

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

50

51

52 INTRODUCTION

53

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

57 1995; Charlwood et al., 1999). Indoor resting mosquitoes are vulnerable to
58 current chemical interventions, which is one of the main reasons for the recent
59 decline in malaria transmission in Africa (Bhatt et al., 2015). Long lasting
60 insecticide treated mosquito nets (LLIN's) prevent feeding, whilst spraying the
61 interior walls of houses with a residual insecticide (IRS) kills and repels
62 mosquitoes that attempt to rest indoors.

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

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

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

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

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

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

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

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

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

131 METHODS

132 STUDY AREA

133 The village of Kakindo/Kyamyorwa B in Muleba District, Kagera Region in
134 northwest Tanzania (02°04'27.5"S, 31°34'10.8"E), described by LeClair *et al.*
135 (2017), was used for the study. The village is separated by a floodplain, used for
136 agricultural purposes, from an inlet of Lake Victoria where the majority of
137 villagers' farm. Most of the houses in the village are mud-walled and thatched
138 roofed, although corrugated iron roofs are becoming more common, and a
139 number of houses are made of brick. The region has two rainy seasons: the main
140 rains occur between March-May (average monthly rainfall 300 mm) and the
141 short rains in October–December (average monthly rainfall 160 mm). Malaria is
142 endemic with peaks of transmission at the end of the rainy seasons. At the start
143 of the study a pyrethroid resistant population of *An. gambiae* was the primary
144 vector in the area (Protopopoff *et al.*, 2013; West *et al.*, 2014) although *An.*
145 *arabiensis* was also present. In January of 2015 the interior walls of all houses in
146 the village were sprayed with the residual insecticide pirimiphos-methyl
147 (Actellic 300CS) and villagers were given LLIN's impregnated with a pyrethroid
148 insecticide and PBO (Olyset Plus®).

149 COLLECTION METHODS

150 Host seeking mosquitoes were collected indoors using CDC light-traps hung
151 close to an occupied mosquito bednet and outdoors with a Furvela tent-trap
152 (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

178 (Charlwood et al., 1995). Zippin's (1958) method, based on maximum likelihood,
 179 which provides an estimate of the standard error, was used to estimate the total
 180 population in each site. As described by Southwood (1978) the total catch $T =$
 181 $n_1+n_2+n_3+n_4+n_5$ where $n_1..n_5$ are the number of insects caught on each
 182 respective round of sampling. Then the value of $\sum_{i=1}^k (i - 1)y_i$ is found, where k
 183 = the number of samples and $i = 1$ and $y_i =$ the catch on the i th occasion.

184 Following this the ratio R is determined where

185
 186

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

188

189 and

190

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

191

192 $p =$ the probability of capture on a single occasion and $q=1-p$ and the estimated
 193 size of the total population is

194

195

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

196

197

198 RESTING SITES

199

200 INDOOR COLLECTIONS

201

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

208 OUTDOOR COLLECTIONS

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

219 MOSQUITO PROCESSING

220 Collected anophelines were identified to species or species group using the keys
221 of Gillies and DeMeillon (1968) and Gillies and Coetzee (1987). Samples of the
222 *An. gambiae* s.l. from both collection periods collected indoors with CDC light-
223 traps, were identified to species by PCR following the protocols of Bass et al.,
224 (2008).

225 AGE DETERMINATION

226

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

231
$$p = \sqrt[m]{n}$$

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

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

236 BLOOD MEAL ANALYSIS

237

238 Blood meal analysis of the sample of fed mosquitoes was done by direct ELISA,
239 using a technique based on that of Beier *et al.* (1998). Filter paper blood spots
240 were cut using an 8mm diameter hole punch and eluted with 600 μ l phosphate
241 buffered saline (PBS), centrifuged for 5–10 seconds and incubated at 4°C
242 overnight. The following morning samples were centrifuged again and a 50 μ l
243 aliquot dispensed into each micro-plate well, covered, and incubated at room
244 temperature for 3 hours. Each well was washed twice with washing buffer
245 (PBS/Tween 0.5%), filled with blocking buffer (PBS/Casein in NaOH; 200 μ l) and
246 incubated for 1 hour. Wells were washed twice with washing buffer and a host-
247 specific conjugate added. Conjugates were: Goat anti-human IgG H&L (50 μ l)
248 diluted 1:4000; Goat anti-dog IgG H-L (50 μ l) diluted 1:2000; Goat anti-bovine
249 IgG H-L (50 μ l) diluted 1:2000, and Rabbit anti-goat IgG H-L (50 μ l) diluted
250 1:2000 (Kirkegaard and Perry Laboratories). After 1 hour, wells were emptied
251 and washed four times with washing buffer, and ABTS peroxidase substrate
252 (100 μ l; Kirkegaard and Perry Laboratories) was added to each well. Initially
253 thirty minutes after addition of the substrate absorbance was read at 405 nm in
254 an ELISA reader (Multiskan FC® Thermoscientific). Each sample was run in
255 duplicate and was tested against two antibodies per run (initially anti-human
256 paired with anti-dog). All samples with negative results were tested using anti-

