Ecology and Behaviour of Vectors of *Plasmodium knowlesi* Malaria in Sabah, Malaysian Borneo

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Abstract

Over the last decade, the transmission of zoonotic malaria from non-human primates to humans has emerged as a public health problem and possible threat to malaria elimination in Southeast Asia. A major outbreak of the macaque malaria parasite *Plasmodium knowlesi* in humans began in Malaysian Borneo in 2004 and is now the primary cause of malaria in this region. This simian parasite is transmitted by mosquitoes in the *Anopheles leucophyrus* species complex. The emergence of *P. knowlesi* has been tightly linked to land-use change, particularly the widespread deforestation occurring in the state of Sabah where the largest focus of human infection is found. Efforts to combat this disease and understand its emergence and future spread in humans are hindered by limited knowledge of mosquito vector ecology and behaviour; and the risks of exposure to vectors in changing landscapes. This PhD aimed to address these knowledge gaps by carrying out a series of field studies near the epicentre of human *P. knowlesi* cases in Sabah, Malaysian Borneo, to elucidate *P. knowlesi* vector ecology, behaviour and transmission potential, verify associations between land-use type and human exposure risk, and characterize the dynamics of transmission within reservoir macaque populations. In combination this information will deepen understanding of *P. knowlesi* transmission and emergence, and provide insights for the control of this and other emerging zoonotic malarias.

My initial study evaluated new sampling methods for collecting resting *P. knowlesi* vectors. Resting collections are valuable for characterization of mosquito habitat and host species choice, however no standard methodology is currently available for *P. knowlesi* vectors. I evaluated two simple traps, resting buckets and sticky resting buckets, for sampling resting *P. knowlesi* vectors within two villages in Kudat District, Sabah. The performance of traps was evaluated, and the relative abundance and host choice of resting mosquito vectors was compared across eight different habitat types representing a gradient of deforestation. In 5748 trap days, a total of 2212 mosquitoes were collected in resting collections, but none were malaria vector species. *Culex* and *Aedes* genera dominated collections; with the former being most abundant in resting bucket traps and CDC aspirator catches, and the latter in sticky resting bucket traps. Several other vector species were collected including the sylvatic

dengue vector *Aedes albopictus*, and *Culex* vectors of filarasis and Japanese encephalitis. Consequently these simple resting traps could be effective for studying the ecology of a range of other important mosquito vectors in Sabah even if not those responsible for malaria.

In a following study I investigated associations between habitat and human exposure to *P. knowlesi* vectors, and tested for associations between vector abundance and human infection risk across a broad geographic range in Sabah. Previous studies indicated that the primary *P. knowlesi* vector was *An. balabacensis*. This vector was more abundant in a village than forest site, conflicting with the original hypothesis that humans are at greatest risk of infection in forests and suggested the possibility of peri-domestic transmission. However this inference was drawn from a limited number of sampling sites in only one district, Kudat, within Sabah. To test this hypothesis over a broader geographical scale, I conducted extensive entomological sampling across four districts in Sabah. Human landing catches were performed to measure human biting rates in forest, farm (plantation) and peri-domestic habitats in 11 villages. Prior to entomological sampling survey of human sero-positivity to *P. knowlesi* was conducted in all villages, carried out as part of a larger research programme. Making use of this data, I tested for associations between vector abundance and human infection risk at the village level. The primary vector *An. balabacensis* was found in all four districts, but at much lower relative abundance than in pilot work from Kudat. Additionally this vector was more abundant in forest and farm habitats than in peri-domestic settings. Only 1 of the 32 *An. balabacensis* collected in this study tested positive for *P. knowlesi*; an individual caught in a forest site. No significant association between the mean abundance of *An. balabacensis* and human *P. knowlesi* sero-positivity was detected in this study. However the relatively small sample size of mosquitoes and sites used here meant there was relatively low power to detect such an effect. This study highlights the importance of incorporating geographical heterogeneity and replication when assessing mosquito-habitat associations, and the need for more intensive longer-term sampling to establish potential entomological indicators of *P. knowlesi* infection in humans.

A final study was conducted to investigate the transmission dynamics of *P. knowlesi* in macaque reservoir populations. Most studies of *P. knowlesi* vectors

have been conducted in or near disturbed forest, where both humans and macaques are in contact. It is unknown whether the same vector species involved in human-macaque infection also mediate transmission between macaques. To investigate this and other aspects of macaque-mosquito interactions, I conducted a field study within the Danau Girang Field Centre in Sabah where there is a large population of long-tailed macaques. First I evaluated the use of Mosquito Magnet Independence Traps (MMIT) as a non-invasive means to sample mosquitoes host seeking near macaque sleeping sites. The MMIT performed well relative to the human landing catch, with both methods collecting *An. balabacensis* and other malaria vector species. Second, MMITs were used to sample mosquitoes host seeking near trees where macaques were sleeping and at unoccupied control trees. Additionally, macaque faecal samples were tested for malaria as an estimate of infection rate in the reservoir population. *Anopheles balabacensis* was more abundant at macaque sleeping sites than control trees indicating this vector has a specific propensity for feeding on macaques. Approximately 37% (n = 17/46) of macaque stool samples tested positive for *Plasmodium* infection but none of these were identified as being *P. knowlesi*. Two *Anopheles* vectors tested positive for *Plasmodium* which was subsequently confirmed as the primate parasite *P. inui*. Thus *P. inui* is likely the major source of malaria infection in this primate population. This study indicates that not all macaque populations pose a *P. knowlesi* risk, but other malaria parasites are common and should be monitored to assess for future spillover.

In combination, this research expands knowledge of *P. knowlesi* transmission in Malaysian Borneo, and has implications for planning surveillance and control. Notably it emphasizes the value of larger-scale surveillance of vector and macaque populations to assess human exposure risk, as and requirement of an integrated One Health approach to tackle zoonotic malaria.

Table of Contents

List of Tables

List of Figures

line indicating the predicted association. Error bars and dashed lines are 95% confidence intervals.

Figure 4.10 Predicted relationship between mean *Anopheles* abundance collected by Mosquito Magnet Independence Traps (MMIT) and average nightly temperature (from the subset of 28 sampling nights at control trees and 29 sampling nights at macaque sleeping sites for which environmental data were available). Points indicate observed data, with the line indicating the predicted association. Dashed lines represent upper and lower 95% confidence intervals.

Figure 4.11 Influence of A) macaque presence/absence, B) number of macaques present and C) daily rainfall on the mean nightly *An. balabacensis* abundance collected by Mosquito Magnet Independence Traps (MMIT). Points indicate observed data in B and C, with the line indicating the predicted association. Error bars and dashed lines are 95% confidence intervals and * represents $P < 0.05$.

Figure 4.12 Predicted relationship between mean *An. balabacensis* abundance collected by Mosquito Magnet Independence Traps (MMIT) and average nightly temperature from a subset of 28 sampling nights at control trees and 29 sampling nights at macaque sleeping sites for which environmental data were available. Points indicate observed data, with the line indicating the predicted association. Dashed lines represent upper and lower 95% confidence intervals.

Figure 4.13 Influence of A) macaque presence/absence, B) number of macaques present and C) daily rainfall on the mean nightly *An. donaldi* abundance collected by Mosquito Magnet Independence Traps (MMIT). Predicted mean *An. donaldi* abundance based on data from sampling 33 nights at control trees and 34 nights at macaque sleeping sites. Points indicate observed data in B and C, with the line indicating the predicted association. Error bars and dashed lines are 95% confidence intervals

Figure 4.14 Predicted relationship between mean *An. donaldi* abundance collected by Mosquito Magnet Independence Traps (MMIT) and average nightly temperature from a subset of 28 sampling nights at control trees and 29 sampling nights at macaque sleeping sites for which environmental data were

List of Additional files

Table S1. Description of habitat types, number of traps and collections made to investigate mosquito resting behaviour in study area.

Table S2. Total number of resting *Aedes* mosquitoes collected using CDC, RB and SRB trapping methods in eight habitats.

Table S3. Total number of resting *Culex* mosquitoes collected using CDC, RB and SRB trapping methods in eight habitats.

Table S4. List of medically important *Culex* species collected using CDC, RB and SRB trapping methods in eight habitats.

Table S5. Total number of blood-fed female resting mosquitoes obtained throughout the study.

Table S6. Blood meal hosts of engorged female mosquitoes. Hosts were identified using PCR and sequencing of the vertebrate cytochrome *b* mitochondrial gene.

Figure S1. Habitats selected to represent a gradient of different microhabitats arising from deforestation. Resting mosquito collections were performed A: inside houses; B: under houses; C: in the peri-domestic area around houses; D: palm plantations; E: rubber plantations; F: forest edge; G: forest interior at ground level; and H: forest canopy.

Figure S2. Map of Tuboh village. Icons indicate sampling areas of different habitat types: yellow pentagons - houses; orange stars - palm plantations; purple squares - rubber plantations; blue triangles - forest patches. Each symbol signifies a different sampling area and habitat, and thus was assigned an individual spatial cluster in analysis.

Figure S3. Map of Paradason village. Icons indicate sampling areas of different habitat types: yellow pentagons - houses; orange stars - palm plantations; purple squares - rubber plantations; blue triangles - forest patches. Each icon signifies a

different sampling area and habitat, thus was assigned an individual spatial cluster in analysis.

Figure S4. Physiological status of female *Aedes* collected.

Figure S5. Physiological status of female *Culex* collected.

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Author's Declaration

I declare that the research described in this thesis is entirely my own work unless otherwise stated. All chapters in this thesis have been compiled in co-authorship with my supervisor, Professor Heather Ferguson, with the intention for all to be published. The first chapter was published in Parasites and Vectors (June 2018) with input from our collaborators Professor Chua Tock-Hing and Kimberley Fornace. The second chapter is in preparation for submission to PLoS Neglected Tropical Diseases.

1 General Introduction

1.1 Background

Global malaria incidence has declined by 18% since 2010, but since 2014 in some regions, this rate has either reduced or reversed [1]. The recent World Health Organisation (WHO) 'Global Technical Strategy for Malaria (2016 – 2030)' aims to reduce the global malaria burden by 90% by 2030 from the rates reported in 2015 [2]. This was 212 million cases of malaria in 2015, which rose to 216 million cases by 2016 [1,3]. Countries in Africa account for 90% of malaria cases, with 7% occurring in Southeast Asia (SE) and 2% in the East Mediterranean regions [1]. The greatest decline in malaria incidence since 2010 (48%) was reported in SE Asia however from 2014 to 2016, a slight increase was noted [1]. The SE Asia region is met with specific challenges relating to the control of the malaria parasite *Plasmodium vivax* as it can cause recurring infections. For this reason, a network now consisting of 18 countries, called APMEN (Asia Pacific Malaria Elimination Network) was established to strengthen the fight towards eliminating malaria across the Asia Pacific Region by 2030 [4]. Numerous challenges threaten malaria elimination such as insecticide resistant mosquitoes, parasite resistance to artemisinin, environmental change and political instability [5]. A lesser known obstacle is the recent emergence of new zoonotic malaria species in humans, namely *P. knowlesi* in SE Asian countries and *P. brasilianum* in South America. There is a significant lack of inclusion of these zoonotic malarias in elimination plans as yet, which will need more recognition if the 2030 targets set out are to be met.

Plasmodium knowlesi naturally infects monkeys across SE Asia and recently has been recognised as the fifth species, in addition to *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale,* to naturally infect man [6]. The global burden of malaria is assessed based on reports of the main human malaria species (*P. falciparum* and *P. vivax*) [1] however excludes infections caused by spillover from our non-human primate relatives. Since 2004, Malaysia has experienced a significant outbreak of *P. knowlesi* in humans but these statistics are not included within the WHO 2016 elimination strategy [5]. A decline in *P. falciparum*/*P. vivax* malaria cases from 1092 in 2013 to 606 in 2014 is reported for Malaysia, but all that is mentioned about *P. knowlesi* is that it has been 'increasing in recent years and may require

a different approach' [5]. Recent calls have been made to acknowledge *P. knowlesi* within the WHO World Malaria Report [7] so that its significance is recognised along with the need for improved diagnosis, case reporting and control. *Plasmodium knowlesi* infection in humans is widespread across several countries in SE Asia however until its significance is recognised within the World Malaria Report it remains an unacknowledged barrier to elimination. In the following sections, a general overview of the biology of *P. knowlesi* and its mosquito vectors will be given. I will start with an outline of malaria transmission before focussing in on current knowledge about *P. knowlesi* specifically.

1.2 Life cycle of *Plasmodium* spp.

Plasmodium parasites require both a definitive and an intermediate host to complete their life-cycle [8]. Vertebrates are intermediate hosts [8]. Definitive hosts are blood-feeding insects and for human infecting malarias, these are female *Anopheles* mosquitoes [9]. Organisms which transmit pathogens between vertebrates are called vectors [10] thus for malaria the vectors are mosquitoes. A mosquito feeds by probing host skin and injecting anticoagulants from the salivary glands into the vertebrate capillaries [11]. Infectious parasites called sporozoites reside in the mosquito salivary glands and enter the host's bloodstream when mosquitoes feed (Fig. 1). Sporozoites travel through the bloodstream to the liver where they invade hepatocytes and undergo an asexual period of replication to develop into schizonts [12]. Schizonts mature and rupture releasing the next parasite form, known as merozoites, into the blood stream [12]. Thousands of merozoites then invade red blood cells and either continue the cycle of asexual reproduction or differentiate into gametocytes. The asexual cycle of reproduction occurs inside the red blood cell where merozoites grow into rings, trophozoites, then schizonts which contain 16-20 identical merozoites [8]. Some merozoites differentiate into sexual stage parasites called gametocytes [13]. When a mosquito feeds on an infected host and ingests gametocytes, males and females fuse inside their midgut to form a zygote [9]. The zygote differentiates into an ookinete which traverses the midgut wall forming an oocyst that matures over a period of 10-15 days. The oocyst develops thousands of sporozoites which invade the mosquito salivary glands where they remain to

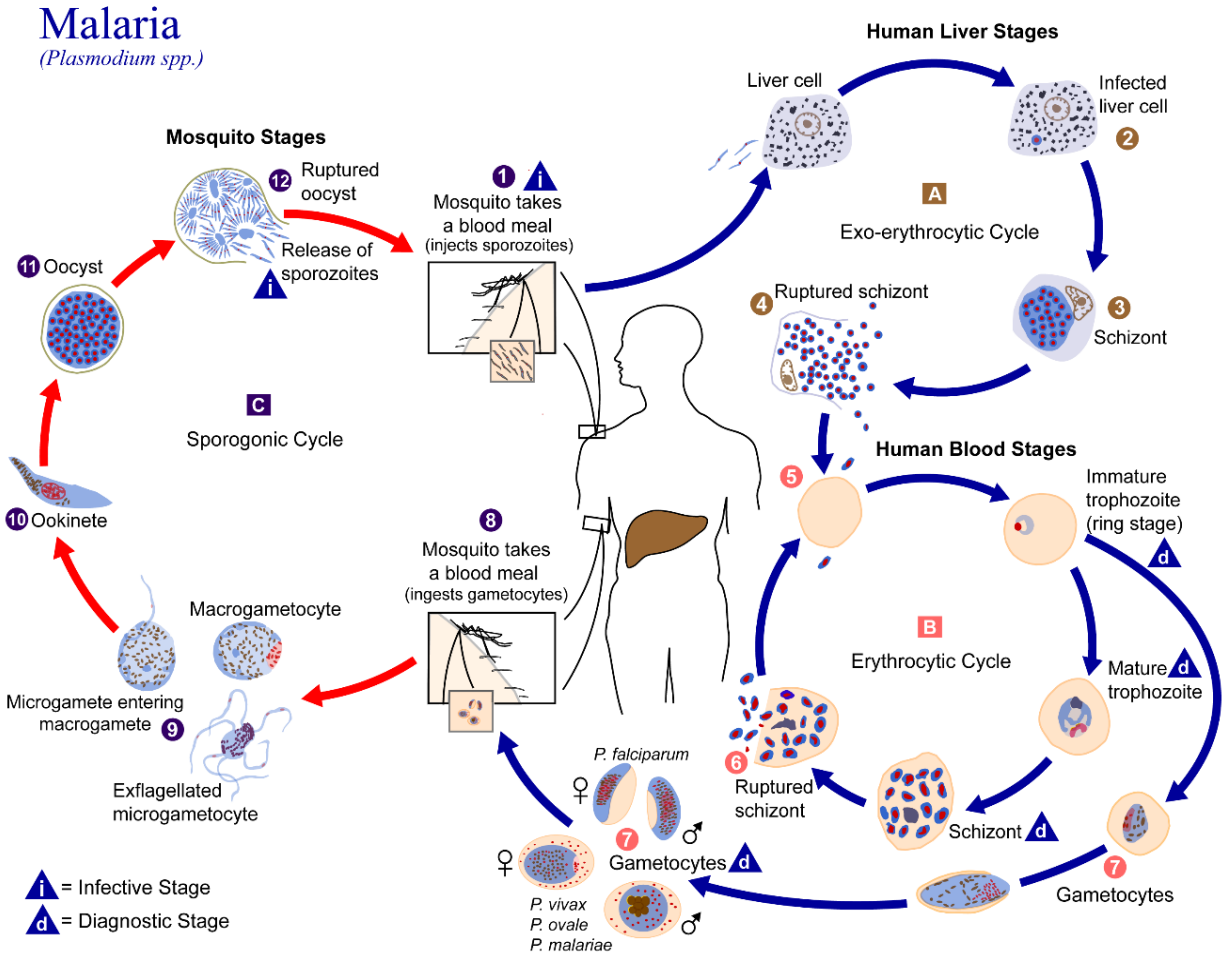

Figure 1.1 Centres for Disease Control and Prevention: Life cycle of *Plasmodium spp*. (https://phil.cdc.gov/Details.aspx?pid=3405) [14].

be injected into a new host the next time the mosquito feeds [15]. The interaction between parasite and host is highly complex and has evolved over a significant period of time, thus parasites are often strictly specific to their host species [8].

1.3 Biology and pathology of *P. knowlesi*

The life-cycle of *P. knowlesi* follows the same general pattern as described above but with some specific characteristics that differ from other human-infecting malarias. All human-specific malarias have a similar life-cycle with the main difference being the time taken to complete different developmental stages. For example *P. knowlesi* parasites require 5 days to complete the stage within the liver hepatocyte [12] whereas *P. falciparum* and *P. vivax* require 6 – 8 days [16]. The developmental time for parasites inside mosquito vectors, also known as the 'extrinsic incubation period' (EIP), is highly temperature dependent and varies between 10 – 18 days in human-specific malaria species [17]. The EIP for *P. knowlesi* is approximately 10 days [18]. Furthermore, *P. knowlesi* completes its asexual cycle of multiplication in hosts within 24 hours whereas other human-specific malarias require 48 to 72 hours [12]. Additionally the development of transmission-stage gametocytes occurs much faster in *P. knowlesi* (within 48 hours after merozoites invade erythrocytes [17]) compared to a much longer period of 7-15 days for *P. falciparum* [19]. The consequence of *P. knowlesi*'s more rapid replication and development in hosts is that parasitaemia can rise quickly in vertebrate hosts, and mosquito vectors can become infectious in a relatively short period of time.

The clinical symptoms associated with malaria infection stem from the asexual cycle of the parasite [20]. In humans, uncomplicated malaria often manifests as periodic fevers and chills which align with the timing of parasite growth within the red blood cell [21]. The most virulent human malaria parasite, *P. falciparum,* can also cause respiratory distress, convulsions, circulatory collapse, kidney injury, abnormal bleeding, severe anaemia and loss of consciousness [22] which can lead to death. *Plasmodium knowlesi* infection in people also potentially results in severe malaria with symptoms identical to those of severe *P. falciparum* with the exception of loss of consciousness [22]. As *P. knowlesi* has a rapid intra-erythrocytic multiplication rate, the likelihood of hyper-parasitemia in patients with this type of malaria is high [8]. The danger of hyper-parasitemia is

that mortality risk increases when parasite load exceeds 100 000 parasites/µl blood or 2.5 % parasitaemia [23]. *Plasmodium knowlesi* has the same mortality risk as the most lethal human parasite species *P. falciparum* [24]. For example in 2010 - 2013 in Sabah, Malaysia, *P. falciparum* and *P. knowlesi* human mortality rates were 4.4/1,000 (95% CI 2.3-7.7/ 1000) and 4.1/1,000 (95% CI 2.1-7.2/1000) respectively, with those calculated for *P. vivax* being much lower at 0.9/1,000 (95% CI 0.1-3.1/ 1000) [24]. Accurate and early diagnosis is therefore critical to treat *P. knowlesi* infection effectively. Severe *P. knowlesi* malaria can be effectively treated with intravenous artesunate and uncomplicated *P. knowlesi* infections can be easily treated with chloroquine or artesunate-mefloquine [25]. Therefore *P. knowlesi* is recognized as a significant public health problem with disease severity being equivalent to *P. falciparum* infection in humans.

1.4 *P. knowlesi* reservoir hosts

The natural hosts of *P. knowlesi* are long-tailed macaques, *Macacca fasicularis* [26], pig-tailed macaques, *M. nemestrina* [27] and some leaf monkeys *Presbytis spp* [28]. These two macaque species are widely distributed across SE Asia, with *M. fasicularis* being the most widespread, found in Brunei, Cambodia, Indonesia, Thailand, Peninsular Malaysia, Sumatra, Java, Borneo, the Philippines, Singapore and Vietnam [29]. *Macaca fascicularis* are classified as least concern on the IUCN Red List of Threatened species [30]. Long-tailed macaques live in troops of 10 – 100 individuals [31] and survive in a variety of habitats: primary rainforests, freshwater swamp forests and mangrove forests [32]. Ongoing human population expansion and encroachment on forested areas has increased the frequency of contact between macaques and humans [30]. In response to deforestation, macaques move to forest fringes where they are likely to encounter people [18]. In general, macaques have been the most successful group of monkeys to co-habit with humans [30]. They adapt well to secondary forest habitats and can often be seen foraging in areas cultivated with fruit trees, rubber trees and nipah palm [30,32,33]. Macaque-human conflict often arises due to monkeys stealing crops and raiding garbage in more urban areas [30]. This occurs frequently in Malaysia, with *M. fasicularis* being responsible for 64 % of human-wildlife conflict complaints [33]. The ecology of reservoir hosts is vital to *P. knowlesi* transmission, and here the main reservoir is widespread, with frequent contact with humans in urban areas, farmland and forest fringes.

In general, natural hosts have a mild long-term infection and are able to control parasitaemia so it is maintained at low levels [34]. More detail on the current knowledge about *P. knowlesi* prevalence in wild macaque populations in Malaysia is given in Chapter 4.

1.5 The life cycle of *Anopheles* mosquito vectors

Like all other malaria parasites that can infect people, *P. knowlesi* is transmitted by female *Anopheles* mosquitoes. Of the 400 species in the *Anopheles* genus, 30 – 40 are vectors of human malaria [35]. Mosquito development time is highly dependent on temperature, larval density, species and food resources [36,37]. The first three stages of the mosquito life cycle are aquatic and involve the development from eggs, to larvae and then pupae which usually takes from 10 to 14 days [17]. Adults hatch from pupae and although females can live for one month in the lab, in nature they likely only survive for 1-2 weeks [17]. *Anopheles* larvae can be distinguished from other mosquito species because they rest flat at the surface of the water to breathe because they lack a respiratory siphon that would allow them to remain deeper [17]. The larvae have four stages, which they develop through moulting of the exoskeleton [17]. Larvae feed on plant matter and microorganisms in water [38], whereas the relatively short-lived pupal stage (1-2 days) does not feed. After hatching, male and female adults mate in swarms at dusk then females seek out a blood source using sensory and olfactory cues [39]. Only females blood-feed because they require protein from the host blood-meal to produce eggs [14]. Post blood-meal, females display resting behaviour such as resting on walls inside houses if they are indoor feeders (endophagic) or amongst vegetation if they are outdoor feeders (exophagic). Some species may rest indoors for a brief time but leave to find a daytime resting habitat before morning [39]. The blood-meal is typically digested and eggs are formed within 2-4 days; this is called the gonotrophic cycle [39]. Gravid females then respond to species specific oviposition cues based on smell, touch, taste and vision to find a suitable body of water to lay their eggs [40]. Approximately 50 - 200 eggs are laid by a female at each oviposition [17] and the cycle continues. Male anophelines feed mainly on nectar and do not require a blood meal. Males can also be found in habitats where the females rest post-feed [39]. All *Anopheles* follow the same general life-cycle but feeding, resting, mating and oviposition behaviours vary with species, and developmental times

and demography with environmental conditions. Vector 'bionomics' includes information about both the ecology (ie. adult and larval habitats) and behaviour (ie. biting and resting) of a mosquito species [41].

1.6 **Vectors of *P. knowlesi***

Mosquitoes in the *An. leucosphyrus* species group are responsible for transmission of *P. knowlesi* [42]. The Leucosphyrus group is well distributed across SE Asia with 20 species being described from Indonesia, Malaysia, Thailand, Philippines, Brunei, Cambodia, China, Vietnam, Laos, Bangladesh, India, Taiwan and Sri Lanka [42]. These mosquitoes are highly associated with tropical rain forests where they breed in partially shaded temporary pools of water [42,43]. The *Leucosphyrus* group comprises three sub-groups: Hackeri, Leucosphyrus and Riparis [42]. Mosquitoes which are confirmed to transmit *P. knowlesi* reside in the Leucosphyrus (*An. balabacensis, Anopheles cracens*, *Anopheles dirus*, *Anopheles introlatus*, *Anopheles latens* and *Anopheles leucosphyrus)* and Hackeri (*Anopheles hackeri*) sub-groups [8,18,44]. The vector responsible for *P. knowlesi* transmission varies with geographic region; with different Anopheline species being incriminated in different settings. For example, *An. cracens, An. introlatus* and *An. hackeri* have been implicated in Peninsular Malaysia [18,45–47], *An. dirus* in Vietnam [48], and *An. latens* in Sarawak, Borneo [49]. In the Malaysian state of Sabah in Borneo where this research is focussed, *An. balabacensis* has been identified as the likely vector of human infection [50].

Key aspects of mosquito and parasite biology influencing malaria transmission are mosquito survival, parasite 'extrinsic incubation period' (EIP), and mosquito biting behaviour. These measures combine to determine "vectorial capacity", a classic measure of malaria transmission defined as the 'average number of inoculations with a specific parasite originating from one case of malaria in unit time' [51]. Malaria transmission is tightly linked to mosquito survival because after taking an infected bloodmeal, mosquitoes need to survive the parasite EIP which is the time required for gametocytes ingested in a bloodmeal to develop into infectious sporozoite stages in mosquito salivary glands [52]. Factors which influence the EIP are temperature, parasite and vector genetics, and larval and adult mosquito nutrition [52]. Even small changes in the EIP can have a large impact on the number of mosquitoes that become infectious and thus malaria

transmission [53]. The effect of temperature on EIP has been widely investigated to understand how climate predicts malaria risk [36,54,55]. This is particularly relevant with rising global temperatures associated with climate change and deforestation [53,56]. The EIP for *P. knowlesi* is ten days [18] but as with other *Plasmodium* species, is likely to fluctuate with environmental conditions [52]. Parous females are those that have previously produced eggs, thus parity is used as a measure of age through examination of the ovaries [57]. Entomological studies in Sabah have indicated a relatively high survival rate for *An. balabacensis*, where 50 % (n = 1791) of a population collected were parous [50]. Based on the daily survival of *An. balabacensis*, as estimated from parity rates (the proportion of mosquitoes that have laid at least one blood meal), it was estimated that ~16 – 24 % females live long enough to transmit *P. knowlesi* [50]. For other *P. knowlesi* vectors in Malaysia, *An. cracens* has been found with a higher survival rate in the forest (31 %) than farm (25 %) [46], whereas in another study the survival of *An. latens* was estimated to be lower in a forest (13 %) than at a longhouse or farm site (both 25 %) [58]. Current knowledge about *P. knowlesi* vectors indicates that mosquito survival is likely to differ with habitat type, but there is a lack of consistency in results about what habitats are most conducive to survival, and how habitat variation and associated temperature changes influence the EIP of *P. knowlesi* in its primary vectors.

In addition to vector survival and EIP length, vectorial capacity is also influenced by mosquito biting behaviour in terms of the number of bites on humans per night. This biting rate is influenced by mosquito abundance and host species preference. *Leucosphyrus* group mosquitoes tend to bite in the early evening, e.g. 18:00 - 20:00 hrs for *An. latens, An. cracens, An. balabacensis* and *An. dirus* [41,46,58,59]. Vectors like *An. cracens*, *An. balabacensis* and *An. dirus,* demonstrate strong outdoor biting behaviour, also known as exophagy [41,46,59–61]. *Anopheles balabacensis* and *An. cracens* will only bite outdoors [61,62] however *An. latens* is known to enter houses at night to bite [41]. The early outdoor biting activity of most *P. knowlesi* vectors coincides with the times when people are carrying out evening activities in the outdoor area of homes including cooking or socialising. Thus people are unlikely to be receiving protection from standard vector control measures like Long lasting Insecticidal Nets (LLINs) at the time when vectors are most active. The outdoor biting activity of *P. knowlesi* vectors is also conducive

to biting monkeys and picking up simian malaria infections. The exophagy and early biting behaviour of *P. knowlesi* vectors facilitate zoonotic transmission and are a major impediment to control with standard methods.

The transmission of host-specific parasites is enhanced when vector feeding is also highly specialised [63]. However zoonoses like *P. knowlesi* benefit from a more generalist vector that will regularly bite both the reservoir macaque host and humans. Mosquito vectors of disease often display strict host preferences, but information about this for *P. knowlesi* vectors is limited [63]. Studies have indicated that *An. latens* show no preference for man or long-tailed macaques [49], *An. balabacensis* favours man and monkeys over domestic animals [64,65] and *An. hackeri* feed largely on monkeys [49]. *Anopheles dirus* is reported to have an overall preference for human blood [66,67], however other studies indicate it can be zoophiliic, biting cattle more frequently than humans [68]. *Anopheles balabacensis* have also been found five times more likely to be attracted to five men than one buffalo, and for individuals to return to the same host species on their second blood meal [69]. *Anopheles cracens* is reported to have a 2:1 preference for feeding on humans than monkeys [45]. Vectors of *P. knowlesi* demonstrate an affinity for humans and monkeys, however information about the frequency of human bloodmeals vs. other hosts for all species is lacking and is likely to differ between environments depending on host availability [63]. In the context of *P. knowlesi* transmission, mosquito host preference has a crucial influence both on human infection but also for maintaining transmission within reservoir hosts.

1.7 History of *P. knowlesi* emergence

Plasmodium knowlesi was first discovered in the 1930s in long-tailed macaques [26]. At that time, Napier and Campbell experimentally infected one *M. mulatta* (Rhesus monkey) and two *M. fasicularis* with *P. knowlesi*. While a mild infection resulted in the long-tailed macaques, the rhesus monkeys suffered a severe and uncontrolled infection [70]. Also in 1930s, Knowles and Das Gupta performed experimental transfer of *P. knowlesi* infected macaque blood into a person which revealed that humans could be infected by this type of malaria [26]. Although this experimental work demonstrated that it was theoretically possible for humans to be infected by *P. knowlesi*, this was considered unlikely to occur

in nature. The first natural human *P. knowlesi* infection was reported in 1965 in a US army surveyor who was stationed in a forest in Peninsular Malaysia [71]. Consequent entomological investigations concluded that transmission of *P. knowlesi* to humans was not a significant public health concern. This was because the vectors of simian malaria, *An. introlatus* and *An. leucosphyrus*, were found biting man and monkeys in the forest however neither were caught feeding in the village [72]. The only mosquito caught in both village and jungle was *Anopheles maculatus*, but it was considered to be at too low abundance to act as a bridge vector between monkeys and humans [72]. Thus the lack of simian malaria vectors in the village led Warren and colleagues to dismiss *P. knowlesi* malaria as a threat to public health.

Decades later, in 1999, reports of unusual *P. malariae* infections in Kapit, Sarawak sparked further molecular investigation into patients admitted to hospital [73]. Typically, *P. malariae* infections are long-term, asymptomatic and parasite load is rarely higher than 5000 parasites per µl blood [73]. However in that year, patients were referring themselves with symptoms of malaria such as fever and high parasitaemias of > 5000 parasites per µl blood [73]. Patient slides which had been microscopically diagnosed as *P. malariae* were negative by PCR and instead were identified as *P. knowlesi* [73]. Overall, *P. knowlesi* was confirmed as being responsible for 58% of 208 malaria admissions to Kapit hospital in 2000 - 2002 [73]. Initially, *P. knowlesi* infection in humans had gone largely undetected because the methods used to test for *P. knowlesi* DNA had only recently been designed [73,74] and molecular detection in hospitals was not yet available. Species identification was performed using microscopy alone however morphological diagnosis for *P. knowlesi* is unreliable as ring stage parasites cannot be easily differentiated from *P. falciparum,* and later trophozoites closely resemble those of the more benign *P. malariae* [75]. Following on from the human *P. knowlesi* outbreak in Sarawak, further hotspots were identified in Malaysia [6,18,24,45], Kalimantan [76], Vietnam [77], Laos [78], Sumatra [79], Singapore [80], Cambodia [81], the Philippines [82] and Thailand [83]. The advent of molecular diagnosis of hospital malaria cases led to the awareness of the extent of *P. knowlesi* in the human population and its emergence as a zoonotic malaria across numerous countries in SE Asia.

1.8 *P. knowlesi* epidemiology

Since the first outbreak of *P. knowlesi* in Malaysia [73], several countries in SE Asia have reported sporadic human cases of human *P. knowlesi* [78,80-83]. However in Malaysia, this disease is more widespread and entrenched, occurring in numerous states across Peninsular Malaysia and Malaysian Borneo (Fig. 1.2) [6]. All human malaria species are endemic to Malaysia, and historically most human infections were due to *P. falciparum* and *P. vivax* [24]. However in 2012, *P. knowlesi* constituted the highest proportion of all human malaria infections (38.4 %), followed by *P. vivax* (30.9 %), *P. falciparum* (19 %), *P. malariae* (10.3 %), *P. ovale* (0.1 %*)* and mixed infections (1.35 %) [6]. High levels of *P. knowlesi* transmission occur in Sabah and Sarawak, Malaysian Borneo, with the highest percentage localised to Kudat in northern Sabah (Fig. 1.2) [6,84]. Historical records indicate that the while the incidence of *P. falciparum* and *P. vivax* malaria substantially decreased during 2004 - 2013 in Sabah, the number of *P. knowlesi* cases increased from 59 to 996 between 2004 and 2013 [24,85]. *Plasmodium knowlesi* notifications were increasing rapidly across all divisions in Sabah from 2001 – 2013 with the highest frequencies in the Interior, West-coast and Kudat Divisions [24]. From 2004 to 2016, the number of *P. knowlesi* cases confirmed by PCR/sequencing in peer-reviewed articles was highest for Malaysian Borneo (4,553), followed by Indonesia (465) and Peninsular Malaysia (204) [86]. The most up-to-date reports state that in 2016, *P. knowlesi* was responsible for 69 % of all malaria cases in Malaysia [87].

Transmission to humans is primarily thought to occur in the forest, where people are exposed to *P. knowlesi* vectors which have been feeding on infected macaques. This view was supported by a range of risk factors identified in patient case studies conducted in the Kudat area in North-western Sabah [84]. Adults (over 15 years), particularly males, were found to be at highest risk of *P. knowlesi* malaria [88]. Additionally, being aware of the presence of monkeys in the previous 4 weeks, and farming or plantation work was also strongly associated with cases [88]. Further to these, travel, outdoor sleeping or having homes with open eaves was common among patients [88]. However, *P. knowlesi* malaria occurs across all age groups, and familial clustering of cases have been reported, suggesting transmission can also occur around the home [84]. In agreement with the original hypothesis proposed by Warren *et al* [72], that only *Anopheles* present

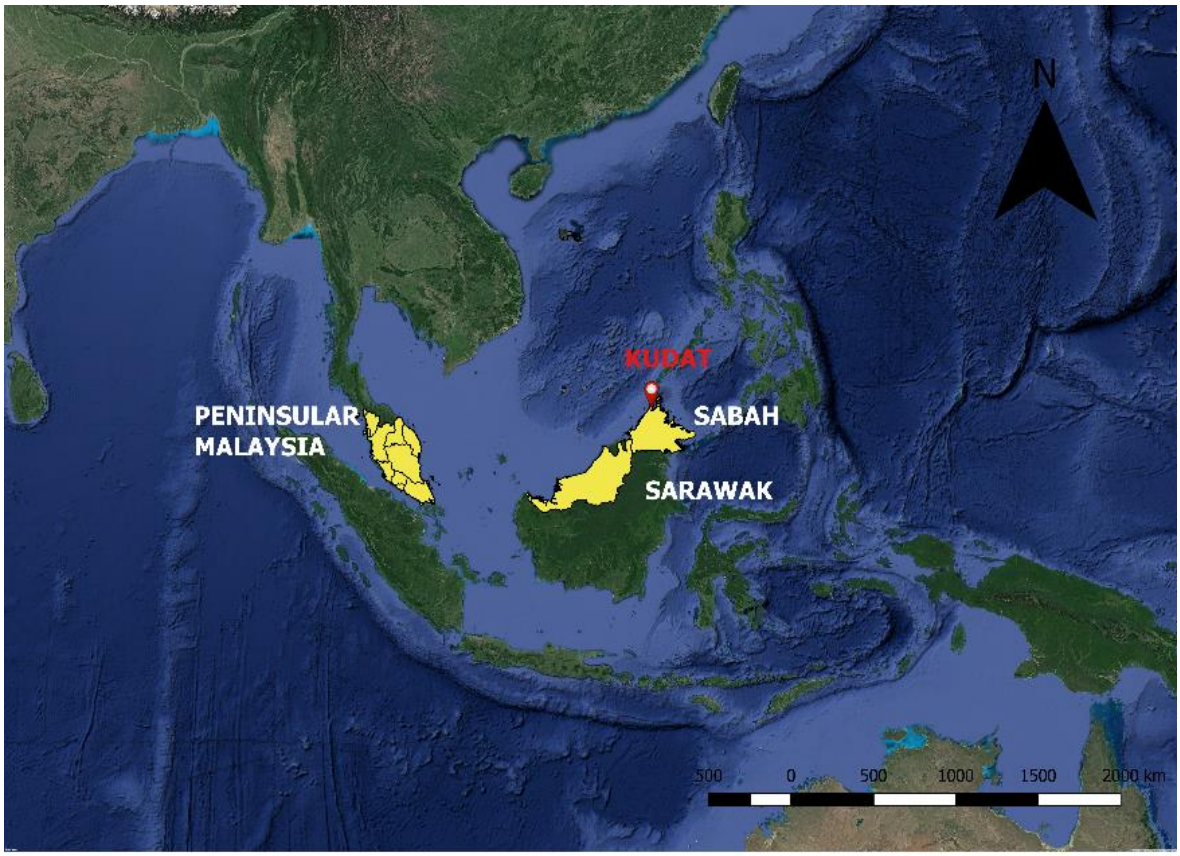

Figure 1.2 Map of Malaysia including mainland Peninsular Malaysia and the states of Sabah and Sarawak in Malaysian Borneo.

in the forest transmit monkey malaria, it seems that most transmission to humans occurs in forested areas however, reports of peri-domestic transmission highlight that this is not always the case.

Human infections are believed to occur as spillover from the monkey reservoir [89]. As yet, there is no definitive evidence of human to human transmission, however gametocytes have been detected in *P. knowlesi* patients (n = 4/10), suggesting that it could be a possibility [75]. Studies in Vietnam detecting co-infections of *P. falciparum*/*P. vivax* and *P. knowlesi* malaria in *An. dirus* mosquitoes strongly suggests that humans can infect vectors with *P. knowlesi* [77]. This is because the chance of one mosquito getting first infected with *P. falciparum* or *P. vivax* malaria and then by a monkey infected with *P. knowlesi*, as well as surviving long enough to develop sporozoites of both, is very low. Asymptomatic infections have been detected in southern Sarawak [90], in addition to Kudat and Kota Marudu districts where 6.9 % of individuals from all age groups were infected [91]. Human to human transmission may play a part in infection dynamics however the spread to humans is most likely dependent on the force of infection coming from macaques [92].

