STUDIES ON THE RESTING BEHAVIOUR AND HOST CHOICE OF *ANOPHELES GAMBIAE* AND *AN. ARABIENSIS* FROM MULEBA, TANZANIA

J.D. CHARLWOOD[1], E. KESSY[2], K. YOHANNES[1], N. PROTOPOPOFF [2,3], M. ROWLAND[3] AND C LECLAIR[3].

1 Pan African Malaria Vector Research Consortium (PAMVERC), P.O. BOX 10, Muleba, Tanzania.

2 Kilimanjaro Christian Medical College, Moshi, Tanzania.

3 London School of Hygiene and Tropical Medicine, London WC1E, U.K.

e mails:

JD Charlwood jdcharlwood@gmail.com

E Kessy kessyenock@gmail.com

K Yohannes c/o jdcharlwood@gmail.com

N Protopopoff nprotopopoff@gmail.com

M Rowland Mark.Rowland@lshtm.ac.uk

C LeClair corey_leclair@hotmail.com

Running Head. *Resting and feeding in Anopheles*

KEY WORDS

Anopheles; gambiae; arabiensis; resting; indoors; outdoors; host choice; prokopack

ABSTRACT

An understanding of the resting and feeding habits of malaria vectors can inform control decisions. We compared the behaviour of *An. gambiae* before, and *An. arabiensis* 11 months after, an intervention of long lasting insecticide treated nets and indoor residual spraying with insecticide. The relative efficacy of a mechanical (Procopack) collection versus manual aspiration was evaluated. Sequential removal samples from three sites indicated that a single sample with the Prokopack collected more than a third of the available insects. Prior to the intervention *An. gambiae* constituted 97.7% of the 312 *An. gambiae* complex identified, with 2.3% being *An. arabiensis*. After the intervention *An. arabiensis* constituted 83% of the 183 specimens identified. In both species gonotrophic development was estimated to take two days. In grass roofed, smoke free houses, mosquitoes were collected from both roof and walls. In smoky houses there was a preponderance of mosquitoes on the walls. Whilst *An. gambiae* was endophilic, completing gonotrophic development inside houses *An. arabiensis* completed egg development in outdoor sites. From one of these sites unfed, part fed and engorged insects were dissected for age determination. With the exception of two parous mosquitoes unfed mosquitoes were virgins whilst the majority of engorged insects were parous (with well contracted sacs). Most (81%) of the 191 engorged, outdoor resting *An. arabiensis* tested had fed on cows and only 9% had fed on humans. It is concluded that the Prokopack sampler is better than manual collection of resting mosquitoes and that the reduction in malaria prevalence seen in the village following the intervention was due to a change in dominant member of the *An. gambiae* complex in the village, from the anthropohilic/endophilic *An. gambiae* to the catholic/exophilic *An. arabiensis*. Monitoring the proportions of the two species may be a sign of impending breakdown in control.

INTRODUCTION

The principal malaria vectors in Africa, *Anopheles gambiae*, *An. coluzzii* and *An. funestus,* tend to feed and rest inside houses whilst the other vector, *An. arabiensis*, is more plastic in its, feeding and resting behaviour (Charlwood et al.,

1995; Charlwood et al., 1999). Indoor resting mosquitoes are vulnerable to current chemical interventions, which is one of the main reasons for the recent decline in malaria transmission in Africa (Bhatt et al., 2015). Long lasting insecticide treated mosquito nets (LLIN's) prevent feeding, whilst spraying the interior walls of houses with a residual insecticide (IRS) kills and repels mosquitoes that attempt to rest indoors.

In some IRS campaigns, it is only the walls that are treated but if the roof is made of grass, and should the insects predominately rest there, then these also need to be sprayed when undertaking such control (WHO 2015). Where the mosquito rests may depend on whether the house is smoky due to people cooking inside (Bockarie et al., 1994). Whether the numbers of mosquitoes successfully obtaining a blood meal, and resting indoors differs between traditional homes without separate kitchens as compared to modern homes with separate kitchens may, therefore, also impact the efficacy of IRS programs, with considerable cost and logistic implications. Information on the favoured resting site(s) of malaria vectors can, therefore, help guide the implementation of potential control measures.

In many cases resting mosquitoes are blood fed (Silver, 2008). Knowledge of the feeding preference also inform models of transmission because host choice is an important component of vectorial capacity (Dye 1992). Anthropophagic behaviour appears to be responsible for *An. dirus* being a vector in Cambodia (Charlwood et al., 2016) whilst reduced feeding on humans was responsible for a reduced transmission by *An. coluzzii* on the archipelago of São Tomé and Príncipe (Sousa et al., 1998). Endophilic mosquitoes collected inside houses, that

do not contain domestic animals, have primarily fed on humans (Charlwood et al., 1995; Gillies & De Meillon, 1968 – and references therein). When people use LLIN's the mosquitoes are more likely to feed outside on a variety of hosts, including man (Charlwood and Graves, 1987; Lefevre et al., 2009). Feeding outside may also mean that they rest outside depending upon the availability of suitable outdoor shelters (White, 1974; Charlwood and Graves, 1987; Githeko et al., 1994). The analysis of the stomach contents of mosquitoes resting outdoors provides information on the host range (and possibly host choice) of the mosquito population. Determining the range of preferred hosts also becomes important when undertaking control, since a propensity to obtain a blood meal from alternative hosts to humans may enable mosquito populations to be sustained until the efficacy of indoor control decreases and transmission indooors is resumed.

In order to undertake assessments of feeding and resting behaviour suitable collection methods are required. The methods include manual aspiration, spray collections, CDC light-trap collection and Prokopack sampler (Silver, 2008; Vazques-Prokopek et al., 2009; Maia et al. 2011). Manual aspiration (using a sucking tube and torch) is a common way of collecting mosquitoes resting indoors. Although it may depend on the proficiency of the collector and type of resting site (Charlwood et al., 1995). The Prokopack sampler is efficient and less bulky compared to CDC backpack (CDC-BP) sampler and is much easier to use (Maia et al., 2011). It is a better method than pyrethrum spray catches, clay pots or urine baited traps (Onyango et al., 2013). Although it is becoming a standard

method for collection of resting mosquitoes (Killeen et al, 2017) its efficacy at collecting anophelines has yet to be assessed.

