Supporting online material

Materials and Methods

Study site

The studies were carried out in Lupiro village (Ulanga District), 40 km south of Ifakara, in the southern Kilombero valley, a seasonal flood plain some 20 km wide in south-eastern Tanzania at latitude 8.38°S and longitude 36.67°E as described previously (*1-4*). In five households, *M. anisopliae*-impregnated sheets were fitted (henceforth called 'test' houses) and five households received untreated sheets ('control' houses). One room in each house was selected for fitting of a sheet. Of the 10 selected houses, 8 had occupants that used bednets, all of which were maintained poorly, having numerous holes.

Ethics

Ethical clearance for the study was obtained from the Institutional Review Board of the Ifakara Health Research and Development Centre (IHRDC) and the Medical Research Coordination Committee of the National Institute for Medical Research (NIMR) in Tanzania. Prior to any practical work the Lupiro community, its village leaders, and Government representatives of Kilombero and Ulanga Districts were sensitized to the intended study. A community meeting was organized by the village chairman where the project was discussed with the research team and permission was granted to begin enrolling study participants. Houses were enrolled only after obtaining informed consent from the head of the household and the individual household members.

Fungus

Metarhizium anisopliae var. *anisopliae* (Metsch.) Sorokin was maintained on oatmeal agar (OA) and placed in an incubator to grow for two weeks, after which fresh conidia were harvested using a 0.05% Triton-X solution and a glass rod. These conidia were used to infect the Njage laboratory strain of *A. gambiae s.s*. The conidia grown from these fungus-killed mosquito cadavers were again inoculated on OA. This procedure was repeated three times in an attempt to enhance virulence before cultivation on OA of the final batch for field application. Conidia were harvested using a glass rod and 0.05% Triton-X. After counting, dilutions were made using 0.05% Tween-20. For both experiments we used a stock solution of 8.0×10^7 conidia ml^{-1}.

Experimental set-up

Thirty local houses were visited for suitability for inclusion in the study. From this group a selection was made of houses i) in which mosquitoes were abundant, ii) insecticide was not used either on walls or on bednets, iii) that were constructed similarly of mud walls and thatched roofs, and iv) were located in the eastern part of the village near the rice fields. From these, 10 households were eventually included in the study after written consent was obtained.

Indoor-resting mosquitoes were collected using torches and mouth aspirators six days a week for a period of 6 weeks (3 weeks pre- and intervention) at the end of the long dry season. There was no rainfall during the 6-week study period. Resting catches were carried out for 15 minutes per house between 07.00 and 10.30 hr by two of the investigators (EJS, KN). In cases where the selected house consisted of multiple rooms, mosquito catches were restricted to only one room. These mosquitoes were

placed gently in polystyrene cups covered with netting material. On each cup the date of collection and the house number were written, and a wad of cotton wool, soaked in 6% glucose solution was placed on top. These cups were placed in a cardboard box to be protected from the sun while walking from one house to the next. Each day, directly after finishing the resting catches in all study houses, a layer of 1 cm of ground water from a nearby borehole and pump was added to the cups, providing humidity and the opportunity for gravid females to oviposit. All cups were placed in an improvised on-site shelving system where the boxes were protected from direct sunlight and predatory ants. A moist towel was placed on top of each box to maintain a sufficiently high humidity in the boxes. The cotton wool containing the glucose solution was moistened daily with fresh solution and replaced with a fresh wad when needed. Mosquito mortality was checked daily. Dead mosquitoes were removed from the cups. From dead *A. gambiae s.l.*, the heads were removed and placed in Eppendorf vials containing silica gel for species identification (*5*). The remaining part of the mosquito cadavers was then placed individually in glass tubes containing a piece of wet clean filter paper, sealed off, and examined for fungal growth four days later (*6*).

