



RESEARCH ARTICLE

New evidence of mating swarms of the malaria vector, *Anopheles arabiensis* in Tanzania [version 1; referees: 1 approved, 2 approved with reservations]

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v1 First published: 22 Sep 2017, 2:88 (doi: [10.12688/wellcomeopenres.12458.1](https://doi.org/10.12688/wellcomeopenres.12458.1))

Latest published: 22 Sep 2017, 2:88 (doi: [10.12688/wellcomeopenres.12458.1](https://doi.org/10.12688/wellcomeopenres.12458.1))

Abstract

Background: Malaria mosquitoes form mating swarms around sunset, often at the same locations for months or years. Unfortunately, studies of *Anopheles* swarms are rare in East Africa, the last recorded field observations in Tanzania having been in 1983.

Methods: Mosquito swarms were surveyed by trained volunteers between August-2016 and June-2017 in Ulanga district, Tanzania. Identified *Anopheles* swarms were sampled using sweep nets, and collected mosquitoes killed by refrigeration then identified by sex and taxa. Sub-samples were further identified by PCR, and spermatheca of females examined for mating status. Mosquito ages were estimated by observing female ovarian tracheoles and rotation of male genitalia. GPS locations, types of swarm markers, start/end times of swarming, heights above ground, mosquito counts/swarm, and copulation events were recorded.

Results: A total of 216 *Anopheles* swarms were identified, characterized and mapped, from which 7,142 *Anopheles gambiae* s.l and 13 *Anopheles funestus* were sampled. The *An. gambiae* s.l were 99.6% males and 0.4% females, while the *An. funestus* were all males. Of all *An. gambiae* s.l analyzed by PCR, 86.7% were *An. arabiensis*, while 13.3% returned non-amplified DNA. Mean height (\pm SD) of swarms was 2.74 \pm 0.64m, and median duration was 20 (IQR; 15-25) minutes. Confirmed swarm markers included rice fields (25.5%), burned grounds (17.2%), banana trees (13%), brick piles (8.8%), garbage heaps (7.9%) and ant-hills (7.4%). Visual estimates of swarm sizes by the volunteers was strongly correlated to actual sizes by sweep nets ($R=0.94$; $P<0.001$). All females examined were nulliparous and 95.6% [$N=6787$] of males had rotated genitalia, indicating sexual maturity.

Conclusions: This is the first report of *Anopheles* swarms in Tanzania in more than three decades. The study demonstrates that the swarms can be identified and characterized by trained community-based volunteers, and highlights potential new interventions, for example targeted aerosol spraying of the swarms to improve malaria control.

Open Peer Review

Referee Status:

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version 1 published 22 Sep 2017	report	report	report

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Competing interests: No competing interests were disclosed.

How to cite this article: Kaindoa EW, Ngowo HS, Limwagu A *et al.* **New evidence of mating swarms of the malaria vector, *Anopheles arabiensis* in Tanzania [version 1; referees: 1 approved, 2 approved with reservations]** Wellcome Open Research 2017, 2:88 (doi: [10.12688/wellcomeopenres.12458.1](https://doi.org/10.12688/wellcomeopenres.12458.1))

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Grant information: This work was supported by the Wellcome Trust [100540, 102350 and 087547], the Innovative Vector Control Consortium (IVCC) [Project No: 43 – Mating Swarms], the Carnegie Corporation of New York [B 8606.R02], and Sida [54100029].

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

First published: 22 Sep 2017, 2:88 (doi: [10.12688/wellcomeopenres.12458.1](https://doi.org/10.12688/wellcomeopenres.12458.1))

Background

Recent successes in malaria control have been mostly attributed to vector control measures, in particular, long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS)¹. These tools have contributed to ~78% of all gains achieved since 2000^{1,2}. However, effectiveness of these interventions is compromised, partly due to spread of insecticide resistance³ and increased outdoor exposure to mosquito bites among other challenges⁴⁻⁶. Consequently, persistent malaria transmission still occurs in many places, even where high and sustained coverage of LLINs has been achieved.

To develop new options for malaria vector control, it is important to re-examine the overall ecology of the malaria vectors⁷, including not only the blood-feeding and resting habits commonly targeted by LLINs and IRS, but also other mosquito habits indoors and outdoors. Mating behavior is one of the most important aspects in the maintenance of species⁸, yet it is a widely under-investigated aspect of the mosquito biology. Improved understanding mosquito mating systems could possibly provide new opportunities for expanding vector control options. Most insects mate in swarms, whereby dispersed populations aggregate at specific times and places^{9,10}. In mosquitoes, including the malaria vectors, swarming flights are influenced by presence of visual markers on the ground^{11,12}. Targeting swarms to deplete mosquito densities indeed offer unrivalled opportunities to drastically reduce mosquito-borne pathogen transmission¹³. Such an approach has proven effective against some *Anopheles* mosquitoes on a limited scale in Burkina Faso¹⁴, but needs to be validated for other vector species in other areas. Besides, mosquito swarms are known to occur perpetually in the same locations at approximately the same time each day^{10,15}, thus targeting them could be easily achieved by trained community volunteers. The concentrations of males in swarms, predictability and accessibility of the swarming sites and the fact that swarms can be artificially manipulated¹⁶, makes the male mosquitoes vulnerable and an easy control target.

While swarms have been commonly identified in west and central Africa¹⁷⁻¹⁹, observations have rarely been reported in East and Southern Africa, most probably because mosquito swarming has not been as thoroughly investigated in this area, as it has been in West Africa. Prior to this current report, there was only a handful of reports, including the seminal work by R.P. Marchand in Tanzania in the early 1980s²⁰, the work by J.D. Charlwood in Mozambique in the early 2000s²¹, and unpublished observations of *An. arabiensis* swarms in the Kilombero valley in south eastern Tanzania (Dr. Kija Nghabi and Japhet Kihonda, Personal Communication).

This current study was designed to explore the *Anopheles* mating systems in rural south-eastern Tanzania, by assessing occurrence of natural swarming and outdoor nocturnal behaviors of *Anopheles* mosquitoes. The assessment involved accurate identification, mapping and characterizing of mosquito swarms in three study villages in Ulanga district, Tanzania. We assumed that despite lack of previous records, *Anopheles* swarms do indeed occur in the area, and that they can be readily identified and characterized. We used a combination of crowd-sourced community

knowledge²², intensive field surveys and expert advice. Our initial objective was therefore to demonstrate natural occurrence of swarms of malaria vectors in rural, south eastern Tanzania and characterize these swarms.

