Supplementary material

Materials and Methods

Mosquito rearing and maintenance

Anopheles stephensi were reared under standard insectary conditions at 27^{o}C, 70% humidity and a 12L: 12D photo-period. Eggs were placed in plastic trays (60 cm x 30 cm x 10 cm) filled with 2.5 L of distilled water. To reduce variation in adult size at emergence, larvae were reared at a fixed density of 800 larvae per tray. Larvae were fed on Liquifry® for 5 days and then on ground TetraFin® fish flakes. Pupae were collected from day 11 to 15, placed in emergence cages and provided with an ad libitum supply of 10% glucose solution supplemented with paraminobenzoic acid (PABA). 6-10 day old adult females were used in all experiments either in experimental cages (16 cm x 16 cm x 16 cm) or in 0.5 L wax coated tubs with mosquito mesh covers. The bottom of these tubs had a hole cut out of the base through which water filled 35 mm diameter Petri dishes were inserted to allow mosquitoes to lay eggs. This rearing and maintenance regime was used for all experiments detailed below.

Fungal isolates, formulations and application

Fungal Isolate Maintenance

Fungal isolates were taken from the insect pathology collection held either at CABI Bioscience, Ascot, UK, Centraalbureau voor Schimmelcultures (CBS), Netherlands, or from the USDA-ARS Collection of Entompathogenic Fungal Cultures (ARSEF). Isolates were maintained on Potato Carrot Agar and stored at 10^{o}C until used for experiments.

Production of conidia

Prior to use, conidia were taken from a PCA slope and used to prepare a spore suspension of approximately 1 x 10^6 conidia ml $^{-1}$ in 0.05% w/v Tween 80 (Sigma) in distilled water. This suspension was then used to inoculate Oxoid Sabouraud-dextrose agar (SDA) in 9 cm diameter Petri dishes by pipetting 0.2 ml aseptically on to the surface and spreading evenly over the agar with a glass rod. Inoculated plates were incubated at 25^{o}C for 11 days. Following incubation, conidia were harvested from the plates using a micro spatula to carefully separate the spore layer from the agar. The harvested conidia of each isolate were placed in separate pre-weighed, glass, weighing dishes and placed in a glass dome desiccator over dried non-indicating silica gel at room temperature (appox 18^{o}C).

Formulation

Once the conidia powder had reached constant weight (approx 4% moisture content), oil formulations were prepared by suspending approximately 0.5 g of conidia powder in 10 ml of oil formulation comprising 12.5% Ondina EL and 87.5% Shellsol T (both supplied by Alcohols Ltd. Bishops Stortford, UK). The concentrated conidia formulations were sonicated in a bath sonnicator (Branson, UK) for 3 min to break up any conidial chains and remove conidia from any remaining mycelial fragments. The formulations were then sieved through a 106 µm sieve (Endecotts Ltd., UK) to remove any large particles from the formulation. A dilution series was then prepared in the formulating oil and conidial counts performed using an Improved Nuebauer heamacytometer. All formulations were adjusted to 1 x 10^8 conidia ml^{-1}in 50 ml oil formulation.

Passaging and re-isolation of IMI 391510

A small number of *A. stephensi* adults were infected with *B. bassiana* IMI 391510 and the pathogen re-isolated from the sporulating cadavers by removing a sample of conidia from the exterior of the cadaver and streaking on to PCA plus 50,000 units Penicillin and 0.05 g Chloramphenicol (Sigma) L^{-1} in 9 cm Petri dishes. These isolation plates were incubated at 25^{o}C for 10 days. Following incubation, the plates were verified microscopically to confirm the presence of *B. bassiana* and any plates containing contaminants were discarded. Conidia from un-contaminated plates were used to prepare a spore suspension for inoculation of SDA plates for production and formulation as described above for all tests.

Biopesticide application

Spray applications employed a hand held artist's air brush which produced an aerosol of the spore formulation from a 20ml glass jar attached to the spray nozzle. Pots were opened out and the bottoms removed. Both the sides and base of the pots as well as the mesh cover were taped flat to a vertical spray surface for application. Application was made with one spray pass of the air brush from a distance of 50 cm. Pots were then left for 24 hours before being reconstructed and the mosquitoes introduced and fed. Control pots received only the carrier oil sprayed in exactly the same way as above. Cages were sprayed with the same application methodology though the cage was rotated so that each side of the cage received a spray pass. The application volume with this methodology was 9.36 (± 0.267) ml m^{-2} with a dose of 9.36 x 10^{8} (±2.67 x10^{7}) conidia m^{-2}.

