

Lethal and Pre-Lethal Effects of a Fungal Biopesticide Contribute to Substantial and Rapid Control of Malaria Vectors

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Abstract

Rapidly emerging insecticide resistance is creating an urgent need for new active ingredients to control the adult mosquitoes that vector malaria. Biopesticides based on the spores of entomopathogenic fungi have shown considerable promise by causing very substantial mortality within 7–14 days of exposure. This mortality will generate excellent malaria control if there is a high likelihood that mosquitoes contact fungi early in their adult lives. However, where contact rates are lower, as might result from poor pesticide coverage, some mosquitoes will contact fungi one or more feeding cycles after they acquire malaria, and so risk transmitting malaria before the fungus kills them. Critics have argued that ‘slow acting’ fungal biopesticides are, therefore, incapable of delivering malaria control in real-world contexts. Here, utilizing standard WHO laboratory protocols, we demonstrate effective action of a biopesticide much faster than previously reported. Specifically, we show that transient exposure to clay tiles sprayed with a candidate biopesticide comprising spores of a natural isolate of *Beauveria bassiana*, could reduce malaria transmission potential to zero within a feeding cycle. The effect resulted from a combination of high mortality and rapid fungal-induced reduction in feeding and flight capacity. Additionally, multiple insecticide-resistant lines from three key African malaria vector species were completely susceptible to fungus. Thus, fungal biopesticides can block transmission on a par with chemical insecticides, and can achieve this where chemical insecticides have little impact. These results support broadening the current vector control paradigm beyond fast-acting chemical toxins.

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Introduction

Current strategies for malaria control center on the use of chemical insecticides against the adult mosquito vectors [1]. Unfortunately, the sustainability and effectiveness of these front-line technologies is being undermined by the exceptionally rapid spread of insecticide resistance in *Anopheles* populations [2]. This growing resistance problem has led to calls for new control tools to help reduce the reliance on existing chemical insecticides [3–6].

A non-chemical approach that has received interest in recent years is the potential of fungal entomopathogens. The proposition is that these be formulated as biopesticides for use as indoor residual sprays or on treated materials and resting targets placed in and around the home. Since initial reports demonstrated the basic premise of this approach [7,8], studies have explored the impact of fungal pathogens on the survival of a range of mosquitoes that

vector disease [9–15], virulence against insecticide-resistant mosquitoes [16–18], possible methods of biopesticide delivery [19–20], and the impact of sub- and pre-lethal effects of infection on vectorial capacity [7,21]. This largely laboratory-based empirical research has been supported by a number of modeling studies demonstrating the potential for use of fungal biopesticides in novel, sustainable integrated vector management strategies [22–25].

Unlike fast-acting chemical neurotoxins, fungal pathogens do not cause rapid mortality or immediate “knockdown” but rather act over a number of days as the fungal spores penetrate the insect cuticle and then proliferate within the hemocoel [26]. In certain agricultural applications, slow speed of kill has been identified as a constraint to biopesticide adoption [27]. This concern has also been raised with respect to malaria control [28,29]. Indeed, the World Health Organization Pesticide Evaluation Scheme (WHOPES) approves for malaria control only insecticides that achieve greater

than 80% mortality in twenty-four hours [30,31], and others use this threshold to determine candidate compounds for inclusion in product development portfolios [5]. One important feature of malaria biology, however, is that the parasite typically takes 12–14 days to develop within the mosquito before it can be transmitted [32,33] (and can take considerably longer depending on environmental conditions [34]). As such, even a slow acting biopesticide can halt transmission if mosquitoes contact fungal spores in one of their early feeding cycles; with WHO guidelines for IRS recommending treatment of at least 85% of houses [35], standard operational coverage should deliver this. This argument is founded on the fundamentals of malaria transmission [36] and is confirmed through detailed modeling studies [22–25].

Nonetheless, there exists a tension between conventional chemical paradigms, represented by the prevailing WHOPES criteria, which emphasize fast acting products for “mosquito control”, and what have been termed Late Life Acting products [24], which emphasize “malaria control” by reducing mosquito longevity.

Here we test the performance of a candidate biopesticide as if it were a conventional chemical insecticide. To date, no fungal studies have strictly followed WHOPES test protocols. We adopted the standard WHOPES assay methods [31] to test the residual action of a candidate fungal biopesticide applied to clay tiles. Clay is one of the standard substrates prescribed in the WHO guidelines and was selected here as the use of mud/clay in house construction is commonplace and its absorptive properties have proved challenging for conventional chemical insecticides [37–39]. We ask three questions. First, how do dose and exposure time affect efficacy on a natural substrate sprayed with a simple formulation of fungal spores? Second, what is the combined impact of lethal and pre-lethal effects of fungal infection on the capacity of mosquitoes to transmit malaria? Third, are insecticide resistant mosquitoes vulnerable to fungal attack? We find that through a combination of lethal and pre-lethal effects, the candidate biopesticide can produce extensive transmission blocking within a single feeding cycle. Additionally, we find that mosquitoes resistant to chemical insecticides are fully susceptible to fungus. Thus, from a disease control perspective, the biopesticide and existing chemical insecticides are similarly effective against susceptible mosquitoes, but the biological can sustain this performance against insecticide-resistant mosquitoes.

Results

Effect of substrate, application dose and exposure time

We followed the standard WHO ‘cone test’ methodology [31] to expose adult female *Anopheles stephensi* mosquitoes to clay tiles sprayed with an oil formulation of spores of the entomopathogenic fungus *Beauveria bassiana*. For the standard dose, spores were applied at an equivalent application rate of 5×10^{11} spores/m² with mosquitoes exposed for 30 minutes one day after spraying. We first compared this dose with serial dilutions of 50%, 10%, 5% and 1%. We then varied exposure time, with periods reduced to 10, 5, 1 minute, and 30 seconds.

Median Lethal Time \pm 95% confidence interval (MLT hereafter) of mosquitoes exposed to the standard dose was 4 (3.66–4.34) days, with 100% mortality occurring by day 5 (Fig. 1A). Reducing dose by up to 2 orders of magnitude had only moderate effects. At 50% dose there was no change in MLT and further dilutions increased MLT by one day only (see Supporting Information S1 for further details). 100% mortality ranged from 6–10 days across the declining doses (Fig. 1A).

