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Evaluation of entomopathogenic fungi *Metarhizium anisopliae* against dengue virus mosquitoes *Aedes aegypti* (Diptera: Culicidae)

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ABSTRACT: In this study, the bio-potential of the entomopathogenic fungus *Metarhizium anisopliae* was tested against *Aedes aegypti* under laboratory conditions. The study includes the analysis of the attractive response, survival and fecundity rate of non-blood and blood fed female mosquitoes exposed to the volatiles of two *M. anisopliae* strains. The attractive response was analysed using a two-choice behavioural bioassay, with three different sizes of dry spore plates (full, 1/4 and 1/16 plates). The survival and fecundity bioassay was conducted simultaneously in plastic pots. Log-rank survival curve analysis was used for statistical comparisons of the attractive response, survival and fecundity. Non-blood and blood fed mosquitoes were highly attracted to *M. anisopliae*-30 volatiles compared with that of the *M. anisopliae*-131 strain. Moreover, attraction was dependent on the size of the dry spore plate. Survival was completely abolished in unfed mosquitoes 5 and 6 days after treatment with 10^9 spores/mL of *M. anisopliae*-30 and *M. anisopliae* -131, respectively, whereas almost 80% of untreated unfed females survived more than 28 days. Survival in blood fed mosquitoes treated with same dose of *M. anisopliae*-30 and *M. anisopliae*-131was abolished after 6 and 7 days, respectively, while over 80% of untreated blood fed females survived more than 28 days in the controls. Mean number of eggs laid by blood fed mosquitoes treated with 10^9 spores/mL of *M. anisopliae*-131 was 26 ± 3 compared to control (67 ± 4). However for *M. anisopliae*-30, 19 ± 3 eggs were laid compared to control 72 ± 5 eggs. This study concludes that both the strains of *M. anisopliae* reduce egg laying capacity and survival rate in *Ae. aegypti*. As such, these strains can be useful for the development of mycoinsecticides for the control of the dengue fever vector mosquito, *Ae. aegypti.*

KEY WORDS: *Aedes aegypti,* attractive response, fecundity rate, *Metarhizium anisopliae*, survival rate, vector control

INTRODUCTION

Mosquito born-diseases are a major tropical health challenge, world-wide. Anthropogenic activities in tropical and subtropical countries play a significant role in increasing number of mosquito breeding sites (Scott *et al.,* 1997; Chareonsook *et al.,* 1996). Consequently, a high proportion of people suffer from viral transmission, including Japanese encephalitis, dengue fever and yellow fever

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(Heddini *et al.,* 2007; Nagi *et al.,* 2011; Chakravarti *et al.,* 2012) as well as the transmission of other pathogens causing diseases such as malaria and filariasis, (Ghosh *et al.,* 2012; Kundu *et al.,* 2013). Chemical pesticides are extensively used to control adult mosquitoes as well as immature stages in their breeding sites. However, due to their negative impact on the environment, non-target organisms, and development of resistance by target species, alternative measures are needed to replace chemical pesticides. As an alternative, the utilization of potential entomopathogenic fungi in pest control is considered an eco-friendly approach.

Entomopathogenic fungi are safe for human and other non-target organisms, do not have residual problem through food chain, enhance biodiversity, reduce the development of resistance (Scholte *et al.,* 2007), attract and kill the target organisms (George *et al.,* 2013), and reduce the fecundity and survival rate of mosquitoes (Paula, 2008; Scholte *et al.,* 2005; Paula *et al.,* 2011; Darbro *et al.,* 2011; Blanford *et al.,* 2012). The bio-potential of entomopathogenic fungi is variable and dependent on the developmental stage of the mosquito species. Earlier report highlight that the application of fungal pathogens, such as *Lagenidium*, *Coelomomyces* and *Culicinomyces*, are effective against the larval stages, whereas *Hypomycetes*, *Beauveria bassiana* and *Metarhizium anisopliae* are highly pathogenic to adult mosquitoes (Scholte *et al.,* 2003). The time from infection until the death of the host depends on the host species, host physiological state, fungal species and virulence of the strain, dose of conidia suspension and abiotic factors (Ferron, 1978; Gillespie and Claydon, 1989; Blanford *et al.,* 2005). In order to regulate mosquito population size, researchers are focussing on increasing the understanding of the biology and ecology of pathogen-mosquito interaction (Roy *et al.,* 2006).

The *Hypomycetes* fungus *M. anisopliae* is recommended as one of the management approaches against insecticide resistant and susceptible mosquitoes (Scholte *et al.,* 2006; Farenhorst *et al.,* 2010). Based on the toxicity tests, Environmental Protection Agency declared no risk to humans when using mass products of microbial biopesticides containing *M. anisopliae* (EPA, 2003). The volatiles released from entomopathogenic fungi may alter behavioural response of the mosquitoes (Mburu *et al.,* 2011; George *et al.,* 2011). Some of the fungal volatiles are not repellent to *Anopheles* and *Culex* mosquitoes (Mnyone *et al.,* 2010), and some fungal dry spores may attract *An. stephensi* (George *et al.,* 2013). Several studies have investigated the effectiveness of fungal pathogens and how these are affected by salinity, temperature, relative humidity and breeding sites with variable water quality, in the context of low cost mass-production and formulation techniques, long shelf life, killing effect on target mosquitoes and non-target organisms (Zimmermann, 2007; Scholte *et al.,* 2004; Blanford *et al.,* 2011; Read *et al.,* 2009).

Dengue fever is one of the most rapidly spreading mosquito-borne diseases worldwide (WHO, 2009), and is transmitted by female *Ae. aegypti* mosquitoes. The behaviour of adult mosquitoes is highly anthropophilic, endophilic and endophagic. Due to the development of insecticide resistance there are several challenges to develop effective control methods (Darbro *et al.,* 2011). Dengue fever vector control strategies include chemical-based control measures, non-chemical-based control measures and biological control agents (Poopathi and Tyagi, 2006). Previous studies have reported that in *M. anisopliae* reduce the survival rate of *Ae. aegypti* mosquitoes, and also kill the insecticide-resistant mosquitoes (Blanford *et al.,* 2005; Farenhorst *et al.,* 2009; Howard *et al.,* 2010). With the background information given above, the present study was undertaken to evaluate impact of *M. anisopliae*-131 and *M. anisopliae*-30 strain on the attraction, survival and fecundity rate of non-blood and blood fed female *Ae. aegypti.*

MATERIALS AND METHODS

a) Mosquito culture establishment:

Aedes aegypti (Rockefeller strain) eggs were collected from the culture maintained at the insectary, at the University of Sweden Agricultural

Science, Alnarp, Sweden. The eggs were kept in plastic trays (25 cm x 25 cm x7 cm) filled to a depth of 5 cm with distilled water. After eclosion, the larvae were provided with finely powdered Tetramin® fish food as a source of food. After the larvae reached the pupal stage, the pupae were separated every day and introduced into a beaker half-filled with distilled water and kept inside the adult emergence cages. Filter paper was provided in distilled water-filled cups for oviposition. The adult mosquitoes were provided with 10% sucrose solution (w/v) as food. At 4 to 5 day old, adults were first starved at eight hours and then provided with a sheep blood meal for 30 min, using the Hemotek® membrane feeding system. The culture was maintained at 27 ± 1°C, 65-70% RH and on a 12:12 h photo-period. From the culture, blood and non-blood fed female adults were taken and used for the behavioural, survival and fecundity bioassays.

b) *Metarhizium anisopliae* culture establishment:

The *M. anisopliae*-131 strain was obtained from Addis Ababa University Ethiopia, whereas and *M. anisopliae*-ICIPE-30 (*M. anisopliae*-30) was a gift from the International Centre of Insect Physiology and Ecology, Kenya. Both strains were cultured on Sabouraud dextrose agar media (dextrose 10 g; peptone 2.5 g; yeast extract 2.5 g; agar 20 g in 1 L H_2O) and kept at 27°C in an incubator for 15 days until sporulation (Plate 1). After sporulation, different sized dry spore plates (full, 1/4, 1/16) were prepared under aseptic condition and used for the behavioural bioassay (Plate 2). Survival and fecundity bioassays were carried out by using spore suspension initially prepared with 0.05% Triton X-100 and conidial concentration of 10^5, 10^7 and10^9 spores/mL (Figure 2A). The conidial concentration was determined by using a Neubauer haemocytometer. The fungal suspension was mixed vigorously by using a vortex mixer, and then 0.2 µl of the suspension was applied on the thoracic region of individual adult mosquitoes using a micropipette inside a safety cabinet.

c) Test of spore germination:

The viable conidia of the two strains were determined by sub-culturing the conidial suspension. The conidial suspension was serially diluted, so that 0.1 mL of 10^{-2} spore suspension inoculated three SDA plates. The spore suspension was uniformly spread on the surface area of the SDA plates by using L-shaped glass rod. After inoculation, plates were covered with sterilized cover slips, sealed with parafilm, and stored in a temperature-controlled incubator for 20-24 h. Percentage germination of spores was examined after the incubation period. From each plate, 300 spore counts were examined under a compound microscope (at 16X magnification). The conidial growth of more than 95% spores out of 300 conidia per plate produced visible germ-tube length at least three times the width (diameter) of the conidium in both strains of *M. anisopliae*.

d) Behavioral bioassay:

The behavioural response of female *Ae. aegypti* mosquitoes exposed to the volatiles emitted by *M. anisopliae* was studied in four treatment groups. In first treatment, non-blood fed mosquitoes were exposed to volatiles emanating from dry spores of *M. anisopliae*-131, and pure SDA media. In a second treatment, non-blood fed mosquitoes were exposed to volatiles emanating from the dry spores of *M. anisopliae*-30 and pure media. In a third treatment, non-blood fed mosquitoes were exposed to the volatiles emanating from the dry spores of *M. anisopliae*-131 and *M. anisopliae*-30. In a fourth treatment, blood fed mosquitoes were exposed to the volatiles emanating from dry spores of *M. anisopliae*-131 and *M. anisopliae*-30. Before each experiment, 10 mosquitoes were kept in each of eight individual release cages and exposed to 24 h of starvation with access to water, and allowed to acclimatize in the bioassay room. Two-choice bioassays were carried out from 6:00 pm to 7:00 pm to analyse the behavioural response of non-blood and blood fed mosquitoes. After a one-hour exposure to the volatiles emanating from the dry spores, the mosquitoes from the treatment and control arms were collected, killed and counted. The experiment was replicated eight times by using new dry spore plates, mosquitoes and viable conidia.

e) Dry spore plate preparation:

The *M. anisopliae*-131, *M. anisopliae*-30 and pure SDA media plates were prepared at full, 1/4 and 1/16 dry spore plates for paired comparisons to test the attraction of non-blood and blood fed *Ae. aegypti*. The differently sized plates of dry spores and pure SDA media plates were prepared in a sterilized safety cabinet by using sterilized knife. After preparation, the plates were immediately transferred to the bioassay room and used for the experiment.

f) Description of two choice behavioral bioassay method (Figure 1):

Computer fans were used to draw the volatiles from dry spore and SDA plates from the treatment and control arms, at the upwind end of the bioassay. The end of the two arms was closed with Plexiglas cylinder (8.9 cm diameter, 5 cm long) with one end sealed with nylon mesh. The most upwind section of two-choice bioassay consisted of two samples chambers of Plexiglas cylinders (9.9 cm diameter, 14.5cm long) into which the plates were placed. Dry spore plates of *M. anisopliae* versus pure SDA plates, or dry spore plates of *M. anisopliae*-131 versus *M. anisopliae*-30, of equal size, were placed into the two parallel arms at the same time for each experiment. In the Plexiglas cylinder, a nylon mesh screen was used to prevent the entry of mosquitoes to the treatment and control chambers. The rotatable nylon mesh screen valve at the other end of a mosquito collecting chamber (9.9 cm diameter, 6.5 cm long) was closed at the end of the experiments to count number of mosquitoes in each arm. A pair of Plexiglass cylinders were used as flight chambers (8.9 cm diameter and 25 cm length). At one end, both flight chambers were attached to a Plexiglass box, known as the decision chamber (30.5 cm × 22 cm × 13 cm). At the other side of the decision chamber, a single Plexiglass cylinder (9.9 cm diameter, 29.5 cm long) extends and is connected to the release cage (8.9 cm diameter, 10 cm long). One end was covered with rotatable nylon mesh and other end sealed with nylon mesh. The flow of *M. anisopliae* volatile was controlled by computer fan into the upwind end of the two arms in two choice bioassay systems. The air flow current was linear until it reached the downwind wall of the decision chamber with an airspeed determined at 10 cm s^{-1}.

g) Conidial suspension preparation for survival bioassay:

Fungal spore suspensions were prepared from 15 days old surface sporulation fungi in which 0.05% Triton X-100 was added. The homogenous spore suspensions were prepared using a vortex mixer. Unwanted material from the spore suspensions were removed by a series of three centrifugations at 300 rpm for 3 minutes. Conidial concentrations of 10^5, 10^7 and 10^9 spores/mL were prepared for the survival and fecundity experiments through serial dilutions using distilled water supplemented with 0.05% Triton X-100. The conidial concentration was determined by using a Neubauer haemocytometer (Figure 2A).

h) Effect of infection on the survival of mosquitoes:

Female adult *Ae. aegypti* mosquitoes were anaesthetized on ice for 7-10 min and inoculated with the spore suspensions, as described above. After inoculation, individual mosquitoes were transferred to plastic pots (12 cm diameter × 8 cm height), and provided with 10% sucrose *ad libitum*. Blood fed mosquitoes were provided with an oviposition substrate identical to that supplied for rearing (Figure 2B). Ten replicates for each spore concentration were run in total. The plastic pots were placed in a controlled climate cabinet at 27 ± 1°C and 65-70% RH until the completion of the experiment.

i) Detection of mycosis:

The fungal infection and its impact on mosquito survival and mortality was recorded. Cadavers (Figure 2C) were removed, dipped in 70% ethanol, and rinsed with distilled water to remove the remaining spores associated with the cuticle, and then each cleaned cadaver was placed on moist filter paper inside Petri dish and sealed with

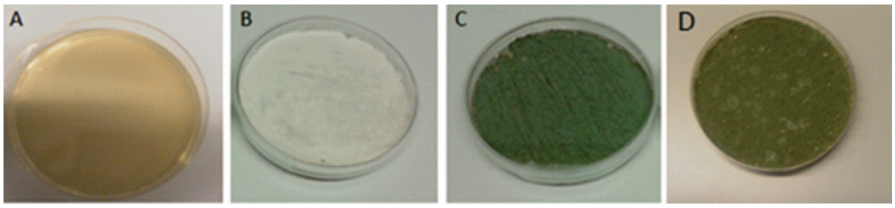

Plate 1. *Metarhizium anisopliae* growth on Sabouraud dextrose agar media at 25ºC.
A) Sabouraud dextrose agar media; B) Mycelium of *M. anisopliae* before sporulation, C) Sporulation of *M. anisopliae*-131; D) Sporulation of *M. anisopliae-ICIPE*-30

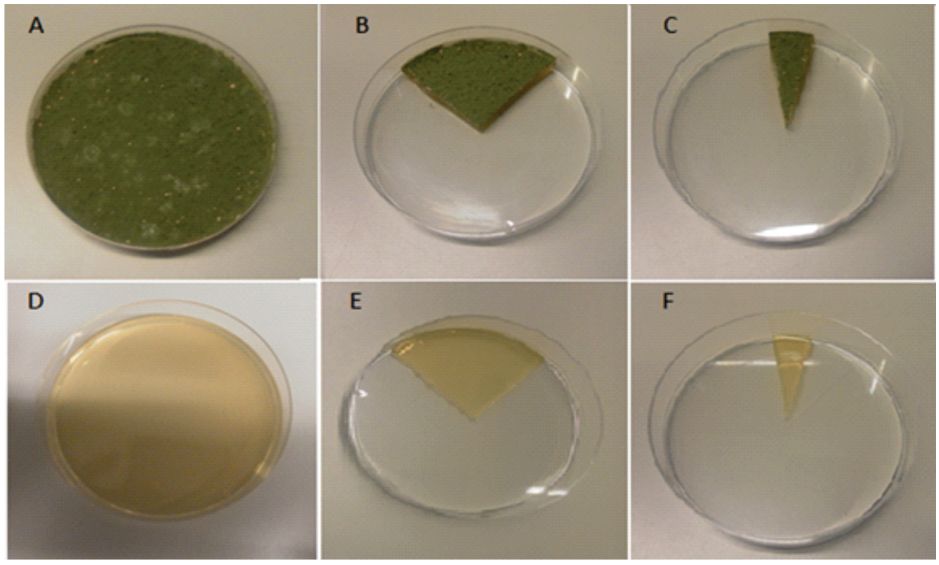

Plate 2. Dry spore plates of *M. anisopliae* and SDA media plates.
A, B and C showing full, 1/4 and 1/16 plates of *M. anisopliae*
D, E and F, showing full, 1/4 and 1/16 plates of SDA media

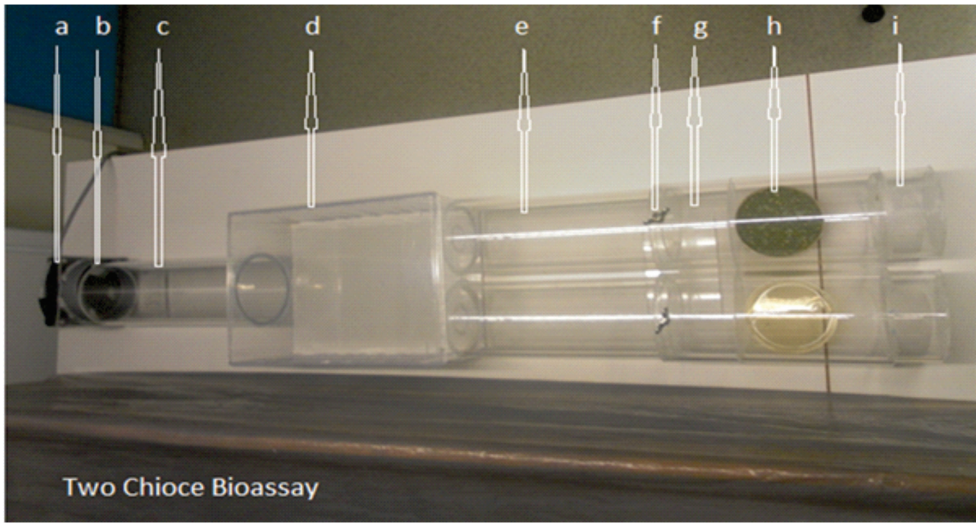

Figure 1. Two choice bioassay chambers
a) Computer fan regulating the air flow, b) Place to release adult mosquitoes, c) Release chamber, d) Decision chamber, e) Decision arm, f) Circular mesh, g) Mosquito collecting chamber, h) Dry spore and control Petri-plates keeping chambers, i) Air inlet covered with mesh

