EXPERIMENTAL INFECTION OF MALARIA MOSQUITOES WITH THE ENTOMOPATHOGENIC FUNGUS, *BEAUVERIA BASSIANA* UNDER FIELD CONDITIONS

By

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DECLARATION

I, Busula Obukosia Annette, declare that the work presented herein is my original work and has not been presented for award of any degree anywhere.

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We confirm that the candidate under our supervision performed the work presented in this thesis.

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DEDICATION

To the family of Mr. and Mrs. Benn B. M. Shihugwa (Maxwell, David, Bilha, Isaac and Solomon).

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LIST OF ACRONYMS AND ABBREVIATIONS

WHO	World Health Organization
MOH	Ministry of Health
ITNs	Insecticide Treated bed-Nets
IRS	Indoor Residual Spraying
NIB	National Irrigation board
ACTs	Artemisinin Combination Therapies
DDT	Dichlorodiphenyltrichloroethane
GHA	Global Healthcare Association
RH	Relative Humidity
AMDTI	Ahero Multipurpose Development and Training Institute
ICIPE	International Centre for Insect Physiology and Ecology
KEMRI	Kenya Medical Research Institute
SE	Standard Error
HR	Hazard ratio
CI	Confidence Interval
sl	Sensu lato

ABSTRACT

Many successful laboratory studies have demonstrated the potential of using the entomopathogenic fungus, *Beauveria bassiana* against malaria mosquitoes. This study focussed on infection of wild malaria mosquitoes with the entomopathogenic fungus *B. bassiana* under field conditions. Four experiments were carried out under laboratory conditions by use of World Health Organization bioassay cones, for exposure of 3-6 days old, non-blood fed laboratory-reared *Anopheles arabiensis* mosquitoes on 100% black cotton cloths impregnated with spores of *B. bassiana* : from stocks of i) 80% viability for 10-70 minutes ii) 78, 51 and 50% including decayed spores of initially 80% viability iii) and 35% cotton cloths to determine virulence of fungus 3, 7, 14, 21, 28, 35 and 42 days post application. Two field studies were carried out in three experimental huts located near the Ahero substation of the National Irrigation Board (NIB) (0° 08' S, 34° 55' E), Kisumu County in Western Kenya to find out whether an increase in surface area of fungus-treated cloth panel led to an increase in fungus infection rates in wild malaria mosquitoes, and to determine efficacy of indoor wall spraying technique by use of *B. bassiana* formulated in oil, against wild malaria mosquitoes. Results showed that 10 minutes were sufficient for *B. bassiana* to infect and kill *An. arabiensis* within 14 days. Further, results indicated that the risk of dying for mosquitoes was higher, 5.47 and 6.28 times when spores from stocks of 80 and 78%, respectively, were used, than when spores from stocks of 51 and 50% were used (Hazard Ratio 80%: HR=5.47 [95% CI: 3.13 – 9.54] Hazard Ratio 78%: HR=6.28 [95% CI: 3.66 – 10.79] Hazard Ratio 51%: HR=1.04 [95% CI: [0.62 - 0.62] Hazard Ratio 50%: HR=1.27 [95% CI: [0.76 - 2.12], respectively. Conidia were infective up to 28 and 7 days post impregnation on 100 and 35% cotton cloths respectively with an overall fungus infection of 337 (60%) and 161 (40%), respectively.

Results from field trials show that conidia from one piece and two pieces of fungus-treated cloth panels were equally effective in killing wild malaria mosquitoes, with 30% and 35% (p=0.203) infection rates, respectively. Additionally, 39% of wild malaria mosquitoes were infected with conidia following indoor wall spraying with *B. bassiana*, and the infection rate of 39% was not significantly different from 30 and 35% infection rates (p=0.07). In conclusion, these findings reveal that 10 minutes are sufficient to kill *An. arabiensis* within 14 days by use of *B. bassiana*-treated cloths, and fungal spores whose viability is 78% and above are infective to mosquitoes only when impregnated on 100% black cotton cloth. Additionally, increase in surface area of *B. bassiana*-impregnated cloth does not lead to increase in fungus infection rates in wild malaria mosquitoes, and spraying of indoor walls of a hut with *B. bassiana* is not an effective technique in infection of wild malaria mosquitoes.

CHAPTER ONE : INTRODUCTION

Malaria is the world's most prevalent vector borne disease (Philips, 2001; Kimani *et al.*, 2006) and a major public health problem (Breman *et al.*, 2011). It is caused by protozoan parasites of the genus *Plasmodium* and is transmitted through bites of female mosquitoes belonging to the genus *Anopheles*. Malaria parasite species that infect humans are *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium Ovale* and *Plasmodium knowlesi*. *Plasmodium falciparum* is considered the most dangerous parasite as it can cause severe disease and fatal complications, including liver failure, cerebral disease and coma (Alonso *et al.*, 2011).

Females of *Anopheles gambiae* sensu lato (*An. gambiae* and *An. arabiensis*) and *Anopheles funestus* are the principal vectors of malaria in most African countries (Gillies and De Meillon, 1968; Coetzee, 2004; Okara *et al.*, 2010). *Plasmodium* parasites undergo sexual reproduction inside the mosquito and need to develop in the insect for several days, generally 10-14 days (Koella *et al.*, 2009). To be capable of transmitting malaria, a mosquito must therefore survive for longer than the extrinsic incubation period of the pathogen (Beier, 1998). Malaria transmission can be blocked by shortening the life span of mosquitoes (MalERa C group, 2011).

Each year, over one million deaths from the direct effects of the disease occur in the African continent (World Bank, 2007) and it is regarded as the leading cause of morbidity and mortality in the Sub-Saharan region (Day 2005). In 2009, there were 3.28 billion people living in areas that have some risk of malaria transmission and 1.2 billion people living in areas with high risk of transmission (WHO, 2010). About 225 million cases of clinical

malaria and 781, 000 malaria related deaths were reported in 2009 (Alonso *et al.*, 2011). In the year 2010, there were 696 290 014 people at risk of malaria infection, 68 925 435 confirmed malaria cases and 111 885 malaria attributed deaths in the African continent (WHO, 2010). These mainly occur in children below five years old and pregnant women (Greenwood and Mutabingwa, 2002; WHO, 2009; 2010).

A report by Kenya Ministry of Health. (2001-2010) states that malaria is the leading cause of morbidity and mortality (in Kenya), with close to 70 percent (24 million) of the population at risk of infection. Kenya Demographic and Health Survey (2008-2009) report that although malaria affects people of all age groups, children under five years of age and pregnant women living in malaria endemic regions are most vulnerable. The report further reveals that the disease becomes a self-perpetuating problem, where it prevents growth of the human and economic capital necessary to bring the disease under control.

Control of malaria mosquitoes relies mainly on indoor residual spraying (IRS) and use of insecticide-treated bed nets (ITNs) while control of *Plasmodium* parasites is by use of antimalarial medicine such as Artemisinin and its derivatives (Roll Back Malaria, 2005; Nauen, 2007; Chandra *et al.*, 2008; Enayati *et al.*, 2010). However, sustainable use of chemicals is undermined by the following problems: resistance in parasite and adult mosquito populations, environmental contamination and risks to human health (Hargreaves *et al.*, 2003; Kikankie *et al.*, 2007; N'Guessan *et al.*, 2007) hence, threatening malaria control (Zucker *et al.*, 2003; WHO, 2009). These problems therefore have increased the interest in alternative and integrated implementation of vector control strategies to include biological control (Takken and Knols, 2009).

Although several effective biological larvicides exist (Fillinger *et al.*, 2003) there have been no biological agents effective against adult mosquitoes. To address this gap, several laboratory and small scale field studies have demonstrated the potential of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* to infect and kill adults of *Anopheles*, Aedes and Culicine mosquitoes (Farenhorst *et al.*, 2009). A study that involved trials with cotton sheets impregnated with fungal spores and suspended in some rural Tanzanian houses showed that mosquitoes rested on the sheets and were infected with fungus. According to Scholte *et al* (2005), survival of mosquitoes decreased due to infection with the entomopathogenic fungi.

Furthermore, they estimated the number of infective mosquito bites per person per year and found that there was a drop from 262 bites to 64 bites. Increasing the coverage of mosquito resting sites could bring this number down to ten (Michalakis and Renaud, 2005). Additionally, theoretical studies have demonstrated that the relatively slow speed of kill of entomopathogenic fungi can be sufficient to impact on malaria transmission since the extrinsic incubation period of the malaria parasite within the mosquito (typically 10-14 days in high transmission settings) creates a window of several days for the fungus to act (Hancock, 2009; Koella *et al.*, 2009).

A successful control of malaria vectors using the entomopathogenic fungus ensures that the host or mosquito contacts a treated surface and receives a sufficient dose of infectious conidia when doing so (Hughes *et al.,* 2004), otherwise the vectors might escape the negative effects of fungal infection (Hancock, 2009). In most studies, fungal infections have been induced by exposure of mosquitoes to various surfaces treated with conidia (Garcia-Munguia *et al.*, 2011). Delivery tools are essential factors to be considered before entomopathogenic fungus can be integrated into a control programme. These could be used to target either host-seeking

and or resting mosquitoes. Most houses in Africa are built with open eaves to allow flow of air within houses. However, it is through these eaves that mosquitoes enter houses (Charlwood *et al.*, 2003; Njie *et al.*, 2009).

Host-seeking mosquitoes could thus be targeted when entering a house through the eaves (Njie *et al.*, 2009) or when attacking a host under a bed net (Hancock, 2009). This study was designed in order to find a suitable tool that ensures maximum infection of wild adult malaria mosquitoes with the entomopathogenic fungus. Field trials were conducted in three experimental huts which were built near the Ahero substation of the National Irrigation Board (NIB), Kisumu County, western Kenya, whereas other experiments were carried out in a field insectary, located 100 metres from the huts.

CHAPTER TWO: LITERATURE REVIEW

2.1. Transmission of Malaria

Malaria is a complex disease and its severity is a function of the interaction between the *Anopheles* mosquitoes, the parasite, the hosts and the environment. Malaria is caused by protozoan parasites of the genus *Plasmodium* namely, *P. falciparum*, *P. vivax, P. malariae* and *P. ovale*. A fifth human parasite *P. knowlesi* was discovered with an animal reservoir (Singh *et al.*, 2004; Cox-Singh *et al.*, 2008; Luchavez *et al.*, 2008). *Plasmodium* parasites are transmitted through bites of female adult mosquitoes belonging to the genus *Anopheles* (Diptera: Culicidae). Principal malaria vectors in most African countries are *An. gambiae*, *An. arabiensis* and *An. funestus*. Their dominance as malaria vectors is largely due to preference for human blood, high vector competence and high daily survival rates. *Anopheles gambiae* and *An. arabiensis* are sympatric; however, *An. arabiensis* is more widely distributed in drier areas whereas *An. gambiae* prefers humid areas (Gillies and Coetzee, 1987; Toure *et al.*, 1994; Okara *et al.*, 2010).

2.1.1. Behaviour of adult mosquitoes

Anopheles gambiae and *An. funestus* are anthropophilic, biting almost exclusively humans (Constantini *et al.*, 1999) although in West Africa, *An. gambiae* are less discriminating and will feed readily on other animals like horses and cattle (Diatta *et al.*, 1998; Bogh *et al.*, 2001). Seasonal changes in *An. gambiae* populations tend to follow the seasonal pattern of rainfall (Gillies and De Meillon, 1968). *Anopheles funestus* is highly anthropophilic and endophilic making it susceptible to control by indoor residual spraying (Gillies and De Meillon, 1968). *Anopheles arabiensis* on the other hand have a wide distribution in Africa. Their range and relative abundance tend to be influenced by climatic factors, especially total

annual precipitation. They tend to be more tolerant to high temperatures and are able to survive in drier conditions, which explains why they are found biting during the dry season (Coetzee *et al.*, 2000). *Anopheles arabiensis* may feed on humans as well as on animals, depending on the availability of both hosts (Gilles and Coetzee 1987; Constantini *et al.*, 1999; Tirados *et al.*, 2006).

