

observed at the genus or species level may be the result of colonization resistance by the more abundant members within similar functional groups (23). Whether the gut microbiota undergoes such nonrandom assembly remains unclear.

Ecological statistical approaches reveal previously unrecognized irregularities in the architecture of complex microbial communities. High-resolution spatial, temporal, and functional analyses of the adherent human intestinal microbiota are still needed. In addition, the effects of host genetics and of perturbations such as immunosuppression, antimicrobials, and change in diet have yet to be carefully defined. We anticipate that microarrays, single-cell analysis, and metagenomics [e.g., a “Second Human Genome Project” (24)] will complement the approach we have illustrated and hasten our understanding of human-associated microbial ecosystems.

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Fungal Pathogen Reduces Potential for Malaria Transmission

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Using a rodent malaria model, we found that exposure to surfaces treated with fungal entomopathogens following an infectious blood meal reduced the number of mosquitoes able to transmit malaria by a factor of about 80. Fungal infection, achieved through contact with both solid surfaces and netting for durations well within the typical post-feed resting periods, was sufficient to cause >90% mortality. Daily mortality rates escalated dramatically around the time of sporozoite maturation, and infected mosquitoes showed reduced propensity to blood feed. Residual sprays of fungal biopesticides might replace or supplement chemical insecticides for malaria control, particularly in areas of high insecticide resistance.

The use of pyrethroid insecticides on bednets or on walls and ceilings is the mainstay of malaria vector control. However, some forms of resistance are a threat to the long-term effectiveness of such measures (1, 2). With practical implementation of novel molecular interventions still years off (3–6), there is a pressing need for practical alternatives for malaria control (7).

Several studies have investigated the use of microbial agents for biological control of mosquitoes (see 8–10 for reviews). The most successful approach has been the use of microbial biopesticides, such as *Bacillus thuringiensis*, for control of the larval stages (8, 9). Here, we report the potential of fungal entomopathogens for indoor use against adult mosquitoes to reduce malaria transmission. Oil-based formulations of fungal entomopathogens are a relatively recent innovation that enables economically viable spore application in ultralow-volume sprays under a wide range of environmental conditions (11, 12). These formulations create an opportunity to apply fungal pathogens for use on indoor surfaces of houses or curtains where some malaria vector species rest after a blood meal or on bednets to which mosquitoes are attracted

by the odor of the occupant. Fungal entomopathogens infect through external contact, with spores germinating and penetrating through the cuticle before proliferating in the hemocoel. Natural mosquito-mosquito transmission is unlikely, because this would require contact between an uninfected adult and a sporulating cadaver. In other pest-control contexts, contact with fungal spores in a spray residue has proved to be a highly efficient means of infecting insects (13, 14) because it does not rely on direct hit of the target and enables accumulation of high doses of pathogen over time through continued or repeat exposure.

Our experimental system comprised *Anopheles stephensi* and the rodent malaria *Plasmodium chabaudi*. Our first experiment was a basic mortality screen of eight Hyphomycetes fungal isolates from two common species, *Beauveria bassiana* and *Metarhizium anisopliae*. The specific isolates were selected based on their known biological activity, that is, either known generalists or those originally isolated from dipteran hosts (15). The basic assay technique exposed blood-fed adult female mosquitoes to oil-based spray residues inside replicated cardboard pots (16). Mosquitoes were introduced 24 hours after the pots were sprayed and, for this initial screen, remained in the sprayed pots for 14 days. The eight fungal isolates varied in virulence to *A. stephensi* (Fig. 1 and table S2), six of which produced >80% mortality by day 14, with >70% of the cadavers bearing sporulated fungi. High mortality by day 14 is encouraging because that is about the time taken for *Plasmodium* to develop from ingested gametocytes to infective sporozoites.

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After initial screening, we selected the *B. bassiana* isolate IMI 391510 for further evaluation. Although it was not the most virulent isolate of those tested, this isolate is used in an existing agricultural biopesticide product, and the provision of a registration dossier would accelerate progress to field-scale evaluation.