1.9 Hypotheses for the emergence of *P. knowlesi* in humans

Several different hypotheses have been proposed for the emergence and expansion of *P. knowlesi* in humans including improved diagnosis, reduction of other human malaria infections and deforestation. There are varying degrees of evidence for each, as discussed below, which may not be mutually exclusive and might act in conjunction with one another.

1.9.1 Improved diagnosis

The cheap and rapid diagnosis of malaria by microscopy makes it the most used technique in rural hospitals [8]. In SE Asia, microscopists are adept in identifying the human malarias *P. falciparum*, *P. vivax* and *P. malariae* however because the attributes of *P. knowlesi* are reminiscent of both *P. falciparum* and *P. malariae*, mis-diagnosis by microscopy is commonplace [8]. Numbers of *P. knowlesi* cases reported in Malaysia rose significantly following the development of

molecular methods for *P. knowlesi* diagnosis, by Singh *et al*. in 2004 [73] using PCR of the small-subunit RNA gene. The increase in *P. knowlesi* notifications could partly be attributed to better diagnostic techniques however, the increase was so substantial (16-fold from 2004 to 2013) that it is unlikely to be attributed to improved diagnosis alone.

1.9.2 Reduction of other human malaria infections

Malaria control in Malaysia over the last few decades has resulted in a notable decline in the incidence of *P. falciparum* and *P.vivax* [24]. It has been hypothesised that this reduction of human malarias was a contributing factor to *P. knowlesi* emergence because it freed up humans as a host where previously there had been competition between malaria species [93]. There is also speculation that declines in other human-specific malarias caused a loss of relative immunity in people which would have given cross-protection against *P. knowlesi* [85,86]. Antibodies to some recombinantly produced *P. vivax* antigens are known to cross-react with *P. knowlesi* [94,95] however this gives little indication about protection offered by antibodies naturally generated in humans. It is unknown if there is competition between *P. knowlesi* and other malaria species within mammalian hosts however mixed infections have been detected in both people [96–99] and vectors [77,100]. Thus there is a lack of information about the immune protection to *P. knowlesi* provided by previous human malaria infection to know if the reduction in human malarias contributed to *P. knowlesi* emergence, but it was unlikely prevented previously by competition between parasite species as mixed infections in hosts are a common occurrence.

1.9.3 Deforestation

A significant ecological change has been occurring in Sabah concurrently with the upsurge in human *P. knowlesi* cases. This change is the large-scale deforestation of land for agriculture. In 1973, 75.7 % of Borneo was covered by intact old growth forest, which had reduced by 30.2% by 2010 [101]. High rates of forest loss occurred in Sabah, where 39.5 % of its original forest became non-forest by 2010 [101]. Of the area that was deforested across Borneo between 1973 to 2010, 38.5 % is now occupied by oil plantation and 6.3 % by timber plantation industries [101]. In depth study of Kudat and Kota Marudu districts in Sabah from

2008 to 2012 found a 4.8 % reduction in forest cover [91]. Fifty-one percent of villages in these districts lost > 10 % forest cover in a 5 km radius in the previous five years [102]. Thus rapid deforestation has been occurring and is still on-going in Sabah.

Recent investigations have identified a significant association between incidence of human *P. knowlesi* infection in Sabah and forest loss [102]. In the past, felling of trees in Malaysia for agriculture has resulted in malaria increases. A WHO report analysing *P. falciparum*/*P. vivax* malaria epidemics in Peninsular Malaysia from 1900 to 1940 discovered a correlation between replanting of rubber crops in response to economic demand and rising malaria incidence, and noticed a decline in malaria cases at periods when trade was low [103]. The situation with *P. knowlesi* is more complex however because a wild animal reservoir (macaque) is incorporated into transmission.

Deforestation has been proposed to trigger *P. knowlesi* emergence through a variety of mechanisms involving humans, macaques and mosquitoes [89]. With deforestation, people are more likely to spend more time in or around the edge of forests leading to increased contact with infected vectors [89]. Second, macaques often move out of forests that are being logged into new forest patches (Salgado-Lynn *et al*, personal communication), and as a consequence may bring the infection into human settlements. Habitat removal and reduction in food availability could cause monkeys to over populate remaining forest patches or even forage within human settlements increasing potential for crossover to humans if competent vectors are present [89]. Third, deforestation may change the abundance, species composition, behaviour and survival of mosquito vectors in a way that enhances their transmission potential. Currently, there is relatively limited understanding of the ecology of *P. knowlesi* vectors in Sabah and how they respond to changes in land use. However there are several potential mechanisms through which deforestation could impact vectors.

One such mechanism by which deforestation can alter vector populations is by changes in microclimatic conditions. Higher temperatures have been noted in land which has been deforested compared to areas of forest cover [56,104–108]. Increased temperatures act to alter vector ecology by speeding up adult and larval growth, shortening gonotrophic cycles and parasite EIP [56,104,107,109].

Clearing of land can result in poor drainage and can create a multitude of larval habitats [110]. In turn, this can lead to higher mosquito densities, increased biting activity and malaria cases as shown in the Amazon with increased populations of the human malaria vector, *An. darlingi* [110]. Furthermore, vector behaviour may be altered if monkeys leave post-deforestation as they may switch to feed on humans more often, leading to zoonotic transmission. When monkeys are present, the vectors may not bite humans as much, a concept which is known as 'zooprophylaxis' [111]. Due to a lack of entomological surveillance prior to the significant deforestation across Sabah, it is unknown how vector populations have been altered, however it is likely that deforestation changing microclimates, vector ecology and behaviour has been the main trigger for *P. knowlesi* emergence in humans.

1.10 An interdisciplinary approach to investigating *P. knowlesi* transmission in Sabah

The significant outbreak of zoonotic malaria in humans in Sabah prompted the creation of a Medical Research Council UK-funded programme which ran from 2012-17:

'Defining the biomedical, environmental and social risk factors for human infection with Plasmodium knowlesi; opportunities for prevention and control of an emerging zoonotic infection'.

This programme, with the abbreviated title of "Monkeybar" took an interdisciplinary approach involving clinicians, epidemiologists, primatologists, entomologists and social scientists to investigate the potential determinants of *P. knowlesi* emergence. The programme focussed on two areas with very distinct *P. knowlesi* transmission: the Kudat division of Sabah, Malaysian Borneo where a large outbreak (hundreds per year) of human *P. knowlesi* infections were occurring, and in Palawan Island, the Philippines, where very few human cases are reported. Operating within the confines of the grant, Monkeybar selected entomological studies to establish baseline information on *P. knowlesi* vectors in Sabah. These included trap evaluation and host choice studies [112], investigating seasonal patterns in vector abundance and infections [50], adult and larval distribution across land-use types and mosquito collections at case (previous

infection) and control (no previous infection) houses to identify *Anopheles* species associated with *P. knowlesi* infection [105]. However several key aspects of vector ecology remained to be investigated to allow design of appropriate surveillance and control measures against *P. knowlesi* infection. These were details on vector behaviour and habitat associations across changing landscapes in Sabah, and the effect these have on *P. knowlesi* exposure risk to humans, in addition to vector ecology associated with *P. knowlesi* transmission among macaque reservoir hosts. Thus this PhD was set up in conjunction with Monkeybar to address these additional knowledge gaps relating to the ecology and transmission potential of *P. knowlesi* vectors.

1.11 **Aims and objectives of research**

The aim of this PhD research was to investigate the ecology of *P. knowlesi* within Sabah with a specific focus on understanding how changes in habitat and host distribution influence vector populations. Elucidation of this will improve understanding about risk factors for emergence of *P. knowlesi* in Sabah, and in other settings, and reveal important information about avenues for vector control. Operating in conjunction with Monkeybar, this PhD study addressed key knowledge gaps about vector ecology necessary for a fuller understanding about *P. knowelsi* transmission and risk to humans in Sabah. This was a four-year funded Biotechnology and Biological Sciences Research Council Doctoral Training Programme (BBSRC-DTP) that included a three-month Professional Internship for PhD Students (PIPS) placement. The research followed the format of three separate studies conducted on subsequent years, each including a period of fieldwork in Sabah. Each study was designed independently to address a specific set of objectives as outlined below. This thesis follows the same format, with each study written up as a separate chapter.

Aim 1: Characterise the resting behaviour of *P. knowlesi* vectors.

Objective 1.1: Test new sampling methods to collect resting *P. knowlesi* vectors.

Objective 1.2: Use resting traps to assess the habitat preference of *P. knowlesi* and other mosquito vectors in Sabah province.

Objective 1.3: Use resting traps to assess the host species choice of *P. knowlesi* and other mosquito vectors.

Aim 2: Investigate the distribution of *P. knowlesi* vectors over a wide geographical scale in Sabah.

Objective 2.1: Determine if *P. knowlesi* vector-habitat associations found from small scale sampling are repeated at larger scales.

Objective 2.2: Test for environmental determinants of *P. knowlesi* vector abundance.

Objective 2.3: Use *P. knowlesi* vector abundance and Monkeybar human sero-prevalence data to identify entomological indicators of infection.

Aim 3: Elucidate malaria transmission dynamics within reservoir host populations.

Objective 3.1: Evaluate methods for collecting malaria vectors host seeking near macaque populations.

Objective 3.2: Determine the abundance and diversity of malaria vectors nearby macaque troops.

Objective 3.3: Identify circulating *P. knowlesi* and other primate malaria infections in vectors and macaque hosts.

2 Evaluation of resting traps to examine behaviour and ecology of mosquito vectors in an area of rapidly changing land use in Sabah

2.1 Abstract

Widespread deforestation occurring in the tropics is hypothesized to impact the transmission of vector-borne diseases (VBD). Predicting how environmental changes will impact VBD transmission is dependent on understanding the ecology and behaviour of potential vector species outside of domestic settings. However there are few reliable sampling tools for measuring the habitat preference and host choice of mosquito vectors; with almost none suitable for sampling recently blood-fed, resting mosquitoes. This study evaluated the use of two mosquito traps: the resting bucket (RB) and sticky resting bucket (SRB) traps relative to CDC backpack aspiration (CDC) for sampling mosquitoes resting in a range of habitats representing a gradient of deforestation. Eight habitats were selected for sampling around two villages in Kudat District, Malaysian Borneo, to reflect the range of habitats available to mosquitoes in and around human dwellings, and nearby forest habitats where reservoir hosts are present: secondary forest (edge, interior and canopy); plantations (palm and rubber); and human settlements (inside, under and around houses).

Over 31 days, 2243 mosquitoes were collected in 5748 discrete collections. Nine mosquito genera were sampled with *Aedes* and *Culex* species being present in all habitats and most abundant. RB and CDC backpack aspiration were most efficient for sampling *Culex* whereas CDC backpack aspiration and SRB were most efficient for *Aedes*. Most *Aedes* identified to species level were *Ae. albopictus* (91%), with their abundance being highest in forest edge habitats. In contrast, *Culex* were most abundant under houses. Most blood-fed mosquitoes (76%) were found in human settlements; with humans and chickens being the only blood source.

RB and SRB traps proved capable of sampling mosquitoes resting in all sampled habitats. However, sampling efficiency was generally low (*c*.0.1 per trap per day), necessitating traps to be deployed in high numbers for mosquito detection. None of the traps were effective for sampling zoonotic malaria vectors;

however, SRB collected relatively higher numbers of the dengue vector *Ae. albopictus*. The higher abundance of mosquitoes in forest edge habitats indicates the potential value of these traps for investigating sylvatic dengue transmission. This study has demonstrated the merits in application of simple resting traps for characterising mosquito vector resting behaviour outside of the home.

2.2 Introduction

Vector-borne diseases are responsible for 17% of all infectious diseases contracted worldwide, impacting the public health and economic growth of primarily developing countries [113]. Vital to the control of vector-borne disease (VBDs) is an understanding of the ecology and behaviour of species responsible for pathogen transmission [114]. This is particularly crucial for tackling emerging VBDs where data on vector biology are scarce. One such example is the emergence of the primate malaria causative agent *P. knowlesi* in human populations in Southeast (SE) Asia over the past decade, with an epicentre in the State of Sabah in Malaysian Borneo [6,24]. *Plasmodium knowlesi* is a simian malaria parasite whose primary hosts are long-tailed and pig-tailed macaques, and leaf-monkeys [115]. Human infection with *P. knowlesi* was previously thought to be rare [72]; however, the number of human infections reported in SE Asia has substantially increased in recent years [24,85]. *Plasmodium knowlesi* now accounts for the largest proportion of malaria cases in people in Malaysian Borneo [6]. Other mosquito-borne diseases are present in this area including human malaria (*P. falciparum*, *P. vivax*, *P. malariae* [6]), filariasis [62,116–121], Japanese encephalitis [122], dengue [123–129], and chikungunya [130]. Cases of Zika were also recently reported [131]. Development of integrated vector control approaches with capacity to target this suite of mosquito VBDs would be of benefit in Malaysia and the numerous other settings where they co-occur.

The emergence of *P. knowlesi* in Sabah has been associated with rapid changes in land use [102]. From 1980 to 2010, the area of land covered by forest in Sabah decreased from 60% to 51% [132]. This change is largely attributable to conversion of forest to plantation to meet the increasing demand for palm oil [132]. Changes in land-use for agriculture have been associated with outbreaks of mosquito VBDs in other settings [133–135]. Proposed mechanisms for these increases include changes in soil conditions and drainage following deforestation that alter the

availability of aquatic habitats for mosquito larvae [135–137]. Ground and water temperatures are higher in cleared than in forested areas [79,107] which can speed up mosquito larval development and reduce the length of the adult gonotrophic cycle. Both these changes are expected to increase mosquito fitness and abundance [104,105,138]. Higher temperatures can also increase the rate of pathogen development in mosquitoes (e.g. malaria parasite development [56,104,138] and dengue virus [139]). Additionally, following forest removal, humans often migrate to new, cleared areas leading to an increase in frequency of contact between human and animal hosts [89]. Consequently deforestation has potential to increase a range of mosquito VBDs of public health importance [136]. This occurred in the Peruvian Amazon where *Anopheles* biting rates increased in deforested areas causing an upsurge in malaria cases [140] and also in Sarawak, Malaysia, where development of a palm oil plantation led to a reduction in malaria vectors but an increase in vectors of dengue virus [135].

The increase in *P. knowlesi* poses a significant challenge because the mosquito vector species responsible for transmission are unlikely to be targeted by conventional control strategies. For example, the primary vector of *P. knowlesi* in Sabah is *An. balabacensis* [50]; a species that bites almost exclusively outdoors (exophilic) and has a relatively high survival rate [141]. Additionally, this vector species feeds extensively on the non-human primates that act as a reservoir for *P. knowlesi*. The two common methods of vector control in Malaysia, insecticide-treated nets and indoor residual spraying [142,143], only provide protection from mosquitoes attempting to feed on people inside houses; and are thus unlikely to have much impact against exophilic and zoophilic species like *An. balabacensis*. These challenges are not unique to *P. knowlesi*. Several of the mosquito species responsible for other VBDs in the area are also exophilic and/or become infected from an animal reservoir. For example, Borneo experiences a sylvatic dengue transmission cycle between macaques and silver langurs [144], driven by forest *Aedes* species [145]. Currently evidence suggests that sylvatic dengue transmission is restricted to forests; however, several spillover cases into the human population have occurred [146,147]. *Aedes niveus* is expected to be responsible for transmission in the forests of Sarawak and spillover to humans is driven by the exophilic *Ae. albopictus*, acting as a bridge vector, spanning a wider range of habitats including villages, agricultural areas and forests [147]. However,

information about key vectors transmitting sylvatic dengue in Sabah is unknown. The human dengue serotypes spread by *Aedes aegypti* and *Ae. albopictus* in urban areas are believed to have originated from sylvatic dengue strains [146] and although currently sylvatic strains seem to be largely restricted to the forest, evidence suggests that these viruses do not require any adaptation time to replicate efficiently in humans [146]. This highlights the potential for epidemics to arise and stresses the need for reliable tools that can be used across a range of habitat types to characterise *Aedes* mosquito ecology and host preference to understand sylvatic dengue transmission in Sabah. Furthermore, both Japanese encephalitis (pigs, horses and donkeys [148]) and filariasis (e.g. cats, dogs and leaf monkeys [149,150]) can be spread to humans from an animal reservoir. The control of this group of VBDs is clearly dependent on the development of novel vector control tools which can target vectors in multiple habitat types outside of the home [151].

The development of such control strategies is impeded by a lack of appropriate sampling tools for investigation of mosquito vector ecology outside of homes. Characterization of mosquito feeding behaviour and habitat use requires tools that sample both the host-seeking and resting population. However, most standard sampling methods can only be applied indoors. For example, host-seeking mosquitoes are frequently sampled using CDC light traps indoors (malaria vectors) [152–154] or BG sentinel traps (dengue vectors) [155–157]. Similarly resting mosquitoes are usually targeted by aspiration of mosquitoes from the inside of house walls (e.g. *Aedes* [158–160] and *Anopheles* [161]) or pyrethrum spray catch indoors [60]. Whilst host-baited traps have shown some success for sampling mosquitoes host-seeking on animals and humans outdoors [46,58,60,69,112,162], there are few methods for sampling mosquitoes resting in forest or other non-domestic habitats. Sampling resting mosquitoes is particularly vital for characterizing mosquito host choice. This is inferred by analysis of the blood meal of recently fed females to identify host preference. There are several methods for sampling mosquitoes resting in and around the home [161,163–166] but these often give biased estimates of host choice by favouring humans and peri-domestic animals [63,167]. These techniques are rarely used to sample mosquitoes in wilderness areas away from homes. As yet, resting collections have largely been used to investigate diseases transmitted around the home, not ones that

could be transmitted in forested habitats or that have a wild animal reservoir host. Recent work in Africa has evaluated standardized, portable and low-cost resting traps for collecting resting *Anopheles* in peri-domestic settings [161,166]. These have yet to be trialled for sampling mosquitoes resting in forest and other non-domestic habitats. Further to defining habitat use and host choice of vectors, there is a need for standardised resting collection techniques to monitor and detect alterations in mosquito behaviour. Changes to the environment and use of control methods can drive adaptations and shift patterns of behaviour in vector populations. An example of this is the use of insecticide-treated bed nets in Tanzania and Papua New Guinea which resulted in shifts to outdoor biting, time of biting and changes in host feeding behaviour [63,168]. Land-use changes such as deforestation for cultivating palm oil also induce changes in mosquito behaviour [135,140]; however, in order to detect shifts in host choice or resting behaviour, new methods are required that can span all available habitats, such as those arising from deforestation, to detect any differences occurring between them.

The aim of this study was to evaluate two new trapping methods for sampling mosquitoes resting within domestic, peri-domestic, agricultural and forest settings in an area of Malaysian Borneo where multiple VBDs are present. Whilst the study encompassed investigation of the mosquito community in general, our focus was on known vectors of malaria, dengue and filariasis. I trialled a simple bucket trap [161] and sticky trap [166] that were originally developed for sampling outdoor resting malaria vectors in Africa. These methods were compared with collections made using a CDC backpack aspirator. This is a standard method for sampling vectors resting inside houses [147,164] and is occasionally used to collect insects resting on vegetation [169]. These techniques were compared across eight different habitat types representing a gradient of deforestation, with the aim of characterising the resting habitat preferences and host choice of potential mosquito vectors. This information will highlight the suitability of these novel techniques for understanding mosquito behaviour and ecology.

2.3 Methods

2.3.1 Study site selection

This study was conducted in the Kudat District of Sabah State in Malaysian Borneo (Fig. 2.1). Kudat was the focus of a successful community engaged and intersectoral approach to control *P. falciparum* malaria from 1987 to 1991 [170]. In recent years however, this district has experienced a high burden of human *P. knowlesi* cases [85]. Dengue incidence is also high and has been increasing considerably in Malaysia since 2000 [123]. Starting in 2012, Kudat was the focus of an extensive, interdisciplinary research project aiming to identify the social and ecological drivers of *P. knowlesi* emergence [171]. As part of this project, a 2 × 3 km grid (Fig. 2.1) encompassing a range of habitats reflecting different land cover types was selected for detailed study of macaque and mosquito vector ecology. This study was based in two villages situated within this grid: Tuboh (06.76467, 116.76953) and Paradason (06.76957, 116.78618). Tuboh is a small village of approximately 20 houses surrounded by patches of clearing, palm trees, rubber trees and secondary forest. Paradason village is situated 1.5 –2 km from Tuboh and is also composed of approximately 20 houses. Palm and rubber fields comprise most of the land surrounding Paradason in addition to a large area of secondary forest.

2.3.2 Resting collection techniques

Three different methods were used to sample resting mosquitoes. The first was the resting bucket trap (RB) [161] which is made from a 20l black plastic bucket lined with black linen cloth (Figure 2.2A). RBs were set by placing them horizontally on the ground, with a black cloth soaked in water inside to increase humidity. Mosquitoes were removed from RB's using a CDC backpack aspirator (John W. Hock, model 1412). The performance of the RB was contrasted with another recently developed method for passive sampling of resting mosquitoes: the Sticky resting bucket (SRB) (Figure 2.2B). This trap is a modification of the Sticky Resting Box [166] in which the inner surface is lined with sticky surfaces to trap mosquitoes that land on them. The SRB is an RB with an inner lining made of four A4 acetate sheets coated in DeBello rat glue. This was developed as an improvement to the standard RB because it was hypothesized that the sticky

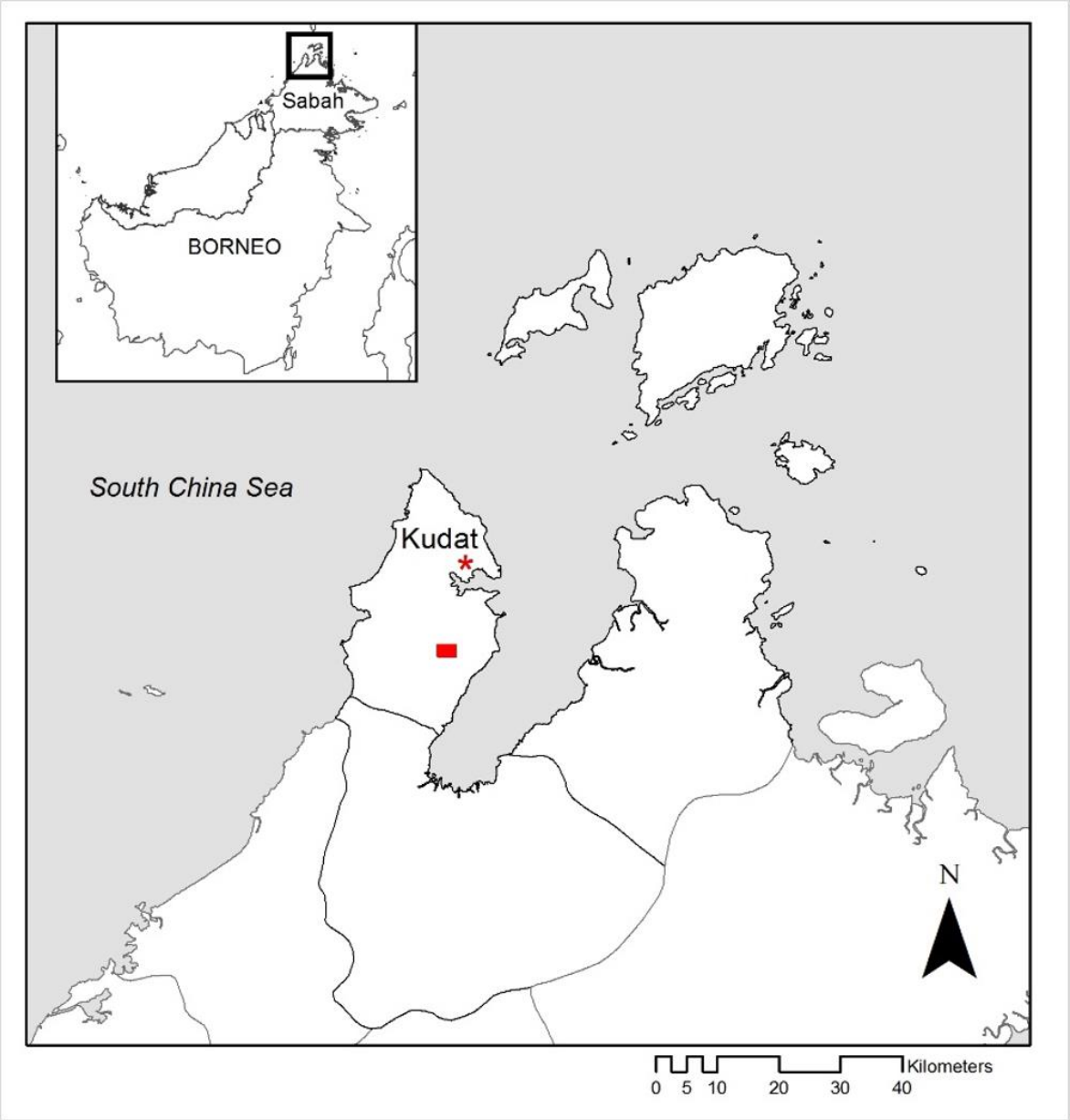

Figure 2.1 Map of Sabah Province in Malaysian Borneo with a red rectangle indicating the location of the study site for investigating resting mosquito behaviour in Kudat District. The rectangle represents a 2 × 3 km grid intensively studied for macaque and mosquito ecology as part of the Monkeybar programme.

A

B

Figure 2.2. Photo of A) Resting bucket (RB) and B) sticky resting bucket (SRB) traps.

surfaces would increase the catch. Mosquitoes affixed to sticky surfaces were removed by cutting out a small square from the acetate sheet. The same acetate sheet was used throughout the week but replaced when more than 5 mosquitoes had been cut from one sheet or if it had become dusty. Both types of resting traps were set up between 12:00–17:00 hrs on the first day and were re-set after collections each subsequent morning between 6:00–11:30 hrs.

RB and SRB collections were made daily in all habitat types except for inside houses because of potential intrusion to residents. Instead, mosquitoes resting inside houses were collected using a CDC backpack aspirator [172–174]. A CDC backpack aspirator was used to aspirate mosquitoes inside houses by moving the nozzle in a steady up and down motion along the walls. As the houses were of differing sizes, the time required for full aspiration varied between 3–10 min. Whilst CDC backpack aspiration is regularly used for mosquito surveillance inside houses, its value for sampling mosquitoes resting in outdoor environments, particularly in wilderness areas away from houses, is unknown. To evaluate this, we also conducted a 2-min timed aspiration of all vegetation/objects within a 2 m radius of each RB trap. The height of aspiration was confined to the reach of the aspirator nozzle, i.e. *c.*2 m from the ground. All surfaces and features of vegetation were searched: plant bases, trunks, axils, dorsal sides of leaves and tree holes. In the forest canopy, RB and CDC backpack aspiration collections were not conducted because the operator could not access the forest canopy with the aspirator and lowering the RB traps from the canopy would cause any mosquitoes resting inside to fly out.

RB and SRB traps were set up in pairs positioned 0.3–1.0 m from each other. Traps were placed facing opposite directions to avoid direct competition, whilst being close enough to be exposed to the same environmental conditions. Pairs were positioned 5–10 m from one another and GPS-marked. Maintaining 5 – 10 m between each SRB-RB pair was not always achievable when they were placed under small houses. Each RB, SRB and 2 min CDC backpack aspiration were single replicates and were used in each habitat type except inside houses and the forest canopy where only CDC backpack aspiration and SRB were used, respectively. Chicken wire mesh with wide holes of one square inch was fixed to the front of SRBs located under and around houses to prevent any larger animals

entering and getting stuck. The order in which traps were checked each morning was selected at random to avoid order effects; with some exceptions made to avoid sampling inside houses early the morning when residents were still sleeping.

2.3.3 **Experimental design**

Surveillance of mosquitoes resting in domestic, peri-domestic and forest settings was carried out over an 8-week period in 2015, with the first 4 weeks spent in Tuboh and the following 4 weeks in Paradason. Within each village, mosquito surveillance was conducted in 8 different habitat types selected to reflect the range of habitats available to mosquitoes in and around human dwellings, and nearby forest habitats where reservoir hosts are present (Table S1 and Figure S1). These habitats also represent a gradient arising from deforestation, including mature secondary forest of approximately 10–15 years-old (inside forest, in the canopy and forest edge), palm and rubber plantations, and human settlements (inside, under, and immediately around houses).

Eight households that were easily accessible by motorbike and who consented to participate were recruited from both Tuboh and Paradason. Within each village, the eight households were subdivided into one group of four houses in the north and one group of four houses in the south. The northerly group of houses were sampled on week one and three of the month and the southerly group on weeks two and four. Four nights of trapping were conducted per week. In some instances, a household sampled in the first week could not participate again, therefore a new house in the nearby area was substituted in its place. A total of 19 different households took part in the study, but in each week of sampling a maximum of four houses were visited. Resting collections were performed under and in the peri-domestic area around each of the four homes totalling 12xCDC, 12xRB and 12xSRB collections per night. Only CDC aspiration was performed inside homes. The nearest accessible (by foot or motorbike) forest patch and palm/rubber plantation to the group of homes was selected for simultaneous sampling. Again 12xCDC, 12xRB and 12xSRB collections per night were performed in plantation, forest edge and forest interior habitats. Only 12xSRB collections were performed in the forest canopy. Each house (n = 19), palm plantation (n = 5), rubber plantation (n = 4) and forest patch (n = 5) sampled over the study

were assigned a code so that RB, SRB and CDC backpack aspiration collections made in the same area could be identified (Figure S2 and Figure S3). These different areas used for sampling were defined as 'spatial clusters'.

2.3.4 **Mosquito processing**

Mosquitoes collected from traps were transported to the central field laboratory in Pinawantai village (8 km from Tuboh). All specimens were then examined under a stereomicroscope and identified to the genus level using the illustrated keys by Rattanarithikul et al. [175–178]. *Aedes* and *Culex* individuals were identified to the subgenus and species level where possible. The sex and gonotrophic stage (unfed, blood-fed, semi-gravid and gravid) of female mosquitoes was recorded. All samples were stored in 95% ethanol at room temperature in microcentrifuge tubes after morphological identification.

2.3.5 **Blood meal analysis**

All females categorised as recently blood-fed, based on the presence of blood visible in the abdomen were subject to blood meal analysis by conducting PCR on their stomach contents, following methods of Kocher et al. [179] and Kent [180]. Primers used were FOR (5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3') and REV (5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3') to amplify a 358 bp fragment of the vertebrate cytochrome b gene [180].

2.3.6 **Data analysis**

Statistical analyses were conducted in R version 3.4.2, with the packages glmmADMB and multcomp. Analyses were performed for specific taxonomic groups that are associated with disease transmission: (i) *Aedes* mosquitoes (including vectors of dengue, chikungunya and Zika virus: *Ae. albopictus* and *Ae. aegypti*); and (ii) *Culex* mosquitoes (including vectors of JE and filariasis: *Cx. quinquefasciatus*, *Cx. fucocephala* and *Cx. sitiens*). GLMMs with a binomial distribution were used to test whether the probability of detecting a mosquito (presence/absence) varied between habitat and trap types. Here the response variable was binary with 0 indicating mosquitoes were absent, and 1 that they were present ($\geq$ 1 individual) in the trap. Fixed explanatory variables fitted

habitat and trap type, with additional random effects for sampling date and spatial cluster.

The significance of variables were tested by backward elimination using likelihood ratio tests. A similar approach was taken to model how the abundance of mosquitoes varied between trap and habitat type. Here, the response variable was the number of mosquitoes caught in a single trapping event, with a negative binomial model used to account for the overdispersion in count data.

2.4 Results

2.4.1 General trends in resting mosquito abundance

Over 31 nights of sampling, 5748 trapping events were conducted from which 2243 mosquitoes were collected (Table 2.1, Table S1). Resting mosquitoes were found in all habitat types, with *Culex* spp. (*n* = 1666) and *Aedes* spp. (*n* = 483) being the most abundant (Table 2.1). Only a few individuals from other genera were collected (*n* = 94, Table 2.1). These were *Tripteroides* (*n* = 38), *Armigeres* (*n* = 20), *Uranotaenia* (*n* = 9), *Lutzia* (*n* = 5), *Hodgesia* (*n* = 2), *Anopheles* (*n* = 1), *Toxorhynchites* (*n* = 1) and unidentified specimens (*n* = 18). Both male and female mosquitoes were found in resting collections, with the proportion of females being highest in SRB collections (69.6% of 381 specimens) and lowest in RB (29.6% of 1067) and CDC collections (30.9% out of 795). Of the 483 *Aedes* mosquitoes, only 264 could be morphologically identified to species level. Of these, 90.9% were identified as *Ae. albopictus* (*n* = 240) and 9.1% *Ae. aegypti* (*n* = 24) (Table S2). The remaining specimens were missing key diagnostic features such as scales which prohibited identification. Assuming the species composition was similar in the sample that could not be morphologically identified, the majority of remaining *Aedes* were likely to be *Ae. albopictus*. The proportion of *Aedes* specimens that could be identified to the species level was highest in SRB (*n* = 140, 81.9%), then RB (*n* = 45, 56.3%) and lowest in CDC backpack aspiration collections (*n* = 79, 34.1%); indicating that aspiration methods were more likely to damage specimens during collection.

Only a small proportion (122/1666) of *Culex* mosquitoes were identifiable to the subgenus level; 14.9% of those that were trapped with RB were distinguishable

to subgenus, 21.2% for SRB and 6.9% for CDC (Table S3). Thus, the trapping methods followed a similar trend for enabling *Aedes* species identification and *Culex* subgenus identification, with SRB allowing greatest accuracy, followed by RB and then CDC. Within the group of specimens that could be identified to subgenus, the medically important subgenus *Culex* was highly represented (45.1% of those that could be identified). Species within this subgenus were *Cx. quinquefasciatus* (*n* = 29); *Cx. fuscocephala* (*n* = 3) and *Cx. sitiens* (*n* = 3; Table S4). Members of the subgenus *Culex* were found in all trapping methods (SRB: *n* = 20; RB: *n* = 22; CDC: *n* = 13) and in most habitat types (underneath houses: *n* = 32; around houses: *n* = 9; rubber plantations: *n* = 6; forest at ground level: *n* = 4, inside houses: *n* = 3; palm plantation: *n* = 1) except for the forest canopy and edge (Table S3).

Only one anopheline mosquito, *An. umbrosus*, was collected (in the forest interior). Pooling across habitat types, SRB collections sampled mosquitoes of a higher number of genera (*n* = 8) than those made by CDC (*n* = 7) or RB (*n* = 5) (Table 2.1). As a result of low sample sizes of other mosquito genera, statistical analysis was restricted to the genera *Aedes* and *Culex*. Mosquitoes were analysed at the level of genus, given that species identification was only possible for part of the sample.

Table 2.1 Abundance of nine genera of resting mosquitoes (males and females combined) collected using CDC backpack aspiration (CDC), Resting bucket (RB) and Sticky resting bucket (SRB) methods over 8-week sampling period in 8 habitat types.

Trap	Genus	Inside house	Under house	Around house	Palm plantation	Rubber plantation	Forest edge	Forest ground level	Forest canopy
RB	*Culex*	×	636	163	52	10	13	94	×
	Aedes	×	8	20	0	14	18	20	×
	Tripteroides	×	1	1	1	0	2	1	×
	Armigeres	×	1	0	0	0	0	2	×
	Uranotaenia	×	0	1	1	2	0	1	×
	Lutzia	×	0	0	0	0	0	0	×
	Hodgesia	×	0	0	0	0	0	0	×
	Anopheles	×	0	0	0	0	0	0	×
	Toxorhynchites	×	0	0	0	0	0	0	×
	Unknown	×	0	2	1	0	1	1	×
SRB	*Culex*	×	31	69	16	5	9	33	12
	Aedes	×	8	6	10	33	67	33	14
	Tripteroides	×	7	1	0	2	1	3	2
	Armigeres	×	1	0	0	0	0	1	1
	Uranotaenia	×	0	0	0	0	1	1	0
	Lutzia	×	2	0	0	0	0	2	0
	Hodgesia	×	1	0	0	1	0	0	0
	Anopheles	×	0	0	0	0	0	0	0
	Toxorhynchites	×	0	0	0	0	0	0	1
	Unknown	×	3	0	1	0	1	1	1
CDC	*Culex*	63	336	79	5	12	9	19	×
	Aedes	3	22	48	9	31	58	61	×
	Tripteroides	0	2	3	0	1	2	8	×
	Armigeres	0	3	1	1	0	4	5	×
	Uranotaenia	0	0	0	0	2	0	0	×
	Lutzia	0	1	0	0	0	0	0	×
	Hodgesia	0	0	0	0	0	0	0	×
	Anopheles	0	0	0	0	0	0	1	×
	Toxorhynchites	0	0	0	0	0	0	0	×
	Unknown	1	1	1	0	3	0	0	×
Total		67	1064	395	97	116	186	287	31

2.4.2 *Aedes* spp.

The probability of collecting an *Aedes* mosquito using each of the three trapping methods was very low (0.01) and differed with trap type (*Dev* = 58.3, *df* = 2, *P* < 0.001) but not habitat (*Dev* = 13.76, *df* = 7, *P* = 0.06). *Aedes* were most likely to be trapped using CDC, then SRB and least likely with RB (Table 2.2). The mean abundance of *Aedes* per trap was low (< 0.05 mosquitoes/trap), and varied with trapping method (*Dev* = 43.92, *df* = 2, *P* < 0.001) and habitat (*Dev* = 17.94, *df* = 7, *P* = 0.01). It was not possible to test for interactions between trap and habitat type in the full data set as only 1 trap type was used in two of the habitat types (e.g. CDC backpack aspiration - inside houses; SRB - forest canopy). However, a second round of analysis was conducted on the subset of data where all 3 collection methods were used. Here, the abundance of *Aedes* was significantly influenced by an interaction between trapping method and habitat (*Dev* = 187.10, *df* = 8, *P* < 0.001). The mean abundance of *Aedes* collected in RB and CDC did not vary between habitats (Table 2.3); however, SRBs placed in forest edge habitats collected significantly more than those placed around houses (*P* = 0.01).

2.4.3 *Culex* spp.

As with *Aedes*, the probability of collecting a *Culex* mosquito was low on each trapping event (*c*.0.01). Analysis of data collected from all 8 habitat types indicated that the probability of capturing *Culex* differed with trap type (*Dev* = 68.34, *df* = 2, *P* < 0.001) and habitat (*Dev* = 39.58, *df* = 7, *P* < 0.001). Here the probability of sampling a *Culex* mosquito was significantly influenced by an interaction between trapping method and habitat (*Dev* = 175.60, *df* = 8, *P* < 0.001). *Culex* were most likely to be trapped using RB than CDC and SRB (Fig. 2.3). All three trap types followed the same trend of having the highest probabilities of collecting *Culex* underneath and around houses, and inside the forest, and the lowest in the forest edge and plantations. The probability of sampling *Culex* was similar across all habitats for both CDC and SRB traps. RB positioned underneath homes were more likely to collect *Culex* than those placed at the forest edge (*P* < 0.05).

Table 2.2 Probability of encountering a resting *Aedes* mosquito per CDC backpack aspiration (CDC), Resting bucket (RB) and Sticky resting bucket (SRB) trap as predicted by binomial generalised linear mixed models (GLMM).

Trap	**Predicted probability of *Aedes* presence**	**Lower 95% CI**	**Upper 95% CI**	**Tukey's test between means**
CDC	0.029	0.016	0.053	RB *vs* CDC, $P < 0.001$
RB	0.009	0.004	0.018	SRB *vs* CDC, $P < 0.001$
SRB	0.017	0.008	0.033	SRB *vs* RB, $P = 0.01$

Table 2.3 Abundance of resting *Aedes* mosquitoes per CDC backpack aspiration (CDC), Resting bucket (RB) and Sticky resting bucket (SRB) traps as predicted by negative binomial generalised linear mixed models (GLMM) for 6 habitat types.

