Correlations between age, distance from aquatic habitats and pyrethroid resistance status of field populations of the malaria vector, Anopheles funestus in south-eastern Tanzania

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Research Article

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Abstract

Background: Malaria transmission can be highly heterogeneous between and within villages, and is influenced by factors such as survival and biting frequencies of *Anopheles* mosquitoes. This study investigated the correlations between biological age, distance from aquatic habitats and pyrethroid resistance status of *Anopheles funestus* mosquitoes in south-eastern Tanzania; and how these correlations may inform improved vector control strategies.

Methods: Female *An. funestus* were collected in houses located 50-100m, 150-200m or over 200m from the nearest known aquatic habitats. The mosquitoes were exposed to 1×, 5× and 10× the diagnostic doses of either deltamethrin or permethrin, or to the synergist, piperonyl butoxide (PBO) followed by the pyrethroids, then monitored for 24hr-mortality. Ovaries of exposed and non-exposed mosquitoes were dissected to assess parity status as a proxy for biological age. Adults emerging from larval collections in the same villages were exposed to the same insecticides at 3-5, 8-11 or 17-20 days old to compare resistance.

Findings: Mosquitoes collected nearest to the aquatic habitats (50-100m) had the lowest mortality compared to other distances, with a maximum of 51% mortality at 10× permethrin. For the age-synchronized mosquitoes collected as larvae, the percentage insecticide-induced mortality, assessed at both the diagnostic and multiplicative doses (1×, 5× and 10×) increased with mosquito age. The highest mortalities at 1× doses were observed among the oldest mosquitoes (17-20 days). At 10× doses, mortalities were 99% (permethrin) and 76% (deltamethrin) among 8-11 day-olds compared to 80% (permethrin) and 58% (deltamethrin) among 3-5 day-olds. Pre-exposure to PBO restored the potency of both pyrethroids. The proportion of parous females was highest among mosquitoes collected farthest from the habitats.

Conclusion: In this specific setting, older *An. funestus* and those collected farthest from the aquatic habitats (near the centre of the village) were more susceptible to pyrethroids than younger ones and those caught nearest to the habitats. These findings suggest that pyrethroid-based interventions may remain at least moderately effective despite widespread pyrethroid-resistance by killing the older, less-resistant and potentially-infective females. Further studies should investigate whether fine-scale targeting of key interventions (e.g. preferentially spraying homes near the centres of villages) would be more impactful.

Background

The risk of malaria in endemic communities is influenced by multiple environmental and biological factors, often leading to heterogeneous distribution, which can be further influenced by intervention use [1–4]. These factors include the distribution of mosquito breeding habitats and human dwellings [5–7], as well as mosquito-related factors such as blood-feeding behaviours, dispersal range and non-random host selection [8–11]. When human settlements are close to the breeding sites, the range at

which mosquitoes can disperse is limited [12], and vector densities tend to be highest where the settlements are most concentrated [11]. These variations can occur at all levels of malaria endemicity and geographical scales. However, they tend to be greater in low-transmission compared to high-transmission areas, and can be significant over fine geographic scales such as between or within villages [1].

The landscape-level variations, especially as influenced by non-random mosquito-biting frequencies, have been elucidated broadly both *in situ* and *in silico*, and are known to have significant epidemiological implications [1,9,13]. Understanding these variations of malaria transmission risk can enable more strategic approaches and better resource allocation for malaria control, even though the actual impact of such targeting remains unclear and poorly understood [14]. It is well known that female *Anopheles* mosquitoes can travel for varied distances [12,15], but final aggregations of these vectors tend to be highest where human densities and household occupancy are greatest [11]. Also, while mosquito densities tend to be highest near their aquatic habitats, it is the females found far from their larval habitations that are more likely to be old and infected with malaria parasites [7]. Furthermore, the infectivity of wild-caught mosquitoes depends on their biological age and the density of the parasite at ingestion [16]. This implies differential infectivity since malaria vectors generally require over ten days to ensure complete parasite development and maturation [17].

