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Effect of insecticide-treated bed nets on house-entry by malaria mosquitoes: The flight response recorded in a semi-field study in Kenya

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ABSTRACT

Insecticide-treated nets are currently a major tool to reduce malaria transmission. Their level of repellency affects contact of the mosquito with the net, but may also influence the mosquito's entry into the house. The response of host-seeking malaria mosquitoes approaching the eave of an experimental house was recorded within a large screen house. We compared entry- and exit rates in relation to the presence in the house of different insecticide-treated bed nets (ITNs) with an untreated net. Mosquitoes were lured towards the house by dispensing a synthetic host-odour blend from within the net in the house. Complementary WHO bioassays revealed that the treated nets caused high knock-down- and mortality responses to the *Anopheles gambiae* sensu stricto strain tested.

The proportion of mosquitoes that came into view of the cameras and subsequently entered the house did not differ between treated nets and the untreated net. Treated nets did not affect proportions of mosquitoes that exited the house and departed from view around the eave. However, the percentage of house-leaving and re-entering mosquitoes when an insecticide- treated net was present, was lower than in the presence of an untreated net.

Our results indicated that there was no spatial repellent effect from pyrethroid-treated nets that influences house-entry at eave level. It is argued that the toxic effect of treated bed nets resulted in a reduced number of mosquitoes re-entering the house, which could thereby affect malaria transmission in neighbouring, unprotected houses.

1. Introduction

Declines in malaria cases over the last decade are mainly attributed to the use of insecticide- treated nets (ITNs), indoor residual spraying (IRS) and better case management (Bhatt et al., 2015). Increased resistance against insecticides and drugs is considered a major threat to the ability to sustain or further decrease malaria and therefore the continuous assessment of the efficacy of existing tools and the development of new products have been put high on the agenda (WHO, 2016). For example, lure-and kill technologies, repellents and genetic tools promise to provide tools complementary to the existing insecticide-based strategies (Benelli and Mehlhorn, 2016; Hammond et al., 2016; Homan et al., 2016; von Seidlein et al., 2017).

ITNs have been proven effective by reducing contact between humans and mosquitoes infected with the *Plasmodium* parasite (Lengeler, 2004; Lindsay et al., 1989). The effectiveness of ITNs is dependent on the susceptibility of the local mosquito population to the chemicals used (e.g. Malima et al., 2009). In addition, house design and the quality of housing are critical (Tusting et al., 2015). The design of houses together with the use of IRS and the presence of ITNs inside houses are other important factors influencing the vector capacity of mosquitoes (Grieco et al., 2000; Koffi et al., 2015; Massue et al., 2016). Continuous monitoring of intervention tools is necessary to measure whether the methods used are still effective or can be optimized. Behavioural adaptions of malaria vectors to control measures have been observed, but there are few studies done that provide supporting data (Gatton et al., 2013; Mathenge et al., 2001; Takken, 2002). Such adaptations could reinforce residual malaria transmission through mosquitoes biting outdoors. This is of concern for further improving malaria control or eradication (Bradley et al., 2016; Killeen, 2014; Killeen et al., 2016; Russell et al., 2013).

Early studies on the effectiveness of ITNs reported the repellent and

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killing effect of pyrethroids on these nets (Miller et al., 1991; Lengeler, 2004). Malima et al. (2009) reported that carbosulfan, a carbamate, prevents mosquitoes from entering a house. Tungu et al. (2010) found a deterrent effect of unwashed PermaNet 3.0, but not for PermaNet 2.0 or washed nets. Behavioural data supporting these effects are scarce (Miller and Gibson, 1994), and conclusions on repellent effects sometimes differ from studies based on indirect behavioural observations that rely on collections from experimental huts (Siegert et al., 2009). More recent studies implemented behavioural recording techniques, and in these studies the repellent effect of pyrethroids is less evident (Cooperband and Allan, 2009; Parker et al., 2015; Spitzen et al., 2014; Sutcliffe and Yin, 2014; Sutcliffe and Colborn, 2015). The change in interpretations of the repellency of nets may not only be due to more advanced recording techniques. The production process of bed nets has also evolved and repellent effects described earlier could possibly be explained by the emulsifiers used to impregnate the nets (Lindsay et al., 1991). Current nets often lack these components and the active ingredients are incorporated into the net (Spitzen et al., 2014). There is an urgent need for evidence-based studies on the repellent effect of pyrethroids on bed nets, given the rapid development of insecticide resistance in a large number of malaria vectors (Hemingway et al., 2016; Ranson and Lissenden, 2016; WHO, 2016).