Habitat	CDC (95% CI)	RB (95% CI)	SRB (95% CI)
Around house	0.033 (0.011–0.095)	1.944×10^{-2} (7.095×10^{-3}–5.328×10^{-2})	0.006 (0.002–0.021)
Under house	0.017 (0.005–0.059)	9.329×10^{-3} (2.010×10^{-3}–4.145×10^{-2})	0.010 (0.002–0.047)
Palm	0.020 (0.002–0.179)	1.880×10^{-7} (5.880×10^{-108}–6.012×10^{93})	0.016 (0.002–0.136)
Rubber	0.051 (0.005–0.521)	2.924×10^{-2} (4.478×10^{-3}–1.910×10^{-1})	0.057 (0.008–0.415)
Forest edge	0.022 (0.002–0.212)	1.611×10^{-2} (1.092×10^{-2}–2.375×10^{-2})	0.071 (0.011–0.463)
Forest ground	0.026 (0.003–0.253)	1.955×10^{-2} (1.413×10^{-2}–2.704×10^{-2})	0.037 (0.005–0.246)

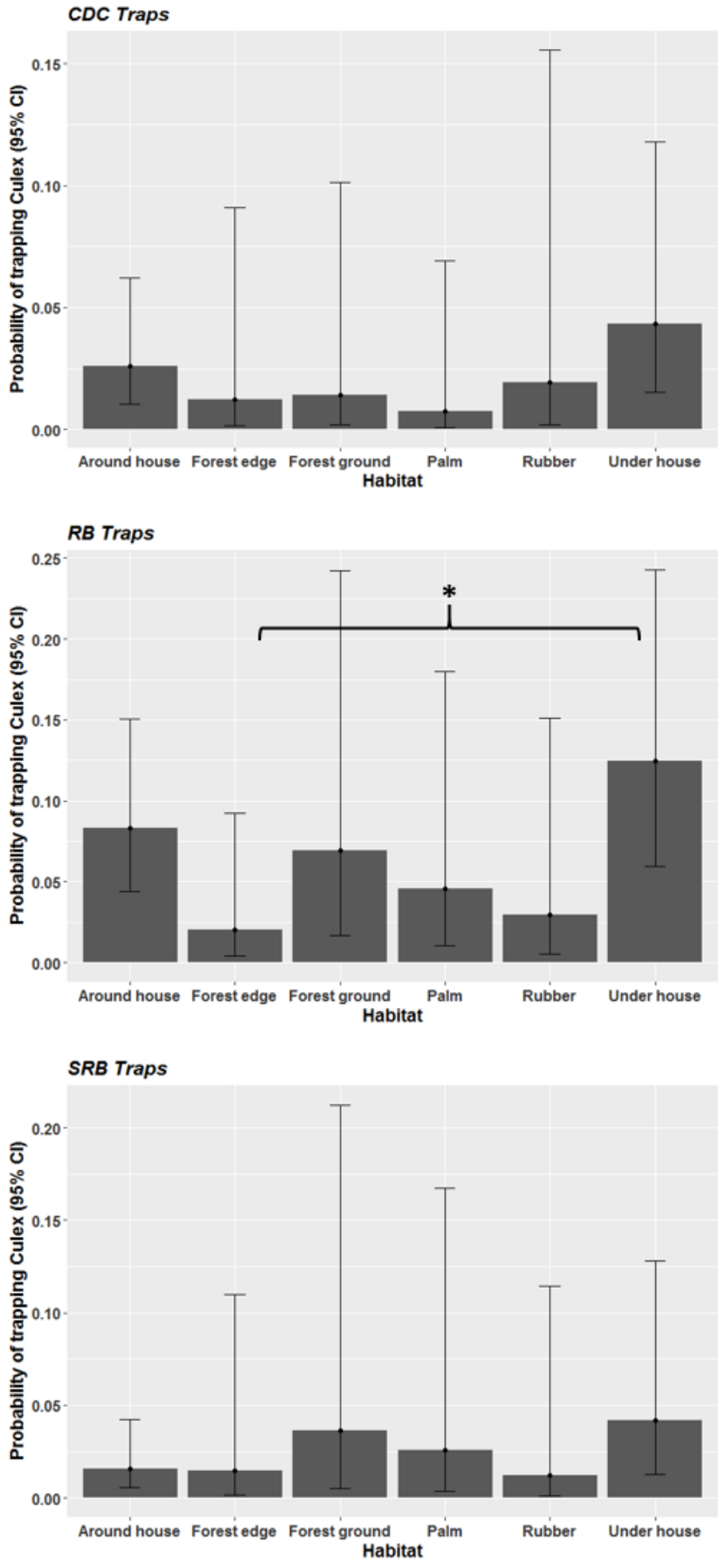

Figure. 2.3 The probability of catching a resting *Culex* mosquito with CDC backpack aspiration (CDC), Resting bucket (RB) and Sticky resting bucket (SRB) methods as predicted by binomial generalised linear mixed models (GLMM). ****P* < 0.05 (*post-hoc* Tukey's test).

The abundance of resting *Culex* collected per trap was low (0.1) and differed substantially between habitat (Dev = 60.76, *df* = 7, *P* < 0.001) and trap types (*Dev* = 60.24, *df* = 2, *P* < 0.001). Analysis of the subset consisting of data from habitats in which all 3 traps were tested (6 out of 8 habitats) indicated there was a significant interaction between trapping method and habitat (*Dev* = 246.92, *df* = 8, *P* < 0.001). All three trapping methods followed the same general trend with mean *Culex* abundance being highest in traps placed underneath houses, and lowest in plantations and at the forest edge (Fig. 2.4). In domestic settings, more *Culex* were found in collections made underneath than around houses with all three trap types (CDC: *P* <0.001; RB: *P* < 0.01; SRB: *P* < 0.05). More *Culex* were collected in RB placed under houses than those at the forest edge (*P* < 0.05). Additionally, more *Culex* were collected from RB placed in the forest interior at ground level than at the edge of the forest (*P* < 0.05).

2.4.4 **Physiological status and blood meal identification**

Resting collections are typically used to sample female mosquitoes that have recently blood-fed so that blood meal identification can be performed to confirm host choice. Of the 846 female mosquitoes sampled in this study, 833 were in acceptable condition to assign a feeding status. The majority of these females were unfed (63.3%, *n* = 527/833), with only 15.2% (*n* = 127) appearing to have recently blood-fed. Similar proportions of blood-fed females were obtained with SRB (16.1%, *n* = 43/266), CDC (15.1%, *n* = 38/251) and RB (14.6%, *n* = 46/316) (Table S5). However SRB traps collected more gravid female mosquitoes (23.3%, *n* = 62/266) than CDC (14.7%, *n* = 37/251) and RB (13.6%, *n* = 43/316). Most blood-fed females (both *Culex* and *Aedes*) were found in collections made under and around houses (Figure S4 (*Aedes*) and Figure S5 (*Culex*)).

Vertebrate DNA was amplified in only thirty percent of the blood fed mosquitoes that were tested (*n* = 38/127). The majority of these were *Culex* mosquitoes, with most collected around and underneath houses. Blast searches using assembled forward and reverse sequences matched 36 *Culex* with *Gallus gallus* (jungle fowl), 1 *Culex* and 1 *Aedes* (*Stegomyia*) *w*ith human DNA (Table S6). Blood meals of specimens caught in the forest and plantations did not amplify.

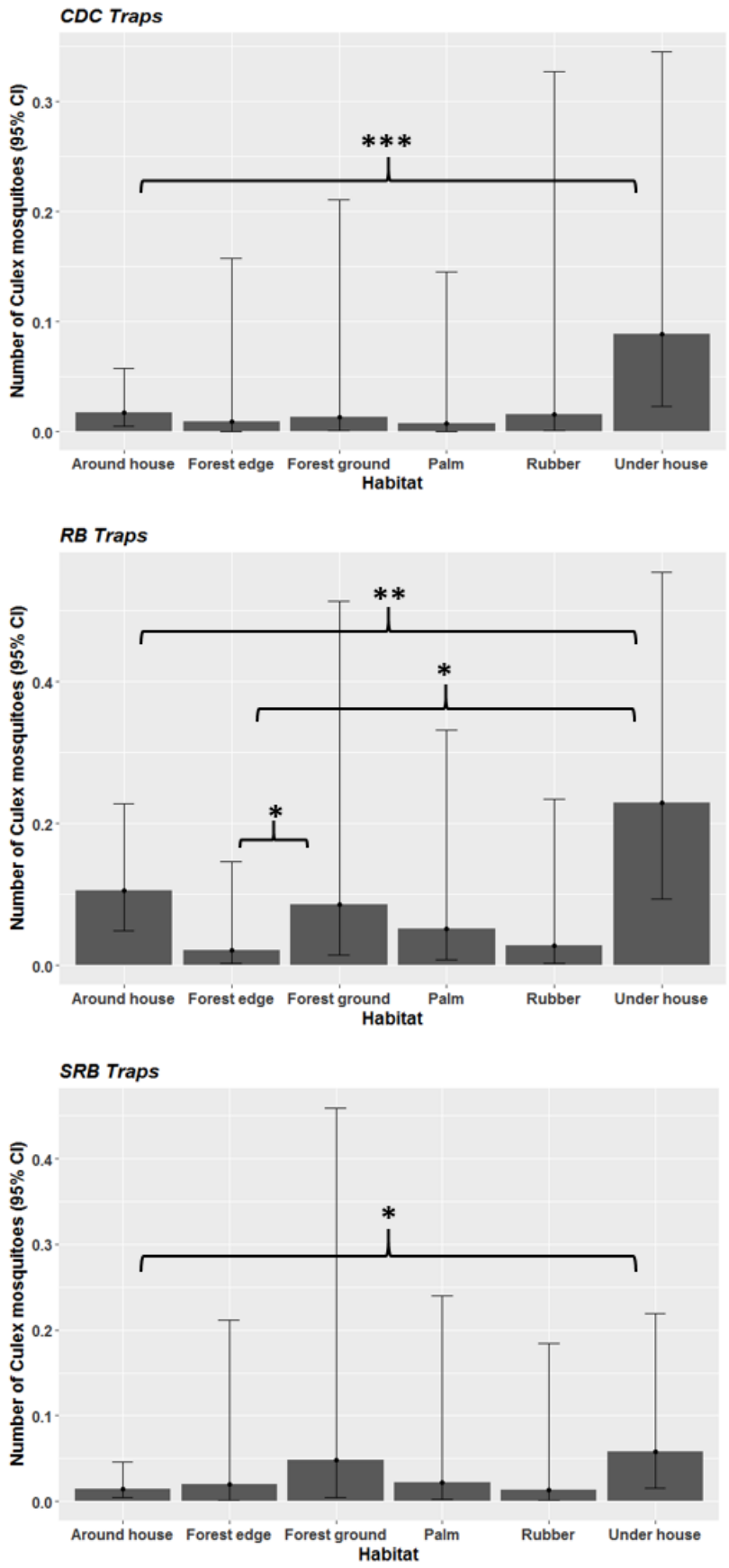

Figure 2.4 The predicted abundance of resting *Culex* mosquitoes collected using CDC backpack aspiration (CDC), Resting bucket (RB) and Sticky resting bucket (SRB) methods in six habitat types. Predicted values obtained with negative binomial generalised linear mixed models (GLMM). **P* < 0.05, ***P* < 0.01, * *P* < 0.001 (*post-hoc* Tukey's test).**

2.5 Discussion

This study represents the first evaluation of two novel methods for sampling mosquitoes resting in a range of domestic, agricultural and forest habitats. Overall these trapping methods had a relatively low probability of detection (*c*.0.1), with mosquitoes being found in < 10% of collections. All resting collection techniques however were successful at trapping mosquitoes in the full range of habitats sampled. *Aedes* and *Culex* mosquitoes were the most abundant and included the known vector species (*Ae. albopictus*, *Cx. quinquefasciatus*, *Cx. fuscocephala* and *Cx. sitiens*). However none of the methods showed promise for collecting malaria vectors, including those responsible for transmitting *P. knowlesi*. Our results provide useful proof-of-principle of the value and limitations of these tools for sampling mosquito vectors and characterizing their resting habitat preferences.

Previous studies had warned of the challenges of collecting outdoor resting blood-fed anophelines in Malaysia [32,50,181]. In a previous study in Paradason village where mosquitoes were sampled by Human Landing Collections, *An. balabacensis* was the dominant *Anopheles* and found at a mean rate of 7.84 per person per night [50]. In trapping methods such as HLC, mosquitoes are actively seeking the host thus commonly collected much higher numbers than passive collection methods such as resting collections. Although the sampling efficiency of the resting traps here was quite low, a substantial number of mosquitoes (*n* = 2243) were collected because traps were deployed at high sampling effort (5748 trapping events). Although these trapping methods were unsuccessful for sampling malaria vectors, genera containing other important vector species (*Culex* and *Aedes*) were caught at comparatively high frequency. Members of these genera were widely distributed and found within all habitat types. More *Aedes* were collected in SRBs placed in forest edge habitats than in SRBs placed around houses. Significantly higher abundances of *Culex* were found in collections made under houses than around houses. It is common for the space below houses in Sabah to be utilised by livestock or domestic pets which could explain the higher numbers of mosquitoes resting under houses. Due to the high variability in mosquito catch rates within habitat types, few other clear statistical differences between habitats were detected. A much greater sampling effort and larger sample sizes would likely be required for a robust test of

differences between habitats. However, the generally wide distribution of resting mosquitoes across all habitats sampled indicates that there is no single location where most of the resting population could be targeted (e.g. through the spraying of insecticides).

Whilst differences in mosquito abundance between trap types were modest, the three trapping methods compared here did have some differences in efficiency. RB traps and CDC backpack aspiration were more efficient than SRB for sampling *Culex*, whereas more *Aedes* were collected with CDC backpack aspiration and SRB than RB traps. It is unclear why the SRB were not consistently better than the other methods, as we hypothesized the sticky surfaces used in this trap may give it an advantage. In summary, our results indicate that the suitability of specific resting traps differs between mosquito genera, though generally, resting bucket traps and CDC collections caught more mosquitoes than SRB.

One explanation for the differential performance of trapping methods is that they target different sections of the vector population. Here we found that the proportion of gravid mosquitoes (*Aedes* and *Culex*) was higher in SRB than RB or CDC backpack aspiration collections. A previous study in Tanzania also found that the proportion of *Culex* mosquitoes that were gravid was higher in sticky traps than resting buckets (outdoors) and backpack aspiration (indoors) [161]. The authors hypothesized that this may be because the polybutylene-based adhesive mimicked an oviposition odour cue. The glue used in SRBs here was also composed of polybutylenes and polyisobutylenes, and may also have acted as an oviposition cue. The choice of trap therefore likely depends on the target species and required physiological state in certain settings. Further examination of the data gathered in this study on the physiological status of resting mosquitoes trapped, particularly *Culex* specimens due to the large sample size generated, could be performed to identify preferred habitat types for blood fed/gravid/unfed mosquitoes.

All three trapping methods were relatively quick and easy to set up and operate. The SRB involved minimal manual labour to retrieve specimens (as mosquitoes were affixed to a sticky sheet) but required slightly more set-up time for preparation of the glue and acetate. An advantage of the SRB is that they can be left for longer periods of time which is beneficial when placing in difficult to

reach habitats such as a forest canopy. RB performed similarly to fixed bursts of two minutes of CDC backpack aspiration in most habitat types. The RB method is more convenient than CDC because only the resting bucket needs to be aspirated instead of a two-minute search by CDC backpack aspiration which is more time-consuming and less standardized.

In making decisions on mosquito trap choice, it is also important to consider the quality of specimens obtained from different methods, and whether they meet requirements for further processing. This study relied on morphological features to identify mosquito species. Scales and hairs are crucial traits for morphological identification to species level. However, we noted that many of these were lost during the trapping process, with a high proportion of *Culex* specimens collected from all three methods being unidentifiable (> 80%). *Aedes* specimens generally remained in better condition, but with notable differences in the proportion that could not be identified between trapping methods. SRB generally kept mosquitoes in a better condition for morphological identification.

The low amplification success of mosquito blood meal hosts was a limitation for the study. A likely explanation could be that the quality of the host DNA was compromised before extraction and amplification. Mosquitoes were examined upon return to the central field station after all resting collections were performed, therefore blood-fed mosquitoes were preserved in 95% ethanol several hours after being collected. There is the possibility that host DNA could have been damaged in this time, thus we recommend to alternatively store immediately in the field upon collection. Previous studies noted that an increase of eight hours after blood meal ingestion significantly reduced the proportion of hosts that could be successfully identified (less than 50% at 15 hours) [182]. Our collections were performed daily, thus exceeding this very short period. As a result, there is a high chance that host DNA in some mosquito blood meals was partially digested in advance of mosquitoes being trapped. Additionally, different habitats may influence blood meal amplification success due to host availability. Around homes there was a notable abundance of blood meal sources e.g. humans, chickens and dogs, therefore mosquitoes collected in those areas would have had the opportunity to feed more recently than mosquitoes collected in areas away from the home such as plantations or forest where there

were fewer hosts available. Blood meals of mosquitoes collected further away from the home were more likely to be advanced in digestion which was confirmed with no amplification of blood meals from mosquitoes collected in the plantations and forest. Minor technical issues may have caused low amplification success in our study however mosquito digestion of host DNA within the blood meal is a more prominent concern. Several medically important mosquito vector species were found in this study. This included known vectors of filariasis and Japanese encephalitis [148,150] (e.g. *Cx. quinquefasciatus*, *Cx. fuscocephala* and *Cx. sitiens*) which are known to be circulating in the study area. These *Culex* species were mainly collected under and around homes, and in palm plantations. In the nearby Ranau District, the most abundant *Culex* species were *Cx. quinquefasciatus* and *Cx. pseudovishnui* [124]. *Culex vishnui*, *Cx. tritaeniorhynchus* and *Cx. gelidus* were also common and all have been incriminated as vectors of JE in Peninsular Malaysia [124]. In Bengkoka Peninsula, neighbouring the Kudat District, *Cx. pseudovishnui*, *Cx. quinquefasciatus* and *Cx. tritaeniorhynchus* are abundant [149,183]. In Sarawak, Kunjin virus was isolated from *Cx. pseudovishnui* [184] and JE virus was isolated from *Cx. tritaeniorhynchus* and *Cx. gelidus* [185]. The variation in *Culex* species between districts may be explained by local ecology and differences in agriculture between regions, e.g. rice fields in Bengkoka.

The majority of *Aedes* mosquitoes that could be identified were *Ae. albopictus*, a suspected vector of dengue virus [147] and also of Zika virus in Singapore [186]. This species was found at highest abundance in forest edge and plantation habitats, possibly due to the availability of both natural shaded breeding sites and artificial containers used for rubber tapping [187]. The increase in availability of domestic breeding habitats such as artificial water containers was previously related to the substantial increase in the abundance of host-seeking *Ae. albopictus* females recorded between the cultivation (1993) and maintenance (1994) stages in an oil palm estate in Sarawak [135]. A further study in Sarawak reported *Ae. albopictus* to be more abundant in agricultural fields (black pepper, cocoa and banana) than in forest sites [147]. Our finding differs from a previous study in Southern Sabah where surveys with oviposition traps found *Ae. albopictus* to be present only near houses, and absent from old growth forest and oil plantations [188]. Similarly, low numbers of host-seeking *Ae. albopictus* were reported in hilly areas covered by primary and secondary forests with

alternating areas of scrub and open grass in Bengkoka Peninsula east of Kudat District [183]. *Aedes albopictus* is known to use vegetation for resting [189], and prefer cool, shaded areas for breeding [190]. In combination, this highlights the relatively plastic and exophilic nature of *Ae. albopictus* [150], which allows it to exploit a range of domestic, agricultural and forest settings. Whilst data on sylvatic dengue transmission is not available for this area, it has been reported in other areas of Borneo in patients with a shared history of forest activities (trekking or tree clearance) [147]. More investigation is required to confirm the extent of sylvatic dengue transmission in this area; however, our finding that *Ae. albopictus* is abundant in forested areas flags up its role as a likely vector.

This study has potential implications for vector control. First, it demonstrates that a range of vector species rest underneath houses thus vector control programmes should target these areas with peri-domestic insecticide spraying. Secondly, we conclude that resting catches are insufficient for examining malaria vector populations in this area. Resting traps should therefore be used as a supplementary tool in conjunction with host-seeking methods. Lastly, important vector species such as *Ae. albopictus* can be found in a range of habitats away from the immediate domestic area. Therefore, efforts to control sylvatic dengue transmission for example would benefit by including habitats away from the home.

2.6 **Conclusions**

This study demonstrated the new resting buckets and sticky resting buckets can be used to sample a taxonomically diverse range of mosquitoes in a variety of different habitats. However, a limitation of these methods is that they have relatively low sampling efficiency, meaning that they must be deployed at large-scale to generate robust data on mosquito vector resting behaviour and habitat choice. These sampling methods were not successful in trapping malaria vectors but were effective for some *Culex* and *Aedes* mosquitoes. In particular, the sticky resting buckets hold promise for future studies characterising sylvatic dengue transmission. Despite the relatively small numbers of mosquitoes found in these traps, sample sizes were sufficient to indicate that a substantially higher number of *Culex* rest underneath than around homes in this area. Local

vector control programmes should consider also targeting these areas with IRS to improve success.

3 Investigating associations between vector habitat and human *P. knowlesi* exposure risk over a wide geographic range in Sabah

3.1 Abstract

Current understanding of the bionomics (ecology and behaviour) of mosquito vectors responsible for *P. knowlesi* transmission in Malaysian Borneo comes from investigations near the epicentre of human cases in Kudat district of Sabah. Previous investigations in this district identified *An. balabacensis* as the vector likely responsible for transmission to humans; with infection risk being highest in forest environments. However, these findings are based on sampling at a limited number of sites with little spatial replication, generating uncertainty about how generalisable these findings are to wider geographical regions where humans may be exposed to *P. knowlesi*. Establishing how these predictions about risk withstand over a wider geographic area is necessary to plan any region-wide control activities. Additionally, so far the relationship between vector bionomics and human infection has been investigated only on the basis of entomological outcomes such as vector abundance and infection rate, but it is unclear how well these variables predict clinical infection risk.

To characterise the transmission of *P. knowlesi* over a wider geographic area and investigate potential entomological indicators of human infection risk, entomological surveillance was conducted across four districts of Malaysian Borneo. In conjunction with this study, a large cross-sectional survey of human *P. knowlesi* infection was performed in the same area. Human-landing catches in peri-domestic, farm and forest sites in a subset of 11 villages selected for a large epidemiological survey of human *P. knowlesi* exposure. The putative *P. knowlesi* vector, *An. balabacensis*, was found in all districts and 6/11 villages sampled. The abundance of *An. balabacensis* was low (< 0.01 per person per night); but significantly more were collected in farm (0.094) and forest (0.082) habitats than in peri-domestic areas (0.007). Only one *An. balabacensis* was infected with *P. knowlesi* (infection rate: n = 1/32). Controlling for habitat variation, there was no significant association between the abundance of *An. balabacensis* and *P. knowlesi* sero-positivity rates among residents at the village-level .

Whilst this study confirms *An. balabacensis* is still the most likely vector of *P. knowlesi* in Sabah, its overall abundance was considerably lower across this wider study area than found in previous focal studies in Kudat district. This demonstrates that caution should be taken before extrapolating findings on vector ecology from a geographically limited area to wider regions. Whilst some associations between *An. balabacensis* abundances and habitat type detected here matched those inferred from smaller studies in Kudat, overall vector densities and infection rates were much lower making it difficult to confirm which habitat poses the highest *P. knowlesi* exposure risk to humans. More investigation is required to establish the role of *An. balabacensis* abundance as a useful indirect indicator of predicting *P. knowlesi* sero-positivity in humans and thus human infection risk.

3.2 Introduction

Malaria transmission in forested areas worldwide is highly sensitive to anthropogenic alterations in land-use [191]. In recent years Malaysia has experienced a shift in the predominant species of *Plasmodium* infecting humans from *P. vivax* and *P. falciparum* to a simian malaria species, *P. knowlesi* [85]. *Plasmodium knowlesi* typically infects macaques and leaf monkeys with *Anopheles* mosquitoes in the Leucosphyrus complex being responsible for transmission [8]. The emergence of *P. knowlesi* in humans has coincided with a significant change in land-use due to the conversion of primary and secondary forest to palm oil plantations [192]. Recently, investigations within a major focus of human infection in the Sabah province of Malaysian Borneo concluded that *P. knowlesi* incidence was significantly associated with forest cover and historical rates of forest loss [193]. The mosquito *An. balabacensis* was also confirmed as the primary vector of *P. knowlesi* within this focal area, with the survival and *P. knowlesi* infection rate in this mosquito vector being significantly higher at a forest and farm site in this district than around people's houses [50]. These observations support the hypothesis of *P. knowlesi* primarily being a forest-associated malaria; with human exposure occurring when people are working outside of their homes [88].

Identification of the habitats and vector species responsible for *P. knowlesi* transmission to humans is a crucial first step for planning control measures.

However, most of our current understanding of *P. knowlesi* ecology comes from intensive study of one location. Specifically in Sabah, human *P. knowlesi* cases have been reported in all regional divisions [24], but detailed study of vector ecology has been restricted to a 2x3 km intensive study site in one district (Kudat, Fig. 3.1) and two sites on the neighbouring Bangii island. Investigations in this area have focussed on *An. balabacensis* abundance, survival and seasonal dynamics, malaria infection rates, and larval ecology in relation to land-use. While useful for understanding the current hotspot of transmission centred in Kudat [50], it is unclear how generalizable these findings are to other areas of Sabah, Malaysia or SE Asia in general where *P. knowlesi* is emerging. For example, the landscape in Kudat is a fragmented mix of forest, farm and deforested areas, but is relatively similar in altitude, with no major urbanization. Longitudinal sampling at three sentinel sites in this area demonstrated that *An. balabacensis* is the dominant *Anopheles* species (95.1%) [50]. However, other members of the Leucosphyrus complex have been implicated in *P. knowlesi* transmission in different parts of Malaysia [18,45–47,50,58,194] and in different Asian countries [48,195–197], and the relationship between vector abundance and human exposure risk is poorly understood. Analysis of entomological risk factors and their association with human exposure across larger spatial scales is required to understand the drivers of *P. knowlesi* emergence both within and beyond Sabah.

The transmission of other malaria species in Southeast Asia exhibits significant heterogeneity characterized by high variation in *Anopheles* populations at a range of spatial scales [198–203]. Notable differences in vector diversity and abundance exist between [198,199,202] and within countries [201,203], and even between villages 2km distance from each other [201]. Such heterogeneity in vector abundance and diversity has been associated with environmental factors such as land-cover, type of agriculture, availability of breeding sites, temperature, topography and elevation [202,203]. Across the state of Sabah, there is substantial variation in elevation, the size and distribution of forest areas, and type of local agriculture. Thus it is likely that the snapshot of *P. knowlesi* vector ecology obtained from Kudat district may not fully represent the state as a whole.

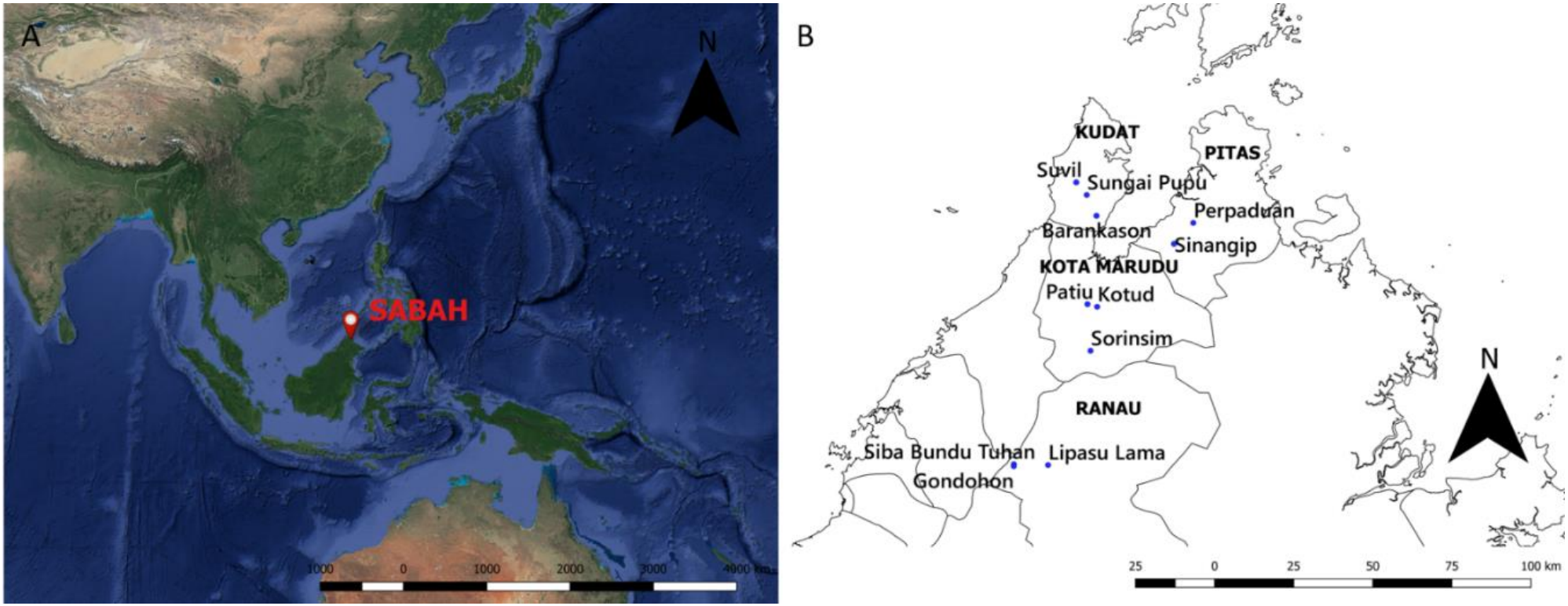

Figure 3.1 A) Location of Sabah in Malaysian Borneo B) Map of Northern Sabah indicating the eleven villages across 4 districts where entomological sampling was conducted in this study between March to June 2016.

It is unknown whether general predictions about associations between habitat and *P. knowlesi* vectors made from a limited geographic area hold true over larger areas of Sabah and Malaysia in general. In Sabah, *An. balabacensis* abundance was high and variable in a peri-domestic site [50], whereas in Sarawak, the primary vector associated with *P. knowlesi* transmission, *An. latens*, was most abundant in the forest [58]. Studies conducted in Western Malaysia found *An. cracens*, another member of the *An.* Leucosphyrus group implicated in transmission for that area, was most abundant in the fruit orchard in comparison to village and forest habitats [46]. Thus studies conducted outwith Sabah indicate vector-habitat associations can vary between geographical settings. Furthermore, previous work on *P. knowlesi* vectors have focussed primarily on the impact of landcover on abundance and transmission potential [46,50,204–206], with little consideration of additional environmental factors that could explain or modify these impacts. For example, studies of malaria vectors often find a strong association with altitude [163,207–209]. Therefore, altitudinal variation was specifically incorporated into the study design to examine how this may affect vectors and expected relationships with habitat.

Understanding environmental drivers of vector ecology is useful but may not directly translate to predicting human infection risk. Vector abundance and sporozoite infection rates are key entomological indicators frequently investigated as proxies of human exposure risk [210–212]. These entomological indicators however may not always be good predictors of risk, particularly when investigating zoonotic malaria transmission where vectors may be highly infected but only bite monkeys instead of humans. Previously there have been some attempts to link *P. knowlesi* vector bionomics and human cases. For example, species incriminated as vectors are often the dominant *Anopheles* species in mosquito collections made in the vicinity of human cases (e.g. *An. cracens* in Peninsular Malaysia [45,46,213], *An. balabacensis* in Sabah [50,194], *An. latens* in Sarawak [58] and *An. dirus* in Vietnam [48]). These studies are limited because no collections were performed concurrently in areas where no malaria cases were reported, thus are lacking suitable controls. Additionally, as yet none have been performed at a large enough spatial scale to be able to link vectors to population-level epidemiological risk factors. In other malaria systems, there has been more success in defining good, robust entomological indicators of clinical

incidence or prevalence arising from studies assessing the impact of vector control methods during randomised control trials. Vector density often correlates with EIR [214-216] and parasite prevalence [214] or malaria incidence [215,217]. However higher vector abundances don't always mean more malaria cases [218] or higher frequencies of parasite detection in people [216]. Studies may fail to detect any association between entomological indices and epidemiological outcomes due to lack of variation in human parasite prevalence or vector densities between treatment groups [218-220]. Additionally, there may be cases when no sporozoite infected mosquitoes are trapped or sporozoite infections are too low to detect changes after vector control is implemented [217,218]. However, in general strong predictions can be made about malaria risk with information on vector densities and sporozoite rates but as yet this has not been established for *P. knowlesi* malaria.

Definition of entomological indicators of human malaria infection requires high resolution, spatially and temporally concurrent data on human malaria exposure and/or infection and vector bionomics. As yet, it has not been possible to do this within the context of *P. knowlesi* transmission due to a lack of large scale data on human *P. knowlesi* incidence. This would be difficult to achieve with *P. knowlesi* because typical measures of human population-level malaria incidence or prevalence are not viable because infection rates are so low. Therefore, encountering active infections is likely to be rare and instead a focus on serology as a more indirect measure of previous infection is appropriate. Recently a large interdisciplinary MRC project 'Monkeybar' (UK MRC ESEI Grant #G1100796) conducted a mass epidemiological survey to estimate human prevalence and antibody responses to *P. knowlesi* in Sabah. The overall aim of Monkeybar was to define the biomedical, environmental and social risk factors for human infection of *P. knowlesi* malaria. As part of this project, a cross-sectional survey for *P. knowlesi* was conducted in communities, from 135 villages distributed across 4 districts in Sabah: Kudat, Pitas, Ranau and Kota Marudu (September to December 2015). The occurrence of this large-scale epidemiological survey provided a unique opportunity to set up complimentary entomological surveillance throughout the four districts to assess the environmental determinants of *P. knowlesi* vectors across a wider region and to test their association with human infection. Other mosquito-borne diseases are common in this area of Malaysia,

with dengue being the most important. Thus in addition to collecting data on malaria vectors, this study also aimed to contribute to knowledge about dengue transmission around human settlements by simultaneously collecting data on dengue vectors.

The goal of this study was to investigate environmental determinants of *P. knowlesi* vector abundance and infection rates across wider spatial scales in Sabah. Key aims were to identify associations with habitat type and test whether results from small-scale sampling in one district (Kudat) are generalizable across the state. In addition, this study set out to test for associations between entomological variables and human exposure to *P. knowlesi* as measured in the Monkeybar sero-prevalence study. This was done through intensive study of mosquito vector ecology and biting behaviours in eleven villages representing four districts of Sabah in the year following a large human malaria sero-prevalence study.

3.3 Methods

3.3.1 Study sites

Entomological surveillance, especially in areas like Sabah where mosquito densities are low and dispersed, is time consuming and expensive, thus difficult to implement on the same scale as an epidemiological survey. Therefore, a subset of villages investigated in Monkeybar's cross-sectional survey in September to December 2015 were selected for follow-up entomological sampling. The intention was to select sites of a wider spatial range, encompassing more ecological diversity than sites where vectors have been previously studied in Sabah, such as Kudat and within this subset, test for associations between community prevalence of *P. knowlesi* and mosquito vector abundance and diversity. The 135 villages investigated in the Monkeybar cross-sectional survey were divided into district and arranged in order of increasing altitude. Three villages were then selected to span the elevation range available within each district. In advance of arranging sampling, the villages were visited to assess suitability based on ease of access. At this stage, some villages had to be replaced as terrain was too challenging to cross at night, however care was taken to ensure villages exchanged represented the altitudinal range available

within that district. Entomological sampling took place six months after the human survey from March to June 2016 (Fig. 3.1). One village in Pitas was missed as the village leader was not available at the proposed time thus overall, eleven villages were visited for entomological sampling.

3.3.2 **Mosquito collection**

Mosquitoes were collected using the Human Landing Catch (HLC) technique. Volunteers were positioned in teams of two with their lower legs exposed, and trapped mosquitoes which landed on them to feed using 30ml plastic screw-top vials. One mosquito was trapped per vial and the hour and habitat of collection were recorded on each. Catches were performed between 18:00 – 00:00 to include the peak biting time of Sabah's primary *P. knowlesi* vector, *An. balabacensis* [50,221]. All HLC took place outdoors because *P. knowlesi* vectors are known to exhibit exophilic biting behaviour [46].

3.3.3 **Experimental design**

Mosquito sampling was conducted to investigate associations between habitat type (farm, forest or peri-domestic areas), altitude, forest cover, and *P. knowlesi* sero-prevalence rates in village residents. To achieve this, all 11 villages were consecutively sampled over a 3-month period (21/03/16 - 16/06/16). One village was sampled per week, with mosquito collections being conducted over four consecutive nights. The research team attempted to visit a village from a different district on each week, so that district-level differences were not confounded by seasonality. However this was not always logistically possible (see Table 3.1 for sampling dates).

Villages were accessible by tertiary or dirt track roads. All villages were rural, with small populations of < 750 residents. These were generally structured into a group of houses surrounded by a mosaic of crops (usually largely palm oil and rubber trees) and secondary forest patches. Thus there was a range of domestic, farm and forest environments available at each village. Within each village, mosquitoes were collected in three habitat types: forest patch, farm and peri-domestic environment (e.g. as shown in Fig 3.2) to replicate the sampling design used in Kudat previously [50]. The peri-domestic environment was defined as the

Table 3.1 Description of eleven villages in which mosquito vectors were sampled in this study. "Crops" describes the dominant types of subsistence farming occuring in the village. "Approximate area of forest patch" refers to the size of the forest patch (estimated from map) in which mosquito collections were conducted within the forest habitat type. "Population size" refers to the estimated number of residents derived from household enumeration conducted as part of the Monkeybar cross-sectional survey in September to December 2015.

District	Village	Elevation (m)	Crops	Approximate area of forest patch (m^2)	Population	Date of first sampling night
Kudat	Barankason (BAR)	128	Rubber	NA	84	23/05/16
Kudat	Sungai Pupu (SUN)	57	Rubber	10 000	75	19/04/16
Kudat	Suvil (SUV)	9	Rubber, palm	500	77	25/04/16
Kota Marudu	Kotud (KOT)	543	Rubber, palm	75	245	30/05/16
Kota Marudu	Patiu (PAT)	260	Rubber	250 000	231	03/05/16
Kota Marudu	Sorinsim (SOR)	180	Rubber	10 000 000	158	05/04/16
Pitas	Perpaduan (PER)	14	Palm	1 600	284	09/05/16
Pitas	Sinangip (SIN)	218	Rubber, palm	500	375	11/04/16
Ranau	Gondohon (GON)	1275	Rubber, cabbage	500 000	460	13/06/16
Ranau	Lipasu Lama (LIP)	897	NA	250 000	311	16/05/16
Ranau	Siba Bundu Tuhan (SIB)	1084	Cabbage, lettuce	10 000 000	737	21/03/16

outdoor garden area immediately surrounding a house (outside, < 5m from a house). Farm sites were located in small plantations and forest sites were in patches of secondary forest comprising non-agricultural trees. Due to the wide geographical range of our sampling, the farm habitat varied between villages depending on what was locally cultivated (Table 1). Forest was distributed patchily throughout the area with patch sizes varying significantly between villages (0.075 - 10km^2, Table 3.1).

