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Larval Ecology of *Anopheles* Mosquitoes in Kudat, Sabah

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ABSTRACT

The emergence of human *Plasmodium knowlesi* malaria appeared to have been precipitated by the displacement of the natural environment of macaques and *Anopheles* mosquitoes resulting from deforestation and land-use changes in Malaysia. A longitudinal survey of larval habitats was conducted from May 2015 to April 2016 in the District of Kudat, Sabah to better understand how these changes have affected mosquitoes across six land use categories. Larvae were collected by dipping and reared in the laboratory for the identification of adults. Five anopheline and three culicine species were present: *Anopheles balabacensis, An. barbirostris, An. lesteri, An. borneensis, An. umbrosus, Aedes albopictus, Culex gelidus*, and *Toxorhynchites sp. An. balabacensis* was found in all six land-use types. Biodiversity by genera was high in all land-use types. The relative importance of land use types and larval habitats as sources of potential vectors was analyzed by the Kruskal-Wallis H test by ranks. In decreasing order *Anopheles* larvae were found in rubber tree plantation > coconut plantation > clearing site > palm oil plantation > forest > settlement area. Important larval habitats were intermittent stream > ditch > pond > artificial container > puddle > river > slow-flowing stream. Eighteen breeding sites of *An. balabacensis* were within (500 m) the average maximum flight range of the species and houses at risk for malaria. Knowledge gained from the study can be used to assess the need for vector control in preventing the spread of *P. knowlesi* in vulnerable areas.

INTRODUCTION

The first human case of *Plasmodium knowlesi* malaria was recorded in an American working in the jungle in Peninsular Malaysia in 1965 (Chin et al. 1965). A second case was documented in 1971 (Fong et al., 1971). It was not until 2004 that this simian malaria became a public health concern when many naturally acquired cases occurred in Kapit, Sarawak in Malaysian Borneo (Singh et al., 2004). Across all divisions of Sabah, notifications of *P. knowlesi* malaria increased in Kudat and West Coast from 2004 onwards (Barber et al., 2012; Barber et al., 2013; Cox-Singh et al., 2008; Cox-Singh & Singh, 2008). With reports of increasing cases from 2010 – 2014, the country had the highest incidence of *P. knowlesi* in the WHO Western Pacific Region (Joveen-Neoh et al., 2011; Ministry of Health, 2016; Yusof et al., 2014). Henceforth, *P. knowlesi* has surpassed *P. falciparum* as the prominent cause of severe malaria in Malaysia (William et al., 2014). There is evidence that deforestation and land conversion for agricultural and industrial purposes have been the driving forces for the emergence of this enzootic mosquito-borne disease in Malaysia (Fornace et al., 2018; Friedrich, 2016). Several studies have been initiated to link deforestation, *P. knowlesi,* and risk to humans from the biomedical, environmental, and sociological standpoints (Fornace et al., 2018; Ramdzan et al., 2019; Vythilingam et al., 2005).

The first pillar of disease control is clinical cure based on active case finding and treatment of infected persons with antimalarial drugs; the second is vector control by eliminating mosquitoes or interrupting man-vector contact. Since most studies about vectors dealt with adult mosquitoes, interest began to focus on the ecology of *Anopheles* larvae having been displaced from their sylvatic habitats following land-use changes (Hii & Vun, 1985; Hii et al., 1988). Chua et al. (2019) showed that the abundance and biting habits of *An balabacensis* were not affected by diverse habitats, that this species was present throughout the year in all habitats, including those found in agricultural and farming areas following deforestation. The significant land-use changes occurring in this area were the conversion of primary and secondary forest to crop farming and human settlements, which may be more conducive for mosquito breeding, survival, and disease transmission (Ahmad et al., 2018; Gottdenker et al., 2014; Hawkes et al., 2019; Patz et al., 2004; Patz & Norris, 2004;). Knowledge of the distribution and determinants of larval habitats of *P. knowlesi* vectors could help augment entomological surveillance and disease control efforts. These observations in a changing environment as they were in Kudat is in line with the declaration of the 2017 World Health Assembly in Geneva that "a new approach is needed to tackle the global emergence and re-emergence of vector-borne diseases which account for over 17% of all infectious diseases worldwide" (WHO, 2017).