Recent advances in tracking techniques make it possible to provide detailed information on flight behaviour around hosts, bed nets and human dwellings without the need for interception traps (Parker et al., 2015; Spitzen et al., 2014; Sutcliffe and Colborn, 2015; Angarita-Jaimes et al., 2016; Spitzen et al., 2016). We investigated if the presence of three different types of pyrethroid-treated nets affected house-entry behaviour of mosquitoes by recording their flight paths near the eave of an experimental house. We hypothesize that, based on short range studies as mentioned above, mosquitoes approaching the eave do not distinguish between the presence inside an experimental house, of an untreated net or a net with insecticides incorporated in the net fibre. The technique used provides information on mosquito behaviour prior to house entry, without the barrier of an interception trap, and can be further developed to support testing and evaluating new generation ITNs, push-pull systems (Menger et al., 2015) and house designs (Tusting et al., 2016).

2. Materials and methods

2.1. Experimental set-up

The study was carried out in West Kenya, Mbita Point township, at the Thomas Odhiambo Campus of the International Centre of Insect Physiology and Ecology (*icipe*-TOC). We video-recorded the approach and house-entry of female mosquitoes on one side of an experimental house that was constructed inside a large screen house. The experimental house had, like many African houses, a wide gap between the top of the wall and the roof. These so-called 'eaves' measured approximately 15 cm in height and were found at both sides of the house. Two windows and a door remained closed during the experiment. For technical details about the set-up we refer to Spitzen et al., 2016 and Supplementary online material, Fig. S1). Exit and entry rates were compared in relation to the presence of different bed nets inside the house: 1) an untreated net, 2) PermaNet 2.0, a long lasting insecticidal net (LLIN) with deltamethrin (dose of 1.4–1.8 g/kg ± 25%) incorporated into a coating on the filaments, 3) PermaNet 3.0, a LLIN with deltamethrin in the side panels (2.1–2.8 g/kg ± 25%) and deltamethrin (4.0 g/kg ± 25%) and piperonyl-butoxide (PBO) (25 g/kg ± 25%) in the top panel and 4) Olyset, a LLIN with permethrin (20 g/kg ± 15%) incorporated into the filament material. Bed nets were kindly provided by Vestergaard Frandsen SA (Lausanne, Switzerland) or obtained from the local market in Kenya. The samples were tested in random order and kept strictly separate in aluminium bags (Lifesystems®, United Kingdom) between experiments. Host odour was standardized using an attractive synthetic blend based on procedures described by Menger et al. (2014) and Smallegange et al. (2010) and consisted; Ammonia 2.5% (v/v), L-(+)-lactic acid 85% (w/w), Tetradecanoic acid 0.0025 g/l, 3-methyl-1-butanol 0.000001% (v/v), Butan-1-amine 0.001% (v/v) including CO_2 produced via sugar fermenting yeast which was dispensed from inside the bed net using an MM-X trap (Supplementary Fig. S1).

2.2. Experimental procedures

2.2.1. Mosquitoes

Mosquitoes were obtained from the *Anopheles gambiae* Giles sensu stricto (Mbita strain) insectaries. Three times a week, the colony was blood fed on a human arm for ten minutes. Rearing procedures are described in Spitzen et al., 2016.

2.2.2. WHO bio-efficacy tests

The bio-efficacy of the tested nets (top panels) was examined using 3 min WHO tube assays (WHO, 2006) and these were done with non-blood-fed, 5–7 day old *An. gambiae* s.s. Ten replicates per treatment were done with an average group size of five mosquitoes per tube. After exposure, the groups of mosquitoes were placed in a single 1 L cup and provided with 6% glucose solution *ad libitum*. Their knock-down status was measured 60 min post exposure and mortality was recorded after 24 h.