Beyond the environmental factors and their influence on mosquito dispersal, experimental observations have also shown that the susceptibility of mosquitoes to insecticides can increase with age; and that older mosquitoes are generally more likely to be killed than younger ones [18–20]. Moreover, resistance in major malaria vectors can vary seasonally and spatially, in some cases at fine geographical scales [21,22]. The observations may also have direct implications on the effectiveness of the insecticidal interventions commonly used against malaria vectors, namely insecticide-treated nets (ITNs) and indoor residual spraying (IRS). Unfortunately, there is currently limited research on how these interactions play out in communities such as rural south-eastern Tanzania, where ITNs have been widely used for more than two decades; and malaria transmission risk has become highly fragmented on a spatial scale, with greater than 10 fold differences in transmission intensity over short distances less than 50 km [23–27]. In these areas, the dominant vector species is *An. funestus*, which mediates over 80% of all malaria transmission events [25,26]. In particular, there has not been any detailed analysis that integrates observations of the environmental factors (e.g. distance from habitats) and biological factors (e.g. mosquito infectivity and dispersal rates) with intervention-related factors (e.g. insecticide resistance levels in the dominant malaria vectors); and how such relationships may influence disease transmission and control.

The aim of this current study was therefore to conduct a fine-scale assessment of the interplay between key biological and environmental factors that influence malaria transmission by *An. funestus* in rural south-eastern Tanzania. The key factors investigated were: a) age of *An. funestus* mosquitoes, b) the susceptibility of these mosquitoes to pyrethroid insecticides, and c) the spatial distances from nearest aquatic habitats.

Methods

Study site

Adult female *An. funestus* mosquitoes were collected from the spatially isolated hamlet in Ikwambi village in Kilombero district, south-eastern Tanzania (location: 7.98033°S, 36.81701°E; altitude: 400 m above the sea level; annual rainfall: 1200-1800 mm; temperatures: 20-33 °C) [28] (**Fig. 1**). *An. funestus* dominates this village in both densities and malaria transmission activity. The collections were done in the dry months from August to early December 2020.

Mosquito collections

Initial surveys were undertaken to identify and mark all possible aquatic habitats of *An. funestus*, based on attributes and methods previously described by Nambunga *et al* [29]. The distance from confirmed habitats of *An. funestus* to human habitations were calculated based on geo-locations data obtained using a handled GPS receiver (eTrex, Vista, Garmin, USA). Three distance ranges were defined from the aquatic habitat as follows; a) 50-100 m (edge of the village), b) 150-200 m (intermediate distance), and c) over 200 m (at the centre of the village) (**Fig. 1**).

Centre for Disease Control and Prevention (CDC) light traps placed beside volunteer-occupied bed nets were used to collect host-seeking female mosquitoes indoor from 18:00 to 07:00 hours each night. The collections, were grouped by distance and acclimatized for 24 hours in an insectary where they were supplied with 10% glucose, and only the survivors used for subsequent tests. Additional collections were done using volunteer-occupied miniaturized double net (DN-Mini) traps placed near human dwellings [30]. The identification of the collected mosquitoes was done using dichotomous keys of African *Anopheles* [31].

Besides the adult collections, *An. funestus* larvae were also collected using procedures described by Nambunga *et al*, [29] and transported in their natural water for onward rearing in the insectary (at 80 ± 10% relative humidity and 27 ± 2°C) at the Ifakara Health Institute vector biology laboratory, the VectorSphere. Tetramin® fish food (Tetra GmbH, Melle, Germany) was provided to supplement the aquatic diet, and the pupae collected were placed in separate cages supplied with 10% glucose. The emergent mosquitoes were collected in three age groups, as follows; i) 3-5 days, ii) 8-11 days and iii) 17-20 days.

Insecticide resistance bioassays

Two candidate pyrethroids (deltamethrin and permethrin) were evaluated using WHO guidelines [32], starting with standard insecticide doses (1×) for phenotypic resistance evaluation, followed by 5× and 10× the standard doses to assess intensities of resistance. Since pyrethroid ITNs were widespread in the area, additional tests were done using the synergist, piperonyl butoxide (PBO), to investigate the role of cytochrome P450 enzymes, and the potency of PBO in reversing resistance [21,22].

Each assay consisted of six replicates with 20 individual mosquitoes per replicate, totalling 120 mosquitoes per bioassay. In the first round of bioassays (using adults collected from houses), the tests were done separately for each distance range from aquatic habitats (50-100 m, 150-200 m and over 200 m); and in the second round (using age-synchronized adults collected as larvae) the tests were done separately for the different age classes (3-5, 8-11 and 17-20 days). Mosquitoes were exposed to the insecticides for 60 minutes and moved to non-insecticidal holding tubes with 10% glucose, then monitored for 24 hours post-exposure mortalities. Since the mosquitoes were resistant to the standard concentrations of both pyrethroids, the additional tests for resistance intensity were completed as prescribed by WHO [32].