Methods

Study area

The study was conducted in rural Ulanga and Kilombero districts, in south eastern Tanzania. Initially, the surveys covered multiple villages in the two districts, but we eventually focused on just three villages of Kivukoni (8.2135°S, 36.6879°E), Minepa (8.2710°S, 36.6771°E) and Mavimba (8.3124°S, 36.6771°E) in Ulanga district, on the Kilombero river floodplain (Figure 1). The main malaria vectors in the area include *Anopheles funestus* and *An. arabiensis* with minor contributions from *An. rivulorum*²³. Overall EIR was last estimated at 4.2 and 11.7 infectious bites/person/year by *An. arabiensis* and *An. funestus* respectively²³. The main vector control approach used in the area is LLINs, and the villages benefited from universal LLIN coverage campaigns both in 2011 and in 2016. The main malaria vectors are resistant to pyrethroids used in the LLINs, but still susceptible to organophosphates^{23,24}. Mean household size is 4.2²⁵. Most of the houses are mainly mud and brick walled, with thatched or iron-sheet roofs. The communities rely mainly on substance farming, cultivating rice and maize but also fishing.

Trainings on swarm surveys and characterization

Ten participants were selected to support the project in the three study villages, with the help of community leaders. All participants were male adults aged 18 to 35 years, who had provided written informed consent prior to being enrolled in the study. Initially, the project leaders and research assistants were trained on how to identify mosquito swarms and swarm markers by experienced entomologists from Burkina Faso. The first brief training was conducted in Ifakara, Tanzania, in December 2014, at which stage this project had just been conceived. The second training was done for project leaders during a visit to Burkina Faso in September 2016. All participants in this study, including volunteers searching the swarms, provided written informed consent prior to being enrolled.

In April 2016, five months before the second training of project leaders, exploratory swarm surveys were initiated, as an extension of ongoing entomological studies in the study area. During and after these exploratory surveys, the project leaders trained the local community-based swarm searching volunteers on how swarms are identified and characterized, focusing on major features of both the swarms and their associated markers (Figure 2). This training was conducted over several weeks, and included: i) swarm searching techniques; ii) swarm sampling techniques; iii) swarm characterization and iv) various entomological procedures essential for examining male and female *Anopheles* mosquitoes collected from swarms. All trainings for local community volunteers were conducted in Kiswahili language, which is the common language in Tanzania, and in this specific study area. These volunteers were then relied upon to identify swarms, which the researchers would then follow up for species confirmation.

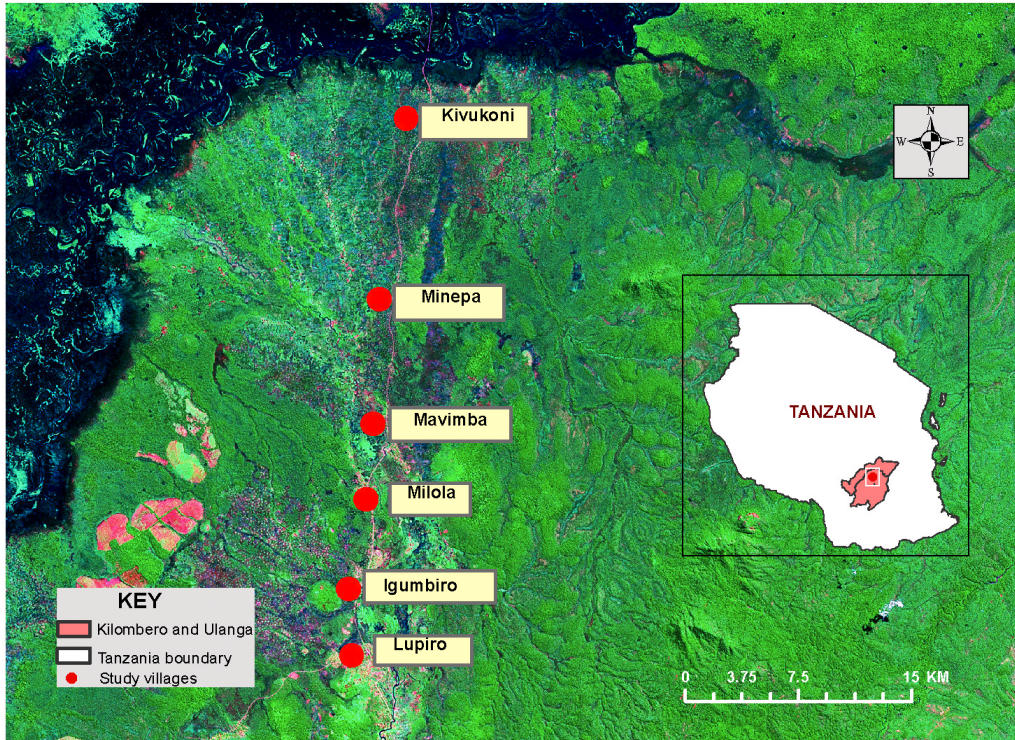


Figure 1. Map of the study area, showing villages in south-eastern Tanzania, where swarm surveys were conducted. Image description: Pan-sharpened mosaic, acquired by Enhanced Thematic Mapper Plus (ETM+) sensor on Landsat 7 Satellite (Courtesy of Donall Cross, University of Aberystwith, UK).



Figure 2. Pictures of volunteers and researchers learning how to identify and sample mosquito swarms in one of the study villages. All participants in the picture provided consent to being photographed.

Further advanced training on swarm surveys and characterization was conducted in Tanzania by a team of experts from Institut de Recherche en Sciences de la Santé (IRSS), Burkina Faso. This advanced training covered: i) swarm searching techniques in dry and wet seasons; ii) swarm visualization techniques; iii) GIS-based analysis of swarms iv) swarm markers and associated environmental features; v) swarm sampling techniques vi) photographic survey of swarms, vii) swarm characterization (including key features to consider); and viii) identification of mating couples in swarms and examination of male and female mosquitoes to identify mated and non-mated individuals.

Initial exploratory surveys of mosquito swarms

To aid logistical assignment of the swarm searching activities, volunteers were assigned one sub-village to search and report the occurrence of swarms. The trained community volunteers were dispatched to search and report any types of insect swarms in their areas, and report these to the researchers. Every time a swarm was reported by the volunteers, the researchers visited the site immediately (if nearby) or the next day (if in far locations): i) first, to verify whether the swarms were actually present, ii) second, to determine whether they were swarms of mosquitoes as opposed to other insects, and iii) third, to assess whether they were actually *Anopheles* swarms. The first confirmed *Anopheles* mosquito swarm was identified by a community-based swarm searcher in mid-August 2016 in Minepa Village. This provided our initial verified evidence of presence of *Anopheles* swarms in this area. As a result of this initial discovery, the team was reorganized for more intensive swarm searching expeditions. From this point forward, the key objectives now included demonstrating that local community members could be trained to readily identify and characterize *Anopheles* swarms and conduct comprehensive mapping and characterization of *Anopheles* swarms in selected villages. Subsequent efforts were restricted to just three villages (Mavimba, Kivukoni and Minepa) in Ulunga district (Figure 1), to enable detailed swarm studies.

Detailed mapping and characterization of *Anopheles* mosquito swarms and swarm markers in three selected villages

After the initial swarm searching, a comprehensive mapping and characterization of the *Anopheles* swarms was conducted in three selected villages, i.e. Minepa, Mavimba and Kivukoni. We worked with volunteers from the different sub-villages, who had been trained on how to spot mosquito swarms and swarm markers, time of day when swarms typically occur, and how to collect mosquitoes using sweep nets to identify the swarming mosquitoes. We relied on lessons learned during the initial exploratory surveys, and progressively optimized our swarm survey techniques. The training provided by experts from Burkina Faso significantly improved the swarm characterization capabilities of the Tanzania team.