Survival and reproductive success of mosquitoes depend on a series of characteristic behaviours such as mating, foraging and oviposition (Takken and Knols, 1999). These behaviours are governed by internal and external factors to which the mosquitoes respond to in a particular manner. To locate food and oviposition sites successfully, insects must integrate visual and olfactory cues. Interactions between olfactory and visual cues guide the insects during this search behaviour (Frye, 2003). Vision is considered more important in diurnally active mosquitoes while physical and olfactory cues are important for nocturnal species (Takken and Knols, 1999).

Dispersal in insects fits five primary needs: finding a resting site, finding a mate, finding a nectar source, finding a host and finding an oviposition site. Mosquitoes mate within 24 – 48 hours after adult emergence. Mating occurs once in the entire life of most mosquito species. However, multiple mating has been reported in some *Anopheles* females (Tripet *et al.*, 2003). In *Anopheles* species, males form swarms and females entering the swarms are recognized by their lower wing-beat frequency (Brogdon, 1998).

Sugar is the basic food of adult mosquitoes, and it is the only nutrient consumed by male mosquitoes (Foster W.A, 1995). The sugar meal provides energy for female mosquitoes during host seeking flight and enhances reproduction (Foster W.A, 1995). Mosquitoes are believed to locate sugar sources by odours emitted by flowering plants (Foster and Takken,

2004). Female mosquitoes require a blood meal for their gonotrophic development. Each blood meal leads to the development of a batch of eggs. In most species, females take blood after mating while in others species, blood feeding precedes mating (Charlewood *et al.*, 2003). Host-seeking behaviour of malaria vectors is influenced by human odour; emanations from skin, sweat and breath greatly influence this behaviour (Constantini *et al.*, 1999). Physical cues such as body heat and temperature also play a role in mosquito host seeking behaviour.

2.1.2. Tools to interrupt transmission of malaria

Measures against malaria can either target *Plasmodium* parasites or adult malaria vectors, *An. gambiae, An. arabiensis* and *An. funestus*.

2.1.2.1 Antimalarial drugs and vaccines

Antimalarial drugs play an important role in treating and controlling malaria. In the past five years, treatment of uncomplicated malaria had transformed to the use of artemisinin combination therapies (ACTs) (World Health Report, 2009). Efficacy of antimalarial drugs is however undermined by the emergence of drug resistant strains of *Plasmodium* parasites (Ntoumi, 2004; WHO, 2010). The highly effective artemisinin derivatives and their partner drugs are vulnerable to the same risk (Talisuna *et al.*, 2004; Dondorp *et al.*, 2010). Next to antimalarial drug use, development of vaccines is underway. Despite good progress with certain candidate vaccines (Abdulla *et al.*, 2008), there is still no effective vaccine for malaria available to date (Winzeler, 2008).

2.1.2.2 Use of insecticides against adult malaria vectors

The objectives of malaria vector control are two-fold: to protect people against infective malaria mosquito bites by reducing vector longevity, vector density and human-vector contact and to reduce the intensity of local malaria transmission at community level and hence the incidence and prevalence of infection and disease (WHO, 2010). There are four classes of insecticides approved for use in public health interventions namely, Carbamates, organophosphates, pyrethroids and organochlorides (WHO, 2010).

Pyrethroids and an organochloride namely, Dichlorodiphenyltrichloroethane (DDT) are the most important and widely used insecticides against malaria vectors (Nauen, 2007). Insecticides act on the insect's central nervous system by blocking neuronal activity thus causing rapid paralysis and death. Resistance to these insecticides has been reported in key vector species such as *An. gambiae* (Martinez *et al.,* 1998; Diabate *et al.*, 2004; Etang *et al.*, 2006; Ransom *et al.*, 2009; Yadouleton *et al.*, 2010), *An. funestus* (Brooke *et al.*, 2001; Wondji *et al.*, 2009) and *An. arabiensis* (Hargreaves *et al.*, 2003; Abdalla *et al.*, 2007). Resistance can be as a result of enhanced metabolic degradation of the insecticide by specific enzymes (Hunt *et al.*, 2005).

Hunt *et al* (2011) confirmed that resistance to one member of a class of insecticides is good evidence that resistance to other chemicals in the same class will occur. The occurrence of insecticide resistance in insect disease causing vectors and even in agricultural pest species poses potential and actual hindrances to successful insect control (Corbel *et al.*, 2007). Pates and Curtis (2005) have shown that mosquitoes can select for certain behaviorally resistant traits, such as earlier mosquito feeding times and earlier exiting from houses with treated nets. The most recent data clearly indicate that insecticide resistance is widespread and often

at very high frequencies, usually sufficiently high to preclude the use of the few insecticides approved by WHO for malaria control (Hunt *et al.*, 2011). These problems have increased interest in alternative and integrated implementation of vector control methods that include biological control.

There are at present no effective biological control tools for adult mosquitoes. Methodologies that target the adult stage of mosquitoes continue to depend exclusively on the use of chemical insecticides (Chandra *et al.*, 2008), whose continual effectiveness is threatened by mosquitoes developing resistance to the synthetic insecticides (Ransom *et al.,* 2009). Combined with safety and environmental concerns of insecticide use (Cohn *et al.*, 2003; Cohn *et al.*, 2007; Roberts, 2001; Smith, 2000), there is need to develop novel malaria control strategies that can reliably and sustainably be used to complement or replace the existing control measures.

2.2. Entomopathogenic fungi for control of malaria mosquitoes

Entomopathogenic fungi are defined as those fungi which have been shown to be pathogens of insects or cause some level of harm within insects (Samson *et al.*, 1988). The longevity of the adult mosquito has the greatest impact on the vectorial capacity, and transmission intensity of a mosquito population. This can be targeted using biocontrol agents, which ensure that mosquito resistance does not readily develop due to the slow speed of kill (Thomas and Read 2007a, Read *et al.*, 2009). Biopesticides that can therefore be used to control mosquitoes and other insect pests include plants, fish, nematodes, viral, bacterial, protozoan and fungal pathogens (Scholte *et al.*, 2004a). Of these, Hyphomycetous entomopathogenic fungi are the most well suited for development as biopesticides against

vector mosquitoes (Thomas and Read, 2007; Read *et al.*, 2009) and have been evaluated for mosquito control purposes.

Classification of fungi

Fungi pathogenic to insects are found in the following taxonomic groups: Chytridiomycota, Zygomycota, and Deuteromycetes (Hyphomycetes) (Kirk *et al.*, 2001). Eighty-five genera and over 750 species of entomopathogenic fungi are known. Two genera, *Beauveria* and *Metarhizium* (which belong to the class Hyphomycetes in Deuteromycota) and 12 species of fungi are being used as ingredient for 'myco-insecticides' or 'myco-acaricides' (DeFaria and Wraight., 2007). The most used species are *M. anisopliae* and *B. bassiana.* These two species have a worldwide distribution, occurring naturally in soils (Scholte *et al.*, 2004a).

2.2.1. Safety and environmental effects of entomopathogenic fungi

The application of fungal spores has raised concerns over the potential health risks especially when applied indoors (Kanzok and Jacobs-Lorena, 2006; Ward & Selgrade, 2005). At present, this risk is considered low due to the opportunistic nature of the pathogen, inability to survive at human body temperatures and formulation of conidia in mineral oil (such as Ondina, Shellsol and Enerpar currently known as Castrol WOM 14 oil. Oil may give increased spore contact and adhesion to an insect's cuticle (Lomer *et al.*, 2001), Suspension of conidia in oil allows effective spore application in extremely arid conditions e.g. in the Sahel (Langewald *et al.*, 1999) by providing essential moisture for the developing fungus (Lomer *et al.*, 2001; Prior and Greathead, 1989). Use of oil also maintains viability of stored fungi above that of dry conidia (Batta, 2003) and at high temperatures (Thomas *et al.*, 1995). Oil formulations may also improve spore survival by enhancing tolerance to UV irradiation (Moore *et al.*, 1993, Alves *et al.*, 1998). These may indicate that different oils can 'extract' or

dissolve different components from the cuticle to promote fungal growth, and this may be another consideration in formulation choice.

In recent laboratory studies, two *Beauveria* species (*B. bassiana* and *B. brongniartii*) were found in surface- drinking water in Norway but not in underground water (Hageskal *et al.*, 2006). Airborne spores of common fungi such as *Aspergillus species, Penicillium species* and *Fusarium* species are abundant indoors and exposure to fungal spores is commonplace both in tropical and temperate climates (Simon-Nobbe *et al.*, 2008). It is therefore thought that applications of *Beauveria* or *Metarhizium* spores indoors would not significantly increase exposure levels (Thomas *et al.*, 2005). *Beauveria bassiana* was cultured from corneal scrapings from a male who had suffered abrasion to the eye (Sachs *et al.*, 1985).

No adverse effects, infection or distress was reported following inhalation, oral or eye exposure, or following subcutaneous or intraperitoneal injections of rats, mice and rabbits with either dry spores or conidial suspensions of *M. anisopliae* or *B. bassiana* (e.g. Shadduck *et al.*, 1982; Zimmermann, 2007). Within treated chicken houses in the USA, *B. bassiana* had no adverse effects on house fly pupal parasitoids nor coleopteran predators (Kaufman *et al.*, 2005). Potential allergens have been identified in some fungi (Ward *et al.*, 1998; Westwood *et al.* ,2006,) but there are no reports of asthmatic conditions amongst workers applying fungi (Roberts, 1977), nor any reports of harmful effects in those who have used *M. anisopliae* and *B. bassiana* for indoor pest control

Based on toxicity tests of *B. bassiana* strain GHA in various animals, this fungus poses no risk to humans and the use of this fungus does not result in toxin levels harmful to the environment (Strassser *et al.*, 2000; US Environmental Protection Agency, 2006). *Beauveria bassiana* has been identified as a medically important fungal pathogen on recipients. For

example, its extracts have anticoagulant and immune system modulating activities, which could provide beneficial physiological activities for humans (Yoon *et al.*, 2003b). This fungus could also be used as an additive to wheat flour for preparation of bread and noodle (Yoon *et al.*, 2003a). *Beauveria bassiana* itself or fungus diseased larvae of *Bombyx mori* have been used as medicine for hundreds of years in China.

Advantages of using *B. bassiana* and *M. anisopliae* fungi

These fungi are host specific, can be cost-effectively mass-produced even locally, they can readily live as saprophytes in nature, and do not require ingestion by the host (Michalakis and Renaud, 2005, Zimmermann 2007a, b). The effect of *B. bassiana* and *M. anisopliae* on mosquitoes is not immediate; they infect and kill adult mosquitoes slowly through external contact (Blanford *et al.*, 2005; Scholte *et al.*, 2007). Compared to insecticides, fungi have low virulence as they kill an insect in 6-14 days after infection depending on the fungal species and isolate used. The slow killing mechanism of the fungus imposes a limited selection pressure on the mosquitoes, thus reducing the likelihood of anti-fungal resistance (Knols and Thomas 2006; Thomas and Read 2007a).

