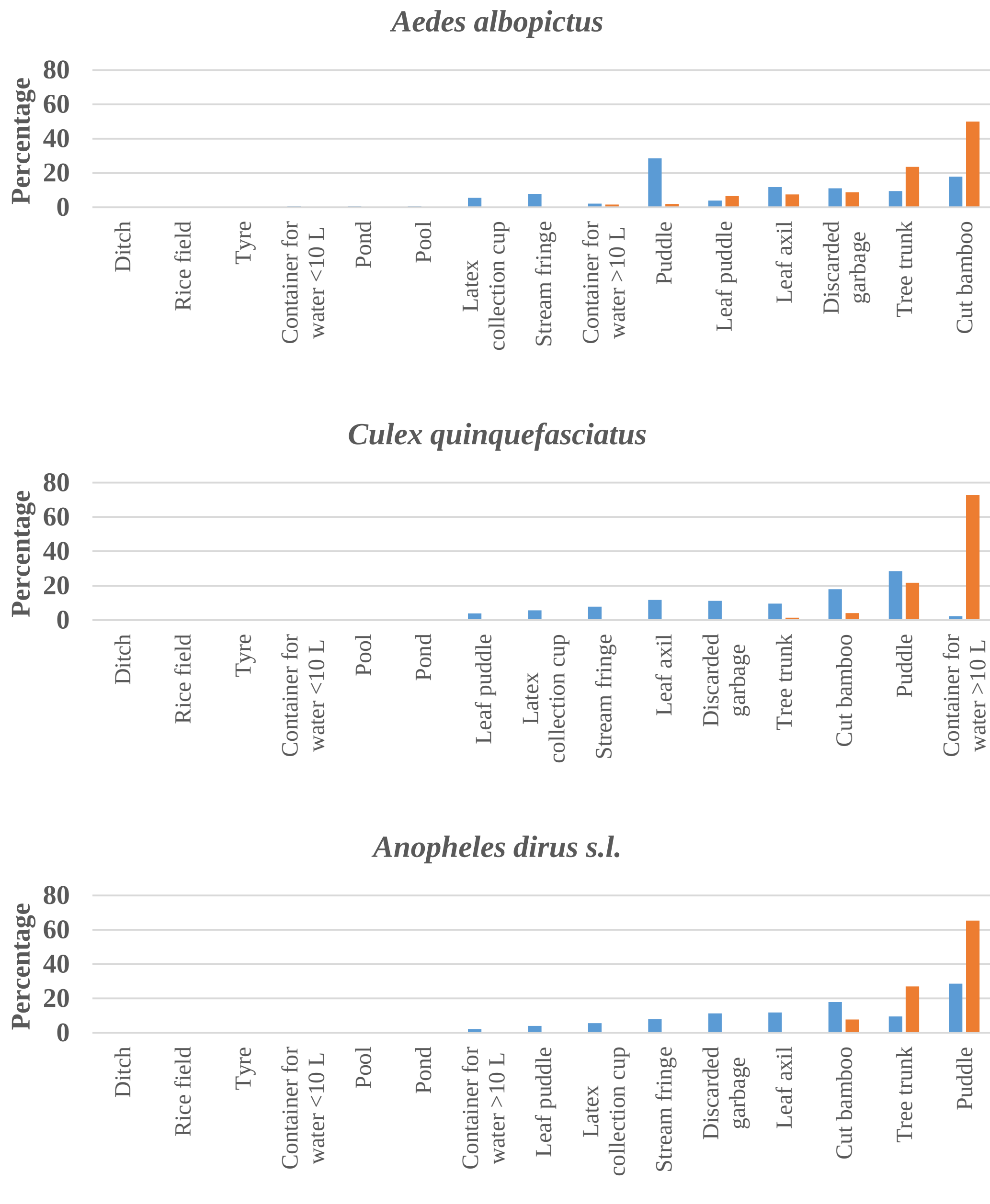
Appendix 17 Relative importance of the waterbody types for vector species in mature rubber plantations