In a series of collections in Tanzania Maia et al*.,* (2011) found that CDC-BP and Prokopack aspirators were equivalent in efficiency for collecting mosquitoes in general but that the Prokopack, being less bulky, was much easier to use. Moreover, there was increased consistency between the numbers of mosquitoes collected between four different collectors operating the Prokopack compared to the CDC-BP. Among their samples, however, only 46 (2%) of the 2000+ mosquitoes collected were anophelines and only a small proportion were collected inside houses. Similarly, Onyango et al. (2013) found that the Prokopack was the most suitable method for the collection of indoor resting *Culex quinquefasciatus* compared to pyrethrum spray catches, clay pots or urine baited traps. Like Maia et al., (2011) they caught too few anophelines to undertake such a comparison for this genus. Thus, although it is becoming a standard method for collection of resting mosquitoes (Killeen et al, 2017) its efficacy at collecting anophelines has yet to be assessed.

Manual aspiration (using a sucking tube and torch) is a common way of collecting mosquitoes resting indoors. It may depend on the proficiency of the collector and type of resting site, although Charlwood et al., (1995) in a series of collections from Tanzania, found that there was little variation in the efficiency of different collectors.

Here we provide information on the host range and age among vectors collected from a village in Northern Tanzania, after an intervention (of LLIN's with a synergist and IRS) was introduced into the village. We also provide comparisons

between the Prokopack collection and manual aspiration of resting mosquitoes inside houses undertaken before the intervention was introduced into the village.

METHODS

STUDY AREA

The village of Kakindo/Kyamyorwa B in Muleba District, Kagera Region in northwest Tanzania (02°04′27.5″S, 31°34′10.8″E), described by LeClair *et al*. (2017), was used for the study. The village is separated by a floodplain, used for agricultural purposes, from an inlet of Lake Victoria where the majority of villagers' farm. Most of the houses in the village are mud-walled and thatched roofed, although corrugated iron roofs are becoming more common, and a number of houses are made of brick. The region has two rainy seasons: the main rains occur between March-May (average monthly rainfall 300 mm) and the short rains in October–December (average monthly rainfall 160 mm). Malaria is endemic with peaks of transmission at the end of the rainy seasons. At the start of the study a pyrethroid resistant population of *An. gambiae* was the primary vector in the area (Protopopoff *et al*., 2013; West *et al*., 2014) although *An*. *arabiensis* was also present. In January of 2015 the interior walls of all houses in the village were sprayed with the residual insecticide pirimiphos-methyl (Actellic 300CS) and villagers were given LLIN's impregnated with a pyrethroid insecticide and PBO (Olyset Plus©).

COLLECTION METHODS

Host seeking mosquitoes were collected indoors using CDC light-traps hung close to an occupied mosquito bednet and outdoors with a Furvela tent-trap (Charlwood et al., 2017).

153 Resting mosquitoes were collected with a Prokopack sampler and by manual
154 aspiration. Collections took place between 7 and 11am. The Prokopack was
155 systematically passed in an up and down direction over the walls or a side to
156 side direction close to the roof, at approximately 1m per second, so that all of the
157 surface of each was sampled once for each replicate collection. Manual collection
158 was performed by experienced collectors with the aid of a torch and an
159 aspirator. As with the Prokopack collection all surfaces to be collected in the
160 sampled room were carefully scanned once for each replicate sample.
161
162 Multiple samples were taken from roof and walls. In order to compare the two
163 collection methods; manual aspiration and Prokopack were alternated. Thus, if
164 the roof was sampled initially with the Prokopack a second sample was taken
165 immediately afterwards manually, and if in that house, on that day, the roof had
166 been sampled initially with the Prokopack then the first collection off the walls
167 was undertaken manually to be followed by a sample with the Prokopack.
168 The efficiency of the Prokopack sampler was determined by removal sampling,
169 as described by Southwood (1978), from three outdoor sites in 2017. In this
170 technique, the rate at which collections decline is directly related to the size of
171 the total population and the number removed. For removal sampling to function
172 adequately a number of assumptions must be met: the catching procedure must
173 not affect the probability of an animal being caught; the population must remain
174 stable during the catching period and, most importantly, the chance of being
175 caught must be equal for all animals. A relatively large proportion of the
176 population must also be caught to obtain reasonably precise estimates. Numbers
177 collected on each trapping interval must decline for estimates to be meaningful

(Charlwood et al., 1995). Zippin's (1958) method, based on maximum likelihood, which provides an estimate of the standard error, was used to estimate the total population in each site. As described by Southwood (1978) the total catch T = *n1+n2+n3+n4+n5* where *n1..n5* are the number of insects caught on each respective round of sampling. Then the value of $\sum_{i=1}^{k}(i-1)y$ is found, where k = the number of samples and i = 1 and y_i = the catch on the *i*th occasion.

Following this the ratio *R* is determined where

$$R = \sum_{i=1}^{k}(i-1)y\mathrm{i}$$

and

$$R = \frac{q}{p} - kq^{k}/(1-q^{k})$$

p = the probability of capture on a single occasion and *q*=1-*p* and the estimated size of the total population is

$$\tilde{N} = T/(1-q^{k})$$

RESTING SITES

INDOOR COLLECTIONS

Indoor collections largely took place before the interventions were introduced into the village. Indoor sites consisted of village houses that either had an outdoor kitchen or where cooking, with a wood fire, took place indoors. Collections from the roof and from the walls and by collection method were kept and scored separately. Collections from the roof were made to the height of the first two cross-beams rather than to the apex of the roof.

