Tropical Medicine and International Health

VOLUME 9 NO 6 PP 664–672 JUNE 2004

# Measuring the efficacy of insecticide treated bednets: the use of DNA fingerprinting to increase the accuracy of personal protection estimates in Tanzania

**Seyi Soremekun[1,2], Caroline Maxwell[1,3], Martin Zuwakuu[3], Cynthia Chen[2,4], Edwin Michael[2] and Christopher Curtis[1]**

1 *London School of Hygiene and Tropical Medicine, London, UK*
2 *Department of Infectious Disease Epidemiology, Imperial College London, Norfolk Place, UK*
3 *Ubwari Field Station of the Tanzanian National Institute for Medical Research, Muheza, Tanga, Tanzania*
4 *Human Reproductive Sciences Unit, University of Edinburgh, Edinburgh, UK*

**Summary** Insecticide-treated nets have proved successful in the prevention of malaria as a result of both the personal protection with which they provide the sleeper and also the 'mass effect' on the local mosquito population when they are used on a community-wide basis. Personal protection estimates are normally based on comparisons of the numbers of bloodfed mosquitoes found in rooms with and without nets, however it seemed possible that a number of those mosquitoes may not have fed on the occupants of the rooms in which they were found but had entered after feeding elsewhere. To address this possible source of error, we used an 8-locus microsatellite system to identify the source of bloodmeals of *Anopheles gambiae s.l.* and *A. funestus* mosquitoes collected in rooms and window traps in Tanzanian villages with and without nets treated with alphacypermethrin. DNA fingerprints were produced from blood samples taken from people who had slept in these rooms and were matched to fingerprints obtained from the mosquito bloodmeals. We were able to type successfully over 90% of the bloodmeals collected and found that proportions of bloodfed mosquitoes that had fed on occupants of the rooms in which they were found were high and only slightly greater in villages without treated nets than those with them (95% and 88%, respectively). When these percentages were used to adjust estimates of personal protection, it was found that the error due to mosquitoes not feeding in the rooms in which they were collected is negligible.

**keywords** DNA fingerprinting, insecticide-treated bednet, mass effect, mosquito biting patterns, mosquito bloodmeal, personal protection

## Introduction

An insecticide treated net (ITN) aims to prevent access of biting insects to the individual sleeper and can also have a 'mass effect' on the local mosquito population when a whole community uses ITNs (Maxwell *et al.* 1999, 2002; Takken 2002; Gimnig *et al.* 2003). The personal protection to a sleeper as a result of ITN use has generally been calculated as the percentage decrease in the number of bloodfed mosquitoes found in rooms with nets, compared with rooms without nets, corrected for any difference in mosquito populations in the villages concerned (Maxwell *et al.* 2002, 2003). The additional mass effect has been calculated to be as great if not greater than the protection effect to the individual (Maxwell *et al.* 2002, 2003). However, these estimates in village houses have not previously taken into account the possibility that a proportion of the bloodfed mosquitoes collected resting in rooms may not have fed on the occupants of those rooms, and whether this proportion is greater in rooms with nets as fewer mosquitoes can feed in such rooms. It is important to correctly assess the relative extent of the personal protection and mass killing effects of an ITN as this has an important bearing on the debate concerning the efficiency of the free provision of ITNs to whole communities in comparison to social marketing (World Health Organization 2002; Curtis *et al.* 2003; Lines *et al.* 2003).

In the present study we therefore aimed to increase the accuracy of estimates of the personal protection afforded by ITNs, and addressed the issue by identification of the blood from mosquito bloodmeals as either from people who slept in rooms where the mosquitoes were collected, or from bites elsewhere. Coulson *et al.* (1990) and Gokool *et al.* (1993) attempted this in experimental huts in

Tanzania but only a relatively small proportion of the bloodmeals were successfully identified. Furthermore, the use of experimental huts where sleepers are paid to remain under the net for the whole night may not reflect the real situation in village houses. Here we demonstrate how by the use of a multiplex PCR technique developed by Michael *et al.* (2001) we were able to successfully type blood in a very high proportion of cases at 8 human microsatellite loci. We used genotyping software in order to generate individual-specific 'fingerprints' for occupants of rooms in Tanzanian villages with and without treated nets. We could then match these to 'fingerprints' obtained from bloodmeals from the mosquitoes collected in these rooms and from exit traps (ET) on the room windows. From this we were able to ascertain the proportions of bloodfed anopheline mosquitoes, which had or had not fed in the house in which they were collected and compare these proportions in villages with and without community-wide use of ITNs. The typing technique also allowed us to make some preliminary observations on mosquito biting patterns and discuss epidemiological implications. By the use of ET on the windows we were able to examine the increases in the tendency of fed and unfed mosquitoes to exit from rooms with ITNs.

## Materials and methods

### Study site and field sample collection

Field samples for the study were collected during July 2003 from two villages in the Muheza District of North-Eastern Tanzania, which has lowland areas holoendemic for malaria transmission. This was just after the long rainy season that commonly occurs between April and June.