Mosquito sampling sites were selected by walking in and around each village at the start of each visit to identify all accessible locations within each of the 3 habitat types. One location per habitat type was haphazardly selected based on the following stipulations: peri-domestic- consent from household residents, farm- a point at least 25m from the nearest house so as to differentiate from peri-domestic sites, forest- minimum patch size of 10x10m, site 20m from forest edge (if not possible, then centre of forest patch). On each night of sampling, one team of two people performed HLC in each of the 3 habitat types, then the teams rotated between habitats on subsequent nights. Across all four sampling nights, a different sampling point was selected within each of the 3 focal habitat types. Each sampling point was at least 25 m from the location used the previous night. Only three nights of collections were performed for Sungai Pupu and Patiu villages due to heavy rainfall and fogging (for dengue control) taking place.

3.3.4 **Mosquito processing**

At the end of each 6-hour sampling period, mosquitoes trapped inside the vials were transported to the central field station by vehicle and put in a -20˚C freezer. Mosquitoes were killed by storing at -20˚C overnight and identified to genera the following day using the Rattanarithikul *et al* (2005) key to the mosquitoes of Thailand [175]. *Anopheles* were further identified to species level using the Rattanarithikul *et al* (2005) key to the *Anopheles* mosquitoes of Thailand [178] and species belonging to the Leucosphyrus group were identified using the Leucosphyrus group of *Anopheles* key [222]. All identifications were performed under a field stereomicroscope. Specimens were kept at -20˚C until further processing.

Figure 3.2 Photos showing examples of typical peri-domestic, farm and forest habitats where mosquito collections were conducted in this study.

3.3.5 *Plasmodium* detection in *Anopheles*

DNA was prepared from all Leucosphyrus group *Anopheles*, *Anopheles donaldi* and *An. maculatus* (human malaria vectors) following removal of the ethanol preservative. DNA was extracted using the QIAGEN DNeasy Blood and Tissue Kit following the manufacturer's instructions with the following minor modifications. Specimens were initially ground in 180 µl buffer ATL using a pestle and hand held homogenisor, and lastly eluted in a volume of 25 µl TE buffer. Extracted DNA was stored at -20 °C until further processing. Nested PCRs were conducted to screen samples for *Plasmodium* DNA using the method of Snounou and Singh [223], which identifies DNA of any species within the *Plasmodium* genus.

Two µl of genomic DNA was subjected to an outer amplification reaction with 0.4 µM of each of the SSU-rRNA *Plasmodium* genus specific primers rPLU1 and rPLU5, 200 µM dNTPs, 3 mM $MgCl_2$, and 1.7 U GoTaq Flexi DNA polymerase (Promega) with 1 x Green GoTaq Flexi Reaction Buffer in a total volume of 25 µl. The nested reaction was identical except for substitution of the primers with rPLU3 and rPLU4, and the use of 2 µl of outer PCR product instead of genomic DNA. PCR conditions for both reactions were: initial denaturation at 95 °C for 5 min; followed by 35 cycles of denaturation at 94 °C for 1 min, annealing for 1 min and extension at 72 °C for 1 min; and a final extension at 72 °C for 5 min. The annealing temperature was modified from [223] to 55 °C in nest 1 and 62 °C in nest 2. PCR products were run on 1.5 % agarose gel in 1x TAE buffer and samples which yielded a band at 235 bp were subjected to a further PCR to identify the species of *Plasmodium* present.

Nine separate reactions were set up following the method of Ta *et al* [224] (to detect *P. falciparum, P. vivax, P. malariae* and *P. ovale*), Lee *et al* [225] (*P. coatneyi, P. inui* and *P. cynomolgi*) and Imwong *et al* [226] (*P. knowlesi*). 2ul product from rPLU1/rPLU5 (nest 1) PCR was subjected to an alternative nest 2 reaction with 0.4 µM of each of the species-specific primer pairs (Table 2), 200 µM dNTPs, 3 mM $MgCl_2$, and 1.7 U GoTaq Flexi DNA polymerase (Promega) with 1 x Green GoTaq Flexi Reaction Buffer in a total volume of 25 µl. PCR conditions were as in nest 1 with different annealing temperatures for each species-specific

reaction (Table 3.2). PCR products (see Table 3.2 for sizes) were run on 1.5 % agarose gel in 1x TAE buffer.

3.3.6 Dengue detection in *Aedes*

All *Ae. albopictus* and *Ae. aegypti* were screened for dengue virus using the SD Bioline NS1 antigen strip test following the methods of Lau et al [227]. Here, mosquito specimens were placed on ice to keep samples cool during manipulation. Mosquito heads were separated from abdomens using sterile scalpels. The abdomens of five individuals were ground in 250 ul PBS using a hand-held homogenisor then pipetted onto an NS1 Ag strip.

3.3.7 *Plasmodium knowlesi* sero-prevalence in humans

Data on malaria prevalence in humans within the study area was obtained during a large cross-sectional study conducted by the Monkeybar project in the districts of Kudat, Kota Marudu, Pitas and Ranau in September to December 2015. This was a study of 135 clusters of 20 households in which all individuals who had resided in the selected households for the previous month were tested for current infections (via blood smears and PCR) and previous exposure to malaria through serology (Fornace *et al*, in prep). The number of *P. knowlesi* PCR positive samples were too low (4/2503 active infections) to use as a measure of human prevalence [228]. Thus, serological measures of previous *P. knowlesi* exposure were used to examine associations with the abundance of Leucosphyrus group *Anopheles* (vectors of *P. knowlesi*) caught in the study. Serology is considered an appropriate alternative to PCR for detection of previous malaria infection, in low transmission settings [229] and has been shown to correlate with entomological inoculation rates [230,231]. The maximum duration of *P. knowlesi* specific humoral responses is unknown, however IgG for the newly developed PkSERA3 antigen 2 can be detected in 63.8 % patients at 28 days post infection [232]. Antibodies for PkSSP2 antigen peaked on day 7 post infection and were detected in only 33.3 % patients [232]. Consequently, the serological indicators measured here can be used to estimate the presence of *P. knowlesi* infection within the previous month.

Following the protocol developed by Herman *et al* [232], Fornace *et al* (in prep) screened human samples by ELISA using *P. knowlesi* specific antigens: SERA3 antigen 2 and SSP2 [232]. Measures of village level sero-positivity (the proportion of individuals from the total screened per village that were IgG positive for *P. knowlesi*) were estimated for all of the 11 villages in which entomological surveillance was conducted.

3.3.8 **Data analysis**

3.3.8.1 ***Anopheles* diversity across habitat types**

Data were analysed using the R statistical programming software, version 3.4.2. The "vegan" package was used to measure four species diversity indices: species richness, rarefied species richness, Simpson's index and the Shannon index. These measures were used to estimate and compare *Anopheles* diversity across habitat types (peri-domestic area, farm and forest). Species richness is the total number of different *Anopheles* species collected in each village. The rarefied species richness is the species richness if collections had the same *Anopheles* abundance (ie. set to the group with the lowest total abundance). Rarefaction is a method used to standardise unequal sampling sizes [233,234]. The Simpson's index,

$\lambda = n/n-1 \times \sum ps\ (1-ps)$ [235], where

n = total *Anopheles* abundance

ps = each species count/n,

measures the probability that two individuals randomly sampled from the dataset will be of the same species [236]. The Simpson's Index is noted to be sensitive to abundant species [237], thus the Shannon Index was also calculated as a comparison. The Shannon index,

$H = -\sum (ni/N) \log (ni/N)$ [236], where

N = total *Anopheles* abundance

ni = each species count,

measures the uncertainty in predicting the species of an individual randomly sampled from the dataset [237]. Confidence intervals for Simpson's Diversity Index were calculated following Zhang [235].

3.3.8.2 **Analysis of environmental variables**

Percentage forest cover in a 100m buffer (circle of radius 100m) around each sampling location for HLC was calculated using the Hansen global forest cover 2014 map [238]. GLMMs were constructed in R using the lme4 package to extract the mean elevations and proportion of forest cover at mosquito collection sites for each habitat type. A negative binomial model was used to predict mean elevation and a model with a binomial distribution was used for percentage forest cover. Elevation or percentage forest cover were the response variables and habitat was the explanatory variable, with date and village set as random effects.

3.3.8.2.1 Mosquito presence and abundance analyses

Statistical analysis was performed on two sets of mosquito data: 1) *An. balabacensis* only, and 2) All Leucosphyrus group *Anopheles*. The second group was inclusive of *An. balabacensis* (n = 32), *An. latens* (n = 7) and mosquitoes (n = 2) which were either of these two but could not be designated to one species due to loss of fragile scales on the wings necessary for morphological identification. Both *An. balabacensis* or *An. latens* are implicated in the transmission of *P. knowlesi* in Malaysian Borneo [50,58] thus were analysed as a whole. The packages lme4 and multcomp were used to analyse mosquito presence and abundance. GLMMs were constructed to test for associations between the two response variables of mosquito presence (binary outcome, 0 = absent, 1= present) and abundance (mean number caught per site per night), and the following explanatory variables: elevation, habitat type and forest cover. To relieve issues with scaling, elevation was converted from a continuous to a categorical variable by splitting into three elevation ranges: low (0 – 375m), medium (376 – 750m) and high (751 – 1125m). Models were fit with a negative binomial distribution for mosquito abundance and a binomial distribution for mosquito presence. In all models, random effects were included for village and

date. The significance of explanatory variables in each of the models was tested by backward elimination using likelihood ratio tests. A Tukeys' post hoc test was performed to assess differences between each of the 3 habitat types. GLMMs were tested in a similar way to examine the abundances of *Aedes* mosquitoes (of which 90.3 % (n = 383) were *Ae. albopictus* and *Ae. aegypti,* vectors of dengue virus).

3.3.8.2.2 Biting rates of malaria vector species

The lme4 package was used to construct generalised linear mixed models (GLMM) in R to extract hourly biting rates of different malaria vector species caught. Only *An. balabacensis, An. donaldi* and *An. maculatus* were examined because the overall abundance of *An. latens* (n = 7) was too low to analyse in this way. The number of mosquitoes of each species caught per hour throughout the night was examined, with the first hour as 18:00 – 19:00 and the last as 23:00 – 00:00. Hourly mosquito abundance was treated as the response variable with the main fixed effect being biting hour. A negative binomial distribution was used with date and village set as random effects. A Tukey's post-hoc test (package multcomp) was used to assess differences in biting rates between hours within each species.

3.3.8.3 Associations between vector abundance and human *P. knowlesi* exposure

GLMMs were constructed to test for associations between mosquito presence and abundance for 1) *An. balabacensis* only and 2) Leucosphyrus group *Anopheles* (*An. balabacensis*/*An. latens*) and village-level *P.knowlesi* sero-positivity. Using data only for each village, models were constructed to obtain mean mosquito abundance and predicted probabilities of detection for each village. A GLMM with negative binomial distribution was used to predict mean mosquito abundance from each village where mosquito abundance per night was the response variable and habitat and date were fit as random effects. A binomial GLMM was used to predict the probability of detecting a mosquito in each village where mosquito presence (1) or absence (0) per night was the response variable and habitat and date were fit as random effects.

Then, village specific predicted mean mosquito abundances and probabilities of detection were used to test for associations with the proportion of individuals sero-positive for *P. knowlesi* antigens in each village. A binomial GLM was used with village sero-positivity as the response variable and mosquito presence or abundance as the fixed effect. A further binomial GLM was used with village sero-positivity as the response variable and mosquito presence or abundance as the fixed effect fit as a quadratic function (eg. abundance +I(abundance^2)). Entomological collections began ~ 6 months after the cross-sectional survey thus did not run in parallel with human sampling. However, an assumption of this analysis is that entomological measures were assumed to be reflective of general differences between villages at the time the cross-sectional survey took place. A power analysis was conducted to assess whether the lack of association between village level human *P. knowlesi* sero-positivity rates and *P. knowlesi* vector abundance was due to the low overall sample size. For this, data gathered for the 11 villages was repeated (x1, x2, x3…. x14) and the new datasets analysed using the binomial GLM with village sero-positivity as the response variable and mosquito presence or abundance as the fixed effect until a significant relationship ($P < 0.05$) was detected.

3.3.9 **Ethics**

This project was approved by the Malaysian Ministry of Health (NMRR-12-786-13048) and by the research ethics committees of the London School of Hygiene and Tropical Medicine (Ref. 6302). All volunteers who carried out mosquito collections signed informed consent forms and were provided with antimalarial prophylaxis during participation. One month after performing HLC, volunteers were screened for malaria parasites by giemsa stained thick and thin blood smears. Participants were to immediately report if feeling ill or feverish and would be taken to the nearest medical facility for check-up and treatment.

Table 3.2 Primer pairs used in nested PCR to detect parasites from *Plasmodium* genus and specific human and simian malaria species.

Target	PCR	Primer	Sequence (5' - 3')	Annealing temp.	Product size
Plasmodium genus	1/ 2	rPLU1	TCAAAGATTAAGCCATGCAAGTGA	55°C	1.6-1.7kb
		rPLU5	CCTGTTGTTGCCTTAAACTCC		
Plasmodium genus	1	rPLU3	TTTTTATAAGGATAACTACGGAAAAGCTGT	62°C	235bp
		rPLU4	TACCCGTCATAGCCATGTTAGGCCAATACC		
P. coatneyi	2	PctF1	CGCTTTTAGCTTAAATCCACATAACAGAC	62°C	504bp
		PctR1	GAGTCCTAACCCCGAAGGGAAAGG		
P. inui	2	PinF2	CGTATCGACTTTGTGGCATTTTTCTAC	60°C	479bp
		INAR3	GCAATCTAAGAGTTTTAACTCCTC		
P. fieldi	2	PfldF1	GGTCTTTTTTTTGCTTCGGTAATTA	66°C	421bp
		PfldR2	AGGCACTGAAGGAAGCAATCTAAGAGTTTC		
P. cynomolgi	2	CY2F	GATTTGCTAAATTGCGGTCG	60°C	137bp
		CY4R	CGGTATGATAAGCCAGGGAAGT		
P. knowlesi	2	PkF1140	GATTCATCTATTAAAAATTTGCTTC	50°C	424bp
		PkR1550	GAGTTCTAATCTCCGGAGAGAAAAGA		
P. falciparum	2	NewPLFshort	CTATCAGCTTTTGATGTTAG	53°C	370bp
		FARshort	GTTCCCCTAGAATAGTTACA		

Table 2 continued on next page

Table 3.2 continued. Primer pairs used in nested PCR to detect parasites from *Plasmodium* genus and specific human and simian malaria species.

Target	PCR	Primer	Sequence (5' - 3')	Annealing Temp.	Product size
P. vivax	2	NewPLFshort	CTATCAGCTTTTGATGTTAG	53°C	476bp
		VIRshort	AAGGACTTCCAAGCC		
P. malariae	2	NewPLFshort	CTATCAGCTTTTGATGTTAG	53°C	241bp
		MARshort	TCCAATTGCCTTCTG		
P. ovale	2	NewPLFshort	CTATCAGCTTTTGATGTTAG	53°C	407bp
		OVRshort	AGGAATGCAAAGARCAG		

3.4 Results

3.4.1 General trends in mosquito vector abundance and diversity

In 42 nights of sampling, a total of 5588 mosquitoes belonging to eight genera were collected (Table 3.3). The majority of specimens were from the *Culex* and *Armigeres* genera, with only a small percentage made up by potential malaria (*Anopheles* 4 %) and dengue vectors (*Aedes* 8 %). Five genera were found in the peri-domestic habitat, six were found in the farm and seven genera were found in the forest (Fig. 3.3). Known malaria vector species (*An. balabacensis*, *An. latens*, *An. donaldi* and *An. maculatus)* comprised 2.5 % of the total catch and 68 % of all Anophelines. Six species of *Anopheles* were collected (Table 3.4) with *An. maculatus* and *An. barbumbrosus* being the most abundant. Two previously described vectors of *P. knowlesi* were detected, *An. balabacensis* and *An. latens*. Four known vectors of the human malaria parasites, *P. falciparum* and *P. vivax,* were also found: *An. balabacensis*, *An. donaldi, An. latens* and *An. maculatus*. Known dengue vector species (*Ae. albopictus* and *Ae. aegypti*) comprised 6.9 % of mosquitoes collected. The majority of *Aedes* specimens, were *Ae. albopictus* (~90%) with only a few *Ae. aegypti* (~1%). The remaining *Aedes* specimens could not be identified to species level.

Anopheline species diversity was lower in peri-domestic and farm sites than the forest sites (Table 3.5). Both the rarefied species richness, Shannon and Simpson Indexes estimated similar trends with forest sites having higher *Anopheles* species diversity, followed by farm sites and then peri-domestic sites (Table 3.5).

The mean elevation of the mosquito collection sites was ~350-480m across peri-domestic, farm and forest habitats (Table 3.6). There was no significant association between habitat type and altitude, meaning that the impacts of these two variables could be evaluated independently (Table 3.6; $P > 0.05$). In general, there was more tree cover at farm and forest sites than at peri-domestic sites but still at peri-domestic sites there some degree of tree cover (Table 3.6).

Figure 3.3 Proportional representation of different mosquito genera within collections made in peri-domestic, farm and forest habitats across 11 villages in this study.

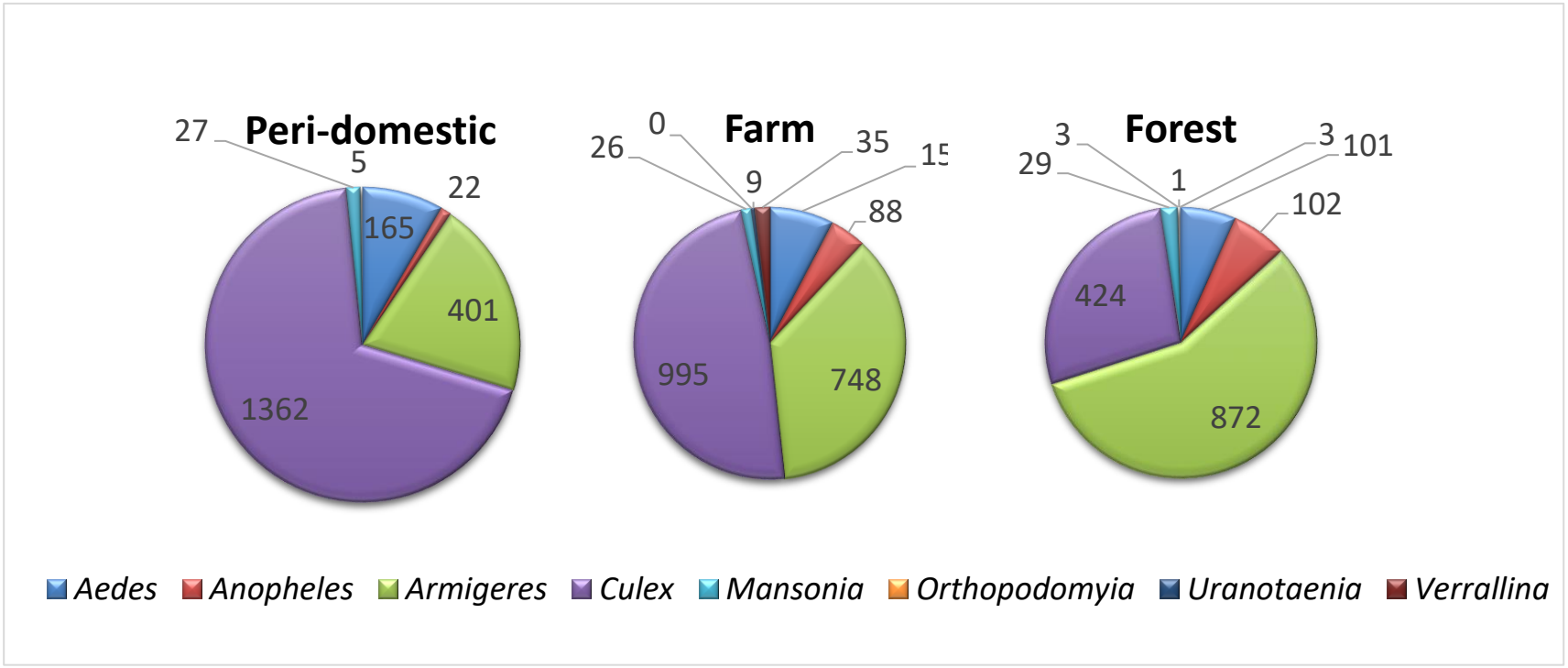

Table 3.3 Relative frequencies of eight mosquito genera caught in eleven villages within the four districts: Kudat, Kota Marudu, Pitas and Ranau in Sabah, sampled from March to June 2016.

	District of sampling											
	Kudat (villages)			Kota Marudu (villages)			Pitas (villages)		Ranau (villages)			
Mosquito genera	**SUV**	**SUN**	**BAR**	**SOR**	**PAT**	**KOT**	**PER**	**SIN**	**LIP**	**SIB**	**GON**	**Total (%)**
Aedes sp.	6	17	14	20	46	72	86	56	19	34	54	424 (7.6)
Anopheles sp.	3	34	1	34	8	33	2	43	19	31	4	212 (3.8)
Armigeres sp.	162	64	313	124	19	97	581	612	34	14	1	2021 (36.2)
Culex sp.	115	78	1663	55	118	354	172	16	54	142	14	2781 (49.8)
Mansonia sp.	46	0	0	0	2	0	31	0	3	0	0	82 (1.5)
Orthopodomyia sp.	0	0	0	0	0	0	3	0	0	0	0	3 (0.1)
Uranotaenia sp.	0	0	0	1	0	0	0	9	0	0	0	10 (0.2)
Verrallina sp.	0	0	2	0	0	0	41	0	0	0	0	43 (0.8)
Unknown	2	1	2	0	1	1	2	1	0	2	0	12 (0.2)
Total	335	195	1999	236	196	558	917	736	129	225	73	5588

Table 3.4 *Anopheles* species caught in eleven villages within the four districts: Kudat, Kota Marudu, Pitas and Ranau in Sabah, sampled from March to June 2016.

	District of sampling											
	Kudat (villages)			**Kota Marudu (villages)**			**Pitas (villages)**		**Ranau (villages)**			
Mosquito genera/ species	**SUV**	**SUN**	**BAR**	**SOR**	**PAT**	**KOT**	**PER**	**SIN**	**LIP**	**SIB**	**GON**	**Total (%)**
Leucosphyrus gp.	0	1	0	8	1	1	0	10	19	0	0	41 (19.3)
*An. balabacensis**#	0	1	0	3	1	1	0	7	12	0	0	32 (15.1)
*An. latens**	0	0	0	0	0	0	0	0	7	0	0	7 (3.3)
*An. balabcensis or An. latens**	0	0	0	1	0	0	0	1	0	0	0	2 (0.9)
Barbirostris *gp*	3	33	1	19	3	3	0	23	0	1	3	89 (42.0)
An. barbumbrosus	0	16	1	14	3	3	0	22	0	0	2	61 (28.8)
An. donaldi#	3	16	0	2	0	0	0	0	0	1	1	23 (10.9)
An. maculatus#	0	0	0	8	3	29	0	10	0	29	1	80 (37.7)
An. tesselatus	0	0	0	0	0	0	2	0	0	0	0	2 (0.9)
Total *Anopheles* sp.	3	34	1	34	8	33	2	43	19	31	4	212

Table 3.5 *Anopheles* diversity measures across different habitat types sampled in eleven villages in Sabah from March to June 2016.

Habitat	*Anopheles* abundance	Species richness (vegan)	Rarefied species richness (vegan)	Shannon index (vegan)	Simpson's index (vegan)	Simpson's index ± 95% confidence intervals (manual)
Peri-domestic	22	4	2.380	0.969	0.5	0.52 ± 0.22
Farm	85	4	2.858	1.276	0.694	0.73 ± 0.05
Forest	98	5	3.139	1.477	0.750	0.79 ± 0.04

Table 3.6 Mean values of elevation and percent forest cover within each of the 3 habitat classes where mosquito sampling occurred in this study.

Habitat	Mean elevation (m) (range)	Mean percentage forest cover (%) (range)	Predicted mean percentage forest cover
Peri-domestic	427.1 (14 – 1109)	10.2 (0 – 70.5)	3.8
Farm	358.5 (13 – 1107)	17.3 (0 – 62.5)	14.3
Forest	478.1 (15 – 1125)	24.2 (0 – 61.4)	8.8

3.4.2 **Vector abundance and distribution**

3.4.2.1 ***Anopheles***

3.4.2.1.1 Analysis of probability of detection

The probability of collecting *An. balabacensis* was approximately 0.1 % and did not differ with habitat (X^2 = 5.33, *df* = 2, *P* = 0.07), percentage forest cover (X^2 = 3.16, *df* = 1, *P* = 0.08) or elevation (X^2 = 0.21, *df* = 2, *P* = 0.90). The probability of collecting an *Anopheles* from the Leucosphyrus group differed with habitat (X^2 = 7.42, *df* = 2, *P* = 0.02) but not forest cover (X^2 = 3.31, *df* = 1, *P* = 0.07) or elevation (X^2 = 0.34, *df* = 2, *P* = 0.85). Leucosphyrus group mosquitoes were more likely to be caught in farm (*P* = 0.02) and forest (*P* = 0.02) sites than in the peri-domestic environment (Fig. 3.4).

3.4.2.1.2 Analysis of mean abundance

The abundance of *An. balabacensis* varied with habitat (X^2 = 9.82, *df* = 2, *P* < 0.01) but not with elevation of HLC site (X^2 =0.13, *df* = 2, *P* = 0.93) or percentage forest cover (X^2 = 3.16, *df* = 1, *P* = 0.08). *Anopheles balabacensis* was significantly more abundant in farm (*P* < 0.01) and forest (*P* < 0.01) habitats than in peri-domestic areas (Fig. 3.5A).

The mean abundance of the Leucosphyrus group did not vary with forest cover (X^2 = 4.12, *df* = 1, *P* = 0.04) or elevation of HLC site (X^2 =1.64, *df* = 1, *P* = 0.20). Habitat was found to be a significant predictor of Leucosphyrus group abundance (X^2 = 12.92, *df* = 2, *P* < 0.01); with their density being significantly lower in peri-domestic environments than at farm (*P* < 0.001) or forest (*P* < 0.001) habitats (Fig. 3.5B).

3.4.2.2 ***Aedes***

The probability of collecting an *Aedes* mosquito did not vary with habitat (X^2 = 5.51, *df* = 2, *P* = 0.06), forest cover (X^2 = 1.45, *df* = 1, *P* = 0.23) or elevation (X^2 = 5.97, *df* = 2, *P* = 0.05). However *Aedes* abundance was positively associated with forest cover (X^2 = 4.36, *df* = 1, *P* = 0.04) (Fig. 3.6). *Aedes* abundance did not vary with habitat (X^2 = 3.51, df = 2, P = 0.17) or elevation (X^2 = 1.66, df = 2, P = 0.44).

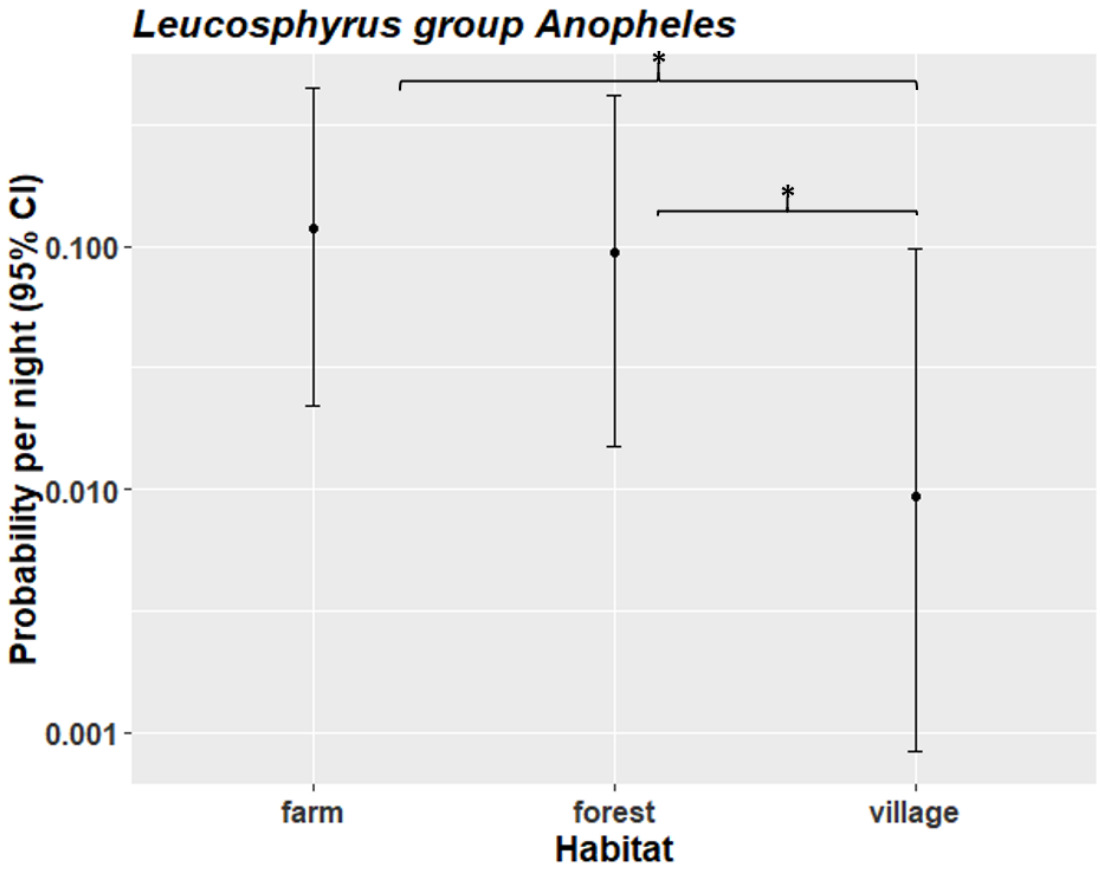

Figure 3.4 Predicted probability of catching Leucosphyrus group *Anopheles* in farm, forest and peri-domestic habitats sampled in this study. Error bars represent 95% confidence intervals.

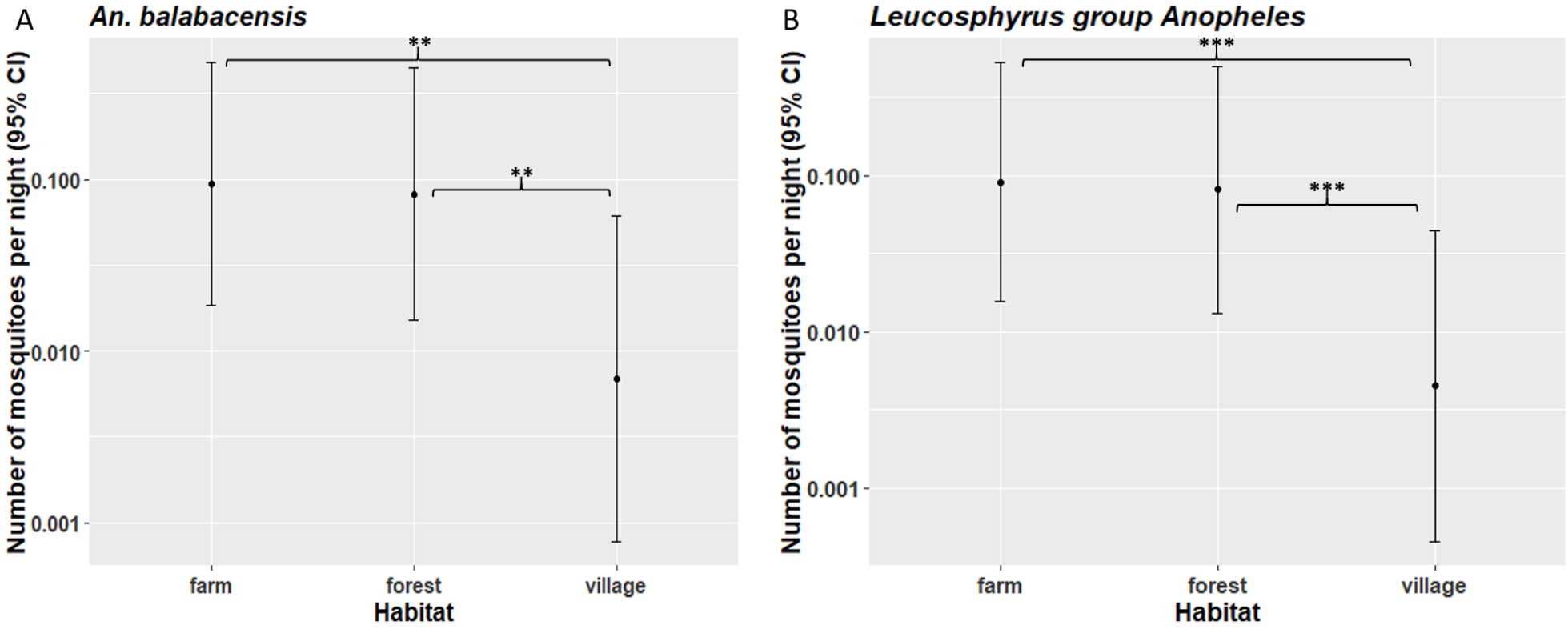

Figure 3.5 Predicted mean abundance of different vector groups within 3 different habitats in this study: A) *An. balabacensis* and B) Leucosphyrus group *Anopheles.* Error bars represent 95% confidence intervals.

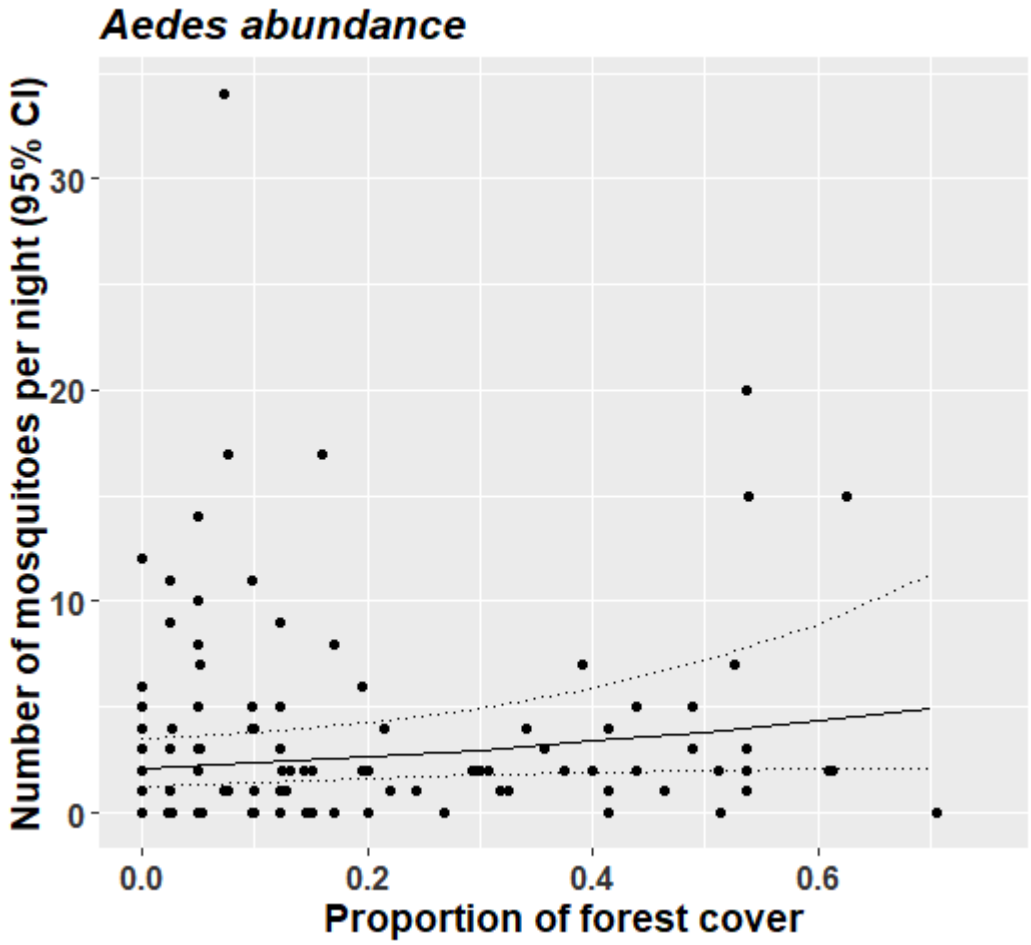

Figure 3.6 Influence of proportion of forest cover in 100m buffer around trapping site on the mean abundance of *Aedes* collected per night. Error bars represent 95% confidence intervals.

3.4.3 Biting patterns of malaria vector species

The hourly rates of malaria vector species were generally very low (~0.005 – 0.038 per species per hour), and higher in *An. maculatus* than *An. donaldi* or *An. balabacensis*. Biting activity was slightly higher during the early evening hours (18:00 – 20:00 hrs) but there was large variability making it difficult to detect clear peaks (Fig. 3.7). No Tukey's test comparison of mean biting rates between hours for each species were significant

3.4.4 Malaria and dengue infection rates

Of the 144 female mosquitoes that were potential malaria vector species, only one tested positive for any malaria parasite species. This was an *An. balabacensis* collected in a forest patch in Sinangip village, Pitas, which was infected with *P. knowlesi*. This represents an infection rate of n = 1/32 *An. balabacensis* (Table 3.4) collected during the study. All *Aedes* specimens (n=424) were negative for the dengue NS1 antigen.

3.4.5 Association between malaria vector abundance and human *P. knowlesi* exposure

Seroprevalence rates of *P. knowlesi* in people across the study area are reported in detail in (Fornace *et al*, in prep). Within the subset of 11 villages where entomological surveillance was conducted here, sero-positivity rates ranged from a low of 0 % (Sib and Sun) to a high of 13.9 % (in Sor). The probability of trapping *An. balabacensis* per village per night was 0.11 – 0.42 (Fig. 3.8A), and 0.11 – 0.50 for the Leucosphyrus group overall (Fig. 3.8B). The abundance of *An. balabcensis* per village per night ranged from 0.11 to 0.73 mosquitoes (Fig. 3.9A) and for the Leucosphyrus group this was 0.11 to 0.91 (Fig. 3.9B). No significant relationship ($P > 0.05$) was detected between the probability of detection and abundances, for both *An. balabacensis* and Leucosphyrus group *Anopheles,* and human *P. knowlesi* sero-positivity rates. The power analysis indicated that this may have been due to the low number of villages and mosquitoes sampled in the study. A minimum number of 160 *An. balabacensis* and 164 Leucosphyrus group *Anopheles* would be required to detect a positive significant relationship ($P < 0.05$) between the presence of *P. knowlesi* vectors and human *P. knowlesi* sero-positivity rates (Table 3.7). To detect a significant positive relationship ($P <$

0.05) between the abundance of *P. knowlesi* vectors and human *P. knowlesi* sero-positivity rates, a minimum sample size of 192 *An. balabacensis* and 574 Leucosphyrus group *Anopheles* would be required (Table 3.7).