To complement adult mosquito bionomics studies, a longitudinal survey of larval breeding habitats was conducted to ascertain anopheline vectors of *P. knowlesi* in the Kudat District in Sabah. The objectives were: Firstly, to determine the abundance and diversity of mosquito larvae with particular reference to *Anopheles* in six land use classes; secondly, to estimate the relative importance of land-use types and different aquatic habitats as sources of potential vectors of malaria; thirdly, to identify other species of public health importance found existing with *Anopheles* in the same land-use type; fourthly, to characterize the relationship between rainfall and larval density; and finally, to approximate the location of *Anopheles*-positive larval habitats vis-à-vis houses at risk for malaria.

MATERIALS AND METHODS

Kudat, comprising 1,287 km^2 in the north-eastern tip of Borneo (Figure 1), is where the forest has been substantially reduced, giving way to rubber trees, oil palm, and coconut plantations. A longitudinal survey of mosquito larval habitats was conducted in Kampung Marabahai, Nangka, Paradason, and Tuboh in Kudat District from May 2015 to April 2016. The National Medical Research Register approved this study of the Malaysian Ministry of Health (NMRR, Reference No. NMRR-12-786-13048. Consent to carry out mosquito larval collection was obtained from the village council, the village headman, and the landowners.

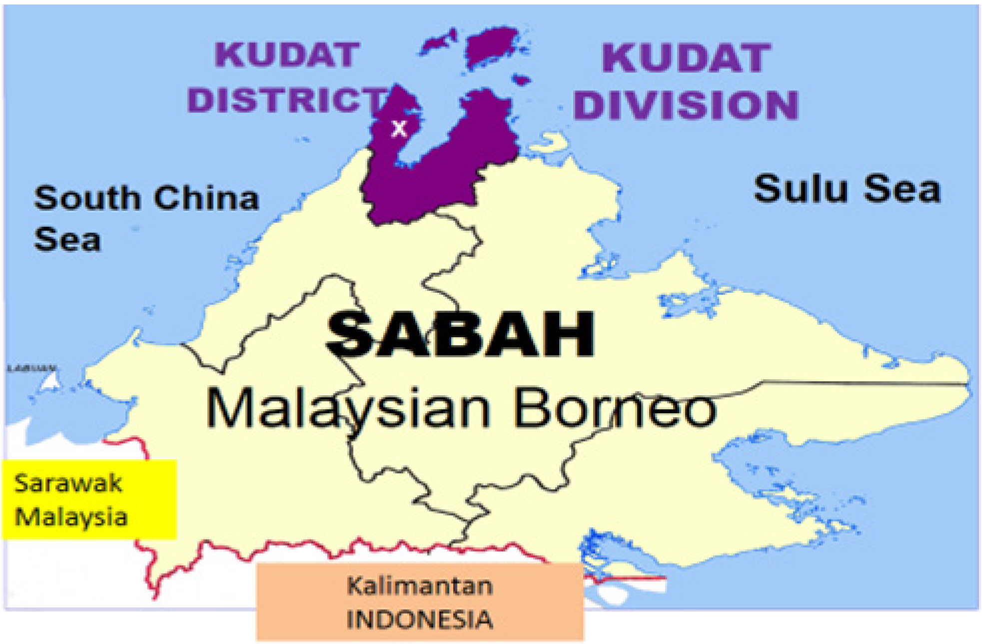

Figure 1 Location map of the study site (X) in Kudat District, Sabah, Malaysia

From 4500 survey cells or blocks, generated with ArcGIS mapping and analytics platform (Environmental Systems Research Institute (ESRI), USA (http://www.esri.com/software/arcgis), a grid consisting of 600 sampling blocks within a 2 × 3 sq km area was overlaid on the land cover and designated as the study site. Thereupon 358 blocks were allocated into 54 – 64 sampling blocks each per land-use class. Individual survey block measured 100 × 100 m, corresponding to one sampling unit. Six land use types were identified from images using a drone and verified by ground-truthing, namely, clearing site, an abandoned area or one that is being prepared for development; coconut plantation; secondary forest, with woody vegetation and indigenous species and what is left of the primary forest following land-use changes; oil palm plantation; rubber tree plantation; and settlement area, an aggregation of residences and other structures built on the ground. An inspection identified twelve categories of aquatic habitats, i.e., artificial container, ditch, irrigation canal, intermittent stream, puddle, pond, rock pool, river, slow-flowing stream, tree hole, leaf axil, and borrow pit (Figure 2). Larvae were collected weekly by the conventional 10-dipping method, sorted by land-use type/habitat, and brought to the field laboratory for rearing. Larval counts from five randomly selected sampling blocks per land-use type were collated monthly for data analysis.

