Optimization of formulation and delivery technology of entomopathogenic fungi for malaria vector control

Ladslaus L. Mnyone

Thesis committee

Thesis supervisors
Prof. dr. ir. W. Takken
Personal Chair at the Laboratory of Entomology,
Wageningen University

Prof. dr. M. Dicke
Professor of Entomology,
Wageningen University

Other members
Prof. dr. ir. R.H. Wijffels
Wageningen University

Prof. dr. J.M. Vlak
Wageningen University

Dr. G.S. de Hoog,
CBS-KNAW Fungal Biodiversity Centre, Utrecht

Dr. ir. C.J.H. Booij
Plant Research International, Wageningen University and Research Centre

This research was conducted under the auspices of the C. T. de Wit Graduate School for Production Ecology and Resource Conservation

Optimization of formulation and delivery technology of entomopathogenic fungi for malaria vector control

Ladslaus L. Mnyone

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 15 December 2010
at 11 a.m. in the Aula

Ladslaus L. Mnyone (2010)
Optimization of formulation and delivery technology of entomopathogenic fungi for malaria vector control
PhD thesis, Wageningen University – with references – with summaries in Dutch and English
ISBN 978-90-8585-787-7

Table of contents

Abbreviations

ACT	Artemisinin-based combination therapy
ALU	Artemether/lumefantrine
ANOVA	Analysis of variance
a_w	Water activity
Bs	*Bacillus sphaericus*
Bti	*Bacillus thuringiensis* var. *israeliensis*
C:N	Carbon nitrogen ratio
CI	Confidence interval
CSIRO	Commonwealth Scientific and Industrial Research Organization
df	Degrees of freedom
EIR	Entomological inoculation rate
FeEDDHA	Iron chelate of ethylenediamine diacetic acid
GDP	Gross domestic product
HR	Hazard ratios
ICIPE	International Centre of Insect Physiology and Ecology
IHI	Ifakara Health Institute
IHRDC	Ifakara Health Research and Development Centre
IMI	International Mycological Institute
IRB	Institutional Review Board
IRS	Indoor residual spray
ITN	Insecticide treated net
IVM	Integrated vector management
KCl	Potassium chloride
LC_{50}	Conidia concentration required to kill 50% of mosquitoes
LC_{90}	Conidia concentration required to kill 90% of mosquitoes
LLINs	Long lasting insecticide treated nets
LT_{50}	Time it takes for 50% of mosquitoes to die
MSG	Sodium l-glutamate monohydrate
MST	Mean survival time
NIMR	National Institute of Medical Research
OBS	Odour baited stations
PDAY	Potato dextrose agar with yeast extract
PE&RC	Production ecology and Resource conservation
RH	Relative humidity
s.l.	sensu lato
s.s.	*sensu stricto*
SDA	Sabourauds dextrose agar
SE	Standard error
Spp	Species (plural)
SPSS	Statistical Package for the Social Science
SP	Sulphadoxine-pyrimethamine
USA	United States of America
UV	Ultraviolet light
WHO	World Health Organization

Abstract

Vector control is one of the most effective means of controlling mosquito-borne diseases such as malaria. The broad goal of this strategy is to protect individuals against infective mosquito bites and, at the community level, to reduce the intensity of disease transmission. With the deployment of mainly insecticide-treated nets (ITN) and indoor residual spraying (IRS), aided by effective drug treatment, certain countries particularly those within the low endemic zones have documented more than 50% reduction in malaria cases over the past decade. To keep up the pace and expand effective malaria control, in line with the global effort to eliminate malaria, IRS and ITN need to be complemented with alternative control methods. Indeed, neither long lasting insecticide nets (LLINs) nor IRS alone will be sufficient to achieve and maintain interruption of transmission in malaria holoendemic and hyperendemic areas. Besides, the sustainability of both methods is inescapably threatened by mosquito resistance to insecticides. Scientific evidence indicates that biological control based on entomopathogenic fungi has the potential to complement existing vector control methods. Two species of entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana*, have demonstrated ability to infect and kill adult malaria vectors.

This thesis describes the results of a series of laboratory investigations followed by small scale field trials in Tanzania in an area of high malaria endemicity, with abundant populations of the malaria vector *Anopheles gambiae* sensu lato. The overall aim was to optimize fungal formulations, develop delivery techniques that maximize fungus infection rates in wild malaria populations, evaluate impact on survival of these mosquitoes and asses the impact on malaria transmission levels. A series of variables that we hypothesized affect the efficacy and persistence of the fungal isolates *Metarhizium anisopliae* ICIPE-30, *M. anisopliae* IP 46 and *Beauveria bassiana* I93-825 against adult *An. gambiae* were assessed. These included a) conidia concentration ($1 \times 10^7 - 4 \times 10^{10}$ conidia m^{-2}, b) exposure time (15 min - 6 h), c) delivery substrates (netting, cotton cloth & mud wall), d) mosquito age (2 - 12 d), e) time since blood meal (3 - 72 h) as well as f) mosquito behaviour (repellency by conidial formulations). Co-formulations of *M. anisopliae* ICIPE-30 and *B. bassiana* I93-825 in ratios of 4:1, 2:1 & 1:1 were also tested. *Metarhizium anisopliae* IP 46 was exposed to *An. gambiae* and *An. arabiensis* to determine its pathogenicity on these mosquito species before being used for the field trials. Mosquitoes were exposed to fungal formulations applied on paper inside holding tubes, except when different delivery substrates were assessed. For the delivery substrates, sections of netting and black cotton cloth were joined using Velcro strips to fit over 20 × 20 × 20 cm wire frame cages; and mud-lined plywood panels were similarly assembled into 20 × 20 × 20 cm cages. Laboratory experiments were performed using laboratory reared mosquitoes at the Ifakara Health Institute, Ifakara, Tanzania. Following the laboratory experiments, fungal formulations were assayed in experimental hut trials in a field setting at Lupiro village (Ulanga District, Tanzania), a rural hamlet 30 km south of Ifakara. Five different techniques that each exploited the behaviour of mosquitoes when entering (eave netting, eave curtains, eave baffles), host-seeking (cloth strips hung next to bed nets) or resting (cloth panels) were assessed.

The degree at which mosquito survival was reduced varied with conidia concentration; 2×10^{10} conidia m^{-2} was the optimum concentration above which no further reductions in survival were detectable. Co-formulations exerted neither synergistic nor additive effect in reducing mosquito survival. The exposure of mosquitoes to fungal formulations for time periods as short as 15 and 30 min was adequate to achieve 100% mortality of mosquitoes within 14 d post exposure. Longer exposure times did not result in a more rapid killing effect. Conidia impregnated on papers remained infective up to 28 d post application, and such trait did not seem to be influenced by the conidia concentration. Mosquitoes of the age between 2-12 d equally succumbed to fungus infection, with them, however, being relative more susceptible when non-blood fed. Oil-formulations of the fungi did not exhibit any repellency to mosquitoes. *Metarhizium anisopliae* IP 46 was pathogenic to both *An. gambiae* and *An. arabiensis*. Conidia were more effective when applied on mud panels and cotton

cloth compared with polyester netting. Cotton cloth and mud, therefore, represent potential surfaces for delivering fungi to mosquitoes in the field.Two delivery techniques, cotton cloth eave baffles and strips hung next to the bed net were successful in exploiting the behaviour of wild anopheline mosquitoes. Up to 75% of house-entering mosquitoes became infected with fungus applied with either technique. By contrast, eave netting, eave curtains and cotton panels placed next to the bed net were ineffective in infecting mosquitoes with sufficiently high doses of fungi to affect their survival. Based on the survival data of the mosquitoes infected with fungus by means of eave baffles, model estimates indicated that fungus alone can reduce EIR by more than 75%.

In conclusion, these findings indicate that with well-optimized fungal formulations and correctly-designed delivery techniques, a high proportion of house-entering wild malaria mosquitoes can be infected with entomopathogenic fungi to achieve considerable reduction in their survival and possibly malaria transmission. More importantly, these findings provide baseline information that is highly relevant for designing and conducting large-scale field trials to validate the projected impact of fungal infection under realistic field situations.

Chapter 1

General Introduction

Ladslaus L. Mnyone

Introduction

This thesis describes studies aimed at developing entomopathogenic fungi as bio-control agents against mosquito vectors of infectious diseases, malaria in particular. Before addressing the major research question and the specific objectives, an overview of the global burden and epidemiology of malaria is given, describing major malaria parasites and mosquito vectors. The currently available malaria control methods are explained together with their potential limitations, which then clearly emphasize the need for complementary control tools, particularly, those that are less likely to suffer from resistance by parasites and vectors. Biological control agents, which could potentially be an option, have been pointed out, with a major focus on entomopathogenic fungi. Entomopathogenic fungi are most likely to be available for programmatic use within the short-term future.

Malaria burden

Malaria is a major cause of mortality and morbidity in humans, especially in low-income countries (Snow et al. 2005). The disease causes more than 243 million clinical cases and about 863,000 deaths annually, with majority of the cases (85%) and deaths (89%) occuring in Africa (WHO 2009). Children below five years and pregnant women are most at risk (Black et al. 2003; Adam et al. 2005; Lawn et al. 2005). Weak economic growth and investment in Africa has largely been attributed to malaria (Hay et al. 2005). Sachs and Malaney (2002) estimated that from 1980 to 1995 thirty one African countries suffered a combined economic loss of US$ 73.6 billion. The average Gross Domestic Product (GDP) of malarious countries in 1995 was five-fold less than of countries without high malaria incidence (Gallup and Sachs 2001). The economic growth of malarious countries (0.4%/yr) was significantly lower than that of countries without the disease (2.3%/yr) (Gallup and Sachs 2001). Malaria, furthermore, limits trade and foreign direct investments through suppressing linkages between malarious and non-malarious regions (Sachs and Malaney 2002). Malaria-related social consequences include work and school absenteeism, poor scholastic performance, lack of labour force, decline of child care and rise in orphanhood. Sadly, the burden still persists despite huge efforts geared at controlling and possibly eliminating malaria.

Malaria parasites and vectors

Plasmodium falciparum is the most common and clinically serious of the five malaria parasite species that are known to infect humans (Snow and Gilles 2002). Other species include *P. malariae*, *P. vivax* and *P. ovale* and *P. knowlesi*. *Plasmodium knowlesi* is considered a cause of monkey malaria, but has now been associated with several human infections in Asia (Luchavez et al. 2008). Three other Plasmodium species, *P. reichenowi*, *P. schwert* and *P. rodhani*, have been described in African great apes, however, to date, they have been associated with human infections (Duval et al. 2009). *Plasmodium falciparum* is the most widespread, most virulent and life-threatening of the five species. Malaria parasites spread from person to person by the bite of adult female anopheline mosquitoes. To maintain their population, malaria parasites display a complex life cycle that alternates between human and mosquito hosts.

All human malaria vectors belong to the genus *Anopheles* (Diptera: Culicidae). Of over 400 species of *Anopheles* mosquitoes that have been described worldwide, 70 species have been incriminated as vectors of malaria (Service 1993). Members of the *Anopheles gambiae* Giles and *An. funestus* Giles are the most important malaria vectors in Africa (Fontenille and Simard 2004; Okara et al. 2010). Members of the *An. gambiae* complex, synonymous as *An. gambiae* Giles sensu lato are morphologically indistinguishable. Within this complex, species that can transmit malaria parasites include *An. gambiae* Giles *sensu stricto*, *An. arabiensis*, *An. bwambae* White, *An. merus* Dönitz and

and *An. melas* Theobald. *Anopheles gambiae s.s* and *An. arabiensis* are both the most widely distributed and most efficient, particularly in the sub-Saharan Africa (Coetzee et al. 2000; Fontenille and Simard 2004). Where the distribution of both species overlaps, they breed in almost similar habitats and their larvae have the same environmental requirements (Gimnig et al. 2001). Range and relative abundance of *An. arabiensis* and *An. gambiae s.s.* appear to be strongly influenced by climatological factors especially total annual precipitation (Lindsay et al. 2000). Populations of *An. arabiensis* survive the dry season better than *An. gambiae s.s*, whose population peaks shortly after the onset of the rainy season (Lemasson et al. 1997). *Anopheles arabiensis* predominates in arid savannas, while *An. gambiae s.s.* predominates in humid forest zones (Coetzee et al. 2000; Lindsay and Martens 1998; Charlwood et al. 2000). However, it is not uncommon to find the latter species of mosquitoes in savanna areas (Coluzzi et al. 1979; Onyabe and Conn 2001). *Anopheles funestus* is also an important malaria vector in Africa, particularly in areas with large and permanent water bodies i.e. swamps, ponds, marshes and rice fields (Gillies and Coetzee 1987). The larvae of *An. funestus* can survive better in these habitats. Therefore, adult densities of this species are less affected by rainfall, thus are capable of maintaining malaria transmission throughout most of the year. In many parts of tropical Africa, several vector species transmit malaria in each location, in some cases at the same time and in other cases during different seasons (Fontenille et al. 1997). As many as five different anopheline species can function as malaria vectors, either simultaneously or seasonally (Service 1993). *Anopheles gambiae s.s.* is considered largely endophagic, taking blood meals mostly indoors, while *An. arabiensis* shows a greater tendency to feed outdoors. *Anopheles arabiensis* prefers outdoor resting (exophily) after blood feeding (Tirados et al. 2006). As with *An. gambiae s.s.*, also *An. funestus* is an indoor resting mosquito that is highly anthropophilic (Gillies and Coetzee 1987), and both prefer indoor resting after a blood meal. A combination of high susceptibility of *An. gambiae* and *An. funestus* to *P. falciparum* and the endophily/endophagy makes these mosquitoes the most effective malaria vectors globally.

Malaria control

Control strategies basically constitute of the control of malaria parasites and vectors. A variety of anti-malarial drugs are available for the treatment of malaria. For years, chloroquine and other quinoline derivatives have been the drugs of choice for treatment of malaria in endemic countries. However, in the past five years, treatment of uncomplicated malaria has transformed to the use of artemisinin combination therapies (ACT) (WHO 2009). Switching to ACT was spurred by alarming levels of drug resistance to previously used drugs such as chloroquine and sulphadoxine-pyrimethamine (SP) (Dodoo et al. 2009). For example, mainland Tanzania was forced to change its policy in 2001 from chloroquine to SP as the first line drug for treatment of uncomplicated malaria in children, and subsequently in 2007, to artemether/lumefantrine (ALU). Severe malaria is treated with intravenous or intramuscular quinine or, increasingly, the artemisinin derivative artesunate (Dondorp and Day 2007). The introduction of these drugs, however, faces considerable logistic difficulty (Snow et al. 2005). This poses a risk of heightening availability and use of substandard artemisinins and consequently acting as a driving force in the selection of the resistant phenotype. Recently, partial artemisinin-resistant *P. falciparum* malaria has emerged on the Cambodia–Thailand border (Dondorp et al. 2010). As such, a multifaceted containment approach are required, and these may include early diagnosis and appropriate treatment, decreasing drug pressure and optimizing vector control tools (Dondorp et al. 2010). To achieve these, among many other ways, the available drugs and vector control tools need to be highly subsidized or provided for free. In addition, efforts to develop new cheap but effective alternatives should remain a high agenda for continued consideration.

Next to anti-malaria drug use, much research is devoted to the development of malaria vaccines (Kilama et al. 2007). Despite good progress with certain candidate vaccines, for example, RTS, S/AS02D vaccine (Abdulla et al. 2008) there is still no effective vaccine for malaria available to date (Winzeler 2008).

In the absence of vaccines and effective treatment for the majority of mosquito-borne diseases, vector control remains essential as a prevention strategy to disease transmission (Killeen et al. 2002; Utzinger et al. 2001; Lengeler 2004). Vector control reduces malaria transmission by reducing adult vector populations, the life span of adult mosquitoes, and preventing vector-human contacts. The currently available vector control methods include environmental management (Sharma 1987; Liu et al. 2004), biological control (Liu et al. 1996), larviciding (Shililu et al. 2003; Fillinger and Lindsay 2006; Chaki et al. 2009; Geissbühler et al. 2009), zooprophylaxis (Saul 2003; Mahande et al. 2007) and adult insecticides (Roberts et al. 2000; Sharp et al. 2007). Vector control in Africa depends primarily on the use of insecticides and repellents applied via indoor residual spraying (Roberts et al. 2000; Zhou et al. 2010) or insecticide treated bednets (ITNs) (Maxwell et al. 2002; Fegan et al. 2007; Killeen et al. 2007). Insecticide-treated nets are effective in reducing malaria morbidity and mortality (Lengeler 2004), and may provide community protection through mass impact on vector mosquito populations, when used at a high coverage rate (Killeen et al. 2007). Currently, ITNs and IRS are advocated to be the most effective and frequently used control strategies (WHO 2009). Clearly, as effective as these tools are, they are not sufficient on their own to eliminate malaria from intensely endemic regions. Besides, the existence of multiple resistance mechanisms by major malaria vectors against the commonly used chemicals emphasizes a threat to the efficacy of ITN and IRS (Corbel et al. 2007). The development of additional control tools to combat this disease therefore, is necessary.

In seeking replacements for insecticides, industry and government agencies are placing increasing emphasis on alternative strategies that rely less on insecticides (Takken & Knols 2009). One of these concerns biological control agents such as the use of insect pathogens. Despite their potential, these methods have not been considered a key point for the current research direction by the major malaria research institutions. Biopesticides that can be used to control mosquitoes and other insect pests include insects, viruses, bacteria, protozoa, fungi, plants, nematodes and fish (Scholte 2004). Integrated with environmental management, biological control aimed at both the larval and adult stage of mosquitoes may contribute profoundly to mosquito control. There are a few efficient bio-control agents that are able to reduce larval mosquito populations. Larval control using bacterial agents is one of the few methods with potential and has been explored in different places. *Bacillus sphaericus* (Bs) and *B. thuringiensis* var. *israelensis* formulations have been shown to be promising tools for the control of the major vectors of malaria in Africa (Fillinger et al. 2003; Fillinger and Lindsay 2006). Several programmes and large-scale trials in tropical and temperate countries have confirmed the efficacy of *B. thuringiensis* var. *israelensis* (Bti) (Karch et al. 1991; Kumar et al. 1998; Barbarazan et al. 1997). Other larval control methods that have gained attention in addition to bacteria include the use of predatory fish. Almost 200 fish species are known to feed on mosquito larvae (Jenkins 1964). *Gambussia affinis*, however, is the best known and widely distributed mosquito fish. This species of fish played great role in reducing malaria cases in southeastern Turkey (Legner 1995), California (Kramer et al. 1987), India, Afghanistan, and Italy (Bellini et al. 1994). Recently, *Oreochromis niloticus* (Linn.) has been found to be useful in controlling mosquito larvae. The fish was found to cause more than 94% reduction in both *Anopheles gambiae* s.l. and *Anopheles funestus* in the treated ponds, and more than 75% reduction in culicine mosquitoes after 15 weeks (Howard et al. 2007). In tropical Africa the main malaria vectors breed in transient sites created by rainfall, which are difficult to target and cover (Service 1993). It can be argued, therefore, that control of adult mosquitoes might be more useful. Strategies which are under development include engineered densoviruses (Carlson et al. 2006), bacteria such as *Wolbachia* spp (Cook et al. 2008; McMeniman et al. 2009), and entomopathogenic fungi (Blanford et al. 2005; Scholte et al. 2005; Read and Thomas 2007; Farenhorst et al. 2009). Of these strategies, entomopathogenic fungi are most likely to be available for programmatic use within the short-term future.

Entomopathogenic fungi

Entomopathogenic fungi are those fungi that cause the premature death of an insect host (Glare and Milner 1991). Out of over 100,000 species of entomopathogenic fungi thought to exist in different global ecosystems, about 750 have been identified to be pathogenic to insects. Most of these fungi can survive saprophytically in the soil for an extended period of time (Keller and Zimmerman 1989). They can also exist in their susceptible insect hosts, and can cause natural epizootics under favorable environmental conditions. Entomopathogenic fungi are basically classified on morphological characteristics. However, more and more classifications are being reassessed using molecular techniques (Deacon 1997; Driver et al. 2000). The known species of entomopathogenic fungi are distributed over several taxa. These include two kingdoms, 5 divisions and a form division (Scholte et al. 2004). The kingdom Chromista contains one division with entomopathogenic species: Oomycota is the only division under the kingdom Chromista that bears entomopathogenic species. In this division, there are two genera (*Leptolegnia* and *Lagenidium*) with species that are strongly associated with mosquitoes. Other divisions of fungi include Ascomycota, Basidiomycota, Chytridiomycota, and the form-division Anamorphici/Deuteromycota. Class-families which have repeatedly been reported as being pathogenic to insects or which show considerable adaptation to causing disease in insects are Chytridiomycota-Chytridiales, Chytridiomycota-Blastocladiales, Oomycota-Lagendiales, Oomycota-Saprolegniales, Zygomycota- Entomophthorales, Zygomycota-Mucorales, Ascomycota, Deuteromycota and Mycelia sterilia (Samson et al. 1988). Many genera of entomopathogenic fungi are under the family Deuteromycota. This group is largely constituted of hyphomycetes. They mostly have the widest host ranges among the entomopathogens, including various mosquito species. The most important genera of this class of fungus include *Culicinomyces*, *Beauveria*, *Metarhizium* and *Tolypocladium*. Of these, *Beauveria* and *Metarhizium* have received the widest attention in many attempts of developing fungal biological control of different arthropod pests.

Beauveria bassiana is a facultative pathogen, and mostly found as a natural inhabitant in the soil environment (Keller and Zimmerman 1989). This fungus has been used to control pests of a wide variety of crops (Ferron 1981). In laboratory tests, the fungus has proven virulent against larvae of *Culex pipiens*, *Cx. tarsalis* (Thomson), *Cx. tritaeniorhynchus* (Gilles) and *Anopheles albimanus* (Wiedemann) (Clark et al. 1968; Sandhu et al. 1993; Geetha and Balaraman 1999). Also, the fungus has been demonstrated to successfully infect and kill puparia and adults of the Mediterranean fruitfly *Ceratitis capitata* (Wiedemann) (Quesada-Moraga et al. 2006), immature and mature stages of various species of ticks (Frazzon et al. 2000; Onofre et al. 2001; Mwangi et al. 1995; Samish et al. 2004), Triatominae (Luz et al. 1997, 2005; Lecuona et al. 2001) and adult anopheline mosquitoes (Blanford et al. 2005; Farenhorst et al. 2008; Mnyone et al. 2009).

Metarhizium anisopliae is also a soil-borne fungus with a worldwide distribution, and predominantly infects the soil-dwelling insects. The distribution and abundance of many strains of the fungus were found to vary with rainfall, soil type, and pH (Rath et al. 1992). *Metarhizium anisopliae* consists of four varieties of which only two are currently considered important. These are *M. anisopliae* var*. acridum,* found mainly in Homoptera and *M. anisopliae* var *anisopliae*. *Metarhizium anisopliae* has a large host species range, including arachnids and five orders of insects (Boucias and Pendland 1998). *Metarhizium anisopliae* has been extensively studied as a regulatory organism for biological control (Frazzon et al. 2000; Dutra et al. 2004). Although mosquitoes are not listed as natural hosts of this fungus, it has been successfully tested against their larval and adult stages (Farenhorst et al. 2008; Mnyone et al. 2009; Bukhari et al. 2010). This fungus was also found highly virulent in immature and mature stages of ticks (Kaaya et al. 1996; Onofre et al. 2001; Alonso-Dıaz et al. 2007; Bittencourt et al. 1999) and adults of *Anopheles* species (Scholte et al. 2005; Farenhost et al. 2008; Mnyone et al. 2009, Howard et al. 2010).