We assessed the impact of IMI 391510 by direct topical application against *A. stephensi* and as a spray residue on cage mesh, partially simulating treatment of bednets (but with a longer exposure than would occur with free-flying mosquitoes approaching an occupied bednet). The treatments killed 91% and 93% of mosquitoes, respectively, by day 14. This compared with 38% mortality in the control mosquitoes sprayed with oil only. Significant differences in median survival times were seen between treatments: controls, >14 days; topical application, 8.0 days; treated netting, 7.0 days (log-rank statistic = 5.43, $P = 0.02$).

Anopheline mosquitoes tend to rest on structures such as walls and ceilings for less than 24 hours after a blood meal (17, 18). Thus, we simulated short-term exposures after repeat blood feeds by exposing adult *A. stephensi* to the fungal spray residue for various combinations of 6 hours at 3-day intervals [3 days representing the approximate length of the feeding cycle (19)]. No difference was detected between any of the various control batches at the end of the assessment period (table S3, $F_{4,10} = 0.24$, $P = 0.91$). For the fungal treatments, there was a significant difference in survival between exposure regimes 14 days after their first exposure ($F_{4,10} = 6.0$, $P = 0.01$), although this was entirely due to the more rapid mortality of the day 0 regime compared with the day 12 regime (Tukey HSD, $P = 0.005$). No other comparisons revealed significant differences. Thus, limiting the time that mosquitoes were exposed to the spray residue did not alter overall survival compared with lifetime exposure shown in Fig. 1. For example, the day 12 treatment, with just one 6-hour exposure to a 12-day-old spray residue, showed 89% mortality 14 days after exposure (Fig. 2).

To assess the potential of this biopesticide to reduce malaria transmission, we tested *A. stephensi* infected with the CW clone of the rodent malaria *Plasmodium chabaudi* (16) (Malaria), compared with uninfected controls (Control), a *B. bassiana* only treatment (*Beauveria*), and a treatment combining *P. chabaudi* and *B. bassiana* (*Beauveria*+Malaria). Subsamples of mosquitoes were dissected to estimate *Plasmodium* prevalence at the oocyst (day 7) and sporozoite (day 14) stages.

Median survival times for the Control and Malaria treatments were not significantly different from one another (both treatments >14 days; log-rank statistic = 0.06, $P = 0.81$), nor was there a difference between the two fungal

treatments (*Beauveria* and *Beauveria*+Malaria both 9.0 days; log-rank statistic = 0.34, $P = 0.56$). By day 14, 90% of the mosquitoes in the *Beauveria* treatment had died, with 95% mortality in the *Beauveria*+Malaria treatment (Fig. 3A). From day 11, there was a marked escalation in the mortality rate in the

Beauveria+Malaria treatment, such that by day 14, daily mortality rate was about 65 times as high as that for the malaria-only treatment (Fig. 3B).

There were no differences in the concentrations of gametocytes in mice fed the two malaria treatments ($F_{1,11} = 0.19$, $P = 0.67$).