Fungal spores of *B. bassiana*, whose viability is high, are of good quality in terms of persistence and virulence compared to *M. anisopliae*. This makes it highly competitive and an attractive alternative (Mnyone *et al.*, 2009; Howard *et al.*, 2010). Results of experiments carried out in the laboratory using *Anopheles* mosquitoes show that an insecticide-resistant mosquito infected with *B. bassiana* and *M. anisopliae* becomes sensitive again to the insecticides (Farenhorst *et al.*, 2009). Therefore, there is a synergistic effect in using both the fungi and indoor residual spraying (IRS) with insecticides.

2.2.2. The fungal infection process

The host cuticle is the first line of defense against infection and has a central role in determining fungal infectivity. The entomopathogenic fungi infect mosquitoes through direct contact with the cuticle. The fungi penetrate thinner, non-sclerotised areas of the cuticle, like joints, between segments, or the mouthparts, and through tarsal contact. First, conidia (spores) adhere to the insect cuticle (see Figure 1) by use of adhesive mucus secreted as the conidium swells during the pre-germination period (Boucius and Pendland, 1998). Conidia then germinate on the insect cuticle (Khachatorians, 1991). Before penetration, germ tubes produce appressoria (penetration structure; see Figure 1) and infection pegs. The cuticle is multilayered and tough but fungus is able to surmount this obstacle by mechanical means and by action of cuticle-degrading enzymes which include proteases, chitinases and lipases (Hajek and St.Leger, 1994; Boucius and Pendland, 1998).

Fungus grows by vegetative growth in the insect haemocoel. They absorb and deplete nutrients and the fat body and finally release toxins which lead to destruction of insect cells (Clarkson and Chamley 1996). The blastospores (yeast-like cells as shown in Figure 1) are responsible for circulation within the insect haemolymph and toxin production (Shah and Pell, 2003). Granulocyte numbers dramatically reduce three days after fungal challenge (Hajek and St.Leger, 1994). Infected mosquitoes die before they have a chance to mature and pass on the malaria parasite. After the insect's death, the fungus emerges from the dead host and sporulation or conidiogenesis occurs on the outside of the cadaver (Shah and Pell, 2003). Fungus that grows on the outside of the insect produces aerial conidial spores that can be harvested and recycled (Eyal *et al.*, 1994).

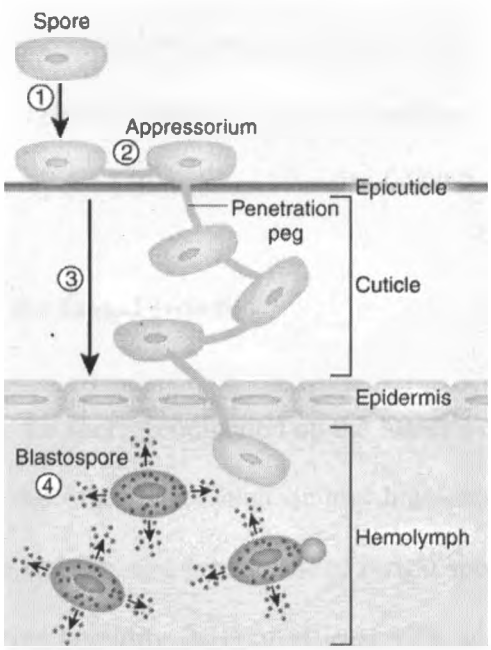

Figure 1: Mode of action of entomopathogenic fungi on contact with the arthropod host (source: Thomas and Read 2007b)

2.2.3. Conditions favorable for fungal growth

For microorganisms to propagate and survive in the environment, certain abiotic factors should be considered. The most important factors are temperature and humidity or moisture content. Each of these factors is as discussed below:

2.2.3.1 Effect of temperature on fungal growth

This influences germination, growth and viability of the fungus on and in the host insect and in the environment. High temperatures may inactivate an entomopathogen before contact with the insect or may reduce or accelerate growth within an insect. In contrast, low temperatures may reduce or stop germination and growth, thus impair or prolong a successful infection. Therefore, the optimal growth temperature for most isolates is 27-28°C (Ferron, 1981; Tefera and Pringle, 2003), although some exceptions of cold-resistant and heat-resistant isolates have been reported (Boucias and Pendland, 1998).

2.2.3.2 Relative humidity for fungal growth

High moisture is necessary for spore germination on the insect's cuticle and sporulation after death of a mosquito. On the other hand, high or low humidity in conjunction with high temperature may affect the viability and persistence of fungal spores. Generally, germination of conidia requires a relative humidity (RH) of at least 97% and they survive longest at a combination of moderate temperatures and high relative humidity (26°C-97% RH or 19°C-97% RH) or low temperatures and low relative humidity (4°C-0% RH). When relative humidity is high, conidia can be quite tolerant to high temperatures although its viability decreases rapidly when exposed to UV light (Hallsworth and Magan, 1999).

2.2.4. Effects of fungi on adult mosquitoes

The sub-lethal fungus infections might possibly reduce both the probability of a female mosquito engaging in host-seeking behaviour and blood feeding, and it might also reduce the probability of a gravid female searching for, locate, and reaching a suitable oviposition site. In the case of malaria epidemiology, a reduction in the number of bites per individual mosquito will reduce the risk of malaria transmission (Scholte *et al.*, 2006).

Fungi have a direct effect on adult mosquitoes and development of *Plasmodium* in mosquitoes (Blanford *et al.* 2005). This was demonstrated in a laboratory experiment using mouse malaria as a model system. Blanford and colleagues observed that only 8% of mosquitoes infected with both the parasite and fungi contained transmissible parasite offspring 14 days after exposure to fungi, compared with 35% of mosquitoes infected with *Plasmodium* alone (Blanford *et al.* 2005). Combining the effects on mosquitoes and *Plasmodium* development, fungi could reduce malaria transmission by approximately 80-fold. The effect might even be greater, given that
fungal infection also decreases the propensity of infected females to feed on blood (Blanford *et al.*, 2005).

Laboratory trials (Farenhorst *et al.*, 2008; Mnyone *et al.*, 2009) and small scale field trials (Scholte *et al.*, 2005; Lwetoijera *et al.*, 2010) show that malaria vectors are susceptible to entomopathogenic fungal infection. Successful infection of adult mosquitoes with the entomopathogens depends on conidial formulation, the dose, exposure time and the fungal strain used (Blanford *et al.*, 2005; Thomas and Read, 2007). Previous studies have used many different combinations of formulations or substrate to demonstrate the effectiveness of the

entomopathogenic fungus to infect and kill mosquitoes (Howard *et al.*, 2010). However, not all of the application methods previously used can be deployed easily in the field. It is therefore important to test potential methods that can be used to disseminate fungus to large populations of wild mosquitoes in the field.

Laboratory experiments by Scholte *et al.* (2005) demonstrated the potential of oil-based formulations of fungal entomopathogens to reduce malaria transmission; this alters the survival or maturation of *Plasmodium* in mosquitoes. Oil-formulated conidia are more efficacious than water-formulated conidia. Therefore, conidia are formulated in oil in order to avoid the repelling effect of conidia towards adult mosquitoes (Mnyone *et al.*, 2010), because oil offers better adhesion and spreading of the formulation on the insect cuticle. Furthermore, the oil film formed on the insect cuticle creates good conditions for fungal invasion and germination in the host (Luz and Batagin, 2005). Oil can also improve the tolerance of conidia to extreme temperatures. Another benefit of using oil is that it prevents conidia from free dispersion in the air and thus reduces the probability of flying mosquitoes and humans getting in contact with it (Scholte *et al.* 2003; 2004a; Darbo and Thomas, 2009).

The propensity to select humans for blood feeding is arguably the most important component of mosquito vectorial capacity (Zwiebel and Takken, 2004). This aspect further determines the success of mating, blood-feeding and oviposition. It takes ten days for ingested gametocytes to develop into infective sporozoites, with estimated values of 3.4-5.8 for the average number of feeding cycles required for parasite development (Killeen *et al.*, 2000). Results obtained by Scholte *et al.* (2006) indicate that if female malaria mosquitoes become infected with fungus early in their lives, it becomes far less likely that they will transmit malaria.

A laboratory study in which infected female mosquitoes were blood fed by arm in small cups revealed that fungal infection reduces (but does not eliminate) feeding propensity and fecundity (Ffrench-Constant., 2005). Reduction in feeding upon infection with fungus might be attributed to degradation of tissues (including the midgut) in combination with the production of secondary metabolites (Amiri *et al.*, 1999; Scholte *et al.*, 2006). If a mosquito survives certain time periods without blood feeding before it dies, it remains, in epidemiological sense, inactive as a vector (Hajek and Saint Leger., 1994; Scholte *et al.*, 2006). In another laboratory study, adult female *An. gambiae* mosquitoes that were three days old after infection with fungus (*M. anisopliae*) showed a decrease in feeding propensity (Ondiaka *et al.*, 2008). Such an impact on mosquito behavior will result in a reduction of female lifetime vectorial capacity and hence malaria transmission risk.

The primary effect of fungus in increasing mosquito mortality substantially reduces the probability of mosquitoes' survival to any given feeding cycle. This therefore reduces the probability of a fungus-infected mosquito reaching the infectious state (Scholte *et al.*, 2006). This was verified in a study by Blanford *et al.* (2005) in which mosquitoes infected with the fungus *B. bassiana*, next to having a high fungus-induced mortality, expressed a strong inhibitory effect on *P. chabaudi*. Scholte *et al.* (2006) observed an increase in mortality in *An. gambiae* around the time of sporozoite maturation. In another laboratory study, mosquitoes were exposed to high conidial concentrations (2×10^{10} and 4×10^{10}conidia/m^2) in a relatively short time (15-30 min). Results from this experiment showed 100% mortality of mosquitoes within 10 days (Farenhorst *et al.*, 2008; Mnyone *et al.*, 2009).

Further laboratory experiments show that both *M. anisopliae* and *B. bassiana* could be used in resistance management and integrated vector management programmes to targetinsecticide-resistant and insecticide-susceptible malaria vectors (Howard *et al.*, 2010).

Insecticide-resistant mosquitoes being simultaneously bombarded with both fungus and an insecticide probably overwhelms the enzymes that normally make a mosquito resistant to the insecticide (Farenhorst *et al.,* 2009), rendering a much lower risk of resistance development (Scholte *et* al., 2007). Another laboratory study shows that horizontal transfer of fungal inoculum between mosquitoes is possible during copulation and may contribute to spreading of the fungus within target mosquito populations in the field (Scholte *et al.,* 2004). A small scale field trial carried out by Lwetoijera *et al,* (2010) showed that effective use of odor-baited stations as a delivery method for entomopathogenic fungus may be considered as an option to target mosquitoes while they are outdoors.

2.3. Justification and significance of the study

Malaria is a global health problem and its burden is high in the Sub-Saharan countries. The mainstay control of malaria is by use of chemical insecticides against malaria mosquitoes. However, resistance to pyrethroids has been reported in both *An. gambiae* and *An. funestus* in many malaria endemic countries in Africa (Zaim and Guillet, 2002; Hunt *et al.*, 2011). Additionally, parasite resistance has rendered previous antimalarial medicines ineffective in most parts of the world threatening malaria control (WHO, 2009; 2010). These scenarios call for alternative strategies for malaria control. To date, many successful laboratory studies have demonstrated the potential of using entomopathogenic fungi against malaria mosquitoes.

However, infecting wild adult malaria mosquitoes with *B. bassiana* (as an environmental-friendly and a non-resistant tool against malaria vectors) in the field has not been investigated. This study provides a useful and practical tool (one piece of black cotton cloth measuring $3m^2$) for applying fungi against wild adult malaria mosquitoes in the field. It also gives information on efficacy of spraying indoor hut walls with spores of *B. bassiana* against

wild malaria mosquitoes. Additionally, information on how often surfaces need to be re-treated with fungus is provided in this thesis. Application of information obtained from this study will lead to reduction in malaria mosquito population, and eventual reduction in malaria transmission. The tools could be incorporated into integrated vector management programmes for control of malaria.