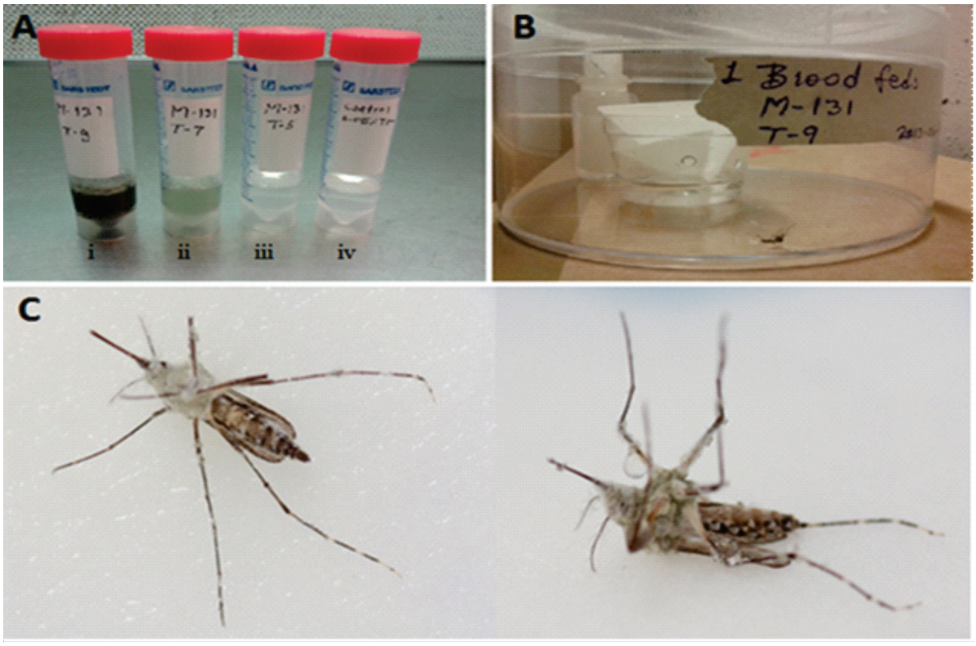

Figure 2.
A) Three different doses [(i). 10^9 spores/mL (ii). 10^7 spores/mL (iii). 10^5 spores/mL, and (iv). zero spores/mL)] of *M. anisolpiae* and control group
B) Bioassay chamber containing 10% sugar solution and moist filter paper in the small beaker for oviposition
C) *M. anisolpiae* mycelium emerged from cadavers of female *Aedes aegypti*

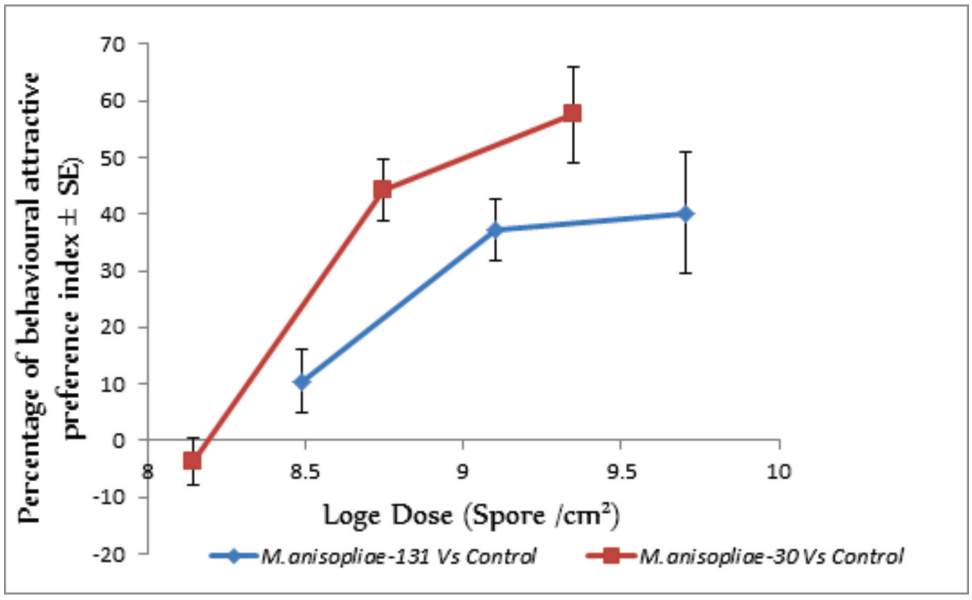

Figure 3. Behavioural responses of *Aedes aegypti* against dry spore volatiles of *Metarhizium anisopliae*

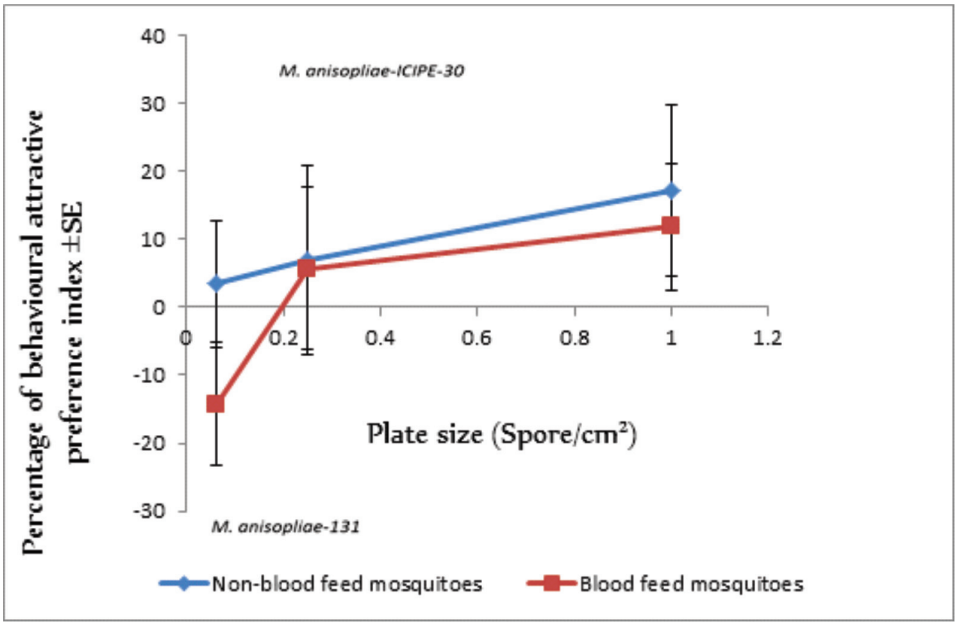

Figure 4. Comparison of *M. anisopliae*-131 and *M. anisopliae-ICIPE*-30 dry fungal spores volatiles attractive response against *Ae. aegypti* mosquitoes