Although entomopathogenic fungi appear promising candidates for the biological control of mosquitoes, so far studies with mosquitoes and such fungi have been mostly limited to laboratory studies or tightly-controlled field studies.

Justification and problem statement

Malaria remains the pre-eminent parasitic disease and one of the top three killers among communicable diseases in the tropics (Sachs and Malaney 2002, Snow et al. 2005). The control of malaria is largely dependent upon vector control strategies using ITNs and IRS (WHO 2009). Irrespective of the achievements associated with the use of chemical insecticides, in many regions of the world particularly in Africa, the pressure to seek effective alternative control tools continues. The main reasons for this are insecticide resistance (Hemingway et al. 2002; Ranson et al. 2002), elimination of natural enemies and disruption of "natural" ecosystems, and fear by the public that the continued use of insecticides will further contaminate the environment, particularly food and water (Callaghan et al. 2001). There is an urgent need, therefore, to develop novel malaria control strategies that can be used to complement the use of ITNs and IRS. Recently, entomopathogenic fungi have demonstrated to be effective in killing adult mosquitoes under field conditions, thus showing great promise for further development (Scholte et al. 2005). These biocides have advantages over chemical insecticides including a minimum likelihood of host resistance (Ffrench-Constant 2004; Read et al. 2009), cost effectiveness (Ffrench-Constant 2005), and minimum risk to the environment and living organisms (Strasser et al. 2000). Interestingly, entomopathogenic fungi have demonstrated a potential of being used in managing insecticide-resistant populations of adult *Anopheles* mosquitoes (Farenhorst et al. 2009; Kikankie et al. 2010; Howard et al. 2010). Although entomopathogenic fungi appear promising candidates for the biological control less has been done to develop formulations and delivery techniques that can effectively be used to evaluate and apply these agents under field settings. The current thesis research, therefore, was aimed at testing the efficacy of *Metarhizium anisopliae* ICIPE-30, *Beauveria bassiana* I93-825 and *M. anisopliae* IP 46 to develop formulations and delivery methods capable of suppressing malaria transmission in the field.

Objectives of the study

General objective

The broad objective is to provide base-line information and develop formulations of entomopathogenic fungi, which are efficacious and capable of suppressing malaria transmission in real life situations; and to assess their effect on the survival of nocturnal anopheline populations under real life situations.

Specific objectives

The specific objectives were to

1. Optimize the effect of *Metarhizium anisopliae* ICIPE-30 and *Beauveria bassiana* I93-825 formulations against *Anopheles gambiae s.s.*;
2. Determine the pathogenicity of *M. anisopliae* IP 46 against *Anopheles gambiae s.s.*, and *An. arabiensis*;
3. Determine the effect of the mosquito's physiological characteristics (age and blood feeding) on infection with entomopathogenic fungi;

4. Assess the behavioural response (repellency/attraction) of anopheline and culicine mosquitoes to various formulations of entomopathogenic fungi;
5. Determine the efficacy and persistence of formulations of entomopathogenic fungi applied on different delivery substrates;
6. Develop techniques that would effectively expose wild malaria vectors to formulations of entomopathogenic fungi.

The outline of this thesis is as follows: In **Chapter 2** a review of the environmental factors whose effects can impede the use of entomopathogenic fungi as bio-control agents against mosquito vectors is provided. The review collates evidence of how temperature, relative humidity, solar radiation, growth media and water content may constrain the key life cycle and bio-control processes of entomopathogenic fungi. Means that can potentially be used to control such constraints are also explained. **Chapter 3** describes the experiments conducted to study the effect of conidia concentration, co-formulation, exposure time and persistence of the isolates *M. anisopliae* ICIPE-30 and *B. bassiana* I93-925 on infection and survival rates of female *An. gambiae.* **Chapter 4** The success of entomopathogenic fungi against mosquito vectors in any situation may depend on the choice of fungal isolate. Therefore, screening more isolates to identify those with the greatest potential for development is extremely useful. In this chapter bioassays are described in which the pathogenicity of *M. anisopliae* IP 46 (Brazilian isolate) on adult *An. gambiae s.s.*, and *An. arabiensis* was first determined. **Chapter 5** describes experiments which aimed at examining if efficacy of entomopathogenic fungi varied with mosquito age and human blood-feeding, as this information will be essential for designing delivery mechanisms to expose the most susceptible portion of the mosquito population to fungal infection. **Chapter 6** Successful fungal infection depends on the host contacting treated surface and receiving a threshold dose of infective fungal conidia. It is essential thus those mosquitoes are not repelled by the conidia. In this chapter experiments are described in which *An. gambiae s.s.*, *An. arabiensis* and *Culex quinquefasciatus* were exposed to surfaces treated with conidia suspended in mineral oil to determine presence of any repellency effect. **Chapter 7** concerns about the experiments whose aim was to test different surface substrates (mud, polyester netting and black cotton cloth) that can be used to disseminate fungal conidia, and to examine whether the substrates affected the availability, efficacy and persistence of the conidia. **Chapter 8** This chapter describes small scale field experiments that employed experimental huts to test different techniques which could be used to deliver fungal formulations against wild anopheles mosquitoes. Five different techniques were tested: eave netting, cotton cloth eave curtain, cotton cloth panels, cotton cloth eave baffles, and cotton cloth strips hung next to bednets. **Chapter 9** This chapter is the summarizing discussion based on the results of preceding chapters. It discusses the overall implications of the obtained results in relation to the use of entomopathogenic fungi for field control of mosquito vectors. Above that, it provides a set of recommendations on issues that need to be addressed to further improve and upscale the use of entomopathogenic fungi.

Chapter 2

Environmental factors affecting the use of entomopathogenic fungi as bio-control agents for mosquitoes: A review

To be submitted as: Mnyone LL, Koenraadt CJM, Takken W and Kirby, MJ: Environmental factors affecting the use of entomopathogenic fungi as bio-control agents for mosquitoes: A review.

Abstract

Entomopathogenic fungi, particularly of the genera *Metarhizium* and *Beauveria*, have demonstrated a great potential for field use against insect vectors of infectious diseases. The success of these agents, however, depends on how environmental constraints can be overcome, whilst conidial viability and virulence are maintained. An understanding of the interactions between environmental conditions, fungal pathogens and host organisms is essential for improving entomopathogenic fungi and matching them to the micro-climatic conditions at the site of their application. This review collates evidence of how environmental factors may constrain the key life cycle and bio-control processes of these agents. Life cycle processes include germination, growth, host invasion and colonization. Bio-control processes include production, drying, storage and formulation. Means that can potentially be used to minimise or possibly mitigate environmental constraints are discussed. It is argued that the usefulness of entomopathogenic fungi can be enhanced by selecting isolates that are best suited to their intended geo-climatic regions.

Introduction

Vector control remains an important component of malaria control, particularly in Africa where most malaria related deaths occur. Indoor residual spraying (IRS) and insecticide treated bednets (ITNs) are currently the most frequently deployed malaria vector control methods (Rowland et al. 2000; Sharp et al. 2007). Although employment of these methods has led to reductions in the disease, problems associated with chemical insecticides, resistance of mosquitoes in particular, have increased the interest in alternate methods (Blanford et al. 2005, Scholte et al. 2005; Ffrench-Constant 2005). Specifically, the growing interest is on late-acting mortality factors which would certainly help maintain low malaria transmission while minimizing selection for resistance (Ffrench-Constant 2005; Thomas and Read 2007). At present, entomopathogenic fungi hold much promise as one of the late-acting biocides. A range of 'proof of concept' studies in both laboratory and field have demonstrated high levels of infection of adult malaria vectors that are exposed to the fungi (Blanford et al. 2005; Scholte et al. 2005). These agents have the potential to reduce malaria transmission rates, owing to their sub-lethal and lethal effects at different points in the mosquito life cycle. Fungal infection can cause a reduction in the blood feeding rate and life time fecundity (Scholte et al. 2006). There is also evidence that co-infection with a fungal pathogen and malaria parasites can cause greater mortality and reduced transmissibility of the malaria parasite (Blanford et al. 2005). Entomopathogenic fungi may be most effective when combined with established vector control methods in a strategic manner. A theoretical model by Hancock (2009) emphasizes this fact. The model demonstrates that when fungi and ITN are combined they can have a strong effect on malaria transmission even where each of the two methods has little impact. Moreover, the model suggests that the performance of combined interventions would be improved even in areas of high transmission intensity and widespread insecticide resistance. Interestingly, malaria vectors with high resistance levels against the commonly used groups of insecticides (pyrethroids, organochlorines, or carbamates) were shown to be highly susceptible to entomopathogenic fungal infection (Farenhorst et al. 2009; Kikankie et al. 2010). Moreover, fungal infection restored part of the insecticide susceptibility of kdr-resistant anopheline mosquitoes (Farenhorst et al. 2010; Howard et al. 2010) suggesting that fungal infections may extend the lifetime of insecticidal control strategies. All these facts provide more insights into the potential of the use of entomopathogenic fungi against disease vectors. However, one important challenge that needs to be addressed as a pre-requisite for developing this technology for field applications is how to overcome effects of environmental factors on fungal efficacy and persistence. Environmental factors, such as temperature, humidity, solar radiation, growth media, water content as well as the carbon and nitrogen levels of the growth media are critical to bio-control processes (production, drying, storage and formulation) and the life cycle processes (germination, growth, host invasion and colonization) of entomopathogenic fungi (Figure 1). The purpose of this review is, therefore, to collate the available information on the named factors and processes, which can then be used as guidance to improve the efficacy of entomopathogenic fungi, *M. anisopliae* and *B. bassiana* in particular.

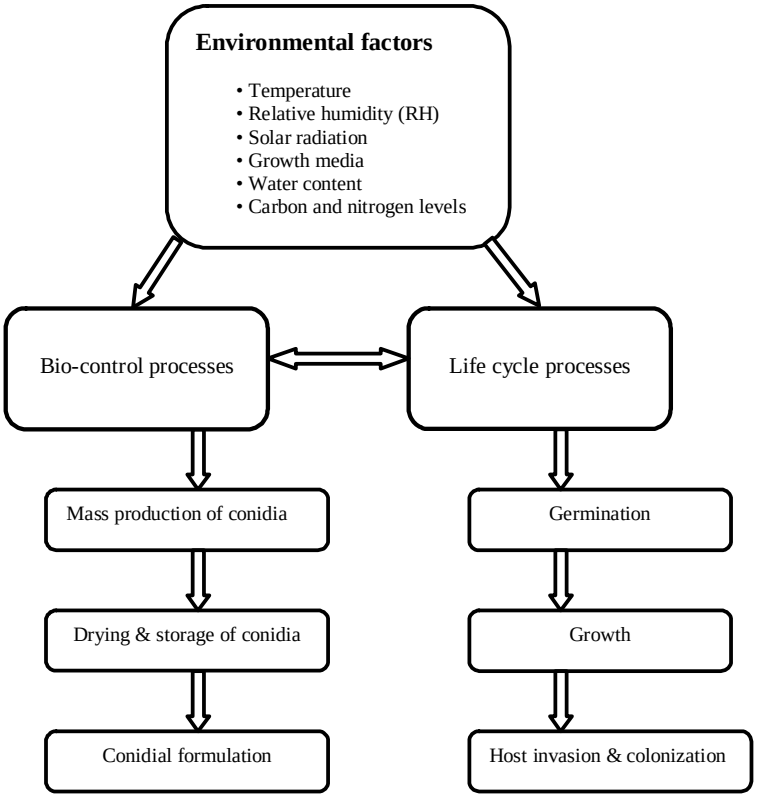


Figure 1: A summary of the environmental factors affecting the bio-control and life cycle processes of entomopathogenic fungi

Conidia germination and growth

Germination of conidia is an essential life cycle process in fungi. This forms the first step in establishing infection of terrestrial arthropods by most entomopathogenic fungi (Luz and Fargues 1997). Conidial germination is strongly influenced by environmental factors, the most important of which are temperature (Dimbi et al. 2004; Rangel et al. 2005; Fernandez et al. 2008a), relative humidity (RH) (Hallsworth and Magan 1999; Fargues and Luz 2000; Luz and Batargin 2005), solar radiation (Braga et al. 2001; Rangel et al. 2006; Fernandez et al. 2008b), and water content of the growth media (Kiewnick 2006).

Temperature

Generally, germination rate increases with temperature until a particular threshold is reached (Fernandez et al. 2008a). Although *M. anisopliae* and *B. bassiana* isolates have shown the ability to germinate at temperatures as low as 5°C, and as high as 35°C and above, maximum germination has consistently been observed within the temperature range of 20°C to 30°C (Ekesi et al. 1999; Fargues and Luz 2000; Dimbi et al. 2004; Fernandez et al. 2008a). Many isolates, however, have been found to germinate faster at 25°C relative to 20°C and 30°C (Luz and Fargues 1997; Dimbi et al. 2004;

Fernandez et al. 2008a). Even with those isolates, germination rate under such extremes is delayed and occurs in very small proportions. The germination rates of six isolates of *M. anisopliae* at the temperatures range of 15-30°C varied between isolates and temperature levels, with fastest isolates being ICIPE-20, -32 and -62 (Dimbi et al. 2004). While germination of 86.6 to 94.9% was observed after incubation at 25°C, less than 5% germination was observed at 5°C incubation. Similarly, Ekesi et al (1999) observed maximum germination (80-100%) for *B. bassiana* and *M. anisopliae* isolates at 25°C, except for *B. bassiana* isolate TP-GHA, which had maximum germination at 30°C. Incubation of *B. bassiana* F-263 at 25°C to 33°C resulted in almost 100% germination within 20h (Shimazu 2004). The incubation of a similar isolate at 34°C delayed germination within a similar time frame but the germination rose to > 90% after 2 d or longer. A similar observation was made by Luz and Fargues (1997) where germination was delayed at 15 and 35°C, but reached more than 95% after 48 h. Overall, *B. bassiana* isolates have been shown to have a wider range of germination temperatures than *M. anisopliae* isolates.

Sub-optimal temperature levels may delay or completely cease germination (Zimmermann 1982; Rangel et al. 2005; Fernandez et al. 2008a) and such scenarios are dictated by the severity of temperatures and the amount of time at which fungal conidia are exposed to such temperatures (Fernandez et al. 2008a). Considering findings by Fernandez and co-workers, the exposure of 60 isolates of *B. bassiana* to 45°C for 1 h separated isolates with medium and low tolerance, whereas exposure for 2 h further separated isolates with medium and high tolerance. As such, the percentage of germination was reduced for every hour of heat exposure, and germination almost ceased after 3 h of exposure (Fernandez et al. 2008a). This study revealed three most thermo-tolerant isolates: CG138, GHA and ARSEF 252. Isolate ARSEF 252 germinated by 90% even after 2 h of exposure to 45°C. The thermal death point of the conidia of the three isolates was recorded at 46°C after 6 h of exposure. Thermal death points as high as 45 °C and 50°C (Liu et al. 2003) and 55°C (Varela and Morales 1996) have been reported in isolates of *B. bassiana* after 10 min of exposure.

Short exposures of entomopathogenic fungi to sub-optimal temperatures have been shown to have a smaller inhibitory effect on germination when this was followed by a return to an optimum temperature (Ruel and Ayers 1999). *Metarhizium anisopliae* and *B. bassiana* isolates evaluated by Fernandez et al (2008a) attained 100% germination after they were transferred to 28°C from temperatures below 5°C. Of twenty nine *B. bassiana* isolates evaluated by Devi et al (2005) fourteen showed >90% germination at a temperature cycle of 35/25°C, whereas when the temperature was increased to 38°C only nine out of the fourteen maintained similar levels of germination. The isolates were exposed to sub-optimal temperatures for 8 h before being transferred to 25°C for germination. Interestingly, at 42±1°C isolate ARSEF 2860 showed ≈78% germination. Particular fungal isolates may be thermo-tolerant but not cold active or the other way round. However, it is not uncommon to find isolates that are both cold active and thermo-tolerant, for example *B. bassiana* ARSEF 252 (Fernandez et al. 2008a).

Similar to germination, optimal growth for many *M. anisopliae* and *B. bassiana* isolates occur at the temperature range of 25 and 30°C (Hallsworth and Magan 1999; Ekesi et al. 1999; Dimbi et al. 2004; Devi et al. 2005). Of this range, however, a temperature of 25°C has most frequently been recorded (Quedraogo et al. 1997, Ekesi et al. 1999; Dimbi et al. 2004). Maximum growth at 30°C has been observed for a few isolates; examples include *M. anisopliae* V90 (Hallsworth and Magan 1999) and *B. bassiana* F-263 (Shimazu 2004). *Metarhizium anisopliae* V90 showed the fastest growth (≈1.7 mm per day) at 30°C, and was surprisingly able to grow at 40°C (Hallsworth and Magan 1999). *Beauveria bassiana* isolate F-263 showed the largest growth rate (50 mm per 14 d) at 30°C (Shimazu 2004). Bugeme et al (2009) also recorded optimum growth at the temperature range of 25 to 30°C for most *M. anisopliae* and *B. bassiana* isolates tested. Fungal growth at temperatures as low as 5°C, and as high as 35°C or above, has been observed in certain isolates; however, at these temperatures the growth rate is greatly reduced. It is around these extreme temperatures that most of the variation in growth amongst fungus isolates is most evident. Fargues et al (1997) recorded maximum thermal thresholds for 65 isolates of *B. bassiana* at the range of ≥30-37°C. The lower threshold temperature

was ≤8°C. Among the 65 isolates, INRA 438 showed the highest growth rate at the optimum temperature, 25°C (6.2 ± 0.06 mm/day). At the same temperature, Dimbi et al (2004) recorded the highest mean growth rate of between 2.1–3.2mm/d for six *M. anisopliae* isolates, with the fastest growing isolates being ICIPE -20, -32 and -62. Although, growth was slower at 15 and 35°C, *M. anisopliae* ICIPE-69, interestingly, had faster growth rates compared with the other isolates. Understanding germination and growth responses in relation to temperature can assist in selecting fungal candidates suited to the intended macro and microclimates. For example, isolates active at temperatures <15°C have been selected for controlling curculionids in temperate regions (Soares et al. 1983), whereas those active at temperatures >28°C may be suitable for warmer regions (McClatchie et al. 1994).

Relative humidity

Relative humidity (RH) influences conidia viability and germination (Daoust and Pereira 1986; Luz and Fargues 1997; Daoust and Pereira 1986). Optimum germination and germ tube elongation occur at RH close to saturation, >95.5% RH (Luz and Fargues 1997; Hallsworth and Magan 1999). At humidities below 95%, the latent period of conidial germination becomes much longer. While germ tube elongation of >30µm after 24 h was observed at 95.5 and 97% RH, it took 72 h at 93% RH for the germ tube to grow to a length of 10 µm (Luz and Fargues 1997). Therefore, entomopathogenic fungi require periods of high humidity or free moisture for sporulation, spore discharge, and invasion of susceptible hosts (Roberts and Campbell 1977; Millstein et al. 1982). The fungus can persist longer in the environment as conidia rather than as mycelium. Conidia have high amounts of saturated fatty acids, trehalose and mannitol which decrease the permeability of the cellular membrane (Pupin et al. 2000; Guerzoni et al. 2001) and prevent denaturation of proteins (Rangel et al. 2006) respectively. Although RH has frequently been considered as a much more critical factor than temperature (Luz and Fargues 1997), optimum RH conditions need to be correctly matched with temperatures to attain a measurable impact by entomopathogenic fungi. Isolates of *M. anisopliae* and *B. bassiana* produced more conidia at ≥95% RH and 20 and 30°C than at ≥95% RH and 15°C (El Damir 2006). This may be the case for most of the *Metarhizium* and *Beauveria* isolates, although slight but important variation exists between both fungal species. At ≥95% RH and 15°C, El Damir (2006) showed conidia production in several isolates of *M. anisopliae* but none in *B. bassiana*. Luz and Fargues (1997) recorded germination at temperatures ranging from 25-30°C under saturated atmosphere. Germination of fungal conidia is dependent not only on the relative humidity and temperature, but also on the amount of time at which they are exposed to such conditions. While germination occurred within 20 h at 95.5% RH, an additional 52 h were required to attain germination at 90% RH (Luz and Fargues 1997).

Water content

Water activity (a_w) (the vapor pressure of water divided by that of pure water at the same temperature) is also a critical factor for conidia germination and growth (Hallsworth and Magan 1999). Studies have demonstrated that many fungi can germinate and grow at water activities of 0.90-0.998a_w (Hallsworth and Magan 1994, 1999; Lazzarini et al. 2006). The optimum a_w is higher than 0.96. Luz and Fargues (1997) observed germination within 16 and 20 h at a_w values ranging from 0.96 to 0.99. Below 0.96 a_w, i.e. 0.93-0.92, germination was markedly delayed and reduced. Intra and interspecies variation has been documented, and has been more apparent at unfavorable a_w levels. For example, Lazzarini et al (2006) found more variation between *B. bassiana* and *M. anisopliae* isolates at 0.93a_w than >0.99 a_w. Tolerance to unfavorable levels of water activity is well linked to the type of growth media. While *M. anisopliae* and *B. bassiana* isolates on Sabourauds dextrose agar developed at a_w of 0.93 (Hallsworth and Magan 1999), that of *M. anisopliae* in liquid media was completely inhibited at a_w higher than 0.96

(Milner et al. 1998). However, it has been shown that germination and growth can be achieved at sub-optimal levels if growth media is amended with certain types of carbohydrates. *Beauveria bassiana* 206 and *M. anisopliae* V90 grown in glycerol-emended media tolerated a wider range of water activity levels than in PEG 600- and KCl-amended media (Hallsworth and Magan 1999). Although a_w of the insect hemolymph is usually high (0.994-0.996), it may decrease during times of starvation and extreme cold (Mullins 1985), or following stress due to fungal infection. Therefore, fungal isolates that are also active at suboptimal a_w levels should be considered as suitable biocide candidates.

Solar radiation

Solar UVA (320 – 400 nm) and UVB (290 – 320 nm) impairs viability of conidia (Moore et al. 1993), probably caused by lethal and mutagenic damage to DNA (Wosten and de Vocht 2000). Conidia can also succumb to indirect effects of solar radiation such as heat stress and desiccation (Rangel et al. 2006). The effect of solar radiation depends on both intensity and length of time on which conidia are exposed to such radiations (Fernandez et al. 2008b). Inter- and intra-specific variation in fungal tolerance to solar radiation has frequently been reported. Fernandez et al (2008b) observed UVB radiation tolerance in *B. bassiana* isolates ranging from 0 to 80% and thus it was possible to separate the isolates into those with low, medium and high tolerance.