Tests with the synergist used four cohorts of mosquitoes, exposed to: i) only deltamethrin or permethrin at standard concentration, ii) 4% PBO followed by the pyrethroids, iii) 4% PBO only, or iv) silicone oil coated papers (control group). Each test was repeated thrice, and the mosquitoes provided 10% glucose. Mortality was recorded 24 hours post-exposure.

Dissections to assess parity status and gonotrophic cycles

The mosquitoes exposed to insecticides, if alive or freshly dead after the 24 hours of mortality monitoring, were immediately dissected (n=1,577), the rest being too dry to be dissected. The dissections were done using a stereo-microscope and the dissected ovaries were observed under a compound microscope for the presence or absence of the coiled tracheolar skeins indicating nulliparous or parous status respectively [33,34]. An additional cohort of mosquitoes collected using the CDC light traps (n=560) and the DN-Mini trap (n=78), and not tested for resistance were dissected for more detailed identification of the ovariole dilations to determine how many times the mosquito had laid eggs (i.e. the number of gonotrophic cycles) [34,35]. The results of the dissections were recorded by distance from the aquatic habitat(s) and method of collection.

Molecular identification of the mosquitoes

A sub-sample of the tested mosquitoes (at least 10% from each replicate) was packed in micro-centrifuge tubes containing silica desiccant. Sibling species of *An. funestus* were identified by PCR using nucleic acid material extracted from the legs, to screen for species-specific nucleotide sequences (internal transcribed spacer 2) in the ribosomal DNA (rDNA) [36].

Data analysis

The percentage mortality of mosquitoes was calculated as a fraction of the total number exposed and interpreted according to the WHO guidelines [32]. Since no control mortalities exceeded 5%, no statistical correction. The mosquitoes were considered susceptible if mortality was 98%, resistant, if mortality was < 90% or possibly resistant and requiring additional tests if mortality ranged between 90% and 97% [32]. The mean proportions of parous mosquitoes at different distances were compared using the analysis of

variance (ANOVA). To determine which specific distance pairs were significantly different, a Tukey's post hoc test was applied.

Additional analysis was done using statistical software, R-Software version 3.6.0 [37]. A generalized linear mixed effect model (GLMM) with binomial distribution and logit link function was used to assess the proportion of mosquitoes died 24hrs post-insecticide exposure at different age groups and distances. Age and distance were added as fixed factors, whereas replicate was added as a random factor. The nearest distance to the habitats (50-100 m) and the youngest age class of mosquitoes (3-5 days) was considered as references for these analyses. Odd ratios with their corresponding 95% confidence intervals were reported. Statistical significance was considered when the p-value is less than 5%.

Results

Relationship between insecticide susceptibility and distance from aquatic habitats

Mosquitoes collected in different houses were resistant to both deltamethrin and permethrin at the standard doses (1×) but the percentage of mortality varied by distance from the aquatic habitats (**Fig. 2**). Mosquitoes collected farthest from the habitats showed significantly higher mortality than those collected nearest to the habitats (Table 1). A similar trend was observed when the mosquitoes were exposed to five and ten times the standard doses of both insecticides, with mosquitoes from the nearest distances showing the lowest mortality and those at the farthest distance showing the highest mortality. Overall, there was lower mortality in tests against deltamethrin compared to permethrin (**Fig. 2**). Further analysis showed that the association of 24-hour mortality with distance was statistically significant for deltamethrin only at the standard dose, and for permethrin at all doses (**Table 1**).

Pre-exposure to the synergist, PBO, significantly increased the potency of the candidate pyrethroids against the resistant mosquitoes, achieving more than 80% mortality at all distances (**Fig. 3**). However, the association of 24-hour mortality with distance in these PBO tests was statistically significant only for deltamethrin (**Table 1**).

Table 1: Summary of 24-hour mortality of *Anopheles funestus* mosquitoes in tests of adults collected at different distances from aquatic habitats. The table also shows the odds of dying among mosquitoes collected at different distances and exposed to deltamethrin or permethrin with or without the synergist, piperonyl butoxide (PBO).

Relationship between biological age of mosquitoes and distance from aquatic habitats

A total of 1397 female *An. funestus* were dissected from the first cohort. Although the overall difference was statistically significant, only the mosquitoes collected by CDC light traps farthest from aquatic habitats (over 200 m) had a significantly greater proportions of parous mosquitoes compared to those collected nearest to the habitats (50-100 m) (p=0.016, **Fig. 4**). Some cohorts of mosquitoes collected beyond 200 m were 100% parous. When the additional cohort of mosquitoes collected by the light traps

was assessed for the number of gonotrophic cycles (n = 560), the majority were found to be nulliparous; and those with multiple gonotrophic cycles were equally distributed over the distances (50-100 m, 150-200 m, over 200 m) (**Fig. 5**). On the other hand, among the mosquitoes collected by DN-Mini (n = 78), the proportion that was nulliparous dropped while the proportion with multiple gonotrophic cycles was slightly higher at 150-200 m and 200 m distances compared to 50-100 m (**Fig. 5**).