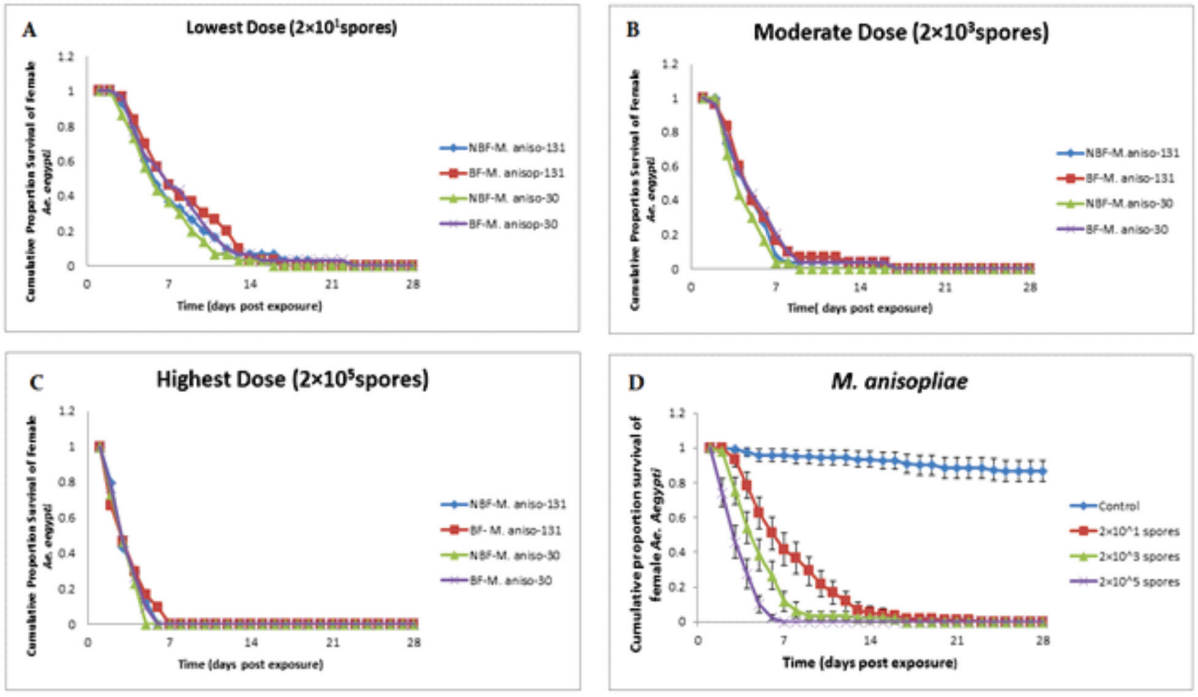

Figure 5. Overall pathogenicity of the two strains of *M. anisopliae* on blood fed and non-blood fed *Ae. aegypti*
A) Low dose ($2x10^1$ spores), B) Moderate dose ($2x10^3$ spores), C) High dose ($2x10^5$ spores), D) Combined survival curves of two strains and two physiological states at all three doses

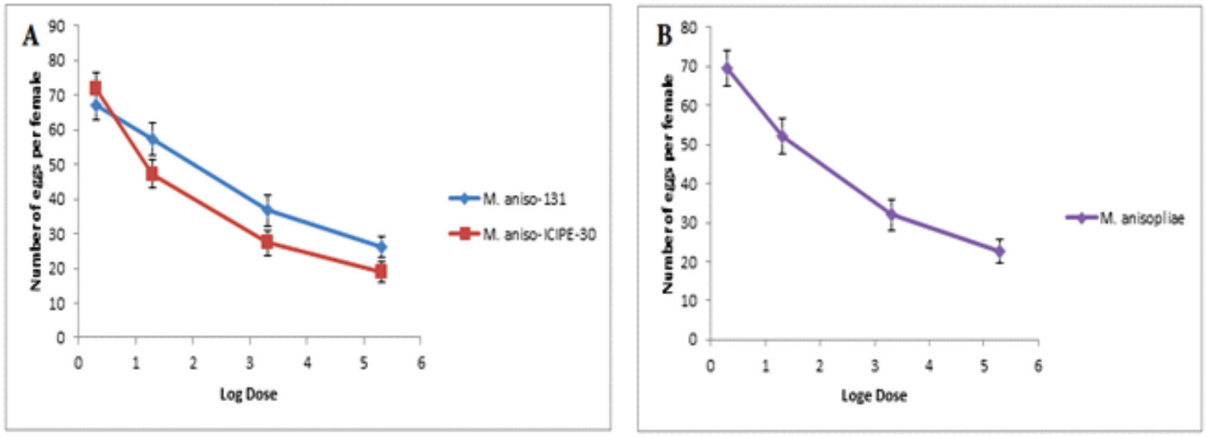

Figure 6. Effect of *M. anisopliae* on egg deposition by blood fed female *Ae. aegypti*
A) *M.* ansopliae-131 and *M. anisopliae-ICIPE*-30. B) Comparison between the two strains of *M. anisopliae* on the fecundity of infected *Ae. aegypti* showed no significant difference (P> 0.05) and the pooled one slope for all the data are significant different (P <0.05).

parafilm. To verify the infection, Petri plates were placed inside an incubator at 27 ± 1°C for 5 days. Pathogens were re-isolated for detection of mycosis and to assess the survival agents from emerging mycelium from the cadaver. The spore structure was examined under a compound microscope.

j) Fecundity bioassay:

The total number of batches and number of eggs per female from uninfected and infected mosquitoes were recorded for both fungal strains. The fecundity and survival bioassays were conducted simultaneously in the same plastic pot setup described above inside the cabinet.

k) Data analysis:

A preference index (PI) indicating the attractive response of female *Ae. aegypti* to the volatiles emanating from the two strains of *M. anisopliae* was calculated according to the formula: PI = (T-C)/(T+C), where T is the number of mosquitoes collected in the treatment arm, and C is the number of mosquitoes collected in the control arm. A linear regression analysis was used to determine the functional relationship between log spores concentration and attractive preference index (PI) using PROBAN (Van Ark, 1995). The homogeneity of the replicated experiments was determined by using a Log-rank Test (Elandt-Johnson, 2009) at a level of 95% significance. Subsequently, the results were pooled to calculate the average preference index and standard deviation. Mean percentage survival and survival curve analyses were calculated for the survival and fecundity experiments and the results were compared by using one-way analysis of variance followed by a Duncan's post-hoc test. Mean survival curve comparison was carried out using a Log-rank (Mantel-Cox) test.