Outdoor collections

A variety of outdoor sites including, vegetation, were searched prior to the introduction of the intervention. A pit-shelter (WHO, 1965) was dug close to a house known to have a high density of mosquitoes (in light-trap collections), and two cardboard boxes (50cm on a side) with their interior walls painted black and openings partially obscured were placed close to a corral where five cows spent the night. After the intervention a latrine, with walls of dried banana leaves and a roof of grass, was sampled for 19 days, only with the Prokopack collector. Subsequently an abandoned mud-walled house, a cow shed and a further latrine were sampled in March 2017. The species, age and blood meal source of mosquitoes collected from these outdoor resting sites were determined.

Mosquito Processing

Collected anophelines were identified to species or species group using the keys of Gillies and DeMeillon (1968) and Gillies and Coetzee (1987). Samples of the *An. gambiae* s.l. from both collection periods collected indoors with CDC light-traps, were identified to species by PCR following the protocols of Bass et al.., (2008).

Age determination

The ovaries of samples of unfed, blood-fed and semi-gravid female *An. gambiae* s.l. collected outdoor resting were dissected according to the protocols described by Charlwood *et al*., (2003) and daily survival rates (p) determined according to the formula:

$$p = \sqrt[\mu]{m}$$

where *m* is the parous rate and μ is the duration of the oviposition cycle.

Following dissection, the abdomens of part-fed, engorged and semi-gravid females were squashed onto filter papers and preserved in a sealed plastic bag containing silica gel.

BLOOD MEAL ANALYSIS

Blood meal analysis of the sample of fed mosquitoes was done by direct ELISA, using a technique based on that of Beier *et al.* (1998). Filter paper blood spots were cut using an 8mm diameter hole punch and eluted with 600 μl phosphate buffered saline (PBS), centrifuged for 5–10 seconds and incubated at 4°C overnight. The following morning samples were centrifuged again and a 50 μl aliquot dispensed into each micro-plate well, covered, and incubated at room temperature for 3 hours. Each well was washed twice with washing buffer (PBS/Tween 0.5%), filled with blocking buffer (PBS/Casein in NaOH; 200 μl) and incubated for 1 hour. Wells were washed twice with washing buffer and a host-specific conjugate added. Conjugates were: Goat anti-human IgG H&L (50 μl) diluted 1:4000; Goat anti-dog IgG H-L (50 μl) diluted 1:2000; Goat anti-bovine IgG H-L (50 μl) diluted 1:2000, and Rabbit anti-goat IgG H-L (50 μl) diluted 1:2000 (Kirkegaard and Perry Laboratories). After 1 hour, wells were emptied and washed four times with washing buffer, and ABTS peroxidase substrate (100 μl; Kirkegaard and Perry Laboratories) was added to each well. Initially thirty minutes after addition of the substrate absorbance was read at 405 nm in an ELISA reader (Multiskan FC® Thermoscientific). Each sample was run in duplicate and was tested against two antibodies per run (initially anti-human paired with anti-dog). All samples with negative results were tested using anti-

bovine and anti-goat. Plates included two positive and four negative controls, hence a maximum of 16 samples were run per plate. Subsequently plates were scored visually by two independent readers (in the manner described by Charlwood *et al*., 2014).

Data Analysis

Data was entered into Excel ® spreadsheets and analysed using Stata 12 (Statacorp, 2011).

Ethics

The study was conducted as a component of the Pan African Malaria Vector Research Consortium project 'Evaluation of a novel long lasting insecticidal net and indoor residual spray product, separately and together, against malaria transmitted by pyrethroid resistant mosquitoes' which received ethical clearance from the ethics review committees of the Kilimanjaro Christian Medical College (certificate number 781 on 16/09/2014), the Tanzanian National Institute for Medical Research (20/08/2014), and the London School of Hygiene and Tropical Medicine (reference 6551 on 24/07/2014). The trial is registered with ClinicalTrials.gov (registration number NCT02288637) on 11/7/2014. Prior to beginning collections, informal sensitization sessions were conducted with village members to explain sampling-related activities. Written and verbal informed consent was obtained from all participants who could withdraw from the study at any time should they wish to do so.

Results

No mosquitoes were collected from the outdoor sites prior to the intervention and it was only from samples made 11 and 13 months' post intervention that

outdoor resting mosquitoes were found. Eleven months after the intervention a member of the *An. gambiae* complex, could still be collected inside bedrooms using CDC light-traps when these were hung next to an occupied bednet but the mosquito did not rest indoors. With the exception of a small number of insects collected indoors twelve months after the intervention no insects were collected from a series of *ad hoc* collections from inside houses after the application of the insecticide, including those sampled prior to the IRS. In 2014 (before the intervention) 303 (97.7%) of the 310 of the *An. gambiae* s.l. identified to species, collected from CDC light-traps in the village, were *An. gambiae*. Hence, we assume that our results from this period apply to this species.

Eleven months' post intervention the species ratio had changed. Only 26 (17%) of the 135 *An. gambiae* s.l identified to species from light-traps between January and February 2016 were *An. gambiae*. A similar proportion was identified from the 48 insects identified from Furvela tent-traps from this period. The other 83% of the insects were *An. arabiensis*. Thus, following the intervention this member of the complex had apparently supplanted the original *An. gambiae*.

INDOOR COLLECTIONS

Prior to the intervention collections were made on 25 days between the 5th June and 16th October 2014. Two hundred and seventy-seven samples from 20 houses were collected in this time, most from just three of the houses. A total of 893 female and 392 male *An. gambiae* s.l., 101 female and 13 male *An. funestus,* three female *An. zeimanni* as well as 36 female *Culex* sp. and 18 female *Mansonia* sp. were collected. Most of the *An. gambiae* s.l. and the *An. funestus* females collected

were blood-fed or semi-gravid although unfed, semi-gravid and gravid females as well as males were also collected (Table 1). The proportion of the collection that were unfed, part-fed and gravid were similar between manual aspirator and Prokopack collections (Table 2) although more were considered to be engorged and fewer semi-gravid in Prokopack collections than in the manual collections. The two categories combined were, however, identical (comprising 61% of the sample in both collections).