Mortality assessment

Mosquito survival was determined daily. Any cadavers found were removed each day and placed in Petri dishes with moistened filter paper. The Petri dishes were sealed with Parafilm to maintain high humidity and the cadavers monitored for fungal sporulation. Median survival data were analysed using Kaplan-Meier survival analysis. Within treatment variation between replicates were assessed using log rank tests. For all experiments reported here there was no significant variation between any replicate within a treatment ($P > 0.12$ or higher). Subsequently replicates were pooled for each treatment and a log rank test used to determine the equality of the survival distribution between treatments.

Malaria infection and mosquito dissection

We used a genotype of *Plasmodium chabaudi*, designated CW, (*S1*) from the World Health Organisation's Registry of Standard Malaria Parasites, University of Edinburgh. Mice (C57BL/6J) were inoculated with 10^6 parasites. Blood smears were taken on days 5 and 6. Mosquito feeds took place on day 7 after mouse infection when all mice had sufficiently high gametocytaemia (proportion of red blood cells infected with gametocytes >0.1%). For the blood meals one mouse per pot was anaesthetised and mosquitoes allowed to feed for twenty minutes. Control mosquitoes received a blood feed from a sham-infected mouse. Subsequently only mosquitoes with clear evidence of a blood meal were used in experiments. All mosquitoes were provided with an ad libitum supply of glucose (10%) and PABA in both cages and pots. Mosquitoes [a minimum of 40 where sufficient mosquitoes survived. Otherwise all remaining mosquitoes were

dissected] were dissected under a binocular microscope in 100µl of M phosphate buffered saline (PBS) and examined under a compound microscope on day 7 from blood feed for determination of oocyst prevalence and day 14 for sporozoite prevalence.

Repeat of the transmission blocking experiment

To test the results reported in Fig 3 with a different parasite clone, we used the CR (*S1*) clone of *P. chabaudi.* All mosquito rearing and infection procedures are as detailed in the material and methods. We used two treatments, a Malaria only treatment and a *Beauveria*+Malaria, exposed to blank oil or *B. bassiana* treated pots, respectively, for six hours every three days as for treatments described in the main text. By day 14, survival in the *Beauveria*+Malaria treatment was only 22%, compared with 72% in the malaria-only treatment. Median survival times were >14 days and 12 days for Malaria and *Beauveria*+Malaria, respectively (Log rank statistic – 50.85; $P < 0.001$)), with 85% of cadavers in the *Beauveria*+Malaria treatment sporulating after incubation. The daily mortality rate pattern was similar to the experiment reported in the main text with the *Beauveria*+Malaria treatment rate escalating to 42% on day 14, whereas the Malaria only mortality rate was just 2% on day 14 and remained relatively constant throughout the experimental period. There was again no difference in either oocyst prevalence (Malaria 50.0 ± 0.13% and *Beauveria*+Malaria 65.0 ± 8.7%: $F_{1,7} = 0.93$; $P = 0.37$) nor the number of oocysts per midgut (Malaria 2.3 ± 1.34 oocysts/midgut and *Beauveria*+Malaria 3.2 ± 1.27 oocysts/midgut: $F_{1,7} = 0.26$; $P = 0.63$) between the two treatments. However, on day 14 there were considerably fewer surviving mosquitoes found to be sporozoite positive in the *Beauveria*+Malaria treatment (4.3%) than in the Malaria treatment (35%). In relation

to the starting population, this equates to 23.8% of the mosquitoes in the Malaria treatment potentially able to transmit malaria at day 14, compared with only 0.69% in the *Beauveria*+Malaria ($F_{1,7}$ = 40.3; P = 0.001). This finding is similar to that described in the main text and demonstrates both repeatability and generality between *P. chabaudi* clones.

Propensity to feed

To assess whether *Beauveria* exposed *A. stephensi* blood feed to the same extent as uninfected mosquitoes we fed females on an uninfected mouse on day 0 and then split those with clear evidence of a blood meal between a Control (exposed to oil-only sprayed pots) and a *Beauveria* treatment replicating each four times with about 40 mosquitoes per pot. We exposed the mosquitoes to either the oil sprayed or *Beauveria* sprayed pots for six hours every three days, starting immediately after the initial feed on day 0, as in the protocol outlined in the main text. On each of day 4, 8, and 14 the two treatments were provided with an anaesthetised uninfected mouse and allowed to feed for 20 minutes. Immediately after these feeds the number of mosquitoes in each pot with a clear blood meal in the abdomen was counted.