Altering exposure period also had only moderate effects on mosquito survival. A 30 minute exposure resulted in 100% mortality in 5 days as did a 10 minute exposure. Reducing exposure

times further to 5 minutes, 1 minute and 30 seconds resulted in 100% mortality in 6, 6 and 7 days respectively (Fig. 1B). All exposure times had the same MLT of 4 days (see Supporting Information S1). Overall, all doses and exposure periods were sufficient to reduce survivorship to at least 80% (the key WHOPES criterion) within 4–6 days.

Survival of blood fed and sugar fed mosquitoes and propensity to feed

Impacting mosquito survival is one way of reducing vectorial capacity. However, fungal infection also reduces feeding propensity [7,21]. If a mosquito won’t feed, then it cannot transmit malaria. To investigate this pre-lethal effect under the current assay system we exposed mosquitoes to treated clay tiles (standard dose and exposure) and then monitored propensity to blood feed during the fungus incubation period. We examined mosquitoes maintained on glucose water only and mosquitoes that had just taken a blood feed.

In line with the baseline assays (Fig. 1), mosquitoes maintained on glucose water died rapidly with MLT of just 4 days (95% CI = 3.91–4.09) and 100% of all exposed insects dead by day 5. Over the same time period control mortality was $16 \pm 4.7\%$ (Fig. 2A). When offered a feeding stimulus (see Materials and Methods), around 90% of control mosquitoes initiated feed behaviors on each day. In contrast, fungal exposed mosquitoes showed a declining response to the feeding stimulus over time, with 77, 60 and 50% of mosquitoes initiating feeding behaviors on days 1, 2 and 3, respectively and no mosquitoes responding on day 4 (repeated measures ANOVA gave a significant effect of both treatment, $F_{1,4} = 94.98$, $P = 0.01$, and time $F_{4,16} = 75.86$, $P < 0.001$, on feeding propensity). Combining the proportion of mosquitoes alive with the proportion attempting to feed gives a measure of overall transmission blocking (biting risk) on any given day. For treated mosquitoes, this combination of pre-lethal and lethal effects revealed reductions in biting risk of 36, 52, 72 and 100% on days 1–4, respectively (Fig. 2B). This represents complete transmission blocking within a feeding cycle.

Adding the effects of blood feeding increased the effective impact of fungal infection further (Fig. 2C, D). While the survival pattern was very similar (control mortality was again $16 \pm 2.61\%$ by day 5, and all fungal-exposed blood-fed mosquitoes dead by day 5 with an MLT of 4 days (95% C.I. = 3.92–4.08)) feeding response of all mosquitoes was strongly down regulated on days 1 and 2 following the blood feed, presumably because mosquitoes were digesting the blood meal and developing eggs. On day three, control mosquitoes showed increased responsiveness ($38.4 \pm 17.6\%$) and on day 4, $81.4 \pm 2.6\%$ of the control mosquitoes took a second blood meal (Fig. 2D). By contrast no fungal-exposed mosquitoes responded to the feeding stimulus on day 3 (survival at this point was $65.8 \pm 7.22\%$) and none of the surviving mosquitoes took a blood meal when it was offered on day 4 (Fig. 2D). Again this represents complete transmission blocking within a single feeding cycle; blood feeding essentially results in a “knockdown” in biting risk within 24 hrs from which fungal infected mosquitoes never recover.

Delayed fungal exposures

One of the strengths of fast acting chemical insecticides is that malaria transmission can be blocked at any feeding cycle as long as the mosquito is contacted before it becomes infectious. This contrasts with slow acting products that require contact early on in the parasite incubation period (see earlier arguments). However, while fungal biopesticides have traditionally been viewed as slow acting, the results from the assays above demonstrate that, in fact,