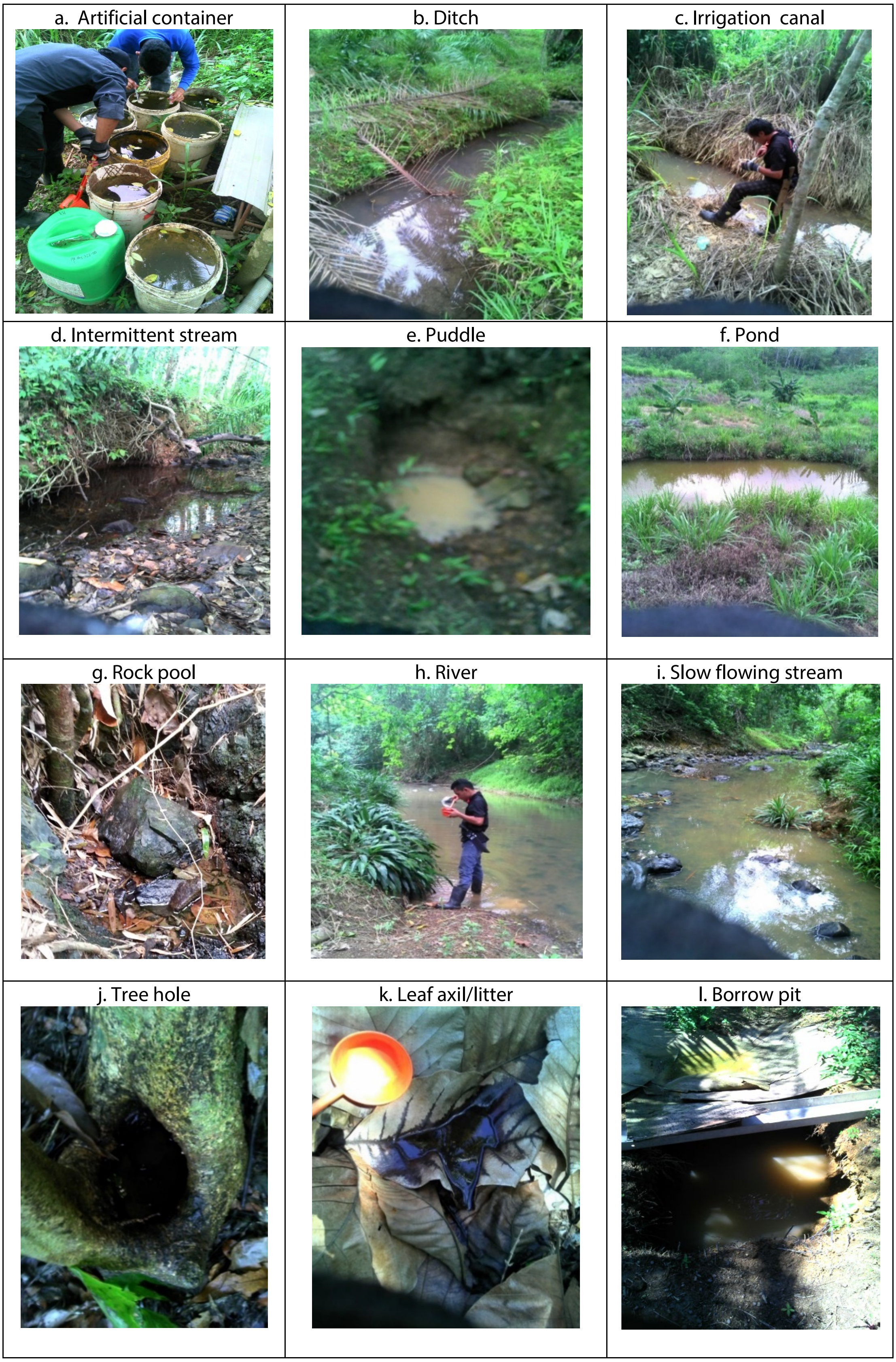

Figure 2 Larval habitat types inspected for mosquito larvae in different land-use types