Two villages were chosen for the study; Kwafungo which had been provided with alphacypermethrin-treated nets in 1999 which have since been re-treated annually (the netted village), and Zenet, a control village with no bednet coverage.

Houses were randomly selected for sampling in both villages, and ET placed on the windows of the chosen houses in the evenings. It was requested of the inhabitants to ensure that as far as was possible, any other available mosquito exit routes were covered by the time they retired to bed each evening. In the mornings, between 0900 and 1100 hours, mosquitoes were collected from the ET and mosquitoes resting on the walls and ceilings inside the same houses were collected by the spray catch technique using pyrethrum dissolved in kerosene in spray pumps to knock down mosquitoes onto sheets spread over all horizontal surfaces within the rooms (Rozendaal 1997). House sampling continued until a total of 100–150 bloodfed anopheline mosquitoes had been collected from each village. Mosquitoes were transported back to the field station in an icebox for species sorting and all bloodfed ones were stored individually in 96% ethanol in 1.5 ml microcentrifuge tubes and refrigerated until the time for transportation to the UK.

Finger-prick samples were collected onto labelled Whatman filter paper from all the people who had slept in or entered rooms from which bloodfed mosquitoes were collected. These blood samples were dried and stored over silica gel individually in sealed plastic envelopes for transportation to the UK. To ensure that all humans relevant to this study were accounted for, the names of the villagers who slept in rooms where mosquitoes were subsequently collected were verified daily, to allow for changes from night to night.

### Ethical consideration

The objective of the study was outlined to the chairpersons of both villages and also to all the participating households and verbal consent was obtained from all the villagers involved. The team also screened participants for malaria, and any found positive for parasites were administered anti-malarials by the nurse on the team. Ethical clearance for this project was obtained from the Tanzanian National Institute for Medical Research, and the ethics committee of the London School of Hygiene and Tropical Medicine.

### DNA extraction

DNA was isolated from the finger-prick blood samples using the Qiagen® DNA Mini Kit protocol (Qiagen, West Sussex, London, UK) for dried blood spots as described by the manufacturer with a slight modification at the elution step as described below. In brief, after incubating 3–6 3 mm circles from the dried blood spots in ATL lysis buffer at 85 °C for 10 min, 20 µl of proteinase K was added to the samples which were then incubated at 56 °C for 1 h, after which 200 µl buffer AL was added and the samples incubated at 70 °C for another 10 min. This was followed by the addition of 200 µl 100% ethanol and two washes in spin columns to elute proteins and other unwanted residues. The DNA was then eluted in two parts with 25 µl distilled water to make a final volume of 50 µl which differed from the 200 µl detailed in the Qiagen Protocol, giving a more concentrated sample of DNA.

The blood-fed mosquitoes were graded as fully fed (FF), 3/4 fed, 1/2 fed or 1/4 fed and the abdomens separated from the heads and thoraces. In the majority of cases it was possible to individually dissect out the bloodmeals from the abdomen tissue to minimize the amount of possibly

interfering mosquito DNA. DNA was then extracted from the bloodmeals following the Qiagen® DNA Micro Kit Tissue protocol, with overnight incubation with ATL lysis buffer and proteinase K option. This was followed by addition of 200 µl AL buffer and 200 µl 100% ethanol and, after a 5 minute incubation at room temperature, two washes in MinElute® spin columns and elution with 20 µl distilled water giving a final volume of 15 µl DNA. Binding of the few target DNA molecules to the spin column membrane was enhanced by the addition of 1 µl carrier RNA (1 µg/µl) to each sample with the AL buffer, as recommended in the protocol.

### PCR amplification and visualization using Genescan® and Genotyper®

An 8-locus short tandem repeat (STR) system developed and optimized by Michael *et al.* (2001) was used to create individual DNA fingerprints for the human blood and mosquito bloodmeal samples. The eight markers used were HUMVWA31/A, D8S1179, D18S51, HUMTHO1, HUMF13A1, HUMFES/FPS, D3S1358 and HUMFIBRA, and primers for these loci were labelled with ABI fluorescent tags 6FAM (HUMVWA31/A, D18S51, HUMTHO1, and HUMFIBRA), NED (D8S1179 and D3S1358) and VIC (HUMF13A1 and HUMFES/FPS), which were used for visualization of the amplified product during electrophoresis on an ABI Prism® DNA Analyzer 3700 (Applied Biosystems, Warrington, UK). The PCR reaction comprized a 25 µl total volume containing 1–50 ng genomic DNA, 1× PARR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCL, 1.5 mM MgCl, 1% Triton-X 100: Cambio Laboratories, Cambridge, UK), 200 µM dNTPs (Qbiogene, Livingston, UK), 1.25 U AmpliTaq Gold (Applied Biosystems), 0.25 µM of each HUMVWA31/A, D8S1179, D21S11 primer, 0.2 µM of each HUMTHO1, HUMF13A1, HUMFES/FPS primer, 0.15 µM of each D3S1358 primer and 0.10 µM HUMFIBRA primer. The DNA was amplified using a DNA Engine® Peltier Thermal Cycler (MJ Research Inc., MA, USA), with 9 min at 95 °C, 30 s denaturing at 93 °C, 1 min 15 s annealing at 57 °C, 15 s extension at 72 °C for 32 cycles followed by an elongation step at 72 °C for 10 min.