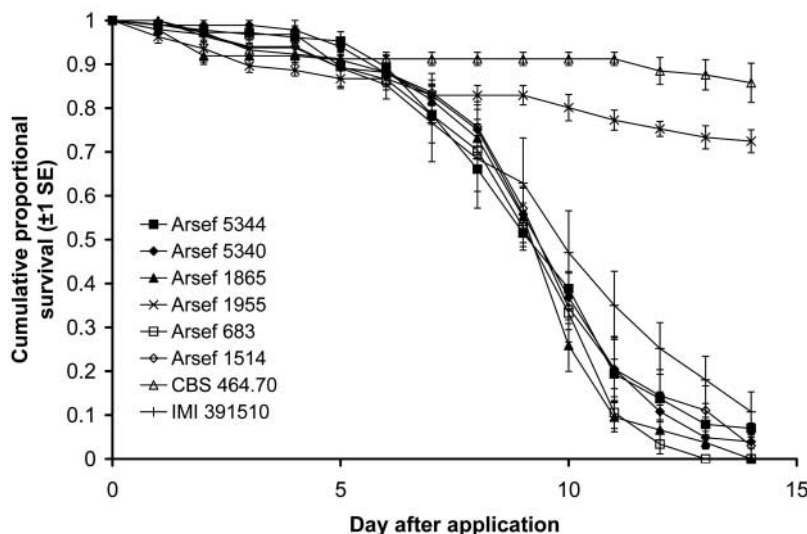


Fig. 1. Cumulative proportional survival of adult *A. stephensi* after exposure to oil-based spray residues containing spores of different fungal entomopathogens. Data represent means \pm SEM from four replicates, each containing 40 female *A. stephensi*. Details on the fungal isolates are provided in (16).

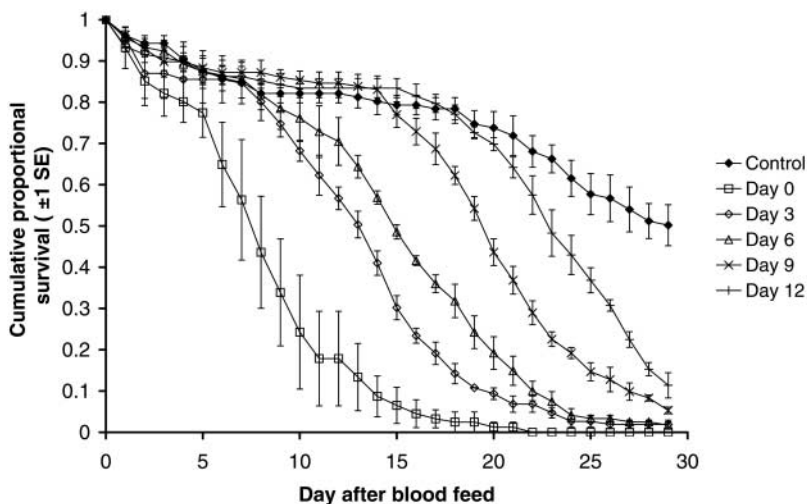


Fig. 2. Cumulative proportional survival of adult *A. stephensi* after exposure to oil-based spray residues containing the fungal pathogen *B. bassiana*, isolate IMI 391510. The lines indicate temporal exposure treatments. For the day 0 treatment, blood-fed *A. stephensi* were introduced to pots sprayed with *B. bassiana* for 6 hours and then removed to an unsprayed pot. On day 3, these mosquitoes were returned to the original sprayed pot for a further 6 hours and then removed again. This continued every three days until day 12. The remaining treatments were staggered such that the day 3 treatments were not introduced to a sprayed pot until day 3 and were subsequently exposed every 3 days for 6 hours until day 12. Days 6, 9, and 12 treatments were first introduced to sprayed pots on those days, and subsequently every 3 days as above. The day 12 treatment was, therefore, exposed only once, for 6 hours, to a pot sprayed 12 days previously. Matching controls for these treatments were moved between blank oil-sprayed pots and unsprayed pots at equivalent frequencies and exposure periods. Data for the respective treatments represent means \pm SEM from four replicates, each containing ~40 female *A. stephensi*. The control line shows the control data for the day 0 exposure treatment (other control lines have been omitted for clarity because there were no significant differences between control treatments).