Kruskal-Wallis H Test by ranks analyzed the relative importance of each land use type and larval habitat (Kruskal &Wallis, 1952). The test was run to determine if there were significant differences among land-use types and aquatic habitats using the formula:

$$H = 12/n\,(n+1)\,\sum Ri^2/ni - 3(n+1)$$

where n is the total number of observations, ni is the number of *Anopheles* larvae per land-use type/larval habitat, and R^2 is the sum of ranks of each land-use type/larval habitat squared. H is statistically significant if it is equal to or larger than the critical value of Chi-square for a given degree of freedom. Simpson's diversity index was used to characterize the mosquito population in terms of genera and species present in each land use type according to the following expression:

$$D = 1 - (\sum n\,(n - 1)/N(N - 1)$$

where n = the total number of organisms of a particular genus or species and N = the total number of organisms of all genera or species (Barcelona Field Study Centre, 2021). Precipitation was measured by a rain gauge (Brannan Thermometers & Instrumentation, UK). The location of *An. balabacensis* and relative distances of larval habitats from houses at risk were approximated from the coordinates on the ArcGIS grid (ESRI, Redlands, USA).

RESULTS

Composition, Abundance and Distribution of Mosquitoes by Land-Use Type

One anopheline and four culicine genera comprised a total of 984 mosquito larvae collected, as follows: 476 (48.4%) *Anopheles*, 352 (35.8%) *Culex*, 132 (13.4 %) *Aedes*, 23 (2.3 %) *Armigeres* and one (0.1%) *Toxorynchites.* The composition by mosquito genera per land-use type was: *Anopheles, Culex,* and *Aedes* were found in all land use categories. *Toxorhynchites* was from a clearing site only. *Armigeres* were from the rubber tree and oil palm plantations only. The main limitation of the study was the difficulty of identifying species, particularly *An. balabacensis* based on the morphology of larvae. Only 126 larvae brought to the laboratory for rearing reached the adult stage for identifying species. The species of surviving laboratory-reared adults were: *An. balabacensis* (70.6%), *An. barbirostris* (1.6%), *An. lesteri* (9.5%), *An. borneensis* (1.6%), *An. umbrosus* (0.8%), *Ae. albopictus* (15%), and *Culex gelidus* (0.8%). *An. balabacensis*, a primary forest-dweller, was present in all land use types and co-existed with other anopheline species in clearing sites and coconut plantation only (Figure 3).

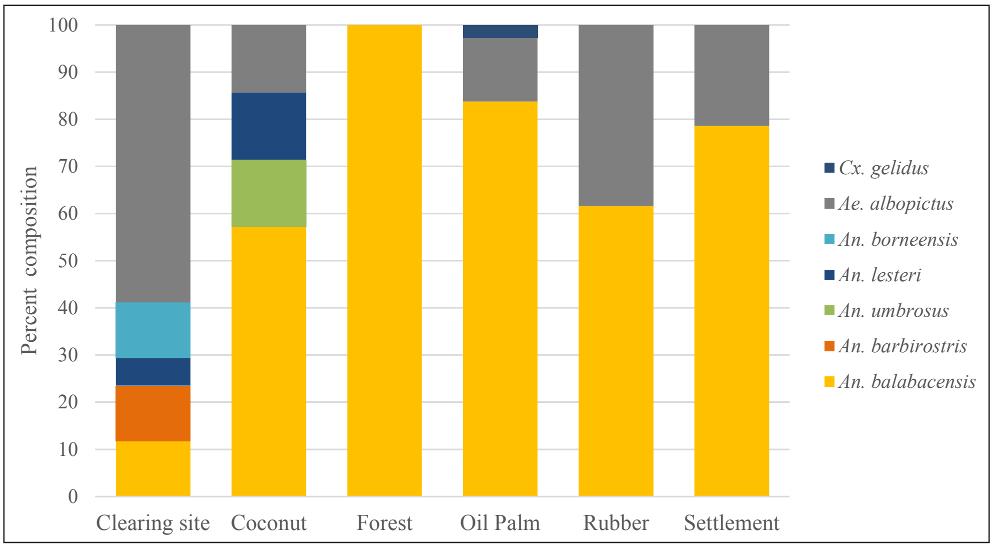

Figure 3 Species composition of laboratory-reared adult mosquitoes per land-use class

Mosquito Diversity and Land-Use Types

Based on Simpson's diversity index, biodiversity by genera was high in six land use types because of both anopheline and culicine larvae thereat. The Richness index was four each in clearing site, oil palm, and rubber tree and 3 in coconut, forest, and settlement (Table 1).