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

261 DATA ANALYSIS

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

264 ETHICS

265 The study was conducted as a component of the Pan African Malaria Vector
266 Research Consortium project 'Evaluation of a novel long lasting insecticidal net
267 and indoor residual spray product, separately and together, against malaria
268 transmitted by pyrethroid resistant mosquitoes' which received ethical
269 clearance from the ethics review committees of the Kilimanjaro Christian
270 Medical College (certificate number 781 on 16/09/2014), the Tanzanian
271 National Institute for Medical Research (20/08/2014), and the London School of
272 Hygiene and Tropical Medicine (reference 6551 on 24/07/2014). The trial is
273 registered with ClinicalTrials.gov (registration number NCT02288637) on
274 11/7/2014. Prior to beginning collections, informal sensitization sessions were
275 conducted with village members to explain sampling-related activities. Written
276 and verbal informed consent was obtained from all participants who could
277 withdraw from the study at any time should they wish to do so.

278

279 RESULTS

280

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

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

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

299 INDOOR COLLECTIONS

300

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

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

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

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

322 OUTDOOR COLLECTIONS

323

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

330 A total of 654 female and 804 *An. gambiae* s.l. and four female and one male *An.*
331 *funestus* group were collected from 33 collections from the latrine in December
332 2016-January 2017. The high proportion (55%) of male *An. gambiae* s.l.
333 collected from the latrine contrasts with the 31% of *An. gambiae* males (283 of
334 901 collected) in, earlier, indoor collections ($X^2=126$, $p<0.001$).

335 Sixty-one of the 68 unfed female mosquitoes dissected from these collections
336 were virgins, five had a mating plug whilst the two remaining unfed mosquitoes
337 were parous with D sacs (Table 4). Most (64%) of the 83 blood-fed insects
338 dissected were parous and most of these (66%) had D sacs (Table 4). This was
339 significantly different to the part fed and unfed insects ($X^2=87.75$ $p=<0.001$).
340 Assuming that the engorged females were the equivalent of the biting population
341 (therefore discounting the unfed insects) the estimated parous rate was 0.64.
342 Assuming a two-day gonotrophic cycle the estimated daily survival rate was
343 80%.

344 BLOOD MEAL SOURCE

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

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

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

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

362 DISCUSSION

363
364 The propensity to feed inside houses and to rest in them once they have done so
365 may be independent adaptations. For example, Hayes and Charlwood, (1977)
366 describe how, in Brazil *An. darling* came into the open houses used on the newly
367 opened Manaus-Caracari highway, fed and left when engorged without touching
368 a single surface in the room, other than the underside of people's hammocks.

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

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

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

389

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

395 Among the small number of *An. arabiensis* collected indoors after the
396 intervention there were significantly fewer gravid and semi-gravid insects
397 compared to those collected from the latrine. This indicates that the insects
398 completed gonotrophic development outside, rather than indoors, but overall
399 the duration of egg development would also have been two days. Estimated daily
400 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

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

433

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

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

451

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

458

459 CONCLUSION

460

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

465

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467

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473

474 REFERENCES

- 475 Bass, C., Williamson, M.S. & Field L.M. (2008) Development of a multiplex real-
476 time PCR assay for identification of members of the *Anopheles gambiae* species
477 complex. *Acta Tropica*, **107**, 50-53.
- 478 Bayoh, M.N., Mathias, D.K., Odiere, M.R., Mutuku, F.M., Kamau, L., Gimnig, J.E.,
479 Vuvule, J.M., Hawley, W.A., Hamel, M.J. & Walker, E.D. (2010) *Anopheles gambiae*:
480 historical population decline associated with regional distribution of insecticide-
481 treated bed nets in western Nyanza Province, Kenya. *Malaria Journal*, **9**, 62.
- 482 Beier, J.C., Perkins, P.V., Wirtz, R.A., Koros, J., Diggs, D., Gargan, T.P. 2nd & Koech,
483 D.K. (1988) Bloodmeal identification by direct enzyme-linked immunosorbent
484 assay (ELISA), tested on *Anopheles* (Diptera: Culicidae) in Kenya. *Journal of*
485 *Medical Entomology*, **25**, 9–16.
- 486 Bhatt, S., Weiss, D.J., Cameron, E., Bizanzio, D., Mappin, B., Darlymple, U., *et al.*
487 (2015) The effect of malaria control on *Plasmodium falciparum* in Africa between
488 2000 and 2015. *Nature*. **526**(7572), 207-211.
- 489 Biran, A., Smith, L., Lines, J., Ensink, J. & Cameron, M. (2007). Smoke and malaria:
490 are interventions to reduce exposure to indoor air pollution likely to increase
491 exposure to mosquitoes? *Transactions of the Royal Society of Tropical Medicine*
492 *and Hygiene*, **101**, 1065–1071.
- 493 Bockarie, M.J., Service, M.W., Barnish, G., Momoh, W. & Salia, F. (1994) The effect
494 of woodsmoke on the feeding and resting behaviour of *An. gambiae* s.s. *Acta*
495 *Tropica*. **57**, 337-340.