Relationship between insecticide susceptibility and chronological age of mosquitoes

The mosquitoes collected as larvae were used to assess variations of insecticide susceptibility in the different chronological age classes. For both pyrethroids, at the standard dose (1×), the percentage of mortality increased with age, suggesting that the older *An. funestus* were more susceptible than younger ones from the same locations (**Fig. 6 & Table 2**). Similarly, in the intensity bioassays conducted with 5× or 10× the standard doses, the levels of resistance varied by age, but mortality significantly increased, reaching 99% for permethrin and 81% for deltamethrin (**Fig. 6**). Further analysis showed that the association of 24-hour mortality with mosquito age was statistically significant for both deltamethrin and permethrin at all doses (**Table 2**).

Table 2: Summary of 24-hour mortality of *Anopheles funestus* mosquitoes collected as larvae and exposed to pyrethroids at different age classes (3-5, 8-11 or 17-20 days old). The table also shows the odds of dying among mosquitoes exposed at different ages to deltamethrin or permethrin.

Molecular identification of the mosquitoes

A total of 409 *An. funestus* mosquitoes were tested by PCR to identify sibling species in the *An. funestus* group, including 60% (n=245) from mosquitoes collected as adults; and 40% (n=164) of the adults emerging from larval collections. The amplification rate for the adult collected mosquitoes was 95% (n=123), and all of them (100%) were identified as *An. funestus sensu stricto* (*s.s.*). On the other hand, the amplification rate for those collected as larvae was 90% (n=148), of which 99% (n=147) were *An. funestus s.s*, the remaining 1% being *An. rivulorum*.

Discussion

This study demonstrated a positive association between the mortality outcomes of *An. funestus* females exposed to the pyrethroid insecticides and the distance between the sites where these mosquitoes were collected and the nearest aquatic habitats (**Table 1**). The positive association was observed in tests with the standard dose of insecticides as well as in tests with the five and ten-fold increases in the standard dose. The highest mortalities were obtained among mosquitoes farthest from the aquatic habitats and nearest to the centre of the village. Compared to the baseline distance (50-100 m), mosquitoes collected at over 200 m and exposed to permethrin and deltamethrin died in the greatest proportions (**Fig. 2**). Also, while the statistical relationship between distance and mortality outcomes was apparent for both deltamethrin and permethrin, statistical significance was observed mostly for deltamethrin (**Table 1**).

A more detailed understanding of these variations and their potential epidemiological implications would require a concurrent analysis of the age distribution of the mosquitoes over the same distances. Mathematical predictions by Smith *et al* had shown that human-biting densities would be greatest near breeding sites where adult mosquitoes emerge, even as the proportions old enough to transmit malaria would be found far from the habitats [7]. An important question, therefore, was how the insecticide resistance profiles influence this system, and whether these potentially infectious adults would also be the most susceptible as suggested by the first experiments in this current study. Indeed, in this current study, the proportion of parous females was greatest among the mosquitoes collected farthest from the aquatic habitats, compared to those collected nearest to the aquatic habitats (**Fig. 4**). Based on this data the age distribution of *An. funestus* appeared to increase from the edge of the village where the mosquitoes emerged (**Fig 1 & Fig 4**) towards the centre of the village. These findings validate previous studies using the mark-release and recapture techniques, where older female *Anopheles* mosquitoes were collected at the furthest distance, over 600m from a point of release and survived over 17 days in the wild [15]. Such non-random age distribution of mosquito populations could have a direct impact on the disease transmission risk [6,7,9]. This current study provides improved understanding of these aspects by illustrating both the age distribution and mosquito responses to insecticide exposure over the same distances.