2.4. Hypothesis

1. Short exposure times of malaria mosquitoes to *B. bassiana*-treated cloths do not result in high fungal infection rates.
2. Spores of low viability states do not infect and kill malaria mosquitoes.
3. Fungal spores impregnated on cotton cloth panels are not virulent on malaria mosquitoes.
4. An increase in surface area of *B. bassiana*-impregnated cloth does not increase infection rates in wild malaria vectors.
5. Indoor spraying hut walls with spores of *B. bassiana* formulated in oil is not an effective technique of infecting and killing wild adult malaria mosquitoes.

2.5. Objectives of the study

2.5.1. General objective

This thesis aimed to investigate efficacy of the entomopathogenic fungus *Beauveria bassiana* against wild malaria mosquitoes, under field conditions in Ahero, Western Kenya.

2.5.2. Specific Objectives

1. To determine the relationship between exposure time and infection of mosquitoes with *Beauvaria bassiana.*
2. To determine the relationship between spore viability and infection of mosquitoes with *Beauvaria bassiana.*
3. To determine virulence of fungal spores impregnated on cotton cloth panels.
4. To determine the effect of surface area on rate of infection of wild malaria mosquitoes with fungi.
5. To determine efficacy of infecting malaria mosquitoes by spraying indoor hut walls with *Beauvaria bassiana.*

CHAPTER THREE: MATERIALS AND METHODS

3.1. Study Area

This study was carried out in three experimental huts constructed on grounds of Kigoche Technical college in Kamagaga village (0° 08' S, 34° 55' E) located near Ahero town in western Kenya. Kamagaga village is approximately 24 Km South-east of Kisumu city and 4.3 Km away from Ahero town. This is a high altitude area lying approximately 1166 m above sea level, with a mean annual temperature range of 17-33°C, average annual rainfall of 1,000–1,800 mm and average relative humidity of 64%. Malaria is highly endemic in the area, with transmission occurring throughout the year (Atieli *et al.*, 2009). Mean annual *Plasmodium falciparum* sporozoite inoculation rates (EIR) of 0.4-17 infective bites per year have been reported in the area (Ndenga *et al.*, 2006). Ahero is dominated by *Anopheles gambiae* and *Anopheles funestus* mosquitoes (Mukabana *et al.*, 2012; Githeko *et al.*, 1994), both of which are competent vectors of *Plasmodium* parasites (Ibrahima *et al.*, 2003).

The main economic activity of local residents, who are ethnic Luos, is rice farming, which creates numerous mosquito larval habitats resulting in high densities of malaria vectors in the area. Maize is also cultivated on a small scale. Cattle, sheep, goats and chicken are kept in the village. Many houses in the area are mud-walled with roofs made of corrugated iron sheets or thatch. Eaves of most houses are open due to the high daytime temperatures (Atieli *et al.*, 2009).

3.2. Fungal isolates, formulations and application

Spores (conidia) of *B. bassiana*, with viability states of 80%, 78%, 51%, and 50%, were provided by the Department of Bioprocess Engineering, Wageningen University, the Netherlands. Those with higher viability of above 80% *i.e.* isolate 193-825 (IMI 391510) were

kindly provided by Professor Matthew Thomas of Penn State University, U.S.A. Fungal spores were stored as dry spores in Falcon™ tubes that were placed in an airtight container containing silica gel and refrigerated at 4°C. The spores were formulated in 100% CASTROL WOM 14 oil (BP Castrol, Durban, South Africa) before application. In all experiments, 0.75 grams of spores were formulated in 300 milliliters of oil, on every three (3) square metres of cotton cloth. This was equivalent to a concentration of 5.0×10^{10} spores per square metre. A spray gun filled with oil-formulation of *B. bassiana* was used during application on cloth panels and on walls of experimental huts. Application was at 1830 hours and thereafter, cloth panels were left to dry for 48 hours before exposure to mosquitoes.

3.3. Experimental huts

Three experimental huts were constructed by a Fungus for Malaria Control in Africa (FMCA) project, funded by the Adessium Foundation. Like most local houses the huts had mud walls and roofs (gable shaped) made of corrugated iron sheets. The huts measured approximately 3.5m long, 3.5m wide and 1.9m high. The roof measured 2.7m high at the apex. Each hut had two windows on opposite sides fixed with an exit trap, measuring 51cm long, 40cm wide and 41cm high, and with a sleeve measuring 12cm. The slots where mosquitoes entered the exit traps measured 20.0cm long and 2.5cm wide.

3.4. Mosquito rearing

Anopheles arabiensis mosquitoes were reared in an insectary at the Ahero Multipurpose Development and Training Institute (AMDTI), located approximately 1 Km from Ahero town. The seed material was obtained from the Thomas Odhiambo Campus of the International Centre of Insect Physiology and Ecology (*icipe*) located near Mbita point

Township in western Kenya. The mosquitoes were maintained under standard insectary conditions of 25±2°C temperature and 80%±10% relative humidity (RH). Humid conditions were maintained by placing wet woolen mats on the insectary floor. Larvae were dispensed into clean plastic, rearing trays (diameter=40cm each) half filled with filtered rain water. The larvae were fed on Tetramin® baby fish food until adult emergence. Each larval rearing tray was topped up every day with filtered rain water to make up for the water lost due to evaporation. To avoid algal growth and waste accumulation on larvae and on rearing trays, respectively, the larvae were removed from the slightly turbid water by use of a small sized gauge sieve and transferred into clean trays half-filled with filtered rain water.

Pupae were collected during mornings (0630 – 0730 hours) and evenings (1830 – 1930 hours) using pipettes and transferred in paper cups measuring 5cm in diameter. The cups were topped up with filtered rain water, immediately put into a mosquito cage measuring 30 cm length × 30cm width × 30cm height and covered with netting material to prevent emerged adult mosquitoes from escaping. One wet (non dripping) cotton towel was placed on top of the cage net to allow mosquitoes to take water *ad libidum* and to maintain humidity within the cage. The towel was replaced every day with a clean one. Washing of the dirty towel was done daily with an odourless soap. The towel was sun-dried, folded and kept in a dry place.

Emerged adult mosquitoes were maintained on six percent sugar solution prepared by dissolving 60 grams of sugar in 940 millilitres of distilled water. A piece of velvex® tissue paper was cut, folded into four pieces and rolled, then put inside a Falcon™ tube. The tube was filled with 6% sugar solution and put inside the cage for mosquitoes to feed on. Velvex® tissue acted as a wick which absorbed the sugar solution from the Falcon tube. Mosquitoes obtained sugar meals by perching on to the tissue extending out of the Falcon tube. The sugar solution and tissue were replaced with fresh preparations at 0630 hours every two days.

Falcon tubes were thoroughly cleaned with an odourless bar soap (brand name-Menengai), air dried and re-used.

Four day old mosquitoes present inside holding cages were starved for between 6 and 8 hours. The mosquitoes were supplied with water during starvation. Female mosquitoes were fed on rabbit blood in darkness at 1900 hours. Before feeding, the back of the rabbit was shaved, cleaned and the animal enclosed in a wooden box. The top centre part of the box had an opening (10cm diameter) through which mosquitoes accessed and fed on the rabbit to repletion. Thereafter, the rabbit was removed from the mosquito cage and released from the wooden box. Male mosquitoes were entirely fed on 6% sugar solution.

Egg cups were prepared by putting a thin layer of cotton wool lining in Petri dishes (8 cm diameter). The cotton was wetted with distilled water from a wash bottle. Whatman filter paper circles (diameter, 8cm) were placed on top of the wet cotton wool and served as oviposition surfaces. The egg cups were introduced in mosquito holding cages on the third and fourth day after the mosquitoes had blood fed. The egg cups were put in the cages at 1900 hours and collected the next morning after mosquitoes had laid eggs on them. Laid eggs were transferred into larval rearing trays containing filtered rain water (5 litres). A lining of adsorbent paper was placed along the upper meniscus of the water to prevent eggs from desiccating when the level of water reduced due to evaporation. Hatched first instar larvae were distributed into more rearing trays to avoid intraspecific competition. Trays containing mosquito larvae were covered with mosquito netting material to prevent wild gravid females from laying eggs into the trays. The mosquito larvae were fed on Tetramin® baby fish food until adults emerged. The cycle was continued until a stable colony was established. Newly emerged mosquitoes were placed in mosquito holding cages ready for use in experiments.

3.5. Relating between exposure time and infection of mosquitoes with *Beauvaria bassiana*

The relationship between exposure time and infection of *An. arabiensis* mosquitoes with spores of the entomopathogenic fungus *B. bassiana* was determined. Spores of *B. bassiana* (80% viability) were formulated in CASTROL WOM 14 oil and applied on cloth panels at a concentration of 5.0×10^{10} spores per square meter. Groups of twenty (10 male and 10 female) laboratory-reared *An. arabiensis* mosquitoes were then brought into contact with the cloth panel for variable lengths of time using the World Health Organization (WHO) cone bioassay test. The periods of time over which experimental mosquitoes were exposed to the treated surfaces included 10, 20, 30, 40, 50, 60 and 70 minutes. Survival and percentage of mosquitoes infected following exposure to the treated surface for a specific period of time were noted. Measurements (survival rates and percent infection) were replicated four times for each period of exposure. Follow up of mosquitoes was done in separate mosquito holding cages which measured 10 cm length × 10cm width × 10cm height. All mosquitoes were maintained on 6% sugar solution. Infection was determined by examining mosquito cadavers for growths of *B. bassiana* (mycosis test).

To carry out a mycosis test cadavers were dipped in 70% ethanol (Farenhorst *et al.*, 2010; Lwetoijera *et al.*, 2010) and immediately rinsed in sterile distilled water. The sterilized cadavers were then placed in Petri dishes lined with dry filter papers and left to dry for two days. Filter papers containing sterile, dry cadavers were moistened with sterile distilled water thereafter. The Petri dishes were sealed (Howard *et al.*, 2010) and placed in a humid airtight plastic container containing towels moistened with distilled water. The fungus, if present, germinated out of the cadaver after 4-6 days (Scholte *et al.*, 2005; Lwetoijera *et al.*, 2010).

The percentage of mosquitoes that showed fungal growths (per treatment) was calculated using the following formula below.

$$\text{Percent fungal infection} = \frac{\text{Number of mosquitoes with fungal growths}}{\text{Total number of mosquitoes}} \times 100$$

3.6. Relating between spore viability and infection of mosquitoes with *Beauvaria bassiana*

The capacity of infecting laboratory-reared *An. arabiensis* mosquitoes was determined by using spores of *B. bassiana* of different viability states in two different ways. In the first case four cloth panels (each measuring 3 m^2) were separately treated with 80%, 78%, 51% and 50% viable spores of *B. bassiana*. The spores were formulated in CASTROL WOM 14 oil and applied on the cloth panels at a concentration of 5.0×10^{10} spores per square meter. Groups of twenty (10 male and 10 female) laboratory-reared *An. arabiensis* mosquitoes were then brought into contact with the cloth panels for 30 minutes. These experiments were replicated four times. Survival and percentage of mosquitoes infected following exposure to the treated surfaces were noted. Infection was determined by examining mosquito cadavers for growths of *B. bassiana* (see section 3.5.). The standard WHO cone bioassay test was used in all cases.