For the visualization process, a 2 µl aliquot of PCR product was added to 19.05 µl Hi-Di™ Formamide and 0.95 µl GeneScan ROX-500 size standard (both from Applied Biosystems), denatured then electrophoresed on the ABI PRISM 3700. Scoring of the fragment sizes and genotyping of the microsatellite markers was performed using the GENESCAN™ and Genotyper™ programs (Applied Biosystems).

### Statistical analysis

Data were analysed using logistic regression (adjusting SE for clustering in individual houses) to compare the proportions of blood fed mosquitoes that fed in rooms where they were caught in the two villages. Proportions of ET and spray catches (SC) according to species and feeding within the collection houses or elsewhere (called 'home' and 'away' in this paper) were also compared using categorical tests. To analyse mosquito biting patterns according to the ages and gender of the human subjects, the number of bites per person was modelled on the negative binomial distribution and logistic regression was used for incidence of biting. A $\chi^2$ test for trend was used to assess the possible effects of bloodmeal size on the success of the PCR amplification of the blood meal DNA. All analyses were carried out using the statistical programmes EpiInfo 6 (2001) and Stata 8 (2002).

## Results

### Anopheline catches

Overall 118 bloodfed mosquitoes were collected from the test village, Kwafungo (with nets) and 131 from the control village, Zenet (without nets), representing 74.0% and 24.8%, respectively of the total numbers of mosquitoes (fed and unfed) collected in the villages (Table 1), a difference which is highly significant ($P < 0.001$). These relative proportions are in agreement with other data from this region, with values of 74–86% for villages without ITNs and 28–53% in those with ITNs for the proportions of bloodfed mosquitoes observed in indoor catches (Lines *et al.* 1987; Maxwell *et al.* 2003). *Anopheles funestus* made up 58% of the total anopheline catches in Zenet but only 31% in Kwafungo. Blood feeding success of *A. funestus* was higher than for *A. gambiae* in the presence of treated nets in Kwafungo (35% and 20% respectively, $P < 0.001$).

To obtain the sample numbers of 131 and 118 bloodfed mosquitoes, 73 house collections were required in the netted village (made in 16 houses over 8 days) but only six catches (in five houses over 2 days) were required in the control village. The difference in the number of bloodfed mosquitoes per collection in the netted and control villages was thus in the ratio of 1:13.6 (1.6/21.8) (Table 1). This value includes both the personal protection and mass effect on mosquitoes (which also causes a reduction in sporozoite rate) of the use of treated bednets. To estimate personal protection alone, we compared the overall proportions of mosquitoes which successfully obtained bloodmeals in the two villages (the 74.0% and 24.8% bloodfed collections

**Table 1** Species of anopheline mosquitoes and numbers found bloodfed inside houses and in exit traps in July 2003 in villages with or without treated nets

| Village | Species | Totals | Mean number mosquitoes per collection | Mosquito status | | % Fed | Bloodfed per collection |
|---|---|---|---|---|---|---|---|
| | | | | Unfed | Fed | | |
| Zenet (no ITNS) | *A. funestus* | 103 | 17.17 | 30 | 73 | 71 | 12.17 |
| | *A. gambiae* | 74 | 12.33 | 16 | 58 | 78 | 9.6 |
| Anopheline totals/summary | | 177 | 29.5 | 46 | 131 | 74.0 | 21.8 |
| Kwafungo (with ITNs) | *A. funestus* | 142 | 1.97 | 92 | 50 | 35 | 0.68 |
| | *A. gambiae* | 333 | 4.56 | 265 | 68 | 20 | 0.93 |
| Anopheline totals/summary | | 475 | 6.51 | 357 | 118 | 24.8 | 1.6 |

A total of 73 house collections were made in the former and six in the latter.

mentioned previously). The reduction in the mosquito biting rate in the houses with treated nets was therefore estimated at 66.5% [(1 − 24.8)/74.0] in this study, which is attributable to the personal protection factor. By identification of the sources of bloodmeals in the mosquitoes found in these rooms, we were able to correct this estimate of personal protection to allow for mosquitoes which had fed elsewhere. It was also possible to use the PCR tool to make some observations on mosquito biting patterns within households and villages and the effect of the use of ITNs on these features.