Table 1 Number of mosquito Genera in 6 land-use types

Genera	Number of individuals of each Genera in each land-use types					
	Clearing site	Coconut	Forest	Oil palm	Rubber tree	Settlement
Anopheles	91	93	68	102	73	49
Culex	40	72	40	111	55	34
Aedes	15	48	13	18	11	27
Armigeres	0	0	0	20	3	0
Toxorhynchites	1	0	0	0	0	0
Total (*N*)	147	213	121	251	142	110
Genera richness	4	3	3	4	4	3
Simpson's diversity index	0.535	0.647	0.568	0.630	0.583	0.652
Simpson's reciprocal index	2.155	2.836	2.315	2.705	2.400	2.871

Richness was directly proportional to diversity. These suggested a suitable environment being shared by different taxonomic groups and the probability of any two genera being different when collected was high. Genus *Anopheles* was most numerous except in oil palm. Species diversity was highest in coconut, 0.714; nil in the forest. Richness by species was highest in coconut, 5; lowest in the forest, 1. *An. balabacensis* was prominent except in the clearing site (Table 2).

Table 2 Number of mosquito SPECIES in 6 land-use types

Species	Number of individuals of each species in each land-use type					
	Clearing site	Coconut	Forest	Oil palm	Rubber tree	Settlement
An. balabacensis	2	4	25	31	16	11
An. barbirostris	2	0	0	0	0	0
An. lesteri	11	1	0	0	0	0
An. umbrosus	0	1	0	0	0	0
An. borneensis	2	0	0	0	0	0
Ae. albopictus	0	1	0	5	10	3
Cx. gelidus	0	0	0	1	0	0
Total (*N*)	17	7	25	37	26	14
Species Richness	4	5	1	3	2	2
Simpson's diversity index	0.574	0.714	0.000	0.287	0.492	0.363
Simpson's reciprocal index	2.34	3.50	1.00	1.40	1.97	1.57

Land Use Types and Larval Habitats as Sources of Potential Vectors of Malaria

Seventy-four out of 358 blocks examined were positive for *Anopheles* larvae, where each block is equivalent to one sampling unit (Table 3). These consisted of 13 or 21.6 % clearing sites, 11 or 18.6% coconut plantations, 11 or 18.3% forest, 15 or 24.1% oil palm plantations, 13 or 24% rubber tree plantations, and 11 or 17.18% settlement areas. Ninety-five out of 368 larval habitats were positive for *Anopheles* larvae. These were located as follows: 15 or 27.7% in clearing site, 17 or 27% in a coconut plantation, 13 or 20% in the forest, 18 or 25% in the oil palm plantation, 18 or 40.9% in rubber tree plantation, and 14 or 20% in the settlement area. Therefore, land-use types and larval habitats were not equally distributed throughout the study area.

Table 3 Sampling blocks and breeding habitats examined for *Anopheles* larvae

Land-use type	Clearing Site	Coconut	Forest	Oil palm	Rubber	Settlement	Total
Number of blocks sampled	60	59	60	62	54	64	358
Number of blocks positive for *Anopheles* larvae (%)	13 (21.6%)	11 (18.6%)	11 (18.3%)	15 (24.1%)	13 (24%)	11 (17.18%)	74 (20.67%)
Breeding habitats sampled	54	63	65	72	44	70	368
Breeding habitats positive for *Anopheles* larvae (%)	15 (27.7%)	17 (27%)	13 (20%)	18 (25%)	18 (40.9%)	14 (20%)	95 (25.6)

Data were not normally distributed depending on the number of sampling visits per month per land-use type or breeding habitat and the total number of larvae collected from each of six land-use types and twelve larval habitats. Larval counts were low and uneven. The distribution of *Anopheles* larvae was not similar for all groups. As assessed by visual inspection of a boxplot, the differences among land-use types were not statistically significant (χ^2=4.15, df=5, p-value=0.219) but significant among larval habitats (χ^2=20.208, df =11, p=0.043), which remained to be studied in greater details. Ahmad *et al.* in 2018 examined larval habitats in nine villages of Kudat and found *An. balabacensis were* preferring to breed in muddy ground pools and tire tracks located within 100 m in the vicinity of plantations and forest fringe houses. In this present study, mean ranks obtained by the Kruskal-Wallis test indicated the comparative importance of land use type and larval habitat as sources of potential vectors of malaria. In decreasing order these were: rubber tree plantation > coconut plantation > clearing site > palm oil plantation > forest > settlement area (Table 4).