Volunteers were assigned specific area (sub-villages) to search for mosquito swarms, by first identifying potential swarm markers in these areas during the day, and then actively searching of mosquito swarms across the area every evening from 18:00Hrs, with the aim of identifying swarms. During the swarm surveys, the volunteers continued to receive supportive supervision and training on

how to identify, locate and sample mosquitoes using sweep nets. This continuous training was important since this is the first time *Anopheles* swarms were being recorded in this area. To facilitate the swarm surveys, we also identified, characterized and recorded all potential swarm markers in the study villages. The swarm markers were classified based on the criteria developed by Diabate *et al.*,²⁶ into four categories as follows: a) flat-contrast markers, such as bare land and footpaths, b) flat no-contrast markers, such as grasses and rice fields, c) elevated contrast markers, such as anthill, woodpiles, bricks and garbage bin, and d) elevated no-contrast markers such as banana tree. Then these markers were subsequently monitored in the evenings to identify and record those with swarms.

Following the initial identification and mapping, each verified swarm was comprehensively characterized by the expert team, by repeatedly visiting and examining the swarms at least three times in different days. In addition to recording date, month, and name of village, we followed a systematic process and recorded various pre-determined characteristics as follows: a) heights at which the swarms occurred, (measured as a distance between the base of the swarm and the ground level), b) time of day when the swarming started appearing, recorded to the nearest minute, c) time of night when the swarms were completely dispersed, also measured to the nearest minute; d) physiological status of a sample of mosquitoes collected in the swarm, e.g. mating status and parity, e) any other mosquito species and insects occurring in the same swarm, f) exact geo-location of the swarm marker; measured using handheld GPS receiver (Magellan eXplorist GC, USA), g) ratio of males to females collected in the sample; Detailed mapping and characterization of h) number of copulation events observed in the swarm; i) number and proportion of females caught that have evidence of having been inseminated, j) type of swarm marker; k) parity status of female mosquitoes collected in the swarms, including number and proportion of females that were parous or nulliparous; l) morphological and molecular identification of the species of *Anopheles* mosquitoes collected in the swarm; m) any other unique observations made at the swarm site or on the swarms; and n) approximate number of mosquitoes collected in each swarm. Swarm sizes were estimated in two ways: by sampling with a large sweep net (190 cm diameter), approximately 10 minutes after the start of the swarm, and visually, by the trained volunteers counting the flying mosquitoes and approximating the counts. Lastly, we observed other features which may affect the occurrence of mosquito swarms, time of sunset such as human movements and other insects.

Laboratory processing of mosquitoes collected in the swarms

Collected mosquitoes (dead or alive) were aspirated from the sweep nets, kept in separate paper cups and maintained on 10% glucose solution so that those still alive could survive till the next morning. The following morning, all mosquitoes were killed in a closed container by freezing, then identified morphologically by taxa and sex, following keys developed by Gillies and Coetzee²⁷. A sub-sample from each collection was further identified by multiplex polymerase chain reaction (PCR) to identify sibling species that were morphologically indistinguishable. The PCR assays were conducted using protocols developed by Scott *et al.*,²⁸.

Physiological age of the wild-caught female malaria vectors was approximated based on the status of their ovaries, i.e. whether they had previously laid eggs or not, by observing the coiling or uncoiling of the ovariole tracheoles²⁹. Each unfed female mosquito was first anesthetized in a refrigerator. A drop of distilled water was added to a slide and each specimen kept still on the slide, then the seventh and eighth abdominal segment was pulled using fine needles under stereo dissecting microscope. The ovarial tracheoles were then observed under a compound microscope at 10X objective lens magnification, to determine whether the mosquitoes were parous (tracheoles uncoiled and stretched) or nulliparous (with tracheolar skeins, i.e. coiled)²⁹.

Assessment of female insemination status and sexual maturity in males:

To determine the insemination rate, female *An. arabiensis* were dissected under a dissecting microscope and their spermathecae examined for the presence of spermatozoon under a compound microscope using 10X objective lenses^{30,31}. To assess whether males had matured sexually, genitalia of *An. arabiensis* males were also observed for signs of rotation³².

Data analysis

Data analysis was done by using R software version 3.0³³. Number of mosquitoes per swarm, duration of swarms, proportion inseminated, proportion parous (or nulliparous) or proportion of males with rotated genitalia were modeled as a function of different variables as follows: village, swarm marker type, month, height of swarms, time of swarming, and volunteer identification. Geolocations of the swarms relative to households and other landmarks were visualized using the ArcGIS 10.0 (ESRI, USA). Percentages of parity status, insemination rate, mean heights of swarms above ground, median duration of swarms, and time of day when swarms started or ended at the different months of surveys, were also calculated. Swarm markers were classified following the classification category developed by Diabate *et al.*,²⁶. Visualized volunteer estimates of swarm sizes, and sweep net swarm size estimates were compared and their linear correlation coefficients estimated.

Ethics statement

Meetings with local leaders were held in the study areas and the main aims of the study were explained by the research team. Written and signed informed consent were obtained from all volunteers participating in the study. All information was given in Kiswahili, the local language. Ethical approval for the study was obtained from Ifakara Health Institute Institutional Review Board (IHI/IRB/No: 38-2016), and from the Medical Research Coordinating Committee (MRCC) at the National Institutes of Medical Research (NIMR), Ref: NIMR/HQ/R.8a/Vol.IX/2428. Approval was also obtained from the Human Research Ethics Committee (Medical) clearance at the University of the Witwatersrand, where EK is registered for PhD (Ethics approval certificate No: M160806). Approval for publishing the manuscript was obtained from the National Institutes of Medical Research (NIMR), Ref: NIMR/HQ/P.12VOL.XXII/24. Printed copies and web links to the publication will be provided to NIMR after publication.

Results

Results of exploratory surveys: initial evidence of *Anopheles* swarms

The initial exploration, conducted in multiple villages, revealed that *Anopheles* swarms do indeed occur in villages along the Kilombero river valley in rural south-eastern Tanzania. The surveys also revealed that community members can be relied upon to identify these swarms and that by combining their local knowledge with expert knowledge, the *Anopheles* swarm surveys could be effectively characterized. The first *Anopheles arabiensis* swarm was observed on 15th August 2016, in the village of Minepa, by one of the local volunteers. To the best of our knowledge, this is the first recorded *Anopheles* swarm in this specific part of Tanzania, and the first in the country since the last report from north-eastern part of the country in 1983³⁰. In the initial exploration, several non-mosquito swarms and also culicine mosquito swarms were incorrectly reported by the volunteers as *Anopheles* swarms, but were dismissed by the expert verification teams. The reliability of the volunteers however increased over the course of the surveys.