In the second case spores from a batch with an initial viability of 80% were used. The percentage of mosquitoes infected was determined following subsequent decay of spores in the batch i.e. at 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10% and 0% viability. Infections were done by applying the spores on cloth panels to which experimental mosquitoes were exposed subsequently. Spores were applied at a concentration of 5.0×10^{10} per square meter in all cases. Groups of twenty (10 male and 10 female) laboratory-reared *An. arabiensis*

mosquitoes were then brought into contact with the cloth panels for 30 minutes. These experiments were replicated four times. Survival and percentage of mosquitoes infected following exposure to the treated surfaces were noted. Infection was determined by examining mosquito cadavers for growths of *B. bassiana* (see section 3.5.). The standard WHO cone bioassay test was used in all cases.

3.7. Virulence of fungal spores impregnated on cotton cloth panels

The aim of this experiment was to determine how viability of spores of *B. bassiana* and their subsequent infectivity to mosquitoes varied with time when impregnated on 100% and 35% cotton cloth panels. The other material contained in the 35% cotton cloth panel was polyester (65%). The cotton cloth panels were selected based on local availability and theoretical potential to hold fungal spores. The cloth panels were impregnated with *B. bassiana* spores formulated in CASTROL WOM 14 oil at a concentration of 5.0×10^{10} per square meter. The panels were then placed inside experimental huts (see section 3.3.). Pieces of cloths which measured 3 × 3 cm were cut from each panel at pre-determined time intervals (once every week) and viability of fungus impregnated thereon tested over time to determine the rate of decay. The infectivity of the fungus to mosquitoes (percent infection) was simultaneously tested to determine how virulence of the fungus varied with time.

Time intervals (post-impregnation) at which viability and virulence of the fungus were tested included 3, 7, 14, 21, 28, 35 and 42 days. Experiments were conducted using 10 male and 10 female-laboratory reared *An. arabiensis* mosquitoes. Virulence was determined using the standard WHO cone bioassay test, which was superseded with follow up on mosquito survival. Infection of mosquitoes was determined by examining mosquito cadavers for

growths of *B. bassiana* (see section 3.5.) A data logger (which recorded ambient temperature and relative humidity) was placed in the experimental hut where the cloth panels were stored.

3.8. Effect of surface area on rate of infection of wild malaria mosquitoes with fungi

The effect of surface area on capacity of infecting wild mosquitoes with spores of *B. bassiana* was tested under field conditions. Tests adopting a 3 × 3 Latin square design were conducted in three experimental huts described in section 3.3. The first hut was provided with one ($3m^2$) piece of fungus-impregnated cotton cloth panel. The second hut was provided with two ($3m^2$) pieces of fungus-impregnated cotton cloths panels. The third hut i.e. the control was left empty with no cotton cloth panel. The cloth panels were impregnated with *B. bassiana* spores formulated in CASTROL WOM 14 oil at a concentration of 5.0×10^{10} per square meter.

Cloth panels could be reached by mosquitoes from two sides, effectively doubling the surface area (note: both front and back sides of the cloths were treated, the actual surface area treated was twice that of the size of the cloth). Each hut was occupied by one volunteer from 1900 to 0700 hours. Each volunteer was protected from mosquito bites by sleeping on a bed covered with two layers of bed net. The volunteers rotated in such a manner that by day nine each had had a chance to sleep three nights in each hut (Table 1). In addition viability of fungal spores applied on the cloth panels was determined. A data logger was provided in one hut to record temperature and relative humidity during the trial.

Table 1: Pattern of rotation of volunteers in experimental huts during experiments designed to study the effect of surface area on rate of infection of wild malaria mosquitoes with spores of *B. bassiana*. Each person slept in any one hut for three nights.

	Hut 1	**Hut 2**	**Hut 3**
Day: 1, 4, 7	One cloth panel	Two cloth panels	No cloth panel
	Person Z	Person Y	Person X
Day: 2,5, 8	Two cloth panels	No cloth panel	One cloth panel
	Person X	Person Z	Person Y
Day: 3, 6, 9	No cloth panel	One cloth panel	Two cloth panels
	Person Y	Person X	Person Z

Male and female adult mosquitoes (*Anopheles* and Culicine) which rested in window exit traps and those resting indoors on walls were separately aspirated from each hut on daily basis between 0700hours and 0800hours using hand-held manual aspirators. Each mosquito was released in individual, clearly labeled plastic tubes (115mm height × 28mm diameter) with mouths covered with small pieces of netting material (10.0 cm^2) secured with rubber bands. Collected mosquitoes were transported to the field insectary constructed 100 metres away from the huts. The tubes that contained wild mosquitoes were then placed on shelves protected from predatory ants. Mosquitoes were maintained on 6% sugar solution. Mortality and survival of collected mosquitoes were monitored and recorded on daily basis until all mosquitoes died. Fungal infection was determined by examining mosquito cadavers for growths of *B. bassiana* (see section 3.5.).

3.9. Efficacy of infecting malaria mosquitoes by spraying indoor hut walls with *B. bassiana*

A before and after experimental design was used to study the efficacy of spraying indoor hut walls as a mechanism of infecting malaria mosquitoes with spores of *B. bassiana*. Three experimental huts (see section 3.3) were used during this experiment. Each of the huts accommodated one human volunteer from 1900 to 0700 hours. Each volunteer was protected from mosquito bites through use of a two layered bed net. In the 'before' phase, walls of the three huts were neither sprayed with pure CASTROL WOM 14 oil nor with spores of *B. bassiana* formulated in CASTROL WOM 14 oil. Three volunteers, each sleeping in a separate hut on an experimental night, participated in the experiment. The volunteers rotated around the huts so that each slept in any one hut for six nights (Table 2). As many mosquitoes as possible were manually aspirated from the huts throughout the sixteen experimental days using hand held mouth aspirators. Collected mosquitoes were released in separate labeled, mosquito holding cages (10 cm length × 10 cm width × 10 cm height) and maintained on 6% sugar solution. Their daily survival was followed up at field insectary. Infection was determined by examining mosquito cadavers for growths of *B. bassiana* (see section 3.5.). The aim of the 'before' phase was to determine any inherent variability in numbers of mosquitoes collected from the three huts.

Table 2: Pattern of rotation of volunteers in experimental huts during experiments designed to study the efficacy of spraying indoor hut walls as a mechanism of infecting malaria mosquitoes with spores of *B. bassiana*. Each person slept in any one hut for six nights.

Day	Hut 1	Hut 2	Hut 3
1, 4, 7,10,13,16	Person Z	Person Y	Person X
2,5, 8,11,14	Person X	Person Z	Person Y
3, 6, 9,12,15	Person Y	Person X	Person Z

In the 'After' phase walls of one experimental hut were sprayed with spores of *B. bassiana* formulated in CASTROL WOM 14 oil, and those of a second hut were sprayed with pure CASTROL WOM 14 oil only. The third hut was not sprayed. Indoor resting mosquitoes were collected using hand-held manual aspirators. Collected mosquitoes were released in separate labeled cages (10 cm length × 10 cm width × 10 cm height) and maintained on 6% sugar solution. Their daily survival was followed in the field insectary. Infection was determined by examining mosquito cadavers for growths of *B. bassiana* (see section 3.5.).

3.10. Ethical clearance

This study was approved by the Kenya Medical Research Institute (KEMRI), National Ethics Review Committee (NON-SSC protocol number 203 dated 23 April 2010).

3.11. Statistical analysis

Data were collected and entered in excel spread sheets (Microsoft Corporation) and mosquito survival data were analyzed using R version 2.13.1 (Development Core Team, 2010). Cox proportional hazard model was used to study the risk of death of mosquitoes infected with fungus while adjusting for other exposure factors. The model with the smallest Akaike Information Criteria (AIC) value was chosen to be appropriate. Median survival times (days) were estimated using the Kaplan-Meier survival function. Survival of mosquitoes was considered not statistically different at $p > 0.05$.

CHAPTER FOUR: RESULTS

Experiments in this thesis were conducted from December 2010 to July 2011. Results from all experiments revealed that infection of both wild and laboratory-reared *An. arabiensis* mosquitoes with spores of *B. bassiana* reduced survival of the mosquitoes as compared to survival of mosquitoes in the untreated or control group regardless of delivery surface or time of exposure post fungus-application. Mycosis test results revealed that mosquitoes from the control groups were not infected with fungus.

4.1. Relating between exposure time and infection of mosquitoes with *Beauvaria bassiana*

High fungus infection rates were observed at all exposure times (Table 3). The difference in infection rates between sexes, at all exposure times was not significant (p=0.633). However, there were significant differences in survival between control and infected mosquitoes (p=0.001, Table 3) and between infected and uninfected mosquitoes (p=0.003). The longest exposure time i.e. 70 minutes resulted in 100% infection rates in both male and female *An. arabiensis* (Table 3). The median survival times for infected and uninfected mosquitoes ranged between 4-7 and 3-7 days, respectively, compared to 16-24 days among mosquitoes in the control group (Table 3). There were significant differences in survival of infected mosquitoes among all exposure times (p=0.001) and between 10 minutes and 20-70 minutes exposure times (Table 3). There was no difference in infection rates and survival of infected mosquitoes between 50 and 70 minutes, and between 60 and 70 minutes exposure times (p=0.167 and p=0.731, respectively).

Table 3: Percent infection rates and median survival time (days) of *An. arabiensis* exposed to *B. bassiana* for different time periods. Probability (*p*) values compare survival of fungus-infected mosquitoes between 20-70 minutes to 10 minutes exposure time.

Treatment	Exposure time (mins)	Number of mosquitoes per sex	Percent infection		Median survival time (days)		Median survival time (days)		
			Females	Males	Control	Treatment	Infected	Uninfected	*p*-value
B. bassiana	10	40	95.0	92.5	17	5	7	7	...
B. bassiana	20	40	92.5	87.5	24	5	7	3	0.010
B. bassiana	30	40	92.5	90.0	17	6	6	5	0.001
B. bassiana	40	40	85.0	95.0	19	7	7	6	0.038
B. bassiana	50	40	95.0	90.0	16	6	5	7	0.001
B. bassiana	60	40	92.5	95.0	16	7	4	6	0.001
B. bassiana	70	40	100.0	100.0	21	8	5	7	0.001

4.2. Relating between spore viability and infection of mosquitoes with *Beauvaria bassiana*

This experiment was conducted in the month of April 2011 in two different ways. In the first case, a total of 320 *An. arabiensis* mosquitoes were exposed to 80%, 78%, 51% and 50% viable spores of *B. bassiana.* Results revealed that an overall infection rate of 72% was achieved. In the second case, a total of 720 *An. arabiensis* mosquitoes were exposed to subsequently decayed spores of 80% viability, *i.e.* 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10% and 0% viability. Overall results showed that 29% of mosquitoes were infected with fungus.

In the first case, 72% (230 of 320) of *An. arabiensis* mosquitoes were infected with fungus from stocks of 80, 78, 51 and 50%. Of 230 mosquitoes, 74, 68, 45 and 43 were infected with fungus from stocks of 80, 78, 51 and 50%, respectively. Overall, 50-88% male mosquitoes and 58-98% females were infected with fungus when spores which were available in stock were used (Table 4). None of the mosquitoes from the control group was infected with fungus. A significant overall effect of spore viability was observed (p=0.001) after controlling for sex and infection status. Results indicated that although the risk of dying was higher at all the other viability states than it was for the control group, it was only significantly higher at 78% and 80%. The risk of death for mosquitoes exposed to 78% and 80% spores was 6.28 times and 5.47 times respectively, higher than that of mosquitoes in the control group, after controlling for infection status and sex of the mosquitoes (Table 4). Additionally, mosquitoes infected with spores of 50% had an increase of 27% risk of dying than the control group, but the increased chance of death was not significant (p=0.369). Similarly, when spores of 51% were used, an increase in the risk of dying for mosquitoes was 4% although the increased chance of death was not significantly different from that of the control group (p=0.889). Compared to uninfected mosquitoes, the infected ones had a 69%

chance of dying, after controlling for sex and viability (Hazard Ratio (HR) = 1.69, 95% Confidence Interval (CI): 1.28–2.22).