Table 4 Kruskal Wallis Test of larval density by land-use types

Variable	Land-use type	n	Mean rank	X^2 statistics (df)	p-value
Number of *Anopheles* larvae	Rubber tree	44	210.08	4.150 (5)	0.219
	Coconut	54	188.66		
	Clearing site	65	187.69		
	Oil palm	63	183.49		
	Forest	70	174.30		
	Settlement	72	172.73		

Anopheles-positive larval habitats were most numerous in rubber tree plantations. No larvae were found in 5 aquatic habitats: borrow pit, irrigation canal, leaf axil, rock pool, and tree hole, while seven Anopheles-positive habitats in decreasing order were: intermittent stream > ditch > pond > artificial container > puddle > river > slow-flowing stream (Table 5).

Table 5 Kruskal Wallis test of *Anopheles* larval density by habitats

Variable	Larval habitat	n	Mean rank	X^2 statistics (df)	p-value
Number of *Anopheles* larvae	Intermittent stream	127	203.46	20.208 (11)	0.043
	Ditch	27	199.91		
	Pond	35	187.24		
	Artificial container	42	185.23		
	Puddle	28	174.09		
	River	56	166.55		
	Slow flowing stream	32	163.31		
	Borrow pit	1	137		
	Irrigation canal	2	137		
	Leaf axil	3	137		
	Rockpool	1	137		
	Tree hole	14	137		

Rainfall and larval density

There was no specific pattern of seasonality between rainfall and larval counts. However, heavy downpours of more than 300 mm between November and December 2015 resulted in the denudation of larval habitats in all land use types by January 2016 (Figure 4).

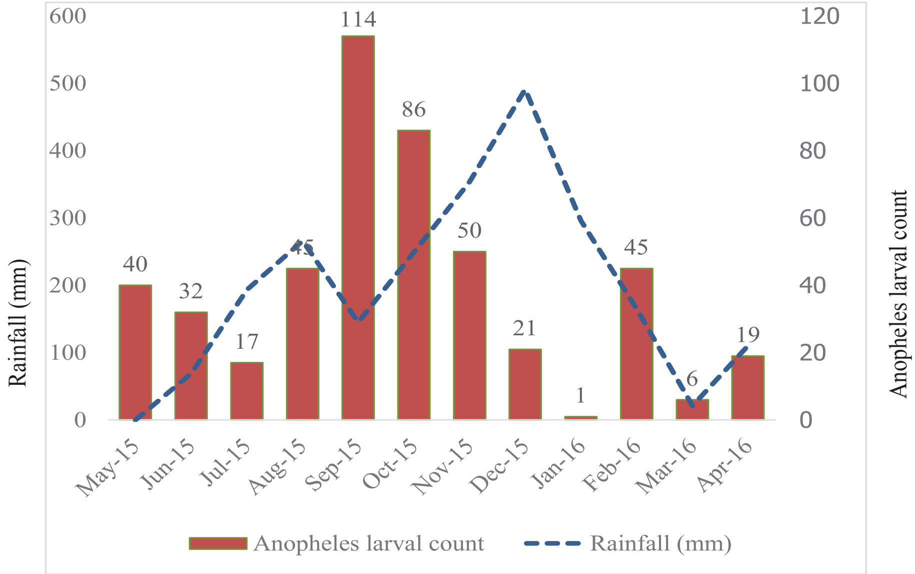

Figure 4 Monthly larval count and rainfall

Spatial distribution

Results showed the widespread distribution of *An. balabacensis* in the study area. Eighteen breeding habitats were positive for *An. balabacensis* larvae from the forest, rubber tree plantation, oil palm plantation, coconut plantation, settlement area, and clearing site. To assess their potential as sources of malaria vectors, the distances from houses per se and those with confirmed cases were approximated from the ArcGIS grid on the Google map (Figure 5). Habitats No. 3, 11, 12, 13, 14, 15, and 17 correspond to the same domicile reported cases (personal communication). *An. balabacensis* is known to have a short flight range of fewer than 500 meters (Verdonschot PFM & Besse-Lototskaya, 2014) (Table 6).