Host invasion and colonization

Transmission of fungus infection hinges on sequential completion of conidiogenesis, conidial discharge, propagule dispersal, host contact and invasion. Conidia attach and invade the host's cuticle mainly through host membranes at the joints and between segments (Ferron 1981; Moore-Landecker 1996). These sites have protected folds onto which conidia remain firmly attached. The hydrophobic nature of the conidia wall, surface topography and chemical properties of the cuticle determine how firm conidia adhere onto it (Bidochka et al. 2000). Once the fungus has penetrated and overcome the host's defensive mechanisms, it colonizes and kills the host. If the fungus fails to overcome the host's defense it may still cause sub-lethal effects rather than killing the host. Examples of such sub-lethal effects are reduced feeding propensity and fecundity (Scholte et al. 2006; Ondiaka et al. 2008). Environmental factors affect ability and speed by which entomopathogenic fungi can infect and colonize their hosts. The most important of such factors are temperature and relative humidity. The rate of disease development increases with a temperature increase until an optimum level is reached (Ekesi et al. 1999; Dimbi et al. 2004). Most fungal isolates have shown higher levels of infection at 25°C, 30°C and 35°C than at 20°C (Dimbi et al. 2004; Bugeme et al. 2009). Ekesi et al (1999), however, showed that some isolates of *B. bassiana* and *M. anisopliae* were highly virulent at 20°C when infecting the legume flower thrips, *Megaluruthrips jostedti* (Trybon). Yeo et al (2003) observed more rapid mortality at temperatures of 18°C and 23°C relative to 10°C when adult aphid species, *Aphis fabae* (Scopoli) and *Myzus persicae* (Sulzer) were exposed to *B. bassiana* and *M. anisopliae* isolates. Bugeme et al (2009) recorded mortalities between 54.4 and 100% at 25°C, 30°C and 35°C compared to mortalities between 8.8 and 72.5% at 20°C, 4 d post infection of adults of the spider mite *Tetranychus urticae* (Koch) by *B. bassiana* and *M. anisopliae* isolates. At 20°C, 100% mortality was observed within 7 and 9d compared to 4d at the temperatures of 25°C, 30°C and 35°C (Bugeme et al. 2009). Dimbi et al (2004) recorded mortalities between 48.8 and 100% at temperatures of 25°C, 30°C and 35°C compared to a mortality of 26% at 20°C, 4d post infection. In this study the adults of tephritid fruit flies, *Ceratitis capitata* (Wiedemann), *C. fasciventris* (Bezzi) and *C. cosyra* (Walker) were exposed to *M. anisopliae* isolates.The speed of fungus-induced mortality, fungal isolate, host species and temperature interact with each other. At 20°C the isolate *M. anisopliae* ICIPE-18 caused a higher mortality than isolates ICIPE-32, -62, -41 and -42, whereas, at 25°C isolates ICIPE-32 and -62 were superior to the rest.

For different host species, at 25°C, *C. cosyra* had the shortest LT_{90} with isolates ICIPE-41 and -62, whereas, *C. fasciventris* had the shortest LT_{90} with isolate ICIPE-32 (Dimbi et al. 2004). Thermal constraints are not only the result of ambient conditions, but may be also achieved through host thermoregulation. Active elevation of body temperature in poikilotherm insects, which can be achieved through habitat selection or basking in the sun (Chappell and Whitman 1990; Heinrich 1993), is a common response in pathogen-infected insects. The ability to thermoregulate provides a considerable therapeutic advantage for the host by slowing the overall rate of disease development (Roy et al. 2006). Grasshoppers, caterpillars (Carruthers et al. 1992), and house flies (Watson et al. 1993) reduced the effects of fungal infection by this means.

Pathogenic activity of entomopathogenic fungi is, in addition to temperature, influenced by relative humidity. High levels of infection have almost always been reported at humidity levels close to saturation (≥95% RH) (Ferron 1981; Luz and Fargues 1998; Fargues and Luz 2000). Fargues and Luz (2000) observed a high dependence of *B. bassiana* INRA 297, highly virulent in *Rhodnius prolixus*, on humidity requiring a moisture threshold of ca. 96%. High and low levels of disease transmission were reported at 93% and 100% RH, respectively (Lazzarini et al. 2006). Similarly, while up to 100% mortality was observed at 98% RH, it did not exceed 25% after incubation at humidity levels of 75 and 43% RH (Lazzarini et al. 2006). Due to discrepancies in terms of environmental requirement for the infection process, it is important to focus research on the interactive effect of different factors. Temperature and humidity, individually or together, determine fungal disease incubation and dose mortality responses. *Schistocerca gregaria* locusts exposed to *M. anisopliae* var *acridum* showed mortality rates that correlated with conidial dose temperatures ranging from 15 to 30°C. However, raising temperature to 35°C resulted in far lower mortality and no correlation with dose (Arthurs and Thomas 2001). Shi et al (2008) found a significant effect of RH on fungal action at 20 and 25°C, but not at 30°C, whereas the effect of temperature was significant at 51 and 74% RH, but not at 95% RH. In a similar study, egg mortalities of 62.5 – 87.5% at high conidial concentration and of 48.9 – 66.6% at the medium concentration varied with temperature and humidity regimes.

To have reliable bio-control agents, we need to link *in vitro* and *in vivo* studies rather than relying only on *in vitro* findings. The host's cuticle presents a very different substrate for fungal germination and growth, thus it is difficult to use results from *in vitro* studies to predict *in vivo* host-pathogen interactions accurately. So far, there are only few studies correlating *in vitro* and *in vivo* performance of entomopathogenic fungi. In such studies, however, the existence of a correlation between conidial germination and growth with pathogenicity is still controversial. Samuels et al (1989) found a positive correlation between isolates, which germinated rapidly and their pathogenicity to *Nilaparvata lugens*. Such correlation has also been reported in other studies (Doberski 1981; Latch and Kain 1983; Matawele et al. 1994). In contrast, Lazzarini et al (2006), Drummond et al (1987), Devi et al (2005) and Bugeme et al (2009) reported a negative correlation between speed of fungal germination and growth with pathogenicity. For example, *M. anisopliae* isolate ICIPE-279 that had a radial growth on medium of 0.6 mm/d at 20°C, caused 72.5% mortality in *Tetranychus evansi*, while the isolate *M. anisopliae* ICIPE-55 with a higher growth rate (2.1 mm/d) caused only 26.3% mortality (Bugeme et al. 2009). There might be a trade-off between growth and pathogenicity of entomopathogenic fungi.

The effect that temperature and humidity have on fungal infection is not only a question of optimal and suboptimal conditions, but also the length of exposure to such conditions. Pathogenic activity of *B. bassiana* on *R. prolixus* was greatly reduced when the time of exposure to optimum relative humidity (≥95%) declined from 12 to 8 h per day (Fargues and Luz 2000). However, daily exposure of the insects infected with fungus to high humidity for at least 12 hours was sufficient for fungus infection and induced mortality. Also, good mortality levels can still be achieved when infected insects are transferred from an adverse to a conducive environment. This suggests that under alternating environmental conditions between day and night for example in the tropics, still considerable infection levels can be achieved.

Mass production

The microbial agents intended for use should be able to produce high amounts of inocula (El Damir 2006; Goettel and Roberts 1992) with ensured virulence, and compatible to formulation and application technology (Jenkins et al. 1998). There are three methods indicated for mass production of entomopathogenic fungi: *in vivo*, submerged and surface culture production. For sustainable use of entomopathogenic fungi in vector control, the focus should be on low-cost production techniques and methods that will ensure high yields of high-quality conidia (Jenkins et al. 1998). Depending on the production methods and conditions, three types of conidia can be produced: aerial conidia, submerged conidia and blastospores. For field use for example in malaria endemic areas where weather conditions are much more severe, submerged conidia are preferable. These conidia have added environmental stability compared to blastospores (Soper and Ward 1981). Understanding the influence of the environmental factors on processes such as germination, growth, sporulation and other production stages is vital to the success of any production system. The effects of temperature, humidity and solar radiation in relation to germination and growth have been discussed in the previous sections.

The type of culture media influences fungal germination and growth (Silman 1993), thus affecting the quantity and quality of the conidia produced (Zhao and Shamoun 2006). A variety of growth media are used for mass production of *M. anisopliae* and *B. bassiana* species. Examples include rice (Daoust and Roberts 1983), maize, crushed grain (Shashi et al. 1999) or bran (Dorta et al. 1990), hemp (M. Jumbe, unpublished data), sugar cane bagasse (Rossi et al. 2003) rice powder, compost and ground corn (Hussy and Tinsley 1981), whole cowpea grain (Shashi et al. 1999) or preserved cadavers of arthropod hosts (Luz and Fargues 1998). Even though fungi can grow on such different media, variation in terms of conidia quantity and quality exist when grown on such types of media (El Damir 2006). *Metarhizium anisopliae* and *B. bassiana* produced relatively large quantities of conidia when grown on rice as compared to wheat and barley (Nelson et al. 1996). However, the highest yield of viable conidia was observed in wheat. Another study recorded more conidia of *B. bassiana* being produced on rice than on cadavers of chinch bugs *Blisssus antillus* Leonard (Hemiptera-Lygaeidae). In that respect, 14.9×10^7 conidia per insect and 10.6×10^9 conidia g^{-1} rice were obtained (Samuels and Coracini 2004). The type of production may also influence the production of toxins within a fungal strain. Wang et al (2004) demonstrated that *M. anisopliae* strains V245 and V275 did not produce destruxins in large scale fermenter or on solid agar, but these toxins were detected when the fungus was grown on rice and in liquid filtrate. For practical reasons, however, cheap and easily acquired growth media would be more appropriate. More so, if such growth media can produce substantial levels of conidia when used in small quantities either singly or in combination. About 5–15 times more conidia were produced when *M. anisopliae* was grown on rice bran and rice husk substrate mixtures than on rice grains (Dorta et al. 1990).

The water content of the growth media (El Damir 2006) also influences fungal production. The water content of the media determines growth, quality and quantity of conidia (Hajek et al. 1990; El Damir 2006). Since different growth media may require different volumes of water (Goettel and Roberts 1992), media types need to be appropriately correlated with water volumes. Depending on the combination of growth medium and water content, differences of up to 240 times per combination were recorded in the production of *M. anisopliae* and *B. bassiana* (El Damir 2006). High conidia production levels were observed at water contents ranging from 1:0.35 to 1:0.60 (substrate: water) (Goettel and Roberts 1992) and 1:0.30 to 1:1.20 (substrate: water) (Magalhaes and Frazao 1996).

The carbon and nitrogen ratio (CN) of the growth medium can affect conidia yield, germination rate, tolerance to desiccation, and storage stability (Cliquet and Jackson 2005). Media with CN ratio of 30:1 produced more conidia than media with CN ratio of 10:1 or 80: 1. These conidia had correspondingly high germination rate, freeze drying tolerance and storage stability (Cliquet and Jackson 2005).

Storage

Conidial storability and longevity is considerably affected by aspects of the conidia production environment such as duration of incubation and rate of drying (Hong et al. 2000). Even minor changes in production methodology can significantly affect the quality of the conidia produced. Fungus may be stored in a dry form or stored as liquid stock cultures. To maintain the quality, both forms need to be stored under optimal storage conditions. Conidia viability declined steadily at 19°C and 0%, while almost no loss in viability occurred at >1 year of storage at 19°C and 97% RH (Daoust and Roberts 1983). Storage at 4°C and 0% RH is also recommended (Roberts and Panter 1985). Regular passage through insects is an important aspect of preservation especially in stock cultures as this process maintains virulence of the fungus; a single passage may increase virulence by almost 2.5 times (Daoust and Roberts 1982). Storage of fungi shows some peculiarities, i.e. transition to pleomorphism (marked variation in shape and size of the cells) as well as contamination by bacteria, mites or other fungi. They, therefore, need to be perfectly and appropriately maintained. The common methods proposed for maintaining fungal cultures are subculturing, suspension in mineral oils, storage in distilled water and lyophilization. Of these, lyophilization has been considered to be ideal. Many strains of fungi can be preserved for up to 23 years by lyophilization if an appropriate lyophilization medium such as skimmed milk is used (Ellis and Roberson 1968). Quangqiang et al (1998) recorded survival rates of fungal cultures of 89.7% and 87.2% for the cultures preserved over a period of 12 years by lyophilization and distilled water, respectively. Storage of filamentous fungi, yeasts and anaerobic actinomycetes cultures in distilled water at 25°C maintained high survival (93%), even after 12-60 months of storage (McGinnins et al. 1974). Drying of conidia is another method that helps to maintain conidial survival and infectivity on storage. While nearly 100% germination was observed on *M. anisopliae* CG423 conidia that were pre-dried after >100 d desiccation, <50% germination was observed in non-dried ones (Magalhaes and Boucias 2004). For the dry conidia, drying techniques, which are less detrimental to the conidia's storage properties need to be employed. The optimal moisture content for storage of *Metarhizium flavoviride* IMI 330189 was found to be ≈ 4% (Moore et al. 1995). This optimum may vary with fungal isolates, and, therefore, specific optima may need to be determined for other isolates. Available drying techniques include vacuum drying, spray drying and lyophilization. Quiangqiang et al (1998) recorded a higher viability and infectivity of vacuum-dried conidia (98%) than lyophilized conidia (49.8%). The main challenge is that the processed organism has to survive both the temperatures that prevail during the drying process and the drying speed (Daemen and Van der stege 1982). As inter and intra-species differences exist, the formulation process must be designed carefully for the particular requirements and limitations of each particular organism (Rhodes 1993). A maximum of 18 months shelf life has been recommended (Couch and Ignoffo 1981), but the criterion may be modified depending on circumstances (Alves et al. 1996).

Formulation

There is a great potential for improving the infectivity and persistence of fungal pathogens as well as their reliable and sustainable field use if formulations are designed to meet a variety of objectives including to i) prevent activity losses during storage; ii) facilitate convenient and efficacious delivery of the agent; iii) promote insect-biocontrol agent compatibility; and iv) improve the effectiveness of the biocontrol agent delivered by enhancing persistence and infectivity. Inclusion of different substances targeted towards controlling stress factors can substantially improve shelf life and efficacy of fungal pathogens on storage and more importantly after application. Conidia are more efficacious when formulated in oil than water (Luz and Batagin 2005). Oil as carrier offers better adhesion and spreading of the formulation on the lipophilic insect cuticle. Oils form a film on the cuticle which retains moisture, thus creating good conditions for elevated numbers of conidia to germinate and invade the host under sub-optimal humidity (Kooyman and Godonou 1997; Luz and Batagin 2005). Oil improves the tolerance of conidia to extreme temperatures (McClatchie et al. 1994). Commercial adjuvants and

additives have shown a potential to improve the persistence of bio-control agents. Examples include Orzan LS (lignin derivative, sodium ligninsulphoate) and skimmed milk (Jackson and Schisler 1996, Behle et al. 1999). Also, using low cost lignin has been reported to potentiate tolerance of bio-control agents against UV radiation (McGuire et al. 2001). These insights can aid in improving fungal formulations. Before particular adjuvants and additives are used with the aim of improving conidial persistence, they should be evaluated for cost effectiveness, and any adverse effect to target and non-target organisms. The interaction between fatty acids and other components of the oil may have either stimulatory or inhibitory effects on fungal development (Luz and Batagin 2005). For example, sesame oil is toxic to the adults of rice weevil *Sitophilus oryzae* (Uttam et al. 2002). Certain oils may be repellent to insects (Begum and Quiniones 1990), in which case they would interfere with the efficacy of fungal formulation since for fungus infection to occur insects need to contact the formulation. Non-repellent or attractive oils without conidiocidal effects would be appropriate for use. Incorporating natural or synthetic chemicals that can attract mosquitoes would equally improve the effectiveness of fungal formulations.

Windows for improvements

The available literature discusses options which could be useful in improving entomopathogenic fungi against the constraints of environmental factors. A few of those options have been discussed in the previous sections.

Growth substrates and nutritional environment can be manipulated to produce more and persistent conidia (Jennings 1993; Rangel et al. 2006). Conidia produced on a medium with arabinose, fructose, glucose, myo-inositol, lactose or mannitol were shown to be at least two times more UV tolerant than conidia producuced on the rich medium potato dextrose agar with yeast extract (PDAY) (Rangel et al. 2006). This is basically exerting nutritional stress, which in turn activates stress-determining genes, which render the produced conidia stress tolerant. Like any other organisms, when the fungus faces stressful conditions, more resistant structures are formed. This is what Jennings (1993) refers to as phenotypic plasticity, and argues that the phenomenon is well known to botanists but rarely considered explicitly by mycologists. Indeed, there have been few attempts to improve the physiological quality of propagules by exploiting phenotypic plasticity of fungi. Under stress, conidia accumulate increased levels of trehalose and polyols (e.g. trehalose, mannitol, inositol, erythritol, polyhydric alcohols). Indeed, trehalose and polyol compounds have been associated with resistance to environmental extremes, accelerated germination, enhanced pathogenicity and improved storage life of fungal propagules (Gornova et al. 1992; Hallsworth and Magan 1995, 1996; Harman et al. 1991). The entomopathogenic fungi, *Aspergillus japonicus* and *Trichoderma harzianum*, retained viability during storage better with increased trehalose than those fungi containing less trehalose (Harman et al. 1991; Gornova et al. 1992). Many reports show that the efficacy of entomopathogenic fungi is limited at low water availability and humidity (Walstad et al. 1970; Moore 1972). Interestingly, manipulation of intracellular polyols has also been found to extend the range of water availability over which fungal propagules can germinate (Hallsworth and Magan 1995). Conidia containing up to 164.6 mg glycerol plus erythritol g^{-1} germinated down to 0.887_{aw} (Hallsworth and Magan 1995). Polyols such as erythritol and glycerol prevent enzyme inhibition due to dehydration, which otherwise restricts conidia germination (Carpenter and Crowe 1988). Trehalose replaces water in membranes at reduced water activity (Crowe and Crowe 1993). Mean trehalose and polyol content declines with increasing age of the culture regardless of fungal species. Therefore, the appropriate harvest time should be identified. Da Costa and Niederpruem (1980) showed that the total polyol content gradually increased within 10 d incubation. Utility of intracellular compounds, conditions required for their accumulation and ideal incubation period may vary between fungal species; therefore, a specific optimization of formulations is necessary. Importantly, there is a tendency to treat each factor separately but this approach is doomed because it implies acceptance of independently acting factors; appreciation of interaction of different factors is extremely critical (Hallsworth and Magan 1996).

Formulation can be used to improve the efficacy and persistence of entomopathogenic fungi. There are additives known to protect and increase the storability of micro-organisms. These include wheat flour (Connick et al. 1991), milk powder (Tadayyon et al. 1997), sodium l-glutamate monohydrate (referred to as MSG) and sucrose (Friesen et al. 2006), trehalose (Elbein et al. 2003), iron fertilizer containing the iron chelate of ethylenediamine diacetic acid (FeEDDHA) (Stover-Muller and Sauerborn 2007), starch and oil (Quimby et al. 1999) as well as extrusion and micro-encapsulation in alginate (Winder et al. 2003). In addition, conidia may also benefit from the additives by utilizing their nutrients during rehydration, thus maintaining stability, which is one of the key determinants of micro-organisms storability (Friesen et al. 2006).

Co-formulations of different isolates have the potential to provide better and more consistent control than a single-strain formulation. Strains with two or more different characteristics could be evaluated for ability to complement each other through synergistic or additive effects (Mnyone et al. 2009). Pathogens with moderate but long lasting pathogenicity could be combined with those with high but short lasting pathogenicity. Formulation containing multiple strains of bacteria (*Enterobacter cloacae* and *Pseudomonas fluorescens*) was shown to perform much better than individual strains did, when subjected to various storage environments (Slininger et al. 2001). *Enterobacter cloacae* and *Pseudomonas fluorescens* differ in their range of antibiotic production, substrate utilization, and growth temperature optima. Research on co-formulations between different isolates of entomopathogenic fungi is worthy considering.

Though it is not always that the origin of fungal isolates relates to their thermo and UV radiation tolerance, there could be an added advantage of screening isolates originating from the environment where control is intended or from any other area with similar environmental conditions. Shimazu (2004) recorded higher optimal growth temperatures in isolates from warmer regions.

Conidial pigmentation, particularly green, has been shown to influence tolerance of fungi to UV radiation (Kawamura et al. 1999; Braga et al. 2006). Braga et al (2006) showed revertants with *M. anisopliae* green conidia, DWR 179 and DWR 176 to be relatively more tolerant to solar radiation than purple and yellow coloured conidia.

Applying fungal products during favorable conditions e.g. during seasons of high humidity and/or rains (Luz et al. 1994), which usually coincide with rises in mosquito population density would heighten the development of fungal infection on mosquitoes. Exploring the possibility of integrating this technology with other available control measures such ITNs and IRS remains necessary to ensure protection during seasons unfavorable to entomopathogenic fungi.

Information on mosquito host-seeking and resting behaviour as well as breeding habitat availability also need to be considered since they will dictate how and where such vectors should be targeted and what sort of improvements need to be performed. Microclimatic data measured in domestic and peri-domestic habitats of the targeted mosquito vectors in order to correctly match certain isolates of fungus to such environments need not to be underestimated. Low persistence of fungal conidia particularly after application has been highlighted as a major problem, which, may impede their use as bio-control agents (Scholte et al. 2005). With exclusive targeting of the indoor biting and resting mosquitoes as with many if not all of the adult mosquito control methods conidia will be less exposed to the environmental extremes than would be with outdoor targeting. However, we should probably be keen at developing products that can be used in either way since not all mosquito vectors rest indoors (e.g. *Anopheles arabiensis*), and even for the indoor resting ones there can be a change in behaviour e.g. spending less time indoors (Jimenez-Arredondo et al. 1997; Pates and Curtis 2005) or completely switching to outdoor resting. Besides, with good mosquito attractants that are being developed the idea will certainly be to use outdoor baited traps which could be impregnated with fungus (lure and kill) (Okumu et al. 2010).

Conclusion

The future of entomopathogenic fungi for use against mosquitoes is bright, but optimization of formulations and delivery technology (this thesis) is necessary in developing them to more robust control tools. Moreover, solutions to key environment related ailments require research contributions from a variety of disciplines including entomology, microbiology, biochemical engineering and formulation science.

Chapter 3

Infection of the malaria mosquito, *Anopheles gambiae s.s.*, with two species of entomopathogenic fungi: effects of concentration, co-formulation, exposure time and persistence

Published as: Mnyone LL, Kirby MJ, Lwetoijera DW, Mpingwa MW, Knols BGJ, Takken W, Russell TL: Infection of the malaria mosquito, *Anopheles gambiae s.s.*, with two species of entomopathogenic fungi: effects of concentration, co-formulation, exposure time and persistence. *Malaria Journal* 2009, **8:** 309

Abstract

Isolates of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* have been shown to infect and reduce the survival of mosquito vectors. Here four different bioassays were conducted to study the effect of conidia concentration, co-formulation, exposure time and persistence of the isolates *M. anisopliae* ICIPE-30 and *B. bassiana* I93-925 on infection and survival rates of female *Anopheles gambiae sensu stricto*. Test concentrations and exposure times ranged between 1 × 10^7 - 4 × 10^{10} conidia m^{-2} and 15 min - 6 h. In co-formulations, 2 × 10^{10} conidia m^{-2} of both fungus isolates were mixed at ratios of 4:1, 2:1, 1:1,1:0, 0:1, 1:2 and 1:4. To determine persistence, mosquitoes were exposed to surfaces treated 1, 14 or 28 d previously, with conidia concentrations of 2 × 10^9, 2 × 10^{10} or 4 × 10^{10}. Mosquito survival varied with conidia concentration; 2 × 10^{10} conidia m^{-2} was the concentration above which no further reductions in survival were detectable for both isolates of fungus. The survival of mosquitoes exposed to single and co-formulated treatments was similar and no synergistic or additive effects were observed. Mosquitoes were infected within 30 min and longer exposure times did not result in a more rapid killing effect. Fifteen min exposure still achieved considerable mortality rates (100% mortality by 14 d) of mosquitoes, but at lower speed than with 30 min exposure (100% mortality by 9 d). Conidia remained infective up to 28 d post application but higher concentrations did not increase persistence. Both fungus isolates are effective and persistent at low concentrations and short exposure times.