RESULTS

a) Attraction of female *Ae. aegypti* to *M. anisopliae* volatiles:

The attraction, calculated as a preference index (PI ± SE), of non-blood fed female *Ae. aegypti* exposed to volatiles emanating from *M. anisopliae*-131versus that of SDA media demonstrates a dose-dependent response (F =11.23; df =1, 22; p = 0.0029; figure 3). Similarly, the attraction of non-blood fed female *Ae. aegypti* to the volatiles emanating from *M. anisopliae*-30 versus that of SDA media was also dose-dependent (F = 31.56; df = 1, 22; p < 0.0001; figure 3), and when the dose-responses of the two strains against SDA media were directly compared they did not significantly differ from one another (F = 2.69; df = 1, 44; p = 0.1084; Figure 3). The resulting shared curve also demonstrated a dose-dependent response (F = 6.60; df = 1, 45; p = 0.0136).

In a direct two-choice experiment, non-blood fed female *Ae. aegypti* were significantly more attracted to the volatiles emanating from *M. anisopliae*-30 compared to those of *M. anisopliae*-131 in a dose-dependent manner (F = 12.11; df = 1.000, 46.00; p = 0.0011; Figure 4). Similarly, the blood fed females also respond to the fungal volatiles in a dose-dependent manner. However, at the lower doses, blood fed females were more attracted to the volatiles emanating from *M. anisopliae*-131 than *M.anisopliae*-30. Conversely, at higher doses, blood fed females were more attracted to the volatiles emanating from *M. anisopliae*-30 than *M.anisopliae*-131, indicating a significant difference between the attraction of the two strains for blood fed females (F = 11.16; df = 1, 45; p = 0.0017). No significant difference in the overall attraction between the physiological states observed (F = 0.08 21; df = 1, 91, p = 0.7751).

b) Effect of *M. anisopliae* on the survival rate of non-blood fed *Ae. aegypti*:

The mean percentage survival of non-blood fed *Ae. aegypti* infected with three standard doses of *M. anisopliae*-131 and that of the controls is presented in figure 5. The mean percentage survival was 0% at 6 days post-infection (dpi) at the highest dose (10^9 spores/mL), 16 dpi at the intermediate dose (10^7 spores/mL) and 20 dpi at the lowest dose (10^5 spores/mL). In comparison, the mean percentage survival of controls (0.05% Triton X-100) was 83 ± 7% up to 28 days (Figure 5A). The survival of *Ae. aegypti* exposed to *M. anisopliae*-131 was significantly different from the controls ($\div^2$ = 66.5, df = 3; p < 0.0001). Similarly, the survival of non-blood fed *Ae. aegypti* infected with *M. anisopliae*-30 was 0% for 5 dpi at the highest dose (10^9 spores/mL), 9 dpi at the intermediate dose (10^7 spores/mL) and 16 dpi at the lowest (10^5 spores/mL), compared to the controls, of which 87 ± 6% survived for for at least 28 dpi. This result also showed statistically significant difference ($\div^2$ = 68.44, df = 3; p < 0.0001) (Figure 5B).

c) Effect of *M. anisopliae* on survival rate of blood fed *Ae. aegypti* :

The mean percentage survival of blood fed *Ae. aegypti* infected with three standard doses of *M. anisopliae*-131 and that of the controls is presented in figure 5. The mean percentage survival of blood fed female *Ae. aegypti* exposed to *M. anisopliae*-131 was 0% for 7 dpi at the highest dose (10^9 spores/mL), 17 dpi at the intermediate (10^7 spores/mL) and 17 dpi at the lowest dose (10^5 spore/mL), which is significantly different from the controls, with 87 ± 6% surviving 28 dpi ($\div^2$ = 74.91, df = 3; p < 0.0001; Figure 5C). Similarly, the survival of blood fed *Ae. aegypti* exposed to *M. anisopliae*-30 was 0% for 6 dpi at the highest dose (10^9 spores/mL), 17 dpi at the intermediate dose (10^7 spores/mL) and 23 dpi at lowest dose (10^5 spores/mL), which was also significantly different from the controls with 90 ± 6% surviving 28 dpi ($\div^2$ = 77.06; df = 3; p < 0.0001). Overall, the survival of the two strains and the two physiological states was significantly different and dependent on ($\div^2$ = 288.5; df = 3; p < 0.0001).

d) Effect of *M. anisopliae* strains and conidia on the fecundity rate of female *Ae. aegypti:*

The number of eggs laid by blood fed female *Ae. aegypti* infected with the two strains of *M. anisopliae* is presented in figure 6. The mean number of eggs laid by blood fed *Ae. aegypti* infected with *M. anisopliae*-131 was 26 ± 3 at the higher dose (10^9 spores/mL), 37 ± 4 at the intermediate dose (10^7 spores/mL), and 57 ± 5 at the lower dose (10^5 spores/mL), which was significantly lower than the control group laying 67 ± 4 eggs (F = 54.19; df = 1.000, 118.00; p < 0.0001). In general, blood fed female *Ae. aegypti* exposed to *M. anisopliae*-30 laid fewer eggs than those exposed to *M. anisopliae*-131, and were significantly different from the control (72 ± 5), with 19 ± 3 eggs laid at the higher dose (10^9 spores/mL), 27 ± 3 at the intermediate dose (10^7 spores/mL),

and 47 ± 4 at the lower dose (10^5 spores/mL). Blood fed females exposed to increasing doses of either of the strains demonstrated a dose-dependent reduction in the number of eggs laid ($F = 104.2$; df = 1.000, 118.0; $p < 0.0001$). All eggs were laid within 5 days post-blood meal.

DISCUSSION

Developing eco-products to control vector mosquitoes and pests are gaining importance in recent times among the scientific communities. The current research was designed to obtain necessary information for the development of a myco-insecticide to control the dengue fever vector mosquito, *Ae. aegypti*. We show that *M. anisoplae* volatiles emanating from dry spore suspensions attract as well as reduce the survival and fecundity rate of female *Ae. aegypti* under laboratory conditions.