Black cotton sheets of 3 m^2 were impregnated with the *M. anisopliae* stock solution described above. In total 700 ml of this stock solution was used to impregnate five sheets, resulting in a conidial density of 3.7 x 10^9 conidia/m^2. Prior to impregnation, vegetable oil was added, resulting in an 8% oil-formulation. Impregnation was done by shaking the flask containing the conidial oil-formulated suspension vigorously and then sprinkling it carefully on the cloths, kneading it manually using latex gloves before the suspension was absorbed by the sheet. This was done in an enclosed room at room temperature with high humidity (>90%) where the sheets were left to dry slowly for 48 hrs. The five control sheets were treated

equally with the difference that the oil formulation did not contain conidia. After drying, all sheets were transported to the houses in Lupiro village and suspended from the lower part of the roofs indoors (Figure S1). Of the five households in which treated sheets were placed, one did not use a bednet. The untreated control sheets were fitted in the other 5 houses (four with and one without a bednet). At the start of the study two dataloggers (TinyTag®) were placed in two different houses between the thatched roof and the cloths to monitor temperature and humidity. Daily indoor mosquito collection continued for 3 weeks after the start of the intervention. Conidial viability during the testing period was determined by two different checks. First, 3 small glass test tubes were stored at room temperature, each containing 1 ml of the original oil-formulated conidial suspension used for impregnation. We inoculated 0.1 ml of these suspensions (with 3 replicates) on SDA-agar plates to count the proportion of conidia that had germinated 18-20 hrs later using a light microscope at 400x magnification. Secondly, conidial viability on the cloths was assessed by cutting off small pieces ($1cm^2$) of 3 different sheets. These three pieces were placed in a small plastic container and transported the same day to the laboratory and placed on SDA-agar. 18-20 hrs later the pieces of cloth were carefully removed from the agar, after which the percentage germination was determined as described above. Both checks were carried out on the day the sheets were placed inside the houses and three times weekly after that.

Data analysis

Mosquito longevity data were plotted on Gompertz survival functions (Genstat 7.0). Mosquito survival was analyzed using Kaplan Meyer survival analysis (SPSS 11.0). Differences in longevity between mosquitoes caught in the 6 different weeks

were analyzed using Cox Regression survival analysis. Differences in the numbers of *M. anisopliae*-infected mosquitoes each week that were caught during the post-intervention period, and the distributions of mosquitoes caught either on cloth or elsewhere in the room were analyzed using Chi-Square tests (SPSS 10.0). Daily survival rates were calculated from Gompertz survival functions.

Adapted malaria transmission model

The impact of vector control interventions upon transmission intensity is typically measured in terms of reducing Entomological Inoculation Rate (EIR), expressed as the number of times one person is bitten by infectious mosquitoes in a year (*7*). At high coverage levels, the community-level impacts of domestically applied insecticides on EIR often exceed the protection afforded to individuals (*8*). We therefore used a specifically adapted model of malaria transmission to estimate the potential impact of fungus-treated targets on EIR, based on the assumption that application would be implemented on a community-wide basis for a lengthy period of time (Figure S2). The experimental data were applied to a cyclical model (*9, 10*) with minimal adaptations (see below). Such feeding-cycle event probability models have the advantages of incorporating impacts on mosquito density (*11*) and allowing consideration of time delay between fungal infection and death. In this model, the predicted mosquito survival to a given feeding cycle (P_i) is calculated as a function of the survival probability per feeding cycle (P_f), the number of feeding cycles completed and cumulative mortality risk that are accrued from the presence of the fungus (M_i):

$$P_i \;=\; (P_f)^i \,(1 - M_i) \tag{1}$$

The potential risk to mosquitoes resulting from the presence of fungus-treated targets is calculated as a function of the effective coverage (C) of the resting population these targets achieve and the time delay, in terms of the minimum number of feeding cycles required between infection of exposed mosquitoes and their ultimate death (D):

$$M_i = M_{i-1} + C (1-M_{i-1}) \text{ if } i>D, \text{ otherwise } M_i=0 \qquad (2)$$

D is estimated by dividing the mean survival time by the expected feeding cycle length. Effective coverage (C) of the applied fungus-methodology is calculated as the proportion of fungus-infected females among the total number of females.