Anopheles mosquito swarms in the three selected villages

After the initial exploratory surveys, and confirmation of occurrence of *Anopheles* swarms, subsequent surveys were concentrated in just the three selected villages of Minepa, Mavimba and Kivukoni (Figure 1). A total of 216 swarms were observed from August 2016 to June 2017. The distribution of mosquito swarms in the study villages is shown (Figure 3), and was as follows; 58.3% of the swarms (n=126) were found in Minepa village, 11.1% (n=24) were found in Mavimba village and 30.5% (n=66) were found in Kivukoni village. There were more swarms distributed in areas close to the rice fields, at the edges of the villages, compared to areas near human settlements (Figure 3). All the swarms observed consisted exclusively of *An. gambiae* s.l. We analyzed a sub-sample of 112 *An. gambiae* s.l mosquitoes by PCR to determine actual sibling species, out of which we obtained 97 successful amplifications (86.6%) and 15 non-amplifications (13.4%). All the successful amplifications were determined to be *An. arabiensis* (100%). In one instance in the early days of the surveys, we also collected 13 male *An. funestus* mosquitoes, in an area where we had *An. arabiensis* swarms. Since no repeat observation of *An. funestus*, we are unable to verify presence of swarms of this species here, until further investigations are completed.

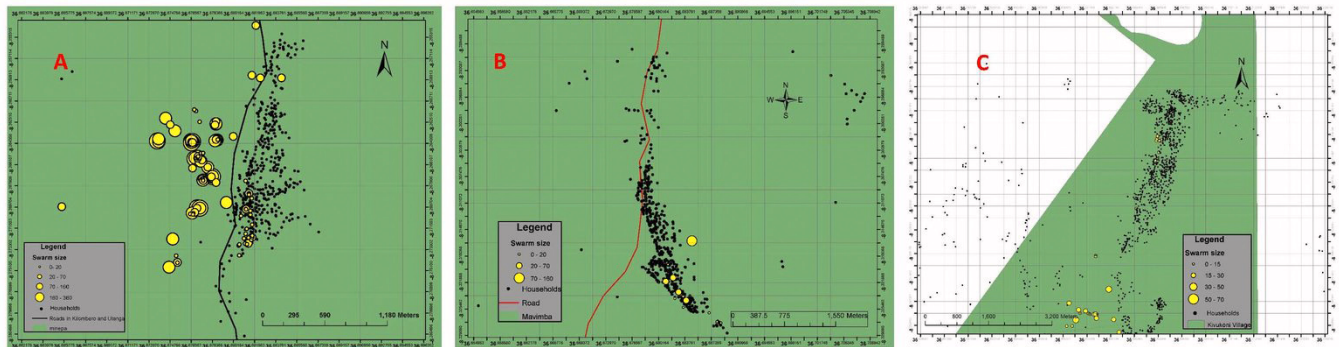
Start times, end times, duration and height of *Anopheles* swarms

It was observed that swarms began with two to three mosquitoes congregating after sunset, and flying above a swarm marker. Then the numbers increased over the next 10 to 15 minutes, with multiple males and slowly decreased in size then disappeared after 20 (IQR;15-25) minutes. Flying mosquitoes were observed by viewing them against the sunset. Mosquitoes tended to congregate at the mean height (\pm SD) of 2.74 \pm 0.64m from the ground. The swarm size increased and became compact above the marker but they disappeared when it became dark, which was also when swarms observations became impossible. Furthermore, we observed that the start and end time of swarming periods varied across months.

Swarms were appearing earlier in the period between August 2016 and January 2017, but later in the period between February and June. Specific start and finish times for swarms during each of the sampling months is shown in Figure 4.

Valley, Tanzania) varied in a similar pattern as the start and end times of the observed *Anopheles* swarm sessions. We considered sunset times for all the months of our data collection, starting August, 2016 to June 2017. Between August and November 2016, the latest time sunset time ranged between 18:32Hrs and 18:42Hrs. However, between December 2016 and March 2017, sunset occurred much later, with the latest sunset

According to weather information archived online by timeanddate.com, the latest time of sunset in the study area (Kilombero



Distribution of *Anopheles* swarms in Minepa Distribution of *Anopheles* swarms in Mavimba Distribution of *Anopheles* swarms in Kivukoni

Figure 3. Comparison of swarm distribution between the three study villages (Original figure created by authors of this article).

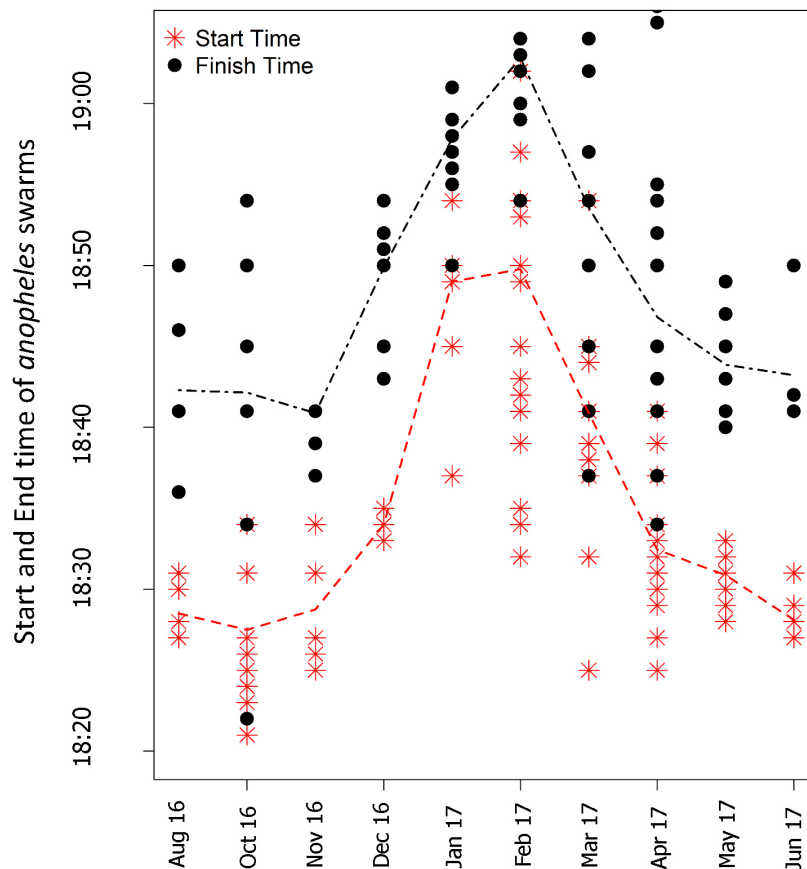


Figure 4. Observed start and end times of *Anopheles arabiensis* swarms in the study villages.

times between 18:56Hrs and 19:03Hrs. The sunset time was early again between April 2017 and August 2017, when the latest sunset times over the valley ranged from 18:26Hrs and 18:35Hrs: <https://www.timeanddate.com/sun/@157407>.