### Bloodmeal identification and its effect on personal protection estimates

We used Genescan® and GENOTYPER software to visualize the allelic differences between humans and to label the alleles according to size. Each human in our study differed at a minimum of two microsatellite loci and produced fingerprint patterns that were easily compared with the fingerprints from the bloodmeals of mosquitoes taken from their houses (Figure 1). Our 8-locus microsatellite system provided unique fingerprint patterns for each the human subjects. 91.2% (227/249) of the mosquito samples were also successfully typed as feeding in the room where they were collected or having fed elsewhere before entering the room, a success rate for bloodmeal typing higher than so far reported in the literature, where the lack of higher success rates has been attributed to factors such as bloodmeal size and time since ingestion (Mukabana *et al.* 2002b). Because of the inverse relationship between time since ingestion and analytical success (Mukabana *et al.* 2002a), we ensured that mosquito collections took place as early in the morning as possible, although it was still observed that PCR success increased significantly with bloodmeal size (Table 3a).

Table 2 shows the proportions of bloodfed mosquitoes which had fingerprint patterns matching humans inside the houses where they were collected ('home' feeding), and those which did not ('away' feeding) in the two villages. In both villages, the 'home' feeds were much more common than the 'away' feeds and among the latter, several of the fingerprints (e.g. mosquito D, Figure 1) were recognizable as from occupants in neighbouring houses where blood samples and mosquitoes had also been collected.

The observed proportion of home feeding was lower in the netted village (Table 2) but over both anopheline species, the difference was not significant as shown by logistic regression ($z = 1.11$, $P = 0.267$ adjusting for house clustering). When the proportions were split by species, *Anopheles gambiae* showed little difference in the home and away feeding proportions between the villages ($P = 0.81$), while *A. funestus* did show significantly more away biting in the netted village compared with the control ($P < 0.001$).

Because there were fewer successful amplifications from mosquitoes which had taken smaller blood meals, we analysed the home/away feeding proportions by bloodmeal size category (Table 3a, columns 5 and 6), and it was observed that the proportions of mosquitoes feeding away in both villages appeared to increase as mosquito bloodmeal size decreased although the difference was not quite significant ($z = -1.82$, $P = 0.069$). It may be that mosquitoes taking smaller bloodmeals are more active and therefore less likely to rest inside a house after they have fed, but the lower success rate in identifying meals from these mosquitoes could also be the true source of the difference. If this is so, it is possible that some distortion to the overall home/away feeding ratio could have arisen as a result of the lower typing success rates of the smaller bloodmeal categories. Table 3b shows the results of a comparison of the distributions of bloodmeal sizes between the villages