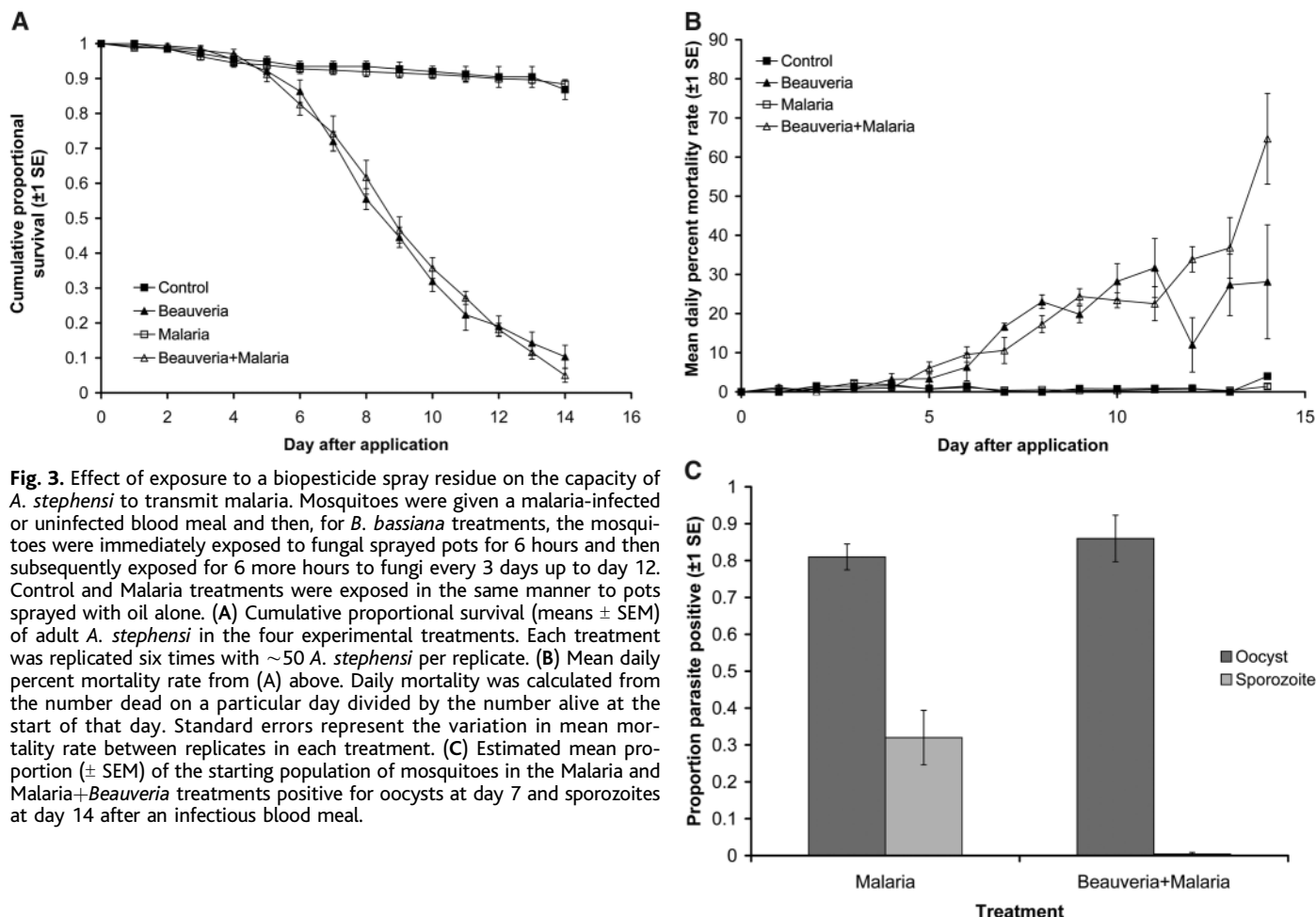


Fig. 3. Effect of exposure to a biopesticide spray residue on the capacity of *A. stephensi* to transmit malaria. Mosquitoes were given a malaria-infected or uninfected blood meal and then, for *B. bassiana* treatments, the mosquitoes were immediately exposed to fungal sprayed pots for 6 hours and then subsequently exposed for 6 more hours to fungi every 3 days up to day 12. Control and Malaria treatments were exposed in the same manner to pots sprayed with oil alone. (A) Cumulative proportional survival (means \pm SEM) of adult *A. stephensi* in the four experimental treatments. Each treatment was replicated six times with \sim 50 *A. stephensi* per replicate. (B) Mean daily percent mortality rate from (A) above. Daily mortality was calculated from the number dead on a particular day divided by the number alive at the start of that day. Standard errors represent the variation in mean mortality rate between replicates in each treatment. (C) Estimated mean proportion (\pm SEM) of the starting population of mosquitoes in the Malaria and Malaria+*Beauveria* treatments positive for oocysts at day 7 and sporozoites at day 14 after an infectious blood meal.

Similarly, oocyst prevalence (Fig. 3C) and mean number of oocysts per midgut did not differ ($F_{1,11} = 0.55$, $P = 0.46$, and $F_{1,11} = 2.35$, $P = 0.16$, respectively). However, on day 14, 35% (109/310) of the mosquitoes remaining in the Malaria treatment were found to be sporozoite positive, compared with only 8% (1/12) in the *Beauveria*+Malaria treatment. Taking into account the difference in survival to day 14 of these two groups (Fig. 3A), this equates to 31% (109/352) of the initial mosquitoes in the Malaria treatment group being alive and potentially able to transmit at day 14, compared with only 0.4% (1/256) in the *Beauveria*+Malaria treatment (Fishers exact test, $P < 0.001$). Thus, fungal exposure led to a reduction of transmission risk by a factor of about 80. This result is supported by a further experiment with a different *P. chabaudi* clone (20).

Our results demonstrate the potential of oil-based formulations of fungal entomopathogens to reduce malaria transmission by reducing adult mosquito survival and altering *Plasmodium* survival/maturation in the mosquitoes. In addition, we have found that between days 8 and 14, fungal infection interferes with the ability of *A. stephensi* to take a blood meal,