Negative binomial regression demonstrated that overall the Prokopack was significantly more efficient at collecting the insects than were the manual collectors [DRR 1.42, 95% CI(1.0,2.0) p=0.05] (Table 3).

Surprisingly, a Kruskal-Wallis test showed that the number of anopheline females collected from smoky houses was not significantly different from smoke-free houses [$X^2(1) = 0.027$, p=0.87] but the numbers of anopheline females collected from the walls and the roof in smoky houses differed significantly [Kruskal-Wallis test $X^2(3) = 8.295$, p=0.04] (Table 4)

Outdoor Collections

The decline in number of mosquitoes collected by sequential removal sampling from the three outdoor sites sampled in March 2017 are shown in Figure 1. The totals collected from each site, differentiated by sex and abdominal condition are given in Table 5, along with the estimated total number of mosquitoes (with standard errors). The initial sample collected 44.2, 45.5 and 48.4% of the total collected and 37.6, 32.0 and 38.8% of the estimated total population in each site.

A total of 654 female and 804 *An. gambiae* s.l. and four female and one male *An. funestus* group were collected from 33 collections from the latrine in December 2016-January 2017. The high proportion (55%) of male *An. gambiae* s.l. collected from the latrine contrasts with the 31% of *An. gambiae* males (283 of 901 collected) in, earlier, indoor collections (X^2=126, p<0.001).

Sixty-one of the 68 unfed female mosquitoes dissected from these collections were virgins, five had a mating plug whilst the two remaining unfed mosquitoes were parous with D sacs (Table 4). Most (64%) of the 83 blood-fed insects dissected were parous and most of these (66%) had D sacs (Table 4). This was significantly different to the part fed and unfed insects (X^2 =87.75 p=<0.001). Assuming that the engorged females were the equivalent of the biting population (therefore discounting the unfed insects) the estimated parous rate was 0.64. Assuming a two-day gonotrophic cycle the estimated daily survival rate was 80%.

BLOOD MEAL SOURCE

The stomach contents of 272 of the mosquitoes from outdoor, and 41 from indoor collections made eleven months post-spray, were identified by ELISA.

At the initial dilutions used a cross-reaction between anti-dog and anti-human samples and a cross-reaction between anti-bovine and anti-goat samples was observed but not vice versa. Dilutions were changed to 1:4000 for anti-human and 1:2000 for the rest of the three antibodies. At these dilutions, the cross-reactions were much weaker compared to the true positives reactions. In order to avoid false positives from the plate reader samples were subsequently scored visually by two independent observers. Given the possibility of false positives

potential mixed feeds were not assessed. This means that potential feeds on humans may have been emphasized compared to other blood-meal sources.

Twenty three of the 272 samples tested failed to produce a reaction. Among the remaining samples most of those from the latrine and cow shed collections were positive for either cows or goats (Table 5). Even among the small sample collected indoors at this time, cows constituted 53% of the positive samples with human fed mosquitoes making up the remaining 47%. From outdoor collections, human fed mosquitoes constituted only 9% of the positive samples.

DISCUSSION

The propensity to feed inside houses and to rest in them once they have done so may be independent adaptions. For example, Hayes and Charlwood, (1977) describe how, in Brazil *An. darling* came into the open houses used on the newly opened Manaus-Caracari highway, fed and left when engorged without touching a single surface in the room, other than the underside of people's hammocks.

Consideration of the pattern of indoor resting behaviour of the malaria vector(s) is an important component or IRS programmes (WHO, 2015). An environmental stimulus, such as surface temperature, has previously been shown to modulate the indoor resting behaviour of *An. gambiae* (Smith et al., 1966).

Before the intervention the resting site of the *An. gambiae* differed between smoke-free and smoky houses. In the former the roof appeared to be the favoured site whilst in the latter it was the walls. This is not surprising (smoke after all rises) and previous studies reported that wood smoke, or environmental conditions associated with wood smoke (e.g. decreased humidity), may modify

the preferred resting location of *An. funestus* (Gibbons 1933) and induce an exophilic response in *An. gambiae* (Bockarie et al., 1994). Thus, the roof of smoky houses may not need to be sprayed in IRS campaigns. Interestingly, the numbers of *An. gambiae* collected from smoke-free and smoky households was not significantly different. The effect of smoke on the numbers of indoor *Anopheles* is inconclusive (Biran et al. 2007, McCann et al. 2017) and the variation observed between localities may be related to specific properties of the biofuel source (Debbun et al. 2006) as well as mosquito species. The results indicate, however, that in addition to achieving a high coverage of sprayable structures smoke-free thatched roofed houses, in areas such as the one under study, both walls and roof need to be sprayed for IRS to work most efficiently.

Indoor resting *An. gambiae* and *An. funestus* collected prior to the intervention showed similar semi-gravid and gravid rates to blood-feds indicating that both species completed egg development inside houses. The semi-gravid mosquitoes collected in the morning might all have become gravid by the afternoon, indicating that overall the mosquitoes had a two day gonotrophic cycle.

Among the small number of *An. arabiensis* collected indoors after the intervention there were significantly fewer gravid and semi-gravid insects compared to those collected from the latrine. This indicates that the insects completed gonotrophic development outside, rather than indoors, but overall the duration of egg development would also have been two days. Estimated daily survival rates of 80% among the engorged mosquitoes were lower than the 84%

401 obtained from more extensive studies elsewhere in Tanzania (Gillies & Wilkes,
402 1965, Charlwood et al., 1994, 1995).

403 The excess of male and unfed *An. arabiensis* collected from the latrine indicates
404 that it was acting as an 'outdoor' shelter hence the blood meals obtained from
405 these mosquitoes should reflect the whole range of hosts used rather than just
406 those inside houses. Human, dog, cattle, and goat blood sources were identified
407 from the outdoor resting collections (Table 6). This substantiates the catholic
408 feeding behaviour and utilization of outdoor resting sites typical of *An.*
409 *arabiensis.* Moreover, the high proportion blood meals of bovine origin, even
410 given the potential emphasis on human feeds due to the order in which the blood
411 meal tests were carried out, support previous studies describing *An. arabiensis*
412 blood meal sources in rural Tanzanian villages (Kweka et al., 2008). The high
413 proportion of blood meals of bovine origin in the study area may be explained by
414 high community LLIN coverage after the intervention in concert with the
415 presence of alternative hosts and suitable outdoor resting sites.