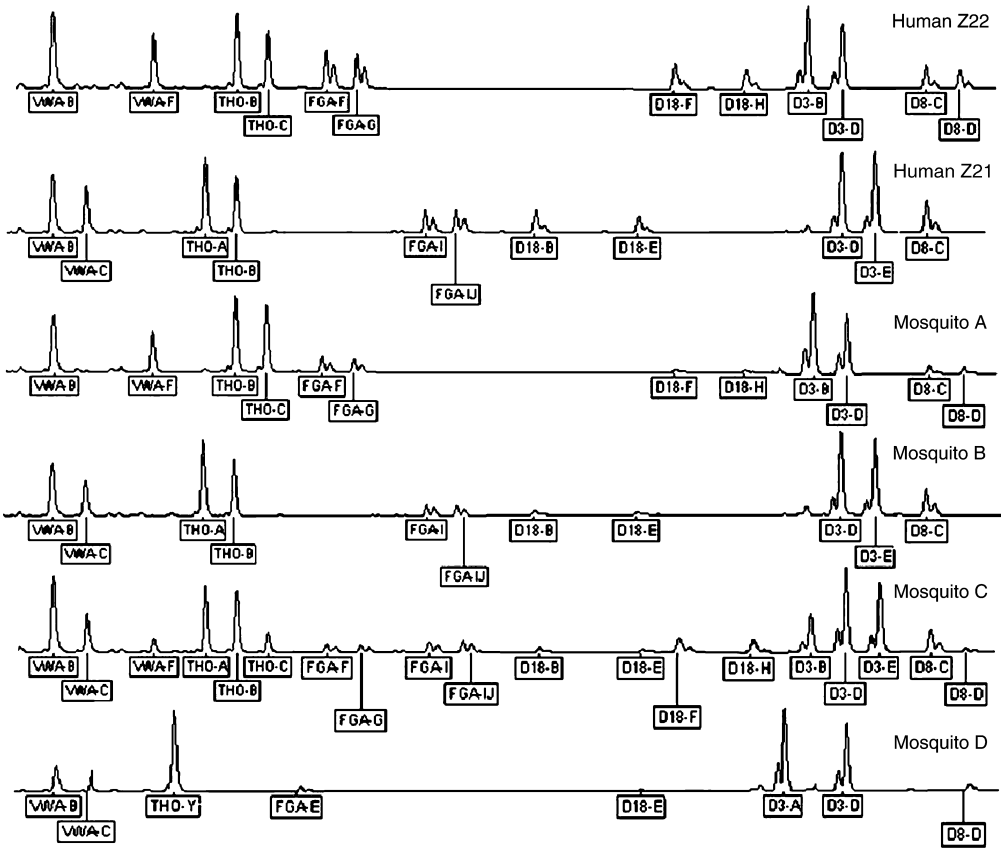


**Figure 1** Example of Genescan®-generated DNA fingerprint patterns from blood spots of inhabitants from one of the study houses and four of the bloodfed mosquitoes collected from inside this house. This diagram shows amplified microsatellite regions from 6 of the 8 loci chosen in the human genome. Within the range of each fluorescent dye, the positions of peaks correspond to the allele fragment sizes (100–300 base pairs) and the height of the peaks to the strength of the signals. Mosquito A and Human Z22 have matching fingerprints indicating that mosquito A fed on this person, as with mosquito B and person Z21. The bloodmeal from mosquito C contained alleles from both persons Z21 and Z22 indicating feeds on both people. The fourth mosquito bloodmeal (mosquito D) shows no similarity to either Z22 or Z21 and clearly had fed on neither of these people but in fact matched with a person from a neighbouring house.

and between species using logistic regression. This distribution was not significantly different either between the species or the two villages (or across the age or gender of the host source of the meals), hence the significance of the differences in the overall home/away feeding outcomes of the villages are also unlikely to be affected by any discrepancies in the smaller bloodmeal size groups.

We used the home and away feeding rates to adjust our previous estimate of the personal protection afforded by nets in the test village. Among the total of 109 successfully amplified bloodmeals from mosquitoes collected in Kwafungo, the data in Table 2 shows that 12% had fed away, indicating that approximately 14 mosquitoes from the overall total of 118 were in this category. Deducting these from both the total number of mosquitoes collected and numbers bloodfed [(118 – 14)/(475 – 14) = 22.6%], leads to an adjusted estimate of the proportion of mosquitoes which entered these netted rooms unfed and succeeded in feeding there. Similarly for the control village, the observation that 5% of the successfully typed bloodfed mosquitoes had fed away leads to an adjusted estimate of 73.0% for the proportion successfully feeding after entering unfed the rooms without nets. Thus a corrected estimate of the % personal protection from mosquito biting provided by the treated nets is 69.0% (1 – 22.6/73.0), which is only slightly different from the uncorrected estimate of 66.5%.

### Exit traps

From the numbers of mosquitoes collected from ET and in SC within the villages (Table 4a), we calculated that 52%

**Table 2** For the successfully amplified mosquito bloodmeals: the proportions deduced to have been from people inside the houses where the mosquitoes were collected (HOME) and from elsewhere (AWAY), divided by species and with overall totals. Double feeding column: represents mosquitoes from HOME groups, which had taken bloodmeals from two people in the house where they were collected

| Village type | Species | Home (%) | Totals home (%) | Away (%) | Totals away (%) | Double feeding (%) |
|---|---|---|---|---|---|---|
| With ITNs | *A. gambiae* | 55 (91.6) | 96 (88)* | 5 (8.3) | 13 (12) | 0/96 (0) |
| | *A. funestus* | 40 (83.3) | | 8 (16.7) | | |
| Without ITNs | *A. gambiae* | 42 (89.4) | 112 (95) | 5 (10.6) | 6 (5) | 10/112 (9) |
| | *A. funestus* | 70 (98.6) | | 1 (1.4) | | |

* The thorax and head from one mosquito was lost from this group and it therefore could not be identified to species level. Thus the total number of 'home' feeding mosquitoes in the netted village of known species is 95, despite the successful amplification of 96 bloodmeals.

**Table 3** (a) Anopheline mosquito bloodmeal sizes (FF = fully fed, 3/4 = 3/4 fed, etc.) in relation to the success of the PCR technique used to identify the source of the bloodmeals in the two villages. $\chi^2$ trend = 13.54, $P < 0.001$. Also showing the numbers and percentages of mosquitoes not feeding in the houses where they were caught (away) sorted by bloodmeal size in two villages in Tanzania. (b) Logistic regression showing that the distribution of the proportions of mosquitoes caught in the four bloodmeal size groups did not differ significantly between the two villages, nor between the sexes nor across two age groups (0–19 years, 20+ years)

(a)

| Bloodmeal size | Totals bloodfed | Number of bloodmeals successfully amplified | % Successful | Away/home feeding | % Away feeding |
|---|---|---|---|---|---|
| FF | 70 | 68 | 97.1 | 4/64 | 6.3 |
| 3/4 | 64 | 61 | 95.3 | 4/57 | 7.0 |
| 1/2 | 68 | 62 | 91.2 | 5/57 | 8.8 |
| 1/4 | 47 | 36 | 76.6 | 5/31 | 16.1 |

(b)

| Parameter (by which bloodmeal size distribution was compared) | *Z*-value | *P*-value | 95% Confidence interval |
|---|---|---|---|
| Village | 0.00 | 0.996 | −1.148 to 1.153 |
| Species | −1.18 | 0.238 | −0.826 to 0.205 |
| Age group | 0.32 | 0.745 | −1.03 to 1.439 |
| Gender | −0.04 | 0.968 | −0.974 to 0.935 |

(24/46) of all the unfed mosquitoes found in houses in the control village were collected from ET, as compared with 91% (324/357) in the netted village. This pattern was repeated for the proportions of blood fed mosquitoes collected in ET in the villages (10.7% and 47.5%, respectively), emphasizing the increased tendency for mosquitoes to exit rooms with ITNs.