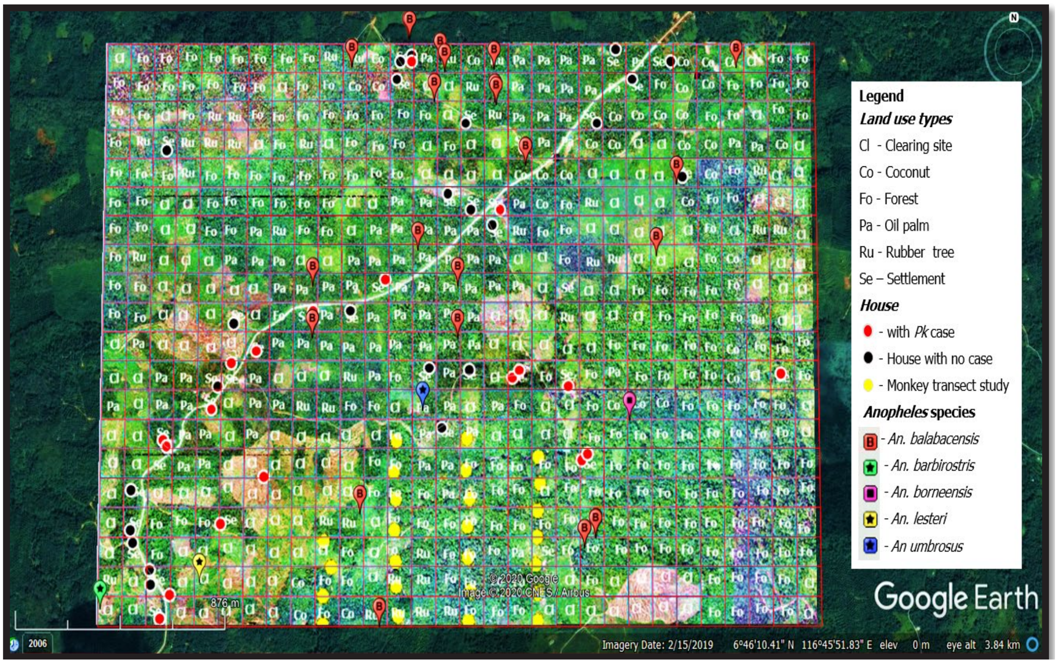

Figure 5 Spatial distribution of mosquitoes showing proximity to houses at risk for P. knowlesi malaria. ArcGIS grid on the Google map, Image Date: 2/15/2019, 6046'10.41" N 116045'51.83" E elev 0 m eye alt 3.84 km

Table 6 Location of *An. balabacensis* larval habitat from domiciles at risk for *Pk* malaria

Aquatic habitat examined	No. of *Anopheles* larvae collected	Coordinates		*An. balabacensis* reared from larva	Land use type	Distance from the nearest house (m)
		N	E			
1	9	6.77959	116.7833	2	RU	218
2	10	6.77845	116.7833	2	RU	144.5
3	11	6.77966	116.7778	5	RU	91.5
4	5	6.76559	116.7782	1	RU	427.6
5	16	6.77854	116.7833	3	RU	138.16
6	17	6.77986	116.7812	2	RU	106
7	19	6.77364	116.7895	1	SE	516
8	1	6.78056	116.78	4	SE	67
9	4	6.78058	116.7799	2	SE	58.6
10	7	6.77591	116.7903	2	SE	392
11	18	6.77106	116.7763	28	PA	83.73
12	6	6.77382	116.7803	3	PA	169
13	12	6.76453	116.7867	6	FO	265
14	13	6.76485	116.7871	2	FO	268
15	14	6.76487	116.7871	17	FO	268
16	2	6.77963	11679264,	1	CO	222
17	3	6.77651	116.7845	3	CO	51.51
18	8	6.77855	116.781	2	CL	152

RU: the rubber tree, SE: settlement, PA: oil palm, FO: forest, CO: coconut, CL: clearing