Several previous reports have shown the bio-potential of *M. anisopliae* and of other of the entomopathogenic fungi, such as *Lecanicillium, Longisporum* and *Beauveria bassiana*, against adult dengue mosquitoes (Milner *et al.,* 2003; Shah and Pell, 2003; Paula, 2008, Scholte *et al.,* 2005). The efficacy of these entomopathogenic fungi may be both increased or decreased depending upon whether the insects are attracted or repelled, respectively (Cory and Hoover, 2006). In this study, we show that both physiological states were attracted by the volatiles emanating from the two strains of *M. anisopliae*, with *M. anisopliae*-30 generally being the more attractive strain. Similar results have been observed by George *et al.* (2013), who showed that the malaria vector *Anopheles stephensi* is also attracted to the volatiles emanating from the dry spores of *M. anisoplae* on the filter-paper. This indicates that as long as *M. anisopliae* is not repellent, the conidia have a greater opportunity to infect mosquitoes using the attract and contaminate principle (Okumu *et al.,* 2010). To achieve this end, spore-treated cloth (Scholte *et al.,* 2005) and resting boxes (Lwetoijera *et al.,* 2010) can be used, as previously demonstrated for wild free-flying *Anopheles* mosquitoes.

Both fungal strains of *M. anisopliae* significantly reduced the survival of the two physiological states of female *Ae. aegypti*. Whereas the highest dose of the fungal treatment (10^9 spore/mL) did not prevent infected blood fed mosquito from laying eggs, it did reduce the average survival of adults to below the incubation period for the dengue virus, which ranges between 10-14 days (Watts, 1987; Paula *et al.,* 2011; Darbro *et al.,* 2011). Interestingly, this study suggested that the survival rate of blood-fed female *Ae. aegypti* is increased compared to that of non-blood fed following *M. anisopliae* infection. For blood fed mosquitoes, the digested blood meal may provide additional nutrients and a stronger immune response, which is a plausible explanation for the reduced mortality. This hypothesis is in agreement with that of Dana *et al.* (2005). Moreover, the increased mortality rate of non-blood fed mosquitoes may be associated with nutrient depletion but also related to immune strength, mechanical damage, and toxicosis (Ferron 1978; Gillespie and Claydon 1989).

Fungal infection significantly reduced the number of eggs laid as well as changed the behaviour of blood fed mosquitoes. Infected mosquitoes laid fewer eggs than the controls, however these eggs were scattered on the filter paper with other eggs placed in other regions of the plastic pot. This change was dependent on the conidia concentration but not strain, and is in line with that reported by Flores *et al.* (2004). In addition, Scholte *et al.* (2006) observed a reduction in fecundity of *An. gambiae* treated with *M. anisopliae*. This study concludes that both strains of *M. anisopliae* tested against *Ae. aegypti* are effective in reducing survival and egg laying. The development of a myco-insecticide using *M. anisopliae* has therefore a potential alternative eco-product for the management of dengue fever mosquitoes.

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REFERENCES

Blanford S., Chan B.H., Jenkins N., Sim D., Turner R.J., Read A.F. and Thomas M.B. (2005) Fungal pathogen reduces potential for malaria transmission. Science 308: 1638-1641.

Blanford S., Jenkins N.E., Read A.F. and Thomas M.B. (2012) Evaluating the lethal and pre-lethal effects of a range of fungi against adult *Anopheles stephensi* mosquitoes. Malaria Journal 11: 365.

Blanford S., Shi W., Riann C., Marden J.H., Koekemoer L.L., Brooke B.D., Coetzee M., Read A.F. and Thomas M.B. (2011) Lethal and pre-lethal effects of a fungal biopesticide contribute to substantial and rapid vector control. PLoS ONE 6: e23591.

Bowen M.F., Davis E.E., Haggart D. and Romo J. (1994) Host-seeking behavior in the autogenous mosquito *Aedes atropalpus*. Journal of Insect. Physiology 40(6): 511-517.

Chakravarti A., Arora R. and Luxemburger C. (2011) Fifty years of dengue in India. Transactions of the Royal Society of Tropical Medicine and Hygiene 106: 273-285.

Chareonsook O., Foy H.M. Teerarakul A. and Silarug N. (1996) Changing epidemiology of dengue hemorrhagic fever in Thailand. Epidemiology and Infection 122: 161-166.

Cory J. and Hoover K. (2006) Plant mediated effects in insect-pathogen interactions. Trends in Ecology and Evolution 21(5): 278-286.

Dana A.N., Hong Y.S., Kern M.K., Hillenmeyer M.E., Harker B.W., Lobo N.F., Hogan J.R., Romans P. and Collins F.H. (2005) Gene expression patterns associated with blood-feeding in the malaria mosquito *Anopheles gambiae*. BMC Genomics 6: 5.

Darbro J., Graham R.J., Kay B.H., Ryan P.A. and Thomas M.B. (2011) Evaluation of entomopathogenic fungi as potential biological control agents of the dengue mosquito, *Aedes aegypti* (Diptera: Culicidae). Biocontrol Science and Technology 21: 1027-1047.

Elandt-Johnson R. and Johnson N.L. (2009) Survival models and data analysis. John Wiley and Sons, New York.

EPA (2003) Environmental Protection Agency website http://www.epa.gov/oppbppd1/biopesticide.

Farenhorst M., Knols B.G.J. and Thomas M.B. (2010) Synergy in efficacy of fungal entomopathogens and permethrin against west african insecticide-resistant *Anopheles gambiae* mosquitoes. PLoS ONE 5(8): e12081.

Farenhorst M. Mouatcho J.C. Kikankie C.K. Brooke B.D. Hunt R.H. Thomas M.B. Koekemoer L.L. Knols B.G. and Coatzee M. (2009) Fungal infection counters insecticide resistance in African malaria mosquitoes. Proceedings of the Natural Academy of Science, USA. 106(41): 17443-17447.

Ferron P. (1978) Biological control of insect pests by entomopathogenic fungi. Annual Review of Entomology 23: 409-442.

Flores A.E., Garcia G.P., Badil M.H., Tovar L.R. and Salas I.F. (2004) Effects of sublethal concentrations of vectovac® on biological parameters of *Aedes aegypti*. Journal of American Mosquito Control Association 20: 412–417.

George J., Blanford S., Domingue M.J., Thomas M.B., Read A.F. and Baker T.C. (2011) Reduction in host-finding behaviour in fungus-infected mosquitoes is correlated with reduction in olfactory receptor neuron responsiveness. Malaria Journal 10: 219.

George J., Jenkins N.E., Blanford S., Thomas M.B. and Baker T.C. (2013) Malaria mosquitoes attracted by fatal fungus. PLoS ONE 8(5): e62632.