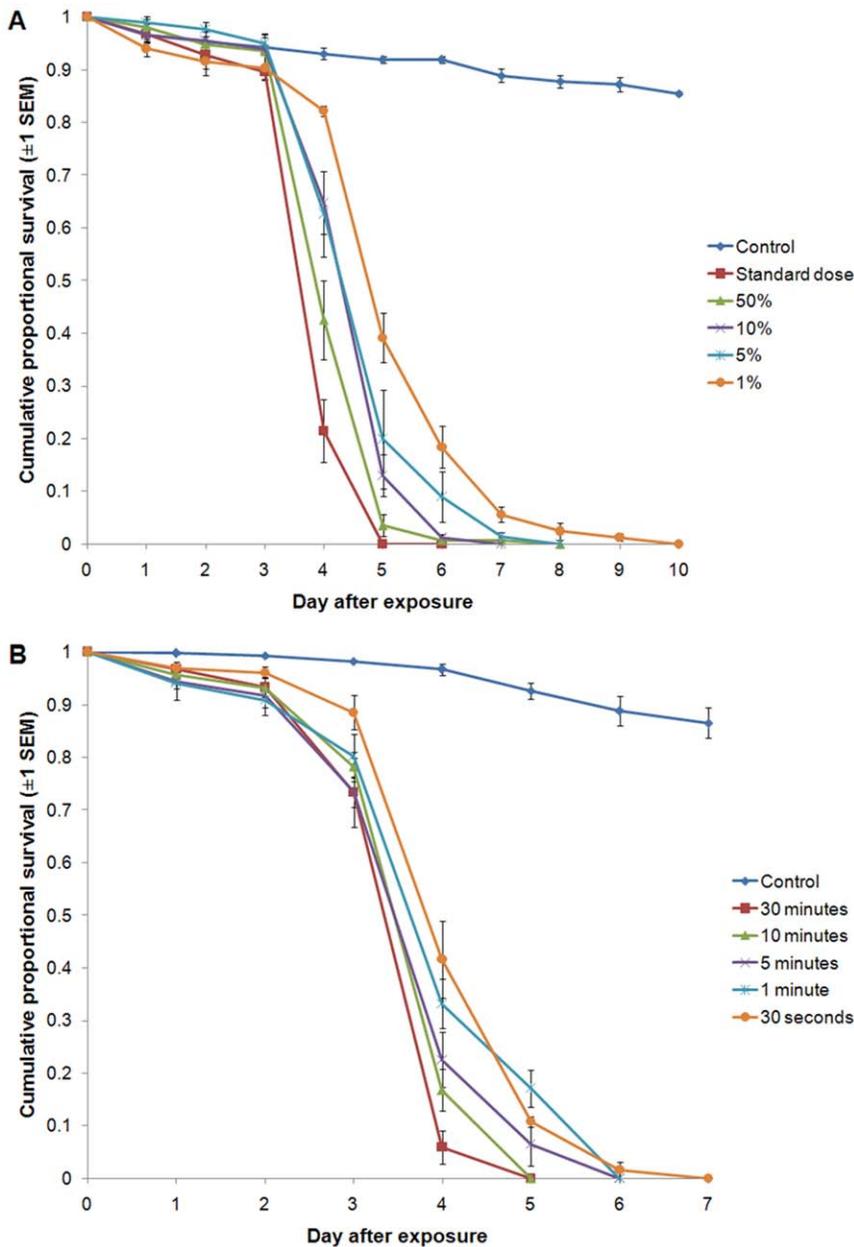


Figure 1. Cumulative proportional survival of *Anopheles stephensi* exposed to the standard dose (see main text) of *Beauveria bassiana* on clay for 30, 10, 5 or 1 minutes or 30 seconds (A); or for 30 minutes with doses reduced by 50, 10, 5 and 1% of the standard on clay tiles (B).

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transmission blocking can be very rapid. In principle, this should enable effective malaria control even if mosquitoes escape fungal infection until late in life. To investigate this we exposed a group of mosquitoes to fungal treated clay tiles on day 0 as (above) but also included groups that were not exposed to treated tiles until the second (day 4), third (day 8), or fourth (day 12) feeding cycles. At all other times fungal treatment groups and the controls were exposed to untreated tiles. All groups were offered a blood meal (as above) on day 0 and every four days subsequently up to and including day 16. In between blood meals the insects' propensity to feed was assessed using the same feeding stimulus described above.

The control group had very little mortality with 80.6 (± 1.24)% surviving to the end of the experiment. Median lethal times ($\pm 95\%$ C.I.) for the fungal exposures were 4 (3.92–4.08), 7 (6.88–

7.12), 11 (10.85–11.15) and 16 (15.92–16.08) days for the groups exposed to the fungi on days 0, 4, 8, and 12 respectively (Fig. 3A). When survival of fungal groups was assessed from the day of exposure, median lethal times were 4 (3.92–4.08), 3 (2.85–3.12), 3 (2.85–3.15) and 4 (3.94–4.06) days for the day 0, 4, 8, and 12 day exposure groups respectively. All fungal exposed mosquitoes in each treatment group were dead within 5 days of exposure time.

Across all blood feeding episodes a minimum of 75% of the control mosquitoes took a blood meal (Fig. 3B). The pattern of response to the feeding stimulus between blood meals was not as clear cut as in the previous assay as we did not clear non-fed and partially fed mosquitoes from the cages. Hence even on the first day after each blood feed some mosquitoes were probing at the feeding stimulus. Nevertheless, the general pattern was similar with