DISCUSSION

Land-use changes have affected the ecology of mosquito vectors in Kudat and have contributed to the introduction of *P. knowlesi* in a vulnerable population (Hawkes et al., 2019; Patz et al., 2004; Patz & Norris, 200; Gottdenker et al., 2004). By and large, Kudat has undergone topographical changes via human intervention, i.e., a large portion of the forest was converted initially to plantation crops (coconut, banana, and other fruit-bearing trees), and more recently, to rubber and oil palm. Both anopheline and culicine mosquitoes have adapted to a variety of conditions in each land-use type. *Anopheles balabacensis* flourished in all land use types and continued to perpetuate the enzootic cycle of *P. knowlesi* in the forest. While *An. balabacensis* co-existed with other anophelines in the Leucosphyrus group, future studies may involve searching for so-called bridge (non-human primates to human) vectors of *P. knowlesi* (Levine, 2018). Other species of public health importance found in the same land-use types were *Aedes albopictus,* the dengue vector in rural habitats, and *Culex gelidus,* a vector of Japanese encephalitis (Rezza, 2012; Vythilingam et al., 1997). The presence of *Toxorhynchites* at least in a clearing site indicated the possible use of predators as biological control agents (Focks, 2007). Evidently, *An. balabacensis* was able to establish its kind in its new environment. The study has shown that all six land-use types and seven larval habitats served as sources of potential vectors of mosquito-borne diseases.

Wong et al. (2015) have done an extensive study of *P. knowlesi* vectors in three endemic sites in Sabah in 2015, indicating that peri-domestic transmission is a possibility in the village given that people usually were exposed when they return from work in or around forested areas and stay outdoors between 1800 – 2000 hours in the evening in the presence of infective (exophilic, exophagic and anthropophilic) *An. balabacensis.* Results showed significantly higher vector densities and seasonal variation in the village than neither farm nor forest, undermining the protective benefit from the use of bednets. Meanwhile, a multi-stage model was developed by Imai et al. (2014) from data obtained in the forest, farm, and village areas. It presumes that *P. knowlesi* infection maintained among macaques in the forest 'spills over' to humans via *Anopheles* mosquitoes in the farm where men work during the day and stay at night and where monkeys usually come foraging for food. Men become infected on the farm and bring home the infection to the village. The proposed transmission cycle is from the forest to the farm to the village with relatively low human cases without macaques. Imai and cohorts claim that active case detection and treatment and the use of insecticide-treated bed nets would suffice in preventing the spread of *knowlesi* malaria if people were to stay indoors and sleep under bed nets at night or at least wear protective clothing with insect repellents outside the house. Simulations showed that LLINs (long-lasting insecticide-treated bed nets) and prompt treatment of cases could provide personal protection to humans with maximal estimated reductions in the human prevalence of 42% and 95%, respectively. For now, *P. knowlesi* malaria control is easier said than done.

Although *P. knowlesi* is presumed to have existed 98,000 to 478,000 years ago, *P.knowlesi* malaria fits the definition of Emerging Infectious Disease (Lee et al., 2011; David & Fauci 2013) or Newly Emerging Disease (NED). These infections have recently appeared within a population or those whose incidence or geographic range is rapidly increasing or threatens to increase in the near future. With the finding of two non-recombining sympatric forms of *P. ovale curtisi* and *P. ovale wallikeri* in Africa, *P. knowlesi* becomes the sixth *Plasmodium* species infecting man (Sutherland et al., 2010). As such, *P. knowlesi* typifies Pavlovsky's five pillars of "landscape epidemiology" (Pavlovsky, 1966) with the presence of an animal donor (macaque), vectors (*Anopheles* mosquitoes), animal recipients (*Homo sapiens*), pathogenic agent (*Plasmodium*), and external environment (fragmented forest); add to these, human behaviour or lifestyle, such as logging for commercial purposes or sports (mountain trekking).

This study thus recommends evaluating deforestation in terms of government policy on land use and supporting operation research on the effectiveness of community-based malaria control. Despite the significant decline of falciparum and vivax malaria, P. knowlesi in control programmes and research to find appropriate and long-lasting interventions in vulnerable areas is recommended.

CONCLUSION

Knowledge gained from the study can be used to assess the need for vector control in preventing the spread of *P. knowlesi* in vulnerable areas. The relevance of changing environments and human behaviour in the evolution and spread of infectious diseases cannot be over-emphasized. Humans, animals, arthropods, parasites, and pathogens migrate, and medical literature is replete with accounts in temperate countries of infectious diseases that used to be considered endemic only in the tropics.

CONFLICT OF INTEREST

The authors declare that they have no competing interests in publishing this article.

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