Background

The control tools currently deployed to reduce malaria transmission in Africa are early diagnosis and prompt treatment, insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS) (Rowland et al. 2000; Sharp et al. 2007). Although these strategies have delivered reductions in childhood disease (Abdulla et al. 2001; Masanja et al. 2008), there remains a high incidence of malaria in many countries, with over 300 - 500 million infections and 1 million deaths each year (Snow et al. 2005). The factors responsible for continuing transmission include the development of *Plasmodium* resistance to drugs (Talisuna et al. 2004), *Anopheles* resistance to insecticides (Hargreaves et al. 2000; Etang et al. 2006), and socio-economic or cultural resistance to control measures (Greenwood et al. 2005). Clearly, as effective as the current tools are, they are not sufficient on their own to eliminate malaria from intensely endemic regions (Killeen et al. 2000). Therefore, additional control tools are needed to combat this disease.

Laboratory and small-scale household studies have demonstrated a great potential to develop entomopathogenic fungi for field control of malaria vectors (Farenhorst et al. 2008, Stevenson 2008). The fungi penetrate the mosquito cuticle through mechanical pressure and/or enzymatic degradation of major cuticle components (Moore-Lendecker 1996; Gillepsie et al. 2000). Once inside the host, the fungi propagate, consuming nutrients and releasing metabolites resulting in mycosis and death (Clarkson and Charnley 1996). In the laboratory, the entomopathogenic fungus *Beauveria bassiana* was able to reduce the number of adult anopheline mosquitoes capable of transmitting malaria by a factor of approximately 80 (Blanford et al. 2005), and one field study on a household scale has shown that *Metarhizium anisopliae* ICIPE-30 can cause a two-fold reduction in the life span of adult mosquitoes (Scholte et al. 2005).

Before entomopathogenic fungi can be scaled-up for use in biological control programmes, it is essential to determine the optimum concentration of conidia (asexual spores shed at maturity) to apply, whether co-formulations offer any advantage over single isolate applications, the exposure time required for conidia to infect mosquitoes, and whether there is a relationship between concentration and persistence. The optimum concentration will be identified as the lowest concentration that is able to achieve the maximum reduction in survival time for each fungal species. Co-formulations could have a synergistic or additive effect due to the different life histories of the fungi involved, or inter-species interactions such as competition-altering activity. Determining the minimum exposure time for infection will guide in developing realistic field dissemination tools having pre-defined whether the intervention can target either host-seeking or resting mosquitoes or both. Examining persistence will allow reapplication rates to be defined. Holistically, this information will provide a sound indication on the viability of this technology for field control of mosquitoes. The study was, therefore, designed to address these issues in assays of the fungal isolates *M. anisopliae* ICIPE-30 and *B. bassiana* I93-925 against adult *Anopheles gambiae sensu stricto*. These isolates were chosen because of their proven efficacy, availability in the market, and minimum risks to non-targeted organisms.

Methods

Mosquito rearing and maintenance

Anopheles gambiae s.s. mosquitoes were reared at the Ifakara Health Institute (IHI), Tanzania. The colony was established from a population caught near Njage village, 70 km from Ifakara, in 1996. Larval and adult stages of the mosquitoes were raised using methods based on those described by Huho et al (2007). Bioassays were conducted using 3-6 d old non blood fed adult female mosquitoes. During all experiments, mosquitoes were supplied 9% glucose solution.

Fungal isolates, formulation and application

Two entomopathogenic fungi species were used in all bioassays: 1) *Metarhizium anisopliae* var. *anisopliae* isolate ICIPE-30, isolated originally in 1989 from the maize stalk borer, *Busseola fusca* (Lepidoptera, Noctuidae) in Western Kenya, and imported as dry conidia from Wageningen University, The Netherlands and 2) *Beauveria bassiana* isolate 193-825 (IMI 391510), isolated from a chrysomelid beetle (Coleoptera) in the USA and imported as dry conidia from the Commonwealth Scientific and Industrial Research Organization (CSIRO), Australia and Penn State University, USA. Before use each batch of conidia was checked for viability by inoculation on Sabouraud dextrose agar (SDA) plates, and only conidia with ≥ 85% germination were used in bioassays.

The conidia were formulated in oil for application. Oil protects conidia from adverse environmental conditions and facilitates adhesion to the insect cuticle. Initially, a fungal stock solution was prepared by suspending 1-2 g of conidia in 20 ml of highly refined mineral oil (Shell Ondina 917®, Shell, The Netherlands) or Enerpar (Enerpar M002®, BP Southern Africa Ltd). To homogenize the mixture it was shaken vigorously, vortexed for 25 sec and then sonicated (ultrasonic bath, Langford Electronics, UK) for 3 min. Dilutions of 1:10, 1:100 and 1:1000 were prepared in oil and the concentration of conidia was calculated using a Neubauer Haemocytometer (Hausser Scientific, USA) with the aid of a compound microscope (Leica ATC2000, USA) at 400× magnification. The solution was adjusted to the desired concentrations for application by diluting with mineral oil. Neither Ondina nor Enerpar oils had any negative effect on conidia germination or mosquito mortality (unpublished data). Unless otherwise stated, the conidia used during the bioassays were formulated in Ondina oil.

Mosquitoes were exposed to conidia applied to sheets of A4 printing paper within plastic exposure tubes (8.2 cm diameter × 12.5 cm height), closed with netting also treated with conidia. The paper and netting were treated using a hand-held pressure sprayer (Minijet®, SATA, Germany) operating at a constant pressure of 2 bars. The nozzle of the spray gun was held 50 cm away from, and at a right angle to, the application surface. A working solution of 23 ml containing the desired conidia concentration was applied to a 1 m^2 area. Treated surfaces were left to dry for 24 h. To avoid cross-contamination, formulations of each fungal isolate were applied in separate rooms.

Bioassay procedures

30-50 adult *An. gambiae s.s.* mosquitoes were introduced to the exposure tubes and held for 6 h (unless stated otherwise in bioassay descriptions below), after which they were transferred to separate untreated cages (9 × 9 × 9 cm) and maintained at 26-27°C and 85-95% relative humidity (RH) with access to 9% glucose solution *ad libitum*. Survival was monitored daily for a maximum of 28 d. Dead mosquitoes were collected individually and put onto moist filter paper in Petri dishes, sealed with parafilm, and incubated at 26-27°C and 85-95% RH for 3-4 d, after which they were examined for evidence of fungal sporulation basing on colour of their conidia. *Metarhizium anisopliae* yields green conidia, whereas, *B. bassiana* yields white conidia. Similar bioassay procedure was carried out for 30-50 mosquitoes in control groups, except that they were exposed to untreated surfaces. During all of the bioassays, four replicates were used for each experimental factor. Four different bioassays were conducted to study the effect of 1) concentration, 2) co-formulation 3) exposure time and 4) persistence on infection and survival of *A. gambiae s.s.*

Bioassay 1: concentration

Mosquitoes were exposed to six different concentrations of the two fungal isolates: (1×10^7, 2×10^8, 1×10^9, 2×10^9, 2×10^{10} and 4×10^{10} conidia m^{-2}). Concentrations were chosen basing on their reported efficacy against mosquitoes and other arthropods, and likelihood of being cost effective.

Bioassay 2: co-infection with M. anisopliae and B. bassiana

Mosquitoes were exposed to co-formulations of 2×10^{10} conidia m^{-2} of both fungus isolates mixed at ratios of 4:1, 2:1, 1:1, 1:0, 0:1, 1:2 and 1:4.

Bioassay 3: exposure time

Mosquitoes were exposed to 2×10^{10} conidia m^{-2} for four different lengths of time: 15 min, 30 min, 1 and 6 h. In a separate experiment two concentrations, 2×10^{10} and 4×10^{10} conidia m^{-2}, formulated in Enerpar oil, were evaluated at 15 and 30 min exposure to determine whether concentration affected time required for infection.

Bioassay 4: persistence

The residual activity of conidia was determined by exposing mosquitoes to the same treated surfaces 1, 14 and 28 d post application. Treated surfaces were kept at 26-27°C and 85-95% RH in-between exposure rounds. Three different concentrations formulated in Enerpar oil (2×10^9, 2×10^{10} and 4×10^{10} conidia m^{-2}).

Statistical analysis

Mosquito survival data were analysed by Kaplan-Meier pair wise comparison using SPSS version 15. Data were stratified by replicate and multiple chi-square pair-wise comparisons were used to examine the effect of treatment on mosquito survival. Survival curves were considered not statistically different at $p > 0.05$. A probit regression (R package version 2.9.1) was used to determine the concentration of conidia required for 50% and 90% mortality 10 d after exposure (lethal concentration: LC_{50} and LC_{90}, Bioassay 1). The Bonferroni method was employed to compensate for multiple comparisons.

Results

Bioassay 1: concentration

Concentrations of 2×10^9 conidia m^{-2} and above of both *M. anisopliae* ICIPE-30 and *B. bassiana* I93-825 resulted in 85-95% mortality of exposed mosquitoes after 10 d. This was higher than 36 ± 3.4% mortality recorded from untreated control after 10 d (Figure 1). The daily survival of mosquitoes exposed to any fungal concentration was significantly reduced compared to that of controls ($p < 0.001$; Figure 1; Table 1). The median survival time (MST ± SE) of control mosquitoes was 11 ± 0.06 d. The MST of fungus-exposed mosquitoes was similar for both isolates and ranged between 4.0 ± 0.09 d at

4×10^{10} to 10 ± 0.47 d at 1×10^{7} for *B. bassiana*. There was no difference between 2×10^{10} and 4×10^{10} for *M. anisopliae* (4.0 ± 0.11, 4.0 ± 0.09 d, $X^2 = 3.54$, $p = 0.07$) or *B. bassiana* (4.0 ± 0.12, 4.0 ± 0.08 d, $X^2 = 3.56$, $p = 0.06$; Table 1). As such, optimum reduction in survival was considered to be reached at a concentration of 2×10^{10} conidia m^{-2} (Figure 2). The concentrations of conidia that were modelled to result in 50% and 90% (LC_{90}) mortality were 1.02×10^{7} (LC50) and 9.77×10^{8} conidia m^{-2} (LC_{90}), *M. anisopliae* and 7.71×10^{7} (LC_{50}) and 2.66×10^{9} conidia m^{-2} (LC_{90}) for *B. bassiana*.

Bioassay 2: co-infection with M. anisopliae and B. bassiana

The survival of mosquitoes exposed to co-formulated conidia was significantly reduced compared with control mosquitoes ($p < 0.001$, Figure 3, Table 2). However, survival was not further reduced compared with the single fungus strain ($p > 0.001$, Table 2). The MST of mosquitoes exposed to co-formulations and single species formulations ranged between 5 - 6 d, while that of untreated controls was 13 d.

In all the co-formulation treatments, sporulation of both fungus species from the same cadaver was never observed. *Metarhizium anisopliae* sporulation only was observed in 78.17% (376/481) of the mosquito cadavers. No sporulation was observed on control mosquitoes.

Bioassay 3: exposure time

The exposure concentration used (2×10^{10}) was selected on the basis of Bioassay 1 results. The ability of conidia of both fungi to kill and reduce the survival of mosquitoes was dependent on the length of exposure. The MST of mosquitoes after a 30 min exposure and above was significantly lower than that after 15 min for both *M. anisopliae* ($p < 0.001$) and *B. bassiana* ($P < 0.001$, Figure 4, Table 3). However, considerable mosquito mortality was still achieved with 15 min exposure (100% by 14 d), but at lower speed than with 30 min exposure (100% by 9 d). Nonetheless, 93.5% and 96.2% mortality was recorded when mosquitoes were exposed for 15 min to *B. bassiana* and *M. anisopliae* by 9 d. The MST for fungus-exposed mosquitoes ranged between 5 - 6 d. Survival of mosquitoes exposed to either *M. anisopliae* or *B. bassiana* for 15 min and above was significantly reduced as compared to controls ($p < 0.001$, Table 3).

In a separate bioassay where conidia concentrations of 2×10^{10} and 4×10^{10} were compared, at each exposure time; 15 and 30 min, the two concentrations equally reduced mosquito survival. This was observed for *M. anisopliae* ($X^2 = 0.63 - 2.92$, $p > 0.05$) and *B. bassiana* ($X^2 = 0.76 - 5.23$, $p > 0.05$), (Figure 5).

Bioassay 4: persistence

Overall, the effect of *M. anisopliae* ($p < 0.001$) and *B. bassiana* ($p < 0.001$, Tables 4 and 5) on mosquito survival declined over time regardless of the conidia concentration used. For *M. anisopliae*, survival of mosquitoes exposed to each concentration 1 d post application were lower than survival of mosquitoes exposed to similar concentrations 14 and 28 d post application ($p < 0.001$). No difference, however, was observed for mosquitoes exposed to each concentration at 14 and 28 d post application ($p > 0.05$, Table 4). For *B. bassiana*, survival of mosquitoes exposed to each concentration 1 d post application was lower than survival of mosquitoes exposed to same concentrations 28 d post application ($p < 0.001$). No difference however, was observed for mosquitoes exposed between 1 and

14d, as well as 14 and 28 d post application ($p > 0.05$, Table 5). Concentration did not tend to influence the decline in conidia persistence ($p > 0.05$, Figure 6). The MSTs of mosquitoes exposed to 2×10^9 conidia m^{-2} of either isolate of fungus at all time intervals post application ranged between 11 - 13 d, while that of 2×10^{10} and 4×10^{10} ranged between 6 - 12 d. The survival of mosquitoes exposed to fungus was always lower than that of controls ($p < 0.001$, Tables 4 and 5). The MSTs of controls ranged between 15 - 16 d.

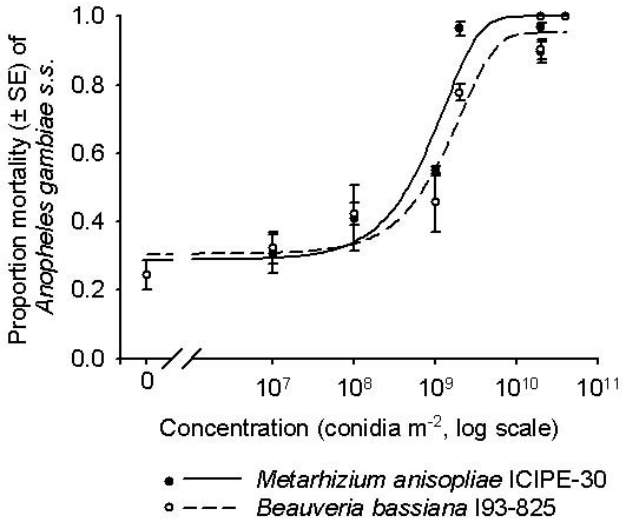


Figure 1: *The percentage mortality of An. gambiae s.s. mosquitoes 3 - 6 d of age, 10 days post exposure to different concentrations of M. anisopliae ICIPE-30 and B. bassiana I93-825. Controls were not exposed to any fungus ('0' concentration). Mosquitoes were exposed to the treatments for 6 h. The sigmoidal models were fitted to the data using probit regression.*

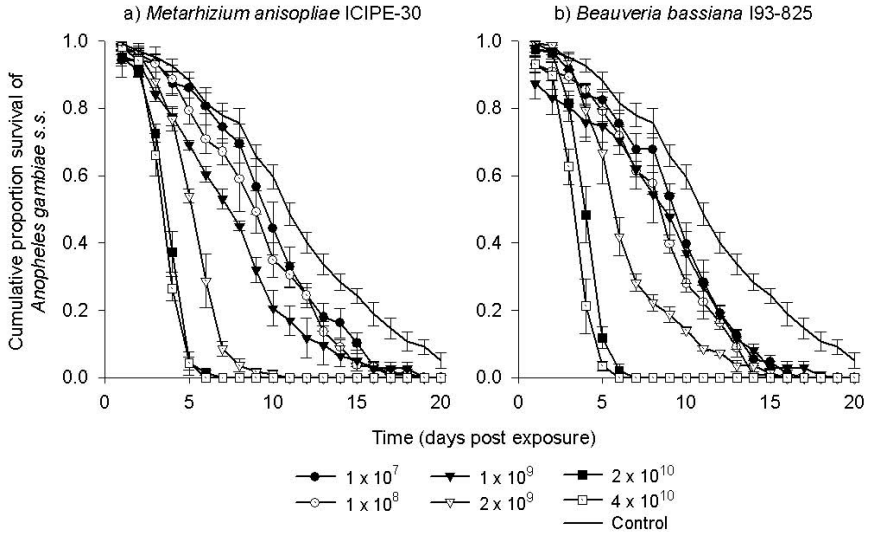


Figure 2: *The survival of Anopheles gambiae s.s. females after 6 h exposure to different concentrations (1×10^7, 1×10^8, 1×10^9, 2×10^9, 2×10^{10} and 4×10^{10} conidia m^{-2}) of a) Metarhizium anisopliae ICIPE-30 and b) Beauveria bassiana I93-825.*

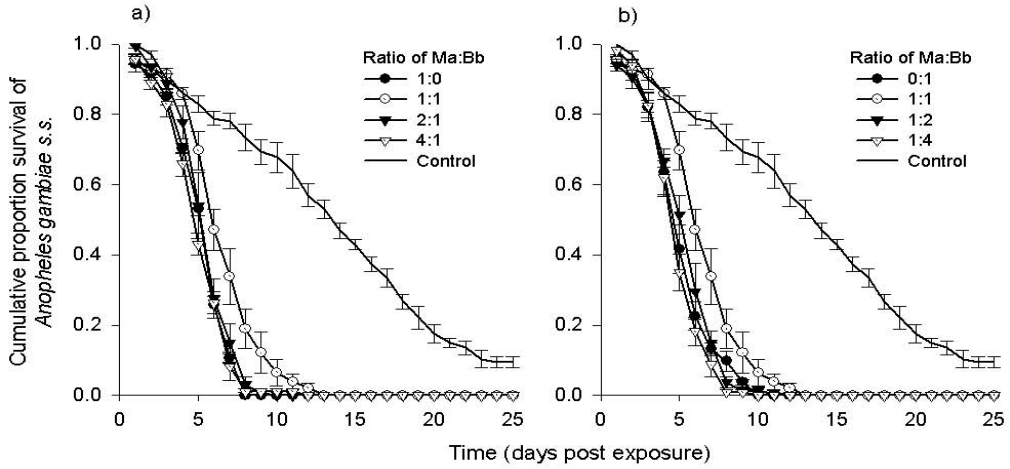


Figure 3: *The survival of Anopheles gambiae s.s. females after exposure to 2 × 10^{10} conidia m^{-2} of co-formulated Metarhizium anisopliae ICIPE-30 and Beauveria bassiana I93-825 under laboratory conditions.* The co-formulation was applied at different ratios of conidia (1:0, 1:1, 2:1 and 4:1) of *M. anisopliae* to *B. bassiana*.

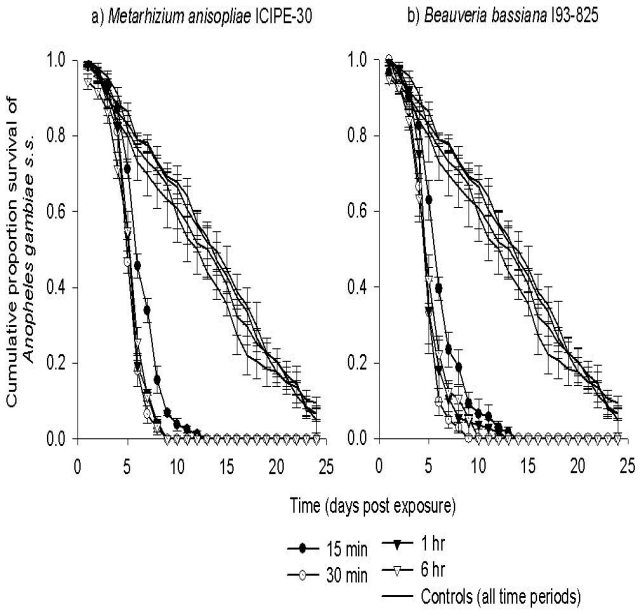


Figure 4: *The survival of Anopheles gambiae s.s. females after exposure to 2 × 10^{10} conidia m^{-2} of a) Metarhizium anisopliae ICIPE-30 and b) Beauveria bassiana I93-825 for different times (15 min, 30 min, 1 h and 6 h).*

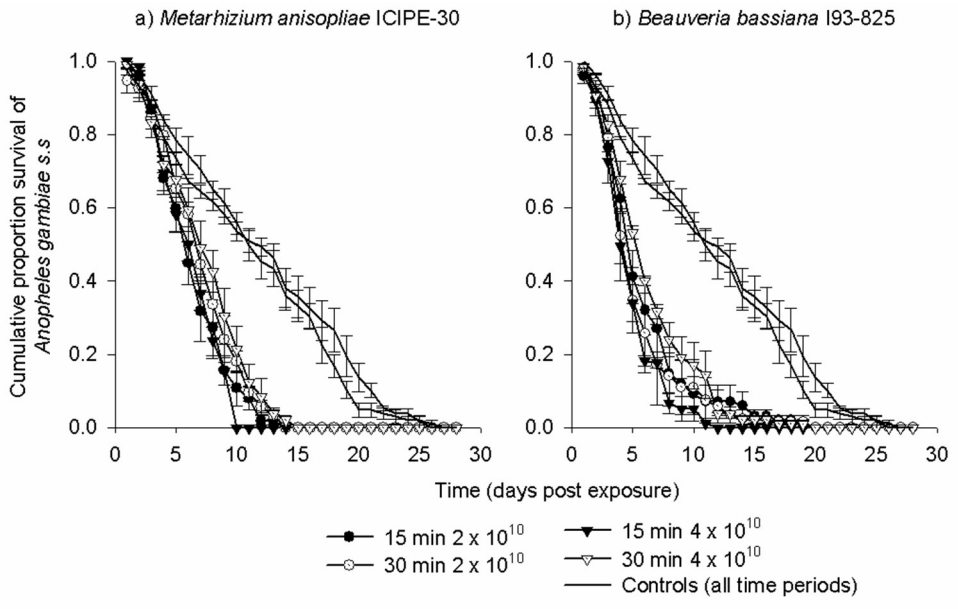


***Figure 5**: Survival curves of Anopheles gambiae s.s. fungus treated and non-treated surfaces. The survival of Anopheles gambiae s.s. females after exposure to 2 × 10^{10} and 4 × 10^{10} conidia m^{-2} of Metarhizium anisopliae ICIPE-30 and Beauveria bassiana I93-825 for different times (15 and 30 min).*

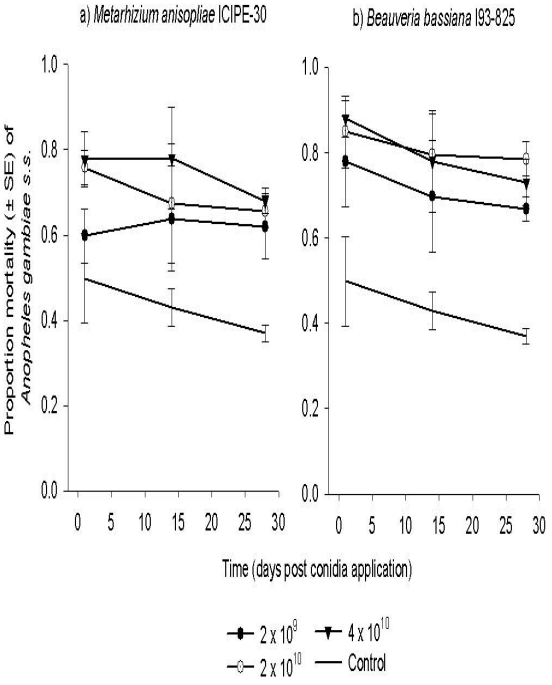


***Figure 6:** Percentage mortality of mosquitoes 12 d post exposure to different concentrations (2 × 10^{9}, 2 × 10^{10} and 4 × 10^{10} conidia m^{-2}) of a) Metarhizium anisopliae ICIPE-30 and b) Beauveria bassiana I93-825 after storage of the treated materials for 1, 14 and 28 d. Treated materials were stored under 26°C-27°C temperature and 85-95% humidity before successive re-exposures.*

Table 1: Kaplan Meier pair-wise comparisons of the median survival times (MST) of *An. gambiae s.s.* females exposed to different concentrations (1×10^7, 1×10^8, 1×10^9, 2×10^9, 2×10^{10} and 4×10^{10} conidia m^{-2}) of *M. anisopliae* ICIPE-30 and *B. bassiana* I93-825 (IMI 391510) for 6 h.