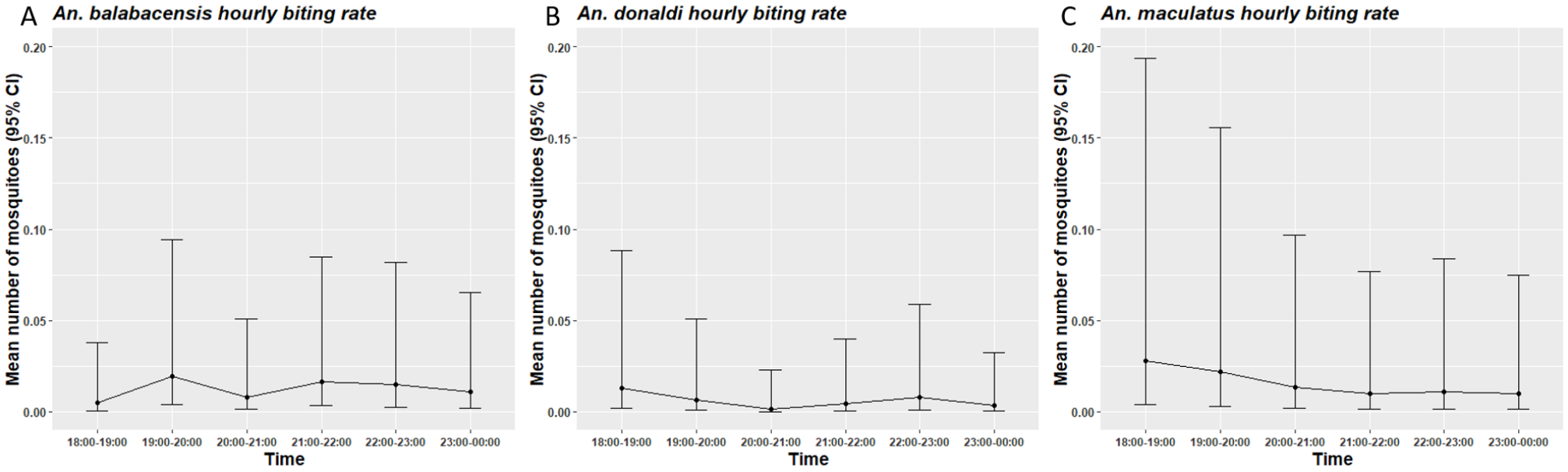

Figure 3.7 Predicted mean number of A) *An. balabacensis*, B) *An. donaldi* and C) *An. maculatus* biting per hour between 18:00- 24:00 hrs, pooled across all sites and habitat types. Error bars are 95% confidence intervals.

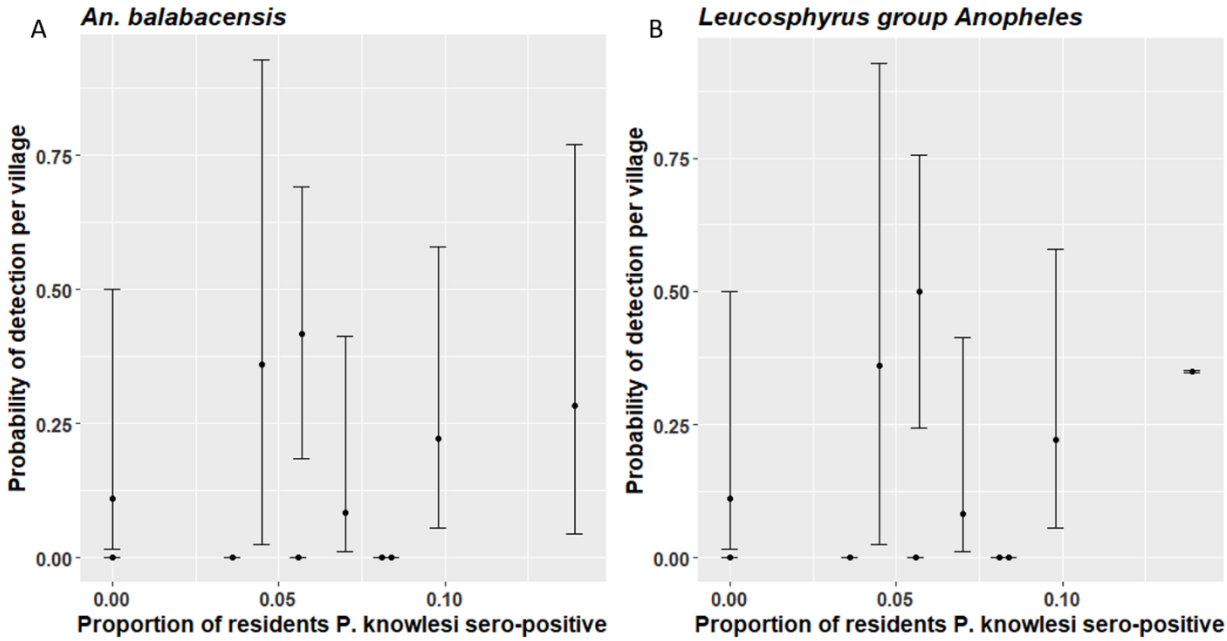

Figure 3.8 Association between the proportion of individuals in a village sero-positive for *P. knowlesi* antigens and the detection of A) *An. balabacensis* and B) Leucosphyrus group *Anopheles* in the village per night. Error bars are 95% confidence intervals.

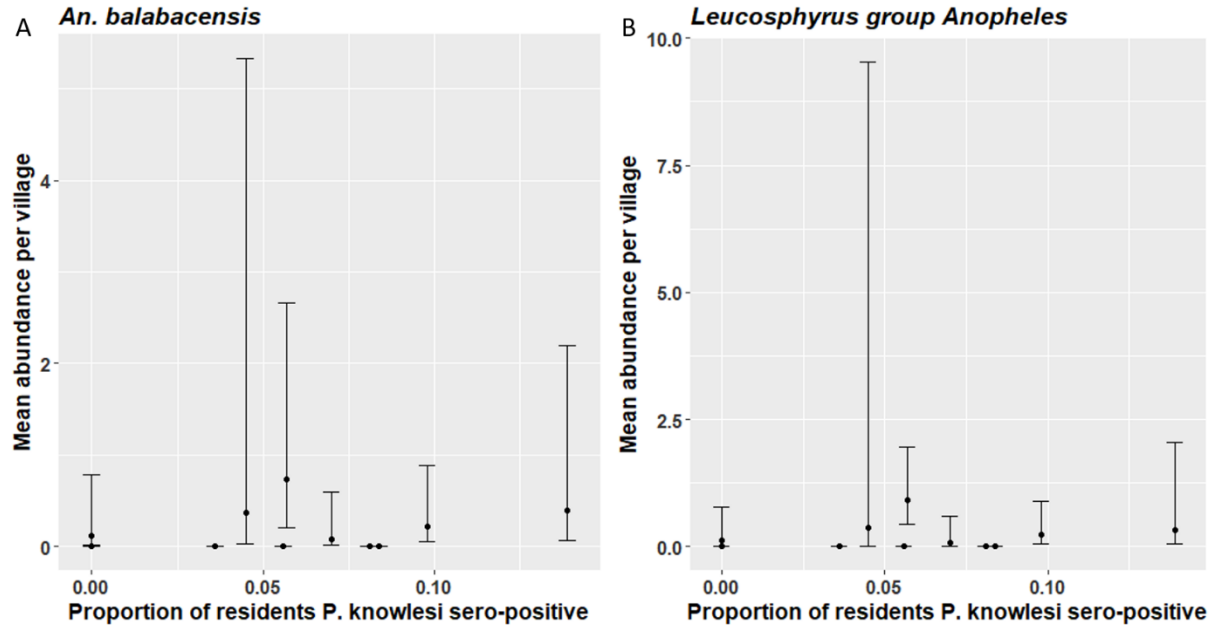

Figure 3.9 Association between the proportion of individuals in a village sero-positive for *P. knowlesi* antigens and the abundance of A) *An. balabacensis* and B) Leucosphyrus group *Anopheles* caught in the village per night. Error bars are 95% confidence intervals.

Table 3.7 Results of power analysis to indicate sample size required to pick up an association between the proportion of individuals in a village sero-positive for *P. knowlesi* antigens and the detection and abundance of *An. balabacensis* or Leucosphyrus group *Anopheles* in the village per night.

Number of replicates of original dataset	Number of *An. balabacensis* mosquitoes	Number of Leucosphyrus gp. mosquitoes	Relationship between human *P. knowlesi* sero-positivity and detection of *P. knowlesi* vectors		Relationship between human *P. knowlesi* sero-positivity and abundance of *P. knowlesi* vectors	
			An. balabacensis	Leucosphyrus group *Anopheles*	*An. balabacensis*	Leucosphyrus group *Anopheles*
1	32	41	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$
2	64	82	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$
3	96	123	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$
4	128	164	$P > 0.05$	$P = 0.049$	$P > 0.05$	$P > 0.05$
5	160	205	$P = 0.049$	$P = 0.027$	$P > 0.05$	$P > 0.05$
6	192	246	$P = 0.031$	$P = 0.015$	$P = 0.039$	$P > 0.05$
7	224	287	$P = 0.019$	$P = 0.008$	$P = 0.025$	$P > 0.05$
8	256	328	$P = 0.012$	$P = 0.005$	$P = 0.017$	$P > 0.05$
10	320	410	$P = 0.005$	$P = 0.001$	$P = 0.007$	$P > 0.05$
12	384	492	$P = 0.002$	$P = 0.001$	$P = 0.003$	$P > 0.05$
14	448	574	$P = 0.001$	$P = 0.0002$	$P = 0.001$	$P = 0.042$

3.5 **Discussion**

Following a large outbreak of the macaque malaria *P. knowlesi* in humans in the Kudat district of Malaysian Borneo, a wider programme of entomological sampling throughout the state of Sabah was conducted. Through this, I demonstrated that the primary vector responsible for transmission, *An. balabacensis*, is relatively widespread but occurs at considerably lower density than estimated in focal studies around Kudat. This wider geographical sampling also indicated that *An. balabacensis* abundance is significantly higher in farm and forest habitats than in peri-domestic sites. This contrasts with earlier work based on sampling of single sites in Kudat that indicated vector abundance was slightly higher in peri-domestic habitats. Only one malaria-infected mosquito was found across the study area, an *An. balabacensis* infected with *P. knowlesi* caught in a forest patch. Whilst this is in line with the expectation that *P. knowlesi* infection rates are highest in *An. balabacensis* found in forests [50], the sample size of infected mosquitoes was too low to draw any significant conclusions about habitat-dependent mosquito infection rates. In collaboration with a large epidemiological survey of *P. knowlesi* infection in humans, a positive association between mean *An. balabacensis* density and sero-prevalence for *P. knowlesi* in people was demonstrated at the village-level. Overall these findings indicate that *P. knowlesi* risk is relatively low and heterogeneous throughout the region, and that studies from just one area (Kudat) may not be universally representative of *P. knowlesi* vector ecology.

On the basis of study at a few sites around Kudat, the primary *P. knowlesi* vector, *An. balabacensis,* was previously reported to be the dominant Anopheline biting humans [50]. This finding was based on longitudinal surveillance of mosquitoes at 3 sites (1 village, farm and forest area). In contrast, I sampled mosquitoes over a considerably wider geographical range, spanning 11 villages from 4 districts, and incorporating replication of forest, farm and peri-domestic habitats within and between villages. Here mosquitoes were sampled at only one 4-day time point, rather than across a year as in [50]. Using this study design, *An. balabacensis* comprised only 15.1% of Anophelines, in contrast to the 95.1% previously reported for Kudat [50]. As part of a *P. knowlesi* case-control study [194], *An. balabacensis* was shown to represent 86.7% of Anophelines within peri-domestic areas at households where cases had been reported in the Kudat

district (73.0% at control houses) [194]. These high proportions of *An. balabacensis* in *Anopheles* collections may be specific to the Kudat district, with the relative abundance of this vector being much lower when measured over a wider spatial scale. Results here demonstrate substantial spatial heterogeneity in vector community structure; with vector densities in Kudat being significantly higher than elsewhere. This could explain why high incidences of human *P. knowlesi* infections were reported from Kudat and that *P. knowlesi* cases contribute the largest proportion of malaria cases in this district [44,85,239,240]. However even within Kudat, our results suggest a considerably lower relative abundance of *An. balabacensis* (2.6 %) than previously reported [50,194]. It is unknown why this is the case, but possibilities include both the non-random selection of sites in early work to target areas with particularly high *An. balabacensis* density; and/or differences in the temporal scale of sampling. Here mosquitoes were sampled for only 3-4 nights per site (in 2016), whereas previous work sampled mosquitoes over 12 months (3 nights/month, 2013-2015). It is possible that the reduction in *An balabacensis* may reflect a longer-term temporal change occurring in the mosquito community or failure to capture seasonal dynamics in the current study.

Habitat type was a major predictor of *Anopheles* presence and abundance in this study. Both *An. balabacensis* and the Leucosphyrus group, were found at significantly higher abundances in farm and forest habitats than in the peri-domestic environment. This differs from a previous study conducted in a single farm, forest and peri-domestic site in Kudat; where *An. balabacensis* was most abundant in the village [50]. Studies in Kapit, Sarawak [58], and in Peninsular Malaysia [46] reported higher abundances of Leucosphyrus group vector species in forest or farm habitats than in peri-domestic sites. Differences reported in Wong *et al* [50] may have been due to site specific factors rather than habitat, highlighting the need for replicated sampling over wide geographical areas for robust habitat prediction [241]. Similar levels of detection and abundances for *An. balabacensis* and Leucosphyrus group *Anopheles* were found for farm and forest sites indicating that these mosquitoes thrive in agricultural areas as well as forests as others have shown [46,58]. Thus people are as likely to encounter a *P. knowlesi* vector when working in the farmland as in the forest. Recent epidemiological studies have identified forest and agricultural-related work

activities as risk factors for *P. knowlesi* risk in Sabah [88,228]. Forest cover and historical forest loss have also been significantly associated with the occurrence of human cases of *P. knowlesi* in this area [193]. People in rural villages in Sabah commonly practice small-scale subsistence farming, thus are at high risk for malaria transmission around villages where malaria vectors are present.

Altitude has long been recognized as a significant predictor of malaria transmission because as elevation increases, temperature decreases causing a reduction in mosquito densities and parasite developmental success and survival [242–247]. In the current study, elevation was not a significant predictor of *Anopheles* presence and abundance. This may be due to the significant additional environmental heterogeneity introduced by sampling over such a wide geographical range which could have swamped any more modest impact of elevation. All sites sampled may also have been within the altitudinal/temperature range suitable for *An. balabacensis*. It could be that this vector can survive a wide range of temperatures associated with an elevation range of 13 – 1125m and that an effect would only be detected if mosquito collections had been performed at the extremes (minimum and maximum) elevations. A modified study design based on sampling vectors across a transect of wider altitudinal gradient may be required to more thoroughly investigate the effect of elevation. After accounting for the effect of habitat, variation in the proportion of forest cover around each sampling site (within 100 m radius) did not further explain any additional variation in vector abundance and distribution. A modelling study indicated that there was a higher risk of human *P. knowlesi* cases in areas of > 65% forest cover in a 2 km radius [193]. Here, percentage forest cover was considered only within a 100m radius of each sampling site, and did not exceed 30%. The lack of association between percentage forest cover and vector abundance here may be reflective of the relatively small spatial scale over which it was considered; with mosquito populations potentially being more dependent on the habitat composition at larger scales.

To test for associations between exposure to vectors and *P. knowlesi* infection risk in humans, the relationship between the presence and mean abundance of *An.* Leucosphyrus group mosquitoes and human *P. knowlesi* sero-positivity was

assessed. Typically, studies investigating entomological indicators of malaria infection in humans use infection incidence or parasite prevalence in blood samples as a primary epidemiological endpoint [212]. It was not possible to use either of these disease indicators here because the rate of *P. knowlesi* infection in the human population is so low that there is limited chance of detecting a new or ongoing infection through random surveillance. Consequently, sero-positivity was measured as an indicator of either current or previous infection; with *P. knowlesi*. The assay used here is able to detect ~60% of *P. knowlesi* infections that occurred within 28 days prior to testing [232]. It is unknown how long the antibodies to *P. knowlesi* used in this assay (SERA3 antigen 2) persist post 28 days however it is unlikely that they are long-lived due to the low prevalences of *P. knowlesi* in the human population [228]. No significant association between the abundance of mosquito vectors (*An. balabacensis* and all *An.* Leucosphyrus group mosquitoes) and human sero-positivity for *P. knowlesi* at the village-level was detected here. This may have been due to the relatively short window in which previous *P. knowlesi* infection could be detected. Few other studies have used sero-prevalence as an indicator of malaria infection in humans. Of these, a study in Tanzania showed that sero-prevalence to *P. falciparum* antigens (MSP1, 2 and AMA1) was positively associated with entomological inoculation rate (EIR) [231]. Similarly, results for *P. falciparum* were obtained in Papua New Guinea [248], and in Senegal a progressive reduction in human IgG antibody response to *P. falciparum* schizont antigens was documented in response to declines in EIR, in a longitudinal study (2000 - 2012) [249].

Thus our results dictate that further study is required to determine if sero-prevalence could be a useful indicator of variation in malaria exposure; particularly in low transmission and elimination settings.

The majority of studies investigating potential entomological indicators of human malaria infection use parasite prevalence or incidence as a primary endpoint [212,214-217,219,220]. Generally entomological variables such as vector density, sporozoite rate and entomological inoculation rates are estimated as potential correlates of infection risk [250]. The relationship between these

entomological indicators and human infection is not always consistent [216,217]. For example, while vector abundance, sporozoite rates and EIR are positively correlated with parasite prevalence in humans in many cases (69–71), sometimes there is a poor association (e.g EIR and prevalence [216,251]). A notable limitation of our study design was that entomological sampling was performed six months after human data was collected. Therefore there was a temporal mismatch in the timing of human and entomological sampling which could have limited the strength of any association. The entomological data was only conducted for a few days thus may not have been accurate representation of vector conditions at the time of human sampling. Further to this, the sero-positivity assay was limited to a short window for detecting previous *P. knowlesi* infections (~1 month) and fails to detect ~40% of infections. These limitations may have prevented the detection of a strong relationship between vector density and human exposure here, and an improved study design may have provided more conclusive evidence of entomological predictors of human *P. knowlesi* risk.

Through mosquito sampling over a wide geographical area in Sabah, this study established that *An. balabacensis* constitutes a lower proportion of *Anopheles* in collections than previously thought. This vector was detected in four districts but found in only 6/11 villages. The results highlight the careful need in making assumptions on vector ecology based on small scale sampling and that wider geographical sampling is preferred. Highest abundances of *An. balabacensis* were detected in farm and forest habitats suggesting that human risk to *P. knowlesi* is greater in these habitats than in peri-domestic areas however too few malaria infections in mosquitoes were detected to make a robust conclusion on this. Furthermore, investigation including a longer range of sampling or incorporation of more villages is required to establish if the abundances of *An. balabacensis* in villages can be used as entomological indicators of *P. knowlesi* exposure in humans.

4 Malaria transmission in macaque reservoir populations in Malaysian Borneo

4.1 Abstract

A significant outbreak of the macaque malaria species, *P. knowlesi*, across SE Asia in the last decade triggered investigations into the vector ecology associated with its transmission. More recently, the natural transmission of a second species found in macaques, *P. cynomolgi*, to humans in Malaysia has highlighted the potential for further emerging zoonotic malarias. Until now, most studies have focussed on vectors responsible for *P. knowlesi* transmission to humans and have largely been done in areas where both primates overlap. Study is lacking into the vectors responsible for the maintenance of malaria parasites within wild macaque reservoir populations which is crucial to understand the infections circulating in monkeys and to identify their potential for spillover and risk to humans.

To examine the abundance and diversity of potential vector species, we evaluated the use of the Mosquito Magnet Independence Trap (MMIT) to passively collect malaria vectors host seeking in the vicinity of macaque sleeping sites. The study site was the Lower Kinabatangan Wildlife Sanctuary of Sabah, Malaysia, an area of protected secondary forest supporting a large macaque population and very few humans. Over 38 nights, one MMIT was placed at trees where macaques were roosting and another at control trees where macaques were absent. Thermal imaging was used to estimate macaque abundance, and temperature and rainfall data were collected to characterise environmental determinants of malaria vector abundance. Malaria vectors and macaque stool samples were screened to measure the diversity and prevalence of primate malaria parasites present.

The MMIT proved to be a reliable method for non-invasive sampling of malaria (also Japanese encephalitis and filariasis) vectors host seeking near macaque sleeping sites. The primary vector of *P. knowlesi* in Sabah, *An. balabacensis*, was caught in low abundances (n = 15) but significantly more were trapped at long-tailed macaque sleeping sites than at control trees ($P = 0.02$) indicating a propensity for feeding on this host species. Screening of macaque faecal samples

noted a high *Plasmodium* prevalence within macaque populations however no *P. knowlesi* malaria was detected here. Additionally, no *P. knowlesi* was found in vectors but two *An. balabacensis* had *P. inui* infections. These results indicate that *P. inui* may be circulating at high prevalence in macaques at LKWS and that there is heterogeneity in *P. knowlesi* prevalence across macaque populations in Sabah. Currently, it is unknown whether natural transmission of *P. inui* can occur to man but due to the infections detected here, and in *An. balabacensis* trapped around human settlements in previous studies, this provokes an awareness of its potential for future spillover into humans.

4.2 Introduction

There are over 200 species of *Plasmodium*, infecting numerous animal species, including birds, reptiles and mammals, as well as humans [252]. Many malaria parasites are known to infect non-human primates, including those in the *Plasmodium* and *Haemosporid* genera [252]. There has been a long-standing interest in studying primate malarias both for use as model systems to understand human malaria [34,253–255] and for assessing the potential for zoonotic spillover from primates. Notably, primate malaria was used in the treatment of neurosyphilis patients in 1935 and primates are used to this day in efficacy testing of new malaria vaccines [256,257]. The first malaria parasite discovered to infect only non-human primates was *Plasmodium pitheci* which was isolated from an orangutan in the early 20th century [258]. Subsequent identification of non-human primate malarias were *P. inui* and *P. cynomolgi*, both described in 1905 infecting the long-tailed macaque in Borneo, *Macaca fasicularis* [259]. In the following 70 years, a further 5 simian parasite species (*P. fieldi*, *P. simiovale*, *P. knowlesi*, *P. coatneyi* and *P. fragile)* were discovered in Asian macaques and another in orangutans (*P. silvaticum* [258]). Malaria parasites have also been found in New World monkeys including (e.g *P. brasilianum* in cacajaos and *P. simium* in howler monkeys) and in African gorillas, chimpanzees and mangabeys [258]. A total of 27 *Plasmodium* species infecting non-human primates have been described [258], with several showing evidence of spillover into human populations [71,224,260]. This potential for zoonotic transmission of simian malaria in addition to its impacts on primate health merits further investigation.

The pathogenic effects of *Plasmodium* infections in monkeys are relatively unknown. There is evidence to suggest that malaria causes fever and anaemia in naïve apes [261]. However as is the case with humans, apes can develop a protective immunity to prevent against malaria-related death in areas of intense transmission [261]. In Asia, five species of macaques have been identified as being natural hosts of two malaria parasites which can also be transmitted and cause disease in humans: *P. knowlesi* and *P. cynomolgi* [195]. These parasite species appear to be benign in macaque hosts and cause few clinical symptoms other than a decrease in hematologic indices [195]. There is little correlation between the outcome of simian malaria in macaques and humans. *Plasmodium knowlesi* infection in macaques is thought to have minimal impact on macaques whereas this parasite infecting humans can result in severe disease and even death [262].

The distribution and prevalence of *Plasmodium* infections in non-human primates is reported to be high [263]. For example, wild chimpanzee and gorilla populations in Africa have high prevalences (24 – 40 %) of *Laverania* and *P. vivax* [261]. Historical studies of macaque populations in west Malaysia (1932 - 1993) indicated a moderate malaria prevalence of 21.5 % in *M. fasicularis* and 20 % in *M. nemestrina* (pig-tailed macaques) [195]. More recently, investigations in Peninsular Malaysia noted an overall malaria prevalence of 97.3 % in 145 *M. fasicularis*; with 13.7 % of those harbouring the species *P. knowlesi* [45]. A further study in Peninsular Malaysia found 50 % of wild *M. fasicularis* were infected with malaria [264]. Thus there is evidence of heterogeneity in malaria infection within and between macaque populations.

Despite growing interest in the distribution and impact of malaria infection on non-human primates [259], until recently there has been limited investigation of transmission ecology. In particular, relatively little is known about the vectors responsible for the transmission of primate malarias. Competent vectors for African ape malaria and South American primate malaria were only identified through experimental infection studies in the 1960s [265]. The natural mosquito vectors of Amazonian primate malaria were discovered through field studies in the late 1980s [266,267]. However only in the last five years have investigations begun to incriminate vectors for African ape malaria in nature [268]. Malaria parasites are widespread in Asian monkeys and more information is available on

their transmission [28,48,100,265,269], particularly in Malaysia due to significant epidemics of zoonotic malaria in people [45,46,49,58]. However, most work has focussed on incriminating the vectors responsible for transmitting primate malaria to humans ([48,50,194], Chapter 2), with much less known about the natural transmission dynamics within macaque populations. An understanding about vectors responsible for transmitting malaria between non-human primates is crucial to identify parasites circulating in monkey populations and to be aware of their potential to cause disease in humans.

The epidemiology of monkey malaria has relevance for public health due to the potential for spillover into human populations. Most notably, the macaque malaria parasite *P. knowlesi* has been documented to infect people in Cambodia [81], Indonesia [76,79], Laos [78], Peninsular Malaysia [6,18,45], Malaysian Borneo [24,262,270], Myanmar [271], Philippines [228], Singapore [80], Thailand [83,272] and Vietnam [98]. Additionally, the first human case of another macaque malaria parasite, *P. cynomolgi,* was reported in Malaysia in 2014 [224]; with 5 further infections documented this year [273]. Outbreaks of the monkey malarias *P. brasilianum* [260] and *P. simium* [274] have also occurred in people in South America. On account of these recent examples of spillovers of primate malaria into humans, zoonotic malaria is increasingly viewed as a public health risk with potential to compromise malaria elimination efforts. A comprehensive understanding of the dynamics of malaria transmission in macaque reservoir populations will be required to tackle the threat of zoonotic malaria and limit spread to humans.

The investigation of primate malaria transmission has been hindered by several logistical and ethical constraints. First, there are numerous challenges to sampling blood from primates, and mosquito vectors attempting to bite them. For example, many primate species are protected such that obtaining blood samples from wild populations is prohibited [258]. Some degree of sampling is possible for less threatened species such as macaques; but only under highly regulated conditions (43,44). Historically, blood samples were obtained from macaques by shooting and killing them; methods that are rightly now considered unethical [276,277]. There is a strong interest in finding alternative non-invasive methods for malaria detection in primates. Recently, there has been good progress with the development of molecular approaches to test for parasite DNA

in primate faecal samples [278–284]. These approaches are promising but are yet to be widely applied and optimized. Similar ethical and logistical constraints make it difficult to sample mosquito vectors attracted to macaques. In the (1960-1970s) this was done using "Monkey Baited Traps" in which macaques were placed in a small cage inside a net with some gaps to allow mosquitoes attracted to enter the net but not escape [27,45,46,58]. Now restrictions on the minimum size and nature of housing for captive primates make such approaches unfeasible. Alternative less invasive approaches such as "e-nets" in which macaques are held in larger cages and have their odour collected to attract mosquitoes have been trialled and show some promise [112], but remain logistically challenging. Additionally, these techniques have been primarily used to sample primate malaria vectors in areas near human settlements [45,46,112,205], with the primary aim of identifying which mosquitoes are responsible for human infection. This may not be reflective of the vectors that sustain transmission between macaques. A better understanding of the dynamics of monkey to monkey transmission is required to identify if and how transmission could be disrupted in the reservoir population. This necessitates reliable non-invasive methods for sampling mosquitoes and parasites within macaque populations.

In the last decade, there has been a substantial outbreak of the macaque malaria *P. knowlesi* in humans within Sabah province, Malaysian Borneo (Chapter 2) [6,24]. Most human cases have occurred within the Kudat District of Sabah. Here, the primary mosquito vector of *P. knowlesi* is *An. balabacensis,* a mosquito that can also transmit many other primate malarias including *P. coatneyi*, *P. cynomolgi*, *P. fieldi* and *P. inui* [50,194], and the human malarias *P. falciparum* and *P. vivax* [118,153]. This mosquito was incriminated as the *P. knowlesi* vector in studies using Human Landing Catches (HLC); a standard method used to sample Asian malaria vectors [18,50,59,112,194]. This technique has been used to sample *An. balabacensis* in the Kudat district, and elucidate its time of biting, malaria infection rate and seasonal dynamics [50,194]. Thus, our understanding of the role of *An. balabacensis* in *P. knowlesi* transmission is largely based on its predominance within samples of vectors attracted to humans. This may give a biased perspective of the range of vectors involved in maintaining transmission within the large non-human reservoir population.

Understanding malaria transmission dynamics in primate-only habitats is essential to understanding the stability of *P. knowlesi*. Here I tested a commercially available Mosquito Magnet Independence Trap (MMIT) to passively sample malaria vectors host seeking in the vicinity of long-tailed macaques within a large wildlife protected area in Malaysia. The MMIT lures mosquitoes using the combination of non-specific mammalian odour bait (CO2 and octenol), heat and water vapour [285,286]. Here traps were placed in the proximity of sleeping sites used by macaque troops within the Lower Kinabatangan Wildlife Sanctuary. This area has a large population of long-tailed macaques, which typically live in groups of 10 - 100 across a home range of 25 - 200 hectares [31]. Troops roost overnight in trees such as *Ficus* and *Colona* sp. near river banks [31]. *Ficus* (fig) trees also provide an important food source for macaques [287]. Relatively few studies have been published describing the use of Mosquito Magnet traps for malaria vectors [285,286,288–291], and the only one trial in Asia has been published [292]. These studies used the MMIT with the aim of sampling human malaria vectors, but here I test it for zoonotic malaria vectors for the first time.

Here I evaluated the MMIT for sampling mosquito vectors attracted to macaque populations within a protected area of secondary forest within Sabah where there are few humans and a high abundance of long-tailed macaques. This work was combined with sampling of macaque faeces to estimate the prevalence of malaria within host populations using a recently developed, non-invasive method [283,284]. These sampling methods were further used to investigate the transmission dynamics of *P. knowles*i and other primate malarias within macaque populations in undisturbed forest habitats. Specific goals were to 1) examine the abundance and diversity of potential vector species within macaque populations, 2) characterise environmental determinants of primate malaria vector abundance 3) measure the diversity and prevalence of primate malaria parasites in vectors and macaque faeces.

4.3 **Methods**

4.3.1 **Study site**

This study was conducted at the Danau Girang Field Centre located in Lot 6 of the Lower Kinabatangan Wildlife Sanctuary (LKWS), Sabah, Malaysian Borneo

(5°24'49.93" N, 118°02'18.58" E) (Fig. 4.1). The Lower Kinabatangan area was used for commercial logging from 1950s to 1987, which was followed by the conversion of 60000 hectares of land to palm and cocoa plantations [293]. In 2005, the Sabah State Government announced the protection of 26000 ha (ten lots, Fig. 4.1) of remaining forest areas in the Lower Kinabatangan to establish a corridor linking 15000 ha of virgin forest reserves [294]. Most forests in the Kinabatangan area have been logged at least once, thus the age of the secondary forest ranges from 10 to 60 years old [295]. The LKWS is a protected secondary disturbed forest area that contains a range of habitat types including primary to secondary lowland dipterocarp forest, floodplains, mangrove and oil palm plantations [295,296]. The sanctuary spans 400km^2, and hosts a wide diversity and abundance of wildlife including ten species of primates: long-tailed macaque (*Macaca fasicularis*), pig-tailed macaque (*Macaca nemestrina*), proboscis monkey (*Nasalis larvatus*), Bornean orangutan (*Pong pygmaeus morio*), red-leaf monkey (*Presbytis rubicunda*), Hose's langur (*Presbytis hosei*), white silvered langur (*Presbytis cristata*), Muller's Bornean gibbon (*Hylobates muelleri*), tarsier (*Tarsius bancanus*) and slow loris (*Nycticebus coucang*). A section of Sabah's largest river, the Kinabatangan (560km, Fig. 1), is located within the Sanctuary. Trees lining this river bank are a key habitat component for several primate species [31,297]. In 2002, an expedition estimated primate population sizes (per km^2) as 16.82 for *M. fasicularis* and 3.30 for *M. nemestrina* [31]. Preferred primate sleeping trees can be easily recognised by field staff and accessed from the river by boat.

4.3.2 **HLC vs MMIT trap comparison**

An initial trap evaluation study was performed to establish whether the Mosquito Magnet Independence Trap (Mosquito Magnet, model: MM3200, supplier: Syarikat Thiam Siong Sdn Bhd, Sabah) was capable of detecting *Anopheles* in the area (Fig. 4.2). As there is no existing reference method for sampling vectors attracted to macaques, this evaluation was based on comparisons with the standard HLC method which has proven efficient for sampling *An. balabacensis* in other parts of Malaysia [46,50,205]. The MMIT was modified before use to run off batteries rather than mains electricity, and on the gas locally available in Malaysia (LPG cooking gas tank, 30% propane and 70% butane). A combustion chamber converts this gas mixture to CO_2, heat and water vapour which is blown

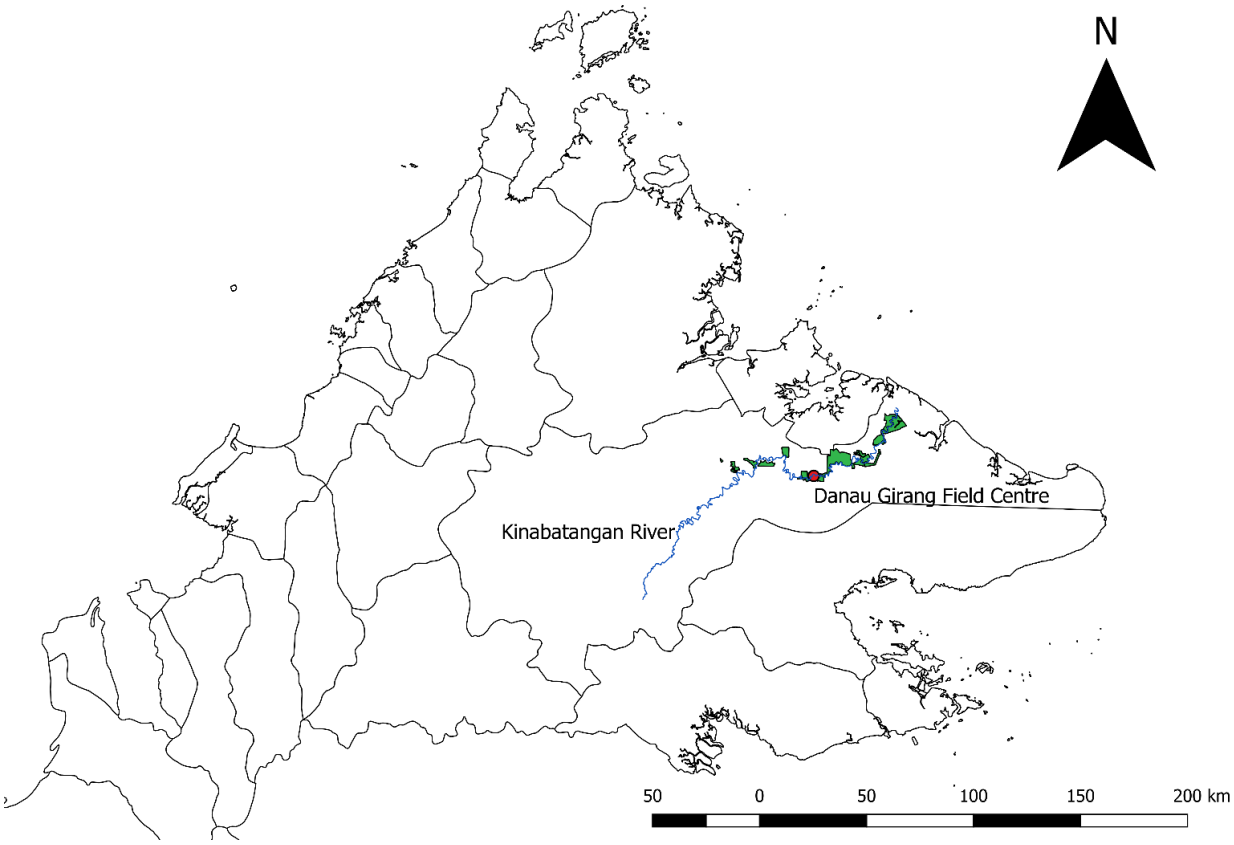

Figure 4.1 Map of Sabah indicating the location of the Danau Girang Field Centre (red) along the Kinabatangan river (blue). Green areas indicate boundaries of the Lower Kinabatangan Wildlife Sanctuary (Lots 1 - 10) and black lines show administrative districts.

Figure 4.2 A Mosquito Magnet Independence Trap (MMIT, left) and a view of the mosquito collection net within the MMIT (right).

out via a fan through an additional synthetic attractant called Lurex3. The active ingredient of Lurex3 is R-octenol, a compound found in mammalian and bovine breath and sweat which in addition to the CO_2, heat and water vapour, attracts mosquitoes to the trap [298–301]. When mosquitoes approach the attractive emissions, they are pulled inside by a reverse current and caught in a net.

Initial comparisons of the HLC and MMIT were conducted on three walking trails within the LKWS (Kingfisher, Ficus and Kayu Malam, Fig. 4.3) which were representative of different ecotypes (wet, wet and dry lowland forest, respectively) surrounding the main building of the research facility. Ten nights of simultaneous HLC and MMIT collections were performed. On each night, two sites were selected 100m apart on one of the three trails (Fig. 4.3). One site was allocated for HLC and the other for MMIT collection. On the following night, trapping methods were rotated between sites in a cross over design. Two rotations (four nights) were completed on the Kingfisher and Ficus trails and one rotation (two nights) on Kayu Malam. Hourly collections were conducted from 18:00 – 00:00 hrs each night to coincide with the peak biting time of *An. balabacensis* (18:00 - 20:00 hrs). One person performed the HLC by exposing the lower legs and trapping mosquitoes which landed to feed in 30 ml plastic specimen vials. They were accompanied by an assistant who noted the collection time on each vial. Each hour comprised 45 minutes of trapping followed by 15 minutes of break to provide a rest period for the individual performing HLC. During this time, the MMIT was switched off and the net removed, stored in a plastic Tupperware then replaced with a new one.