Between the two species, the proportions of mosquitoes collected in SC and ET did not differ significantly (see Table 4a for numbers of *A. funestus* and *A. gambiae* collected) with the exception of the bloodfed mosquito collections from the control village where there were significantly more fed *A. funestus* collected resting indoors than *A. gambiae* ($\chi^2 = 6.0$, $P = 0.014$).

The proportion of 'home' feeding mosquitoes did not differ significantly between ET and SC collections in either village (Table 4b).

**Table 4** (a) Numbers of fed and unfed *A. gambiae* and *A. funestus* mosquitoes collected from exit traps (ET) and by spray catches (SC) in the control and test villages. (b) Significance test for difference in SC/ET 'home' feeding proportions within the villages: control village, *not significant (Fisher's Exact test); Test village, †not significant ($\chi^2$ $P \geq 0.5$ with Yates' correction)

| | Control village | | Netted village | |
|---|---|---|---|---|
| | ET | SC | ET | SC |
| (a) | | | | |
| Unfed mosquitoes | | | | |
| *A. funestus* | 16 | 14 | 87 | 5 |
| *A. gambiae* | 8 | 8 | 237 | 28 |
| Fed mosquitoes | | | | |
| *A. funestus* | 3 | 70 | 24 | 26 |
| *A. gambiae* | 11 | 47 | 32 | 36 |
| (b) | | | | |
| Fed mosquitoes*,† | | | | |
| 'Home' feeding | 7 | 105 | 49 | 47 |
| 'Away' feeding | 0 | 6 | 5 | 8 |

### Observations on mosquito biting patterns according to age and gender of house occupants

The 44 humans who slept in rooms where mosquito collections were made were divided into those <20 years old and those ≥20 years, and by gender. It was observed that whilst most people received few or no bites, less than 20% of the individuals in both villages received more than 80% of the bites – this was most apparent in the netted village where, despite reportedly using a treated net at night, one individual received many bites and 62% of the total number of identified bloodmeals came from him. A check on the bednet belonging to this person revealed that it was quite badly torn, which no doubt increased the ease with which the mosquitoes were able to feed. Because of the heterogeneity in mosquito biting patterns we used the negative binomial model to analyse the distribution of the number of bites received by people in each age group for each village. In the netted village members of the age group ≥20 years received significantly more bites per individual than subjects in the <20 years age group ($z = 4.48$, $P < 0.001$), whilst there was no significant age pattern in biting frequency in the control village ($z = -0.82$, $P = 0.412$), although the observed majority of bites were on those aged <20 years. When the participants were split by gender we observed that in the netted village there were significantly more bites on males ($z = 2.60$, $P = 0.009$), but in the control village there was no significant difference between genders ($z = 0.99$, $P = 0.321$). By comparing the proportion of old and young people in each village who were bitten at least once (as revealed by an identified bloodmeal) with those who were apparently never bitten, it was determined through logistic regression that in both villages there were significantly more younger people who completely avoided being bitten (netted village: $P = 0.002$ and control village: $P = 0.021$). Between the villages this pattern did not differ significantly ($z = -1.44$, $P = 0.151$).

## Discussion

We have demonstrated that by using an 8-microsatellite marker DNA fingerprinting method it is possible to correct estimates of the % personal protection by identification of the source of mosquito bloodmeals to determine what proportion of the bloodfed mosquitoes collected from rooms with or without nets had not fed on the occupants of those rooms but on people or animals elsewhere. It turned out that the necessary correction only changed our estimate by about 3%.

We emphasize that despite the presence of treated nets, a few mosquitoes still successfully obtain bloodmeals from individuals sleeping under them (Tables 1 and 2). The relative rarity of such feeds is partly due to the reduction in the mosquito population size where there is widespread use of treated nets in a village (see 'Mean numbers of mosquitoes per collection' column, Table 1) and also as a result of the personal protection of individual net users (see '% Fed' column in Table 1). In our study, because of these two effects, a much larger number of mosquito collections had to be carried out in the netted village (i.e. the 73 collections as opposed to the six in the control village mentioned in the results Table 1) to obtain the targets of over 100 bloodfed mosquitoes for analysis.

The majority of mosquitoes collected in the netted village were unfed, which emphasizes the excito-repellency effect of treated nets, also reflected in the exit trap data which confirms the increased exiting behaviour of both unfed and fed mosquitoes from rooms with treated nets (Lindsay *et al.* 1989; Mathenge *et al.* 2001; Maxwell *et al.* 2003).