Concentrations	MST±SE	1×10^7	1×10^8	1×10^9	2×10^9	2×10^{10}	4×10^{10}
M .anisopliae							
Control	11 ± 0.32	χ^2 = 15.12 *p* <0.001	χ^2 = 24 *p* <0.001	χ^2 = 50.7 *p* <0.001	χ^2 = 195.11 *p*<0.001	χ^2 =239.68 *p*<0.001	χ^2 = 261.26 *P* <0.001
1×10^7	10 ± 0.32		χ^2 = 1.87 *p* = 0.17	χ^2 =14.46 *p* <0.001	χ^2 =138.37 *p*<0.001	χ^2 = 197.25 *p* <0.001	χ^2 = 219.10 *p*<0.001
1×10^8	9 ± 0.37			χ^2 =5.95 *p*<0.001	χ^2 = 98.86 *p*<0.001	χ^2 =166.80 *p* <0.001	χ^2 = 188.48 *p* <0.001
1×10^9	8 ± 0.43				χ^2 = 53.99 *p* <0.001	χ^2 =119.57 *p*<0.001	χ^2 = 104.37 *p* <0.001
2×10^9	6 ± 0.15					χ^2 = 79.62 *p* <0.001	χ^2 = 104.37 *p* <0.001
2×10^{10}	4 ± 0.12						χ^2 = 3.54 *p* = 0.07
B .bassiana							
Control	11 ± 0.32	χ^2 = 25.09 *p* <0.001	χ^2 = 47.61 *p* <0.001	χ^2 = 27.13 *p* <0.001	χ^2 = 109.37 *p* <0.001	χ^2 = 251.31 *p*<0.001	χ^2 = 251.60 *p* <0.001
1×10^7	10 ± 0.47		χ^2 = 4.53 *p* <0.001	χ^2= 0.319 *p* = 0.57	χ^2 = 38.97 *p* <0.001	χ^2=171.18 *p* <0.001*	χ^2 = 181.16 *p* <0.001
1×10^8	9 ± 0.23			χ^2 = 1.47 *p* = 0.22	χ = 21.40 *p* <0.001	χ^2=158.71 *p* <0.001	χ^2 = 168.32 *p*<0.001
1×10^9	9 ± 0.58				χ^2 =21.90 *p* <0.001	χ^2=112.07 *p* <0.001	χ^2 = 114.26 *p* <0.001
2×10^9	6 ± 0.17					χ^2 = 88.29 *p* <0.001	χ^2=130.05 *p* <0.001
2×10^{10}	4 ± 0.11						χ^2 = 3.56 *p* = 0.06

Table 2. Kaplan-Meier pair wise comparisons of the median survival times (MST) of *An. gambiae s.s.* females exposed to 2×10^{10} conidia m^{-2} of co-formulated *Metarhizium anisopliae* ICIPE-30 and *Beauveria bassiana* I93-825. The co-formulation was applied at different ratios of conidia (1:0, 1:1, 2:1 and 4:1) of *M. anisopliae* to *B. bassiana*.

Ratio of Ma:Bb	MST±SE	1:0	1:1	2:1	4:1
i) Increasing ratio of *M. anisopliae*					
Control	13 ± 0.73	χ^2 = 132.57 *p* <0.001	χ^2 = 109.63 *p* <0.001	χ^2 = 134.20 *p* <0.001	χ^2 = 121.23 *p* <0.001
1:0	6 ± 0.12		χ^2 = 0.15 *p* = 0.70	χ^2 = 0.29 *p* = 0.60	χ^2 = 1.37 *p* = 0.24
1:1	6 ± 0.21			χ^2 =0.65 *p* = 0.34	χ^2 = 2.59 *p* = 0.07
2:1	6 ± 0.16				χ^2 = 0.53 *p* = 0.46
ii) Increasing ratio of *B. bassiana*					
Ratio of Ma:Bb	MST±SE	0:1	1:1	1:2	1:4
Control	13 ± 0.73	χ^2 = 128.01 *p* <0.001	χ2 = 109.63 *p* <0.001	χ2 = 133.22 *p* <0.001	χ^2 = 114.34 *p* <0.001
0:1	5 ± 0.19		χ^2 = 1.63 *p* = 0.20	χ2 =0.004 *p* = 0.93	χ2 = 0.29 *p* = 0.59
1:1	6 ± 0.21			χ^2 = 0.29 *p* = 0.59	χ^2 = 3.10 *p* = 0.08
1:2	6 ± 0.19				χ^2 = 3.24 *p* = 0.06

Note: The co-formulation was applied at different ratios of conidia (1:0, 1:1, 2:1 and 4:1) of *M. anisopliae* to *B. bassiana*.

Table 3: Kaplan-Meier pair wise comparisons of the median survival times (MST) of *An. gambiae s.s.* females exposed to *M. anisopliae* ICIPE-30 and *B. bassiana* I93-825 (IMI 391510) for different exposure times (15 min, 30 min, 1 h and 6 h).

Exposure time	MST±SE	15 min	30 min	1 h	6 h
M. anisopliae					
Control	13 ± 0.32	χ^2 = 124.1 *p* <0.001	χ^2 = 154.65 *p* <0.001	χ^2 = 149.59 *p*<0.001	χ^2 = 150.98 *p* <0.001
15 min	6 ± 0.32		χ^2 = 36.89 *p* <0.001	χ^2 = 26.07 *p*<0.001	χ = 28.39 *p* <0.001
30 min	5± 0.37			χ^2 = 1.15 *p* = 0.28	χ^2 = 0.57 *p* = 0.44
1 h	5±0.43				χ^2 = 0.86 *p* = 0.77
B. bassiana					
Control	13 ± 0.32	χ^2 = 118.5 *p* <0.001	χ^2 = 97.98 *p* <0.001	χ^2 = 79.84 *p* <0.001	χ^2 = 103.81 *p* <0.001
15 min	6 ± 0.17		χ^2 = 42.68 *p* <0.001	χ^2 = 16.28 *p* <0.001	χ^2 = 14.97 *p* <0.001
30 min	5 ± 0.11			χ^2 = 0.50 *p* = 0.23	χ^2 = 0.29 *p* = 0.59
1 h	5 ± 0.10				χ^2 = 0.06 *p* = 0.81

Table 4: Kaplan-Meier pair wise comparisons of the median survival times (MST) of *An. gambiae s.s.* females exposed to surfaces 1, 14 and 28 d after treatment with different concentrations (2×10^9, 2×10^{10} and 4×10^{10} conidia m^{-2}) of *M. anisopliae* ICIPE-30.

Comparison of:		Different concentrations within same time post-application			The same concentration between different times post-application	
	MST±SE	2×10^9	2×10^{10}	4×10^{10}	14 d	28
1 d						
Control	15 ± 0.95	χ^2 =15.15 *p* <0.001	χ^2 = 43.34 *p* <0.001	χ^2 = 55.58 *p* <0.001	χ^2 = 0.08 *p* = 0.77	χ^2 = 0.01 *p* = 0.32
2×10^9	11 ± 0.51		χ^2 = 9.32 *p* <0.001	χ^2 = 15.44 *p* <0.001	χ^2 = 5.17 *p* <0.001	χ^2 = 4.10 *p* <0.001
2×10^{10}	6 ± 0.46			χ^2 = 0.001 *p* = 0.98	χ^2 = 9.80 *p* <0.001	χ^2 = 8.95 *p* <0.001
4×10^{10}	8 ± 0.39				χ^2 = 14.74 *p* <0.001	χ^2 =18.55 *p* <0.001
14 d						
Control	16 ± 1.15	χ^2 = 30.48 *p* <0.001	χ^2 = 58.07 *p* <0.001	χ = 49.02 *p* <0.001		χ^2 = 0.43 *p* = 0.54
2×10^9	12 ± 0.55		χ^2 = 6.59 *p* <0.001	χ^2 = 4.08 *p* <0.001		χ^2 = 0.06 *p* = 0.80
2×10^{10}	10 ± 0.64			χ^2 = 0.21 *p* = 0.22		χ^2 = 0.49 *p* = 0.83
4×10^{10}	10 ± 0.45					χ^2 = 0.003 *p* = 0.96
28 d						
Control	16 ± 1.15	χ^2 = 35.94 *p* <0.001	χ^2 = 77.49 *p* <0.001	χ^2 = 62.29 *p* <0.001		
2×10^9	13 ± 0.33		χ^2 = 11.22 *p* <0.001	χ^2 = 5.76 *p* <0.001		
2×10^{10}	11 ± 0.49			χ^2 = 0.56 *p* = 0.46		
4×10^{10}	11 ± 0.61					

Table 5: Kaplan-Meier pair wise comparisons of the median survival times (MST) of *An. gambiae s.s.* females exposed to surfaces 1, 14 and 28 d after treatment with different concentrations (2×10^9, 2×10^{10} and 4×10^{10} conidia m^{-2}) of *B. bassiana* I93-825.

Comparison of:		Different concentrations within same time post-application			The same concentration between different times post-application	
	MST±SE	2×10^9	2×10^{10}	4×10^{10}	14 d	28
1 d						
Control	15 ± 0.95	$\chi^2 = 5.73$ $p < 0.001$	$\chi^2 = 20.48$ $p < 0.001$	$\chi^2 = 16.50$ $p < 0.001$	$\chi^2 = 0.08$ $p = 0.77$	$\chi^2 = 0.01$ $p = 0.32$
2×10^9	11 ± 0.42		$\chi^2 = 7.03$ $p < 0.001$	$\chi^2 = 5.01$ $p < 0.001$	$\chi^2 = 0.61$ $p = 0.44$	$\chi^2 = 4.09$ $p < 0.001$
2×10^{10}	9 ± 0.55			$\chi^2 = 0.24$ $p = 0.63$	$\chi^2 = 1.74$ $p = 0.19$	$\chi^2 = 6.36$ $p < 0.001$
4×10^{10}	9 ± 0.79				$\chi^2 = 0.09$ $p = 0.77$	$\chi^2 = 6.03$ $p < 0.001$
14 d						
Control	16 ± 1.15	$\chi^2 = 29.08$ $p < 0.001$	$\chi^2 = 52.46$ $p < 0.001$	$\chi^2 = 49.02$ $p < 0.001$		$\chi^2 = 0.43$ $p = 0.54$
2×10^9	12 ± 0.44		$\chi^2 = 4.23$ $p < 0.001$	$\chi^2 = 7.04$ $p < 0.001$		$\chi^2 = 5.12$ $p < 0.001$
2×10^{10}	10 ± 0.67			$\chi^2 = 0.37$ $p = 0.54$		$\chi^2 = 1.79$ $p = 0.18$
4×10^{10}	10 ± 0.45					$\chi^2 = 0.40$ $p = 0.53$
28 d						
Control	16 ± 1.15	$\chi^2 = 47.61$ $p < 0.001$	$\chi^2 = 34.67$ $p < 0.001$	$\chi^2 = 55.76$ $p < 0.001$		
2×10^9	13 ± 0.42		$\chi^2 = 7.56$ $p < 0.001$	$\chi^2 = 4.92$ $p < 0.001$		
2×10^{10}	12 ± 0.54			$\chi^2 = 0.249$ $p = 0.11$		
4×10^{10}	12 ± 0.64					

Discussion

These experiments were designed to provide preparatory information necessary for the use of oil-formulated entomopathogenic fungi in field-based mosquito control. Due to the expense and logistics involved in applying any insecticidal agent on a large-scale, it is essential to first define the concentration and time required to infect and kill mosquitoes. Information concerning persistence is also needed to determine re-application rates.

Low conidia concentrations and short exposure times can result in small infective doses that can be countered by immune responses. Insect responses to entomopathogens involve melanization, encapsulation and phagocytosis of invading fungal blastospores (Bogus et al. 2007), but it is likely that these responses can be overcome at high concentrations. In these experiments it was found that for both fungal species concentration was positively correlated with mortality, and that the maximum and most rapid reductions in mosquito survival were achieved at concentrations of 2×10^{10} conidia m^{-2} and above. With well standardized production systems, formulations, application methods and delivery tools, a concentration of 2×10^{10} conidia m^{-2} can be operationally amenable. The efficacy of *M. anisopliae* in terms of mortality against *An. gambiae* s.s. was slightly, although not dramatically, higher than that recorded by Scholte et al (2003), and these differences may be related to the use of mineral oil to formulate the conidia, instead of sunflower oil. Additionally, the quality of the conidia batch, the method of application and the target species can also impact on the efficacy of fungal application.

For example, in this study the efficacy of these two fungal species against *An. gambiae s.s.* was comparable, yet when a similar range of conidial concentrations were tested against *Anopheles stephensi*, *B. bassiana* was found to be much more effective than *M. anisopliae* (Stevenson 2008). Overall, a similar positive relationship between conidial concentration and mortality, as observed here, is also evident in the published literature (Thomas and Jenkins 1997; Scholte et al. 2003; Stevenson 2008); it is difficult to directly compare studies because of differences in fungal isolate, oil formulation, target species, substrates onto which conidia are applied, bioassay protocols and units used to express conidial concentration.

When *M. anisopliae* and *B. bassiana* were applied as a co-formulation against *An. gambiae s.s.*, neither an additive nor synergistic effect was evident. Similar results have been found when entomopathogens were evaluated in combination against arthropods other than mosquitoes (Leal-Bertioli et al. 2000; Wang et al. 2002; Maranga et al. 2005; Rao et al. 2006). The initial interaction between the two fungal species occurs at the point of mosquito exposure. Even if conidia of both species adhere to the mosquito cuticle, a competitive advantage would be gained if one of the fungi was faster to invade and colonize the mosquito haemocoel. Following colonization, the successful fungus could prevent other fungi from becoming established by competitive exploitation, limiting resource availability, actively synthesizing and releasing inhibitory metabolites or stimulating host immune responses (Read and Taylor 2001). Considering that exposure to the co-formulation had no additive effect, it is likely that the activity of one fungal species was partially or completely redundant. The complete absence of co-sporulation and the predominance of *M. anisopliae* suggest a competitive advantage of *M. anisopliae* over *B. bassiana*. Additive (Thomas et al. 2003) and synergistic (Inglis et al. 1999; Malakar et al. 1999) effects of co-infection have been recorded for other entomopathogens at sub-optimal temperature regimes. Thus the possibility remains that fluctuating temperature and or relative humidity either in the laboratory or field may affect co-formulations of *M. anisopliae* and *B. bassiana*.

The length of time required for conidia to infect and kill mosquitoes is an important consideration for developing dissemination tools for field use. The exposure times tested in the current study were selected to represent realistic exposure periods. Mosquitoes may spend up to 15 min trying to enter a bed net (Clements 1992) and after blood-feeding may rest on a surface for up to 24 h (Gillies 1954), though in areas of high bed net coverage it is likely that mosquitoes spend on average less than six hours inside houses (Lines et al. 1987; Mathenge et al. 2001; Killeen et al. 2007). In this study it was found that exposure times as short as 15 and 30 min were sufficient for conidia of both *M. anisopliae* and *B. bassiana* to infect mosquitoes and reduce survival. Similarly, no effect of increasing exposure time beyond 5 min up to 6 h on infection rates was found when *An. stephensi* were exposed to 2×10^9 conidia m-2 of *B. bassiana* (Stevenson 2008). Increasing the exposure time beyond 6 h and/or concentration did increase mortality of *An. stephensi* (Stevenson 2008) and other arthropods (Maniania 1994; Quesada-Moraga et al. 2006). When the concentration tested against *An. gambiae* was increased, no effect of exposure time was observed, though only relatively short exposure times were tested (15 min - 6 h). However, longer exposure times (24 h, 48 h and continuous) of *An. gambiae* to *M. anisopliae* were tested by Scholte et al (2003) and at high concentrations and no difference between the exposure times tested was observed. Formulations of either *M. anisopliae* or *B. bassiana* could, therefore, be used with dissemination tools/surfaces that target host seeking (short contact) as well as resting (long contact) mosquitoes.

Despite a general decline in the persistence *M. anisopliae* and *B. bassiana* against *An. gambiae s.s.*, conidia were still pathogenic up to 28 d post application. During the cur- rent study, the conidia were stored under constant temperature (26°C-27°C) and RH (85-95%) thus it is unknown if fluctuating environmental conditions would affect the length of residual activity. A slight decline over time in the germination of conidia when applied in the field has been reported elsewhere (Scholte et al. 2005), yet 63% of conidia remained viable after three weeks. Most importantly it was found that increasing the concentration of conidia did not increase the residual activity. Although the residual activity of fungi is

short lived compared with traditional synthetic insecticides, it is comparable with other successful bio-insecticides such as *Bacillus thuringiensis* var. *israelensis* (Karch et al. 1991; Fillinger et al. 2003). The MSTs values recorded during the persistence experiment for exposure immediately after drying (1 d) were lower than MSTs observed when similar concentrations were tested elsewhere in this study. The difference could have been due to variation in the quality of the conidia batch.

To be capable of transmitting malaria, a mosquito must survive for longer than the extrinsic incubation period of the pathogen, 9 - 14 d for *Plasmodium* spp (Beier 1998). This period is longer than the average mosquito life span and, therefore, malaria transmission can be attributed to a small fraction of the mosquito population. Daily survival is actually the most sensitive component of vectorial capacity (Garret-Jones 1964; Miller et al. 1973) and thus control strategies that reduce vector age are highly desirable. The current study recorded large reductions in the daily survival of female *An. gambiae s.s.* when exposed to relatively high (2×10^{10} and 4×10^{10}conidia m^{-2}) concentrations of both *M. anisopliae* and *B. bassiana*. These concentrations were lower than that used by Scholte et al (2005). When mosquitoes were exposed to high conidial concentrations in this and other studies (Scholte et al. 2003; Blanford et al. 2005; Scholte et al. 2005; Farenhorst et al. 2008), 100% mortality was often achieved within 10 d. If these results can be replicated in the field this could lead to a considerable reduction in malaria transmission (Hancock 2009).

Conclusions

Of the few biological control tools targeting adult mosquitoes that are currently under development (including fungal, bacterial, viral and protozoan pathogens), entomopathogenic fungi are likely to be developed for programmatic use. Especially since fungus production and application all involve relatively simple infrastructures and processes, which could potentially be adopted in malaria endemic countries. An application of either *M. anisopliae* or *B. bassiana* at a concentration of 2×10^{10} conidia m^{-2} should be able to infect mosquitoes in a relatively short time (15 or 30 min) for up to one month after application. This concentration should provide a considerable safety margin for application error, exposure time and residual activity. However, there remains a need to test the fungi in large-scale field trials and to develop protocols to ensure simple and economical distribution and application in malaria endemic developing countries. Further developments to increase conidia persistence are still necessary in order to enhance the potential epidemiological impact of fungi on malaria transmission.

Acknowledgements

We are grateful to the Adessium foundation (Reeuwijk, The Netherlands) for funding this research. We thank Jennifer Stevenson, Simon Blanford and Christian Luz for their useful comments and assistance, and Paulina Kasanga, Edgar Mbeyela and Emmanuel Simfukwe for technical assistance. We thank Frank van Breukelen (Wageningen University), Nina Jenkins and Matthew Thomas (CSIRO/Penn State University) for supplying the *M. anisopliae* and *B. bassiana* conidia.

Chapter 4

First report of *Metarhizium anisopliae* IP 46 pathogenicity in adult *Anopheles gambiae s.s.* and *An. arabiensis* (Diptera; Culicidae)

Published as: Mnyone LL, Russell TL, Lyimo IN, Dickson W Lwetoijera DW, Kirby MJ, Luz C: First report of *Metarhizium anisopliae* IP 46 pathogenicity in adult *Anopheles gambiae s.s.* and *An. arabiensis* (Diptera; Culicidae). *Parasites & Vectors* 2009, **2:** 59

Abstract

The entomopathogenic fungus *Metarhizium anisopliae* isolate IP 46, originating from a soil sample collected in 2001 in the Cerrado of Central Brazil, was tested for its ability to reduce the survival of adult male and female *Anopheles gambiae sensu stricto* and *An. arabiensis* mosquitoes. A 6-h exposure to the fungus coated on test paper at a concentration of 3.3×10^6 conidia cm^{-2} reduced the daily survival of both mosquito species (HR = 3.14, $p < 0.001$), with higher risk of dying in *An. gambiae s.s.* relative to *An. arabiensis* (HR = 1.38, $p < 0.001$). Fungal sporulation was observed in >95% of mosquito cadavers in the treatment groups. The results indicate that *M. anisopliae* IP 46 has the potential to be a bio-control agent for African malaria vector species, and is a suitable candidate for further research and development.

Background

Metarhizium anisopliae IP 46 has shown ovicidal effects against the eggs of *Aedes* spp in Brazil (Luz et al. 2008; Albernaz et al. 2009; Santos et al. 2009). However, its pathogenicity against adult malaria vectors has never been explored. As such, we examined the effect of this strain against laboratory-reared adult *Anopheles gambiae sensu stricto* and *An. arabiensis*, with the aim to include IP 46 in the spectrum of fungal candidates available for use as biocontrol agents.