Since studies on landscape distribution of the biological age of mosquitoes can be affected by the methods used for mosquito collection, this current study included an additional set of collections during which both the CDC light traps placed indoors and the DN-Mini traps placed outdoors were used. Here, less than 30% of the mosquitoes caught by the CDC had two or more gonotrophic cycles, and the maximum number of cycles was three (**Fig. 5)**. These low proportions of low old mosquitoes in light traps may reflect the tendency of CDC light traps to catch mostly nulliparous mosquitos in such settings [38,39]; but also the fact that in this study, only live or freshly dead mosquitoes were dissected, yet parous mosquitoes can be more likely to die during collection [38]. However, when the DN-Mini trap was used, the proportion of nulliparous mosquitoes decreased (relative to the CDC light trap collections), and there were significant proportions with at least one or two gonotrophic cycles (**Fig. 5**). Moreover, there was a slight increase in proportions with multiple gonotrophic cycles among mosquitoes collected at the second and third distance ranges (over 200 m) compared to the nearest distance (50-100 m). This comparison also emphasizes the observations that the two traps used here yield biased catches, which must be considered when interpreting the findings [30,40].

In the tests using mosquitoes collected as larvae from the field sites, the age-synchronized females also showed positive correlations between the chronological age classes and the insecticide potency. The youngest *An. funestus* (3-5 days old) were the most strongly resistant to the candidate insecticides (**Fig. 6**), while the oldest mosquitoes (17-20 days old) were the least resistant. These findings suggest that despite widespread pyrethroid resistance, older mosquitoes may remain substantially susceptible, and that insecticidal interventions may retain some degree of efficacy in the communities. This is important since it is the older mosquitoes that are responsible for parasite transmission; mosquitoes need approximately two weeks for parasite maturity in their bodies [7,17]. Future research should therefore

investigate whether this demographic of mosquitoes can or should be selectively targeted and how such a strategy could impact malaria control. Several studies have indeed demonstrated age-related variations in insecticide resistance, in other mosquito species other than *An. funestus* [18–20,41–43], but practical applications of this knowledge remain limited. However, other factors like blood feeding and larval nutrition play a role in insecticide-induced mortality [43,44]. The observation that permethrin was more potent than deltamethrin is also important for consideration, though this itself is unlikely to have major implications as the differences were marginal.

The tests where mosquitoes were pre-exposed to PBO showed potential significant involvement of mixed-function oxygenases in the resistance. Overall, the potency of pyrethroids and mortalities significantly increased after the PBO exposures, reaching between 82% and 98% (**Fig. 3**). The lack of full recovery in some mosquitoes may suggest that additional mechanisms may be involved in the resistance profile of the local *An. funestus* mosquitoes. Interestingly, even in these PBO tests, there was evidence of the effects of distance; the maximum mortality was observed among mosquitoes collected beyond 200 m (98% compared to 85% among mosquitoes collected at 50-100 m from the aquatic habitats). However, when all the PBO tests data were analysed together, there was no significant correlation between the distances and the performance of PBO (**Fig. 1 &Table 1**). These results may corroborate previous findings in which age-related pyrethroid resistance was determined to not be a function of metabolic resistance gene expressions in *An. funestus* [41].

Despite the broad success of this study in analysing the correlations between biological ages, distance from aquatic habitats and pyrethroid resistance status of *An. funestus* mosquitoes, there were a few limitations. Firstly, as previously described by Charlwood *et al.*, the older mosquitoes are more likely to die during collection when the CDC-LT is used during specimen collection [38]. This might have marginally confounded the age evaluation of both insecticide pre-exposed and non-exposed cohorts. However, age synchronised, larval collected mosquitoes showed the same trend as the mortality of mosquitoes increased with the increased age and concentration. Thus, these results could still be considered informative. Secondly, fewer mosquitoes from the farthest distance (over 200 m) were dissected compared to the other distances as most of them were susceptible to the insecticide, and were therefore dead and too dry to be dissected. The effect of insecticide pre-exposure on dissection was rectified by the additional collections done using both CDC light traps and DN-Mini. Lastly, due to the difficulties of collecting *An. funestus* at the larval stage, the PBO pre-exposure tests were done only on the adult collections but not on the age-synchronized mosquitoes collected as larvae.

Conclusion

In south-eastern Tanzania, where *An. funestus* now dominates malaria transmission, the older mosquitoes are more likely to be found farthest from the aquatic habitats and near the centre of the villages, and are also less resistant to pyrethroid insecticides. Similarly, in the age synchronized mosquitoes, the oldest cohorts were the least resistant to the candidate pyrethroids. For permethrin but not deltamethrin, the oldest cohorts attained susceptibility thresholds at five times the standard dose,

compared to the youngest cohorts, which remained resistant even at 10 times the standard dose. These findings provide insight on how the insecticide-based interventions could still provide a level of protection against malaria in the communities irrespective of the strong insecticide resistance currently reported. To achieve the maximum outcomes, the interventions may need to be spatially targeted even at fine-scale, instead of being randomly distributed. The targeting may enable effective tackling of the older population, which drives malaria transmission and is more likely to be most impacted by the insecticides.
Since older, more susceptible mosquitoes are mostly in the middle of the villages, one possible option for achieving targeted control may include a ringed approach involving indoor residual spraying or high-quality ITNs (with either PBO or dual actives) at the centre, and larval source management at the edge of the villages. While such a strategy remains to be evaluated, it could potentially be useful where resources are limited and the vector control programs are logistically challenging to roll out throughout the communities.