Estimated swarm sizes: data collected through direct visualization by community volunteers compared to data collected using sweep nets

We estimated swarm sizes from two data sources. First, we used data provided from visual assessments of the volunteers looking

at swarms and estimating how many mosquitoes were flying in them at that time. Second, we used data from collections done by the large standardized sweep nets, with which trained volunteers collected the mosquitoes approximately 10 minutes after the start of swarming. Though the values provided from the two data sources were not equal (i.e. visual estimates being consistently higher than sweep net counts), there was a significant linear correlation (Table 1 and Figure 5) between the visual estimates provided by the community volunteers and estimates obtained from sweep net counts ($R^2 = 0.94$; $P < 0.001$). There were

Table 1. Swarm sizes estimated by either visual observations by trained volunteers, or sweep net sampling in the three study villages (a total of 216 swarms were observed). C.I: Confidence Interval.

	Total number of swarms (% of all swarms observed)	Visual estimates by volunteers (No. mosquitoes/swarm)		Sweep net sampling estimates (No. mosquitoes/swarm)	
		Mean	C.I	Mean	C.I
Kivukoni	66 (30.5%)	34.0	31.4 - 36.6	13.1	11.5 - 14.9
Mavimba	24 (11%)	38.7	36.3 - 41.3	20.6	19.9 - 22.5
Minepa	126 (58.3%)	95.5	93.8 - 97.3	56.1	54.8 - 57.4

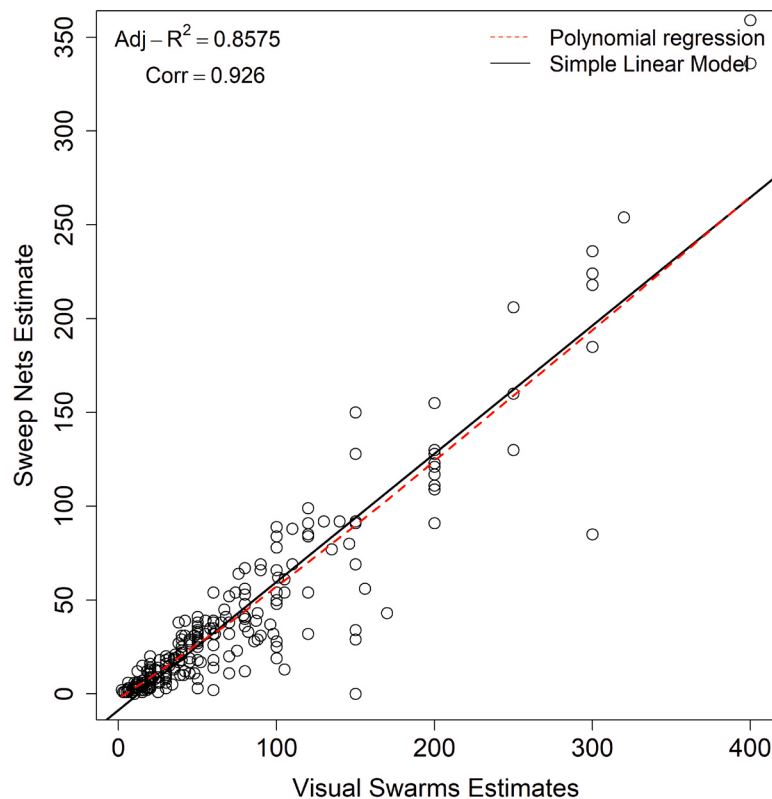


Figure 5. Estimated swarm sizes; and correlation between estimates done by visualization and estimates done using sweep nets.

also variations of swarm sizes observed in different villages, with Minepa village having the largest swarms followed by Mavimba and Kivukoni villages (Table 1).

Results of swarm markers survey

The commonest swarm markers were rice fields (25.6%), burned grounds (17.2%), banana trees (13%), brick piles (8.8%), grass (7.9%) garbage heaps (7.9%), ant hill (7.4%), wood piles (5.6%), demolished houses (1.8%), footpath (1.3%), trenches (1.3%), toilets (1.3) and cowshed (0.5). Table 2 shows the common swarm markers and the frequency of swarms observed in each marker. Most of the swarm locations remained the same, several months after the swarms were first identified and verified.

Copulation events, mating status, sexual maturity and parity rates in mosquitoes collected from the swarms

Throughout the study period, we observed and collected a total of 22 copulation events in the swarms. The proportion of female to male *An. arabiensis* mosquitoes in the swarm was 0.004 (28/7114). Of the 28 female *Anopheles arabiensis* mosquitoes dissected, 54% (N=15) were determined as nulliparous. The remaining 46% (N=13) were not examinable since the specimen had dried up. We also assessed the male *An. arabiensis* collected from the swarms for sexual maturity. Of these, 95.4% (N=6787) had rotated male genitalia, indicating sexual maturity. We assessed the 28 females recovered from the swarms, to assess whether their spermatheca were already filled with male sperms or not. Of all females dissected, 42.9% (N=12) had evidence of having been inseminated, while the remaining 57.1% (N=16) were not inseminated.

Discussion

Malaria vector control remains constrained by lack of comprehensive understanding of mosquito ecology, yet such ecological studies are considered a prerequisite for improved control and eventual disease elimination⁷. Though not widely studied, the mating behavior of malaria mosquitoes may presents some unique opportunities for improved vector control¹³. In a recent trial in Burkina Faso, it has been demonstrated that targeting *Anopheles* mosquito swarms with effective aerosol insecticide spraying could rapidly crash vector populations in local communities¹⁴. Though mating and swarming behaviours of *Anopheles* mosquitoes are among the most important components of the vector reproduction biology⁸, there have been limited field studies of mosquito swarming in Tanzania, the last published field observations having been in 1983²⁰. This neglect is mainly because most scientist have not considered swarming behaviour as having any potential opportunities for disease prevention and vector control. On the contrary, studies on mating and swarming behaviours of *Anopheles* mosquitoes in West Africa have already demonstrated that it could possibly be relied upon to control mosquito densities and associated pathogen transmission, by killing mosquito swarms with effective insecticides^{13,14}.

Our study has yielded evidence of occurrence of the swarms of *An. arabiensis* mosquitoes in the rural south-eastern Tanzania. Furthermore, the study proves that trained volunteers are able to successfully identify and locate swarms in their villages. A map of all swarm locations so far identified has been built-up with the help of the locally recruited volunteers (Figure 3). Interestingly, most of the swarm locations remained the same, several months

Table 2. Common types swarm markers observed in the three study villages. In brackets, the actual number of swarm markers that had *Anopheles* swarms over them.