Methods

The fungus was imported as conidia from the Institute of Tropical Pathology and Public Health, Federal University of Goiás, Goiânia, Brazil (Tropical Pesticides Research Institute Import Permit No. 2471). Before conducting bioassays, the IP 46 isolate was host-passaged through laboratory-reared *An. gambiae s.s.* adults in order to maintain its virulence. Conidia were harvested from cultures grown on autoclaved rice substrate (200 g per bag) in nylon bags at 25°C and 12 h photophase, after 15 d incubation. They were then dried in silica gel at 4°C for 4 d. Preparation of stock- and working-solution concentrations formulated in Enerpar oil (Enerpar M002®, BP South Africa Ltd) followed standard protocols (Blanford et al. 2005). Before each experiment, conidia viability (>95% germination on Sabouraud Dextrose Agar) was confirmed. 1200 µl of the working-solution was applied evenly to 15 × 25 cm proofing paper using a metal bar (0.31 mm diameter; paper and applicator bar from RK Print Coat Instruments, London), giving a uniform concentration of 3.3×10^6 conidia cm^{-2}. The treated paper was left to dry for 12 h at 26 ± 1°C and 80 ± 5% RH, and then used to line the inside of plastic exposure tubes (8.2 cm diameter × 12.5 cm height). Untreated control replicates used paper treated with Enerpar oil only.

A total of 30-40 unfed 3-7 d old adult *An. gambiae s.s.* (colony established in 1996, Njage village, Tanzania) or *An. arabiensis* (colony established in 2007, Sagamaganga village, Tanzania) were introduced to the exposure tubes. Four separate bioassays were run (both sexes for each species) and three replicates were carried out for each bioassay. Mosquitoes were held in the tubes for 6 h, after which they were transferred to (9 × 9 × 9 cm)holding cages at 26 ± 1°C and 90 ± 5% RH, and provided with 9% glucose/ water (w/v) solution. The survival and fungus infection status of mosquitoes were monitored daily for up to 28 d, following procedures described elsewhere (Blanford et al. 2005). Mosquito survival was analysed by Kaplan-Meier pair-wise comparison and Cox regression analysis, using SPSS version 16. Cox regression generated hazard ratios (HR) indicating the daily risk of dying for a mosquito in each bioassay group.

Results and discussion

Metarhizium anisopliae IP 46 was capable of infecting males and females of both mosquito species: >95% of *An. gambiae s.s.* and *An. arabiensis* cadavers showed fungus sporulation after incubation for 5-6 d. The fungus significantly reduced the survival of all exposed mosquitoes compared to controls ($p < 0.001$, Table 1, Fig. 1); >90% of mosquitoes in the exposure groups had died by day 14 while >25% of control mosquitoes were still alive by this time. All of the control mosquitoes in all bioassays had died by day 28. For *An. gambiae s.s.* the daily risk of dying was over three-fold greater in exposed females (HR = 3.18, $p < 0.001$) and males (HR = 3.81, $p < 0.001$) relative to their controls. A similar trend was observed in exposed females (HR = 2.28, $p < 0.001$) and males (HR = 3.31, $p < 0.001$) of *An. arabiensis*. The daily risk for males was higher than for females in both species (*An. gambiae s.s.* HR = 1.11, $p = 0.001$ and *An. arabiensis* HR = 1.13, $p = 0.004$). Overall, daily risk of dying was higher for exposed *An. gambiae* than *An. arabiensis* (HR = 1.38, $p < 0.001$). The controls for *An. gambiae* survived relatively longer (males MST = 14 d; females MST = 16 d) than those of *An. arabiensis* (male

MST = 12 d; female MST = 12 d, Table 1), but this difference was accounted for by Cox regression model which compares relative risks rather than fixed survival time values.

For effective malaria control, entomopathogenic fungi do not need to kill vector mosquitoes instantly (Hancock et al. 2008). If mosquitoes are able to reproduce and pass genes to the next generation before they are killed by an insecticide the selection pressure for the development of resistance is significantly reduced (Thomas and Read 2007; Ondiaka et al. 2008). Here we have shown that the isolate *M. anisopliae* IP 46 kills females of *An. gambiae s.s.* and *An. arabiensis* on average 8-9 d after exposure. By day 14 the majority (>90%) of exposed mosquitoes had been killed. Given that the *Plasmodium* parasite requires approximately 9 to 14 d to infect the mosquito salivary glands, the risk of malaria transmission by fungus infected mosquitoes is minimal (Talman et al. 2004). Similar rates of mortality have been recorded for other entomopathogenic fungi against mosquitoes (Blanford et al. 2005, Farenhorst et al. 2008; Stevenson 2008; Scholte et al. 2005). Perhaps most importantly, *M. anisopliae* IP 46 was effective against both *An. arabiensis* and *An. gambiae s.s.* suggesting that it could be used to target both indoor and outdoor resting anophelines. This is the first study demonstrating the susceptibility of adult *An. arabiensis* to *Metarhizium anisopliae*.

Ultimately the success of entomopathogenic fungi against malaria-carrying mosquitoes in any situation may depend on the choice of fungal isolate. This is because of the inter-isolate variation in virulence, spore production and persistence in relation to their ability to withstand sub-optimal environmental conditions (De La Rosa et al. 2000; Sun et al. 2003; Ansari et al. 2004; Bugeme et al. 2009; Ihara et al. 2009). The long-standing barriers that have prevented the widespread uptake of biological control agents include low virulence and short-term residual activity. In order to overcome such barriers it is necessary to screen an array of fungal strains to identify those with the greatest potential for development. We found that the isolate *M. anisopliae* IP 46 is able to reduce the survival of adult anophelines within the same time frame as other strains, *M. anisopliae* ICIPE-30 and *B. bassiana* IMI 391510 (Scholte et al. 2003; Blanford et al. 2005; Stevenson 2008). We anticipate that our findings will encourage research into other strains and further investigation and development of IP 46.

Table 1: Pair-wise Kaplan-Meier median survival times (MST) for adult *Anopheles gambiae s.s.* and *An. arabiensis* exposed to oil-formulated *M. anisopliae* IP 46 (treatment) or oil only (control).

Species	Sex	MST ± 1S.E.		χ^2 value	*p* value
		Control	Treatment		
***An. gambiae* s.s.**	♀	16 ± 0.51	9 ± 0.23	94.58	<0.001
	♂	14 ± 0.76	8 ± 0.30	133.07	<0.001
An. arabiensis	♀	12 ± 0.79	8 ± 0.38	63.04	<0.001
	♂	12 ± 0.45	6 ± 0.31	113.13	<0.001

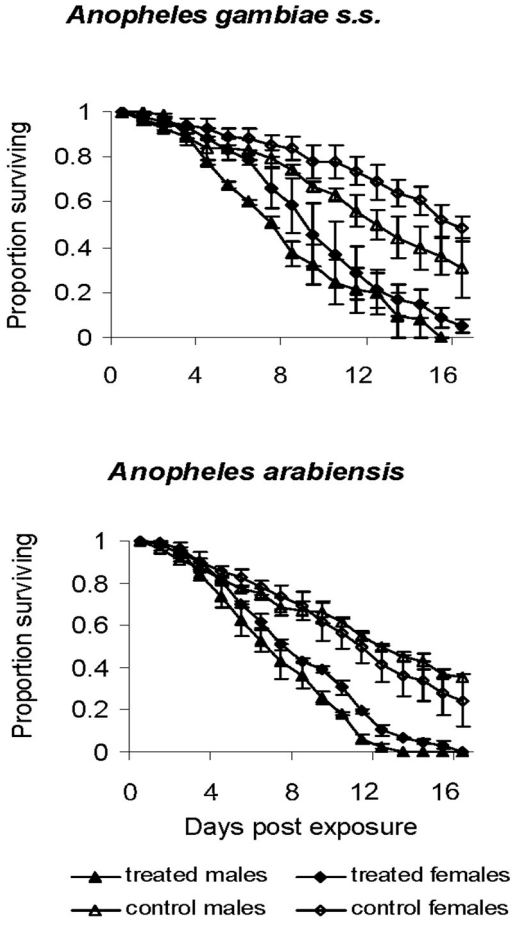


Figure 1: *Survival of adult female and male a) Anopheles gambiae s.s. and b) An. arabiensis mosquitoes after 6 h exposure to Metarhizium anisopliae IP 46 conidia.*

Acknowledgements

We wish to acknowledge Monika Mpingwa, Kassian Mbina, Ally Daraja, Paulina Kasanga and Emannuel Simfukwe for rearing mosquitoes and providing technical assistance.

Chapter 5

Infection of *Anopheles gambiae* mosquitoes with entomopathogenic fungi: effect of host age and blood-feeding status

Published as: Mnyone LL, Kirby MJ, Mpingwa MW, Lwetoijera DW, Knols BGJ, Takken W, Koenraadt CJM, Russell TL: Infection of *Anopheles gambiae* mosquitoes with entomopathogenic fungi: effect of host age and blood-feeding status. *Parasitology Research* 2010, DOI 10.1007/s00436-010-2064-y.

Abstract

Physiological characteristics of insects can influence their susceptibility to fungal infection of which age and nutritional status are among the most important. An understanding of host-pathogen interaction with respect to these physiological characteristics of the host is essential if we are to develop fungal formulations capable of reducing malaria transmission under field conditions. Here, two independent bioassays were conducted to study the effect of age and blood-feeding status on fungal infection and survival of *Anopheles gambiae sensu stricto* Giles. Mosquitoes were exposed to 2×10^{10} conidia m^{-2} of oil-formulated *Metarhizium anisopliae* ICIPE-30 and *Beauveria bassiana* I93-825 conidia, and their survival monitored daily. Three age groups of mosquitoes were exposed: 2-4, 5-8, and 9-12 d since emergence. Five groups of different feeding status were exposed: non-blood-fed, 3 h, 12 h, 36 h and 72 h post-blood feeding. Fungal infection reduced the survival of mosquitoes regardless of their age and blood-feeding status. Although older mosquitoes died much faster than younger ones, age did not tend to affect mosquito susceptibility to fungal infection. Non-blood-fed mosquitoes were more susceptible to fungus infection compared to all categories of blood-fed mosquitoes, except for those exposed to *B. bassiana* 72 h post blood-feeding. In conclusion, formulations of *M. anisopliae* and *B. bassiana* can equally affect mosquitoes of different age classes, with them being relatively more susceptible to fungus infection when non-blood-fed.

Background

Recent studies increasingly demonstrate the potential for controlling malaria vectors by using entomopathogenic fungi (Scholte et al. 2005; Blanford et al. 2005; Farenhorst et al. 2008; Mnyone et al. 2009). These fungi do not cause instant mortality, but cause sub-lethal and late-life lethal effects on different stages of the mosquito life cycle. Due to such properties, fungi can potentially be used as "evolution-proof" agents and overcome mosquito resistance unlike the currently deployed fast-acting chemical insecticides (Ffrench-Constant 2005; Koella et al. 2009, Read et al. 2009). As part of an integrated strategy, fungi could respond to, or avert emergence of serious levels of insecticide resistance. For instance, recent laboratory studies by Farenhorst et al (2009) recorded similar susceptibility to fungal infection between insecticide-resistant and non-resistant colonies of *Anopheles* mosquitoes. Furthermore, theoretical modelling suggests the existence of a synergistic interaction between fungal bio-insecticides and insecticide treated bed nets (ITNs) even in scenarios of high malaria intensity and widespread insecticide resistance (Hancock 2009). This suggestion was validated by a recent study in Benin, where there was a strong interaction of the insecticide permethrin and the fungi *Metarhizium anisopliae* or *Beauveria bassiana* (Farenhorst et al. personal communication) on mosquito survival.

Under field conditions, entomopathogenic fungi target mosquitoes of different physiological characteristics, which may influence their overall effectiveness as biocontrol agents. Host species, age, developmental stage, sex and nutritional status have frequently been reported to affect insect susceptibility to fungal infection (Feng et al. 1985; Maniania and Odulaja 1998; Dimbi et al. 2003). Host age and feeding status are among the most important physiological characteristics. A critical understanding of the host-pathogen interaction with respect to these host physiological characteristics, therefore, is essential for the development of formulations capable of suppressing malaria transmission under real life conditions.

Mosquito populations are known to experience senescence (Harrington et al. 2001; Okech et al. 2003; Styer et al. 2007). As mosquitoes age, adverse changes to major life-history parameters have been recorded including differences in mortality, flight capacity, immune function, salivary gland function and cuticular hydrocarbon composition (Rowley and Graham 1968; Nayar and Sauerman 1973; Beckett 1990; Chen et al. 1990; Brei et al. 2004; Hugo et al. 2006). The susceptibility of mosquitoes to fungal infection could be affected when a) the insect cuticle weakens with age, thereby facilitating more successful fungal penetration, and b) a lowered immune response allows the fungus to rapidly propagate inside the host. Recent research has demonstrated greater susceptibility of older (17-21 d old) than younger (3-7 d old) *An. stephensi* mosquitoes to three species of entomopathogenic fungi (J. Stevenson, unpublished data). This age composition, however, may not be representative of that of mosquitoes under realistic field conditions, where only a small proportion of mosquitoes may survive for 21days or longer (Gillies and Wilkes 1965). More importantly, the wild vector community is normally composed of various species of mosquitoes; and this may dictate their survival and aging effect trends. As such, knowledge about species-specific aging effects in relatively younger mosquitoes will be of added value. Higher susceptibility in older than younger individuals has also been reported in studies of other insects. The susceptibility of tsetse flies to *M. anisopliae* was greater among older than younger individuals (Maniania and Odulaja 1998). Similar phenomena have been observed with regard to insecticides. The mean knockdown times of *Anopheles stephensi* and *An. gambiae* mosquitoes exposed to pyrethroids were significantly shorter if they were 10 d old as compared to those of newly emerged ones (Hodjati and Curtis 1999). Nevertheless, contrasting reports, where greater susceptibility has been recorded in younger than older insects, also exist (Mullens 1985; Dimbi et al. 2003).

Nutritional differences between non-blood-fed and blood-fed mosquitoes may also affect the efficacy of a fungal-based intervention. Various studies have recorded contradicting effects of blood feeding on mosquito longevity. One hypothesis is that mosquitoes which are fed on blood alone, without access to sugar, will have lower survival, possibly because nutrients are diverted for egg production (Okech et

et al. 2003). Alternately, other experiments have recorded that blood feeding can actually increase mosquito survival when compared with mosquitoes fed only on sugar (Scott et al. 1993; Scott et al. 1997). The nutritional benefits of blood feeding could alter the efficacy of fungal infections due to changes in the host's ability to activate an immune response or conversely alter the level of nutrients available for pathogen propagation. *Rhodnius prolixus* bugs that were fed on blood showed a higher immune response to *Enterobacter cloacae* infection compared to *R. prolixus* fed on plasma (Feder et al. 1997). A similar susceptibility trend was observed between freshly blood-fed and non-blood-fed *An. gambiae* (G3 strain, originating from The Gambia) mosquitoes (J. Stevenson, unpublished data). In that study mosquitoes were fed directly on rats.

Under realistic field situations, mosquito vectors of different age and blood feeding status may be encountered. An understanding of host-pathogen interaction with respect to mosquito age and blood feeding status, therefore, is essential if we are to develop fungal formulations capable of reducing malaria transmission. This study was designed to examine if efficacy of *M. anisopliae* ICIPE-30 and *B. bassiana* I93-825 varied with mosquito age and human blood-feeding. This information will be essential for designing delivery mechanisms to expose the most susceptible portion of the mosquito population to fungal infection.

Materials and methods

Mosquitoes

Mosquitoes originated from a colony of *Anopheles gambiae s.s.* established from Njage village, Tanzania in 1996 and maintained at the Ifakara Health Institute. Larvae and adults were reared using procedures described by Huho et al (2007). Bioassays were conducted using fed and unfed adult female mosquitoes.

Fungal isolates, formulation and application

Two fungal isolates were used in this study: *Metarhizium anisopliae* var. *anisopliae* ICIPE-30, isolated in 1989 from the maize stalk borer, *Busseola fusca* (Lepidoptera, Noctuidae) in Western Kenya, and *Beauveria bassiana* I93-825 (IMI 391510), isolated from a chrysomelid beetle (Coleoptera) in the USA. Before each experiment, viability of conidia (i.e. % germination on Sabouraud Dextrose Agar) was confirmed to be >85%.

Conidia were formulated in Enerpar, a highly refined mineral oil (Enerpar M002®, BP Southern Africa Ltd). Stock and working conidia suspensions were prepared using procedures described by Mnyone et al (2009). In all bioassays, a concentration of 2×10^{10} conidia m^{-2} was used. Mosquitoes were exposed to conidia applied to sheets of A4 printing paper within plastic exposure tubes (8.2 cm diameter × 12.5 cm height), closed with netting also treated with conidia. The paper and netting were treated, dried and assembled in plastic exposure tubes as described by Mnyone et al (2009).

Bioassay procedures

Thirty to forty adult *An. gambiae s.s.* mosquitoes were introduced into the fungal exposure tube (8.2 cm diameter × 12.5 cm height) and held for 6 h after which they were transferred to separate untreated cages (9 cm × 9 cm × 9 cm) and maintained at 26–27°C and 85–95% relative humidity (RH)

with access to 9% glucose solution *ad libitum*. Based on earlier observations, 6 h was considered an appropriate exposure period (Mnyone et al. 2009). Mosquito survival was determined by recording mortality in each holding cage for a maximum of 28 d. Cadavers were collected daily, put onto moist filter paper in Petri dishes, sealed with parafilm, and incubated at 26–27°C and 85–95% RH for 3–4 d. After that, they were then examined for evidence of fungal sporulation. Similar bioassay procedures were followed for 30–40 mosquitoes in control groups, except that they were exposed to untreated surfaces. During all of the bioassays, six independent replicates were used for each experimental factor. Two different bioassays were conducted to study the effect of age and blood feeding on infection and survival of *An. gambiae* s.s.

Bioassay 1: Effect of mosquito age on infection and survival

Three different age groups of female mosquitoes were exposed to both fungal isolates: (1) 2-4 days, (2) 5-8 days and (3) 9-12 days post emergence.

Bioassay 2: Effect of mosquito blood feeding on infection and survival

Five groups with differing blood feeding status were exposed to both fungal isolates: (1) non-blood-fed, (2-5) 3, 12, 36, or 72 h post blood feeding. Mosquitoes were starved of glucose for 6 h before blood feeding. For blood feeding, a volunteer placed a bare forearm inside the cage and allowed mosquitoes to feed for 10 min. After blood feeding, all unfed and partially fed mosquitoes were aspirated out of the cage leaving only fully engorged mosquitoes. Blood feeding was scheduled so that when the mosquito groups were exposed to the fungus they were in the age range of 3-6 d.

Data analysis

Mosquito survival data were analysed using Cox regression to determine the relative risk of dying (hazard ratios (HR)) for the specific treatment group compared with the baseline group. The explanatory factors included in the analysis were fungal treatment, mosquito age and blood feeding status, as required. Hazard ratios of each mosquito category and its respective control were generated in independent models to observe if confidence intervals were suggestive of any interaction between fungus related mortality and age or blood meal. Non-blood-fed mosquito data was run against each of the four categories of blood-fed mosquitoes generating four independent models to confirm interaction between fungus-related mortality and a blood meal. Kaplan-Meier pair-wise method was used to obtain median survival times (MST) for treated and untreated group of mosquitoes. The Bonferroni method was employed to compensate for multiple comparisons. SPSS version 17 was applied.

Results

Bioassay 1: Effect of mosquito age on infection and survival

Overall the survival of all three different age groups of mosquitoes was reduced relative to their control counterparts when exposed to both *M. anisopliae* and *B. bassiana* (Table 1). The daily risk of dying was over two-fold greater in mosquitoes exposed to *M. anisopliae* relative to controls (HR = 2.26 [95% CI = 1.86 – 2.74], p <0.001), whereas the daily risk of dying was over three-fold greater in mosquitoes exposed to *B. bassiana* relative to controls (HR = 3.46 [95% CI = 2.85 – 4.19], P <0.001).

Irrespective of fungus infection, the age of mosquitoes affected speed at which they were dying, with older mosquitoes dying faster than younger ones (*M. anisopliae*: Wald = 75.54; df = 2; p <0.001, *B. bassiana*: Wald = 76.16; df = 2; p <0.001). For *M. anisopliae*, the risk of dying was greater in 9-12 d old mosquitoes relative to 2-4 d old (RH = 1.52 [95% CI = 1.27 – 1.82], p <0.001) and 5-8 d old (RH = 1.25 [95% CI = 1.04 – 1.50], p <0.001) mosquitoes. Similarly, for *B. bassiana*, the risk of dying was greater in 9-12 d old mosquitoes relative to 2-4 d old (RH = 1.51 [95% CI = 1.26 – 1.81], p <0.001) and 5-8 d old (RH = 1.51 [95% CI = 1.26 – 1.83], p <0.001) mosquitoes. For both fungal species, the latter two age groups of mosquitoes had a similar risk of dying (RH = 1.22 [95% CI = 1.01 – 1.47], p = 0.06). There, however, was no overall significant interaction between age and speed at which fungus-infected mosquitoes were dying (*M. anisopliae* Wald = 1.76; df = 2; p = 0.42, *B. bassiana*: Wald = 1.60; df = 2; p = 0.21).

Bioassay 2: Effect of mosquito blood feeding on infection and survival

The survival of all fungus-infected mosquitoes was reduced compared to their control counterparts when mosquitoes were non-blood fed or 3, 12, 36 or 72 h post blood feeding (Table 2). The daily risk of dying was over two-fold greater in mosquitoes exposed to *M. anisopliae* (HR = 2.75 [95% CI = 2.27 – 3.33] p <0.001) and *B. bassiana* (HR = 2.28 [95% CI = 1.87 – 2.78] p <0.001) relative to controls.

A blood meal significantly affected the speed at which mosquitoes died; and overall, blood fed mosquitoes had a lower risk of dying relative to unfed ones (*M. anisopliae*: Wald = 95.39; df = 4; p <0.001, *B. bassiana*: Wald = 80.16; df = 4; p <0.001). With independent models where non-blood fed mosquitoes were independently compared with blood fed categories, there was an interaction between blood feeding and fungus-induced mortality except for mosquitoes exposed to *B. bassiana* 72 h since blood feeding (*M. anisopliae*: Wald = 23.25 – 52.56; df = 1; p <0.001, *B. bassiana*: Wald = 16.63 – 50.42; df = 1; p <0.001).

Table 1: Median survival times (MST ± SE) of different age groups (2-4, 5-8 and 9-12 days old) of *Anopheles gambiae* s.s. exposed to *Metarhizium anisopliae* ICIPE-30 and *Beauveria bassiana* I93-825

Fungus species	Age (days)	MST± SE (days)		*p* value
		Control	Treatment	
***M. anisopliae* ICIPE-30**	2-4	15 ± 0.51	8 ± 0.51	<0.001
	5-8	14 ± 0.39	7 ± 0.39	<0.001
	9-12	11 ± 0.25	5 ± 0.25	<0.001
***B. bassiana* I93-825**	2-4	15 ± 0.51	7 ± 0.28	<0.001
	5-8	14 ± 0.39	6 ± 0.22	<0.001
	9-12	11 ± 0.25	6 ± 0.28	<0.001

Table 2: Median survival times (MST ± SE) of *Anopheles gambiae s.s.* exposed to *Metarhizium anisopliae* ICIPE-30 and *Beauveria bassiana* I93-825 when non-blood-fed, and 3, 12, 36 and 72 h since blood feeding

Fungus species	Blood feeding status before exposure (no. of hours)	MST± SE (days)		*p* value
		Control	Treatment	
***M. anisopliae* ICIPE-30**	Unfed	14 ± 0.56	7 ± 0.41	<0.001
	3	14 ± 0.70	7 ± 0.46	<0.001
	12	18 ± 0.06	9 ± 0.50	<0.001
	36	18 ± 0.76	9 ± 0.46	<0.001
	72	16 ± 0.81	9 ± 0.70	<0.001
***B. bassiana* I93-825**	Unfed	14 ± 0.56	7 ± 0.22	<0.001
	3	14 ± 0.70	8 ± 0.41	<0.001
	12	18 ± 0.06	10 ± 0.57	<0.001
	36	18 ± 0.76	10 ± 0.46	<0.001
	72	16 ± 0.81	10 ± 0.32	<0.001

Discussion

When mosquitoes were exposed to either *M. anisopliae* or *B. bassiana* their survival, in all bioassays, was significantly reduced relative to their control counter parts. This supports previous laboratory trials that have demonstrated the potential of entomopathogenic fungi for adult mosquito control (Scholte et al. 2005; Blanford et al. 2005; Farenhorst et al. 2008). Our bioassays investigated the interaction between the pathogen, and host age and blood feeding status, which are key factors affecting initiation and development of fungal infection (Dimbi et al. 2003).