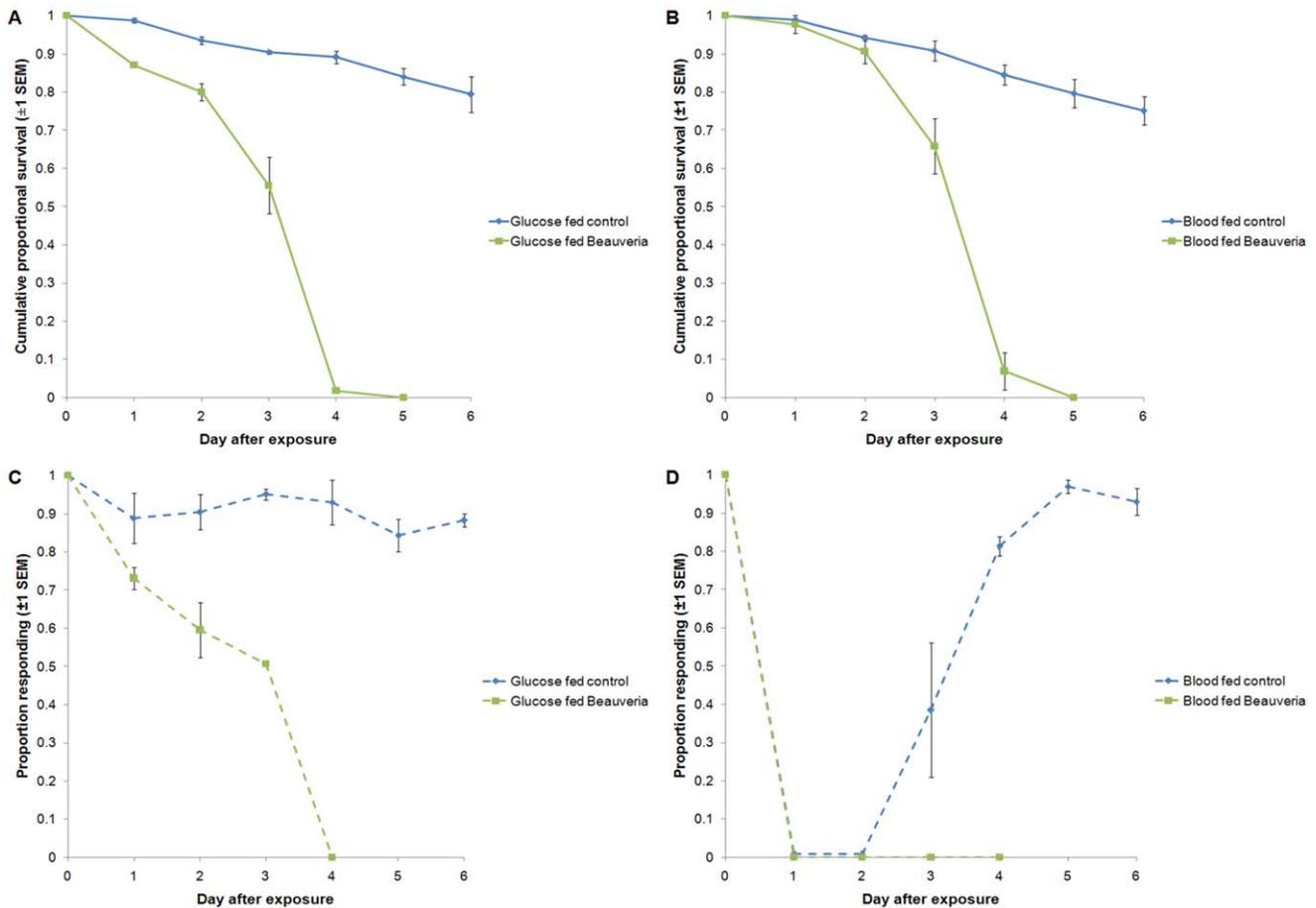


Figure 2. Survival and feeding propensity of *Anopheles stephensi* exposed to *Beauveria bassiana*. A) Survival of mosquitoes maintained on glucose only. B) Survival of blood fed mosquitoes. C) Daily mean proportion of glucose maintained mosquitoes responding to a feeding stimulus. D) Daily mean proportion of mosquitoes taking a blood meal (days 0 and 4) or responding to a feeding stimulus (days 1, 2 and 3). doi:10.1371/journal.pone.0023591.g002

control insects showing a sharp decline in feeding propensity in the two days following a blood meal (generally less than 40% responded - Fig. 3B) followed by a return to the feeding stimulus by day 3 or 4. In contrast, all the fungal-exposed treatments showed substantial reductions in feeding propensity, with no mosquitoes taking a blood meal at 4 days after fungal exposure (see Fig. 3B).

Interestingly, despite the rapid impact on survival and feeding, fungal-exposed insects still realized some fecundity. In the gonotrophic cycle following exposure, the day 0, 4, 8, and 12 day exposure groups laid 59, 53, 53 and 75% of the number eggs produced by the relative controls, respectively.

Metabolic rate and tethered flight performance

The pre-lethal effects of fungal infection on feeding propensity are clearly substantial and important. However, given the nature of the fungal infection process (i.e. physical proliferation within the insect together with production of various secondary metabolites known to impact aspects of insect physiology [40–42]) it is reasonable to expect that an infected mosquito might suffer additional reductions in performance prior to death. One way of looking at the gross impact of infection is to measure respiration via CO₂ output as a proxy for resting metabolic rate (RMR). Here, we measured RMR of uninfected and infected mosquitoes using a flow-through respirometer (see Materials and Methods for full description). Prior to exposure, metabolic rates of a sub-sample of

mosquitoes assigned to control and treatment groups were measured. This baseline RMR did not differ significantly to metabolic rates of control insects measured over the following three days ($F_{3,24} = 0.21$ $P = 0.89$; Fig 4a). Fungal-infected mosquitoes, on the other hand, showed a significant elevation in metabolic rate ($F_{1,33} = 6.31$, $P = 0.017$), 2–2.5 times greater than controls (Fig. 4a). Mortality in the treated group left insufficient insects to do a full run on day 4. No significant effects of insect size, time of day, respirometer chamber, day after exposure, or ‘day×treatment’ interaction were observed (see Supporting Information S1).

The full implications of the elevated RMR are unclear, but the increased energy expenditure might be expected to impact on energetically costly behaviors such as flight. To investigate this we suspended infected and uninfected female *An. stephensi* mosquitoes on fine insect pins and measured three aspects of flight performance (Fig. 5). We found that the time to initiate voluntary flight increased in fungal-exposed insects, becoming significantly longer than controls from day 2 after exposure (Mann-Whitney $U = 11$, d.f. 18, $z = -2.66$, $P = 0.008$) (Fig. 5A). By day 4, only half of the treated insects initiated flight during the whole 5 hour monitoring period, whereas all control insects flew. Additionally, the length of the first voluntary flight was significantly shorter in fungal exposed insects from day 2 onwards ($P = 0.003$ - see Supporting Information S1 for full statistics) (Fig. 5B). Finally, the ability to sustain flight following repeated physical stimulation (i.e.