	Flat contrast swarm markers	Flat no-contrast markers	Elevated contrast markers	Elevated no-contrast markers
Minepa Village	Burned ground (17) Well (0) Cowshed (0) Trench (0)	Rice fields (36) Footpath (1) Grass (8)	Bricks (17) Garbage heaps (10) Woodpile (1) Demolished house (2) Ant hill (7) Toilets (3)	Banana trees (24)
Mavimba Village	Burned ground (4) Well (0) Cowshed (0) Trench (2)	Rice fields (1) Footpath (1) Grass (5)	Bricks (0) Garbage heaps (4) Woodpile (1) Demolished house (0) Ant hill (6) Toilets (0)	Banana trees (0)
Kivukoni Village	Burned ground (16) Well (0) Cowshed (1) Trench (1)	Rice fields (18) Footpath (1) Grass (4)	Bricks (2) Garbage heaps (3) Woodpile (10) Demolished house (2) Ant hill (3) Toilets (1)	Banana trees (4)

after they were first identified. This phenomenon of swarms occurring in the same location is widely reported by other scientists, particularly in west Africa²⁶. In this current study, we characterized the *Anopheles* swarms repeatedly to obtain an initial profile of malaria vector swarms in this area; first evidence of such swarms in the study area.

The commonest swarm markers are shown in [Table 2](#), and were found on landscapes that were not blocked by either trees or houses. Several other studies have also reported the roles of such markers on mosquito swarming¹⁵, and here, we relied on similar swarm marker classification as previously used in Burkina Faso²⁶. Mosquitoes preferred to swarm on areas such as rice fields, burned grounds, banana trees, brick piles, garbage heaps and ant hills. It may therefore be possible to predict the distributions of *Anopheles* swarms based on the spatial distribution of the common swarm markers in the village. In this study, once we had an accurate representation of the main swarm markers, it became easier to conduct training programs for the volunteers, who relied heavily on these swarm markers as a guide to search for mosquito swarms in their assigned areas. Second, with a clear definition of swarm markers, it could be possible, as has been demonstrated in some studies to manipulate swarm markers, or create artificial swarm markers to attract *Anopheles* mosquitoes and enhance mating¹⁶.

Our study also demonstrated that locally recruited and trained non-entomologist volunteers from the study villages are capable of not only identifying the swarm but also estimating the swarm size and the associated swarm markers in the study village. This idea was derived from an earlier study conducted in the villages where community members were able to identify locations where mosquito densities were high²². In this current study, we initially guided the volunteers on how to identify mosquito swarms and their associated environmental features. This approach confirmed that such community-based volunteers can be relied upon to identify, characterize and map *Anopheles* swarms across villages, so that these swarms can potentially be targeted for control and ensuring its sustainability. An added advantage of this approach is that it also increases local community knowledge on mosquitoes, and the level of community engagement in research increased. By using such participatory approach, we expect that in the time of targeting *Anopheles* swarms e.g. by aerosol spraying, high levels of acceptability could be achieved, and the volunteers could be relied upon to lower implementation costs.

This study did not observe any *An. funestus* swarms. However, 13 male *An. funestus* mosquitoes were collected near the *An. arabiensis* swarms, suggesting possible presence of *An. funestus* swarms in the area. Unfortunately, this was only one instance and could not be verified during this study. It may be possible that this was a separate distinct *An. funestus* swarm that was formed close to *An. arabiensis*. Since they were all male *An. funestus*, we speculate that *An. funestus* swarms also occur in the area, and that extended surveys could reveal greater details of their swarming behaviours. It is therefore recommended that more detailed surveys should be conducted to understand the swarming behaviours of *An. funestus*

in the area. Evidence by other studies in Africa suggest that swarms of *An. funestus* appear in a similar way to those of *An. gambiae*²¹. Since it is known that *An. funestus* contributes to the significant amount of the remaining malaria transmission in this area²³, it is therefore suggested that any control measures targeting *An. gambiae* without affecting *An. funestus* will have little impact on the ongoing malaria transmission in this area. More attention should be put on how to identify swarms of *An. funestus* and their associated environmental features.

Consequently presence of dragonflies, though not initially targeted for observation, was an indication of swarm presence in many locations, though in this case we have not considered it as a physical swarm marker. Similar observations were also reported by Sawadogo *et al.*,¹⁵ in their studies in Burkina Faso. Furthermore, human movements, sounds, strong wind and rainfall were found to affect mosquito swarming behaviours. For instance, in some cases when swarm sampling was conducted during the rainfall, we rarely observed swarms even in the previously confirmed swarms. Dragon flies and other predators could thus be considered good indicators of presence of mosquito swarms in certain areas. Lastly, in this study, most of the swarms were found and confined at the edge of the village and in the rice fields, this could mostly be attributed to the fact that swarms prefer to the location that are not disturbed by human activities such as movements.

Overall, the swarming behavior of *An. arabiensis* in the valley is now clearly tractable. Further investigations should explore opportunities on whether new interventions exploiting this phenomenon, such as targeted aerosol spraying of swarms, could significantly disrupt malaria transmission.

Conclusions

This is the first report of *Anopheles* swarms in Tanzania, in more than three decades. The study provides evidence that the swarms can be identified, characterized and quantified by trained community-based volunteers; and opens potential new opportunities for targeting male malaria mosquitoes to improve disease control. Further investigations are recommended to further characterize the swarms in the area and to verify whether other residual malaria vector populations such as *An. funestus* also form similar mating swarms. More importantly, future studies should seek to demonstrate whether new interventions exploiting this phenomenon, such as targeted aerosol spraying of swarms, could significantly disrupt malaria transmission.

Data availability

The data used in this manuscript have been uploaded online to the [Ifakara Health Institute](#), DOI, [10.17890/ihl.2017.06.99](#)³⁴, under a CC0 1.0 license.

The terms and conditions that users need to agree to in order to download the data, can be viewed [here](#).

Competing interests

No competing interests were disclosed.

Grant information

This work was supported by the Wellcome Trust [100540, 102350 and 087547], the Innovative Vector Control Consortium (IVCC) [Project No: 43 – Mating Swarms], the Carnegie Corporation of New York [B 8606.R02], and Sida [54100029].

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We thank the local communities of Ulanga district for allowing us to work in their compounds. We thank Mr. Maxeclin Ngakuka (Kivukoni village) and all volunteers for their assistance during

swarm searching and sampling. We also thank the project team Felician Meza (Ifakara Health Institute) and Said Abbasi (Ifakara Health Institute). We also extend our thanks to the team from Burkina Faso; Dr. Abdoulaye Niang (IRSS, Bobo-Dioulasso, Burkina Faso), Mr. Simon Sawadogo (IRSS, Bobo-Dioulasso, Burkina Faso), Mr. Azizie Millogo (IRSS, Bobo-Dioulasso, Burkina Faso), Mr. Abdourasmane Noufou Diabate (IRSS, Bobo-Dioulasso, Burkina Faso) for provide training on swarm searching. Dr. Jane Bonds, Latham and Dr. Frederic Tripet, all participate in initial discussions where the protocols for this study were developed. Their support is greatly appreciated. All persons acknowledged have been informed. Lastly, we thank all reviewers of this article for their comments and suggestions.