4.3.3 **Experimental design**

Mosquito sampling using MMIT was conducted to characterize the abundance and diversity of potential vector species within the reserve, and investigate the impact of macaque presence, abundance and environmental factors (temperature, rainfall) on the nightly abundance of potential vectors. A 20km stretch of the Kinabatangan River was selected as the study site. This transect included a range of potential microhabitats including various tree species such as *Colona*, Bonkol, Bayur, Tangat and *Ficus*. The 20km was divided into ten 2km transects with the Danau Girang Field Centre (DGFC) situated approximately in the middle (Fig. 4.4). A minimum of 20 m of riparian forest was on either side of

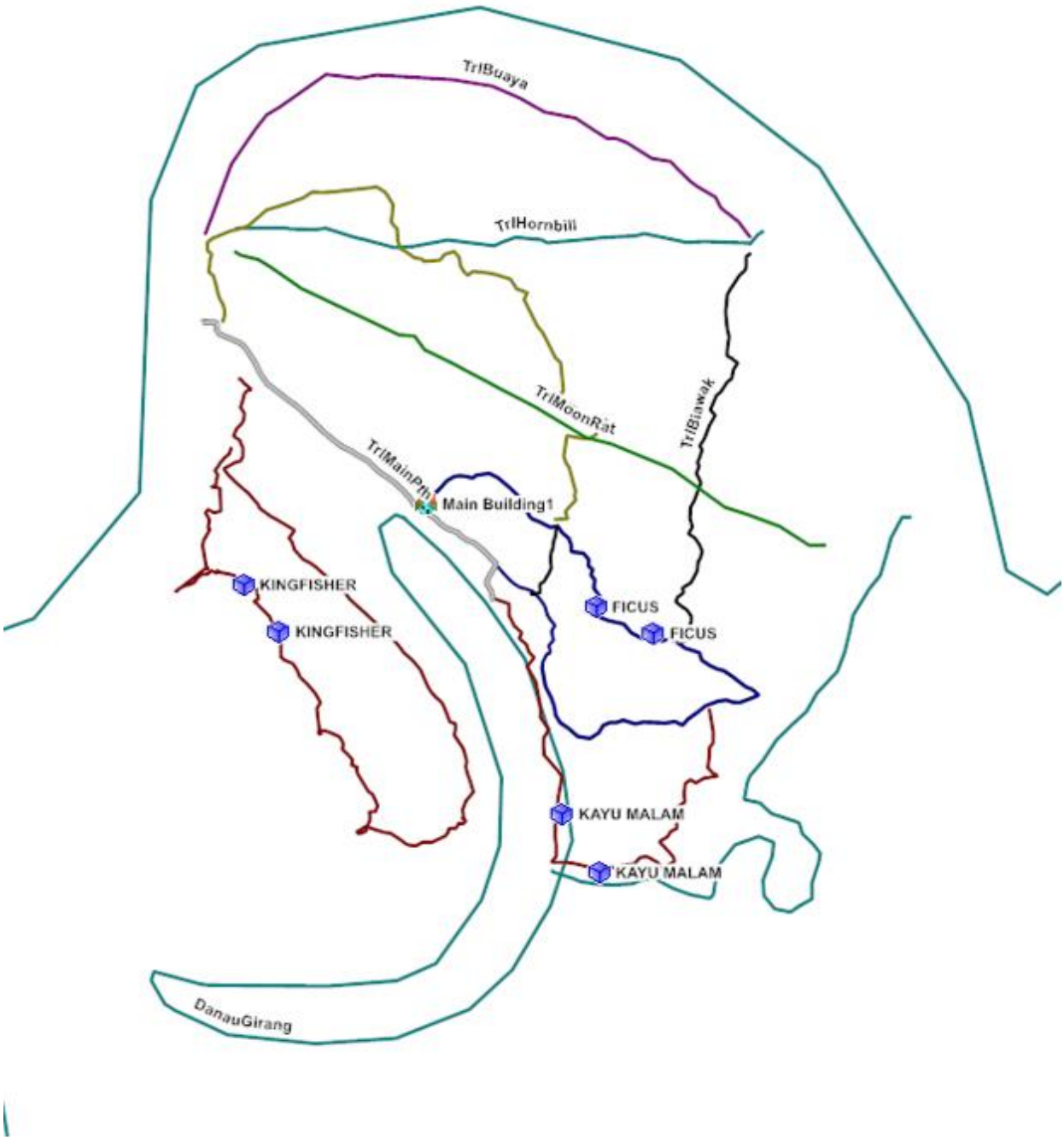

Figure 4.3 Map of forest trails surrounding the main building of Danau Girang Field Centre in the Lower Kinabatangan Wildlife Sanctuary. Boxes indicate the sites used on Ficus (wet lowland forest), Kingfisher (wet lowland forest) and Kayu Malam (dry lowland forest) for human-landing catch (HLC) and Mosquito Magnet Independence Trap (MMIT) evaluation of collecting *Anopheles*. Blue lines depict bodies of water.

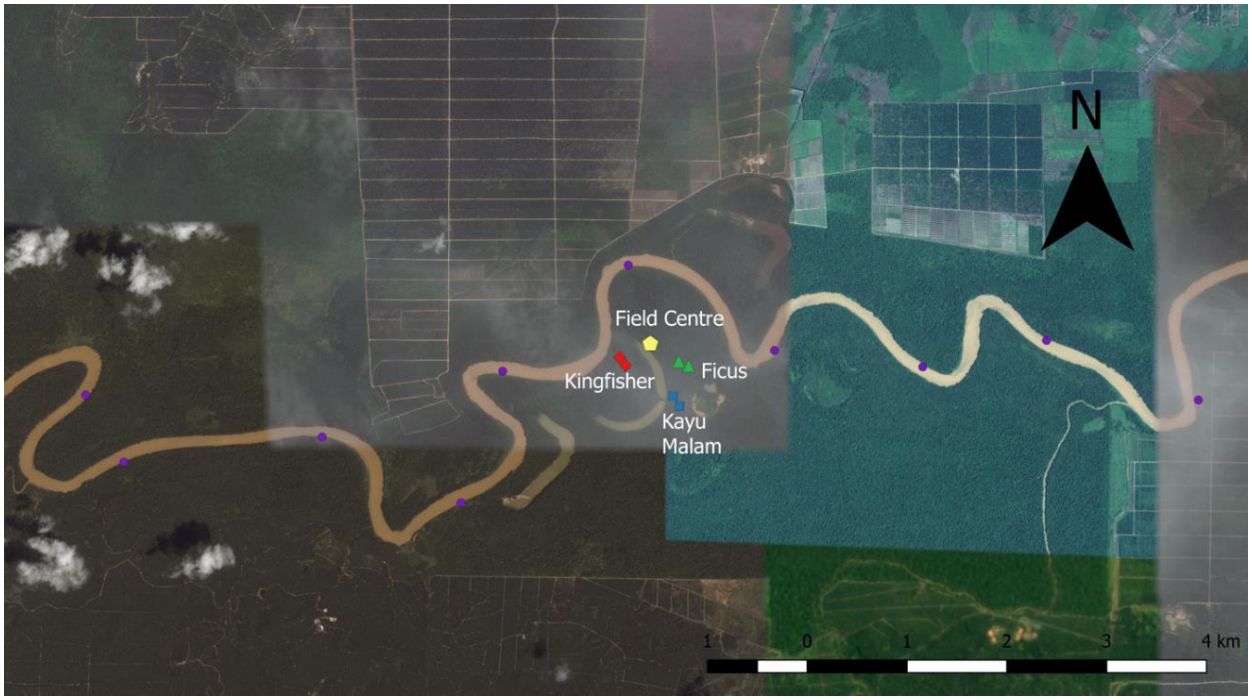

Figure 4.4 The 20km stretch of the Kinabatangan River surrounding Danau Girang Field Centre where macaque roosting sites and control trees were selected for mosquito collection. Purple dots indicate the boundary of each 2km transect that could be randomly selected for mosquito sampling on each night.

the river [302] and the nearest human settlement was at least 15 km downstream from DGFC. The home range of long-tailed macaques in this reserve was estimated as 1.25km^2 in a previous survey [31]. Therefore sampling mosquitoes within different 2km transects each night was considered appropriate to avoid repeated sampling near the same macaque troop. Each transect was visited once every ten nights and was selected by random number generation using the android app: Random UX. Sampling was conducted in blocks of five nights with one night break culminating in a total of 38 sampling nights between September to November of 2017. Thus each block was sampled 3 - 4 times, once every 10 days, with traps placed on alternate sides of the river on each visit.

4.3.4 **MMIT to sample *Anopheles* host seeking near macaques**

On each night of sampling, two mosquito magnet independence traps (MMIT) were used to collect host seeking adult mosquitoes. One trap was positioned at a tree identified as having sleeping macaques and another at an uninhabited tree acting as a 'control' to differentiate mosquitoes specifically attracted to macaques.

We arrived at the selected transect by boat at 17:30 hrs each day. A thermal imaging camera was used to scan river banks to identify potential macaque troops by driving slowly up and down the river. Previous use of thermal imaging systems for wildlife studies have conducted aerial surveys [303–305], therefore this was the first to apply a hand-held technique operating at ground level. When the camera indicated presence of a troop, trees were inspected using binoculars to determine if they were long-tailed macaques (*Macaca fasicularis*). If confirmed, a MMIT was placed near the bottom of the sleeping tree if the bank was accessible (steady incline from the river, level area to position the trap and penetrable vegetation). Macaques would generally move from the selected tree to higher up in the canopy or deeper inside the forest as the boat approached but return after the trap was placed. The position of the MMIT was recorded, and a Tiny Tag data logger (Gemini UK) was fixed to the trap base for hourly temperature recording (Fig. 4.5). Due to the high density of macaques in the LKWS, there was no occasion where groups were not detected within the sampling transect. A tree of similar structure and species, but uninhabited by macaques and other primates, was selected as the control site each night. The

Figure 4.5 The Mosquito Magnet Independence Trap (MMIT) in position on the river bank at the base of a *Ficus* (fig) tree to be used by a long-tailed macaque troop as their overnight resting place.

distance between the test and control MMIT was at least 100m. MMITs were in place and operating by 18:00 hrs each night.

Mosquitoes were collected at macaque sleeping and control sites each night from 18:00 – 06:00 hrs. Before sunrise and movement of macaques from the sleeping site (~ 05:30 hrs), the number of macaques sleeping in the tree where the MMIT was placed were counted using the thermal camera. Daily rainfall data (recorded from a rain gauge) during the study period was provided by DGFC.

4.3.5 **Mosquito processing**

In the trap evaluation study, mosquitoes caught in the HLC were trapped in 30ml plastic screw-top vials. In the MMIT, mosquitoes were trapped in nets which were then were placed into plastic Tupperware containers. On return to the field laboratory, collection vials and boxes containing the MMIT nets were stored at -20˚C for ~12 hrs to kill mosquitoes. Specimens were then morphologically identified to genera and where possible, species using Rattanarithikul *et al* (2005) [175-178]. *Anopheles* belonging to the Leucosphyrus group were further identified using the Sallum *et al* (2005) Revision of the Leucosphyrus group of *Anopheles* key [306]. All identified mosquitoes were stored in 95% ethanol in 1.5ml eppendorfs. *Anopheles* were screened for *Plasmodium* infections as described in Chapter 2.

4.3.6 **Macaque Faecal collection**

On each morning following mosquito collections, trap sites were visited at 06:00 hrs. After emptying traps, the ground within a 20m radius of sleeping trees was inspected for the presence of fresh macaque stools. Here two people conducted a twenty-minute search of the forest floor and canopy (within reach). Efforts were made to ensure each faecal sample corresponded to a different individual based on variation in the colour, consistency and distance of sample from other faeces. As many samples as possible were taken within these limitations, however there were some cases where no faeces could be found. Wearing gloves and a N95 disposable respirator face-mask, a 2 cm^3 faecal sample was deposited into a 50 ml falcon tube and sealed in a zip-lock plastic bag. Tubes were then immediately transported to the laboratory and filled with RNAlater solution. The

sample was homogenized immediately using a sterile chopstick until completely broken. Tubes were then stored at -20˚C until further processing.

DNA was extracted from 200 µl of each macaque stool solution using the QIAamp DNA Stool Mini Kit following the manufacturer's instructions. DNA was eluted in 100 µl buffer AE and stored at -20 ˚C until further processing. Nested PCRs were conducted to screen samples for *Plasmodium* DNA firstly using the method of Siregar [283], which identifies DNA of any species within the *Plasmodium* genus. 2µl of genomic DNA was subjected to an outer amplification reaction with 0.4 µM of each of the SSU-rRNA *Plasmodium* genus specific primers PfF4595 (GATTACAGCTCCCAAGCAAAC) and PfR5019 (GTTTAGCCAGGAAGTCAGCGTC), 100 µM dNTPs, and 0.5 U Phusion High Fidelity DNA polymerase (New England Biolabs M0530) with 1 x High Fidelity buffer in a total volume of 25µl. The nested reaction was identical except that only 1µl PCR product from nest 1 was used for nest 2. PCR conditions were: initial denaturation at 94 ^{o}C for 5 min; followed by 30 cycles of 94 ^{o}C for 15 seconds, 60 ^{o}C annealing for 15 seconds and 72 ^{o}C for 45 seconds; and a final extension at 72 ^{o}C for 5 minutes. 1µl of PCR product from nest 1 was used for nest 2. Nest 2 was an exact replicate of nest 1 but with 16.5 µl dH_2O. PCR products were run on 3 % agarose gel in 0.5x TAE buffer. Samples with a 424 bp band were positive for *Plasmodium* genus.

After initial screening for Plasmodium, all positive samples went through another round of PCR to test for the specific presence of *P. knowlesi* following the method of Kawai *et al* [284]. Here 2µl of genomic DNA was subjected to an outer amplification reaction with 0.5 µM of each of the cytochrome b *P. knowlesi* primers PCBF (ATGCTTTATTATGGATTGGATGTC) and PCBRed (ACATAATTATAACCTTACGGTCTG), 100 µM dNTPs, and 0.5 U Phusion High Fidelity DNA polymerase (New England Biolabs M0530) with 1 x High Fidelity buffer in a total volume of 25µl. The nested reaction was identical except for substitution of the primers with PkCBF (TATTCTTCTTTAGTGGATTATTTA) and PkCBRed (GTATTGTTCTAATCAGTGTA), and the use of 1 µl of outer PCR product instead of genomic DNA. PCR conditions were initial denaturation at 98 ^{o}C for 1 min; followed by 35 cycles of 98 ^{o}C for 10 seconds, 50 ^{o}C annealing for 30 seconds and 72 ^{o}C for 30 seconds; and a final extension at 72 ^{o}C for 5 minutes.

PCR products were run on 3 % agarose gel in 0.5x TAE buffer. A 131bp band indicated that the sample was positive for *P. knowlesi* DNA.

4.3.7 **Statistical analysis**

Data were analysed using the R statistical programming software, version 3.4.2 with the packages lme4 and multcomp. Generalized Linear Mixed Models (GLMMs) were used to compare the sampling ability of the two trapping techniques (HLC and MMIT) for mosquitoes and *Anopheles* in particular. These Generalised Linear Mixed Models (GLMMs) were constructed with a negative binomial distribution to account for overdispersion in mosquito count data [307]. The response variable was the mean abundance of mosquitoes in general or just *Anopheles* per night or hour. The main fixed effect of interest was trap type with random effects fit for date and trail (eg. Kayu Malam, Ficus or Kingfisher). A post hoc Tukeys' test was used to assess differences in mosquito abundances between traps.

Sampling of mosquitoes near trees where macaques were sleeping was conducted for 38 nights. On each night an unoccupied control tree was selected, but on a few occasions, macaques or other primates were present at the control site in the mornings or the traps stopped working overnight due to failure of gas supply or batteries. Excluding these scenarios, data was available from 33 nights of sampling at control trees and 34 nights at trees with sleeping macaques (Table 4.2). With this data, GLMMs were constructed to test for differences in the response variables of 1) *Anopheles* abundance 2) *An. balabacensis* abundance and 3) *An. donaldi* abundance between macaque sleeping sites and control trees. A negative binomial distribution was used with date and river transect set as random effects. Models tested for associations between mosquito abundance and macaque presence and abundance, and rainfall on the day of sampling. The significance of each variable was tested by backward elimination using likelihood ratio tests. Post-hoc Tukey's tests were performed to assess differences in mosquito abundance between sleeping sites and control collections.

On nine sampling nights, the datalogger failed to record the average nightly temperature. Thus a subset of data (28 nights at control trees and 29 nights at

sleeping sites) for which mean nightly temperatures were available was used to test for associations between temperature and mosquito abundance. Here the impact of temperature on 3 different groups of mosquito data was investigated: *Anopheles* only, *An. balabcensis* and *An. donaldi* abundances. Negative binomial GLMMs were constructed with the presence/absence of macaques, number of macaques present, mean nightly temperature and daily rainfall were fixed effects, and date and river transect set as random effects. The significance of each variable was tested by backward elimination using likelihood ratio tests. Post-hoc Tukey's tests were performed to assess differences in mosquito abundance between sleeping sites and control collections.

4.3.8 **Ethics**

This project was approved by the College of Medical, Veterinary and Life Sciences Ethics Committee at the University of Glasgow (Application number: 200160160) and the Medical Ethics Committee of Universiti Malaysia Sabah (Application number: JKEtika 1/16 (3)).

4.4 **Results**

4.4.1 **HLC vs MMIT trap comparison**

Both HLC and MMIT collected mosquitoes belonging to the same eight genera (Table 4.1). In general, more *Aedes*, *Anopheles*, *Culex* and *Verrallina* were collected with MMIT than with HLC. The only genus where more mosquitoes were collected with HLC than with MMIT was *Mansonia*. All mosquitoes were identified to species level where possible, however due to time constraints, the genera with medically important species detected were prioritized: *Anopheles*, *Culex* and *Mansonia*. *Aedes* and *Uranotaenia* mosquitoes were mostly identified to subgenus. The *Aedes* genus contains vectors of dengue (*Ae. albopictus* and *Ae. aegypti*) that are usually detected in mosquito collections in Sabah however the subgenus detected in this area were not of medically important species. *Coquillettidia*, *Orthopodomyia* and *Verrallina* mosquitoes were only classified to genus. In general, mosquitoes trapped by HLC were in better condition for morphological identification than those trapped in the MMIT nets because key characteristics necessary for species determination such as hairs and scales were

better preserved. The *Aedes*, *Anopheles*, *Culex, Mansonia* and *Uranotaenia* specimens that could not be assigned to at least a subgenus represented 5.3 % for HLC and 12.0 % for MMIT. Only 0.3 % of the total catch for HLC and 0.6 % for MMIT were not in a suitable condition for placing to a genus.

With respect to potential malaria vectors, almost all *Anopheles* caught in the HLC could be identified to species, except for one individual that was missing features to distinguish between *An. barbirostris* or *An. donaldi*. Two *Anopheles* from MMIT collections (3.2 % of total caught by MMIT) could not be placed to a subgenus. Five different *Anopheles* species were collected by HLC compared to 8 species with MMIT (Table 4.1). Both methods trapped the known *P. knowlesi* malaria vector, *An. balabacensis,* and *An. donaldi*; with a higher proportion of these species in the *Anopheles* caught by HLC (80.5 %, n = 29) than MMIT (72.6 %, n = 45).

Although there was a tendency towards higher numbers of mosquitoes in MMIT than HLC collections, overall there was no statistically significant difference in the mean abundance caught per night (Tukey's test: *P* = 0.39, Figure 4.6A). Similarly, the mean number of *Anopheles* per night was not significantly different between trap types (Tukey's test: *P* = 0.210, Figure 4.6B). There was no significant difference in the mean number of mosquitoes (*P* =0.212, Figure 4.7A) or *Anopheles* (*P* = 0.299, Figure 4.7B) caught per hour between HLC and MMIT trapping methods.

Anopheles balabacensis and *An. donaldi* were caught in all hours between 18:00 hrs and 23:00 hrs (Fig.4.8) with most activity for *An. donaldi* noted in the first two hours of sampling (18:00 – 20:00).

4.4.2 **MMIT to sample *Anopheles* host seeking near macaques**

Overall mosquitoes from eight genera were collected (Table 4.2). *Mansonia* made up over half of mosquito collections, followed by *Culex* 25 %, *Verrallina* 10 %, *Anopheles* 4 %, *Aedes* 3 %, *Uranotaenia* 1 % and *Coquillettidia* 0.5 %. A total of 9 *Anopheles* species were collected: 125 individuals from 6 species near macaque sleeping sites, and 218 individuals from five species at control trees. *Anopheles epiroticus*, *An. gigas* and *An. tesselatus* were caught only at sleeping

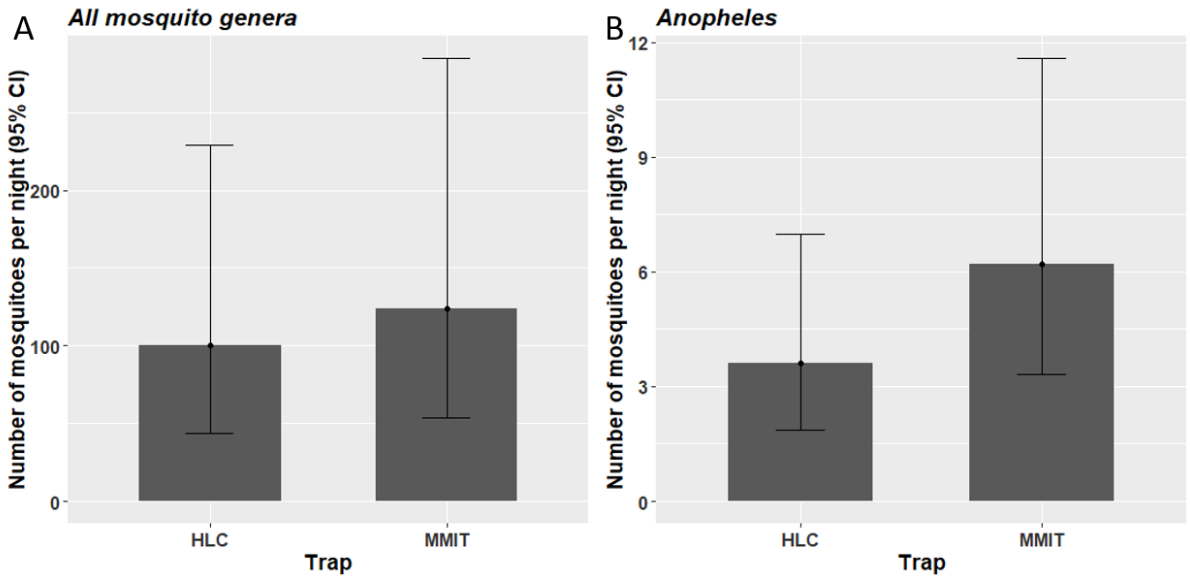

Figure 4.6 Mean abundance of A) all mosquito genera and B) all *Anopheles* caught per night by Human landing catch (HLC) and Mosquito Magnet Independence Trap (MMIT) as predicted by negative binomial generalised linear mixed models (GLMM). Error bars represent 95% confidence intervals.

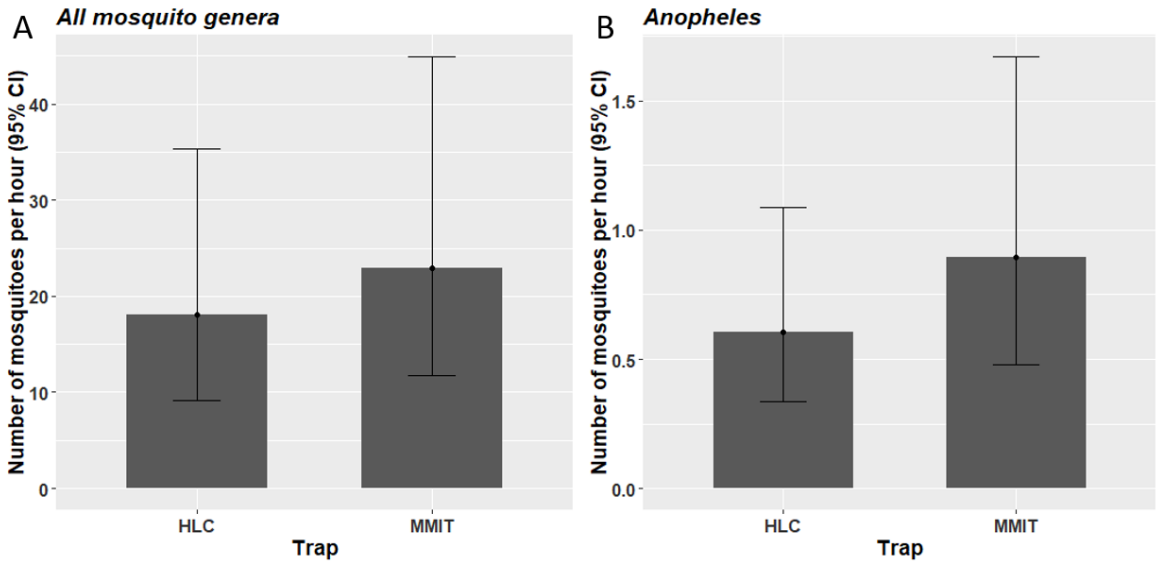

Figure 4.7 Mean abundance of A) all mosquito genera and B) all *Anopheles* caught per hour by Human landing catch (HLC) and Mosquito Magnet Independence Trap (MMIT) as predicted by negative binomial generalised linear mixed models (GLMM). Error bars represent 95% confidence intervals.

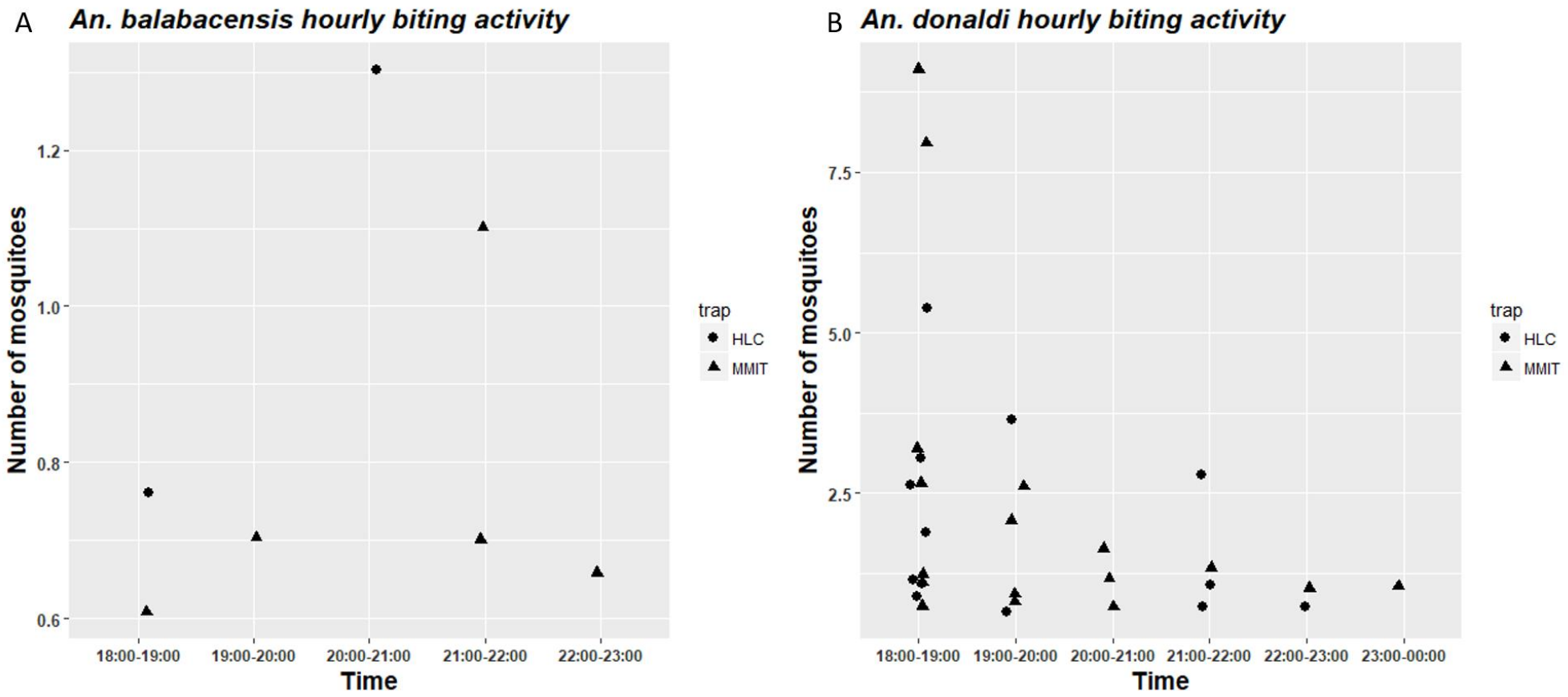

Figure 4.8 A) *An. balabacensis* and B) *An. donaldi* trapped per hour by human-landing catch (HLC) and Mosquito Magnet Independence Trap (MMIT).

Table 4.1 Mosquitoes caught by Mosquito Magnet Independence Trap (MMIT) and human-landing catch (HLC) over ten nights of trap comparison study in Lower Kinabatangan Wildlife Sanctuary, Sabah.

Mosquito genus/ subgenus/ species	Human-landing catch (HLC)	Mosquito Magnet Independence Trap (MMIT)
Aedes	**87**	**151**
Aedimorphus	2	8
Am. Caecus	2	2
Ayurakitia	0	1
Downsiomyia	1	0
Edwardsaedes	3	1
Finlaya	2	12
Paraedes	67	81
Pr. Ostentato	10	0
Ochlerotatus	3	10
Unknown *Aedes* spp.	9	38
Anopheles	**36**	**62**
An. balabacensis	2	5
An. barbirostris	0	2
An. barbirostris/donaldi	1	5
An. barbumbrosus	0	2
An. cellia subgenus	0	1
An. donaldi	27	40
An. kochi	0	1
An. montanus	2	1
An. roperi	1	1
An. tesselatus	3	2
Unknown *Anopheles* spp.	0	2
Coquillettidia	**2**	**23**
Culex	**278**	**532**
Cx. brevipalpis/phangngae	1	0
Cx. foliates	21	5
Cx. fuscocephala	13	13
Cx. gelidus	1	6
Cx. hutchisoni	25	37
Cx. malayi	3	0
Cx. perplexus/Cx. whitei	0	2
Cx. pseudosiniensis	9	5
Cx. quinquefasciatus	8	4
Cx. siniensis	1	2
Cx. sitiens	5	1
Cx. tenuipalpis sub	47	97
Cx. vishnui/ pseudovishnui	31	179
Cx. whitmorei	78	30
Unknown *Culex* spp.	35	151

Table 4.1 continued on next page

Table 4.1 continued. Mosquitoes caught by Mosquito Magnet Independence Trap (MMIT) and human-landing catch (HLC) over ten nights of trap comparison study in Lower Kinabatangan Wildlife Sanctuary, Sabah.

Mosquito genus/ subgenus/ species	Human-landing catch (HLC)	Mosquito Magnet Independence Trap (MMIT)
Mansonia	**396**	**300**
Ma. annulate	122	98
Ma. annulifera	3	3
Ma. bonnae	13	3
Ma. dives	10	15
Ma. dives/ bonnae	105	63
Ma. Indiana	86	63
Ma. uniformis	48	29
Unknown Mansonia spp.	9	26
Orthopodomyia	**3**	**3**
Uranotaenia	**4**	**9**
Ur. Longirostris	0	2
Ur. species 3	1	2
Unknown *Uranotaenia* spp.	3	5
Verrallina	**237**	**746**
Unknown genera	3	11
Total	**1049**	**1846**

Table 4.2 Mosquitoes caught with Mosquito Magnet Independence Trap (MMIT) at trees with and without sleeping macaques (control trees) within the Lower Kinabatangan Wildlife Sanctuary, Sabah.

Mosquito genus/ subgenus/ species	Macaque sleeping sites (34 nights)	Control trees (33 nights)
Aedes	**193**	**169**
Ae. laniger	1	0
Aedimorphus	0	1
Ayurakitia	0	1
Edwardsaedes	29	1
Finlaya	1	0
Paraedes	142	130
Ochlerotatus	15	22
Scutomyia albolineata	1	0
Stegomyia	0	1
Unknown *Aedes* spp.	4	13
Anopheles	**144**	**265**
An. balabacensis	13	2
Barbirostris gp	122	251
An. barbirostris	0	2
An. donaldi	106	211
An. epiroticus	1	0
An. gigas	1	0
An. kochi	0	0
An. montanus	2	2
An. roperi	0	1
An. tesselatus	2	0
An. umbrosus gp	1	2
Unknown *Anopheles* spp.	2	5
Coquillettidia	**37**	**23**
Coq. Nigrosignata	19	18
Unknown *Coquillettidia* spp.	18	5
Culex	**1581**	**1213**
Cx. baileyi	0	1
Cx. brevipalpis/phangngae	23	2
Cx. cinctellus	21	13
Cx. foliatus	4	3
Cx. fuscocephala	0	1
Cx. gelidus	42	27
Cx. hutchisoni	399	350
Cx. infantulus	0	1
Cx. infula	1	8
Cx. mammifer/ wilfredi	2	26
Cx. nigropunctatus	189	132
Cx. pseudosinensis	43	21
Cx. pseudovishnui/vishnui	277	209
Cx. quinquefasciatus	2	1
Cx. scanloni	1	0
Cx. siniensis	5	1

Table 4.2 continued on next page

Table 4.2 continued. Mosquitoes caught with Mosquito Magnet Independence Trap (MMIT) at trees with and without sleeping macaques (control trees) within the Lower Kinabatangan Wildlife Sanctuary, Sabah.

Mosquito genus/ subgenus/ species	Macaque sleeping sites	Control trees
Cx. sitiens	24	16
Cx. tenuipalpis	277	198
Cx. whitmorei	133	53
Cx. whitmorei/gelidus	0	3
Cx. whitei	0	1
Unknown *Culex* spp.	138	146
Mansonia	**3151**	**3036**
Ma. annulate	468	467
Ma. annulifera	254	265
Ma. bonnae	220	153
Ma. dives	695	416
Ma. dives/bonnae	186	232
Ma. Indiana	300	588
Ma. uniformis	191	148
Unknown Mansonia spp.	837	767
Orthopodomyia	**2**	**4**
Uranotaenia	**48**	**63**
Uranotaenia	11	19
Pseudoficalbia	30	33
Unknown *Uranotaenia* spp.	7	11
Verrallina	**631**	**523**
Total	**5787**	**5296**

sites, whereas *An. barbirostris* and *An. roperi* were only caught at control trees (Table 4.2). Both malaria vector species, *An. balabacensis* and *An. donaldi* were trapped at sleeping sites and control trees.

One *An. barbirostris*, a known vector of human malaria in Sri Lanka, Bangladesh, Timor Leste and Thailand [41,308], was caught at a control tree however it is unknown whether this species plays a role as a malaria vector in Borneo.

Combining over all species in the genera, the mean abundance of *Anopheles* mosquitoes caught per night was not dependent on the presence ($X^2 = 0.45$, *df* = 1, *P* = 0.50) or number of macaques ($X^2 = 0.62$, *df* = 1, *P* = 0.43), or daily rainfall ($X^2 = 0.67$, *df* = 1, *P* = 0.41) (Fig. 4.9A, B and C). Based on the subset of data for which full temperature recordings were available, a strong positive association was detected between mean nightly *Anopheles* abundance and temperature ($X^2 = 6.46$, *df* = 1, *P* = 0.01, Fig. 4.10).

The primate malaria vector *An. balabacensis*, however, was significantly impacted by the presence of macaques at sampling sites ($X^2 = 8.25$, *df* = 1, *P* < 0.01). The mean abundance of *An. balabacensis* was significantly higher near macaque roost sites than at control trees (Tukey: *P* = 0.02, Fig. 4.11A). *Anopheles balabacensis* abundance did not vary with the number of macaques present ($X^2 = 1.28$, *df* = 1, *P* = 0.26) or daily rainfall ($X^2 = 0.42$, *df* = 1, *P* = 0.52, Fig. 4.11 B and C). Analysis of the subset of data for which full temperature recordings were available indicated that the abundance of *An. balabacensis* was not strongly associated with mean nightly temperature ($X^2 = 3.75$, *df* = 1, *P* = 0.05, Fig. 4.12).

Fewer of the human malaria vector *An. donaldi* were caught at macaque sleeping sites (n = 106) than control trees (n = 211, Table 4.2). This difference in mean abundance between macaque sleeping sites and control sites was only marginally significant (Tukeys: *P* = 0.052). After accounting for other sources of variation, the abundance of *An. donaldi* was not dependent on the presence or absence of macaques ($X^2 = 0.92$, *df* = 1, *P* = 0.34) (Fig. 4.13A). The abundance of *An. donaldi* was unrelated to the number of macaques present ($X^2 = 0.13$, *df* = 1, *P* = 0.72) or the daily rainfall ($X^2 = 0.23$, *df* = 1, *P* = 0.63) (Fig. 4.13B and C). Based on the subset of data for which full temperature recordings were

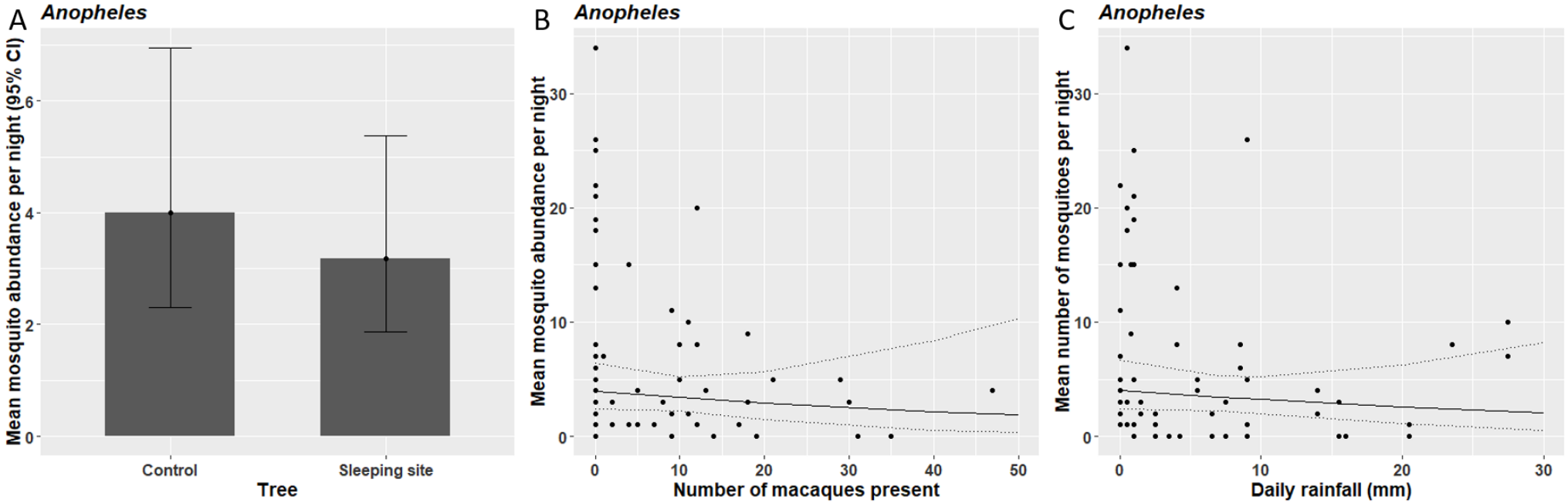

Figure 4.9 Predicted relationship between the mean nightly abundance of *Anopheles* mosquitoes caught in MMIT collections and A) macaque presence/absence at sampling trees, B) number of macaques present at a tree and C) daily rainfall. Points indicate observed data in panels B and C, with the line indicating the predicted association. Error bars and dashed lines are 95% confidence intervals.

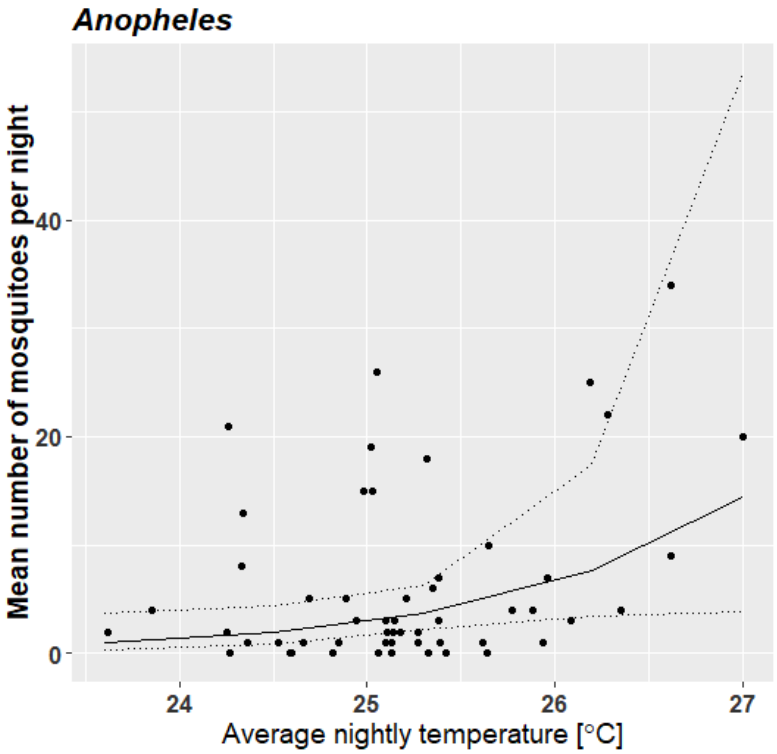

Figure 4.10 Predicted relationship between mean *Anopheles* abundance collected by Mosquito Magnet Independence Traps (MMIT) and average nightly temperature (from the subset of 28 sampling nights at control trees and 29 sampling nights at macaque sleeping sites for which environmental data were available). Points indicate observed data, with the line indicating the predicted association. Dashed lines represent upper and lower 95% confidence intervals.