Abbreviations

ITS2: internal transcribed spacer 2 region; IHI: Ifakara Health Institute; IRB: Institutional Review Board; LLINs: Long-lasting insecticidal nets; NIMR: National Institute for Medical Research; CDC: Centre for disease control and prevention; PBO: Piperonyl butoxide; IRS: indoor residual spraying.

Declarations

Ethics approval consideration

This study was permitted by the Institute Review Board of Ifakara Health Institute; IHI/IRB/No: 26-2020 and Medical Research Coordinated Committee of the National Institute for Medical Research of the United Republic of Tanzania; NIMR/HQ/R.8a/Vol. IX/3495. With these approvals, an ethical approval waiver was obtained from the Animal Research Ethics Committee of the University of the Witwatersrand, where the lead author is a post-graduate student (Waiver 11-10-2021-O).

Consent for publication

This manuscript has been approved for publication by the Institute for Medical Research of the United Republic of Tanzania (NIMR/HQ/P.12 VOL XXXIV/78).

Availability of data and material

Data are available under the Ifakara Health Institute data sharing policy upon request.

Competing interest

The authors declare that they have no competing interests.

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Authors' contributions

PGP, EWK, FOO and LLK conceptualized the idea, designed the study and supervised the experiments; PGP analysed the data and drafted the manuscript; DSM, LLM, IHM, RMN and HSN helped in data analysis and drafting of the manuscript; JK and HB helped with the field mosquito collection and morphological identification. All authors read and approved the final version of the manuscript.

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Tables

Table 1: Summary of 24-hour mortality of *Anopheles funestus* mosquitoes in tests of adults collected at different distances from aquatic habitats. The table shows the odds of dying among mosquitoes collected at different distances and exposed to deltamethrin or permethrin with or without the synergist, piperonyl butoxide (PBO).

Insecticide	Insecticide dose	Distance (m)	Mean mortality (95% CI)	OR (95% CI)	p-value
		50-100	11 % (5.96-20.23)	Ref	Ref
	1×	150-200	14 % (7.78-23.15)	1.26 (0.49-3.22)	0.633
		>200	41 % (31.03-52.29)	5.54 (2.43-12.63)	<0.001*
		50-100	46 % (35.67-57.18)	Ref	Ref
Deltamethrin	5×	150-200	45% (34.50-55.97)	0.95 (0.51-1.77)	0.873
		>200	54 % (42.82-64.33)	1.35 (0.73-2.51)	0.343
		50-100	46 % (35.67-57.18)	Ref	Ref
	10×	150-200	53 % (41.61-63.16)	1.28 (0.69-2.39)	0.429
		>200	60 % (48.95-70.11)	1.74 (0.93-3.26)	0.082
		50-100	28 % (18.84-38.26)	Ref	Ref
	1×	150-200	35 % (25.38-46.02)	1.42 (0.72-2.78)	0.307
		>200	44 % (33.34-54.75)	2.05 (1.06-3.97)	0.033*
		50-100	36 % (25.68-48.10)	Ref	Ref
Permethrin	5×	150-200	50 % (38.31-61.70)	1.76 (0.93-3.31)	0.080
		>200	58 % (45.60-68.70)	2.38 (1.26-4.49)	0.008*
		50-100	51 % (40.41-61.97)	Ref	Ref
	10×	150-200	68 % (56.54-76.83)	1.98 (1.04-3.75)	0.037*
		>200	79 % (68.44-86.36)	3.53 (1.76-7.04)	<0.001*
		50-100	82 % (69.85-89.55)	Ref	Ref
Permethrin +	PBO	150-200	93 % (83.54-97.48)	3.14 (0.94-	0.063

PBO				10.51)	
		>200	92 % (81.49-96.49)	2.47 (0.80-7.61)	0.115
		50-100	85 % (73.61-92.01)	Ref	Ref
Deltamethrin + PBO	PBO	150-200	98 % (89.10-99.77)	10.41 (1.28-85.00)	0.029*
		>200	95 % (85.61-98.38)	3.35 (0.86-13.07)	0.081

Key: OR: Odds ratio; CI: Confidence interval; Insecticide dose: fold increase on standard dose; Reference distance (Ref): 50-100m; * denotes statistical significance at p = 0.05, relative to the reference distance.