Although older mosquitoes died relatively faster than younger individuals, age did not affect mosquito susceptibility to fungal infection. Increased risk of death in older than younger individuals has also been reported elsewhere (Harrington et al. 2001; Styer et al. 2007). Finally, no interaction between age and fungus-induced mortality has been reported in housefly *Musca domestica*, onion fly *Delia antiqua* and blackfly *Phormia regina* exposed to *M. anisopliae* and *B. bassiana* (Rizzo 1977). Contrarily, age-dependent fungus-induced mortality has been observed for example in scarabeid larvae (Ferron 1967), *Heliothis zea* larvae (Mohamed et al.1977), and adult tsetse flies (Maniania and Odulaja 1998). In these three insect species, the fungus-induced mortality was relatively higher in older than younger individuals. Fungus-induced mortality, however, has been shown to decrease with age in noctuid larvae (Getsin 1961; Fargues and Rodriguez-Rueda 1980; Boucias et al. 1984).

Increased speed of mortality among older individuals could be explained by various physiological properties that change with the age of mosquitoes, and possibly other insect species. Older mosquitoes show reduced immune function. Melanization responses in laboratory reared *An. gambiae* declined within a short period of time (Chun et al.1995; Schwartz and Koella 2002), and older individuals were only able to mount strong immune response after they were supplemented with blood (Schwartz and Koella 2002). Enzymes, phenol oxidases, which are suggested to be important for melanization and sclerotization in the hemolymph (Chun et al.1995), and numbers of immune competent hemocytes (Li et al. 1992) decrease with age. A change in the number and morphology of circulating hemocytes was attributed to increased mortality in older *Aedes aegypti* (Hillyer et al. 2005). Moreover, glycogen and total soluble proteins levels decrease with age (Nayar and Sauerman 1973; Mourya et al. 1993).

Non-blood fed mosquitoes were more susceptible to fungus infection than blood fed ones. A digested blood meal increases the nutrient reserves of the host, thus more time may be required for the fungus to deplete nutrients and kill the insect. In addition, insects with a digested blood meal can also mount a stronger immune response. This could have been the reason for the lower mortality in blood-fed relative to unfed mosquitoes. Histopathological studies of tissues infected by fungus suggest that the insect dies due to the combination of nutrient depletion, mechanical damage, and toxicosis (Ferron 1981; Gillepsie and Claydon 1989). The fact that blood-fed mosquitoes are less susceptible to fungal infection could be beneficial in terms of evolution proofing against resistance development. Although fungal infection reduces the fecundity of female mosquitoes (Scholte et al. 2006), they are still able to pass their genes to the subsequent generation reducing selection pressure on resistance against fungi (Thomas and Read 2007). Furthermore, fungal infections suppress the successful development of *Plasmodium* parasites in the vectors (Blanford et al. 2005), and hence both effects (i.e. fungus-induced mortality and parasite resistance) lead to a significantly reduced parasite transmission risk.

Conclusions

Formulations of *M. anisopliae* and *B. bassiana* can equally infect mosquitoes of different age classes, more so when non-blood-fed. Therefore, delivery techniques that target host-seeking or house entering mosquitoes may be more effective than techniques targeting resting (and often blood-fed) mosquitoes. To expect more in terms of reducing wild mosquito populations, however, more efforts should gear towards producing more virulent and persistent fungal products.

Acknowledgements

We are grateful to the Adessium foundation (Reeuwijk, The Netherlands) for funding this research.

Chapter 6

Behavioural response of anopheline and culicine mosquitoes to surfaces treated with the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*

Published in a slightly modified form as: Mnyone LL, Koenraadt CJM, Lyimo IN, Mpingwa MW, Takken W, Russell TL: Anopheline and culicine mosquitoes are not repelled by surfaces treated with the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*. *Parasites & Vectors* 2010, **3:** 80

Abstract

Entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana* are promising bio-pesticides for application against adult malaria mosquito vectors. An understanding of the behavioural responses of mosquitoes towards these fungi is necessary to guide development of fungi beyond the 'proof of concept' stage and to design suitable intervention tools. Here we tested whether oil-formulations of the two fungi could be detected and avoided by adult *Anopheles gambiae sensu stricto, Anopheles arabiensis* and *Culex quinquefasciatus*. The bioassays used a glass chamber divided into three compartments (each 250 × 250 × 250 mm): release, middle and stimulus compartments. Netting with or without fungus was fitted in front of the stimulus compartment. Mosquitoes were released and the proportion that entered the stimulus compartment was determined and compared between treatments. Treatments were untreated netting (control 1), netting with mineral oil (control 2) and fungal conidia formulated in mineral oil evaluated at three different dosages (2×10^{10}, 4×10^{10} and 8×10^{10} conidia m^{-2}). Neither fungal strain was repellent as the mean proportion of mosquitoes collected in the stimulus compartment did not differ between experiments with surfaces treated with and without fungus regardless of the fungal isolate and mosquito species tested. Our results indicate that mineral-oil-formulations of *M. anisopliae* and *B. bassiana* are not repellent against the mosquito species tested. Therefore, both fungi are suitable candidates for the further development of tools that aim to control host-seeking or resting mosquitoes using entomopathogenic fungi.

Background

Laboratory (Blanford et al. 2005; Farenhorst et al. 2008; Mnyone et al. 2009) and small scale field trials (Scholte et al. 2005; Lwetoijera et al. 2010) have demonstrated that malaria vectors can succumb to, and die from entomopathogenic fungus infection. Furthermore, these fungi can equally infect and kill insecticide-resistant and insecticide-susceptible malaria vectors (Farenhorst et al. 2009; Kikankie et al. 2010; Howard et al. 2010). In these views, entomopathogenic fungi are increasingly attracting attention as potential biological control agents against malaria vectors, particularly as they are considered to be evolutionary proof agents, against which resistance is less likely to develop (Read et al. 2009).

For fungal infection to occur, the conidia need to contact the host, after which they attach to, germinate, and penetrate the cuticle (Khachatorians 2001). Once within the host mosquito, the hyphae proliferate whilst exploiting nutritional resources and release toxic metabolites that eventually cause sub-lethal effects to the host and/or kill it (Bush et al. 2001). Examples of the sub-lethal effects are reduced blood feeding propensity and fecundity of the mosquitoes (Scholte et al. 2006).

Success or failure of infection depends on the nature of host-parasite interaction, which can be altered by physiological, ecological and behavioural conditions of the host (Roy et al. 2006). Many studies on host-pathogen interaction in arthropods have focused on physiological and ecological alterations, with little attention paid to behavioral alterations. One of the most important behaviours is the host insect's ability to detect and avoid fungal conidia. Termites (Roy et al. 2006; Yanagawa and Shimizu 2007; Yanagawa et al. 2008), ants (Roy et al. 2006) and groundnut beetles (Ekesi et al. 2001) were all shown to detect and avoid *Metarhizium anisopliae*. Behavioural avoidance was also observed in adults of the common flower bug (*Anthocoris nemorum*) (Meyling and Pell 2006) and ladybirds (*Coccinella septempunctata*) that could both detect and avoid *Beauveria bassiana* conidia (Omond 2007). In the field of mosquito control, if conidia can repel mosquitoes this could minimize mosquito contact with conidia, and thus reduce the efficacy of this control tool. Entomopathogenic fungi that are either non-repellent or attractive would be more desirable, unless their ability to repel is strong enough to prevent mosquitoes from entering human houses and biting people.

Laboratory studies to optimize fungal formulations of *M. anisopliae* and *B. bassiana* have been conducted (Mnyone et al. 2009) and provide a foundation for conducting field-based efficacy studies. Understanding how mosquitoes respond behaviourally to fungal exposure is thus essential. Avoidance behaviour may hamper the efficacy and the overall epidemiological impact of the fungus. We therefore tested the behavioural response of *An. gambiae s.s.*, *An. arabiensis* and *Culex quinquefasciatus* after contacting or detecting conidia of *M. anisopliae* and *B. bassiana*. *Culex quinquefasciatus* are susceptible to entomopathogenic fungi and under field settings they often appear together with malaria vectors, thus both may be targeted. Most importantly, *Culex quinquefasciatus* cause nuisance and are important vectors of filariasis. Therefore, understanding how they respond to the fungus was also deemed important since targeting both vectors would be more cost-effective and possibly enhance societal adoption of the technology. Behavioural responses can vary with conidia dose (Yanagawa et al. 2009); therefore, we tested different conidia doses formulated in pure mineral oil.

Materials and methods

Mosquitoes

Mosquitoes used in this study were obtained from insectary colonies maintained in the Ifakara Health Institute (IHI), Tanzania. The *An. gambiae s.s.* colony was established from a population near Njage village, Tanzania, in 1996. The *An. arabiensis* colony was established from Sagamaganga village,

Tanzania in 2007. Larvae and adults were reared using procedures described by Huho et al (2007). The *Cx. quinquefasciatus* colony was established from Ifakara village, Tanzania in 2009; using similar procedures as with anophelines except that adults were blood-fed on pigeons. The study was performed using 3-7 d old unfed adult female mosquitoes that were starved at least 6 h before use.

Fungal isolates, formulation and application

Two fungal isolates were used: 1) *M. anisopliae* var. *anisopliae* ICIPE-30, isolated in 1989 from the maize stalk borer, *Busseola fusca* (Lepidoptera, Noctuidae) in Western Kenya, and 2) *B. bassiana* I93-825 (IMI 391510), isolated from a chrysomelid beetle (Coleoptera) in the USA. Dry conidia of *M. anisopliae* were produced at IHI, after passaging and re-isolation from infected mosquito cadavers. Conidia were harvested from 15 d old cultures grown on rice grains. Dry conidia of *B. bassiana* were imported from Penn State University, USA (courtesy M.B. Thomas, Penn State University, USA). Conidia were formulated in highly refined mineral oil, Enerpar (Enerpar M002®, BP Southern Africa Ltd). Preparation and application of fungal formulations was done using procedures described by Mnyone et al (2009). After the treatment of exposure netting (conidia formulation or oil) it was left to dry for 24 h at ambient conditions.

Behavioural chamber

A glass chamber with three equally sized compartments (250 × 250 × 250 mm) was used: release, middle and stimulus compartments (Figure 1). The release compartment was separated from the middle compartment by a plywood frame fitted with white paper with a square opening at the middle (50 × 50 mm) to allow mosquitoes to move into the adjacent compartment. The middle and stimulus compartments were separated by a plywood frame fitted with a piece of polyester netting. The netting contained three rows of circular holes (10 mm diameter), with each row containing three holes. The distance between holes within and between adjacent rows was 50 mm. The ends of the glass chamber were covered with a transparent piece of cloth to prevent mosquitoes from escaping. The different treatments (three concentrations of fungal conidia suspended in mineral oil, mineral oil only and untreated) were applied to the netting separating the middle from stimulus compartments. In each replicate, freshly treated netting was used. To attract mosquitoes into the stimulus compartment, via the exposure netting, a host odour in the form of a guinea pig or worn sock was placed into the stimulus compartment. When used, the guinea pig was restrained within a plywood box covered with netting to protect guinea pigs from mosquito bites. The glass chamber was cleaned with distilled water and 70% alcohol in between trials and left to dry in open air to prevent transferring residual effects to the subsequent trials. Four glass chambers were used in parallel, corresponding to the four different treatments as detailed in the experimental procedures below. Air flow inside the experimental room was passive.

Experimental procedures

Experiment 1

Two doses (2×10^{10} and 4×10^{10} conidia m^{-2}) of *M. anisopliae* and *B. bassiana* were tested against *An. gambiae s.s.* and *An. arabiensis* and compared with oil-only and untreated controls. Fungal isolates were tested each at a time; and species of mosquitoes were tested against a particular fungal isolate each at a time. About 70 mosquitoes were placed into the release compartment in the early evening (6:00 pm) and were free to move towards the stimulus compartment overnight. The host in the stimulus compartment was a guinea pig.

The next morning (7:00 am), mosquitoes were collected from each compartment, killed and counted. The trial was repeated four times, each time with fresh mosquitoes, to obtain four replicates for each experimental factor.

Experiment 2

The set up and procedures were similar as described for Experiment 1, however, with the following exceptions. The experiment tested only *B. bassiana* against *An. gambiae s.s.*, *An. arabiensis* as well as *Cx. quinquefasciatus*. The conidial concentrations tested were: 2×10^{10} and 8×10^{10} conidia m^{-2}. The host odour in the stimulus compartment consisted of socks worn by a human volunteer (Pates et al. 2001). One sock was used per each glass chamber. The socks were worn for 12 h; and used immediately after being put off. Each trial was repeated six times to obtain six independent replicates.

Data analysis

The proportion of the mosquitoes released that were collected in the stimulus compartment was the output measure; it was calculated as a ratio of the number in stimulus compartment to the total number of mosquitoes (number in release, middle and stimulus compartments). Data were arcsine transformed to meet the assumption of standard normal distribution; then analysis of variance (ANOVA) was performed to compare different treatments. SPSS version 17 was used.

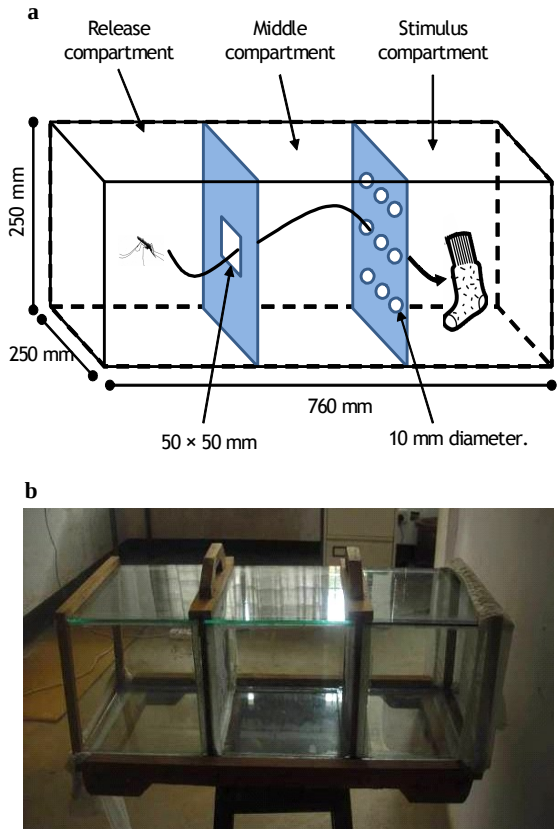


Figure 1. *Behavioural chamber with three equally sized compartments: release, middle, and stimulus compartment. A guinea pig (Experiment 1) or a worn sock (Experiment 2) was placed in the stimulus compartment.*

Results

Experiment 1

For *M. anisopliae,* the mean proportions (±SE) of *An. gambiae* that entered the stimulus (guinea pig) compartment were: untreated control 42.1 ± 4.2%, mineral oil only 37.9 ± 5%, conidia dose 2×10^{10} 41 ± 8.1% and conidia dose 4×10^{10} 44.9 ± 5%. This difference was not statistically significant (F = 0.24; df = 3,12; *p* = 0.87; Figure 2). The mean proportions for *An. arabiensis* were: untreated control 52.8 ± 4.3%, oil only 49.9 ± 5.2%, conidia dose 2×10^{10} 41.6 ± 5.2% and conidia dose 4×10^{10} 49.6 ± 4.2%. This difference was also not statistically significant (F = 1.0; df = 3,12; *p* = 0.43, Figure 2).

For *B. bassiana*, mean proportions of *An. gambiae* that entered the stimulus chamber were: untreated control 36.4 ± 1.2%, mineral-oil-only treated netting 40.9 ± 4.6%, conidia dose 2×10^{10} 47.5 ± 2.8% and conidia dose 4×10^{10} 44.8 ± 6.4%. This difference was not statistically significant (F = 1.27; df = 3,12; *p* = 0.33; Figure 2). Mean proportions of *An. arabiensis* were: untreated control 43.48 ± 4.2%, oil only 45.7 ± 5.6%, conidia dose 2×10^{10} 49.7 ± 4.4%, and conidia dose 4×10^{10} 47.1± 5.1%. This difference was also not statistically significant (F = 0.27; df = 3,12; *p* = 0.84, Figure 2).

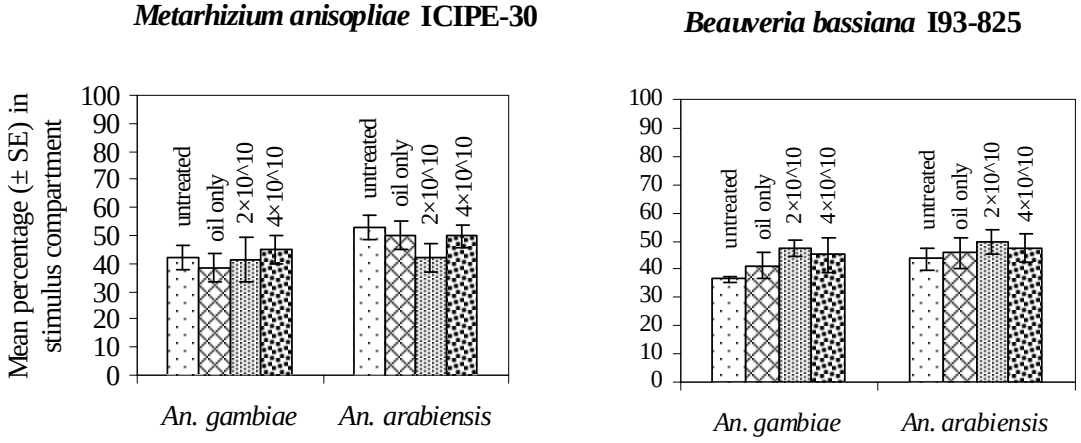


Figure 2. *Proportions (Mean ± SE) of Anopheles gambiae s.s. and Anopheles arabiensis mosquitoes collected in the stimulus compartment with plain control, oil control, and two formulations of Metarhizium anisopliae ICIPE-30 and Beauveria bassiana I93-825.*

Experiment 2

Mean proportions of *An. gambiae* that entered the stimulus (worn sock) compartment were: control 35.4 ± 2.1%, oil-only control 30.7 ± 2%, conidia dose 2×10^{10} 30.5 ± 1% and conidia dose 8×10^{10} 32.7 ± 2.7%. This difference was not statistically significant (F = 1.19; df = 3,20; *p* = 0.34; Figure 3). Mean proportions for *An. arabiensis* were: untreated control 32.1 ± 2.1%, oil only 30.1 ± 2%, conidial dose 2×10^{10} 31.5 ± 2.3% and conidial dose 8×10^{10} 30.1 ± 2.3%. This difference was not significant (F = 0.21; df = 3,20; *p* = 0.89). Mean proportions for *Cx. quinquefasciatus* were: untreated control 41.5 ± 1.1%, oil only 39.2 ± 2.4%, conidia dose 2×10^{10} 36.1± 1.7% and conidia dose 8×10^{10} 36.4 ± 3.2%. The difference was also not statistically significant (F = 1.37; df = 3,20; *p* = 0.28: Figure 3).

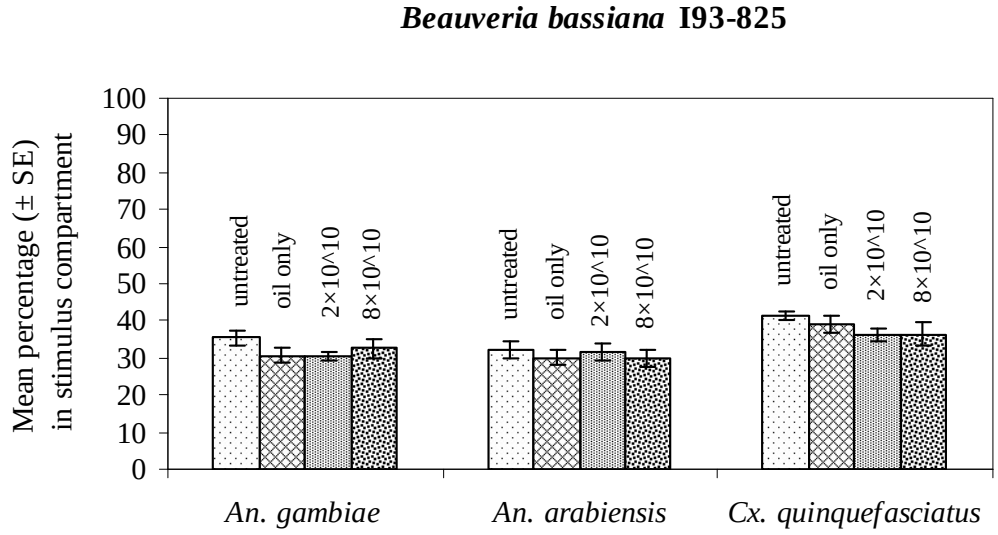


Figure 3. *Proportion (Mean ± SE) of Anopheles gambiae s.s., Anopheles arabiensis and Culex quinquefasciatus collected in the stimulus compartment with untreated control, mineral oil only control, and two formulations of Beauveria bassiana I93-825.*

Discussion

This study was designed to examine whether mosquitoes expressed any behavioural response (either repelled or attracted) to odours or contact with *B. bassiana* and *M. anisopliae* formulations. Successful fungal infection depends on the host contacting treated surface and receiving a threshold dose of infective conidia (Vandenburg et al. 1998; Hughes et al. 2004). If mosquitoes are repelled by conidia, this may reduce the number of mosquitoes that are exposed to the product. Results of our two experimental bioassays do not indicate that the conidia express a repellent action against the three mosquito species tested: Similar proportions of mosquitoes traversed the netting with and without fungus into the stimulus chamber. Scholte et al (2003) observed a moderate repelling effect of *M. anisopliae* dry conidia on *An. gambiae s.s.* The repelling effect, however, disappeared after the conidia were suspended in vegetable oil. In our study, although a different type of oil was used (mineral oil, Enerpar), the oil might have similarly suppressed the moderate repelling effect of the conidia. In a field study in Tanzania, a large proportion of wild anophelines was found sitting on fungus-impregnated sheet (Scholte et al. 2005). Possibly, the oil film prevents conidia from free dispersion in the air and thus reduces the probability of flying mosquitoes encountering conidia (Scholte et al. 2003) or masks the conidia odour. Interestingly, there are several other benefits gained from formulating conidia in oils. Conidia are more efficacious when formulated in oil than water (Luz and Batagin 2005). Compared to water, oil as carrier offers better adhesion and spreading of the formulation on the lipophilic insect cuticle. Furthermore, the oil can form a film on the host cuticle that acts as a humectant, creating good conditions for conidia to germinate and invade the host (Luz and Batagin 2005). Mineral oil can also improve the tolerance of conidia to extreme temperatures.