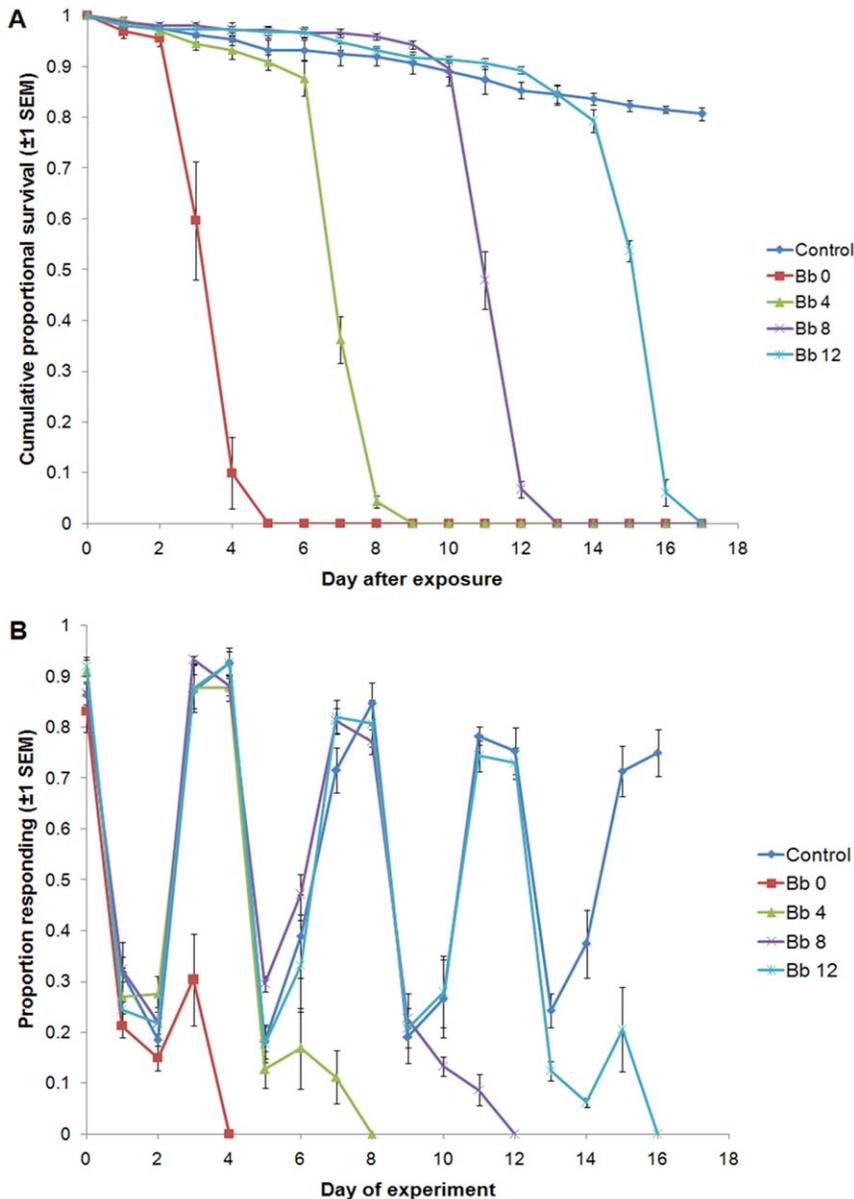


Figure 3. A) Survival of *Anopheles stephensi* exposed to clay tiles treated with *Beauveria bassiana* on either day 0 (Bb 0), or not until day (Bb 4), day 8 (Bb 8) or day 12 (Bb 12). Control mosquitoes were exposed to untreated clay tiles on each of these days. B) Proportion of mosquitoes taking a blood meal on days 0, 4, 8, 12 and 16 and proportion responding to a feeding stimulus in between the blood meal days. doi:10.1371/journal.pone.0023591.g003

flight to exhaustion, providing a measure of the energetic budget available to the insect) was also significantly shorter in treated insects. By day 3 fungal exposed insects could fly for only half as long as control insects ($P=0.009$) and by day 4 this was reduced further to just a third ($P=0.015$; Fig. 5C).

Efficacy against insecticide resistant mosquitoes

To assess the impact of the fungal biopesticide on mosquitoes resistant to chemical insecticides we ran two bioassays against thirteen colonies of *Anopheles* mosquitoes comprising three different species (*An. gambiae* s.s., *An. arabiensis* and *An. funestus*). The colonies were assessed for their resistance to four compounds (an organophosphate, organochloride, pyrethroid and carbamate) at discriminatory doses prescribed by WHO [31] using a standard cylinder

assay. The colonies were found to range from fully susceptible, resistant to one of the compounds, to two, three or in one case all four chemical classes (see Supporting Information S1 for colony description and details of the insecticides and assay performed). Exposure to the fungal biopesticide on clay tiles using the standard dose and thirty minute exposure period as described above showed MLTs of three or four days (see Fig. 6) and 100% mortality by day 6 irrespective of mosquito species or the level of chemical resistance. For example, the *An. gambiae* colony “TONGS”, which was fully resistant to all chemical classes, had an MLT of 4 (3.93–4.07) days and all individuals were dead by day 5 (± 0.0) which was not dissimilar to the fully susceptible *An. gambiae* colony “SUA” which had an MLT of 4 (3.82–4.18) days and were all dead by day 6 (± 0.25) (see Supporting Information S1 for further details).

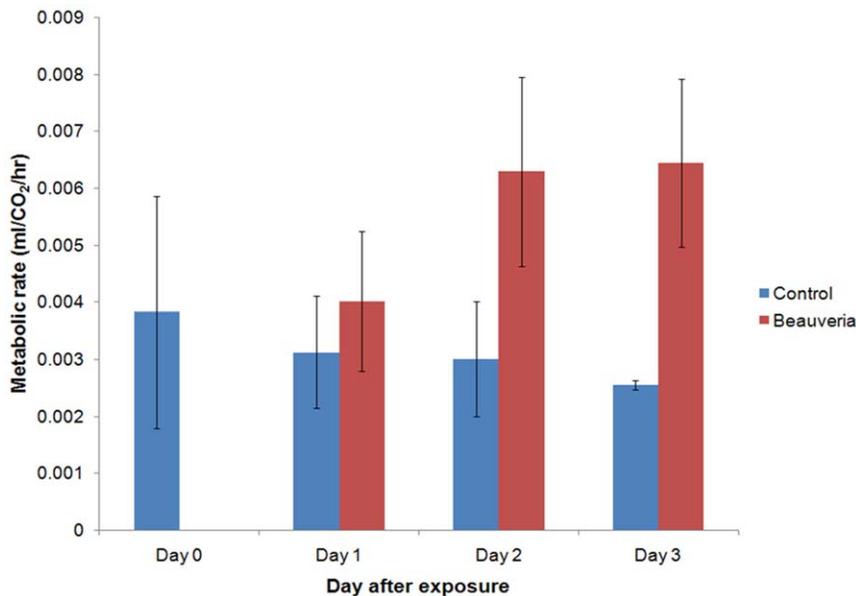


Figure 4. Resting metabolic rate of control and fungal exposed *Anopheles stephensi* as measured by CO₂ output in flow through respirometer. A sample of mosquitoes used in the experiment was assayed for metabolic rate on day 0 to provide a pre-treatment comparison. The measurement variable is CO₂ emission (ml/hr) as body size had no detectable effect on CO₂ output. doi:10.1371/journal.pone.0023591.g004

Discussion

The rapid mortality of mosquitoes following short-term residual contact with fungal spores on a realistic substrate is much faster than reported in many earlier studies [7,8,10,13,16,18]. We are not sure why this should be; the fungal isolate has been tested before, and at the doses we used here. What is different here is that the exposures were made on clay substrates in a cone test and that the spray apparatus used appears to result in more efficient delivery of spores to the substrate ([43] The use of this assay system, which is that recommended by WHO, suggests that previous reports of efficacy were underestimates).