2.2.3. Behavioural assays

Five experimental nights per treatment were scheduled. For the analysis, we included experimental nights that were video-recorded for four hours only. Two-hundred non-blood-fed mosquitoes, 3–8 days old, were selected 8–10 h before release in the screen house and placed in 1L plastic cups, provided with a paper towel soaked in water. The MM-X trap dispensed odour from 20:00 h onwards, just before the moment mosquitoes were released and video recorded. At 24:00 h the cameras were stopped and the bed net was removed from over the trap, allowing the mosquitoes to enter the trap. Mosquitoes caught in the trap were collected and counted the next morning (Spitzen et al., 2016). During control experiments, there was no bed net present and the trap started running at 20:00 h so that mosquitoes could be trapped from the moment of mosquito release. This treatment was added to provide basic information on the number of mosquitoes that enter the house in the absence of a bed net. The odour-baited trap thus served as a proxy for a human being asleep in the house. Due to differences in trapping hours, we did not use data from the house without a bed net to compare the entry/exit rates with the other four treatments.

2.3. Data analyses

For mosquitoes that came into view of the cameras, observations of (re-)entry and exit behaviour were compared between treatments using a Generalized Linear Model (GLM) with binomial distribution and logit link function. The effects of sampling day, mean temperature and humidity over 4 h of filming were estimated and fitted as parameters when significant ($P < 0.05$). The fitted model per response category is presented in Supplementary material, Table S1. A pairwise comparison test of least significant difference (LSD) was used in case a significant effect of net type on entry and exit behaviour was observed. SPSS Statistics version 22 (IBM corp., USA) was used for the statistical analyses.

3. Results

3.1. WHO bio-efficacy tests

The effect of the various bed nets on the knock-down and mortality of *Anopheles gambiae* was recorded after exposure to netting in a WHO

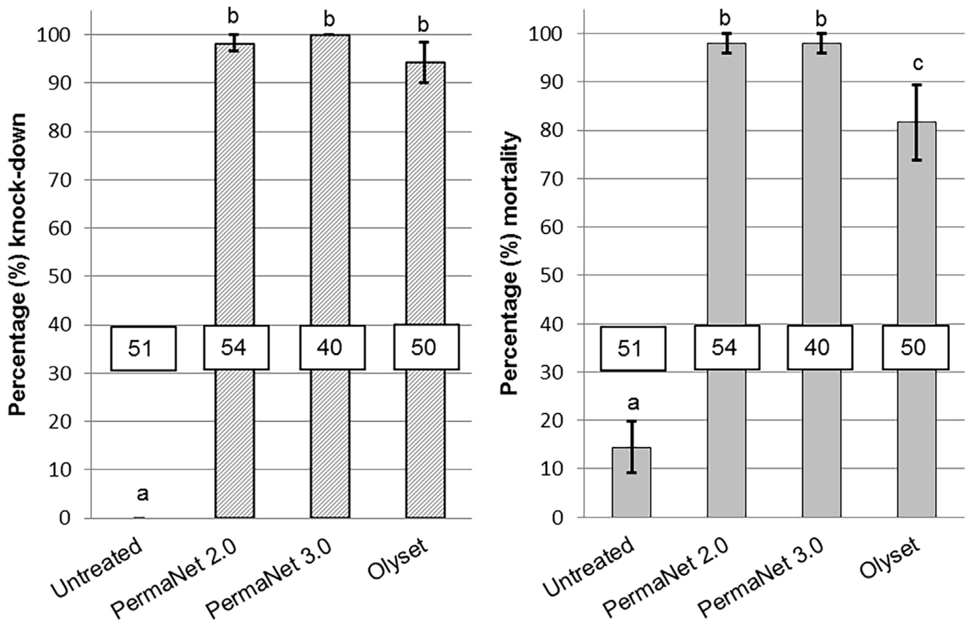

Fig. 1. Mean (± SEM) percentage of knock-down (left panel) and mortality rates (right panel) of *Anopheles gambiae* exposed to an untreated bed net and insecticide-treated bed nets in WHO tube tests for 3 min. Knock-down was measured after one hour and mortality after 24 h. Numbers inside bars represent total number of mosquitoes tested in 10 replicates. Different letters above bars represent significant differences between treatments (Fisher exact test, $P < 0.01$).