416 The deficit of males of *An*. *gambiae* seen in the in the earlier indoor collections is
417 not due to an excess of females (fed-gravid ratios being similar between indoor
418 and outdoor collections ($X^{2=}$ 0.48, p = 0.49, n.s.) indicating that male *An. gambiae*
419 may also rest outside. The deficit of unfed (i.e. newly emerged) female *An.*
420 *funestus* as well as the deficit of males of this species implies that samples were
421 being taken some distance from the breeding site of this, normally endophilic,
422 mosquito. The great majority of unfed female *An. arabiensis* collected from the
423 latrine were unfed virgins. The small number of unfed parous mosquitoes
424 collected may have been insects that just arrived too late at the feeding site to

obtain a blood meal. Most of the parous mosquitoes were collected engorged. An engorged virgin mosquito will not develop eggs (Charlwood et al., 2003). Virgin, engorged mosquitoes would therefore have been pre-gravid. In São Tomé, fed female *An. coluzzii* collected from swarms were all considered to be 'part-fed' rather than engorged (Charlwood et al. 2000). Virgins may, therefore, excrete most of the meal (which impedes flying ability) before joining a swarm in the evening. Whether or not pre-gravid feeds are sufficient for a malaria infection to develop is not known.

Following the intervention in 2015 CDC light-trap collections demonstrated a decline in the *An. gambiae* population and shift in the species ratio, with *An. arabiensis* predominating in 2016. A shift in the relative abundance of sympatrically occurring members of the *An. gambiae* complex has been reported from Kenya (Bayouh et al., 2010) and rural Tanzania following universal distribution of LLINs (Russell et al., 2011, Lwetoijera et al., 2014). The authors attributed this shift to direct mortality and blood feeding inhibition in *An. gambiae*. This may have contributed to fewer egg-laying *An. gambiae* females and presumably reduced *An. gambiae* larval habitat occupancy. Laboratory and semi-field studies (Kirby et al., 2008, Paaijmans *et al*., 2008, Schnider *et al*. 2000) suggest that in the presence of mixed-species larval environments the survival of *An. gambiae* is higher than *An. arabiensis*. The reduced number of *An. gambiae* eggs laid following the intervention may have facilitated the rise of *An. arabiensis.*

The change of hosts and feeding sites (cows, outdoors) rather than just a reduction in survival would appear to be responsible for a reduction in vectorial capacity leading, presumably, to a reduction in malaria transmission.

Although the Prokopack and manual aspiration sampled the different gonotrophic stages and sexes equally, the Prokopack was considerably more efficient than manual collections. During the removal sampling the initial collection with the Prokopack sampled more than a third of the estimated total from each site. This is a measure of the efficiency of the unit and reinforces its suitability for the collection of resting malaria vectors

Conclusion

The replacement of *An. gambiae* by *An. arabiensis* following IRS and distribution of LLINs may be due to the latter species having a propensity to more readily feed on animals outside houses than on humans inside them. The Prokopack is a useful tool for the collection of resting mosquitoes.

Acknowledgements

We would like to thank Judy Cronery, Khalid Florian and Elsa Tómas for help with the collection and sorting of the mosquitoes, and the people Kyamyora from whose houses we collected, especially 'Bibi' and Mzee Ramadan. JDC would like to thank John Morgan of the Liverpool School of Tropical Medicine for the Prokopack sampler used in this study.

REFERENCES

Bass, C., Williamson, M.S. & Field L.M. (2008) Development of a multiplex real-time PCR assay for identification of members of the *Anopheles gambiae* species complex. *Acta Tropica,* **107**, 50-53.

Bayoh, M.N., Mathias, D.K., Odiere, M.R., Mutuku, F.M., Kamau, L., Gimnig, J.E., Vuvule, J.M., Hawley, W.A., Hamel, M.J. & Walker, E.D. (2010) *Anopheles gambaie*: historical population decline associated with regional distribution of insecticide-treated bed nets in western Nyanza Province, Kenya. *Malaria Journal*, **9**, 62.

Beier, J.C., Perkins, P.V., Wirtz, R.A., Koros, J., Diggs, D., Gargan, T.P. 2nd & Koech, D.K. (1988) Bloodmeal identification by direct enzyme-linked immunosorbent assay (ELISA), tested on Anopheles (Diptera: Culicidae) in Kenya. *Journal of Medical Entomology,* **25**, 9–16.

Bhatt, S., Weiss, D.J., Cameron, E., Bizanzio, D., Mappin, B., Darlymple, U., *et al*. (2015) The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature.* **526**(7572), 207-211.

Biran, A., Smith, L., Lines, J., Ensink, J. & Cameron, M. (2007). Smoke and malaria: are interventions to reduce exposure to indoor air pollution likely to increase exposure to mosquitoes? *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **101**, 1065–1071.

Bockarie, M.J., Service, M.W., Barnish, G., Momoh, W. & Salia, F. (1994) The effect of woodsmoke on the feeding and resting behaviour of *An. gambiae* s.s. *Acta Tropica*. **57**, 337-340.

Ceita, J.G.V. (1986) Malaria in São Tome´ and Príncipe. (1986) In: Buck, A.N. (Ed.), *Proceedings of the Conference on Malaria in Africa.* American Institute of Biological Sciences, Washington DC, pp. 142–155.

Coetzee, M., Craig, M. & le Sueur, D. (2000) Distribution of African malaria mosquitoes belonging to the *Anopheles gambiae* complex. *Parasitology Today*, **16**, 74-77.