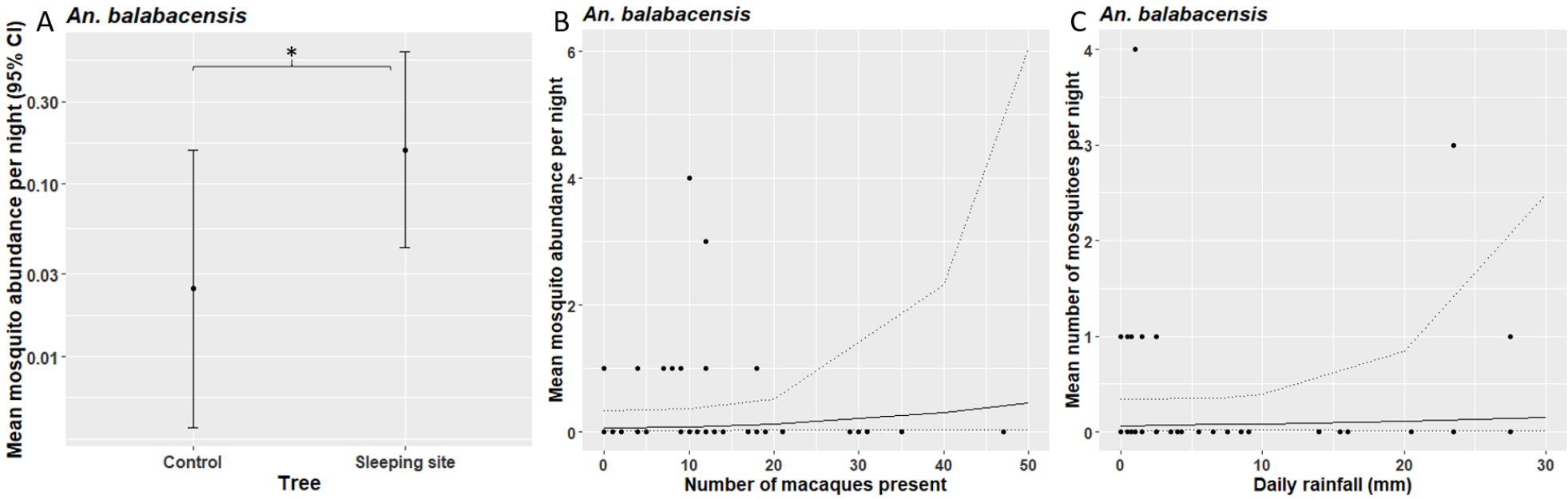

Figure 4.11 Influence of A) macaque presence/absence, B) number of macaques present and C) daily rainfall on the mean nightly *An. balabacensis* abundance collected by Mosquito Magnet Independence Traps (MMIT). Points indicate observed data in B and C, with the line indicating the predicted association. Error bars and dashed lines are 95% confidence intervals and * represents $P < 0.05$.

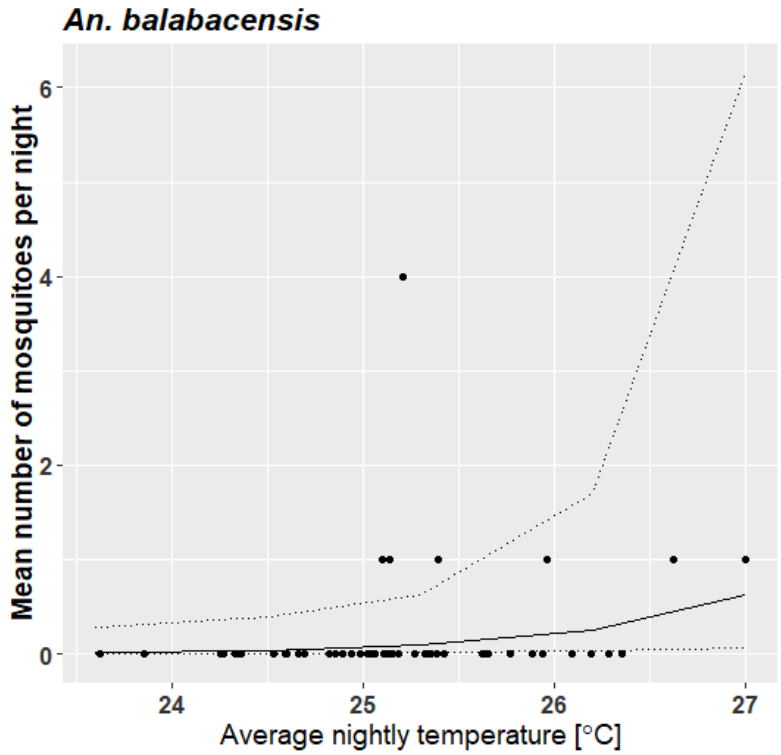

Figure 4.12 Predicted relationship between mean *An. balabacensis* abundance collected by Mosquito Magnet Independence Traps (MMIT) and average nightly temperature from a subset of 28 sampling nights at control trees and 29 sampling nights at macaque sleeping sites for which environmental data were available. Points indicate observed data, with the line indicating the predicted association. Dashed lines represent upper and lower 95% confidence intervals.

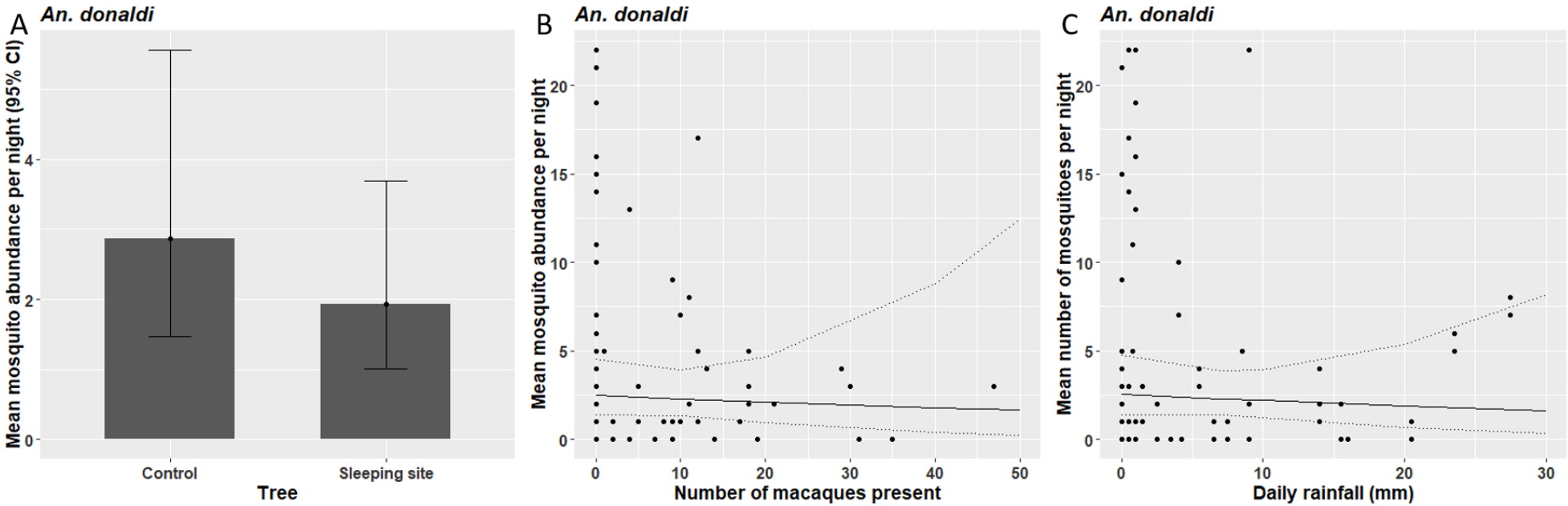

Figure 4.13 Influence of A) macaque presence/absence, B) number of macaques present and C) daily rainfall on the mean nightly *An. donaldi* abundance collected by Mosquito Magnet Independence Traps (MMIT). Predicted mean *An. donaldi* abundance based on data from sampling 33 nights at control trees and 34 nights at macaque sleeping sites. Points indicate observed data in B and C, with the line indicating the predicted association. Error bars and dashed lines are 95% confidence intervals.

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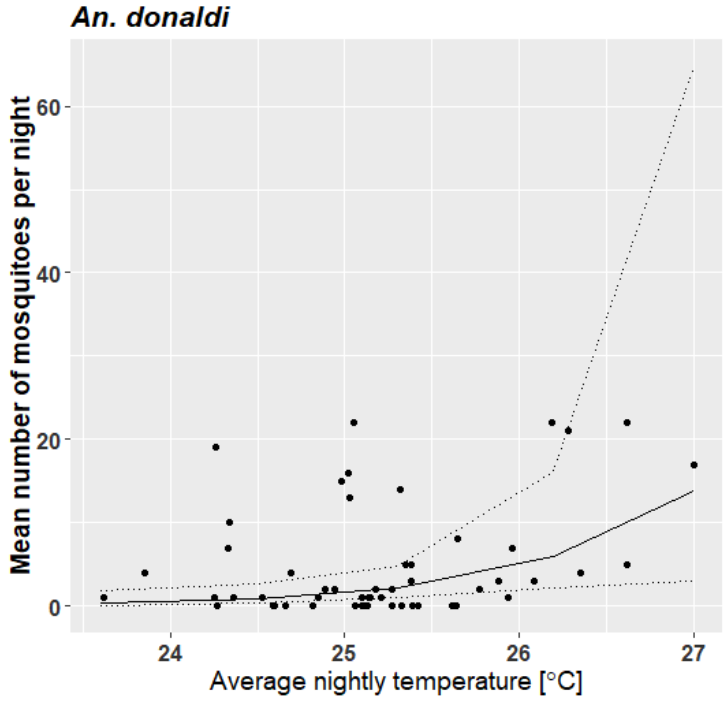

Figure 4.14 Predicted relationship between mean *An. donaldi* abundance collected by Mosquito Magnet Independence Traps (MMIT) and average nightly temperature from a subset of 28 sampling nights at control trees and 29 sampling nights at macaque sleeping sites for which environmental data were available. Points indicate observed data, with the line indicating the predicted association. Dashed lines represent upper and lower 95% confidence intervals.

available, the abundance of *An. donaldi* was positively associated with mean daily temperature (X^2 = 5.86, *df* = 1, *P* = 0.02, Fig. 4.14

4.4.3 *Plasmodium* infections in mosquitoes and macaque stools

Eighty-one *Anopheles* collected in the initial HLC versus MMIT trap comparison were tested for malaria (*An. donaldi* = 61, *An. balabacensis* = 7, *An. barbirostris/donaldi* = 5, *An. tesselatus* = 5, An. celia gp = 1, *An.* unknown = 2). Of these, one tested positive for *Plasmodium* infection (n = 1/81). From the larger study of mosquito abundance at macaque sleeping sites and control trees, 398 *Anopheles* were tested for malaria (Barbirostris gp = 373 (including *An. barbirostris* (2) and *An. donaldi* (317)), *An. balabacensis* = 15, *An. epiroticus* = 1, *An. tesselatus* = 2 and unidenitifed *Anopheles* species = 7, Table 2). Of these, one tested positive for *Plasmodium* (n = 1/398). The *Plasmodium* infections were identified to species-level by subsequent primate-species specific PCR. Both were *P. inui* infections (Fig. 4.15) found in *An. balabacensis* (one trapped by HLC and the other caught at a control tree by MMIT), representing an overall infection rate of n = 2/22 in this vector species. No *P. knowlesi* infected mosquitoes were found in either component of this study.

Of the 46 long-tailed macaque faecal samples collected, 17 (37%) tested positive for *Plasmodium* (Fig. 4.16). However in the subsequent round of PCR analysis to test for *P. knowlesi*, none were positive. Samples were not screened for other specific malaria species in this analysis, thus the identity of *Plasmodium* infections remains unknown.

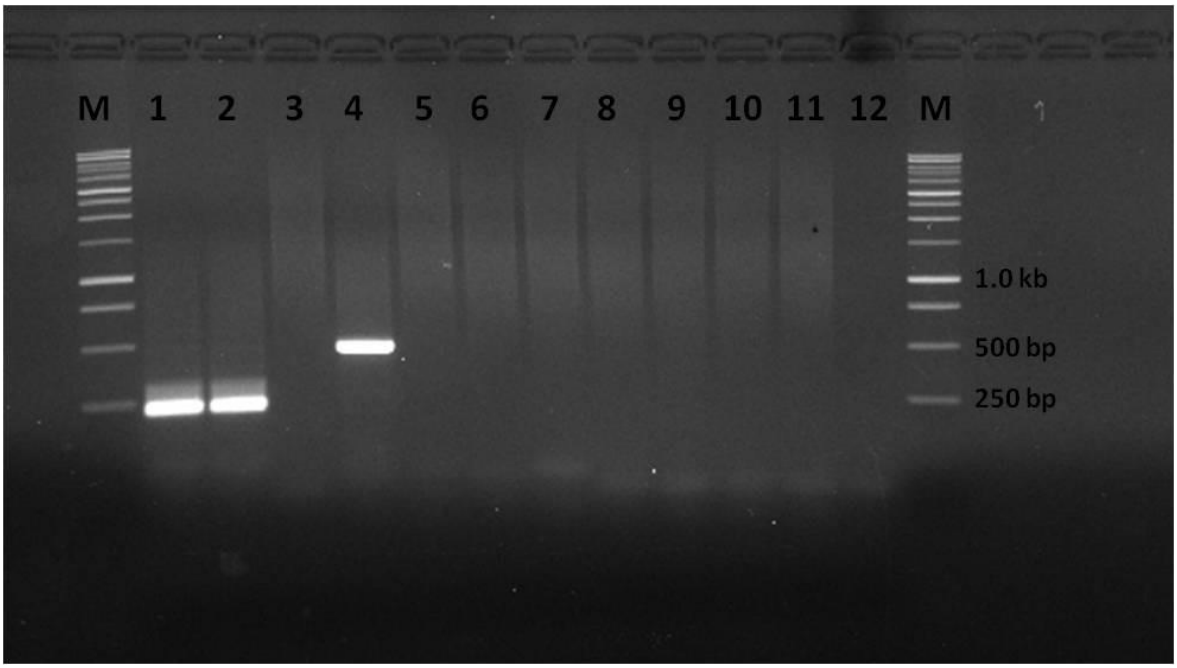

Figure 4.15 Example gel electrophoresis of PCR products from *Plasmodium* screening of *An. balabacensis* specimen. Lane 1 = *P. knowlesi* positive control (*Plasmodium* genus PCR), lane 2 = *An. balabacensis* specimen (*Plasmodium* genus PCR), lane 3 - 11 = *An. balabacensis* specimen (*Plasmodium* species PCR) for *P. coatneyi, P. inui*, *P. fieldi*, *P. cynomolgi*, *P. knowlesi*, *P. falciparum, P. vivax, P. malariae* and *P. ovale*, lane 12 = Negative control.

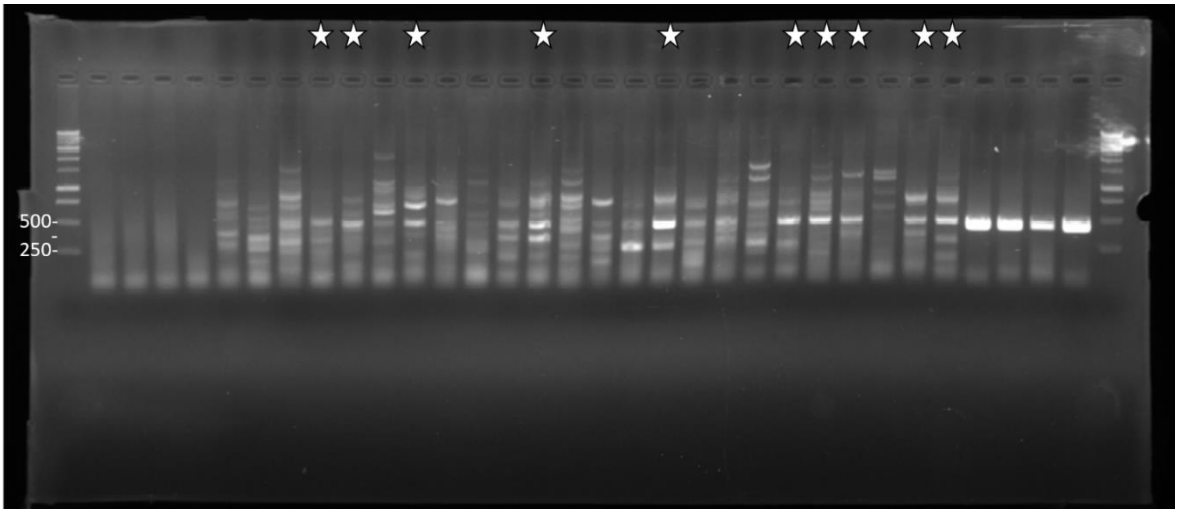

Figure 4.16 Example gel electrophoresis of PCR products from *Plasmodium* screening of macaque faecal samples. Lane 1 = PCR negative control, lanes 2-4 = extraction negative controls, lanes 5 – 28 = macaque faecal samples, lanes 29 – 32 = PCR *Plasmodium* positive control. Stars indicate *Plasmodium* positive faecal samples.

4.5 **Discussion**

This study represents the first evaluation of the use of MMIT for mosquito vectors of primate malaria. In an initial step, this tool was shown to be capable of detecting a similar range and abundance of mosquito species and *Anopheles* as the HLC which was previously demonstrated as the most effective collection method in Sabah [112]. Through using the MMIT in the vicinity of trees where macaques roosted; several potential monkey and human malaria vector species were identified (*An. balabacensis*, *An. donaldi* and *An. barbirostris*). While *Anopheles* density was not higher overall at trees with than without macaques; the abundance of the confirmed primate vector, *An. balabacensis*, was significantly increased near sleeping sites. This is an indication of *An. balabacensis* actively host seeking on macaques. Although macaque density was very high and analysis of their faecal samples indicated significant rates of *Plasmodium* infection; no *P. knowlesi* was detected in either vector or macaque samples here. The only malaria detected was *P. inui*; another known primate parasite that has not yet been documented in humans. Therefore it should not be assumed that all macaque populations are infected with *P. knowlesi* and present a risk of zoonotic transmission in Sabah.

Overall MMIT collections were comparable to HLC in total mosquito abundance and in the range of genera caught. With respect to *Anopheles*, MMIT and HLC collected similar abundances but some differences arose in the species composition between trapping methods, where MMIT caught 8 species and HLC caught only 5. The MMIT is more likely to trap generalist rather than host-specific mosquito species due to the non-specific mammalian R-octenol bait. The concentration of R-octenol emitted and how this equates to that which is released from one mammalian host is unknown. It may be that the concentration of R-octenol emitted by the MMIT is much higher than a single host thus attracts a wider range of species than the scent of one human in the HLC. There may be some additional 'human-specific' odour cues available in HLC not produced by the MMIT, which lure more anthropophilic species. In this case there was only one individual performing HLC and because volatile emissions from humans vary within the population [309], a fairer test would be to incorporate additional people to carry out collections. Specific mosquito species are known to be sensitive to plume structures of carbon dioxide emissions from Mosquito Magnet traps [310]

however in this case, there were no *Anopheles* species trapped by HLC that were not found in the MMIT. With the advantages of not requiring a real host and the ability to operate in a passive collection style, the MMIT proved to be as reliable as HLC in collecting host seeking *Anopheles,* including specific malaria vector species.

In addition to *Anopheles*, the MMIT demonstrated ability to trap other mosquito species of medical importance including vectors of Japanese encephalitis ([176]*;* *Mansonia* spp), and filariasis (e.g *Cx. quinquefasciatus and Cx. sitiens*[177]. *Mansonia uniformis, Ma. annulifera, Ma. dives, Ma. bonnae* and *Ma. indiana* are known vectors of the filarial worm *Brugia malayi* in Malaysia where leaf-monkeys (*Presbytis* spp) have been identified as reservoirs of infection [150]. No *Aedes* vectors of arboviruses were trapped despite confirmation of sylvatic populations of the dengue vector *Ae. albopictus* in other secondary forest areas in Sabah (Chapter 2, [121]). The MMIT is a less invasive method of mosquito sampling, with fewer ethical implications than putting monkeys in cages as bait and has shown to be effective for examining multiple vector groups host seeking near macaques.

Following confirmation of the MMIT's sampling ability, it was used to characterize the community of mosquitoes host seeking near macaque troops sleeping in trees. Paired collections were also made at similar but unoccupied "control trees" to help distinguish mosquitoes specifically attracted to macaques from the general host seeking population. The primary *P. knowlesi* vector species in Sabah, *An. balabacensis* [50], was detected in low densities in this study, but occurred at significantly higher densities near trees with than without sleeping macaques. The increased abundance of *An. balabacensis* near macaques indicates a specific propensity to feed on this host type. Host choice and preference may be impacted for *An. balabacensis* with the availability of human hosts; as indicated in village settings in Sabah where higher abundances were found biting humans than monkeys [112,153]. A host choice experiment conducted in the Cambodian forest also found at ground level *An. balabacensis* were more attracted to men (n =203) compared to monkeys (n = 12), whereas high in the canopy in the absence of men, a large proportion of *An. balabacensis* were trapped (n = 295) [311]. Here I have shown that in a relatively undisturbed forest

with a high density of monkeys, *An. balabacensis* were significantly attracted to feed on macaques. However, this feeding behaviour is likely to vary in different ecological settings depending on the availability of other host species.

The abundance of *An. balabacensis* was significantly associated with macaque presence, but not the overall number sleeping near the collection point. The size of macaque troops varied across sampling nights over a range of 2 – 47 individuals (average ~14 macaques), thus incorporating a substantial variability for testing an association with mosquito density. The lack of association between vector abundance and macaques may be the result of the odour plume of even one macaque being sufficient to lure *An. balabacensis* with no additional response with more hosts. Alternatively, it could be that the trees selected by macaques are themselves an attractive site for mosquitoes. Groups of long-tailed macaques are known to revisit trees used for sleeping [31], possibly due to favourable characteristics such as an abundance of fruits, clear view of predators and comfortable wide branches for sleeping. Macaque odour cues may build up around repeatedly used trees and signal a reliable location for vectors to find a bloodmeal. Additionally, there could be additional environmental characteristics not measured here that contributed to higher *An. balabacensis* abundances at macaque sleeping sites. For example, this study did not assess the availability of suitable larval habitats and as yet, the resting behaviour for this species is unknown. Other studies examining the influence of host density on malaria vector density indicate little correlation between adult Anopheline density and the density of cows or humans [312]. The potential for transmission of primate malaria will therefore be high within groups of macaques if the abundance of *An. balabacensis* does not depend on the number of macaques present.

Temperature and rainfall have been widely demonstrated to influence mosquito vector abundance [313]. Here, the average nightly temperature had a significant influence on the number of mosquitoes trapped by the MMIT. Temperature is known to impact several mosquito development and parasite fitness traits [36,104,242,245,314,315]. Here, higher daily temperatures were associated with an increase in *Anopheles* and *An. donaldi* abundances. Higher temperatures may have caused odour plumes from MMITs to disperse further or could reflect

increased mosquito flight activity on warmer nights. No effect of temperature on *An. balabacensis* was detected likely due to the small sample size for this malaria vector species. Here, daily rainfall did not impact primate malaria species abundance which also may be because of the low statistical power in this study. Investigations have found a negative correlation with mosquito abundance and rainfall on the day of sampling because mosquitoes are less likely to fly in heavy downpours [316]. Whilst higher mosquito densities are usually found with a time-lag after heavy rainfall, due to the generation of novel larval habitats [316-318], this could not be investigated here as it was a short study. To investigate seasonal fluctuations in vector abundance with rainfall, sampling across a full season would be required. A prior study sampling over a one year period in Sabah indicated no clear seasonal patterns in *An. balabacensis* abundance in the forest [50]. At this stage, further investigation including a longer sampling period is required to establish how vector abundance fluctuates seasonally and how this impacts malaria risk to forest dwelling macaques.

The original aim of this study was to investigate the transmission of *P. knowlesi* within its primary macaque reservoir in undisturbed forest. Despite the presence of known mosquito vectors around macaque sleeping sites, no *P. knowlesi* infection was found in either mosquitoes or macaque faecal samples. Only two malaria infected mosquitoes were found in the study; an *An. balabacensis* trapped in an MMIT (control tree) and another in an HLC which both tested positive for *P. inui*. *Plasmodium inui* is a common primate malaria species found in macaques [255]. This indicates there was active transmission of monkey malaria in the area, but contrary to expectation from other work in Sabah, there was no *P. knowlesi* [50,319]. *Plasmodium inui* can be experimentally transferred to people through blood transfusion and by mosquitoes in the lab [115] however it is unknown whether humans can be infected naturally. Relatively high rates of *P. inui* and other primate malaria species (e.g. *P. cynomolgi, P. fieldi and P. coatneyi*) have been detected in *An. balabacensis* within village settings where *P. knowlesi* human cases were reported in Sabah [194]. Thus *P. inui* infected mosquitoes are relatively widespread and can be present at relatively high prevalence in peri-domestic as well as forest settings. Thus *P. inui* could post a significant risk for zoonotic spillover if it becomes adapted to infecting people naturally.

A high proportion of malaria infections (37%, n = 17/46) were detected in PCR screening of fresh macaque stools. However these samples tested negative for *P. knowlesi* in a further analysis with a parasite specific probe. Unfortunately it was not possible conduct further analyses to confirm which malaria parasite species was infecting these macaques. Given its presence in mosquitoes, we hypothesise that the infection could be *P. inui*. Previously, the prevalence of *P. knowlesi* within macaque populations has been reported as 6.9 % and 30 % in Peninsular Malaysia [45,264], and 20 % and 86.6 % in Sarawak [225,320]. These studies identified infections from screening of macaque blood samples which may have greater sensitivity to low density infections than the faecal screening method used here [321]. However, similarly low prevalences of *P. knowlesi* in macaques was found in other studies (e.g. 0.4 % *P. knowlesi* prevalence [322]); indicating macaque infection rates are heterogeneous. *Plasmodium knowlesi* parasites may just be absent from the macaque population in the LKWS perhaps due to population isolation from other infected macaque groups in Sabah. It was assumed that the force *of P. knowlesi* infection coming from macaques in Sabah was high because out of nine wild long-tailed macaques sampled in Kudat, a hotspot of human infection in 2013 - 2014, eight were blood positive for *Plasmodium* with six confirmed as being *P. knowlesi* (Salgado-Lynn, personal communication). Thus there are likely differences in the species of *Plasmodium* infecting macaques across different populations and regions in Sabah. Macaques in general may not be an overall risk factor for *P. knowlesi* transmission as there could be significant heterogeneity in the malaria parasite community. In light of this, recent analyses generating *P. knowlesi* risk maps [89,204,323] based on macaque distribution may have reduced accuracy because of failure to incorporate underlying variation in infection prevalence within macaque populations.

The absence of *P. knowlesi* was the most unexpected result of this study. I can speculate that the absence of *P. knowlesi* in the vector population and macaque samples could be due to the relatively low abundance of *An. balabacensis* in the LKWS compared to other studies. Here the mean abundance of *An. balabacensis* was 0.02 – 0.17 per night; in contrast to nightly rates of 6.8 – 8.8 reported elsewhere [50]. In contrast to studies in other parts of Sabah where *An. balabacensis* was the dominant *Anopheles* species [50,194], here *An. donaldi* was the most prevalent (77.5% of *Anopheles*). Compared to *An. balabacensis*, *An.*

donaldi is thought to be a less efficient vector of malaria [62]. *Anopheles donaldi* can be experimentally infected with *P. falciparum* and *P. vivax* [324] in the lab and has been found with sporozoites morphologically similar to human malaria in nature (although the exact parasite species was not identified [221]). It is unknown what role it plays, if any, in primate malaria transmission in Sabah [221]. In experimental infections, *An. donaldi* developed oocyst stage infections of the monkey parasites *P. cynomolgi* and *P. fieldi* [28], but monitoring was not continued to confirm whether development continued to transmission-stage sporozoites. *Anopheles donaldi* is zoophilic [221,325] with a preference for bovine over human bloodmeals [64]. This relatively generalist behaviour is backed up by the observation that the abundance of this species was not higher at either macaque or control trees and may account for lack of infection. Alternatively, regardless of vector behaviour *P. knowlesi* may simply be absent or in low prevalence in the reservoir population at LKWS.

This study has also given insights into mosquito diversity, ecology and vector-borne disease risk within undisturbed forest areas of Sabah as compared to sites of human disturbance. I note a similar range of mosquito genera were collected here as found in and around villages in other parts of Sabah; particularly Ranau district (Chapter 2). However some of the most abundant vector species caught in LKWS (*Ma. dives*, *Ma. annulata* and *Ma. indiana)* were less dominant in domestic, forest or agricultural settings near villages. Other vector species of zoonotic pathogens including Japanese encephalitis and filariasis were also trapped. This study was the first to report *An. epiroticus*, *An. gigas*, *An. montanus* and *An. roperi* in Sabah. These species may have been missed in previous studies because most of them are based on sampling in and around human settlements; with the diverse ecotypes and multitude of blood-meal wildlife species found at LKWS likely sustaining a different range of mosquitoes. These findings reflect the value of wider ecological sampling of mosquitoes in a range of ecological settings to fully assess the potential for spillover of vector-borne zoonotic diseases.

To conclude, by performing mosquito collections in an area hosting a high frequency of long-tailed macaque groups, this study has demonstrated the suitability of MMIT for catching vectors of primate malaria and other zoonotic

pathogens nearby macaque sleeping sites. The MMIT performed well in comparison to the gold standard HLC technique that is most commonly used to investigate the ecology and behaviour of Asian Anophelines and here we propose it as a reliable alternative to studying these mosquitoes with the advantages of not requiring users to be exposed to infectious bites, or holding macaques captive to act as baits. The primary *P. knowlesi* vector in Sabah, *An. balabacensis,* was found at relatively low density in this study area but was significantly associated with macaque sleeping sites indicating a preference for these hosts. Absence of *P. knowlesi* from mosquitoes and macaques suggests that not all macaque groups in Sabah can be assumed to be infected and pose an infection risk to people.

5 General discussion

5.1 Principal findings

The significant human outbreak of the zoonotic malaria *P. knowlesi* that started in Malaysian Borneo ~ 2004 sparked the research conducted in this thesis. While early work in Malaysia focussed on detection and characterization of clinical infections in humans [6,8,24,73,85,96,239,326,327], there was relatively little understanding of the ecology and drivers of transmission in this area. In particular, there were substantial knowledge gaps regarding the mosquito vectors responsible for transmission, and how changes in land use occurring throughout the region would impact their ability to infect humans. In the absence of an effective vaccine, vector control is the only effective way to reduce malaria transmission. Consequently detailed understanding of the ecology and behaviour of *P. knowlesi* vectors is crucial for both predicting human exposure risk and planning control strategies. To address these knowledge gaps, a large interdisciplinary research programme on *P. knowlesi* emergence in Malaysia (Monkeybar) was initiated in 2012 with the main goals of defining the biomedical, environmental and social risk factors for human infection. Monkeybar incorporated a substantial work package on entomology with the aim of improving on the understanding about *P. knowlesi* vector ecology and surveillance methods. Research in this thesis was designed to complement and expand upon initial findings of the Monkeybar programme by filling in additional gaps related to vector surveillance methods, testing hypotheses about the effect of land-use on human exposure risk, and elucidating the force of transmission coming from the macaque reservoir. These topics were investigated across three independent yet interlinked field studies conducted between 2015 – 2017 in the state of Sabah, Malaysian Borneo. Key findings from these studies are summarized briefly below.

5.1.1 Resting bucket traps are an effective means of sampling non-malaria vector species

Vector surveillance requires monitoring of both host seeking and resting mosquito populations. Sampling of resting mosquitoes in particular provides important information about vector habitat and host preferences. I focussed on

evaluating simple, low cost, flexible traps for sampling *P. knowlesi* vectors in a range of habitats representing a gradient of deforestation. This study confirmed the challenging nature of sampling resting malaria vectors, particularly in tropical forested regions of Southeast Asia. Of the more than 2000 mosquitoes that were collected, only one was an *Anopheles* and it was not a malaria vector species. However, these resting traps proved effective for sampling a number of other locally important mosquito vector species including those implicated in dengue and filariasis transmission. In particular, I showed these vectors can be found in a range of domestic, agricultural and forest settings; but are particularly abundant resting underneath homes in Sabah, which are typically built on stilts. This highlights the potential value of improving the impact of vector control programmes by extending the spraying of residual insecticides to cover the area underneath as well as inside of houses.

5.1.2 Vector density and habitat use across Sabah is not accurately predicted from pilot studies in Kudat

Subsequently, larger-scale sampling of host-seeking mosquitoes was conducted across 4 districts and 11 villages in Sabah with the aim of testing associations between habitat and *P. knowlesi* vector abundance. Pilot studies conducted in a small number of sites in Kudat district indicated that contrary to expectation, *P. knowlesi* vectors may be at higher abundance in village than farming or forested settings. However this inference was based on study of only three sites in one district of Sabah. I conducted entomological sampling over a large geographic scale, incorporating substantial habitat replication, to test this hypothesis. I found that the distribution and abundance of the confirmed *P. knowlesi* vector, *An. balabacensis*, was highly variable between villages and districts. Overall, this vector species was not the dominant member of the anopheline community as was observed in pilot work within Kudat district. The findings of this study reject the original hypothesis by showing that *An. balabacensis* density was significantly higher in forest and farm habitats compared to village settings. Furthermore, the mean abundance of *An. balabacensis* across all 4 districts in Sabah was considerably lower than found around the epicentre of human cases in Kudat. This highlights the risk of over extrapolating results from small studies to larger geographical areas and the crucial value of replication in studies of vector ecology. Although the abundance of *An. balabacensis* was lower in village than

forest settings, it was routinely found in the peri-domestic area around homes. This suggests that the original hypothesis that *P. knowlesi* malaria is only a risk to humans when they are deep in the forest is not true.

5.1.3 Vector abundance and human *P. knowlesi* infection risk

In collaboration with Monkeybar, it was possible to investigate entomological indicators of human infection risk by using data on human *P. knowlesi* exposure gathered in a large cross-sectional survey. This type of large-scale epidemiological data is difficult to obtain, requiring a large amount of funds and personnel. These challenges are even more pronounced for *P. knowlesi* because it's relatively low prevalence in humans means a large number of people need to be tested to detect infection. Given the demands of large-scale epidemiological sampling, it would be of great value to have robust entomological indicators of human infection risk. Here I investigated whether the general abundance of *Anopheles*, and/or the Leucosphyrus group in particular was predictive of *P. knowlesi* seropositivity rates in people at the village level. Unfortunately, no significant association was detected between village-level vector abundance and human *P. knowlesi* sero-positivity rates. Despite the lack of significant association in this study, it would be premature to dismiss the existence of entomological correlates of *P. knowlesi* risk. This study had several limitations which could have confounded or obscured potential relationships between vector abundance and human infection risk. First, the low abundance of vectors and small number of sites meant there was low power to detect an effect. Second, there was a six-month lag between human survey and entomological sampling. Third, ~ 40 % of *P. knowlesi* respondents may have been missed in the sero-prevalence assay. This study can provide useful pilot data on mosquito vector abundance and infection rates, which could be used to guide the design or larger-scale epidemiological studies based on simultaneous sampling of vector and human populations, over more villages and trapping nights.

5.1.4 MMIT is a good method for non-invasive sampling of vectors host seeking on primates in the forest

Until now, investigation of *P. knowlesi* vectors has largely focussed on those with potential to transmit parasites between monkeys and humans; based on sampling

in areas where humans and monkeys co-exist. The third study in this thesis aimed to characterise *P. knowlesi* transmission within macaque populations, with the aim of testing whether the vectors responsible for transmission to humans also mediate transmission between monkeys. This required finding a way to sample mosquitoes biting macaques. Previous work attempted to do this using monkey-baited traps or electrocuting nets, but both of these methods require the capture and handling of wild primates, had moderate performance and were logistically challenging. I aimed to find a less-invasive method for assessing mosquitoes attracted to wild macaque populations, that could reliably detect malaria vector species. For this purpose, I evaluated the use of Mosquito Magnet Independence Traps (MMIT) as a tool for collecting malaria vectors host seeking near macaque sleeping sites. The MMIT performed well relative to the human-landing catch, the best reference method of collecting host seeking *P. knowlesi* vectors. Both methods collected *An. balabacensis* and other malaria vector species. Additionally, the MMIT proved suitable for sampling over medically important vectors species such as Japanese encephalitis and filariasis vectors. Overall the study demonstrated the suitability of the MMIT for non-invasive mosquito sampling of multiple vector groups host-seeking near macaques.

5.1.5 Malaria risk from macaque populations: heterogeneity in *P. knowlesi* infections

I conducted the first investigation of *P. knowlesi* transmission in a wild macaque population in Sabah. This was done by sampling vectors and macaques in a protected area inhabited by a large population of long-tailed macaques, and no humans other than a small number of research staff. The primary *P. knowlesi* vector *An. balabacensis* was found host seeking near macaque roosts in this setting, but at relatively low density compared to areas of disturbed forest in Kudat District, Sabah. Contrary to expectations, no *P. knowlesi* infections were found in vectors in this forest. Furthermore, indirect estimation of *Plasmodium* infection rates from faecal samples indicated malaria prevalence in macaques was relatively high (~37%), but none of these infections were identified as being *P. knowlesi*. The most likely cause of infection was *P. inui,* another primate malaria that was found in vectors. This highlights that there is heterogeneity in *P. knowlesi* infections across macaque populations in different regions in Sabah, and that not all are sources of infection. The large focus of human *P. knowlesi*

malaria occurring around Kudat may therefore be due to a uniquely high prevalence within the local macaque population. Some evidence for this was obtained in the Monkeybar project, where six of the nine macaques trapped in the Kudat area tested positive for *P. knowlesi*. Together, these findings indicate that not all macaque populations across Sabah pose a *P. knowelsi* risk to humans and that outbreaks of human infection are likely dependent on *P. knowlesi* prevalence in the local macaque population.

5.1.6 Malaria risk from macaque populations: other species posing a threat to humans

Plasmodium knowlesi was absent from long-tailed macaques and vectors within the protected area. However, *Plasmodium* infection was commonly found in macaque stool samples (~37%), and another primate malaria species, *P. inui,* was found in vectors. *Plasmodium knowlesi* therefore is not the only parasite species posing a risk to humans. Currently it is unknown if *P. inui* can infect man under natural conditions, but as it appears to be circulating at high frequency in macaques and has been detected in vectors biting in human settlements, there is a high risk of spillover into humans if the parasite adapts to natural transmission. This study has therefore highlighted the need for awareness and surveillance of additional emerging zoonotic malaria species in Sabah.

5.2 Limitations of the study

This PhD study has advanced methods for vector surveillance, identified key relationships between habitat and *P. knowlesi* vector abundance, vector abundance and *P. knowlesi* infection at the community-level, and elucidated aspects of malaria transmission in macaque populations in Sabah. These will contribute to improved understanding of zoonotic malaria transmission, but there were several notable limitations of the study which could have impacted results.

5.2.1 Evaluation of resting traps for collecting vectors of *P. knowlesi*

First, the conclusion that simple resting traps are not effective for sampling malaria vectors in Sabah was based on a relatively short study of only 2 months,

at 2 sites. Some vector control activities (spraying) occurred during the study period which may have impacted mosquito numbers. Additionally it is possible that sampling took place during a relatively low density season for mosquitoes. Little is known about seasonality in *P. knowlesi* vector populations, but it is a characteristic feature of most tropical anophelines. Consequently, greater success may have been obtained from longer term evaluation of traps over ~ 1 year to allow for seasonal fluctuations in mosquito abundance. Additionally, this study did not investigate all of the techniques described for collecting resting mosquitoes, including those such as pit traps or clay pots which have proved effective in African settings [163,165,328–331].

A significant limitation to this study was that during the fourth week of collections, the two study villages were visited by government personnel for the bi-annual spraying of the insecticide, lamda-cythalothrin[332], as part of a vector-borne disease control programme. Insecticide was sprayed underneath, on the outside walls of houses and on vegetation surrounding the home. No insecticide was sprayed in any of the other habitats selected for resting mosquito collections. Whilst this presented a major obstacle, the study was performed to completion, continuing the sampling for the full two months.