Some fed mosquitoes presumably exit houses and avoid ET, but we consider it unlikely that this would affect the significance of the difference in proportions of 'home' and 'away' fed mosquitoes between the two villages. We take this view because the proportions of 'home' feeding mosquitoes collected from either ET on windows or in SC in the rooms were not significantly different (4b). Uncovered gaps in house walls and eaves may provide an escape route for a considerable number of mosquitoes stimulated by the excito-repellency of the pyrethroid on the nets and the numbers of fed mosquitoes in our collections may seriously underestimate the total numbers that fed during the night. This study has excluded 'away' feeders as a serious source of underestimate of the % personal protection due to treated nets, but further studies of mosquitoes escaping from houses despite ET placed on windows might reveal that escapes through uncovered gaps are a more important source of error in estimates of personal protection (Maxwell *et al.* 2003).

There were significant differences in the bloodfeeding and exiting behaviour of *A. funestus* and *A. gambiae* (Table 1 and Table 4). *Anopheles funestus* is a highly endophilic and endophagic species and this was reflected in the higher proportions of fed mosquitoes of this species collected resting indoors in the control village, compared with the corresponding proportion of *A. gambiae*. Although the treated nets induced excito-repellency in both species (Table 4), only in *A. funestus* was there a significant increase in the 'away' feeding in the netted village *vs.* the control (Table 2). This suggests that after pyrethroid contact this species shows a greater tendency to move from building to building after feeding.

Our PCR tool enabled us to unequivocally identify those cases where a single typed bloodmeal contained microsatellite alleles from two people (Figure 1, Mosquito C). However, such cases were only found in the control village (Table 2). This presumably reflects the relative ease with which a mosquito can access human hosts without the physical barrier and insecticidal properties of a net.

The molecular fingerprinting method we have used has applications beyond the assessment of the entomological efficacy of ITNs and is sure to become a very useful method in the future. Success rates in the typing of human blood from mosquito bloodmeals have ranged from 20% to 80% in previous studies of this type (Gokool *et al.* 1993; Koella *et al.* 1998; Michael *et al.* 2001; Ansell *et al.* 2002; Mukabana *et al.* 2002b), where the investigators have relied on different methods of DNA extraction and/or visualization of alleles. We demonstrated that by using forensic-grade extraction kits and automatic genotyping equipment the successful typing of human DNA extracted from bloodfed mosquitoes was possible in over 90% of the mosquitoes collected, using microsatellite loci with high variability in human populations (Litt & Luty 1989; Kimpton *et al.* 1996). The size of the mosquito bloodmeal has been shown here to be related to the success of the PCR technique, but bloodmeal DNA from mosquitoes taking feeds from animals may also fail to amplify due to the specificity of the primers used. The extraction of human DNA from mosquito bloodmeals by a simple forensic kit may enable the use of this technique in the field and minimize the problems associated with storage and transportation of large numbers of bloodfed mosquitoes.

We have also been able to obtain some preliminary data on mosquito biting in relation to age and gender. Michael *et al.* (2001) showed for *Culex quinquefasciatus* mosquitoes in villages without nets that there is a clear age pattern in biting incidence, where incidence tends to increase with age in boys and peaks at age 15 years in girls. Although the observed difference in the numbers of old and young people that were bitten between the netted and control villages was not significant, the mean number of bites per person was higher in those ≥20 years old in the netted village. This differed from the pattern in the control village and suggests better protection of young people by treated nets. This is presumably at least partly because larger (and older) people are more likely to sleep with their bodies touching, or even protruding from their nets. If the better protection of younger people is confirmed in a larger study this would be welcome news as children who have not yet developed immunity are those most affected by malaria in highly endemic areas and are therefore most in need of protection.

## Acknowledgements

We thank the people and village authorities of Kwafungo and Zenet for their excellent co-operation with this project. We are grateful to Mathew Mwaimu, Musa Sudi and Steven Mkongewa for skilful assistance with the field collections. Clearance for this project and publication came from the Tanzanian National Institute for Medical Research and Commission for Science and Technology. Seyi Soremekun is grateful for a travel grant from the London School of Hygiene and Tropical Medicine and a field-work grant from the Chadwick Trust.

## References

Ansell J, Hamilton KA, Pinder M, Walraven GE & Lindsay SW (2002) Short range attractiveness of pregnant women to *Anopheles gambiae* mosquitoes. *Transactions of the Royal Society for Tropical Medicine and Hygiene* **96**, 113–116.

Coulson MR, Curtis CF, Ready PD, Hill N & Smith D (1990) Analysis and amplification of human DNA present in mosquito bloodmeals. *Medical and Veterinary Entomology* **4**, 357–366.

Curtis CF, Maxwell C, Lemnge M *et al.* (2003) Scaling-up coverage with insecticide treated nets against malaria in Africa: Who should pay? *The Lancet Infectious Diseases* **3**, 304–307.

Gimnig JE, Kolczak MS, Hightower AW *et al.* (2003) Effect of permethrin-treated bed nets on the spatial distribution of malaria vectors in western Kenya. *American Journal of Tropical Medicine and Hygiene* **68** (Suppl. 4), 115–120.