Further analysis indicated that although female mosquitoes had an increase of 12% risk of dying, this increased chance of death was not significant (p=0.312). The Median survival time of mosquitoes in the control group was 14 days, while those of mosquitoes infected with spores of 50 and 51% viability states were 11 days each. Median survival times of 14 and 11 days were not significantly different (p=0.360). Finally, the median survival time of infected mosquitoes from stocks of 78 and 80% spore viability were 4 and 3.5 days respectively, and they were not significantly different from each other (p=0.106).

Table 4: Proportions (percent infection), Hazard ratios (HR) plus 95% confidence level (Cl) and median survival time (days) of male and female *An. arabiensis* infected with *B. bassiana* from stocks of 50, 51, 78 and 80% spore viability. Hazard Ratio compares daily risk of death in controls to that of each fungus treatment.

Viability of fungus (%)	Number of mosquitoes per sex	Percent infection rate		HR (95% CI)	*p*-value	Median survival time (d)
		Females	Males			
50	40	58.0	50.0	1.27 (0.76-2.12)	0.369	11.0
51	40	63.0	51.0	1.04 (0.62-0.62)	0.889	11.0
78	40	80.0	90.0	6.28 (3.66-10.79)	0.001	4.0
80	40	98.0	88.0	5.47 (3.13-9.54)	0.001	3.5

Results from the second approach of infecting *An. arabiensis* with spores of *B. bassiana* (a case where subsequently decayed spores from a batch with an initial viability of 80% were used) showed that high infection rates were achieved with 80% viable spores, irrespective of the sex of the mosquitoes, as compared to use of subsequently decayed spores i.e. 70%, 60%, 50%, 40%, 30%, 20%, 10% and 0% (Table 5). Mosquitoes exposed to a 60% and 80% spore viability had a higher risk of dying (33 and 60% respectively) when compared to the controls, after adjusting for infection status, the increased risk was only significant at 80% (60%:

Hazard Ratio (HR) = 1.33, 95% Confidence Interval (CI): 0.95–1.86; 80 %: HR=1.60, 95% CI: 1.09–2.35). Infected mosquitoes were 2.4 times more likely to die than the uninfected ones, after adjusting for spore viability (p=0.001). The risk of dying for mosquitoes exposed to 70%-20% (excluding 60%) spore viability was similar (HR=0.78-0.45) to those exposed to 0% viability (Table 5).

Although significant differences in survival of infected mosquitoes exposed to 10% viable spores were observed when compared to those exposed to 0%, the risk of death for mosquitoes in the two groups was not significantly different(HR=0.78, 95% CI: 0.57–1.07). The Median survival time of infected mosquitoes at 80% was 5 days while those from 70% to 10% ranged between 7 and 12 days. Mosquitoes from the control group had median survival times ranging between 18 and 22 days (Table 5).

Table 5: Proportion (percent infection), Hazard ratios (HR) plus 95% confidence level (CI) and median survival time (days) of male and female *An. arabiensis* infected with subsequently decayed spores (80, 70, 60, 50, 40, 30, 20, 10 and 0%) of *B. bassiana*.

Spore viability	Number of infected mosquitoes (percent infection)		Median survival time (days)		HR (95% CI)
	Males (n=40)	Females (n=40)	Treated	Control	
80 %	38 (95%)	37 (93%)	5	21	1.60 (1.09-2.35)
70 %	21 (53%)	25 (63%)	8	19	0.78 (0.55-1.09)
60 %	22 (55%)	19 (48%)	7	18	1.33 (0.95-1.86)
50 %	15 (38%)	11 (28%)	14	16	0.40 (0.29-0.56)
40 %	4 (10%)	7 (18%)	14	20	0.48 (0.35-0.66)
30 %	5 (13%)	2 (5%)	14	20	0.50 (0.37-0.69)
20 %	0 (0%)	2 (5%)	15	19	0.45 (0.33-0.61)
10 %	3 (8%)	1 (3%)	12	22	0.78 (0.57-1.07)
0 %	0 (0%)	0 (0%)	11	20	-

n is the number of mosquitoes

4.3. Virulence of fungal spores impregnated on cotton cloth panels

This experiment was conducted in the month of April, 2011. Sixty percent (337 of 560) of *An. arabiensis* mosquitoes were infected with fungus when 100% cotton cloth was used. The rate was high as compared to 40% (161 of 400) of *An. arabiensis* infected with fungus when 35% cotton cloth was used. Results indicated that the risk of dying among mosquitoes exposed to 100% treated-cotton cloth, after adjusting for infection status and days post application, was 2.18 times higher when compared to those exposed to 35% treated-cotton cloth (Hazard Ratio (HR) = 2.18: 95% Confidence Interval (CI) 1.85–2.58). Irrespective of the type of cloth used, infection rates declined as days post-fungal spore application increased (Table 6). Survival of mosquitoes exposed to fungus was always lower than that of controls (p=0.001). Overall, there was no significant difference in survival of mosquitoes exposed to fungus, 3 days post application when compared to 7, 14 and to 21 days post application, irrespective of the cloth type (p=0.607, 0.842 and 0.102 respectively). However, a significant difference was observed in survival of mosquitoes between 3, 35 and 42 days post application (p=0.001).

Spores of *B. bassiana* remained infective up to the 28th and 7th days post fungus application by use of 100 and 35% cotton cloths. On the 28th day post application of fungus on 100% cotton cloth, 73% of male and 70% of female mosquitoes were infected. On the 7th day post application of fungus on 35% cotton cloth, 55% male and 85% female mosquitoes were infected (Table 6). On the 28th day post application of fungus on 35% cotton cloth, in comparison to application on 100% cotton cloth, 13% male and 10% female mosquitoes were infected with fungus (Table 6). The number of days survived by mosquitoes in the control groups when 100 and 35% cotton cloths were tested ranged from 14-20 days and those of fungus-infected mosquitoes ranged from 5-15 days for fungus-infected mosquitoes for the two cloths types (Table 6). Survival of mosquitoes within the same treatment was not

significantly different, and 35% cotton cloth lost its viability to zero on the 31st day; before the 35th day post application of fungus (Table 6).

Table 6: Percent infection and median survival times (days ± S.E) of *An. arabiensis* exposed to *B. bassiana* on day 3, 7, 14, 21, 28, 35 and 42 post-application of fungus to 100 and 35 % cotton cloths

Days post fungus application	Number of mosquitoes per sex	Percent infected				Median survival time (days) ± S.E			
		35% Cotton cloth		100% Cotton cloth		Infected		Control	
						100% cotton cloth	35% cotton cloth	100% cotton cloth	35% cotton cloth
		M	F	M	F				
3	40	48	70	90	10	6.0±0.24	7.2±0.30	17.0±1.35	18.0±0.73
7	40	55	85	83	90	5.2±0.23	7.1±0.33	17.0±1.38	18.0±0.73
14	40	35	40	78	78	5.0±0.21	8.2±0.46	17.0±1.73	19.1±1.04
21	40	38	10	80	75	6.0±0.25	7.4±0.56	16.0±1.11	19.0±0.80
28	40	13	10	73	70	6.3±0.29	10.0±0.47	17.0±1.38	14.0±1.22
35	40	-	-	8	13	15.1±0.43	-	17.0±1.54	
42	40	-	-	8	0	10.4±0.55	-	20.0±0.94	

4.4. Effect of surface area on rate of infection of wild malaria mosquitoes with fungi

This experiment was conducted in December 2010. Elevated mortality was observed in fungus- infected mosquitoes as compared to mortality in mosquitoes in the control (untreated) group (Table 7). The median survival times for uninfected mosquitoes collected from the control hut and huts provided with one and two pieces of fungus-treated cloth panels were 30, 26 and 29 days, respectively, while those of infected mosquitoes were 7, 8 and 11 days, respectively. There was no significant difference in survival of uninfected mosquitoes from the control hut and survival of uninfected mosquitoes from huts provided with 1 and 2 treated cloths (p=0.65 and 1.00 respectively). Survival of uninfected mosquitoes collected from huts provided with treated cloths was not significantly different (p=0.57). However, significant

difference was observed in survival of uninfected (control) and infected mosquitoes (p=0.001).

A total of 665 wild Anopheline and Culicine mosquitoes were collected from huts which were provided with: no cloth (control), one and two pieces of fungus-treated cloth panels, during the nine-day experimental period (Table 8). The total number of mosquitoes collected from the control hut, and huts provided with one and two pieces of fungus-treated cloth panels was 162, 250 and 253 respectively (Table 8). There was no significant difference (p=0.09) in mosquito collections among the huts. However, 71% (471 of 665) of *An. gambiae* and 22% of *An. funestus* (149 of 665) were collected during the entire period, irrespective of the treatment provided in each hut (Table 8).

From 162 mosquitoes collected from the control hut, 5 (3%) were infected with fungus, while 78 of 250 (31%) and 94 of 253 (37%) mosquitoes collected from huts provided with one and two pieces of fungus-treated cloth panels were infected with fungus, respectively (Table 8). Overall, 30% of *An. gambiae* and 35% of *An. funestus* (malaria vectors infected with fungus) were collected from huts provided with one and two treated cloth panels (Table 8). No significant difference (p=0.20) was observed in infection rates between mosquitoes collected from huts provided with fungus-treated cloths.

Table 7: Treatment type, median survival time (days) (± S.E) for uninfected and fungus-infected wild mosquitoes collected from control hut and huts provided with one and two-treated cloths panels

No. of fungus- treated cloth	Infection status of mosquitoes	Median survival time (days) ± S.E
0 (Control)	Uninfected	30.0 ± 1.29
1	Uninfected	26.0 ± 1.11
2	Uninfected	29.0 ± 1.23
0 (Control)	Infected	7.0 ± 3.65
1	Infected	8.0 ± 0.68
2	Infected	11.0 ± 0.59

Table 8: Number of treated cloth panels, total number of wild mosquitoes collected per mosquito species and per treatment from control hut and huts provided with fungus-treated cloths panels. In brackets are percentages of wild mosquitoes infected with fungus per treatment and per mosquito species.

Number of cloth panels	Number of mosquitoes				
	An. gambiae	***An. funestus***	***An. coustani***	**Culicine**	**Total**
0	126 (1)	26 (1)	0	10 (1)	162 (3)
1	176 (21)	61 (9)	0	13 (1)	250 (31)
2	169 (28)	62 (7)	3 (1)	19 (1)	253 (37)
	471 (50)	**149 (17)**	**3 (1)**	**42 (3)**	**665 (71)**

4.5. Efficacy of infecting malaria mosquitoes by spraying hut walls with *B. bassiana*

This experiment involved application of the before and after experimental-design. Collection of mosquitoes from experimental huts before treatment of indoor walls of the huts was done in the month of January, 2011, while collection of mosquitoes from the huts after treatment of the huts with formulation of *B. bassiana* was done in the month of February, 2011. A total of 872 wild mosquitoes, to include 608 *An. gambiae*, 135 *An. funestus* and 129 other mosquito species (*An. coustani*, *An. pharoensis* and Culicine) were collected from three untreated huts during the pre-fungus treatment period (Table 9). During the post treatment period with *B. bassiana* formulated in CASTROL WOM 14 oil, a total of 662 mosquitoes, to include 479 *An. gambiae,* 93 *An. funestus* and 90 other mosquito species were collected from three huts (Table 9). There was no significant difference in the total number of mosquitoes collected during the pre- and post-treatment periods (p=0.06). However, female mosquitoes significantly exceeded males in number in all species during both pre and post treatment periods (p=0.02).The number of mosquitoes collected from the hut whose walls were neither sprayed with oil only nor with spores of *B. bassiana* formulated in oil was 310, while a second hut, whose walls were sprayed with pure oil only had 156 mosquitoes, and a third hut whose walls were sprayed with spores of *B. bassiana* formulated in oil had 196 mosquitoes.