likely reducing transmission potential still further (20, 21). Mosquitoes can be infected by direct contact with spray droplets or by contact with spray residues on treated surfaces and netting, with 6 hours exposure sufficient to cause high levels of infection; this is well within the post-blood feed resting period for the majority of *Anopheles* malaria vector species (17, 18). We identified a range of isolates causing $\geq 90\%$ mortality within 12 to 15 days (22–24). Further screening is likely to reveal more virulent isolates, but it is also likely that vector survival in the wild is less than under laboratory conditions (22). Furthermore, mosquitoes cannot transmit sporozoites until about 2 weeks after an infectious blood feed, and rapid killing of the mosquito is not necessary for reducing malaria transmission. As emphasized by MacDonald (25) in justifying the possibility of malaria eradication by indoor residual spraying of insecticide, even limited changes in daily probability of survival of mosquitoes can have a large impact on the prospects of survival through the time required for *Plasmodium* maturation to the infective stage; hence, the substantial reduction in sporozoite-positive mosquitoes and the escalating daily mortal-

ity rate that we observe due to fungal infection are highly significant in terms of malaria control.

Fungal biopesticides are already registered for agricultural use alongside chemical insecticides in a number of African countries (12, 26). Transferring use to mosquito targets could (subject to further safety testing and appropriate registration) be rapid, with biopesticide products slotting readily into existing chemical application methodologies and strategies (22). As part of an integrated strategy, they could be used to respond to, or avert, the emergence of serious levels of insecticide resistance. Development of resistance against fungal pathogens has not been reported for insects, but even if resistance does occur, cross-resistance with chemical insecticides seems extremely unlikely. In the longer term, there would seem to be additional promise for using residual sprays of fungal pathogens for novel paratransgenic approaches to deliver toxins or effector molecules that block sporogony within the vector (e.g., see 27, 28). Unlike malaria control by genetic modification of mosquitoes, the fitness of biopesticide transgenes could be quite low and, because secondary transfer of fungi from

mosquitoes is very unlikely, fungal transgenes would be much easier to control than mosquito transgenes.

