CHARACTERIZATION OF THE LARVAL BREEDING SITES OF *ANOPHELES BALABACENSIS* (BAISAS), IN KUDAT, SABAH, MALAYSIA

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Abstract. The Malaysian government has a goal of eliminating malaria by 2020 but the increase in the number of cases of *Plasmodium knowlesi* presents a major challenge to this goal. Understanding the biology and breeding sites of vector mosquitoes is important to eliminating this disease. Therefore, we aimed to identify the larval breeding sites of *Anopheles balabacensis* in Kudat, Sabah, Malaysia, examine and define the environmental characteristics of its habitat and determine the association between these environmental characteristics and the biology of *An. balabacensis*. This study was carried out in the nine villages of Kudat. *An. balabacensis* breeding sites were identified. *An. balabacensis* preferred to breed in muddy ground pools and tire tracks formed in plantations and along the forest fringe. The most common breeding site had a surface area < 1 m^2, a depth of 5-10 cm, was in partly shaded area and located approximately 100 m from the nearest house. The breeding sites for *An. balabacensis* were near human settlements and work places, making this mosquito as potential vector for malaria in Kudat. The studied environment characteristics did not significantly influence the abundance of *An. balabacensis*. Our findings suggest comprehensive coverage of breeding sites is necessary for larval control and reduction of malaria transmission.

Keywords: *Anopheles balabacensis, Plasmodium knowlesi*, malaria in Malaysia, mapping of mosquito

INTRODUCTION

The Malaysian Ministry of Health has a goal to eliminate malaria from Malaysia by 2020, but the increased incidence of *Plasmodium knowlesi* malaria from 376 cases in 2008 to 1,604 cases in 2016 (WHO Malaria Policy Advisory Committee, 2017) presents a major challenge to this goal. Therefore, effective control measures for *Plasmodium knowlesi* and its vectors is needed to reach this goal of elimination of malaria from Malaysia.

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P. knowlesi has long been present in Malaysia (Chin *et al*, 1965) but now it has become an emerging cause of zoonotic human malaria (Singh *et al*, 2004; Cox-Singh *et al*, 2008). Cases have been confirmed throughout Malaysia in areas where the ranges of its natural macaque hosts and *Anopheles leucosphyrus* group vectors overlap (Tan *et al*, 2008; Vithilingam *et al*, 2008). The majority of cases are found in Sabah and Sarawak (Singh and Daneshvar, 2010). Since knowlesi malaria is primarily zoonotic Malaysia does not include it in the elimination campaign, but it is monitored continuously, especially in Sabah and Sarawak.

In Sabah the malaria vector control program appears to be effective, judging by the reduction of human malaria cases from 50,500 in 1990 to < 5000 in 2012 (MOH, 2014). While other human malaria species are becoming more successfully controlled, *P. knowlesi*, the most common cause of malaria in Sabah, appears to be increasing (William *et al*, 2013). Deforestation is believed to be the key factor contributing to the spread of this form of malaria in Sabah (William *et al*, 2013). The destruction of the monkey's habitat for development or cash crop cultivation, such as oil palm productions, has forced the monkeys to forage for food and have closer contact with humans in both rural and urban areas. The change in land-use may also lead to more favorable breeding conditions for the mosquito vectors, a change in mosquitoes species or a change in vector biting preference due to declining monkey population (Brant *et al*, 2016).

Anopheles balabacensis and *An. latens* are vectors of human and simian malaria in Sabah (Collins *et al*, 1967; Manguin *et al*, 2008; Vithilingam *et al*, 2013). *An. balabacensis* is a mosquito preferring the forest and of the forest fringe. It has been found in all districts of Sabah (Hii and Vun, 1985). They are primate feeders and highly efficient vectors of human and simian plasmodia (Colless, 1952; Peter *et al*, 1976). Studies carried out in 1990's found *An. balabacensis* was abundant in rural areas such as Papar (Hii *et al*, 1990), Ranau (Rohani *et al*, 2008) and Kudat (Hii *et al*, 1991), Malaysia. In 2010, it was reported nearly half the malaria cases in Sabah were detected among foreigners living and working in plantation and logging areas. Approximately 60% of cases involved people with occupations at higher risk of contracting malaria, such as forestry, plantation and agricultural sectors, near monkey reservoirs of *Plasmodium knowlesi*.