Table 2: Summary of 24-hour mortality of *Anopheles funestus* mosquitoes collected as larvae and exposed to pyrethroids at different age classes (3-5, 8-11 or 17-20 days old). The table also shows the odds of dying among mosquitoes exposed at different ages to deltamethrin or permethrin

Insecticide	Insecticide dose	Age (days)	Mean mortality (95%CI)	OR (95% CI)	p-value
		3-5	25 % (16.73-53.60)	Ref	Ref
	1×	8-11	36 % (26.15-46.76)	1.67 (0.85-3.30)	0.138
		17-20	40 % (29.89-51.05)	2.00 (1.02-3.93)	0.044*
		3-5	45 % (34.50-55.97)	Ref	Ref
Deltamethrin	5×	8-11	54 % (42.82-64.33)	1.42 (0.76-2.65)	0.269
		17-20	70 % (59.12-79.01)	2.85 (1.49-5.46)	0.002*
		3-5	58 % (46.48-67.82)	Ref	Ref
	10×	8-11	76 % (65.73-84.31)	2.37 (1.20-4.68)	0.013*
		17-20	81 % (71.20-88.37)	3.35 (1.57-6.55)	0.001*
		3-5	20 % (12.63-30.19)	Ref	Ref
	1×	8-11	41 % (31.03-52.29)	2.81 (1.39-3.69)	0.004*
		17-20	79 % (68.44-86.36)	14.82 (6.89-31.89)	<0.001*
		3-5	84 % (74.00-90.32)	Ref	Ref
Permethrin	5×	8-11	93 % (84.30-96.59)	2.39 (0.86-6.65)	0.094
		17-20	98 % (90.55-99.37)	7.57 (1.65-34.74)	0.009*
		3-5	80 % (69.81-87.37)	Ref	Ref
	10×	8-11	99 % (91.66-99.82)	19.75 (2.55-25.95)	0.004*
		17-20	96 % (89.01-98.79)	6.42 (1.79-23.01)	0.004*

Key: OR: Odds ratio; CI: Confidence interval; Insecticide dose: fold increase on standard dose; Reference age (Ref): 3-5 days old; * denotes statistical significance at p = 0.05, relative to the reference age.

Figures

Figure 1

Map of the study area as a representative for adult mosquito collections at different distances from aquatic habitats.

Figure 2

Percentage mortality in *Anopheles funestus* mosquitoes collected at different distances from the aquatic habitats, and exposed to deltamethrin or permethrin. The red-dotted lines represent 90% mortality and the blue-dotted lines represent 98% mortality.

Figure 3

Percentage mortality in *Anopheles funestus* mosquitoes collected at different distances from the aquatic habitats, and exposed to either the candidate pyrethroids alone or PBO followed by pyrethroids. The red-dotted lines represent 90% mortality and the blue-dotted lines represent 98% mortality.