As indicated previously, the average feeding cycle of *Anopheles* mosquitoes in the field is 2–4 days and the incubation period of the malaria parasite inside the mosquito at least 12–14 days. In order for a mosquito to transmit malaria, therefore, it needs to pick up the parasite from an infected human host and survive 3 or 4 subsequent feeding cycles before being able to transmit the parasite to another human host. Contacting a lethal insecticide like permethrin or DDT at any one of these cycles would stop transmission – which is why properly implemented ITN and IRS programs work so well (at least in the absence of resistance). While still not an instant knockdown, our results indicate that if mosquitoes contacted a virulent fungus at any feeding cycle within the parasite incubation period the mosquitoes would not survive long enough to transmit malaria either.

Insect death, however, is only part of the story. Our results also show that once infected with fungus, mosquitoes are less inclined to feed. The effect appears stronger as the fungal infection progresses but can contribute to significant reductions in host feeding as early as day two, essentially accelerating the transmission blocking effects of the fungus. Pre-lethal reductions in feeding propensity have been shown before [7,21] and appear a common effect of fungal infection in insects [44–47]. What is striking here is that when the effects of blood feeding are added in, risk of malaria transmission is essentially reduced to zero within a day of fungal exposure and never recovers.

In addition, fungal infection increases mosquito metabolic rate and reduces flight propensity and flight stamina. Again, fungal induced reductions in flight performance and elevated metabolic rate have been shown previously in other insects [47–50] and poor flight performance has been strongly associated with reductions in the mobile energy reserves of the host [48]. Energetic demands in the field associated with host finding, searching for oviposition sites and predator avoidance (previously shown to be compromised in fungal-infected insects [51]) are likely to be considerably higher than in our laboratory setting. We have been dealing with young, healthy insects maintained under ideal conditions. Long range flights to search for nectar sources, blood meals and oviposition sites under variable environmental conditions [52,53], coupled with the agility required to evade death while blood feeding and repeated contact with treated surfaces, makes life hard for a mosquito. Add in the burden of malaria infection [54–58] and age-related senescence [59–63] then there is every reason to think that the behavioral, physiological and survival effects we have found so far are underestimates of the potential of fungi to reduce malaria transmission. Indeed, it is an interesting possibility that in the natural context, simply making mosquitoes sick could be sufficient to disrupt the malaria transmission cycle.

Our comprehensive evaluation of multiple mosquito strains and species covering diverse mechanisms and expressions of insecticide resistance demonstrates that insecticide resistance confers no cross-resistance to fungal pathogens in the key African malaria vectors. This result extends previous studies [16,17] and contrasts the situation with chemical insecticides where there are major problems of cross-resistance, undermining the potential of many resistance management strategies [2,4]. Furthermore, co-exposure to fungus and insecticide has been shown to increase the susceptibility of otherwise resistant mosquitoes to existing chemical insecticides [16,17].

Several previous studies have argued for the evolutionary benefits of Late Life Acting products; slow speed of kill enables mosquitoes to achieve part of their lifetime reproductive output, reducing selection pressure for resistance [24–26]. In a very recent

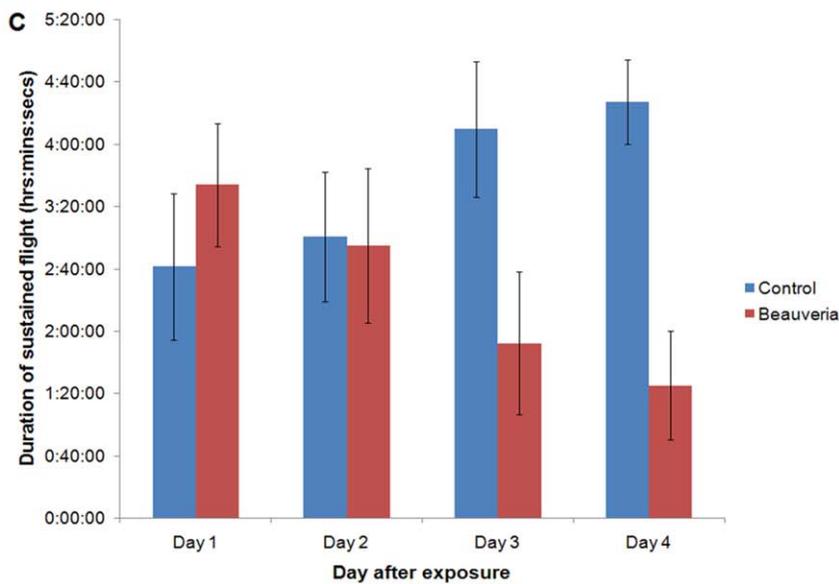
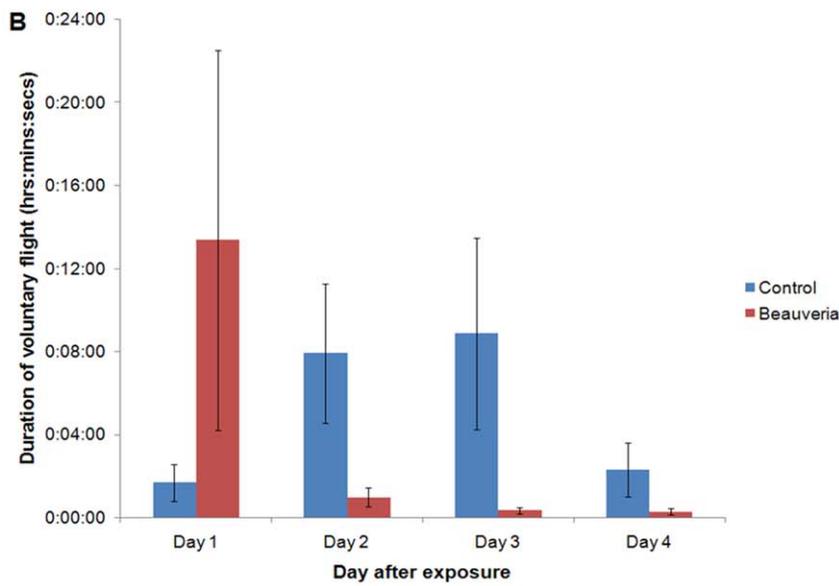
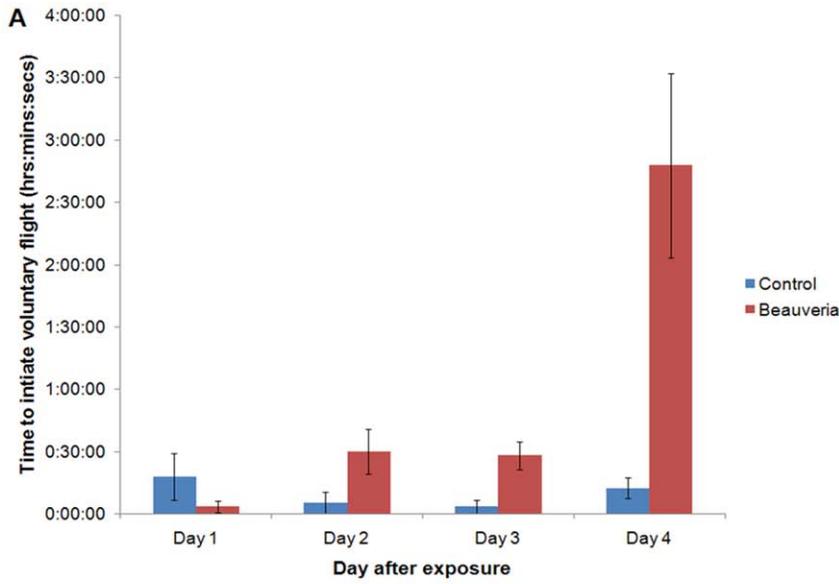