Plasmodium knowlesi is the most common cause of malaria admissions to Kudat District Hospital (Barber *et al*, 2012). Kudat is located 190 km north of Kota Kinabalu, the state capital, and is in northern of Borneo. Kudat is famous for its tourist attractions. A number of ethnic groups reside in Kudat; Rungus being the largest ethnic group (Barber *et al*, 2012). Most of the indigenous populations in Kudat reside in communities close to fruit orchards, palm oil, coconut and rubber plantations. This area is surrounded by forest containing wildlife, including pig-tailed macaques, the host for *P. knowlesi*.

This study aimed to identify *Anopheles balabacensis* larval breeding sites in Kudat, Sabah, Malaysia and describe the characteristics of those sites. This information can improve our understanding of the biology of these vectors and the ecology of their breeding sites in order to inform larval control measures.

MATERIALS AND METHODS

Study sites

Study was conducted in Kudat, Sa-

Fig 1–Map of Sabah and Kudat Division showing the nine study villages.

bah Malaysia, where *P. knowlesi* is the predominant malaria parasite (Barber *et al*, 2012; William *et al*, 2013). This study was conducted in 9 villages: Pomunsukan (N6° 45.227′ E116° 45.948′), Tamalang (N6° 54′23.6′ E116° 49′36.8′), Nangka (N6° 46.967′ E116° 47.541′), Gumandang (N6° 58′32.8′ E116° 47′11.6′), Timug (N6° 43.822′ E116° 46.038′), Lotong (N6° 44.905′ E116° 48.018′), Paradason (N6° 46.023′ E116° 48.312′), Tajau Darat (N6° 56.698′ E116° 46.910′), and Kg. Onduon (N6° 42.893′ E116° 47.835′) (Fig 1). These villages were selected because of their high incidence of knowlesi malaria, large vector populations and easy accessibility by road.

Larval surveillance

We conducted larval surveillance on 6 occasions between February 2014 and January 2015. All potential breeding sites of Anopheline mosquitoes within 1 km radius of the study villages were inspected for *Anopheles* larvae. A dipper was used to collect the larvae which were placed in a white plastic tray. Mosquito larvae were collected with a pipette and transferred into a labelled bottle. Each bottle was covered to ensure the larvae remained undamaged while being transported to the field laboratory. The collected larvae were reared in an insectarium in the white plastic tray on a diet of ground ox liver.

Mapping of mosquito population

The coordinates of the collected mosquito larvae were marked using a hand-held Geographic Positioning System (GPS), (Garmin GPSMAP® 60CSx; Garmin, Olathe, KS) and processed with

MapSource® software (Garmin). Raster images (dated 11 November 2014) of Kudat and the surrounding area obtained from Google map were used. Bodies of water, forest areas, vegetation areas, roads and residential areas were identified on the images and marked as feature layers. The coordinates where mosquito larvae were identified were marked on the map using a GIS database software (ArcGIS 9.3; Environmental Systems Research Institute, Redlands, CA). The images of the studied areas were overlaid with feature layers and larval isolation areas were then added and then displayed with the WGS 1984 Coordinate system.

Characterization of larval habitat

Variables recorded for each habitat were area, water depth, type of canopy cover, distance to the nearest house, pH, dissolved oxygen, water temperature, water turbidity, plant species and surface debris coverage. The study areas were divided by a 1 meter grid. The water depth was obtained for each pool at 3 locations and the mean depth was calculated and recorded. Canopy cover was classified as open, partially shaded and shaded. Plant and debris coverage was recorded as the percentage it was covered using the 1 meter grid to obtain the estimate. The distance from where the larvae were obtained to the nearest house was measured using the GPS instrument mentioned above. The water pH and temperature were measured with the CyberScan (Eutech Instruments®, Thermo Fisher Scientific, Waltham, MA), dissolved oxygen was measured with CyberScan DO300, (Eutech Instruments®, Thermo Fisher Scientific), and water turbidity was measured with the TN-100, (Eutech Instruments®, Thermo Fisher Scientific). All measurements were obtained between 08:00 and 11:30 AM.