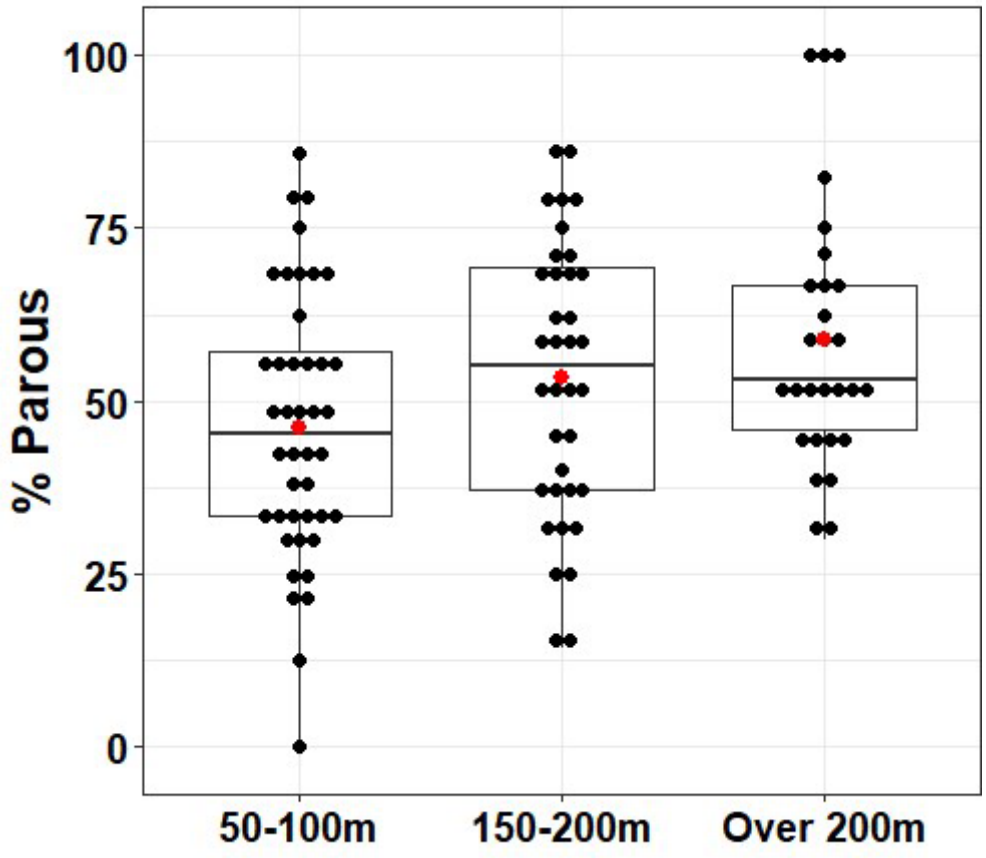

Figure 4

The parity status of *Anopheles funestus* mosquitoes collected at different distances and pre-exposed to insecticide. Red dots indicate the means.

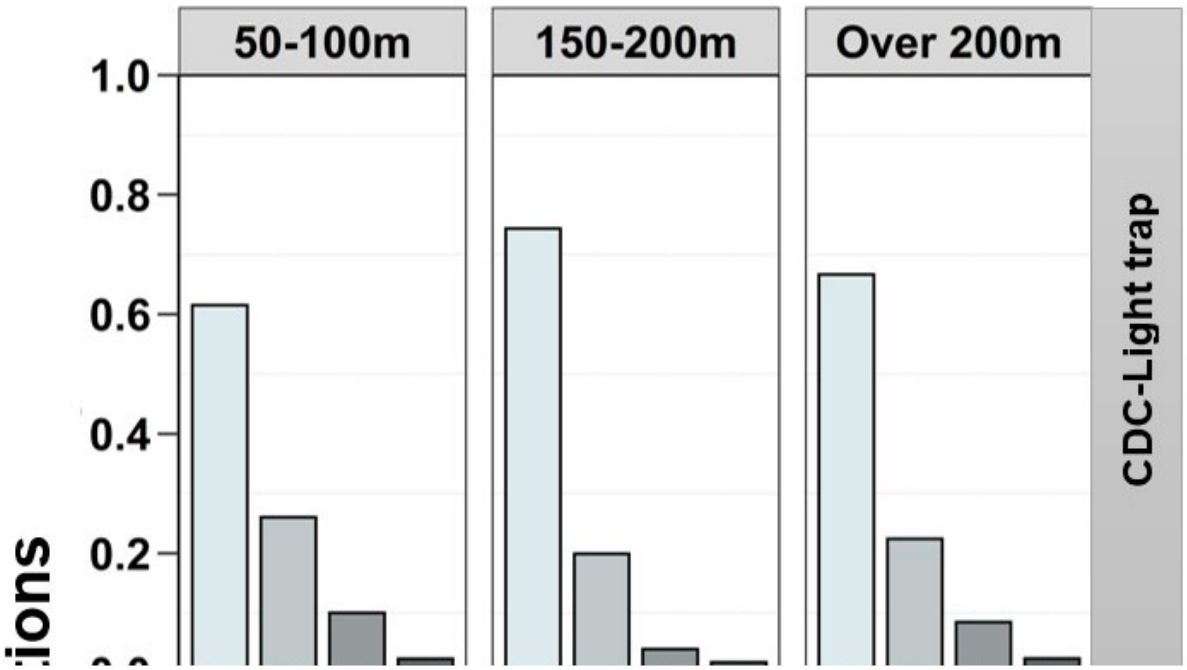

Figure 5

Proportions of mosquitoes with different numbers of gonotrophic cycles, among those collected by either CDC light traps (top panel) or the miniaturized double net (DN-Mini) trap (bottom panel) at different distances.

Figure 6

Percentage mortality in different age cohorts of emergent *Anopheles funestus* mosquitoes collected as larvae and exposed to deltamethrin or permethrin. The red-dotted line represents 90% mortality, the blue-dotted line represents 98% mortality.