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

499 Coetzee, M., Craig, M. & le Sueur, D. (2000) Distribution of African malaria
500 mosquitoes belonging to the *Anopheles gambiae* complex. *Parasitology Today*, **16**,
501 74-77.

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

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

508 Charlwood, J.D., Pinto, J., Sousa, C.A., Ferreira, C., Petrarca, V. & do Rosario, V.E.
509 (2003) A mate or a meal' - Pre-gravid behaviour of female *Anopheles gambiae*
510 from the islands of São Tomé and Príncipe, West Africa. *Malaria Journal*, **2**, 7.
511

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

516 Charlwood, J.D., Pinto, J., Sousa, C.A., Ferreira, C. & do Rosário, V.E. (2002) The
517 swarming and mating behaviour of *Anopheles gambiae* (Diptera: Culicidae) from
518 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) *Insect 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: Culicidae) 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.

567 Lwetoijera, D.W., Harris, C., Kiware, S.S., Dongus, S., Devine, G.J. & McCall, P.J.
568 (2014) Increasing role of *Anopheles funestus* and *Anopheles arabiensis* in malaria
569 transmission in the Kilombero Valley, Tanzania. *Malaria Journal*, **13**,1.

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

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

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

581 Oxborough, R. (2016) Trends in US President's Malaria Initiative-funded indoor
582 residual spray coverage and insecticide choice in sub-Saharan Africa (2008-
583 2015): urgent need for affordable, long-lasting insecticides. *Malaria Journal*. **15**,
584 146.

585 Paijmans, K.P., Huijben, S., Githeko, A. & Takken, W. (2009) Competitive
586 interactions between larvae of the malaria mosquitoes *Anopheles arabiensis* and
587 *Anopheles gambiae* under semi-field conditions in western Kenya. *Acta Tropica*.
588 **109**, 124-130.

589 Protopopoff, N., Matowo, J., Malima, R., Kavishe, R., Kaaya, R., Wright, A. *et al.*
590 (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

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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
<i>An. gambiae</i> s.l.	96	58	409	143	187	392
Proportion	0.11	0.06	0.46	0.16	0.21	1/0.44
<i>An. funestus</i>	8	2	52	18	21	13
Proportion	0.08	0.02	0.51	0.18	0.21	1/0.13

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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					male/female ratio
	Unfed	Part- fed	Engorged	Semi- gravid	Gravid	
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

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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

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662

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

667

Order of collection	Females			
	Aspirator		Prokopack	
	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)

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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			Wall/Roof
		collections	Mean	95% C.I.	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

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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 collected	Estimated total	S. E.
		unfed	Fed/gravid			
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.

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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

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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

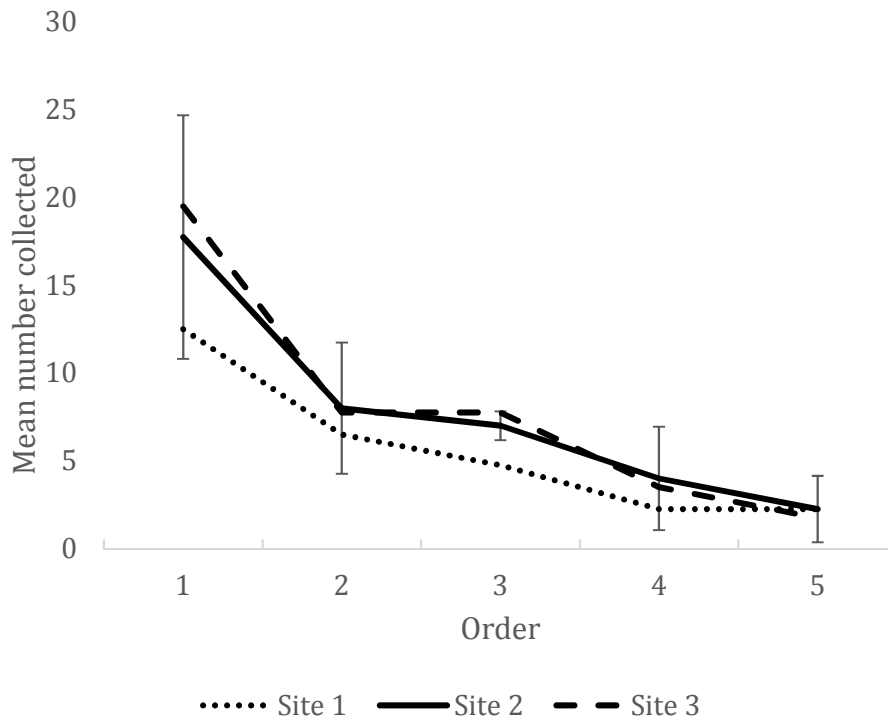
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704 Figure 1. Decline in the number of mosquitoes collected with the Prokopack
705 sampler from three sites in Kyamyorwa village, Tanzania, March 2017. The error
706 bars are the standard deviations derived from the second site.