From mycosis test results, no fungal conidia were observed germinating on the dead mosquitoes during the pre-treatment period. Mycosis test results did not show fungal conidia germinating on mosquitoes during the pre-treatment period, and on mosquitoes collected from the control hut and a hut whose walls were sprayed with pure oil only, during the post treatment period.

Table 9: Proportions of total number of wild mosquitoes collected during the pre and post-fungus treatment periods. In brackets are percentages of wild mosquitoes collected during the pre-fungus period and percentages of wild mosquitoes infected with fungus during the post-fungus-treatment period.

Mosquito species	**Pre-period**	**Post-period**
	Total collection (percent)	**Total collection (percent infection)**
An. gambiae	608 (69.7)	479 (14.0)
An. funestus	135 (15.5)	93 (11.0)
Culicine	116 (13.3)	86 (13.0)
An. coustani	12 (1.4)	1 (0.00)
An. pharoensis	1 (0.1)	3 (0.00)
	872 (100.00)	**662 (48.00%)**

Survival of wild mosquitoes was significantly reduced upon infection with *B. bassiana.* The Median survival time of mosquitoes collected during the pre-treatment period was 24 days (range 1-82 days). Results indicated that the risk of dying among fungus-infected mosquitoes, after adjusting for sex and treatment, was 5.34 times higher than among the uninfected mosquitoes (Hazard Ratio (HR) = 5.34: 95% Confidence Interval (CI) 3.95-7.22). The Median survival times of uninfected and fungus-infected mosquitoes were 20 and 2 days respectively (range 1-82 days). Further analysis indicated that female mosquitoes had a 50% higher chance of dying than male mosquitoes after adjusting for infection status and treatment (HR=1.50: 95% CI 1.25-1.81). Comparing mosquito collections from a hut whose walls were not sprayed, and a hut whose walls were sprayed with pure oil only, while adjusting for sex and infection status of the mosquitoes, results show that the chance of dying

for mosquitoes collected from a hut sprayed with oil only increased by 75% compared to those from a hut sprayed with fungus (HR= 0.75: 95% CI 0.59-0.96).

Although there was a 3.52 % decrease in the chance of dying for mosquitoes collected from the hut which was not sprayed as compared to the hut sprayed with fungus formulated in oil after controlling for sex and infection, the decrease was not significant (HR= 0.96 : 95% CI 0.78-1.20). Overall, there was a significant treatment factor effect on mosquitoes during the post treatment period (p=0.02). Of the mosquitoes collected from the fungus-treated hut, 45% (89 out of a 196) developed fungal growth on the cadavers. This percentage was composed of *An. gambiae* (34%), *An. funestus* (5%) and Culicines (6%). Results show that 68 wild *An. gambiae*, 10 *An. funestus* and 11 Culicine mosquitoes were infected with fungus. Of these, 78 (39%) were malaria vectors i.e. *An. gambiae* and *An. funestus*. Results from the surface area and indoor wall spraying experiments show that there was significant difference in infection rates, in all wild malaria and non-malaria mosquitoes (p=0.02). When three substrates of different surface areas i.e. one piece of fungus-treated cloth panel, two pieces of fungus-treated cloth panels and indoor fungus-treated walls of a hut were treated with fungus, the infection rates achieved were 31, 37 and 45% respectively. When a fungus-treated cloth panel and when indoor walls of a hut were sprayed with formulation of fungus, 31% and 45% infection rates were achieved. The rates were significantly different (p=0.003). However, when two pieces of fungus-treated cloth panels and when indoor walls of a hut were sprayed with fungus, infection rates of 37 and 45% respectively, were achieved and the rates were not significantly different (p=0.07). In conclusion, use of one piece of fungus-treated cloth panel against wild mosquitoes is preferable over using two pieces of fungus-treated cloth panels or spraying indoor house walls with fungus formulated in oil.

CHAPTER FIVE: DISCUSSION

The current study showed that all exposure times led to 100% mortality of *An. arabiensis* by the 14th day when compared to the control mosquitoes and, exposure time as short as 10 minutes was sufficient for *B. bassiana* to infect and kill *An. arabiensis* mosquitoes within 14 days. High fungus-infection rates were achieved when spores from stocks of 80% and 78% were used to infect *An. arabiensis*. Further, results show that the ability of *B. bassiana* conidia to infect and kill *An. arabiensis* mosquitoes was high when 100% cotton cloth was impregnated with the fungus as compared to the use of 35% cotton cloth. This is the first study to report the effect of *B. bassiana* formulated in oil and sprayed on one and two pieces of black cotton cloths, and on mud walls of a hut, against wild malaria mosquitoes. Field trials carried out in experimental huts showed that use of one and two pieces of fungus-treated cloth panels, and spraying of indoor walls of a hut with *B. bassiana* formulated in oil were equally effective techniques against wild malaria mosquitoes.

Controlled experiments showed that exposure time, spore viability and cloth type strongly affected the rate of infection in mosquitoes exposed to *B. bassiana*. The length of time mosquitoes need to rest and pick up sufficient spores to result in substantial impact on survival is an important factor to consider when designing the optimal target for exposing mosquitoes in the field. Exposure times tested in this study were selected to represent realistic exposure periods, considering the fact that mosquitoes may spend less than 10 minutes trying to enter an untreated mosquito net and after blood-feeding, they may rest on a surface for periods up to 24 hours (Clements, 1992a). Overall, results from the current study indicate that longer exposure times led to 100% fungus infection rates. All exposure times led to 100% mortality of *An. arabiensis* by the 14th day when compared to the control mosquitoes. Exposure time as short as 10 minutes was therefore sufficient for *B. bassiana* to reduce

survival and infect *An. arabiensis* mosquitoes. In a study carried out by Farenhorst and Knols (2010) an exposure time as short as 5 minutes was sufficient to reduce mosquito survival. Several other studies support results of the current study. A study by Wright *et al.* (2004) to determine the effects of increased exposure time of adult blowflies to *M. anisopliae* at 1.0×10^8 conidia/ml showed that even with extremely short exposure periods of 10, 30, 60 or 120 seconds, increased infections were achieved. In another experiment, when *B. bassiana* spores were mixed with the date pulp, effective mortalities of adult red palm weevils could be achieved after a treatment time of 15 minutes-within a baited trap (Deadman *et al.*, 2001). However, the dose used is stated as 10 grams of *B. bassiana* spores per 20ml of pulped dates, which may be at saturation levels for a weevil. In another study, no differences in mortality of adult *Glossina morsitans centralis* tsetse flies exposed to *M. anisopliae* were observed when exposure times were increased from 5 to 10 and to 20 minutes (Maniania, 1994). Whilst a trend of higher mortality in both male and female adult *An. gambiae* mosquitoes exposed continuously to dry spores of *M. anisopliae* was noted, reducing exposure time to 48 hours or 24 hours did not significantly reduce the impacts on survival (Scholte *et al.*, 2003; Scholte 2004). In another study, 24 hour exposure of adults of *G. morsitans centralis* to *M. anisopliae* to treated nitrocellulose membranes resulted in 100% mortality within 10 days (Maniania, 1994).

A study by Mnyone *et al* (2009) showed that 15 minutes were sufficient for conidia to infect and reduce survival of mosquitoes (100% mortality by day 14) but at a lower speed of kill than with 30 minutes exposure time (100% mortality by day 9). The current study showed 100% mortality by day 9 at 40-70 minutes exposure times and 100% mortality by day 11, at 10-30 minutes exposure times. However, a concentration of 2.0×10^{10}conidia/m^2 was used in a study by Mnyone *et al.* (2010), whereas a concentration of 2.5 times higher, 5.0×10^{10} conidia /m^2 was used in the current study.

The impact of exposure time is effectively another dose function i.e. the longer the exposure time the greater the reduction in mosquito survival as demonstrated in the current study. However, all exposure times (10-70 minutes) led to 100% mortality and reduced survival within 14 days post fungus infection. Uninfected mosquitoes also showed reduced survival (Median survival time=3-7 days). This reduction in survival indicated that the mosquitoes might have suffered sub-lethal effects from fungus infection that could not be detected from the daily mycosis routine.

Spore viability or dose is another important factor to be considered in infection of mosquitoes with fungi. In the current study, spores from stocks of 80% and 78% achieved high infection rates in both female and male mosquitoes as compared to use of spores from stocks of 51 and 50%. Survival of mosquitoes exposed to conidia of low viability was not different from that of the control (uninfected) mosquitoes. This reveals that the spores were not virulent and cannot be used to infect wild malaria mosquitoes in the field. On the other hand, the risk of mosquitoes dying from exposure on subsequently reducing viability (80-0%) indicates that the risk of mosquitoes dying increased when cloths with spores of 80% viability were used as compared to use of cloths with decayed spore viability (70-0%). Additionally, mosquitoes which were exposed to decayed conidia of 70-0% viability showed similar low risk of dying as mosquitoes in the control group. Therefore, spores with viability of 80% and above should be used for infection of mosquitoes in order to achieve high mortality rates.

Other experiments were carried out elsewhere by several people to determine the effect of spore dosage on mosquitoes and other insects. For example, adult blowflies suffered increasing mortality with exposure to increasing spore concentrations of oil formulations of *M. anisopliae* on cotton cloth up to a limit of 1.0×10^7 conidia/ml (Wright *et al.*, 2004). In another experiment, 10% higher mortalities of *Phlebotomus papatasi* sandflies were recorded 9 days after exposure to 1.0×10^7 conidia/ml, than with 1.0×10^5 conidia/ml of *B. bassiana*

contaminated sucrose solution (Warburg, 1991). Additionally, decreasing mean survival times were associated with increased fungal dose when *Lutzomyia longipalpis* sandflies were exposed to filter paper soaked in suspensions of 1.0×10^6, 1.0×10^7 and 1.0×10^8 conidia/ml of *M. anisopliae* (Briscoe, 1999).

Lower survival of *Schistocerca gregaria* locusts was correlated with increased dose of oil formulations of *M. anisopliae var. acridum* within optimal temperature ranges (Arthurs & Thomas, 2001). *Zonocerus variegatus* grasshoppers also showed reduced mean survival times with increasing does of *M. anisopliae var. acridum* (Thomas & Jenkins, 1997). Quesada-Moraga *et al.* (2006) reported that with higher doses of fungal extracts, the difference in survival was more marked. Further, studies by Mnyone *et al.* (2010 and 2009) also showed that at higher spore viability (≥85%), survival of mosquitoes is reduced while mosquito infection is increased. Similarly, soluble protein extracts of *M. anisopliae* were fed to cotton leafworm larvae for periods ranging from 24 hours to 144 hours, at low and high concentrations. This benefit of encouraging longer resting, in terms of increased mosquito mortality, is more marked at lower doses and so may be more important for 'decaying' treated surfaces.