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Supporting Online Material

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Materials and Methods
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An Entomopathogenic Fungus for Control of Adult African Malaria Mosquitoes

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Biological control of malaria mosquitoes in Africa has rarely been used in vector control programs. Recent developments in this field show that certain fungi are virulent to adult *Anopheles* mosquitoes. Practical delivery of an entomopathogenic fungus that infected and killed adult *Anopheles gambiae*, Africa's main malaria vector, was achieved in rural African village houses. An entomological inoculation rate model suggests that implementation of this vector control method, even at the observed moderate coverage during a field study in Tanzania, would significantly reduce malaria transmission intensity.

Mosquito vector control is an integral part of controlling malaria (1). In Africa, this is almost exclusively based on the use of chemical insecticides for indoor residual spraying and impregnation of bednets for killing adult mosquitoes (2–6). The high efficacy achieved at modest coverages results from the exquisite sensitivity of malaria transmission intensity to the daily survival rate of adult mosquitoes (7). However, the continuing use of both public health and agricultural insecticides has led to a substantial increase in physiological resistance of mosquitoes in recent years (8, 9). These problems have increased interest in alternative

and integrated implementation of vector control methods that include biological control. Although several effective biological larvicides exist (10), there have been no biological control agents effective against adult mosquitoes. Addressing this gap, we have recently reported encouraging results with entomopathogenic fungi from the Hyphomycetes (Imperfect Fungi) infecting and killing adults of the African malaria vector *Anopheles gambiae sensu stricto* through tarsal contact in laboratory containers (11, 12). Unlike other mosquitoicidal biocontrol agents, such as bacteria, microsporidia, and viruses, these fungi can infect and kill insects without being ingested. Tarsal contact alone is enough to kill the insect, a characteristic shared with insecticidal chemicals. Moreover, Hyphomycetous insect-pathogenic fungi, such as *Metarhizium anisopliae* and *Beauveria bassiana*, are produced commercially and used against several agricultural insect pests worldwide (13).

Here, we report the results of a field study in a rural village in Tanzania in which we assessed whether wild mosquitoes became in-

fectured and had reduced life spans after resting on 3 m² *M. anisopliae*-impregnated black (14) cotton sheets (“targets”) suspended from ceilings in traditional houses (fig. S1). Pre- and postintervention mosquitoes were collected, and equal numbers of untreated and treated houses were included (15).

In the 10 study houses, we collected a total of 2939 mosquitoes, 1052 during the preintervention (3 weeks) and 1887 during the intervention period (3 weeks). These were maintained on a 10% glucose diet in paper cups until death, after which fungal infections were detected, retrospectively, by observation of emerging hyphae from mosquito cadavers (16). We found that 88.9% were *A. gambiae s.l.* (17) and 10.7% *Culex quinquefasciatus*. Overall, 53.6% of the mosquitoes were caught on the targets, and 46.4% elsewhere in the rooms (18). None of the mosquitoes that had been collected during the preintervention period, nor any of the mosquitoes collected from the control houses during the entire experimental study period were found to be infected with the fungus. Of the 580 female *A. gambiae s.l.* that were collected in the five treatment houses during the intervention period, 132 were infected with *M. anisopliae*.

There was no significant difference in longevity between mosquitoes that were collected before and uninfected mosquitoes that were caught after the intervention ($F = 2.903$, $P = 0.088$). Similarly, longevity of mosquitoes caught in the control houses was not different from that of noninfected mosquitoes collected in the treatment houses during the intervention period ($F = 0.91$, $P = 0.3411$). By contrast, fungus-infected *A. gambiae s.l.* had significantly shorter life spans compared with those of noninfected mosquitoes (Fig. 1; overall effect pooling both sexes, $F = 178.9$, $P < 0.001$). Median lethal times (LT₅₀) values were 3.70 and 3.49 days for *M. anisopliae*-infected males and females, respectively, and 5.88 and 9.30 days for uninfected males and females, respectively. Of the 188 infected

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