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[Data Source](#)

Open Peer Review

Current Referee Status:   

Version 1

Referee Report 15 November 2017

doi:[10.21956/wellcomeopenres.13490.r27476](https://doi.org/10.21956/wellcomeopenres.13490.r27476)



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This paper represents a lot of work. The problem with studying swarming behavior (as opposed to mating behavior) is the difficulty of having sensible questions to ask. I have watched and filmed swarms of anophelines for many hours (mainly in Mozambique) and still am not sure what the most relevant ones, at least without sophisticated equipment, might be. There is always more perspiration than inspiration. This paper describes the basics of swarms of *Anopheles arabiensis* but I have a number of questions regarding some of the information in the paper. For example, having recently observed swarms of *An. arabiensis* in Eritrea (but only over a period of two weeks and in a single village) I remain at a loss regarding definitions of swarm markers for this species. The swarms I observed occurred over a very slightly darker area of sand in a dry riverbed but the mosquitoes did not alter their behavior when either a light cloth or dark T-shirt was placed under the swarming mosquitoes. I am therefore of the opinion that I have no real clue what the insects were using to maintain station. (This is unlike the situation with *An. coluzzii*, which will follow a dark cloth or *An. pharoensis* which follow light ones). I am, therefore, not convinced that the so-called markers described in the present study were actually being used by the *An. arabiensis*. Their wide variety indicates that they were just in the area and that something else was the actual 'marker'. Only by manipulating apparent markers and seeing their effect on the swarms can we be sure that they are for real.

In Eritrea males left their diurnal resting sites once light levels had fallen to around 520 Lux (which occurred at different times according to whether it was cloudy or not) and immediately started swarming. There was no delay. As the authors indicate swarming did not last long but at the same time, as they also point out, the cessation of swarming was coincident with difficulty of actually observing the insects so it is difficult to be sure that activity had ceased. Swarms of *An. funestus*, monitored with a video camera with infra-red capabilities in Mozambique, continued beyond the time that they could be seen with the naked eye. So a caveat regarding ending time should be included. The change in start times according to sunset times was also recorded in *An. coluzzii* from São Tomé and should perhaps be referenced.

Sweep netting, of course, does not remove all of the insects from a swarm. A better alternative is to take a flash photograph and then use a program like imageJ to count objects of different sizes in the image (which can be compared to counts done by hand). Since most pictures are taken from below the swarm the only objects in such pictures are the mosquitoes. Should two insects overlap then their combined size is larger than individual insects and so they are considered by the program to be a separate category. It may even be possible to use the camera on a smartphone, now widely available, to take such pictures.

Similarly I saw (perhaps) just a single pair in copula in Eritrea. These are normally very easy to see since the pair is larger and flies more slowly than individual insects (the male and female flying in different directions). They almost invariably leave the swarm (the female dragging the male behind her. It is only perhaps when a small female mates with a larger male that they continue in the swarm, the male in this case pulling the female behind him). In all their observations the authors report 'observing and collecting 22 copulation events'. (Difficult to know how you collect an event). I think that this begs the question as to whether, like 'love and marriage' these phenomena really go together (and the ones in Eritrea) really were 'mating swarms'. In São Tomé pairs dropped out of the swarms 'like flies' (if you'll excuse the pun). Something more is going on – but we don't know what it is. I think it is a misnomer to call them mating swarms. Indeed it is worth discussing the fact that so few mating pairs were seen. It is possible that mating was largely taking place in swarms elsewhere.

To watch swarms and note their behavior does indeed not require that much professional input. In São Tomé the swarm with mating was discovered by a two-year-old child who had to really persist to get the 'professionals' attention!

The authors also found that 12 of 28 females collected from swarms had been inseminated. Like one of the other reviewers I also find this very strange. Did these insects have mating plugs? What were they doing there? Elsewhere, as mentioned above, pairs in copula leave the swarm before the male ejaculates. The females do not return (as shown by the absence of multiple mating plugs in the many hundreds of females that have been examined). My own suspicion would be that the volunteers were being bitten and included these females in the sample.

It is also interesting that males with un-rotated terminalia were collected. Since these males would probably have eclosed the previous evening they would have been almost 24hrs old at the time of the swarm it implies that rotation takes longer than a day. But I was of the impression that that was all the time that was needed. Some comment on this might also be informative.

Mosquitoes swarm close to where they rest. Rather than attempting to target swarms per se a better and more sustainable method of control would be to find the resting site (and here the swarming site could be used as an indicator of nearby resting sites) and intervene there. If, as is likely, there are common characteristics between resting sites (a dark background perhaps) then it might also be possible to develop and deploy artificial resting sites that might be treated with a non-repellent insecticide or a factor that the males might transfer to the females that they mate with to affect them. Since newly emerged females also often share the same resting site as the males such an intervention would work against them too.

I agree with the comments of the other referees in that the article could be a lot shorter, that the figures could be improved and the text be made more concise.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Referee Report 20 October 2017

doi:[10.21956/wellcomeopenres.13490.r26301](https://doi.org/10.21956/wellcomeopenres.13490.r26301)



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This is a valuable description of *A. arabiensis* mating swarms in Tanzania based on one year swarm survey in 3 villages. The motivation of the study, as stated by the authors is that after the pioneering work of Marchand and later of Charlwood in East Africa, there have been no studies of swarming behavior of African anophelines in that region. The absence of observations on swarms of *A. gambiae* s.l., despite some efforts, led some to doubt the relevance of swarms to mating in these species, especially in that region. By replicating older results, the authors confirmed the role of swarming behavior to mating across Africa. Thus, the results, mostly replicate previous studies including: swarm formation time after sunset, swarm size range, and association with ground markers. The study relied on trained local village observers/collectors following earlier studies in Mali and Burkina Faso (cited). The paper reads easily (and pleasantly) but it could be improved by a substantial reduction in length, adhering to concise and more precise scientific writing, and possibly addressing specific questions/hypotheses, as suggested below.

Major comments:

1. Because this study is a descriptive survey of swarms that mostly replicate previous studies (it could cite other studies for details on the Methods), it would better fit a "Research Note" or a much shorter article. Suggestions to accomplish that include replacing Fig. 1 with a brief text and removal of Fig. 2, which is not informative. The Methods section could be reduced to 1/4 of its current length. The detailed description of the training for swarm spotters/collectors, should be summarized in 3 sentences including the description of training in standard entomological and field ecology methods, which would better rely on citations. The first section of the Results "Results of exploratory surveys: initial evidence of Anopheles swarms," (which repeats information given in the Methods) could be removed (also from the Methods section) or replaced by a single sentence such as: "The initial exploration, confirmed that Anopheles swarms do occur in the study area." Additionally, there are repetitions and unnecessary details that dilute the main message.

Certain terms must be defined accurately. For example a “swarm” may represent a single observation of males flying stationary above a marker in a single evening or to the site, where such aggregations were observed on multiple evenings. When stated that 216 swarms were observed, it is not clear which definition was used. Likewise, the term “sub-village” is vague. Please define its area.

Despite the long Method section, I could not find a summary of the sampling effort i.e. the number of swarm collectors operating throughout the year in every village, which is essential. Did the same team pass between villages in different times or they were divided between the villages? How were the swarm spotter/collectors been evaluated in terms of quality and efficiency of work?