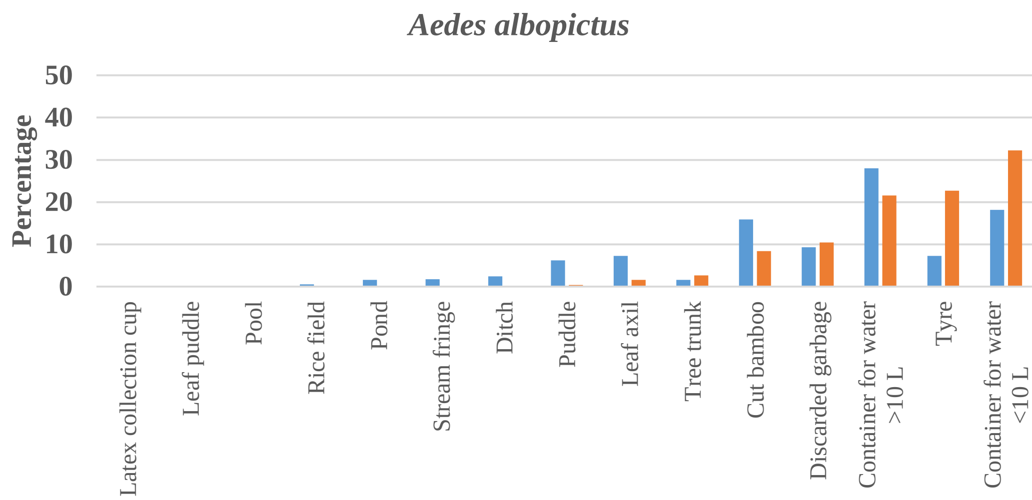
Relative importance of the waterbody types for the breeding of vector species in mature rubber plantations and their relation to the total number of waterbodies surveyed (% total waterbodies surveyed, % contribution to *Ae. albopictus* or *Cx. quinquefasciatus* mosquitoes)

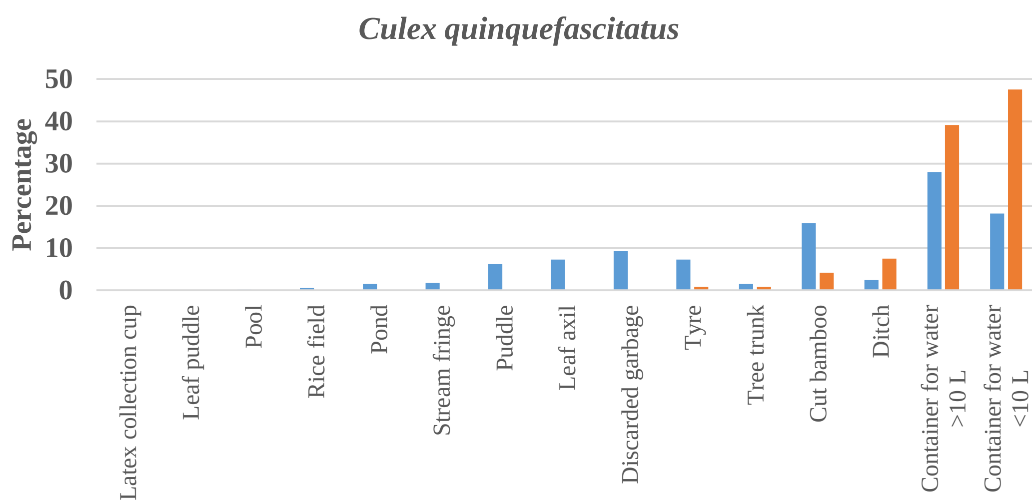
Appendix 18 Relative importance of the waterbody types for vector species in immature rubber plantations



Relative importance of the waterbody types for the breeding of vector species in immature rubber plantations and their relation to the total number of waterbodies surveyed (■ % total waterbodies surveyed, ■ % contribution to *Ae. albopictus, Cx. quinquefasciatus* or *An. dirus s.l.* mosquitoes)

Appendix 19 Relative importance of the waterbody types for vector species in villages





Relative importance of the waterbody types in villages for the breeding of vector species and their relation to the total number of waterbodies surveyed (% total waterbodies surveyed, % contribution to *Ae. albopictus or Cx. quinquefasciatus* mosquitoes)