Gokool S, Curtis CF & Smith DF (1993) Analysis of mosquito bloodmeals by DNA profiling. *Medical and Veterinary Entomology* **7**, 208–215.

Kimpton CP, Oldroyd NJ, Watson SK *et al.* (1996) Validation of highly discriminating multiplex short tandem repeat amplification systems for individual identification. *Electrophoresis* **17**, 1283–1293.

Koella JC, Sorensen FL & Anderson RA (1998) The malaria parasite *Plasmodium falciparum*, increases the frequency of multiple feeding of its mosquito vector, *Anopheles gambiae*. *Proceedings of the Royal Society of London: Biological Sciences* **265**, 763–768.

Lindsay SW, Snow RW, Broomfield GL, Semegah Janneh M, Wirtz RA. & Greenwood BM (1989) Impact of permethrin-treated bednets on malaria transmission by the *Anopheles gambiae* complex in The Gambia. *Medical and Veterinary Entomology* **3**, 263–271.

Lines JD, Myamba J & Curtis CF (1987) Experimental hut trials of permethrin-impregnated mosquito nets and eave curtains against malaria vectors in Tanzania. *Medical and Veterinary Entomology* **1**, 37–51.

Lines J, Lengeler C, Cham K *et al.* (2003) Scaling-up and sustaining insecticide-treated net coverage. *The Lancet Infectious Diseases* **3**, 465–466.

Litt M & Luty JA (1989) A hypervariable microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within the cardiac muscle actin gene. *American Journal of Human Genetics* **44**, 397–401.

Mathenge EM, Gimnig JE, Kolczak M, Ombok M, Irungu LW & Hawley WA (2001) Effect of permethrin-impregnated bednets on exiting behaviour, blood feeding success, and time of feeding of malaria mosquitoes (Diptera: Culicidae) in western Kenya. *Journal of Medical Entomology* **38**, 531–536.

Maxwell CA, Myamba J, Njunwa KJ, Greenwood BM & Curtis CF (1999) Comparison of bednets impregnated with different pyrethroids for their impact on mosquitoes and on re-infection with malaria after clearance of pre-existing infections with chloroproguanil-dapsone. *Transactions of the Royal Society for Tropical Medicine and Hygiene* **93**, 4–11.

Maxwell CA, Msuya E, Sudi M, Njunwa KJ, Carneiro IA & Curtis CF (2002) Effect of community-wide use of insecticide-treated nets for 3–4 years on malarial morbidity in Tanzania. *Tropical Medicine and International Health* **7**, 1003–1008.

Maxwell CA, Chambo W, Mwaimu M, Magogo F, Carneiro IA & Curtis CF (2003) Variation of malaria transmission and morbidity with altitude in Tanzania and with introduction of alphacypermethrin treated nets. *Malaria Journal* **2**, 28.

Michael E, Ramaiah KD, Hoti SL *et al.* (2001) Quantifying mosquito biting patterns on humans by DNA fingerprinting of bloodmeals. *American Journal of Tropical Medicine and Hygiene* **65**, 722–728.

Mukabana WR, Takken W, Seda P, Killeen GF, Hawley WA & Knols BG (2002a) Extent of digestion affects the success of amplifying human DNA from blood meals of *Anopheles gambiae* (Diptera: Culicidae). *Bulletin of Entomological Research* **92**, 233–239.

Mukabana WR, Takken W & Knols BJ (2002b) Analysis of arthropod bloodmeals using molecular genetic markers. *Trends in Parasitology* **18**, 505–509.

Rozendaal JA. (ed) (1997) Mosquitoes and other biting Diptera: control measures. In *Vector Control-Methods for Use by Individuals and Communities*. World Health Organization, Geneva, 52–164.

Takken W (2002) Do insecticide-treated nets have an effect on malaria vectors? *Tropical Medicine and International Health* **7**, 1022–1030.

World Health Organization (2002) Scaling-up insecticide treated netting programmes in Africa. A strategic framework for coordinated national actionWHO/CDS/RBM/2002.43, World Health Organization, Geneva.

**Authors**

**Seyi Soremekun** (corresponding author) and **Edwin Michael**, Department of Infectious Disease Epidemiology, Imperial College London, Norfolk Place, W2 1PG, UK. Tel.: +44 (0)2075943622; E-mail: s.soremekun@imperial.ac.uk, e.michael@imperial.ac.uk

**Caroline Maxwell** and **Martin Zuwakuu**, Ubwari Field Station of the Tanzanian National Institute for Medical Research, Box 81, Muheza, Tanga, Tanzania. Tel.: +255744365776; E-mail: c_maxwell@tanga.net

**Cynthia Chen**, Human Reproductive Sciences Unit, University of Edinburgh, Chancellor's Building, 49 Little France Crescent, Edinburgh EH 169SB, UK.

**Christopher Curtis**, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK.
Tel.: +44 (0)2079272339; Fax: +44 (0)2079272164; E-mail: chris.curtis@lshtm.ac.uk