Correlations between 2 continuous variables (area size, water depth, distance to the nearest house, pH, dissolved oxygen, water temperature, water turbidity, emergent plant and surface debris coverage) were analysed by using Pearson's correlation coefficients, SPSS for Windows® Version 21 (IBM, Armonk, NY). Associations between nominal variables (type of habitat) and continuous variables were analysed using the chi-square, Phi and Cramer's V tests using Statistical Package for the Social Science (SPSS) for Windows® Version 21. Multiple linear logistic regression analysis by the backward elimination method was used to determine associations between studies variables and occurrence of *An. balabacensis* larvae using SPSS for Windows®, version 21.

Identification of adult mosquitoes

Mosquito larvae collected during the survey were reared to adults and identified to species using standard taxonomic keys (Reid, 1968; Sallum *et al,* 2005) and with DNA identification. The DNA was first extracted using the DNeasyR Blood & Tissue Kit (Qiagen, Valencia, CA) and stored at -20°C until analysis and then polymerase chain reactions (PCR) was used to identify specific sequences of the inter-transcribed spacer region 2 (ITS2) rRNA for *Anopheles* mosquitoes. The ITS2 sequence was amplified using primers ITS2A (5' TGTGAACTGCAGGACA 3') and ITS2B (5' TATGCTTAAATTCAGGGGGT 3') (Beede and Saul, 1995). Each reaction mixture of 50 µl contained 5 µl of mosquito DNA template, 0.2 µM of each primer and 25 µl of 2x MyTaq Mix (Bioline, Taunton, MA). The PCR reaction was performed using a Mastercycler Thermal Cycler (Eppendorf, Hamburg, Germany). The PCR conditions were as follows: dena-

Table 1
The number of *Anopheles* mosquito larvae collected by breeding site.

Muddy ground pool	Clear ground pool	Tire track	Slow-flowing stream	Swamp water pocket	Total of no.
82	22	104	10	31	249

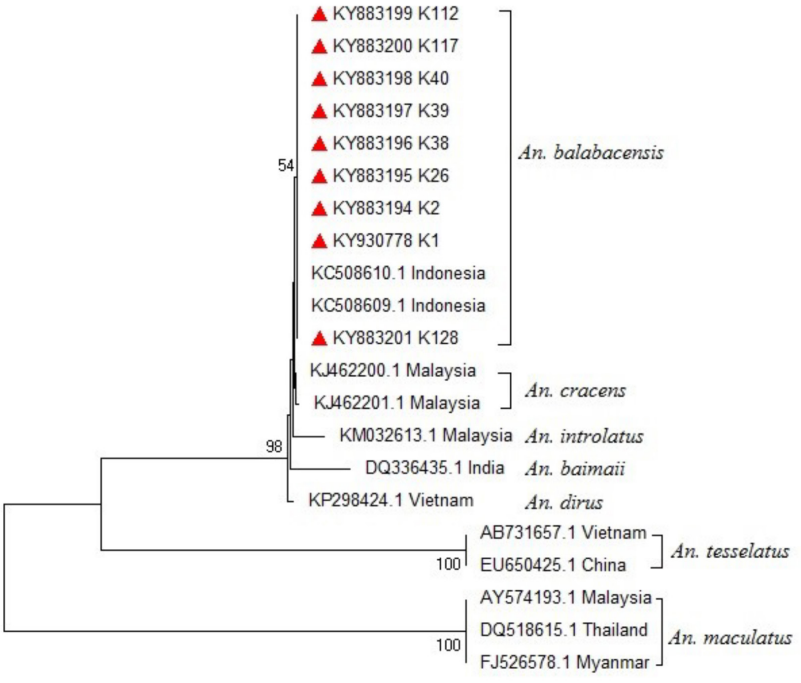

Fig 2–Phylogenetic tree of ITS2 rDNA gene sequences of *Anopheles* species found in our study (▲) compared to our specimens in the GenBank.

turation at 94ºC for 5 minutes, followed by 35 cycles of amplification at 94ºC for 1 minute, annealing at 51ºC for 1 minute, elongation at 72ºC for 2 minutes, and then elongation again for 10 minutes at 72ºC and then the a holding temperature of 4ºC was used. PCR amplicons were subjected to electrophoresis on 1.5% agarose gel (Bioron, Ludwigshafen, Germany).