Figure 5. Tethered flight performance of control and fungal exposed *Anopheles stephensi*. A) Time to start of voluntary flight and B) the length of that initial flight. C) The duration flight was maintained in mosquitoes repeatedly stimulated. doi:10.1371/journal.pone.0023591.g005

development, Fang et al. [64] demonstrated the potential to genetically modify an insect pathogenic fungus to kill malaria parasites within mosquitoes without dramatically impacting mosquito survival, further extending the ‘evolution proof’ potential. In our study, the fungus clearly imposes a fitness cost, although the fact that there is some realized fecundity following infection suggests there would still be reduced selection pressure for resistance compared with the instantaneous action of conventional chemicals [24].

While the resistance management benefits of Late Life Acting products are clear in theory, the approach has been criticized as impractical [28,37] and sits at odds with current WHOPES criteria. Acknowledging these issues, the current study attempts to align the fungal biopesticide approach with the established chemical insecticide paradigm. Our results demonstrate that using the standard WHOPES protocols, short-term residual contact with fungal spores on a realistic substrate can cause extensive mortality of mosquitoes. Important pre-lethal effects including reduced feeding propensity and flight stamina reduce vectorial capacity further; if a mosquito does not want to feed, is less able to sustain flight to search for a host and ultimately dies before the malaria parasite can complete its development, there will be no transmission. Thus the combined pre-lethal and lethal effects of fungi, together with resistance breaking properties, make possible malaria control without fast acting neurotoxins (reasserting the fact that other once successful IRS treatments, such as cyclodiene insecticides in the 1950s, were slower acting [65]). While it would also be possible to enhance speed of kill further by genetically modifying fungi [64,66], the data we report here suggests that natural variation alone may be sufficient.

The fungal isolate used in the current study exists as a commercial pest control product and has full EU and US registration for certain agricultural applications [67,68]. In principle, repurposing this isolate for malaria control could deliver a novel product for operational use in a relatively short timeframe, and at a fraction of the R&D costs of a new chemical entity. Given the emerging insecticide resistance crisis and acknowledged difficulties in getting new chemical insecticides to the market for many years [2], further evaluation of the fungal biopesticide approach under diverse field conditions would seem justified.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of the Pennsylvania State University (Permit Number: 27452). All mice were anesthetized prior to mosquito feeds using a Xylazine:Ketamine (0.15:1) mix at 0.1 ml/10 grams body weight i.p. and all efforts were made to minimize suffering.

Fungal application, mosquito exposure and survival monitoring

Beauveria bassiana spores were formulated in a mix of mineral oils and the concentrations adjusted to give the desired spores/ml of formulation. Formulated spores were applied using a pump sprayer clamped horizontally over the test tile and 10 cm

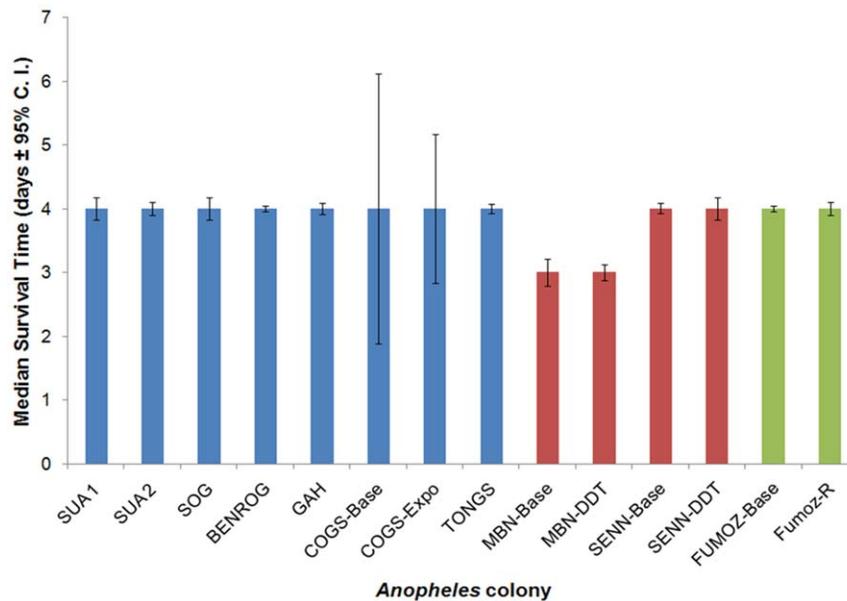


Figure 6. Survival of thirteen colonies of *Anopheles* mosquitoes following exposure to *Beauveria bassiana* on clay tiles. Resistance to chemical insecticides for each colony was determined and ranged between fully susceptible and resistant to all four classes of chemical insecticides currently used for vector control (see main text and Supporting Information S1). Two assays were conducted with the fully susceptible *Anopheles gambiae* s.s. SUA colony acting as a positive control in each assay. Blue bars are *Anopheles gambiae* s.s., red bars *Anopheles arabiensis* and green bars *Anopheles funestus* colonies. doi:10.1371/journal.pone.0023591.g006