tube test. Mosquitoes that were exposed to an untreated bed net were not knocked-down (Fig. 1). PermaNet 2.0 and PermaNet 3.0 caused a 98.33% (SE = 1.67%) and 100% knock-down response, respectively. Knockdown by Olyset was 94.33% (SE = 4.16%). The number of mosquitoes that were knocked down after exposure to PermaNet 2.0, PermaNet 3.0 and Olyset was significantly higher than with the untreated net (Fisher exact test, $P < 0.001$, n = 105, n = 91, n = 101, respectively). There was a significant difference in mortality between the treated nets (Fisher exact test, $P < 0.01$). An average of 98% (SE = 2%) died within 24 h after exposure to either PermaNet 2.0 or PermaNet 3.0. For Olyset, the average mortality after 24 h was 81.67% (SE = 7.78%). Mortality in the group of mosquitoes that was exposed to the untreated net was 14.5% (SE = 5.29%). The number of mosquitoes that died following exposure to PermaNet 2.0, PermaNet 3.0 and Olyset was significantly different from the untreated net (Fisher exact test, $P < 0.001$, n = 105, n = 91, n = 101, respectively).

3.2. The effect of net type on house entry

Mosquitoes were successfully recorded for 19 nights, resulting in 5207 tracks of mosquitoes that came into view of the eave area. As 200 mosquitoes were released per night, it is evident that individual mosquitoes came in view multiple times (19*200 = 3800). Not all scheduled replicates could be completed due to irregularities in power supply. Mosquito activity varied between nights ranging from 68 to 635 tracks per night; however the ratio between the four response categories around the eave showed little variation between nights and treatments (see Table 1). The control, with no net, was not included in the GLM as mosquitoes could be removed from the set-up by entering the trap with odour dispenser. We tested if mosquitoes showed different responses to treatments over a six-week time period by comparing responses within the first, second or third block of two weeks. Testing period had no effect and was left out of the model apart from the response category where mosquitoes came in view and entered (GLM, $P > 0.05$). The mean temperature and humidity over 4 h per testing day was included in the model where an effect was shown (GLM, $P < 0.05$). The fitted model for each response category is given in Supplementary Table S1. The number of mosquitoes that flew towards the house and entered, or exited the house and departed from view did not differ between the treatments, including the untreated net (GLM, d.f. = 3, Wald $\chi^2 = 4.97$ P = 0.174 and Wald $\chi^2 = 2.14$, P = 0.544 respectively). There was a significant effect of net type for mosquitoes that were observed to exit and immediately re-enter the house. More mosquitoes re-entered when an untreated net was present than when there was an insecticide-treated net (GLM, d.f. = 1, pairwise comparison, LSD, $P < 0.05$). Consequently, the proportion of mosquitoes that came into view but did not enter the house depended on net-type, and pairwise comparisons showed that the proportion that did not enter was lower in the presence of untreated nets than insecticide treated nets, GLM, d.f. = 1, pairwise comparison, LSD, $P < 0.001$).

Mosquitoes could enter the trap after recording hours (from 24:00–10:30 h) when the net was removed from the MM-X trap and hung aside. Catches from the MM-X trap are presented in Fig. 2. The day of testing, average temperature and humidity was included in the GLM model but had no effect on numbers caught and were left out of the final model (Supplementary Table S1). Treatment influenced the numbers caught significantly (GLM, d.f. = 3, Wald $\chi^2 = 43.01$, $P < 0.001$) in that fewer mosquitoes were caught when an insecticide-treated net was present. The presence of an Olyset net had an intermediate effect on catch size. Pairwise comparisons showed that catches with the Olyset net were lower than those from the untreated net (GLM, LSD, $P < 0.01$), but higher than the PermaNets 2.0 and 3.0 (GLM, LSD, $P < 0.05$).

Data from the control without a net was not further analysed because of the difference in sampling time (14.5 h vs. 10.5 h).

4. Discussion

Mosquitoes showed susceptibility to the tested ITNs with a nearly 100% knock-down response within one hour after exposure using the WHO tube test. There was no knock-down with the untreated net. Mortality rates after 24 h were significantly lower for the Olyset net, but greater than 80% for all treated nets.

The percentage of mosquitoes that exited and re-entered the house was higher when an untreated net was present compared to ITN's. When exposed over a period of 4 h, and given the susceptibility of the mosquitoes to the insecticide-treated nets, it is likely that a proportion of the tested mosquitoes was affected after touching the treated net.