The amplified product of a reference specimen from the gel was purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sent for sequencing. Sequences were aligned and checked manually using BioEdit software, version 7.2.5 (Applied Biosystems, Warrington, Cheshire, UK). The sequences were compared to representative sequences obtained from the GenBank using Clustal W, version 2. A Neighbour Joining (NJ) phylogenetic tree was constructed using MEGA software (Version 6) with 1,000 bootstrap replicates.

RESULTS

Mosquito identification

A total of 86% of collected larvae emerged into adult mosquitoes. The numbers of *Anopheles* mosquito larvae by breeding site are shown in Table 1. The most common *Anopheles* species identified in this study (with ▲) was *An. balabacensis*. The *Anopheles balabacensis* species found in this study were identical to that found in Indonesia with GenBank accession nos. KC508609.1 and KC508610.1 (Fig 2).

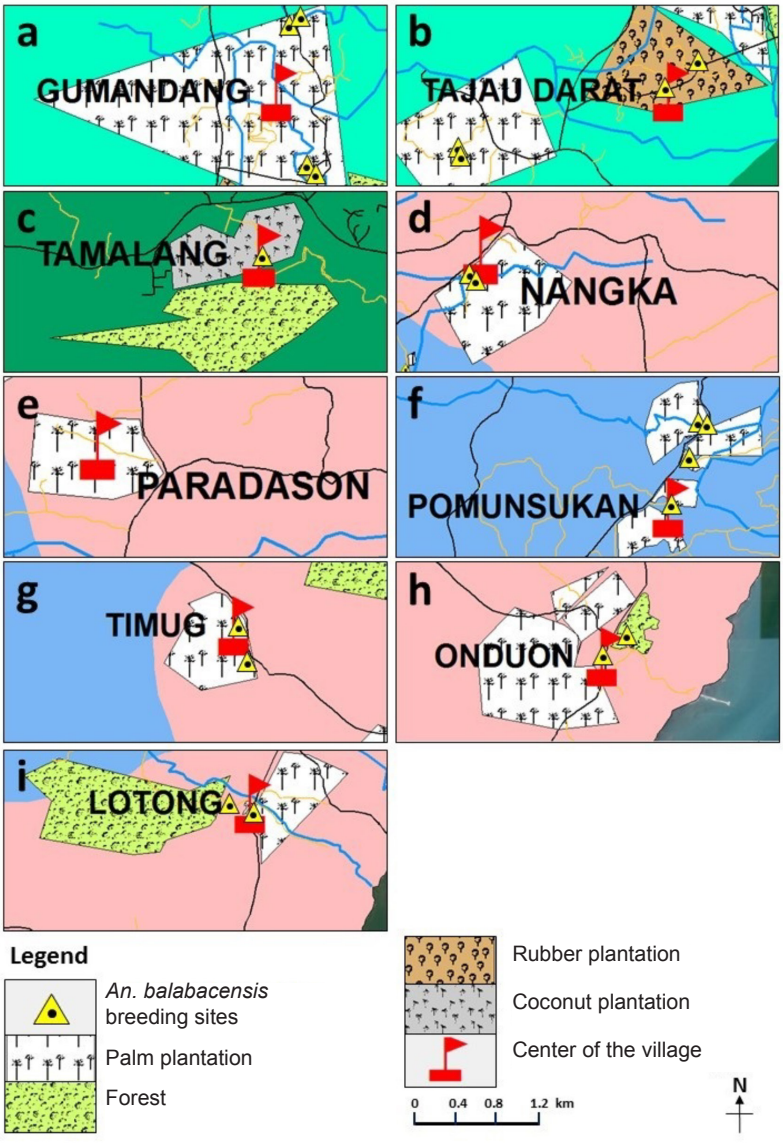

Fig 3–*An. balabacensis* breeding sites and land in the study areas.

ITS2 sequences of *An. balabacensis* from Indonesia were identical to those found in our study.

An. balabacensis habitat characterization

An. balabacensis was found in all the villages studied, except Paradason. *An. balabacensis* was found mostly on palm plantations. Only a few breeding sites were found on coconut plantations, rubber plantations and on the forest fringe (Fig 3).