above it. Each tile received five pumps from the sprayer which delivered 0.7 ml of formulation with each pump. Following application tiles were left to dry at room temperature for 24 hours. A standard WHO cone assay was used for exposing the mosquitoes to the treated tiles. The plastic cone was secured over each tile and between 30 and 60 (depending on assay) unfed female *An. stephensi* were introduced. The mosquitoes were then left for 30 seconds, 1 minute, five minutes, 10 minutes or 30 minutes depending on the assay. Each treatment was replicated four times giving a minimum of 120 mosquitoes per assay. Following exposure mosquitoes were removed to holding cages where they were either blood fed (see below) or maintained on 10% glucose water for the duration. Subsequently each day either until all treated insects had died or for 14 days, whichever was earlier, the number of dead mosquitoes were counted and removed from the cages.

Feeding propensity

Two methods were used to assess feeding propensity. Mosquitoes were offered either an anaesthetized mouse or a glass bottle filled with hot water (temperature range 35–42°C) and then covered with an investigator's recently worn sock. The sock covered bottle was placed next to the side of the cage and in contact with the mesh. On the day of blood feeds, mosquitoes were allowed to feed from mice for twenty minutes and then those not taking a blood meal were counted. On intervening days the "sock-bottle" method was used as a feeding stimulus and the number not actively probing at the bottle were counted. In both cases this number was related to the total number of mosquitoes in that cage at the time of the feeding assessment to give the proportion feeding on that day.

Fecundity assessment

Assays where anaesthetized mice were used for feeding also provided the opportunity to assess fecundity. Two days following the first blood meal egg bowls (35 mm Petri dishes lined with filter paper and soaked with distilled water) were introduced. The following morning these bowls were removed and replaced with fresh bowls and this was repeated until the end of the experiment. Eggs were counted and related to the number of mosquitoes alive to give an eggs/female fecundity estimate.

Estimating Resting Metabolic Rate

Metabolic rates were measured by using groups of three mosquitoes at rest within flow-through respirometry chambers. Dry CO₂ free air was passed through the 20 ml chambers at 0.25 litres/min and then dried and passed through a Li-Cor 6252 carbon dioxide analyser. Within each run, seven experimental chambers containing mosquitoes were sampled in sequential fashion by using a computer controlled valve system. Three chambers containing control mosquitoes and four for fungal exposed mosquitoes were used in the first run and the order was reversed for the second run giving 7 replicate estimates per day of the experiment. An eighth chamber was left empty and sampled between each of the occupied chambers to establish a baseline. A pre-treatment run was performed on day 0 using the mosquitoes to be allocated to treatment groups and then two runs were made every day for the duration of the experiment. All chambers were housed in a reach-in incubator set to 25 (±0.2)°C. Analog signals from the flow meter and carbon dioxide analyzer were converted to digital and recorded on a computer (Sable Systems, Salt Lake City).

Flight performance

To assess flight performance female mosquitoes were briefly immobilized with CO₂ and then placed on a dish under a dry ice curtain. A small amount of dental wax was melted on the blade of a surgical scalpel and the head of an insect pin touched to the wax. Immediately following a mosquito gently held in forceps was manipulated so that the dorsal thorax came into contact with the head of the pin, held briefly to allow the wax to set and then the sharp end of the pin was stuck vertically in a block of polystyrene. Each mosquito was carefully examined to ensure that no wax had spread over the insect, that no damage to the insect had been caused and that the insects' orientation was appropriate (i.e. not cantered to one side, forward or back). In any case where the mounting of the insect on the pin did not satisfy these requirements the mosquito was discarded and a new one set up. Two measures were made: 1) the duration of voluntary flight. In this case the time until first flight was initiated was recorded as well as the duration of this flight. The second measure looked at the duration of sustained flight. In this case and following the end of the voluntary flight the mosquito was stimulated to fly again by gently stroking its legs with a fine paint brush. Each time the mosquito stopped flying it was stimulated again until after five consecutive stimulations the insect could not fly again. Flight assays were censored after five hours.

Assays against chemical resistant mosquitoes

Assays were carried out at the VCRU/NICD/NHLS facility in South Africa where a range of resistant and susceptible *Anopheles* colonies are maintained. Two assays were performed. In each a fully susceptible *Anopheles gambiae* s.s. colony (SUA) was included. Again the WHO cone test was employed and all methodologies, substrate, standard dose, application method and exposure time were as described above. In each assay and following exposure to either untreated or *Beauveria* treated clay tiles mosquitoes were removed to 0.375 ml cardboard cups covered with mesh and supplied with 10% sucrose. Mortality was monitored daily for 14 days. Following the fungal assays mosquitoes from each colony used were exposed to each of four chemical insecticides (DDT, Bendiocarb, Malathion and Deltamethrin) representing the four major classes of compound available for vector control (Organochlorides, Carbamates, Organophosphates and Pyrethroids). A standard WHO cylinder assay was used with four replicates of 25 mosquitoes per replicate for each colony used on the fungal assays. Mortality was assessed after 24 hours and related to the WHO criteria for assessing resistance/susceptibility to chemical insecticides. Further details can be found in the supplementary materials.

Supporting Information

Supporting Information S1 Further details of the material and methods, analyses and results for the experiments described in the main text.

(DOC)

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Author Contributions

Conceived and designed the experiments: SB AFR MBT. Performed the experiments: SB WS RC LLK BDB. Analyzed the data: SB. Contributed reagents/materials/analysis tools: JHM MC. Wrote the paper: SB AFR MBT.

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