A further limitation of this study was the relatively low success in successfully amplifying DNA from bloodmeals in mosquitoes caught in resting collections. Bloodmeal identification is a standard technique for estimating host choice in mosquitoes [63]. This identification can be done using PCR-based methods that amplify DNA from blood in the mosquitoes that have recently fed (~24 - 48 hours). DNA amplification rates from bloodmeals can be as high as 81 % [333], but in this study were only 30 %. This was likely due to poor quality of host DNA in mosquito bloodmeals; either because it had degraded prior to storage or had been partially digested by the mosquito. In future, this event could be mitigated by performing more frequent collections from the resting traps, such as 2-3 times per night, coupled by immediate preservation of bloodmeals in the field.

5.2.2 Investigating associations between vector habitat and human *P. knowlesi* exposure risk over a wide geographic range

The relationship between vector abundance and human *P. knowlesi* exposure was investigated in the second study however no significant association was detected. Collections were performed six months after human sampling, thus may not have been reflective of the true picture of vector populations at the time of human survey. Further to this, *P. knowlesi* prevalence in humans was not based on detection of active infection at the time of sampling, but of indication of prior exposure through sero-positivity.

Current understanding is that there is only a short window of detection for antibodies to *P. knowlesi* (within 1 month of infection) and the sero-prevalence assay only detects ~ 60 % of infections. Thus our measure of *P. knowlesi* infection in humans was not very sensitive, and introduced a further time lag between the epidemiological data and the timing of mosquito collections. An improved design would be simultaneous collection of entomological sampling and epidemiological data, and more sensitive estimates of exposure in humans. Gathering of longer term data over a wide geographic area would also strengthen the power of the study but would require a significantly larger team and resources.

5.2.3 Understanding dynamics of transmission in macaque populations

Identification of *Plasmodium* infections in macaques in the third study could have been enhanced by screening blood instead of stool samples. Previous investigations of malaria in long-tailed macaques in Malaysia have been based on analysis of blood samples [45,225,264,320], which is known to be more sensitive than based on faecal samples [283]. The Siregar *et al* [283] method used here yielded several PCR products as indicated by the high number of bands on some gels. This made it difficult to clearly define *Plasmodium* positive and negative individuals. The non-specific banding was likely due to the higher rate of DNA degradation in stool samples. Analysis based on blood samples would have likely have been more precise and yield higher estimates of prevalence, but would have required capture and tranquilisation of macaques, necessitating veterinary

staff and limiting the number of animals that could be sampled at one time. Despite the poorer specificity, faecal sampling offered a less invasive method of sampling multiple individuals.

An additional limitation to this study was that it was only performed with one macaque population, thus the findings would be strengthened by replication in other macaque populations in other areas. Furthermore, it is unknown if other monkey species are contributing to zoonotic malaria transmission in Sabah. This study may have been limited by restricting analysis to long-tailed macaque populations, and future work should examine pig-tailed macaques and leaf-monkey populations to understand the complete picture.

5.3 General implications for understanding emergence and control of zoonotic malaria

The total body of work presented here has several implications for understanding the emergence and spread of *P. knowlesi* in Malaysian Borneo, and the approaches that could be taken to control it. The key implications arising from this PhD study are described below.

5.3.1 Human exposure to *P. knowlesi*

The work provides a deeper understanding of where and when humans are most likely to be exposed to *P. knowlesi*. The trapping work performed in peri-domestic, farm and forest habitats across a wide geographical area in Sabah indicated that *P. knowlesi* vectors are at highest abundance in farm and forest habitats, but also present to a limited degree in peri-domestic settings. The chance of receiving a bite from a *P. knowlesi* vector was similar in farm and forest settings, indicating that these mosquitoes can thrive in human-altered habitats. Significant changes to *P. knowlesi* vector ecology have therefore occurred since early studies in the 1970s which concluded vectors do not migrate out of the forest. Despite occurring at lower abundance, *P. knowlesi* vectors were also found biting people around the home, stressing that peri-domestic transmission is also possible. These results add to the growing body of evidence that transmission to humans is not restricted to forests. To investigate how the findings from this study are applicable across the whole of Sabah and beyond,

future work should include entomological surveillance in different land-use types with broad spatial replication.

5.3.2 Control of *P. knowlesi* transmission

Classical methods of vector control exploit key aspects of mosquito vector behaviour [114]. For example, Indoor Residual Spraying relies on mosquitoes choosing to rest inside houses, and insecticide treated bed nets rely on vectors biting indoors during sleeping hours. These are the main components of vector control in Malaysia [1]. The research was not successful in identifying where *P. knowlesi* vectors rest but confirmed they actively host seek in in peri-domestic, farm and forest settings. An option for preventing transmission to humans around houses could be the use of outdoor spatial repellents which act to create a 'safe-space', clearing an area of host seeking vectors [334]. In contrast to previous work, I found higher densities of vectors in forests and in farmland than around the home, therefore people may need to be protected in those habitats to control *P. knowlesi* transmission. Attractive toxic sugar baits could be appropriate here, and/or people working in forest or farm areas where exposure is anticipated to be high could be protected by repellent clothing [335].

Furthermore, there is the hypothesis that *P. knowlesi* could be controlled by a broad-brush approach of removing macaques. However, *P. knowlesi* parasites were not circulating in the macaques that I studied in Sabah suggesting that not all present a risk to humans. Culling of monkeys therefore would not be a very ethical or targeted approach to reducing transmission. A more effective approach would be to incorporate surveillance in macaques to identify populations which pose a risk of spillover. Future work evaluating the use of spatial repellents and toxic baits in habitats posing a *P. knowlesi* risk to humans is necessary to establish if these will be effective methods of vector control.

5.3.3 Implications of *P. knowlesi* for malaria elimination

The emergence of *P. knowlesi* has provided a significant challenge in the face of malaria control in Malaysia. The country has made great progress in the substantial decline of *P. falciparum* and *P. vivax* malaria and has been assigned to the 'pre-elimination' phase by WHO. The National Malaria Elimination

Strategic Plan set out in 2011 aimed for malaria to be eliminated from Peninsular Malaysia by 2015 and Malaysian Borneo by 2020 [6]. However, the increase in *P. knowlesi* cases has hindered these goals. Currently, only *P. falciparum* and *P. vivax* are acknowledged in the annual World Malaria Report and there is not yet consensus about whether this should include *P. knowlesi*. *Plasmodium knowlesi* infections are frequently misdiagnosed as *P. falciparum* or *P. vivax* malaria thus scientists stress that an increased awareness of this parasite will lead to more accurate estimation of incidence and progress towards control [7]. The research presented in this PhD study indicates that *P. knowlesi* malaria is a threat to the human population over a wide geographic region in Sabah due to the widespread distribution of competent vectors. Whilst *P. knowlesi* remains circulating in the macaque reservoir host population, the risk of transmission to humans is present.

Due to the more complex nature of zoonotic malaria transmission, involving wild animal reservoir hosts, it is obvious that a change in the approach to control is required for this type of malaria. Treatment and control of the parasite within the human population will reduce the number of cases but until more focus is made on infection dynamics within the reservoir host, the potential for ongoing outbreaks remains. There is a need to understand the force of infection coming from macaques to identify what parasite species are circulating and their prevalence. Recently in Peninsular Malaysia an example of the type of study necessary for deducing this was performed where 781 long-tailed macaques from 77 locations were sampled to identify hotspots of infection. *Plasmodium knowlesi* was present in 13.6 % of the macaques, 26.4 % were infected with *P. inui*, 17.7 % with *P. cynomolgi*, 12.8 % with *P. coatneyi* and 11.8 % had *P. fieldi*. Examination of suburban and urban populations revealed that infections are not only restricted to macaques living in the forest [87]. There is an additional need to understand the potential of parasite transfer from humans back to monkeys in Malaysia. This has been observed in Africa with human *P. malariae*, *P. ovale* and *P. vivax* infections in wild chimpanzees [336] and *P. falciparum* in gorillas [337,338]. The *P. knowlesi* outbreak has introduced a novel challenge for malaria elimination in Malaysia but with adequate reporting (*P. knowlesi* malaria now registered as a notifiable disease by the Ministry of Health [87]) and incorporation

of the macaque reservoir in surveillance, the country will be in a good position to conduct an evidence-based approach to control.

5.3.4 **Other primate malarias posing a spillover risk to humans**

Plasmodium knowlesi is the first primate malaria parasite to emerge in the human population in Sabah but there is evidence to suggest that people in this area are regularly exposed to a wide range of other primate malarias. Entomological studies conducted around human settlements in Kudat by Monkeybar demonstrate that people are routinely exposed to vectors infected with other primate malarias including *P. cynomolgi*, *P. inui*, *P. coatneyi* and *P. fieldi* [50,194]. Natural human *P. cynomolgi* cases have recently been reported in Peninsular Malaysia [339] and Sarawak [273], indicating that this simian species may also be emerging as a public health threat. Whilst no reports of natural transmission of *P. inui* have been made [115], there is still a risk that this could occur. Both *P. cynomolgi* and *P. inui* are morphologically similar to the human malaria species *P. vivax* and *P. malariae*, thus may be misdiagnosed by microscopy. In addition, these parasites tend to be more benign thus infected people may not display symptoms as severe as *P. knowlesi* infections allowing the parasites to go undetected. Thus it would be valuable to incorporate molecular diagnostics for these parasite species in routine surveillance. *Plasmodium knowlesi* may have been the first simian parasite to cause a substantial human outbreak in Sabah, but there is potential for spillover of other simian species in the future.

Considering the variety of other simian parasite species circulating in macaque and mosquito populations, it is unknown why *P. knowlesi* was the only species to make the jump into humans. This could be due to differences in the life-cycles of simian species. *Plasmodium cynomolgi*, *P. coatneyi* and *P. fieldi* are tertian parasites with a 48 hour asexual replication cycle; and *P. inui* is quartan, replicating every 72 hours [32]. *Plasmodium knowlesi* is the only quotidian parasite with the shortest schizogonic cycle of 24 hours which may have made it easier to replicate in humans. Another suggestion for the success of *P. knowlesi* in the human population may be that human to human transmission is happening. It is currently assumed that human infection is only a result of spillover from the macaque population, however it is known that *P. knowlesi* patients harbour the

gametocyte stage and the competent vector, *An. balabacensis,* can be found host seeking in peri-domestic areas. Transmission models indicate very low probabilities of human to human transmission (1 in 1500 simulations or 10 in 2000 simulations) therefore even if the event is possible, it is still only likely to be rare [87]. Then again, higher diversities in PkMSP1 and PkAMA1 sequences from human isolates vs monkey isolates from Thailand and Malaysia has given an indication that this may be possible [340,341]. Further studies are required to establish if this is occurring. Scientists defined the evidence deemed to be acceptable at the recent 2016 WPRO (WHO Western Pacific Region) meeting: presence of mixed human malaria and *P. knowlesi* infections in mosquitoes, human blood in *P. knowlesi* infected mosquitoes, *P. knowlesi* patients without a history of simian exposure, development of drug resistance genes in *P. knowlesi* isolates, or distinct genetic haplotypes in humans not found in monkeys [87]. It is unknown as to why *P. knowlesi* is so successful in infecting humans under natural conditions and it is clear that more investigation is required to establish if human to human transmission is a contributing factor.

5.4 **Remaining questions**

There are key elements still missing which are necessary to fully understand *P. knowlesi* transmission dynamics and risk to humans. Firstly, establishing the host preference of *An. balabacensis* is crucial to know the frequency of human or monkey bloodmeals. Studies have noted *An. latens* as having an overall preference for humans over macaques [205] and *An. cracens* having a higher preference for humans than monkeys [46]. However the degree of *An. balabacensis* preference for humans or monkey, as well as other Leucosphyrus group species, is yet to be established. Within vector populations it should be identified if there are mosquitoes only feeding on monkeys, only feeding on humans and what proportion of vectors have generalist feeding. Advancements have been made in recent years to define aspects of *P. knowlesi* transmission in Sabah relating to human risk such as land-use [102], human demographics [88] and vector bionomics [50,194] but future studies examining human competency under natural conditions, vector host preference and how this is impacted by host density will make further additions to the field.

5.5 Conclusions

The global effort to achieve malaria elimination has made significant progress in many areas but has also been faced with several challenges and complexities making elimination difficult to achieve in some settings. Zoonotic malaria is an example of this, although its overall impact on global malaria elimination may be hard to assess now. However it is clear that climate change and rapid rates of deforestation can radically alter vector-borne disease systems, potentially exposing humans to a wider range of pathogens. Understanding the risk posed by this and how to control it will require a One Health approach based on detailed understanding of the ecology of both vector and reservoir populations.

Additional files

Table S1. Description of habitat types, number of traps and collections made to investigate mosquito resting behaviour in study area.

Habitat type	Description	Traps per sampling night	Resting collections made per week
Inside house	All interior walls of every room in the home	4 x backpack aspiration	16 x backpack aspiration
Under house	Houses were raised on stilts ~0.5-1m above the ground, thus collections were performed in the gap between the ground and the house floor	12 x backpack aspiration 12 x resting buckets 12 x sticky resting buckets	48 x backpack aspiration 48 x resting buckets 48 x sticky resting buckets
Around house	The peri-domestic garden area, within 10m of the home	12 x backpack aspiration 12 x resting buckets 12 x sticky resting buckets	48 x backpack aspiration 48 x resting buckets 48 x sticky resting buckets
Plantations (palm or rubber)	Farming areas of 100-200m^2 where oil palm trees are being cultivated	12 x backpack aspiration 12 x resting buckets 12 x sticky resting buckets	48 x backpack aspiration 48 x resting buckets 48 x sticky resting buckets
Forest edge	The forest fringe at the join between forest patch and area of other land-use	12 x backpack aspiration 12 x resting buckets 12 x sticky resting buckets	48 x backpack aspiration 48 x resting buckets 48 x sticky resting buckets
Forest ground level	20m inside the forest patch on the forest floor	12 x backpack aspiration 12 x resting buckets 12 x sticky resting buckets	48 x backpack aspiration 48 x resting buckets 48 x sticky resting buckets
Forest canopy	20m inside the forest hanging in trees at 2.5-9m above ground level	12 x sticky resting buckets	48 x sticky resting buckets

Table S2. Total number of resting *Aedes* mosquitoes collected using CDC, RB and SRB trapping methods in eight habitats.

		Habitat type								
Trap	***Aedes* species**	**Inside house**	**Under house**	**Around house**	**Palm plantation**	**Rubber plantation**	**Forest edge**	**Forest ground level**	**Forest canopy**	**Sum**
RB	*Ae. albopictus F*	×	4	3	0	0	2	3	×	12
	Ae. albopictus M	×	0	7	0	4	7	6	×	24
	Ae. aegypti F	×	0	3	0	0	0	1	×	4
	Ae. aegypti M	×	0	4	0	0	1	0	×	5
	Unknown *Aedes* F	×	4	2	0	2	1	4	×	13
	Unknown *Aedes* M	×	0	1	0	8	7	6	×	22
	Total	×	8	20	0	14	18	20	×	80
SRB	*Ae. albopictus F*	×	7	3	5	16	27	15	9	82
	Ae. albopictus M	×	0	1	4	13	22	7	2	49
	Ae. aegypti F	×	1	0	0	0	4	2	1	8
	Ae. aegypti M	×	0	0	0	0	1	0	0	1
	Unknown *Aedes* F	×	0	2	1	4	6	6	2	21
	Unknown *Aedes* M	×	0	0	0	0	7	3	0	10
	Total	×	8	6	10	33	67	33	14	171

Table S2 continued on next page

Table S2 continued. Total number of resting *Aedes* mosquitoes collected using CDC, RB and SRB trapping methods in eight habitats.

		Habitat type								
Trap	***Aedes* species**	**Inside house**	**Under house**	**Around house**	**Palm plantation**	**Rubber plantation**	**Forest edge**	**Forest ground level**	**Forest canopy**	**Sum**
CDC	*Ae. albopictus F*	0	6	4	3	1	4	5	×	23
	Ae. albopictus M	0	2	11	1	3	18	15	×	50
	Ae. aegypti F	0	0	1	0	0	3	0	×	4
	Ae. aegypti M	0	0	0	0	0	1	1	×	2
	Unknown *Aedes* F	3	10	16	1	5	5	12	×	52
	Unknown *Aedes* M	0	4	16	4	22	27	28	×	101
	Total	3	22	48	9	31	58	61	×	232
	Overall sum	3	38	74	19	78	143	114	14	483

Table S3. Total number of resting *Culex* mosquitoes collected using CDC, RB and SRB trapping methods in eight habitats.

	Habitat type									
Trap	***Culex* subspecies**	**Inside house**	**Under house**	**Around house**	**Palm plantation**	**Rubber plantation**	**Forest edge**	**Forest ground level**	**Forest canopy**	**Total**
RB	*Culex* F	×	5	3	0	0	0	0	×	8
	Culex M	×	10	1	0	1	0	2	×	14
	Culiciomyia F	×	0	0	0	0	1	0	×	1
	Culiciomyia M	×	0	0	0	0	2	0	×	2
	Eumelanomyia F	×	2	1	0	0	1	2	×	6
	Eumelanomyia M	×	2	1	0	0	0	2	×	5
	Lophoceraomyia F	×	2	0	0	0	1	0	×	3
	Lophoceraomyia M	×	9	5	0	2	0	0	×	16
	Oculeomyia F	×	0	0	0	0	0	0	×	0
	Oculeomyia M	×	0	0	0	0	0	0	×	0
	subgenera unknown F	×	27	46	19	3	5	41	×	141
	subgenera unknown M	×	44	72	16	4	3	34	×	173
	Total	×	101	129	35	10	13	81	×	369
SRB	*Culex* F	×	9	2	1	4	0	1	0	17
	Culex M	×	2	1	0	0	0	0	0	3
	Culiciomyia F	×	0	1	0	0	1	0	0	2
	Culiciomyia M	×	0	0	0	0	0	0	0	0
	Eumelanomyia F	×	1	0	0	0	1	0	1	3
	Eumelanomyia M	×	1	0	0	0	1	1	0	3
	Lophoceraomyia F	×	0	0	0	0	0	0	0	0

Table S3 continued on next page

Table S3. continued Total number of resting *Culex* mosquitoes collected using CDC, RB and SRB trapping methods in eight habitats.

	Habitat type								
Trap	***Culex* subspecies**	**Inside house**	**Under house**	**Around house**	**Palm plantation**	**Rubber plantation**	**Forest edge**	**Forest ground level**	**Forest canopy**	**Total**
SRB	*Lophoceraomyia* M	×	0	0	0	0	0	1	0	1
	Oculeomyia F	×	0	1	0	0	0	0	0	1
	Oculeomyia M	×	0	0	0	0	0	1	0	1
	subgenera unknown F	×	10	36	7	1	4	17	9	84
	subgenera unknown M	×	4	7	4	0	2	12	2	31
	Total	×	27	48	12	5	9	33	12	146
CDC	*Culex* F	3	3	1	0	1	0	1	×	7
	Culex M	0	3	1	0	0	0	0	×	4
	Culiciomyia F	0	1	0	0	0	0	0	×	1
	Culiciomyia M	0	1	0	0	0	0	0	×	1
	Eumelanomyia F	0	1	1	0	0	0	1	×	3
	Eumelanomyia M	0	1	0	0	0	0	0	×	1
	Lophoceraomyia F	0	6	1	0	0	3	1	×	11
	Lophoceraomyia M	*0*	4	0	0	1	1	0	×	6
	Oculeomyia F	*0*	0	0	0	0	0	0	×	0
	Oculeomyia M	*0*	0	0	0	0	0	0	×	0
	subgenera unknown F	*16*	47	28	4	1	3	10	×	109
	subgenera unknown M	*44*	269	47	1	9	2	6	×	378
	Total	*63*	336	79	5	12	9	19	×	523
	Overall sum	*63*	464	256	52	27	31	133	12	1038

Table S4. List of medically important *Culex* species collected using CDC, RB and SRB trapping methods in eight habitats.

		Habitat type							
Trap	***Culex* vectors of medical importance**	**Inside house**	**Under house**	**Around house**	**Palm plantation**	**Rubber plantation**	**Forest edge**	**Forest ground level**	**Forest canopy**
RB	*Cx. quinquefasciatus*	×	5	1	3	0	0	0	×
	Cx. fuscocephala	×	0	0	0	0	0	0	×
	Cx. sitiens	×	0	0	1	0	0	0	×
SRB	*Cx. quinquefasciatus*	×	0	3	7	0	0	0	0
	Cx. fuscocephala	×	0	3	0	0	0	0	0
	Cx. sitiens	×	0	0	1	0	0	0	0
CDC	*Cx. quinquefasciatus*	0	1	0	9	0	0	0	×
	Cx. fuscocephala	0	0	0	0	0	0	0	×
	Cx. sitiens	0	1	0	0	0	0	0	×

Table S5. Total number of blood-fed female resting mosquitoes obtained throughout the study.

		Genera of blood-fed females							
	Habitat type	***Culex***	***Aedes***	***Uranotaenia***	***Armigeres***	***Tripteroides***	***Lutzia***	**Unknown**	**Total**
RB	Under House	13	3	0	0	0	0	0	16
	Around House	18	2	0	0	0	0	0	20
	Palm	5	0	1	0	0	0	0	6
	Rubber	2	0	0	0	0	0	0	2
	Forest edge	1	0	0	0	0	0	0	1
	Forest interior	1	0	0	0	0	0	0	1
SRB	Under House	5	2	0	1	2	1	1	12
	Around House	22	0	0	0	0	0	0	22
	Palm	4	0	0	0	0	0	0	4
	Rubber	0	0	0	0	0	0	0	0
	Forest edge	0	0	0	0	0	0	0	0
	Forest interior	3	1	0	0	0	0	0	4
	Forest canopy	0	1	0	0	0	0	0	1
CDC	Inside House	5	1	0	0	0	0	0	6
	Under House	8	3	0	0	0	0	0	11

Table S5 continued on next page

Table S5 continued Total number of blood-fed female resting mosquitoes obtained throughout the study.

		Genera of blood-fed females							
	Habitat type	***Culex***	***Aedes***	***Uranotaenia***	***Armigeres***	***Tripteroides***	***Lutzia***	**Unknown**	**Total**
CDC	Around House	7	2	0	0	1	0	0	10
	Palm	2	0	0	0	0	0	0	2
	Rubber	2	1	0	0	0	0	0	3
	Forest edge	1	0	0	0	0	0	0	1
	Forest interior	1	3	0	0	1	0	0	5
	Overall sum	100	19	1	1	4	1	1	127

Table S6. Blood meal hosts of engorged female mosquitoes. Hosts were identified using PCR and sequencing of the vertebrate cytochrome *b* mitochondrial gene.

Genera	Subgenera or species	Habitat	Trap	Blood-meal host	Number of mosquitoes
Culex	Unknown	Around house	CDC	*Gallus gallus*	4
Culex	*Culex* (1), *Cx. quinquefasciatus* (1)	Around house	RB	*Gallus gallus*	10
Culex	Unknown	Around house	SRB	*Gallus gallus*	9
Culex	*Culex* (2), *Oculeomyia* (1)	Under house	CDC	*Gallus gallus*	3
Culex	Unknown	Under house	RB	*Gallus gallus*	2
Culex	*Cx. quinquefasciatus* (2)	Under house	SRB	*Gallus gallus*	2
Armigeres	*Arm. moultoni*	Under house	SRB	*Gallus gallus*	1
Lutzia	*Lt. vorax*	Under house	SRB	*Gallus gallus*	1
Culex	Unknown (1), *Cx. fuscocephala* (1)	House indoor	CDC	*Homo sapiens*, *Gallus gallus*	2
Culex	Unknown	Palm	RB	*Gallus gallus*	1
Culex	Unknown	Palm	SRB	*Gallus gallus*	2
Aedes	*Stegomyia*	Rubber	CDC	*Homo sapiens*	1

Figure S1. Habitats selected to represent a gradient of different microhabitats arising from deforestation. Resting mosquito collections were performed A: inside houses; B: under houses; C: in the peri-domestic area around houses; D: palm plantations; E: rubber plantations; F: forest edge; G: forest interior at ground level; and H: forest canopy.

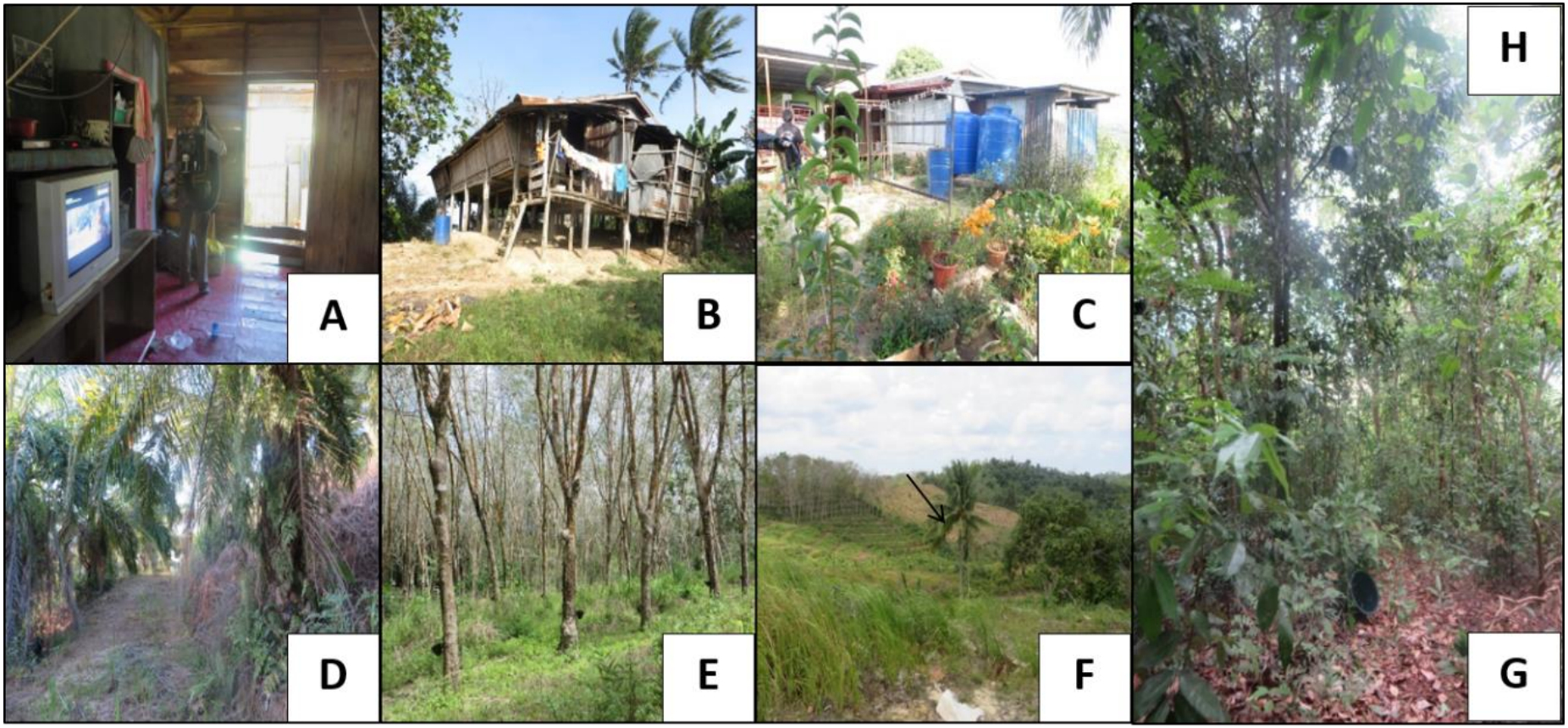

Figure S2. Map of Tuboh village. Icons indicate sampling areas of different habitat types: yellow pentagons-houses; orange stars-palm plantations; purple squares-rubber plantations; blue triangles-forest patches. Each symbol signifies a different sampling area and habitat, and thus was assigned an individual spatial cluster in analysis.

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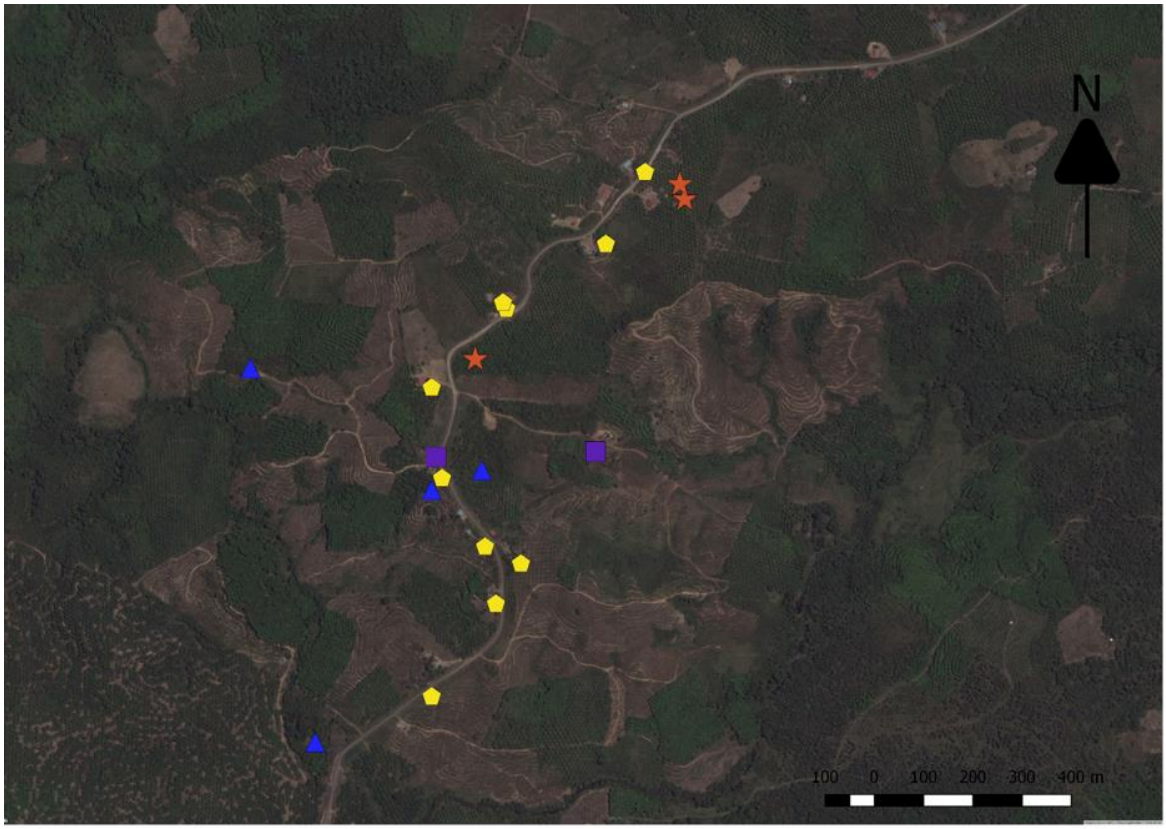


Figure S3. Map of Paradason village. Icons indicate sampling areas of different habitat types: yellow pentagons-houses; orange stars-palm plantations; purple squares-rubber plantations; blue triangles-forest patches. Each icon signifies a different sampling area and habitat, thus was assigned an individual spatial cluster in analysis.

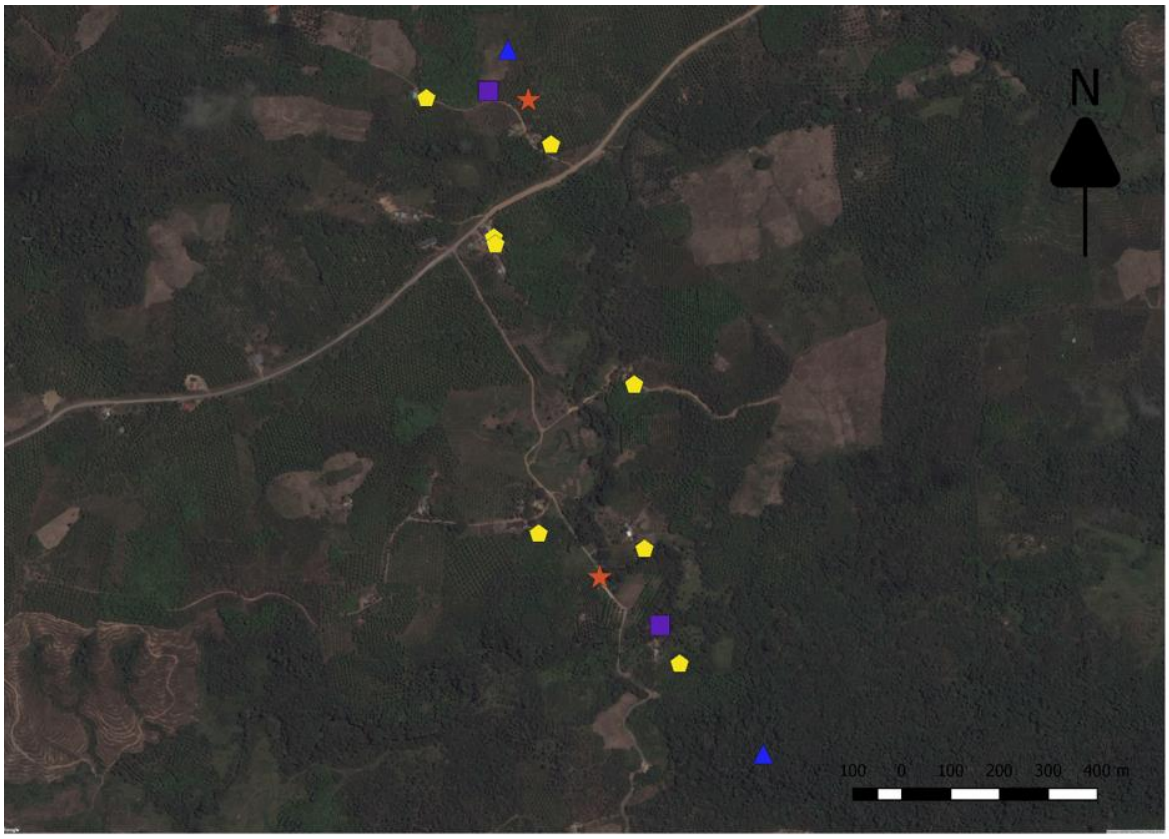


Figure S4. Physiological status of female *Aedes* collected.

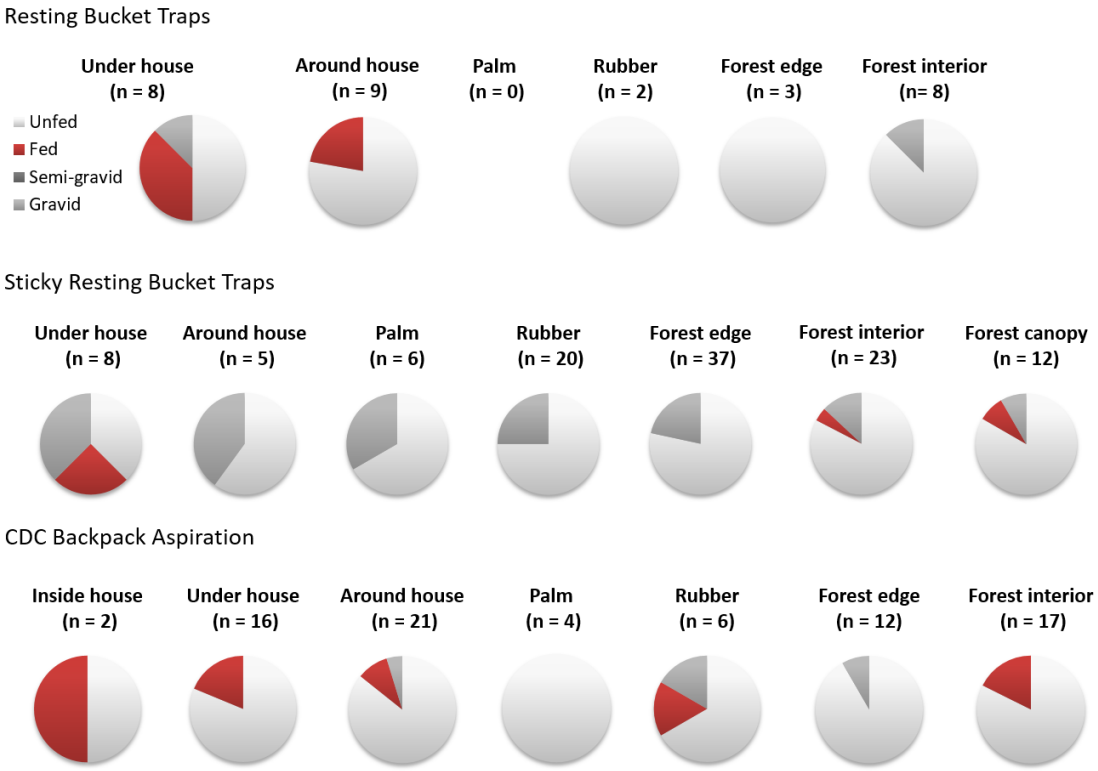
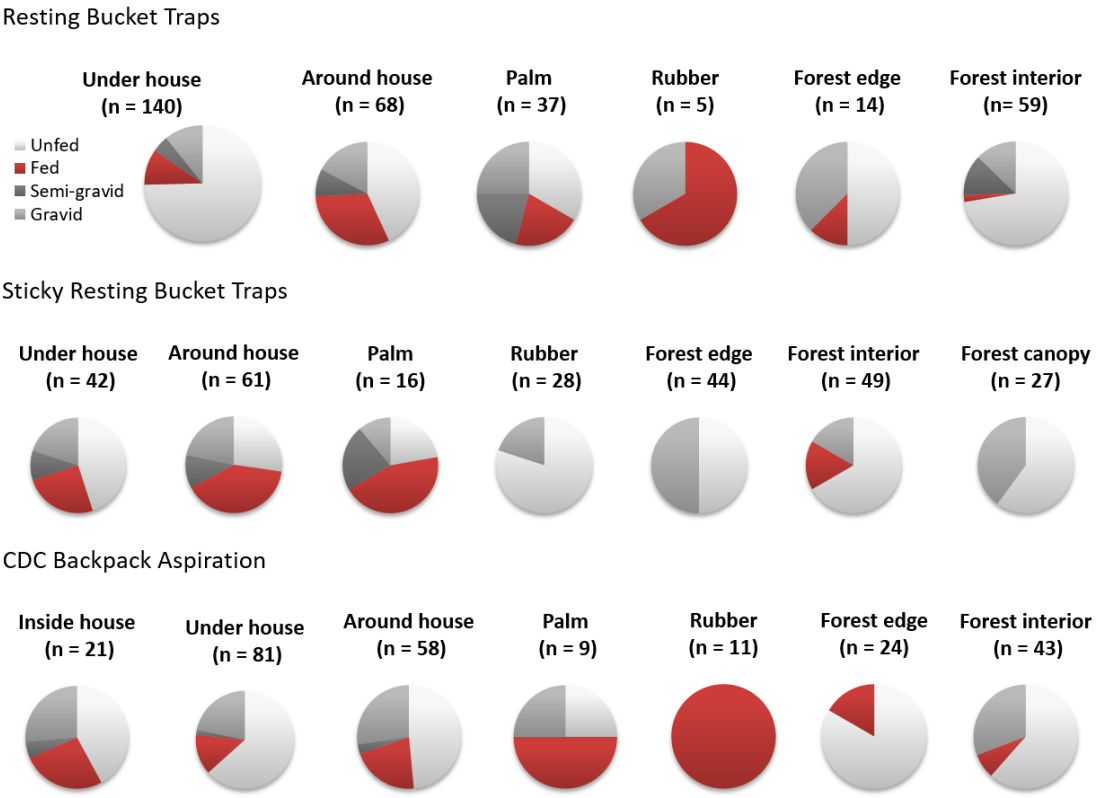


Figure S5. Physiological status of female *Culex* collected.



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