A total of 97 breeding sites was sampled and 29 of them were positive for *An. balabacensis*. The most common breeding site for *An. balabacensis* was muddy ground pools (n=14), followed by tire tracks (n=6), slow flowing streams (n=4),

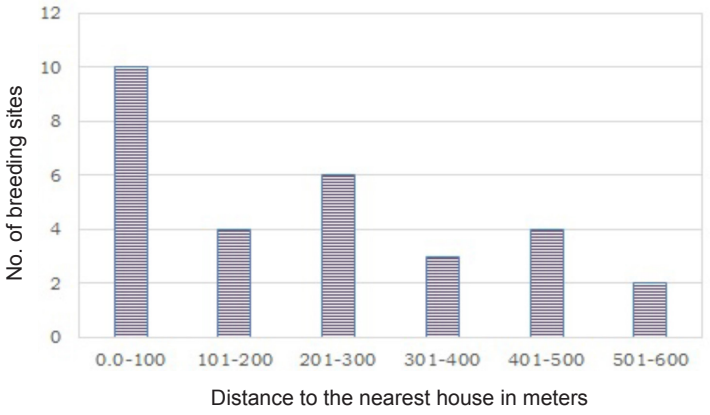

Fig 4–Distance of *An. balabacensis* breeding sites and the nearest house in studied villages.

Table 2
Distribution of the larvae of *Anopheles balabacensis* breeding sites sampled based on habitat type in Kudat, Sabah.

Habitat type	No.
Muddy ground pool	14
Clear ground pool	3
Tire track	6
Slow- flowing stream	4
Water pocket (swamp area)	2
Total	29

clear ground pools (n=3), and swamp water pockets (n=2) (Table 2). Most of the *An. balabacensis* larval breeding sites were located < 100 m from the nearest house (Fig 4). Approximately 80% of the breeding sites were located within 0.4km from the center of each studied village (Fig 5). *An. balabacensis* was found to favor < 1 m^2 in area (Fig 6), fresh, shallow water with a depth of 5.1 to 10.0 cm (Fig 7) and partially shaded (Fig 8).

Water parameters

The water parameters for all types of habitat studied such as pH, dissolved oxygen (DO), water temperature, debris and turbidity of the water were determined on site during the larval survey by means of portable meters. Table 3 shows the range of environmental parameters for the five breeding habitats of *An. balabacensis* found in Kudat. In general, pH of all the breeding habitats that contained larvae of *An. balabacensis* was found to be in the range of 6.4 to 7.9.

The highest DO reading was recorded for the tire track (100.0%). The DO level of water pocket in swamp area was found to be the lowest (0.02%) among all the studied habitats (Table 3). Incidentally the swamp water pocket was also found to be the least preferred breeding site of *An. balabacensis*.

Breeding site water variables of *An. balabacensis* are shown in Table 3. The water temperature range was 22.2 - 32.7°C, the highest being in a slow flowing stream as the lowest being in the clear ground pool. The temperature varied by sampling time. The slow moving stream water had the greatest temperature range. Clear ground pool had the greatest plant and debris coverage (87.5%) and the swamp water pockets has the least (0%). Clear ground pool also had the highest turbidity (132 NTU) and the swamp water pockets has the least (1.1 NTU).

Statistical analysis of environmental variables (Table 4)

Negative correlations were observed between distance to the nearest house and pH (r= -0.331, p<0.05). Similarly, the water depth was observed to be negatively cor-