Mosquito survival was similar to other experiments with 70.7 (± 7.06)% of controls and 29.3 (± 4.67)% of the *Beauveria* treatment surviving at day 14 ($F_{1,4}$ = 20.7; P = 0.01) and 88% of the *Beauveria* treatment cadavers showing sporulation of the fungus on incubation. There was a significant difference between treatments in the propensity of surviving mosquitoes to feed ($F_{1,4}$ = 9.47; P = 0.037), a significant effect of the day of the

feed ($F_{3,4}$ = 32.89; P < 0.001) and an interaction between treatment and day of feed ($F_{3,4}$ = 12.46; P = 0.001). On day 4, 71.2 ± 6.68% of control and 62.4 ± 7.1% *Beauveria* exposed mosquitoes fed ($F_{1,4}$ = 0.77; P = 0.429). The *Beauveria* treatments seemed to begin to show less inclination to feed on day 8 (Control 76.6 ± 7.93%; *Beauveria* 48.4 ± 8.22%) though the difference was not quite significant ($F_{1,4}$ = 6.2; P = 0.068). By day 14, when transmission is expected to be possible there was a large difference between treatments with 85.0 ± 5.83% of the Control mosquitoes taking a blood meal compared with only 28.2 ± 9.87% of those surviving in the *Beauveria* treatment ($F_{1,4}$ = 19.82; P = 0.011). Fungal infection clearly interfered with the ability of *A. stephensi* to take a blood meal by day 14 and this trend appears to have started as early as day 8 following initial exposure to the fungal spores.

References

S1. G.H. Beale *et al.* in *Rodent Malaria,* R. Killick-Kendrick, W. Peters, Eds. (Academic, London, 1978), pp. 213-245.

Supplemental Table S1. Details of fungal pathogens used in the initial mortality screen against *Anopheles stephensi*.

Isolate accession number[1]	Pathogen species	Provenance
ARSEF 5344	*Beauveria bassiana*	Diptera: Muscidae – USA
ARSEF 5340	*Beauveria bassiana*	Diptera: Muscidae – USA
ARSEF 1865	*Beauveria bassiana*	Diptera: Anthomyiidae – France
ARSEF 1955	*Beauveria bassiana*	Diptera: Muscidae – Brazil
ARSEF 683	*Metarhizium anisopliae* var *anisopliae*	Coleoptera: Scarabaeidae – China
ARSEF 1514	*Beauveria bassiana*	Diptera: Muscidae – France
CBS 464.70	*Metarhizium anisopliae* var *anisopliae*	Lepidoptera: *Thaumatopoea* sp. – Israel
IMI 391510	*Beauveria bassiana*	Coleoptera: Chrysomelidae – USA

1. ARSEF: USDA-ARS Collection of Entomopathogenic Fungal Cultures, USA
   CBS: Centraalbureau voor Schimmelcultures, Netherlands
   IMI: CABI Bioscience, UK

Supplemental Table S2. Median survival time (based on observations up to day 14) of *An. stephensi* exposed to fungal isolates in the initial mortality screen.

Isolate	Median survival time (days)	Mortality rate (%) at Day 10 ± SE	Mortality rate (%) at Day 14 ± SE	Percent surviving at day 14	Percent sporulation
ARSEF 5344	10	25.0 ± 2.51	8.3 ± 8.33	7	82
ARSEF 5340	10	34.7 ± 9.02	11.1 ± 11.11	4	76
ARSEF 1865	10	54.9 ± 7.39	83.3 ± 16.67	0	85
ARSEF 1955	>14	3.47 ± 1.17	1.2 ± 1.19	72	3
ARSEF 683	10	37.6 ± 3.96	All dead	0	91
ARSEF 1514	10	41.9 ± 11.07	80.6 ± 10.02	3	71
CBS 464.70	>14	0.0 ± 0.00	2.2 ± 1.28	85	0
IMI 391510	10	27.1 ± 6.16	53.8 ± 16.25	11	73

Supplemental Table S3. *An. stephensi* survival and sporulation results from the intermittent fungal exposure experiment. Mortality rate data refer to the days after first fungal exposure for the respective treatments. Percent surviving refers to the number of mosquitoes surviving 30 days after the onset of the experiment.

Exposure	Treatment	Median survival time (days)	Mortality rate (%) at Day 10 ± SE	Mortality rate (%) at Day 14 ± SE	Percent surviving at day 30	Percent sporulation
Day 0	Control	28	1.3 ±1.33	1.8 ± 1.75	46	0
	B. bassiana	9	37.0 ± 12.8	29.2 ± 15.02	0	74
Day 3	Control	28	0.0 ± 0.00	5.0 ± 1.33	44	0
	B. bassiana	15	11.3 ± 1.39	18.9 ± 5.88	2	61
Day 6	Control	>30	0.0 ± 0.00	1.3 ± 1.28	50	0
	B. bassiana	16	14.1 ± 4.07	21.8 ± 3.21	2	55
Day 9	Control	28	1.2 ± 1.23	0.0 ± 0.00	44	0
	B. bassiana	21	12.9 ± 3.0	21.1 ± 6.76	5	62
Day 12	Control	29	2.2 ± 2.15	0.0 ± 0.00	46	0
	B. bassiana	24	10.7 ± 3.64	16.0 ± 3.88	11	59