Charlwood, J.D. & Edoh, D. (1996) Polymerase chain reaction used to describe larval habitat use by *Anopheles gambiae* complex (Diptera: Culicidae) in the environs of Ifakara, Tanzania. *Journal of Medical Entomology*, **22**, 202-204.

Charlwood, J.D. & Graves, P.M. (1987) The effect of Permethrin-impregnated bednets on a population of *Anopheles farauti* in coastal Papua New Guinea. *Medical and Veterinary Entomology,* **1**, 319-327.

Charlwood, J.D., Pinto, J., Sousa, C.A., Ferreira, C., Petrarca, V. & do Rosario, V.E. (2003) A mate or a meal' - Pre-gravid behaviour of female *Anopheles gambiae* from the islands of São Tomé and Príncipe, West Africa. *Malaria Journal*, **2**, 7.

Charlwood, J.D., Nenhep, S., Sovannaroth, S., Morgan, J.C., Hemingway, J., Chitnis, N. & Briet O.J.T. (2016) 'Nature or Nurture': Survival rate, oviposition interval, and possible gonotrophic discordance among S.E. Asian anophelines. *Malaria Journal,* **15**, 356.

Charlwood, J.D., Pinto, J., Sousa, C.A., Ferreira, C. & do Rosário, V.E. (2002) The swarming and mating behaviour of *Anopheles gambiae* (Diptera: Culicidae) from São Tomé Island. *Journal of Vector Ecology*, **27**, 178-183.

519 Charlwood, J.D., Smith, T., Kihonda, J., Billingsley, P.F. & Takken, W. (1995)
520 Density independent feeding success of malaria vectors in Tanzania. *Bulletin of*
521 *Entomological Research,* **85**, 29-35.

522 Charlwood, J.D., Vij, R. & Billingsley, P.F.B. (1999) Dry season refugia of malaria-
523 transmitting mosquitoes in a dry savannah zone of East Africa. *American Journal*
524 *of Tropical Medicine and Hygiene,* **62**, 726-732.

525 Debboun, M., Frances, S.P. & Strickman, D. (2006) I*nsect repellents: principles,*
526 *methods, and uses*.

527 Dye, C. (1992) The analysis of parasite transmission by blood-sucking-insects.
528 *Annual Review of Entomology*, **27**, 1-19.

529 Gibbins, E.G. (1933) The Domestic Anopheles Mosquitoes of Uganda. *Annals of*
530 *Tropical Medicine & Parasitology*, **27,** 15–25.

531 Gillies, M.T. & Coetzee, M. (1987) A supplement to the Anophelinae of Africa
532 South of the Sahara (Ethiopian Zoogeographical Region). *Publication of the South*
533 *African Institute for Medical Research, Johannesburg. no 55.*

534 Gillies, M.T. & de Meillon, B. (1968) Anophelinae of Africa south of the Sahara,
535 (Ethiopian Zoogeographical Region), 2nd Edition. Johannesburg, *Publication of*
536 *the South African Institute for Medical Research. no 54*.

537 Gillies, M.T. & Wilkes, T.J. (1965) A study of the age composition of populations of
538 *Anopheles gambiae* Giles and *A. funestus* Giles in north-eastern Tanzania. *Bulletin*
539 *of Entomological Research,* **56***,* 237-262*.*

540 Gimnig, J.E., Ombok, J., Kamau, L. & Hawley, W.A.N. (2001) Characteristics of
541 larval Anopheline (Diptera: Culicidiae) habitats in Western Kenya. *Journal of*
542 *Medical Entomology*, **38**, 282-288.

543 Githeko, A.K., Service, M.W., Mbogo, C.M., Atieli, F.K. & Juma, F.O. (1994) Origin of
544 blood meals in indoor and outdoor resting malaria vectors in western Kenya.
545 *Acta Tropica,* **58**, 307-316.
546 Hayes, J. & Charlwood, J.D. (1977) O *Anopheles darlingi* evita DDT numa area de
547 malaria resistente a drogas. *Acta Amazonica,* **7**, 289.
548 Killeen, G.F., Masalu, J.P., Chinula, D., Fotakis, E.A., Kavishe, D.R., Malone, D. &
549 Okumu, F. (2017) Control of Malaria Vector Mosquitoes by Insecticide-Treated
550 Combinations of Window Screens and Eave Baffles. *Emerging Infectious Diseases*,
551 **23**, 5.
552 Kweka, E.J., Nkya, W.M.M., Mahande, A.M., Assenga, C., Mosha, F.W. *et al*. (2008)
553 Mosquito abundance, bed net coverage and other factors associated with
554 variation in sporozoite infectivity rates in four villages of rural Tanzania. *Malaria*
555 *Journal,* **59**, 7.
556 Kirby, M.J. & Lindsay, S. (2009) Effect of temperature and inter-specific
557 competition on the survival of *Anopheles gambiae sensu stricto* and *An. arabiensis*
558 larvae. *Acta Tropica,* **109**, 118-123.
559 LeClair, C., Cronery, J., Kessy, E., Tomás, E.V.E., Rowland, M., Protopopoff, N. &
560 Charlwood, J.D. (2017). 'Repel all biters': Combination mosquito nets enhance
561 collections of endophilic *Anopheles gambiae* and *An. arabiensis* in CDC light-traps.
562 *Malaria Journal XX*
563 Lefevre, L.C., Kounbrobr, R.D., Elguero, E., Fontenille, D., Renaud, F., Costantini, C.
564 & Thomas, F. (2009) Beyond nature and nurture: Phenotypic plasticity in blood-
565 feeding behaviour of *Anopheles gambiae* s.s. when humans are not readily
566 accessible. *American Journal of Tropical Medicine & Hygiene,* **81**, 1023-1029.

Lwetoijera, D.W., Harris, C., Kiware, S.S., Dongus, S., Devine, G.J. & McCall, P.J. (2014) Increasing role of *Anopheles funestus* and *Anopheles arabiensis* in malaria transmission in the Kilombero Valley, Tanzania. *Malaria Journal,* **13**,1.