From the field data, the estimated value of D=1.7, using T=4.48 as the mean survival time (Figure S3) and an expected length of the feeding cycle L=2.7 days (*4*). Effective coverage (C) with the fungus can be calculated as the proportion of fungus-infected females (n=132) among the total number of females collected in the test houses during the intervention period (n=580), resulting in an estimate of C=0.2276. For model simulations, the survival rate per feeding cycle (P_f), human infectiousness (κ), number of human bites per year (H_{Bt}), and sporogonic cycle length were derived from detailed transmission studies conducted in Namawala, a village nearby our study village (Lupiro), with similar environmental and socio-ethnic characteristics (*4, 9*); P_f = 0.62 (probability of mosquito survival for one feeding cycle), κ = 0.018 (infective bites per human bite), Q = 0.95 (human bites per bite), and δ = 3 (number of previous

blood meals that occurred too recently for ingested parasites to have become infectious).

References

1. J.D. Charlwood, R. Vij, P.F. Billingsley, *Am. J. Trop. Med. Hyg.* **62**, 726 (2000).

2. T. Smith *et al.*, *Acta Trop.* **54,** 55 (1993).

3. J.D. Charlwood *et al.*, *Bull. Entomol. Res.* **85,** 37 (1995).

4. J.D. Charlwood *et al.*, *Bull. Entomol. Res.* **87,** 445 (1997).

5. J.A. Scott, W.G. Brogdon, F.H. Collins, *Am. J. Trop. Med. Hyg.* **49,** 520 (1993).

6. D.R. Boucias, J.C. Pendland, *Principles of Insect Pathology* (Kluwer Academic Publishers, Dordrecht, 1998).

7. T.A. Smith, R. Leuenberger, C. Lengeler, *Trends Parasitol.* **17,** 145 (2001).

8. C.F. Curtis, A.E.P. Mnzava, *Bull. World Health Organ.* **78**, 1389 (2000).

9. G.F. Killeen *et al.*, *Am. J. Trop. Med. Hyg.* **62**, 535 (2000).

10. G.F. Killeen *et al.*, *Am. J. Trop. Med. Hyg.* **62,** 545 (2000).

11. D.L. Smith, F.E. McKenzie, *Malar. J.* **3,** 13 (2004).

Figure captions – supporting online material

Figure S1.   A. Position of black cloth (dotted line) treated with conidia of *M. anisopliae* inside a traditional Tanzanian house. B. Blood fed *A. gambiae* mosquitoes resting on a fungus-impregnated cloth.
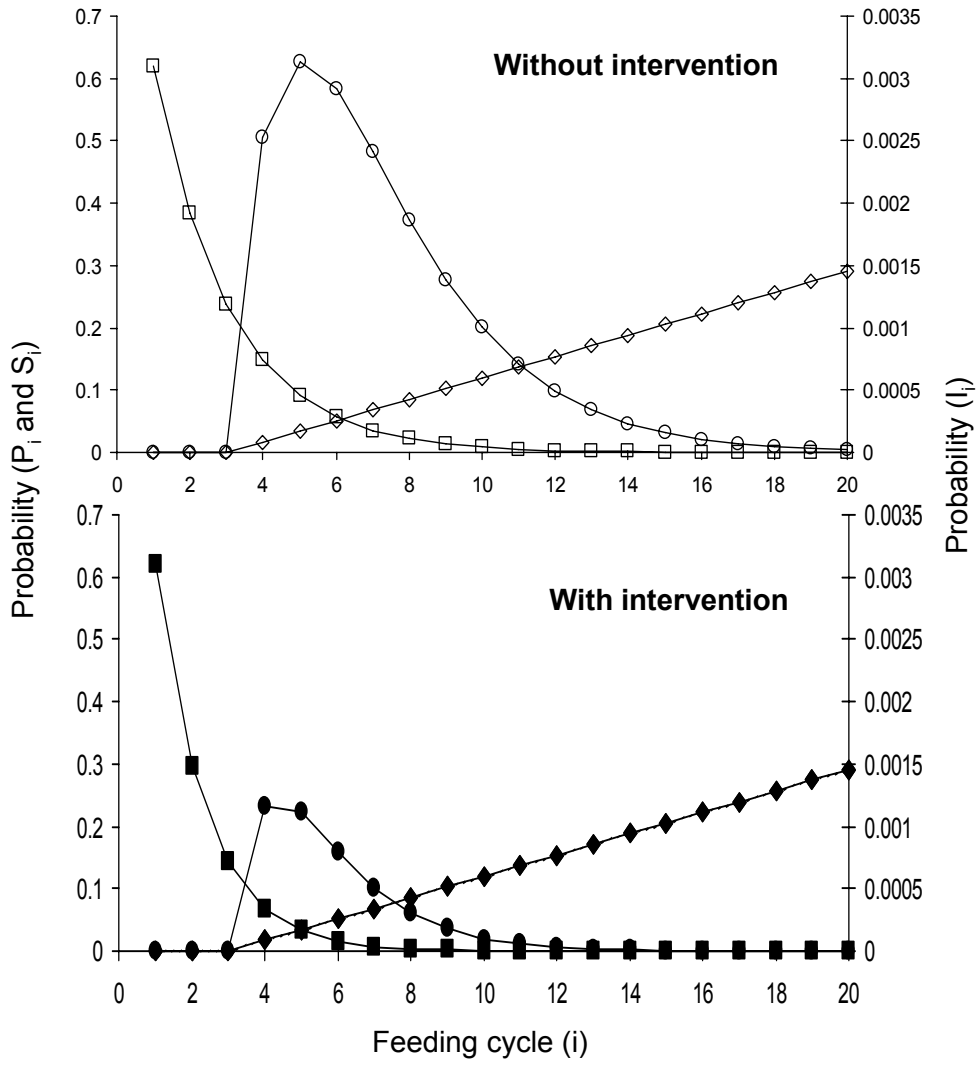