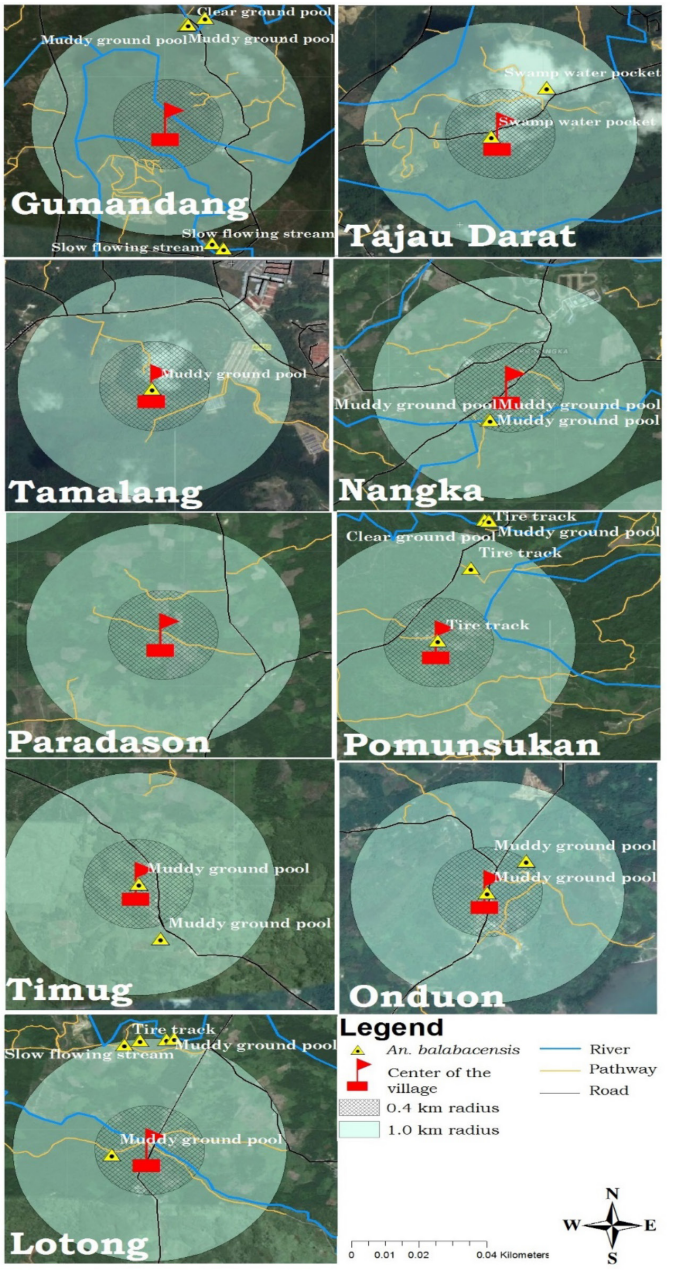

Fig 5–*An. balabacensis* breeding sites in relation to the village center at study sites.

related with turbidity (r= -0.361, p<0.05). On the other hand, positive correlations were observed between debris coverage and dissolved oxygen (r= 0.533, p<0.01). While debris coverage and area size (r= 0.524, p<0.01).

Multiple linear regression analysis revealed no associations between environmental factors and the occurrence of *An. balabacensis* mosquito larvae.

DISCUSSION

In this study, we determined the breeding sites of *An. balabacensis* in Kudat, Sabah, Malaysia and specific ecological factors of those sites. Twenty-nine of *An. balabacensis* breeding sites were sampled from 97 breeding sites of mosquito larvae in these malaria endemic villages. *An. balabacensis* larvae were found by themselves in some sites and found with other mosquito species in other sites. The majority of mosquito bred in water with a surface area <1.0 m^2. A previous study reported that the size of the larval habitat was negatively associated with the *Anopheles* larvae density (Fillinger *et al*, 2009).

The most common *An. balabacensis* breeding site in our study was shallow muddy ground pools found near human settlements and work places where the pools were formed by the foot prints of humans or animals. Our findings are similar to those of Rohani *et al* (1999) who studied the breeding sites of *An. balabacensis* in Ranau, Sabah, Malaysia and found the larvae almost exclusively in shaded, shallow, muddy, turbid freshwater bodies such as animal wallows, tire tracks and at the margins

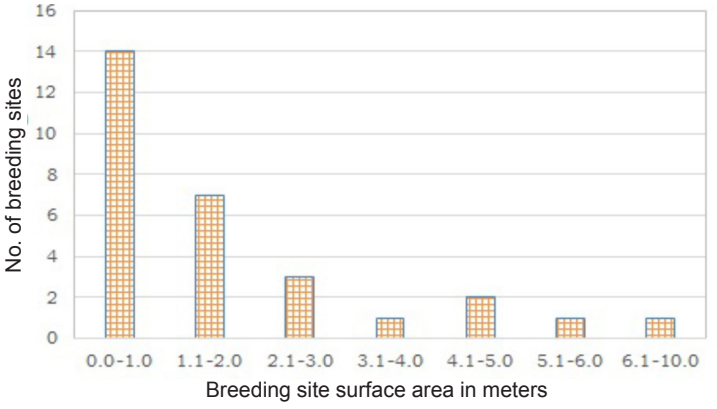

Fig 6–*An. balabacensis* breeding site areas.