2. Interestingly, large differences were found among villages in the number of swarms and their sizes. Could these differences reflect different density of *A. arabiensis* overall in these villages (it would be helpful to include average indoor density if such data exist)? Were the sampling effort the same between villages in terms of the number of collectors/observers, duration of activity, and their work-quality, or hospitality of local residents? Were the sampling conducted at the same time across all villages? For example, if the survey in one village occurred in a dryer period, it might account for the lower number and sizes of the swarms. What other factors might explain that variation (e.g., availability of suitable markers)?

Minor/Specific points:

Background

- “Most insects mate in swarms, whereby dispersed populations aggregate at specific times...” – this statement might mean to refer to diptera or to culicids. I doubt it holds for all insects. The references given focused on dipterans. Please check and confirm.
- “Targeting swarms to deplete mosquito densities indeed offer unrivalled opportunities to drastically reduce mosquito-borne pathogen transmission” – in what way it is unrivalled?
- “Such an approach has proven effective against some *Anopheles* mosquitoes on a limited scale in Burkina Faso¹⁴, but needs to be validated for other vector species in other areas. ...thus targeting them could be easily achieved by trained community volunteers. ... easy control target.” I afraid the scalability of this approach for regional, country and continental control is a heavy challenge that cannot be described as “easy”. There are other control strategies that can be effective in a single village or few villages, but can’t be effectively scaled up. For example draining or insecticide-treating all larval sites in certain villages to drastically reduce transmission, but unless the scalability issue has been addressed, the promise of this strategy is doubtful. Therefore the promise of the proposed new approach should be considered seriously with its limitations.
- “...a combination of crowd-sourced community knowledge²², intensive field surveys and expert advice” – specifically what “crowd sourced community knowledge” was obtained.

Results:

- Figure 3 – the legend is very small and nearly impossible to read. It would help to have on large-font and uniform legend for all 3 villages. Please clarify if (i) all houses in the area depicted are shown, i.e., there are no other houses (not shown) regardless if they “officially belong” to the

village, gpsed etc.. Also, what is the reason that the background to C is different? The landscape around the villages may be better conveyed using Google Earth (than a green color). Figure 3 summarizes large body of spatial information. It might be interesting to extract measures of inter-swarm distances, its seasonal variation, and effect of swarm size, as well as the variation between villages.

- Figure 4. The authors explain the variation in the swarming time by the change in sunset time. Thus, please add the corresponding line (curve) showing the sunset time and include the correlation between these variables in the Figure.
- Figure 5. Unless many observations are on top each other, the number of swarms observed appear to be 4-6/month, which amount to mere ~60 observations total rather than 216 as understood from the top of the results. If there are on top of each other, the author can consider jittering observations and changing their symbol to better appreciate the weight of each value. Please clarify.
- Table 2 shows variation in swarm occurrence on different markers. I suggest that authors reshape the table by listing all the swarm markers along the rows (in groups based on their classification) and have the columns show the percent (and total number) of swarms above each marker across villages. This will allow the reader to compare the ranking of the markers. Moreover, it might be helpful to know how many such markers exist in the surveyed area. For example if there are no rice fields or banana trees in one village and many in another, it will be difficult for the mosquitoes to express their preference for banana tees, etc. The interpretation of this table can be expanded in the discussion.
- The authors may want to comment on whether the collection of a proportion of inseminated females in swarms as well as some funestus specimens might reflect collection of mosquitoes that are not related to the swarming activity.

Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Referee Expertise: Vector ecology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Referee Report 28 September 2017

doi:[10.21956/wellcomeopenres.13490.r26445](https://doi.org/10.21956/wellcomeopenres.13490.r26445)



Edward D Walker

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This quite interesting and pleasantly written article summarizes a useful study combining observational science, citizen science, community engagement, Anopheles mosquito swarming and mating behaviour, and empirical analysis. The study is mensurative, that is, depending upon various methods of various degrees of sensitivity in measuring and recording observations and events. The distribution of locations of mating swarms of male Anopheles arabiensis is presented quite well and reliably. One problem is the issue of recorder bias; how likely are the observers to go to (say) sites most convenient for them to visit as opposed to sites difficult to visit. But this problem seems to have been surmounted here and the authors are not unaware of it. The observations on swarm composition (e.g., sex ratio), timing of formation and deformation, and density (number of mosquitoes comprising them) are all useful. This study might lead to new tools for vector control based upon these observations and related data.

Conclusions from data are again based on observational inferences but this kind of study is useful precisely for that reason.

Certain matters remain unclear and these have to do in particular with the location of swarms and what is meant by swarm markers. For example, a category of swarm marker is rice field and another is banana tree and another is toilet by which we must mean latrine shed. These are all entirely different environmental features with very different shapes, sizes, compositions, origins, and so on. From the observations one cannot make a strong conclusion about the particular configuration of what is meant by "swarm maker" typologically. That is, are swarm markers typically of a certain configuration or do swarms form just about anywhere the initial males starting them begin to fly at a particular spot? The authors should clarify this issue as a point of discussion. Secondly is the problem of what is meant by contrast or no contrast swarm markers. I do not know what this means. Do they mean contrast with a horizon, contrast between two hues or shades, ... ? As a means of providing a particular attribute of a typical swarm marker, the use of this descriptor of contrast is vague.

The writing style is generally quite good and the manuscript needs little adjustment for editing. The caption legends for Figure 5 are awkward. The X axis could simply read Visual Estimate and the Y axis could read Sweep Net Estimate. I see no reason to include a polynomial fit to the data on this figure because the relationship looks plainly linear. There seem to be two data points tucked up high in the upper right corner but they are obscured by the regression information.

In the last paragraph of the Results section, the following sentence should be revised: "Of the 28 female anopheles arabiensis mosquitoes dissected, 54% (N=15) were determined as nulliparous. The remaining 46% (N=13) were not examinable since the specimen had dried up." It should read as follows: "of the 28 female An. arabiensis dissected, fifteen were nulliparous and the remainder were too dry to determine

parity status."

In Table 2, the structure of the table needs some changes reflecting the clarification of the contrast and no contrast comment above. By cowshed must be meant the area of land where cows are stanchioned at night. This is not a 'cowshed' in the usual sense but rather a flat piece of ground where the animals are kept at night. If it is a kraal type of situation, there may be some living or nonliving fence in place in which case it cannot be flat. Otherwise, explain how a cowshed is flat. Under elevated contrast markers, I would like to know if by ant hills is meant termite mounds. Please clarify. My experience is east Africa is that ant hills are quite low to the ground and indiscernible whereas termite mounds are of course tower like structures and clearly elevated.

Some banana trees are quite tall. How did the citizens with the sweep nets manage to sample over them?

It is interesting that Figure 3 shows that in some locations the swarms form independently or distance from houses whereas in other locations the swarms form mostly amongst dense clusters of houses. Compare panel 1 and panel 2, for example. Why is this?

Thank you for giving me this opportunity to review this interesting and useful manuscript.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