Figure S2. Predicted proportions of emerging *A. gambiae* that are alive (P_i - squares), infectious for malaria (S_i - diamonds), and both alive and infectious (I_i - circles) over the course of their lifetime without intervention (top) and with intervention (bottom) of *M. anisopliae* at village level at an effective coverage of 0.228 and a lag-time of 2 gonotrophic cycles.

Figure S3. Daily survival rates of wild *M. anisopliae*-infected (○) or uninfected (●) female *A. gambiae s.l.* mosquitoes.

Scholte et al (SOM) – revision, Figure 1
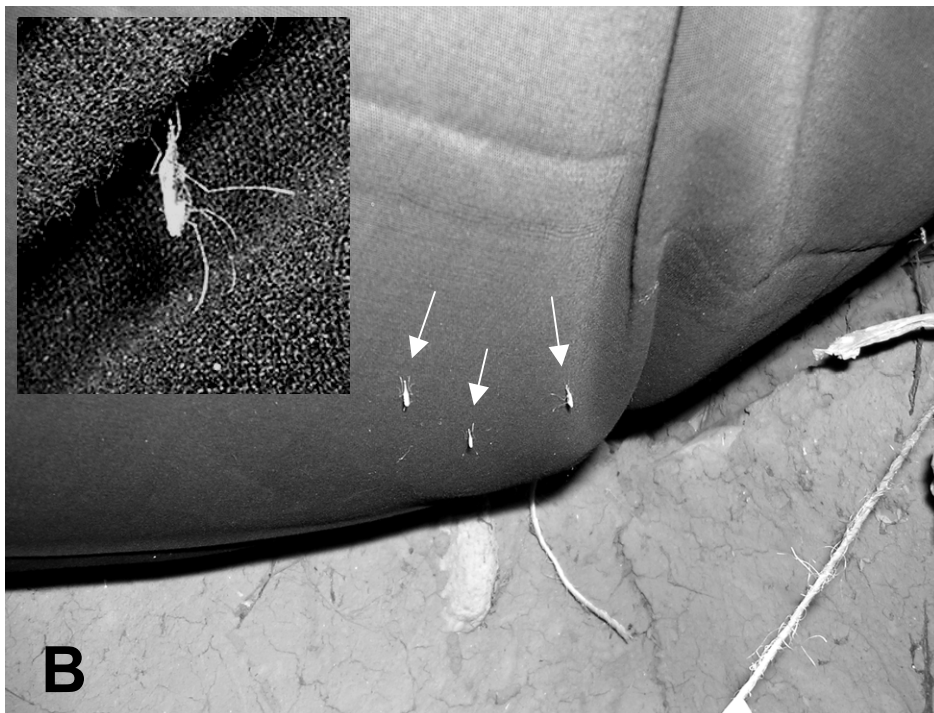
Scholte et al (SOM) – revision, Figure 2

Scholte et al (SOM) – revision, Figure 3