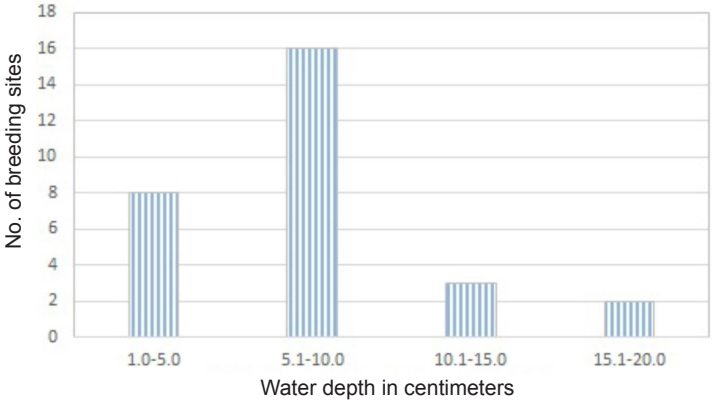

Fig 7–*An. balabacensis* breeding site water depths.

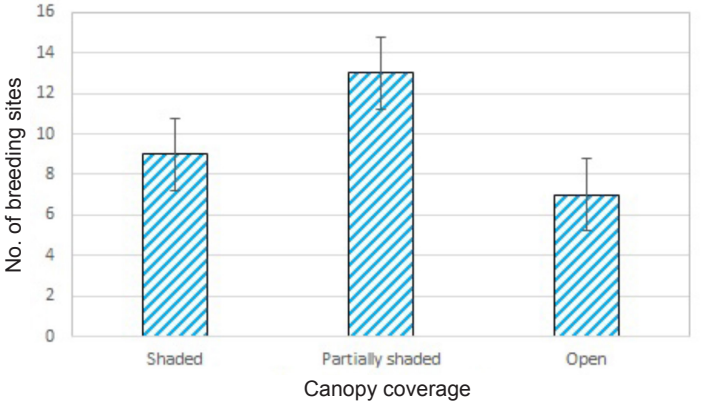

Fig 8–*An. balabacensis* breeding site canopy cover.

of streams. These tire tracks represent a potential transmission area for malaria.

During the 12-month study period most *An. balabacensis* breeding sites occurred during the raining season and disappeared entirely during the dry season. Rainfall is the main water source of mosquito breeding habitats (Oo *et al*, 2002). However, excessive rain can flush out, damage mosquito eggs and kill larvae (Sattler *et al*, 2005; Rohani *et al*, 2010) and inadequate rainfall can reduce breeding sites (Minakawa *et al*, 2005) by 75% during the dry season as compared to rainy seasons (Majambere *et al*, 2008). The density of mosquito larvae and pupae is directly proportional to rainfall (Oo *et al*, 2002).

In our study, *An. balabacensis* preferred larval breeding sites located <100 m from the nearest house. This may be explained by the limited flight distance of gravid *An. balabacensis* females leading the mosquitoes to lay eggs near the source of a blood meal. Our findings are similar to those of Sattler *et al* (2005) who reported 90% of *An. gambiae* were found breeding within 300 m of human habitations. The current study has significant shorter distance as a cut-off point to create a risk map of malaria in Kudat, compared to a dis-

Table 3
Anopheles balabacensis breeding site water variables.

Habitat type	pH of water	Dissolved oxygen (%)	Water temperature (ºC)	Debris coverage (%)	Water turbidity (NTU)
Clear ground pool	7.0-7.7	65.7-79.3	22.2-28.9	45.4-87.5	65.3-132.0
Muddy ground pool	6.4-7.1	12.0-88.4	25.7-27.8	2.4-26.6	32.0-116.0
Swamp water pockets	6.7-6.8	0.0-0.8	27.3-27.6	0-25.0	1.1-9.2
Slow flowing stream/drain	6.7-7.9	20.0-78.7	24.7-32.7	15.4-43.3	40.0-72.0
Tire track	6.4-7.8	15.3-100.0	24.5-30.2	8.5-30.0	35.0-76.0

NTU, nephelometric turbidity unit.