Maia, M.F., Robinson, A., John, A., Mgando, J., Simfukwe, E. & Moore, S.J. (2011) Comparison of the CDC Backpack aspirator and the Prokopack aspirator for sampling indoor- and outdoor-resting mosquitoes in southern Tanzania. *Parasites & Vectors,* **4**, 124.

Muriu, S.M., Coulson, T., Mbogo, C.M., Godfray, C.J. (2012) Larval density dependence in *Anopheles gambiae* s.s., the major African vector of malaria. *Journal of Animal Ecology,* **82**, 166-174.

Onyango, S.A., Kitron, U., Mungai, P., Muchuri, E.M., Kokwaro, E., King, C.H. & Mutuku, M. (2013) Monitoring malaria vector control interventions: effectiveness of five different adult sampling methods. *Journal of Medical Entomology*, **50**, 1140-1151.

Oxborough, R. (2016) Trends in US President's Malaria Initiative-funded indoor residual spray coverage and insecticide choice in sub-Saharan Africa (2008-2015): urgent need for affordable, long-lasting insecticides. *Malaria Journal*. **15**, 146.

Paijmans, K.P., Huijben, S., Githeko, A. & Takken, W. (2009) Competitive interactions between larvae of the malaria mosquitoes *Anopheles arabiensis* and *Anopheles gambiae* under semi-field conditions in western Kenya. *Acta Tropica*. **109**, 124-130.

Protopopoff, N., Matowo, J., Malima, R., Kavishe, R., Kaaya, R., Wright, A. *et al*. (2013) High level of resistance in the mosquito Anopheles gambiae to pyrethroid

591 insecticides and reduced susceptibility to bendiocarb in north-western Tanzania.
592 *Malaria Journal*, **12**, 1.
593 Russell, T.L., Govella, N.J., Azizi,, S., Drakeley,, C.J., Kachur, P.S. & Killeen, G.F.
594 (2011) Increased proportions of outdoor feeding among residual malaria vector
595 populations following increased use of insecticide-treated nets in rural Tanzania.
596 *Malaria Journal*, **10**, 1.
597 Schneider, P., Takken, W. & McCall, P.J. (2000) Interspecific competition between
598 sibling species larvae of *Anopheles arabiensis* and *Anopheles gambiae. Medical*
599 *and Veterinary Entomology*, **14**, 165-170.

600 Silver, J.B. (2008) *Mosquito Ecology, Field sampling methods*. 3rd Edition. Springer,
601 New York.

602 Service, M.W. (1971) Studies on sampling larval populations of the *Anopheles*
603 *gambiae* complex. *Bulletin of the World Health Organisation*, **45**, 169-180.

604 Smith, A., Obudho, W.O. & Esozed, S. (1966) Resting patterns of *anopheles*
605 *gambiae* in experimental huts treated with malathion. *Transactions of the Royal*
606 *Society of Tropical Medicine and Hygiene,* **60**, 401-408.

607 Sousa, C.A., Pinto, J., Almeida, P.G., Ferreira, C., do Rosario V.E. & Charlwood, J.D.
608 (2001) Dogs as a favoured host of *Anopheles gambiae* sensu stricto
609 (Diptera,Culicidae) of São Tomé, West Africa. *Journal of Medical Entomology,* **38**,
610 122-125.

611 Southwood, T.R.E. (1978) *Ecological methods: with particular reference to the*
612 *study of insect populations* Chapman & Hall. ISBN 978-0-412-15880-3

613 Vazquez-Prokopec, G. (2009) A new, cost-effective, battery-powered aspirator
614 for adult mosquito collections. *Journal of Medical Entomology*, **46**, 1256-1259.

615 Statacorp. (2011) *Stata Statistical Software: Release 12.*

616 White, G.B. (1974) *Anopheles gambiae* complex and disease transmission in
617 Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* **68**,
618 278-298.

619 West, P.A., Protopopoff, N., Wright, A., Kivaju, Z., Tigererwa, R., Mosha, F.W., *et al*.
620 (2014) Indoor residual spraying in combination with insecticide-treated nets
621 compared to insecticide-treated nets alone for protection against malaria: A
622 cluster randomised trial in Tanzania. *PLoS Medicine,* **11**, 4.

623 World Health Organization. (1975) Manual on practical entomology in malaria. Part II Methods
624 and Techniques. Geneva: WHO, 1-197.

625 World Health Organization. (2015) *An operational manual for indoor residual*
626 *spraying (IRS) for malaria transmission control and elimination.* Geneva: WHO, 1-
627 134.

628 Zippin, C. (1958) The removal method of population estimation. *Journal of*
629 *Wildlife Management*, **22**, 82-90.

630 Charlwood, J.D., Tomás, E.V.E., Cuamba, N. & Pinto, J. (2015) Analysis of the sporozoite ELISA for estimating
631 infection rates in Mozambican anophelines. *Medical and Veterinary Entomology*, **29**, 10-16

632

633

634 TABLES

635

636 Table 1. Total number (and proportion of the total), by abdominal condition, of
637 *An. gambiae* s.l. and *An. funestus* females and males (and total female/male ratio)
638 collected indoor resting from 25 days of collection between 5th June and 16th
639 October 2014, Kyamyorwa, Muleba, Tanzania.

640

	Abdominal condition					
	Unfed	Part-fed	Engorged	Semi-gravid	Gravid	Male
An. gambiae s.l.	96	58	409	143	187	392
Proportion	0.11	0.06	0.46	0.16	0.21	1/0.44
An. funestus	8	2	52	18	21	13
Proportion	0.08	0.02	0.51	0.18	0.21	1/0.13

641

642

643

644

645

646

647

648

649

650

651

652

653 Table 2. Proportion of the total collection by abdominal condition and ratio of
654 males to females according to collection type (Manual aspiration or Prokopack),
655 Kyamyorwa, Muleba, Tanzania.