The material to be treated is another aspect to consider for delivery of fungal spores to malaria mosquitoes. The material selected must be amenable to fungal treatment, attractive to mosquitoes and readily available in the field at an affordable amount. Insecticides have been very successfully applied to polyester nets (Vatandoost *et al.*, 2006) for review on efficacy of ITNs, but also to cloth and blankets for leishmaniasis and malaria control (Reyburn *et al.*, 2000; Rowland *et al.*, 1999), plastic tarpaulins for mosquito control (Graham *et al.*, 2002), plastic lattice strips for *Aedes aegypti* repellency (Kawada *et al.*, 2006), and cardboard for termite control (Potter, 2007). However, most insecticides are formulated as suspensions in water.

The fungal formulations, being suspensions in oil, are hydrophobic which may affect the binding to certain hydrophilic substances. Black cotton cloth was initially selected for use in the current experiment, as this was the material used for impregnation with oil formulated *M. anisopliae* in the field work in Tanzania (Scholte *et al.,* 2005). An experiment carried out in Tanzania, by Mnyone *et al.* (2010) reveal that mud panels and black cotton cloths strips hung around bed nets present suitable substrates for fungal conidia, and can be used to evaluate and apply fungal formulations in the field. They used *B .bassiana* and *M. anisopliae* spore formulations in their study (Mnyone *et al.*, 2010).

Fungal persistence is also vital for mosquito infection (Enserink, 2005; Thomas & Read, 2007; Knols & Thomas, 2006). In the current study, the ability of *B. bassiana* conidia to infect and kill *An. arabiensis* mosquitoes was high when 100% cotton cloth was impregnated with the fungus as compared to the use of 35% cotton cloth probably because conidia were readily available on 100% cotton cloth panels up to 28 days post application of fungus, but with time, however, conidial viability and virulence decreased, thus the conidia became less efficacious. This decline of spore viability and virulence of spores over time has also been reported in laboratory (Farenhorst and Knols, 2010; Mnyone *et al.*, 2009, and field studies (Scholte *et al.,* (2005). Decline in viability was faster on the 35% cotton cloth used in this study, reaching 0% viability on the 30th day post application, possibly because of poor conidial-attachment on the cloth which might have been a result of smooth fibres of 65% polyester (35% cotton) material contained in it.

From field trials in Tanzania, Scholte *et al.* (2005) collected the highest proportion of infected mosquitoes from *M. anisopliae* treated houses in the first two weeks, with a decline by the third week. Whilst conidial viability of stored suspensions remained high, that of the impregnated sheets dropped from 95% one day after impregnation to 63% after 3 weeks. The observations of 63% viability of *M. anisopliae* spores applied to cloth in the field (Scholte *et*

al., 2005) and low infection of mosquitoes exposed to *B. bassiana*-treated surfaces 3 months post spraying (Blanford *et al.*, 2005), would indicate the need for regular fungal retreatment to maintain high levels of kill.

Despite of the decline in viability, conidia in the current study were still infective up to the 28th and the 7th day for 100 and 35% cotton cloths, respectively. The 28 days post application in the current study could be compared to monthly application of aqueous suspensions of *B. bassiana* and *M. anisopliae* to pastures which resulted in 80-92% suppression of populations of *Rhipicephalus appendiculatus* on cattle (Kaaya & Hassan, 2000). Other similar studies carried out elsewhere show that applications of aqueous suspensions of both *B. bassiana* or *M. anisopliae* spores to the ears of cattle only showed persistence of effects against ticks for 1 or 3 weeks, respectively (Kaaya *et al.*, 1996). The limited persistence was presumably due to desiccation, ultraviolet (UV) damage and reduction in the number of spores adhering to the coat of the animal. However, dry spores applied within plastic contamination devices on traps shielded from direct UV rays were still able to infect 76% of collected tsetse flies after 3 weeks (Maniania and Odulaja, 1998) and did not show significantly reduced efficacy after 31 days (Maniania, 2002).

Mnyone *et al.* (2010) and Howard *et al.* (2010) reported in their studies that polyester netting has the lowest efficacy in disseminating fungus probably due to the chemicals used to soften polyester fibres or chemical effects from the netting itself. Additionally, the small holes in the material (25 holes /cm^2) offer a small surface area for both conidial attachment and exposure to mosquitoes (Mnyone *et al.*, 2010). High fungal infection rates in mosquitoes exposed on 100% cotton cloth are attributed to availability of more spores per unit area, which increased the probability of mosquitoes picking up spores from the cloth. In conclusion, 100% cotton cloth was found to be a suitable substrate to deliver fungus as compared to 35% cotton cloth.

Field trials conducted in experimental huts showed that infection of wild malaria mosquitoes with *B. bassiana* strongly reduced their survival. The initial challenge in making fungal application a viable option for malaria control is to ensure maximum pick up of spores by mosquitoes. From the small scale house trial in Tanzania (Scholte *et al.,* 2005), no overall difference in survival was seen between mosquitoes caught from treated and those caught from untreated houses. This was explicable by the low infection rate of mosquitoes. Field study results by Scholte *et al.* (2005) revealed that mosquitoes caught in the field were more susceptible to fungal infection compared to laboratory reared mosquitoes. Similar findings were obtained in the current study. In this study, the maximum number of days that uninfected wild female *An. gambiae* survived, counted from the day of capture was 81 while uninfected *An. funestus* survived up to 69 days. Males of the same mosquito species survived up to 82 and 66 days respectively. In addition, uninfected wild female and male Culicines survived up to 105 and 81 days respectively. Uninfected *An. arabiensis* did not survive beyond 35 days.

Indoor resting and blood-seeking malaria mosquitoes can be targeted by hanging fungus-treated cloth panels in a hut, as it was done in this study. Fungal infection may have an impact on mosquito population density if only enough wild malaria mosquitoes could be infected with conidia impregnated on cloth panels. This could consequently lead to reductions in malaria transmission risks. From the results obtained in this study, use of one treated cloth panel would be preferred to use of two pieces of treated cloth panels and to indoor wall spraying with fungus, because the difference in fungus-infection rates in wild malaria mosquitoes when the three treatment factors were applied was not significantly different. Consequently, use of cloth would be preferred because cotton cloth is widely available, cheap and easy to spray as compared to treating indoor mud walls of a hut with fungus. Additionally, use of one piece of cloth would be economic in terms of the amounts of oil and spores impregnated onto the cloth. Use of cloth ensures that huts remain clean of oil-

based layer on walls. The cloth can also be easily removed and washed before new impregnation can be done.

A field study conducted in rural villages in Tanzania showed that 23% of wild mosquitoes were infected with fungus when one piece of cotton cloth was used (Scholte *et al.*, 2005). This finding is similar to use of one treated cloth panel in the current study. Therefore, fungus-infection rates in wild malaria mosquitoes were similar when one and two pieces of fungus-treated cloth panels were used. However, the difference between the two studies is that the current study assessed infection of both wild *An. gambiae* sensu lato and *An. funestus* mosquitoes by use of *B. bassiana* formulated in CASTROL WOM 14 oil at a dosage of 5.0×10^{10} conidia/m^2, while the field study in Tanzania assessed use of *M. anisopliae* formulated in vegetable oil at a dosage of 3.7×10^{9} conidia/m^2 against *An. gambiae* s.l and *Culex quinquefasciatus*. The study by Scholte *et al.* (2005) was carried out in local houses; hence some of the mosquitoes might have escaped, while the current study was carried out in experimental huts which contained window exit traps in which escaping mosquitoes were caught.

The current study revealed that a small proportion (3%) of mosquitoes from control huts showed fungal growth. This could have occurred because the mosquitoes could have remained undetected in the treatment huts during collection of mosquitoes, prior to switching the treatments or, mosquitoes from treatment huts visited the control hut a night before or after swapping the treatments. Uninfected mosquitoes collected from huts that were provided with treated cloth(s) could have directly flown into the huts without having contacted the treated cloth panels, and if they happened to contact treated panels, time spent on the panels could have been too short for them to pick up substantial amount of conidia.

In a study carried out in a village house in Tanzania, 37.9% (22[8 males and 14 females] of 58 mosquitoes) of *An. gambiae* were infected with *M. anisopliae* when the fungus was applied by a paint brush on part of a wall (Scholte *et al.,* 2005). The infection rate (37.9) was significantly similar to that achieved by the current study (39%). When survival of male and female mosquitoes in Tanzania was compared, the risk of dying for mosquitoes was similar (p=0.811). However, the risk of dying for female mosquitoes was higher (p=0.001) than that of male mosquitoes in the current study. This is the first field study to report the lowest median survival time of fungus-infected mosquitoes (overall Median survival time=2 days and a range of 1-4 days for both infected *An. gambiae* and *An. funestus*). In a previous laboratory study where dry conidia of *M. anisopliae* were used against *An. gambiae*, the overall median survival times of 3-5 days were recorded. Median survival times for male and female mosquitoes were 7.6 and 7.1 days respectively (Scholte *et al.*, 2003). The reduction in the survival time reported in this study indicates that the fungus had a great impact on the survival of wild mosquitoes.

CONCLUSIONS

1. Exposure time, as short as ten minutes can infect and kill mosquitoes within 14 days.
2. Use of higher doses (78% viable spore and above) result in high infection rates and reduced survival of malaria mosquitoes, hence reduced vector population and malaria transmission.
3. The locally available 35% cotton cloth is a poor substrate while 100% cotton cloth is a suitable substrate to disseminate fungus to malaria mosquitoes and kill the mosquitoes.
4. One piece and two pieces of fungus-impregnated cloth panels are equally effective in infection of wild malaria mosquitoes.
5. Indoor wall spraying of a hut with *B. bassiana* formulated in CASTROL WOM 14 oil is not an effective technique to infect wild malaria mosquitoes.

RECOMMENDATIONS

1. Further investigations should be carried out by use of *B. bassiana*-impregnated cloths, by placing odour-baited traps inside the experimental huts so as to attract outdoor-resting mosquitoes.
2. More studies should be conducted to assess cross contamination effect of mosquitoes exposed to fungus-treated surfaces and those from the controls stored in the same cage.
3. Experiments conducted in experimental huts be repeated during the rainy season to compare fungus infection rates, variability in mosquito catches in terms of numbers and proportions of mosquitoes species.
4. One piece of cotton cloth impregnated with *B. bassiana* be included in the integrated vector management programmes, to complement the existing adult vector control tools.

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APPENDIX: ETHICAL APPROVAL FOR THE STUDY

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KEMRI/RES/7/3/1 **April 23, 2010**

TO: **DR. NGUYA KALEMBA MANIANIA,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. JOHN GITHURE,**
HEAD, HUMAN HEALTH DIVISION,
ICIPE

RE: **NON-SSC PROTOCOL NO. 203 (*INITIAL SUBMISSION*): EFFICACY OF ENTOMOPATHOGENIC FUNGI FOR THE CONTROL OF MALARIA VECTORS IN WESTERN KENYA**

This is to inform you that during the 177th meeting of the KEMRI/ERC meeting held on 20th April 2010, the above study was reviewed.

The Committee notes that the above referenced study aims to develop formulations and delivery methods for readily available entomopathogenic fungi which are efficacious, long-lasting and capable of suppressing malaria transmission when applied to entire communities.

Due consideration has been given to ethical issues and the study is hereby granted approval for implementation effective this **23rd day of April 2010,** for a period of twelve (12) months. Kindly submit the translated (into a language readily understood by the community) Informed Consent Document (ICD) for our records.

Please note that authorization to conduct this study will automatically expire on **22nd April 2011.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **11th March 2011**.

You are required to submit any amendments to this protocol and other information pertinent to human participation in this study to the ERC prior to initiation. You may embark on the study.

Yours sincerely,

R.C. Kithinji

R. C. KITHINJI,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICS REVIEW COMMITTEE