Ghosh A., Chowdhury N. and Chandra G. (2012) Plant extracts as potential mosquito larvicides. Indian Journal of Medical Research 135: 581-598.

Gillespie A.T. and Claydon N. (1989) The use of entomogenous fungi for pest control and the role of toxins in pathogenesis. Pesticide Science 27: 203-215.

Heddini A., Janzon R. and Linde A. (2007) Increased number of dengue cases in Swedish travellers to Thailand. Journal of Infectious Diseases 195: 1089-1096.

Howard A.F.V., Koenraadt C.J., Farenhorst M., Knols B.G. and Takken W. (2010) Pyrethroid resistance in *Anopheles gambiae* leads to increased susceptibility to the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*. Malaria Journal 9: 156-168.

Kundu M., Rawani A. and Chandra G. (2013) Evaluation of mosquito larvicidal activities of seed coat extract of *Cassia sophera* L. Journal of Mosquito Research 3: 76-81.

Lwetoijera D.W.L., Sumaye R.D., Madumla E.P., Kavishe D.R. and Mnyone L.L. (2010) An extra-domiciliary method of delivering entomopathogenic fungus, *Metharizium anisopliae* IP 46 for controlling adult populations of the malaria vector, *Anopheles arabiensis*. Parasites and Vectors 3: 1-18.

Mburu D.M., Ndung'u M.W., Maniania N.K. and Hassanali A. (2011) Comparison of volatile blends

and gene sequences of two isolates of *Metarhizium anisopliae* of different virulence and repellency toward the termite *Macrotermes michaelseni*. Journal of Experimental Biology 214: 956-962.

Milner R.J., Samson P. and Morton R. (2003) Persistence of conidia of *Metarhizium anisopliae* in sugarcane fields: effects of isolate and formulation on persistence over 3.5 years. Biocontrol Science and Technology13: 507-516.

Mnyone L.L., Koenraadt C.J., Lyimo I.N., Mpingwa M.W., Takken W. and Russell T.L. (2010) Anopheline and culicine mosquitoes are not repelled by surfaces treated with the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana.* Parasites and Vectors 3(1): 80.

Nagi A.G., Murad R. and Baig M. (2011) Dengue fever outbreak among children in Karachi: experience at a tertiary care children hospital. The Journal of Bahria University Medicine Dental College 1(2): 44-48.

Okumu F.O., Killeen G.F., Ogoma S., Biswaro L., Smallegange R.C., Mbeyela E., Titus E., Munk C., Ngonyani H., Takken W., Mshinda H., Mukabana W.R. and Moore S.J. (2010) Development and Field Evaluation of a synthetic mosquito lure that is more attractive than humans. PLoS ONE 5: e8951.

Paula A.R., Brito E.S., Pereira C.R., Carrera M.P. and Samuels R.I. (2008) Susceptibility of adult *Aedes aegypti* (Diptera: Culicidae) to infection by *Metarhizium anisopliae* and *Beauveria bassiana*: prospects for dengue vector control. Biocontrol Science and Technology 18:1017-1025.

Paula A.R., Carolino A.T., Silva C.P. and Samuels R.I. (2011) Susceptibility of adult female *Aedes aegypti* (Diptera: Culicidae) to the entomopathogenic fungus *Metarhizium anisopliae* is modified following blood feeding. Parasites and Vectors 4: 91.

Poopathi S. and Tyagi B.K. (2006) The challenge of mosquito control strategies; from primordial to molecular approaches. Biotechnology Molecular Biology Review 1(2): 51-65.

Read A.F. Lynch P.A. and Thomas M.B. (2009) How to make evolution-proof insecticides for malaria control. PLoS Biology 7(4): 1-10.

Roy H.E., Steinkraus D.C. Eilenberg J. Hajek A.E. and Pell J.K. (2006) Bizarre interactions and endgames: entomopathogenic fungi and their arthropod hosts. Annual Review of Entomology 51: 331-357.

Scholte E.J., Bart J.K., Robert A.S. and Willem T. (2004) Entomopathogenic fungi for mosquito control: a review. Journal of Insect Science 4(19): 1-24.

Scholte E.J., Knols B.G. and Takken W. (2006) Infection of the malaria mosquito *Anopheles gambiae* with the entomopathogenic fungus *Metarhizium anisopliae* reduces blood feeding and fecundity. Journal of Invertebrate Pathology 91: 43-49.

Scholte E.J., Ng'habi K., Kihonda J., Takken W., Paaijmans K., Abdulla S., Killeen G.F., and Knols B.G. (2005) An entomopathogenic fungus for control of adult African malaria mosquitoes. Science 308: 1641-1642.

Scholte E.J., Takken W. and Knols B.G.J. (2007) Infection of adults *Aedes aegypti* and *Ae. albopictus* mosquitoes with the entomopathogenic fungus *Metarhizium anisopliae*. Acta Tropica 102: 151-158.

Scholte E-J., Njiru B.N., Smallegange R.C., Takken W. and Knols B.G.J. (2003) Infection of malaria (*Anopheles gambiae S.S.*) and filariasis (*Culex quinquefasciatus*) vectors with the entomopathogenic fungus *Metarhizium anisopliae*. Malaria Journal 2: 1-29.

Scott T.W., Naksathit A., Day J.F., Kittayapong P. and Edman J.D. (1997) A fitness advantage for *Aedes aegypti* and the viruses it transmits when females feed only on human blood. American Journal of Tropical Medicine and Hygiene 57: 235-239.

Shah P.A. and Pell J.K. (2003) Entomopathogenic fungi as biological control agents. Application of Microbiology and Biotechnology 61: 413-423.

Stevenson J.C. (2008) The use of fungi against adult malaria mosquitoes. In Ph.D Thesis. London School of Hygiene and Tropical Medicine, London.

Van Ark H. (1995) Introduction to the analysis of quantal response data. Agrimetrics Institute, Agricultural Research Council, Pretoria, South Africa.

Watts D.M. (1987) Effect of temperature on the vector efficiency of *Aedes aegypti* for dengue virus. American Journal of Tropical Medicine and. Hygiene 36: 143-152.

WHO (2009) Dengue guidelines for diagnosis, treatment, prevention and control WHO/HTM/NTD/DEN

Zimmermann G. (2007) Review on the safety of the entomopathogenic fungus *Metarhizium anisopliae*. Biocontrol Science and Technology 17(9): 879-920.

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