	Abdominal condition					
	Unfed	Part-fed	Engorged	Semi-gravid	Gravid	male/female ratio
Aspirator	0.13	0.08	0.38	0.23	0.18	0.68
Prokopack	0.11	0.06	0.51	0.10	0.23	0.68

656

657 Table 3. Density rate ratio (and 95% confidence interval) of the number of
658 anophelines collected according to the method used, manual aspirator or
659 Prokopack aspirator.

660

Method	n	N	Median	IQR	DRR [95% C.I.]	p-value
Aspirator	113	349	1	0-5	1	-
Prokopack	122	534	3	1-6	1.48 [1.03, 2.12]	0.03

n – number of collections; N – total number of collected anophelines; DRR – density rate ratio; IQR – inter-quartile range

661

Table 4. Mean number (and standard deviation) of female and male *An. gambiae* collected from smoke-free houses by manual aspiration or Prokopack aspirator according to the order in which the collection was undertaken, Kyamyorwa B, Muleba, Tanzania.

	Females			
	Aspirator		Prokopack	
Order of collection	1	2	1	2
Roof	2.36 (1.5)	1.09 (0.9)	3.49 (3.3)	3.33 (3.0)
Walls	4.76 (2.2)	0.94 (0.6)	6.71 (4.0)	3.04 (1.9)
	Males			
Roof	0.93 (1.3)	0.50 (0.5)	1.70 (1.4)	0.95 (1.1)
Walls	1.97 (2.6)	0.67 (1.2)	4.89 (5.0)	1.17 (1.0)

672

673

674 Table 5. Mean numbers (with 95% confidence intervals) of *Anopheles gambiae*
675 collected with the Prokopack mechanical aspirator in smoke-free and smoky
676 houses.

		Number of collections	Mean	95% C.I.	Wall/Roof ratio
No smoke	Wall	25	3.4	2.2-4.6	
	Roof	39	5.0	2.9-7.1	0.68
Smoke	Wall	29	3.1	1.2-4.8	
	Roof	29	1.2	0.5-1.9	2.58

677

678

679

680 Table 6. Number of *Anopheles gambiae* s.l. collected and estimated total
681 population (based on Zippin's (1958) method, as described by Southwood
682 (1978)), during removal samples. (S.E. – standard error)

683

Site	Males	Females		Total	Estimated	S. E.
		unfed	Fed/gravid	collected	total	
1	70*	33*	10	113	133	3.26
2	93	40	23	156	222	8.28
3	81	56	24	161	201	13.85

684 * - includes 1 *Culex* sp.

685

686

687 Table 7. Gonotrophic age, according to abdominal condition, of *An. arabiensis*
688 females collected resting outdoors, January–February 2016, Kyamyorwa,
689 Tanzania.

	Gonotrophic Age							
	Nulliparous				Parous			
	Virgin	Plug	NII	NIII	a-sac	b-sac	c-sac	d-sac
Unfed	61	5	0	0	0	0	0	2
Part fed	1	5	0	1	0	0	0	2
Engorged	6	19	3	2	3	8	7	35

690

691

692 Table 8. Host source of resting *Anopheles arabiensis*, determined by ELISA,
693 collected indoors, outdoors (from a latrine with banana leaf roof and walls) and a
694 cow shed, 2016, Kyamyorwa, Tanzania with percentage of the total analysed.
695 (C.I. - adjusted Wald confidence intervals).

696

Collection	Human	% (C.I)	Dog	% (C.I)	Cow	% (C.I)	Goat	% (C.I)	No result
Indoor	16	47 (34-69)	0	0	18	53 (37-69)	0	0	7
Cow shed	1	4 (1-7)	0	0	19	79 (59-91)	4	17 (6-36)	1
Outdoor	18	9 (6-14)	4	2 (1-7)	154	81(74-86)	15	8 (5-13)	15

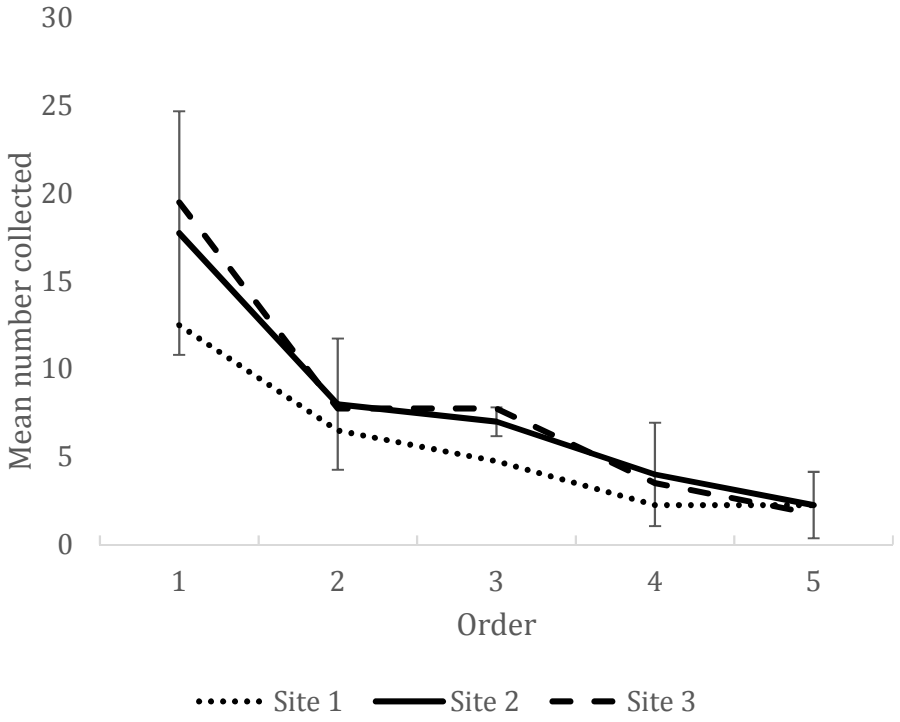
697

698

699

700

701



702

703

704 Figure 1. Decline in the number of mosquitoes collected with the Prokopack

705 sampler from three sites in Kyamyorwa village, Tanzania, March 2017. The error

706 bars are the standard deviations derived from the second site.