Table 1
Response of mosquitoes around the eave in percentages, based on number in view of cameras. The control without a net is not included in the statistical analysis. The fitted model for each response category is given in Table S1 of the Supplementary material. Different letters behind mean responses within a column indicate significant differences between the nets (pairwise comparison, GLM, * $P < 0.05$, ** $P < 0.001$).

Treatment	*No. of tracks (200 mosquitoes released per night)*	*% not entering eave*	*% entering eave*	*% exiting eave & departing*	*% exiting eave & re-entering*
Control	285	73.3	14.7	2.1	9.8
(no net)	267	65.9	16.1	4.9	13.1
	173	65.9	12.7	8.7	12.7
	116	62.1	21.6	7.8	8.6
total/*average* 841	*210.3*	*66.8*	*16.3*	*5.9*	*11.1*
Untreated	198	66.7	18.2	4.5	10.6
	295	51.2	12.5	5.4	30.8
	233	49.4	14.2	10.3	26.2
	635	59.7	18.4	7.2	14.6
	212	49.5	15.1	20.8	14.6
total/*average* 1573	*314.6*	*55.3a* **	*15.7*	*9.7*	*19.4a* *
PermaNet 2.0	205	68.3	16.1	3.4	12.2
	286	67.8	16.1	5.6	10.5
	334	65.0	13.8	9.9	11.4
	105	62.9	12.4	11.4	13.3
total/*average* 930	*232.5*	*66.0b*	*14.6*	*7.6*	*11.8b*
PermaNet 3.0	555	73.3	11.7	4.7	10.3
	508	67.5	13.4	6.5	12.6
	286	63.3	19.6	4.9	12.2
total/*average* 1349	*449.7*	*68.0b*	*14.9*	*5.4*	*11.7b*
Olyset	68	67.6	14.7	7.4	10.3
	319	69.9	14.7	6.6	8.8
	127	67.7	11.0	11.0	10.2
total/*average* 514	*171.3*	*68.4b*	*13.5*	*8.3*	*9.8b*

This is supported by the number of catches in the MM-X trap after the tested net had been removed and hung beside the odour-baited trap (24:00–10:30 h). Fewer mosquitoes were trapped with exposure to ITN treatments than with the untreated net. The deltamethrin containing nets (PermaNet 2.0 and PermaNet 3.0) caused higher knock-down and mortality rates compared to the permethrin-treated net (Olyset) in the tube tests and this is reflected by the higher capture rates following exposure to the Olyset net. As expected, with an untreated net the percentage of mosquitoes that exited and re-entered was higher (Table 1).

The aim of our study was to determine if there was a spatial repellent effect of three different ITNs on house entry of mosquitoes. The number of mosquitoes observed to enter through the eave when an ITN was present inside, was not lower than without a net or when an untreated net was used. Consequently, there was no spatial repellent effect of the tested ITNs at eave level. As recently concluded by Parker et al. (2015), and supported by other studies (Cooperband and Allan, 2009; Spitzen et al., 2014), host seeking is affected by pyrethroid treated nets but not as a result of repellent effects. More likely, mosquitoes are affected by the physical barrier of the net and continue host-seeking as long as they have not had an opportunity to blood feed. A proportion leaves the house to search elsewhere if not affected by the toxic effect of the insecticide after initial contact with the treated nets. A different effect is shown for the carbamate carbosulfan, where