Table 4
Correlation coefficient between environmental variables of 29 breeding sites of *An. balabacensis* in Kudat, Sabah.

	pH	DO	Water temperature	Turbidity	Water depth	Area size	Coverage
DO	0.049						
Temperature	0.238	-0.019					
Turbidity	-0.084	0.246	0.250				
Water depth	0.269	-0.189	-0.226	-0.361*			
Area size	0.227	-0.003	-0.284	-0.121	0.027		
Coverage	0.157	0.533**	0.007	0.095	-0.096	0.524**	
Distance[a]	-0.331*	-0.268	-0.140	0.030	-0.037	-0.720	-0.241

DO, dissolved oxygen; [a]Distance to the nearest house.
*Correlation is significant at the 0.05 level ($p<0.05$).
**Correlation is significant at the 0.01 level ($p<0.01$).

tance of 750 m recommended by Hoek *et al* (2003) in his study in Sri Lanka. Our findings suggest humans are at risk for *P. knowlesi* malaria since the vectors are present nearby as supported by previous study done by Manin *et al* (2016).

In our study, *An. balabacensis* preferred to lay their eggs in partially shaded habitats, similar to the findings of Minakawa *et al* (2005) who studied the breeding sites of *An. gambiae* complex and *An. funestus*. *An. dirus* egg laying sites are also influenced by canopy cover, vegetation and debris (Oo *et al*, 2002). Plant and debris coverage are significantly associated with breeding sites area (Minakawa *et al*, 1999; Ali *et al*, 2012) and dissolved oxygen (Ali *et al*, 2012).

The water temperature in our study varied widely, similar to the findings of Paaijmans *et al* (2008). Warm temperatures tend to accelerates the development of larvae and pupae. Water temperature can also affect the food supply of mosquito

larvae, hence their diversity, density and activity (Paaijmans *et al*, 2008; Zhou *et al*, 2007). Dejenie *et al* (2011) reported both biotic (vegetation and fauna) and abiotic (chemical and physical) factors play a significant role in the breeding site selection of *Anopheles* mosquitoes. Swamp water pockets had the narrowest temperature range (27.3-27.6°C). Swamp water pockets also had the lowest percentage of DO (0.02-0.83%) found in our study, which could be the main reason why *An. balabacensis* choose this the least often to lay their eggs.

Robert *et al* (1998) reported *An. ambiensis* bred in clear shallow (<0.5 m) water. However, Sattler *et al* (2005) found *Anopheles gambaie* also laid their eggs in organically polluted habitats. Sattler *et al* (2005) reported that as long as the turbidity of the water was due to edible particles, turbidity favored *Anopheles* species larval production. Our finding showed that most of the habitats containing larvae of *An. balabacensis* had high turbidity which suggests the turbidity factor may influence the choice of breeding sites.

The transmission of malaria depends on healthy mosquito vectors (Omumbo *et al*, 1998; Wong *et al*, 2015) and is mainly constrained by mosquito flight distance. *Anopheles* mosquitoes have a habitat limited to a maximum flight distance of 2 km (WHO Roll Bank Malaria, 2005). The availability of mosquito breeding sites plays an important role in the transmission of malaria. Changes in land use may have been a factor in malaria epidemics in Kudat. This area has undergone massive deforestation for human settlements, human activities, road construction and crop cultivation, resulting in ecological conditions that increase contacts between humans and monkeys and exposure to mosquito bites, similar to a study by Brant *et al* (2016) who reported the ability for *An. balabacensis* to transmit *P. knowlesi* between canopy-dwelling simian hosts and ground-dwelling humans and that forest distubance increases the abundances of this disease vector.

Malaria control education programs should include mosquitoes breeding sites. Seeking out and eradicating these sites may reduce mosquito numbers. Methods that would help include draining standing water, clearing stream of debris, filling in tire tracks and other areas that filled standing water, especially near housing areas. Plantations also need to be involved in these efforts.

In this study *An. balabacensis* breeding sites were identified and studied. This information can inform mosquito control programs, which need to be consistent and thorough.

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