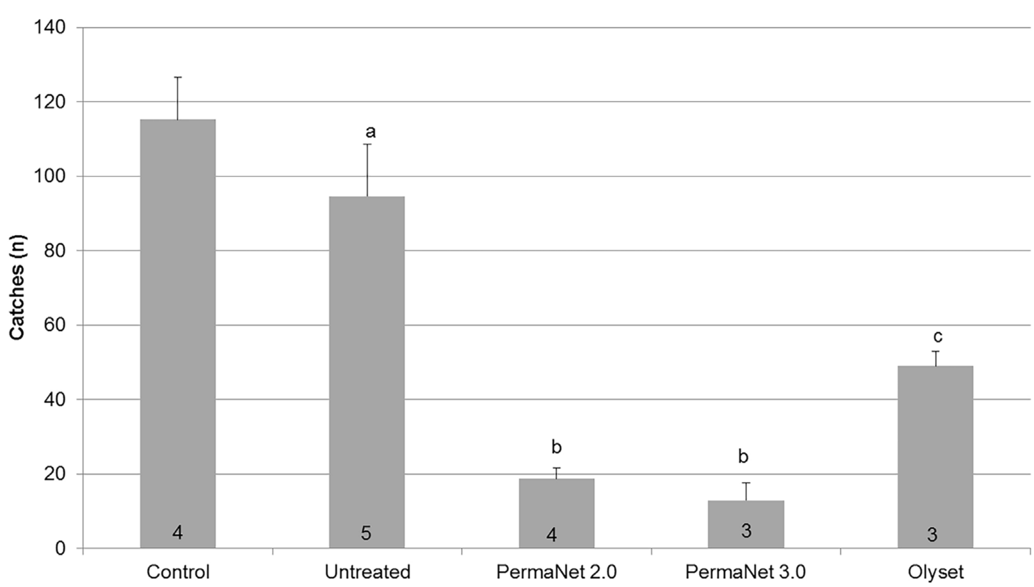

Fig. 2. The average number (+SE) of *Anopheles gambiae* caught in the MM-X traps following exposure to differently treated bed nets. Mosquitoes were trapped from 24:00 h till 10:30 h with the only exception for the control where no net was present. In the control mosquitoes were trapped from 20:00 h till 10:30 h. Different letters above bars indicate significant differences between treatments (GLM, pairwise comparisons, LSD, $P < 0.05$). Numbers inside bars represent number of test nights.

Malima et al. (2009) mention a deterrent effect on house entry when bed nets were treated with this class of chemicals.

Earlier studies by Miller et al. (1991) and Darriet et al. (1984) reported a reduction of mosquitoes entering houses when pyrethroid treated bed nets were used. We allocate this difference to the different production process of modern nets, where previous repellent effects were possibly caused by the emulsifiers used to impregnate the nets (Lindsay et al., 1991). LLINs often lack these components and the active ingredients are incorporated into the net which may affect their repellent properties (Spitzen et al., 2014). Besides, possible repellent effects may be overcome by the natural presence of host odours, as indicated by Kongmee et al. (2012) and further discussed by Sutcliffe and Yin (2014). An exception to this theory seems an experimental hut trial using interception traps where the authors report a deterrent effect of unwashed PermaNet 3.0, but not for PermaNet 2.0 or washed nets. (Tungu et al., 2010). Interpreting results from studies on the repellent effect of IRS on house entry should also consider the emulsifiers or solvents used, the presence and quality of host odours, and whether the observed effect is a result of toxicity rather than repellency (Grieco et al., 2000; Killeen and Moore, 2012; Sutcliffe and Yin, 2014).

Although we could not follow behaviours of individual mosquitoes throughout and focused on the area where most house entry occurs, we obtained some valuable information on the proportion of mosquitoes that moved in and/or out via the eave. Exit and re-entry behaviour could be a result of mosquitoes repeatedly attempting to get to a blood meal while being obstructed from doing so by the bed net. Whether a proportion of these exiting mosquitoes would still suffer from a sub-lethal effect as a result of the insecticides could not be observed in the present study.

When there is no repellent effect of the ITNs, selection for resistance to pyrethroids is even faster than when there is a repellent effect. Mosquitoes that are repelled by an ITN may still be able to find another blood host and survive long enough to contribute to the gene pool of the population (Takken, 2002).

5. Conclusion

This study provides further evidence, based on behavioural recordings of mosquitoes, that bed nets with the insecticides incorporated into the netting do not repel mosquitoes. However, ITNs will remain an important barrier to spread the malaria parasite to humans, especially as long as the mosquito population remains susceptible to the insecticides used. Analysing behaviour of vectors in (semi-)field settings can contribute to the development and effectiveness of new intervention strategies, such as push-pull studies (Okumu et al., 2012; Menger et al., 2015; Homan et al., 2016). Increasing the field of view of the cameras and linking outdoor flight behaviours with behaviours inside a house would further benefit the evaluation of repellents or physical barriers.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2017.05.008.

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