The behavioral responses of the arthropod hosts to the fungus may vary with the species of fungus, its virulence and conidia concentration. For example, termites, *Coptotermes formosanus*, are able to discriminate the species of fungi by their species-specific odours (Yanagawa et al. 2009). It was also found that the antennal response increased with increasing concentrations of suspension in the range from 10^3 to 10^7 conidia ml^{-1} (Yanagawa et al. 2009). As such, species-specific evaluations will need to be undertaken before other fungal species or concentrations can be developed for use against specific disease vectors. Importantly, in the present study none of the two fungal isolates were repellent at three conidia doses tested, which represent dose rates that have been recommended for field use (Mnyone et al. 2009).

The absence of a repellent effect of *M. anisopliae* and *B. bassiana* conidia in our experiments could be beneficial in different ways. There is the possibility for infecting mosquitoes by the lure-and-kill principle (Okumu et al. 2010), using for example odour-baited extra-domiciliary targets (Lwetoijera et al. 2010), since the fungal formulations do not have a repellent affect that would interfere with the attraction to the lures. Such lack of a repellency of entomopathogenic fungi against target mosquitoes will also enable entomopathogenic fungi to be integrated into use alongside the existing control tools. In a combination strategy with insecticide-treated bed nets (ITNs), mosquitoes deflected due to moderate repellency of synthetic insecticides, could be pushed to alternative surfaces treated with entomopathogenic fungus. In this way, the combined impact of ITNs and entomopathogenic fungi could be synergistic. As suggested by theoretical models that found that when ITNs and fungi are combined, the impact on malaria transmission is equivalent to the additive effect of each intervention alone (Hancock 2009).

Conclusion

Oil-formulations of *M. anisopliae* ICIPE-30 and *B. bassiana* I93-825 were not found to repel *Anopheles gambiae s.s.*, *An. arabiensis* and *Culex quinquefasciatus*, thus, emphasizing the potential of using either fungi for the control of vector mosquitoes.

Acknowledgements

We wish to thank Paulina Kasanga, Ally Daraja, Kassian Mbina and Emanuel Simfukwe for rearing mosquitoes and technical assistance. We also thank Seth Irish for establishing the colony of *Culex quinquefasciatus*; and Plasdusi Bakatu for taking care of guinea pigs. This study was funded by the Adessium foundation, Reeuwijk, The Netherlands. We thank Frank van Breukelen (Wageningen University), Nina Jenkins and Mathew Thomas (CSIRO/Penn State University) for supplying the *M. anisopliae* and *B. bassiana* dry conidia.

Chapter 7

Tools for delivering entomopathogenic fungi to malaria mosquitoes: effects of delivery surfaces on fungal efficacy and persistence

Published as: Mnyone LL, Kirby MJ, Lwetoijera DW, Mpingwa MW, Simfukwe ET, Knols BGJ, Takken W, Russell TL: Tools for delivering entomopathogenic fungi to malaria mosquitoes: effects of delivery surfaces on fungal efficacy and persistence. *Malaria Journal* 2010, **9:** 246

Abstract

The infection of malaria vectors with entomopathogenic fungi increases daily mortality rates and, thus, represents a control measure that could be used in integrated programmes alongside insecticide treated bed nets (ITNs) and indoor residual spraying (IRS). Before entomopathogenic fungi can be integrated into control programmes, an effective delivery system must be developed. The efficacy of *Metarhizium anisopliae* ICIPE-30 and *Beauveria bassiana* I93-825 (IMI 391510) (2×10^{10} conidia m^{-2}) applied on mud panels (simulating walls of traditional Tanzanian houses), black cotton cloth and polyester netting was evaluated against adult *Anopheles gambiae sensu stricto*. Mosquitoes were exposed to the treated surfaces 2, 14 and 28 d after conidia were applied. Survival of mosquitoes was monitored daily. All fungal treatments caused a significantly increased mortality in the exposed mosquitoes, descending with time since fungal application. Mosquitoes exposed to *M. anisopliae* conidia on mud panels had a greater daily risk of dying compared to those exposed to conidia on either netting or cotton cloth (p <0.001). Mosquitoes exposed to *B. bassiana* conidia on mud panels or cotton cloth had a similar daily risk of death (p = 0.14), and a higher risk than those exposed to treated polyester netting (p <0.001). Residual activity of fungi declined over time; however, conidia remained pathogenic at 28 d post application, and were able to infect and kill 73 – 82% of mosquitoes within 14 d since exposure to the conidia. Both fungal isolates reduced mosquito survival on immediate exposure and up to 28 d after application. Conidia were more effective when applied on mud panels and cotton cloth compared with polyester netting. Cotton cloth and mud, therefore, represent potential surfaces for delivering fungi to mosquitoes in the field.

Background

To eliminate malaria, vector control programmes will need to incorporate novel tools to complement the use of insecticide treated bed nets (ITNs) and indoor residual spraying (IRS). Both ITNs and IRS are highly effective against anthropophagic and endophilic species, but their efficacy is threatened by the emergence of resistance to synthetic insecticides (N'Guessan et al. 2007; Moreno et al. 2008). Furthermore, the growing demand of the global community for non-chemical control tools has refocused research objectives to address the practical aspects of biological control tools that have previously had limited uptake. Currently, a number of novel tools based on biological interactions are undergoing development including the application of fungal, bacterial, viral and protozoan pathogens (Read and Thomas 2009). Of these, entomopathogenic fungi show considerable promise for development as biopesticides (Zimmermann 1993, Luz et al. 1994; Samuels and Coracini 2004; Scholte et al. 2005; Farenhorst et al. 2008). Fungus production and application all involve relatively simple infrastructure and processes, which can be readily adopted in malaria-endemic countries. Moreover, entomopathogenic fungi are effective, but relatively slow-acting pathogenic agents (late-life acting), enabling genes of infected mosquitoes to be passed onto subsequent generations, thus weakening the selection pressure for resistance development (Ffrench-Constant 2005; Read et al. 2009).

Before entomopathogenic fungi can be integrated into control programmes, additional information regarding isolate selection, optimisation of production and formulation is required. While many successful laboratory evaluations of the efficacy of entomopathogenic fungi have been conducted (Scholte et al. 2003; Blanford et al. 2005; Achonduh and Tondje 2008), more research evaluating various formulations, delivery techniques and formats remains essential. Fungal formulations could be used to target either host-seeking and/or resting mosquitoes. Host-seeking mosquitoes could be targeted when entering a house through the eaves (Lines et al. 1987; Njie et al. 2009), or when attacking a host under a bed net (Hancock 2009). Resting mosquitoes could be targeted indoors on walls (Gillies 1954; Scholte et al. 2005) or both indoors and outdoors using a point source decoy resting site, e.g. resting boxes, clay pots, or black cotton cloth attached to the roof or walls (Farenhorst et al. 2008; Scholte et al. 2005).

The aim of the current study was to test different surface substrates that can be used to infect mosquitoes with fungal conidia and to examine whether the substrate used affected the availability, efficacy and persistence of the conidia.

Materials and methods

Mosquito rearing and maintenance

Anopheles gambiae sensu stricto Giles were reared at the Ifakara Health Institute (IHI) insectary (colony established in 1996, Njage village, Tanzania). Larval and adult stages of the mosquitoes were raised using methods described by Huho et al (2007). All bioassays were conducted on 3-6 d old adult female mosquitoes that had had access to 9% glucose/water (w/v) since emergence. During experiments, mosquitoes were maintained on 9% glucose/water (w/v) solution.

Fungal isolates and formulation

Two fungal isolates, *Metarhizium anisopliae* var. *anisopliae* (Metsch.) Sorokin isolate ICIPE-30 and *Beauveria bassiana* (Balsamo) Vuillemin isolate I93-825 (IMI 391510) were used in all bioassays. *Metarhizium anisopliae* was isolated from the maize stalk borer, *Busseola fusca* (Lepidoptera,

Noctuidae), in 1989 in Western Kenya and imported as dry conidia from Wageningen University, The Netherlands (courtesy F. van Breukelen, Wageningen University). *Beauveria bassiana* was isolated from a chrysomelid beetle (Coleoptera) in the USA and imported as dry conidia from the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia and Penn State University, USA (courtesy M. Thomas & N. Jenkins, Penn State University). Dry conidia of *M. anisopliae* were produced at IHI, after passaging and re-isolation from infected mosquito cadavers. Conidia were harvested from 15 day old cultures grown on rice grains. Before use, the viability (≥ 85% germination) of each conidia batch was confirmed by inoculation on Sabouraud dextrose agar (SDA).

Conidia were formulated in oil before application. Fungal stock solution was prepared by suspending 1-2 g of conidia in 20 ml of a 1:1 mixture of highly refined Enerpar (Enerpar M002®, BP South Africa LTD) or Shellsol (Shellsol T®, South Africa Chemicals) oils. The Enerpar and Shellsol oils have no effect on conidia germination or infectivity (L.M., unpublished data). To homogenise the mixture it was shaken vigorously, vortexed for 25 sec and then sonicated for 3 min in an ultrasonic bath (Langford Electronics, UK). Dilutions of 1:10, 1:100 and 1:1000 were then prepared in the same oil mixture and the concentration of conidia calculated using a Neubauer hemocytometer (Hausser Scientific Horsham PM, USA) and compound microscope (Leica ATC™ 2000, Buffalo, NY 14240, USA) at 400× magnification. The solution was adjusted to 2×10^{10} conidia m^{-2} by further dilution in oil.

Delivery substrates

The delivery substrates examined were:

1) polyester netting (intended to target host-seeking mosquitoes) was obtained from Safi Net™, A to Z Textile Mills Ltd, Arusha, Tanzania. White netting was used.
2) mud panels similar to the walls of adobe houses (intended to target resting mosquitoes). About 20 mm layer of mud, collected from natural soils near Ifakara, was lined onto the inner surface of plywood panels. The panels were kept to dry under room temperature for about 3 weeks. Panels had screws, thus the individual sections could be assembled to obtain mud cages for the bioassays (Figure 1).
3) black cotton cloth (intended to target resting mosquitoes). The cloth was obtained from the local shops, Ifakara, Tanzania.

The fungal solution was applied to the delivery surfaces using a hand-held air compressor sprayer (Minijet®, SATA, Germany) held 50 cm away and at a right angle to the substrate's surface. About 23 ml of fungal formulation (2×10^{10} conidia m^{-2}) was applied onto each of the surfaces. Treated materials were left to dry for 48 h. Sections of netting and black cotton cloth were then joined using Velcro strips, to fit over 20 × 20 × 20 cm wire frame cages. Mud-lined plywood panels were assembled into 20 × 20 × 20 cm cages. Mosquitoes were then exposed to the treated surfaces (Figure 1) as described in the bioassay procedures below.

Bioassay procedures

To test the efficacy of conidia applied to different delivery substrates, 40-55 adult *An. gambiae s.s* mosquitoes were introduced to four replicate conidia-treated and oil-treated control cages for 6 h. After exposure, mosquitoes were transferred to separate untreated cages (9 × 9 × 9 cm) and maintained at 26 ± 1°C and 90 ± 5% relative humidity (RH) with access to 9% glucose/water (w/v) solution *ad libitum*. Mosquito survival was monitored daily for a maximum of 28 d. Dead mosquitoes were collected and placed onto moist filter paper in petri dishes, sealed with parafilm, and kept at 26 ± 1°C and 90 ± 5% RH for 3–4 d, after which they were examined for evidence of fungal sporulation. After the initial treated surfaces were stored at 26 ± 1°C and 90 ± 5% RH and separate mosquito cohorts were exposed 14 and 28 d later to determine the persistence of conidia on different delivery surfaces.

Data analysis

Mosquito survival data were analysed using Cox regression to determine the relative risk of dying (hazard ratios) for the specific treatment group compared with the control group and with each other. The explanatory factors included in the analysis were treatment, delivery surface and time of exposure post-application. The Kaplan-Meier method using SPSS version 17 (SPSS Inc., Chicago, IL), was applied to obtain median survival times for treated and untreated groups of mosquitoes. Survival curves were considered not statistically different at $p > 0.05$.

Results

Overall, fungal infection reduced the median survival time (MST) of fungal-exposed mosquitoes compared to the controls regardless of delivery surface or time of exposure post-application (Table 1). The daily risk of dying for mosquitoes exposed to either *M. anisopliae* (Hazard Ratio [HR] = 2.72 [95% CI = 2.58 – 2.86], $p < 0.001$) or *B. bassiana* (HR = 2.23 [95% CI = 2.12 – 2.34], $p < 0.001$) was more than two-fold higher than that for control mosquitoes (Table 2). For *M. anisopliae*, the risk of death for mosquitoes exposed to conidia on mud panels was higher than that for mosquitoes exposed to conidia on either polyester netting (HR = 1.17 [95% CI = 1.1 – 1.24], $p < 0.001$) or cotton cloth (HR = 1.11 [95% CI = 1.05 – 1.18], $p < 0.001$) regardless of the time at which mosquitoes were exposed post conidia application (Table 2). Mosquitoes exposed to *B. bassiana* on mud panels had a similar risk of death to mosquitoes exposed to this fungus on cotton cloth (HR = 0.96 [95% CI = 0.91 – 1.01], $p = 0.14$) regardless of the time at which mosquitoes were exposed post conidia application. The risk of death for mosquitoes exposed to conidia on the mud panels and cotton cloth was higher than that for mosquitoes exposed to conidia on the polyester netting (mud panel: HR = 1.17 [95% CI = 1.11 – 1.24]; cotton cloth: HR = 1.19 [95% CI = 1.14 – 1.23], $p < 0.001$, Table 2). As such, the poorest performance was consistently recorded for the polyester netting material.

The effect of conidia on mosquito survival generally declined over time post-application for all delivery surfaces examined (Figure 2). Mosquitoes exposed to *M. anisopliae* 2 d and 14 d post-application, had a similar risk of death (HR = 0.95 [95% CI = 0.9 – 1], $p = 0.05$). The residual efficacy of conidia of *M. anisopliae* had significantly declined by 28 d post-application, with the relative risk of death for mosquitoes exposed at 2 d post-application being higher (HR = 1.12 [95% CI = 1.06 – 1.19], $p < 0.001$, Table 2). The residual efficacy of *B. bassiana* declined a little more sharply with mosquitoes exposed at 2 d post-application having a higher risk of death than mosquitoes exposed both 14 d (HR = 1.17 [95% CI = 1.03 – 1.25], $p = 0.01$) and 28 d (HR = 1.17 [95% CI = 1.06 – 1.28], $p = 0.002$) post application. Mosquitoes that were exposed to *B. bassiana* at 14 d and 28 d post-application had similar risk of death (HR = 0.99 [95% CI = 0.93 – 1.06], $p = 0.82$, Table 2). Nonetheless, 28 d post-application conidia of both fungi still infected and killed 73 – 82% of mosquitoes by day 14.

Table 1: Median survival times (MST ± SE) of *Anopheles gambiae s.s.* exposed to *Metarhizium anisopliae* ICIPE-30 and *Beauveria bassiana* I93-825 at day 2, 14 and 28 post-application of fungus to mud panel, polyester netting and black cotton cloth

Fungus isolate	Delivery surfaces	Days post application	MST ± SE (days)		χ^2 value	*P* value
			Treatment	Control		
M. anisopliae	Mud panel	2	6 ± 0.62	13 ± 0.68	125.64	<0.001
		14	7 ± 0.30	16 ± 0.94	70.37	<0.001
		28	9 ± 0.02	14 ± 0.72	62.80	<0.001
	Polyester netting	2	6 ± 0.40	14 ± 0.85	108.04	<0.001
		14	11 ± 0.51	16 ± 1.01	58.04	<0.001
		28	11 ± 0.82	15 ± 0.70	62.89	<0.001
	Cotton cloth	2	5 ± 0.49	14 ± 0.61	61.81	<0.001
		14	9 ± 0.37	17 ± 0.70	80.57	<0.001
		28	11 ± 0.38	15 ± 0.72	50.46	<0.001
B. bassiana	Mud panel	2	8 ± 0.27	13 ± 0.68	86.09	<0.001
		14	9 ± 0.45	16 ± 0.94	62.52	<0.001
		28	10 ± 0.47	14 ± 0.72	41.08	<0.001
	Polyester netting	2	10 ± 0.62	14 ± 0.61	54.90	<0.001
		14	11 ± 0.62	17 ± 0.70	33.01	<0.001
		28	12 ± 0.66	15 ± 0.72	27.88	<0.001
	Cotton cloth	2	5 ± 0.24	14 ± 0.85	156.47	<0.001
		14	10 ± 0.50	16 ± 1.01	76.42	<0.001
		28	10 ± 0.44	15 ± 0.70	52.64	<0.001

Table 2: Mortality hazard ratios of mosquitoes exposed to *Metarhizium anisopliae* ICIPE-30 and *B. bassiana* I93-825 at day 2, 14 and 28 post-application of fungus to mud panel, polyester netting and cotton cloth.

Factor	Comparisons	HR	95% C.I	*P* value
***Metarhizium anisopliae* ICIPE-30**				
Treatment	Treatment vs control	2.72	2.58 – 2.86	<0.001
Surfaces	Mud panel vs polyester netting	1.17	1.1 – 1.24	<0.001
	Mud panel vs cotton cloth	1.12	1.05 – 1.18	<0.001
	Cotton cloth vs polyester netting	0.96	0.91 – 1.02	0.17
Days post-application	2 vs 14	0.95	0.9 – 1	0.05
	2 vs 28	1.12	1.06 – 1.19	<0.001
	14 vs 28	1.05	1 – 1.11	0.11
***Beauveria bassiana* I93-825**				
Treatment	Treatment vs control	2.23	2.12 – 2.34	<0.001
Surfaces	Mud panel vs polyester netting	1.17	1.11 – 1.24	<0.001
	Mud panel vs cotton cloth	0.96	0.91 – 1.01	= 0.14
	Cotton cloth vs polyester netting	1.19	1.14 – 1.23	<0.001
Days post-application	2 vs 14	1.13	1.03 – 1.25	= 0.01
	2 vs 28	1.17	1.06 – 1.28	= 0.002
	14 vs 28	0.99	0.93 – 1.06	= 0.82

Discussion

This study was intended to provide fundamental information for developing delivery formats in advance of field evaluations of entomopathogenic fungi against malaria vectors. Consistent with published literature (Blanford et al. 2005; Farenhorst et al. 2008; Achonduh and Tondje 2008), we found that infection with either *M. anisopliae* or *B. bassiana* significantly reduced the survival of female *An. gambiae s.s.* mosquitoes. The efficacy of fungal formulations (measured by reduction of adult survival) varied among the candidate delivery materials. The efficacy of *M. anisopliae* conidia was highest when applied on mud panels compared with cotton cloth or polyester netting. For *B. bassiana*, the efficacy of conidia was highest when applied to either mud panels or cotton sheets compared with polyester netting. The lowest efficacy of either fungal isolate was consistently recorded for the polyester netting. Rapid drop in the efficacy of conidia applied on the netting has also been reported in other studies (M. Farenhorst, unpublished data; Howard et al. 2010). Variations in efficacy of treatment between surfaces of different material are not unique; previous research into pyrethroid insecticides for impregnating bednets revealed that efficacy is dependent on the type of fabric used, with polyester being more effective than nylon and cotton (Vatandoost et al. 2006). In our case, the polyester netting may have reduced the viability of conidia of both fungal species, possibly through poor conidia attachment due to its smooth fibres and/or chemical effects from the netting itself or chemicals used to soften polyester fibres.

Even though the residual activity of fungal isolates declined over time, conidia remained pathogenic up to 28 d post application (and possibly longer) and were still able to infect and kill 73 – 82% of mosquitoes within 14 d. When conidia were applied on the netting, the residual activity declined much more rapidly compared with mud panels and cotton cloth. Decline in the residual activity of conidia has also been reported elsewhere (Scholte et al. 2005; Farenhorst et al. 2008). The residual activity of fungal conidia appears to decline at comparable rates to other biopesticides, for example *Bacillus thuringiensis* (Karch et al. 1991; Fillinger and Lindsay 2006). The residual activity of chemical insecticides also declines with time, but compared to biopesticides their persistence is often longer (Itoh 2005), which is partially dependent on dose. A fungal formulation that can infect and kill at least 50% of the host-seeking mosquitoes for over two months after field application is desirable, as this would exert considerable epidemiological impact on malaria transmissions (Scholte et al. 2005; Hancock 2009).

It is important to note that the treatable surface area of polyester netting was much smaller than mud and cotton cloth due to the holes in this material (25 holes/cm^2). As such the netting had a smaller surface area for both conidial attachment and exposure of mosquitoes. The higher efficacy of fungus observed when applied to mud and cotton cloth could therefore have simply been due to availability of more conidia per unit area, maximizing mosquito exposure and probability of picking up more conidia.

The pathogenic effect of the fungal conidia declined over time on different substrates, with efficacy declining the fastest on the polyester netting. In the initial exposure (2 d post application), many conidia could have been readily available on the surface of each substrate. With time, however, the bond between conidia and the surface may have gotten stronger such that few conidia were readily available to mosquitoes. This may have accounted for a quick drop in efficacy of fungi applied on cotton cloth and netting between the initial and subsequent exposures. A drop in efficacy could also be explained by reduced virulence of the conidia. For the mud panel, the decline was fairly constant. Others have reported a decline in residual activity resulting from fluctuating environmental conditions (Scholte et al. 2005), but this was unlikely the case here since experiments were done under stable and controlled conditions.

Our aim was to develop a delivery tool(s) that facilitates rapid dissemination of fungal conidia to mosquitoes and remains effective for a prolonged amount of time. The high mortality (82% within 14 d) since exposure of mosquitoes to conidia on mud and cotton cloth 28 d post application emphasizes the potential of these two surfaces as target tools. The delivery surfaces examined in the current study

were selected because they could be easily adapted for practical dissemination of spores under realistic field conditions. Cotton cloth could be placed to partially cover eave openings, on ceilings (Scholte et al. 2005), and on the internal surface of resting traps (e.g. lure and kill with resting stations) (Lwetoijera et al. 2010). For mud panels, conidia could be applied using indoor residual spraying. In an effort to also target outdoor resting mosquitoes, odour-baited traps (Okumu et al. 2010) made from mud panels could be useful. Our results support further research into any of these suggestions that may effectively disseminate mosquito-killing conidia while being practical for end users of the technology.

Conclusions and recommendations

Mosquitoes exposed to entomopathogenic fungi expressed a reduced survival from conidia used 2 up to 28 d after application. Conidia were more effective when applied on mud panels and cotton cloth compared with polyester netting. Cotton cloth and mud, therefore, present useful and practical tools for applying fungi against resting mosquitoes in the field. These tools should be used such that mosquito contact to conidia is maximized in order to correctly predict the efficacy and residual activity of fungi.

Acknowledgements

The authors thank the Adessium Foundation (Reeuwijk, The Netherlands) for funding this research. Many thanks to Jennifer Stevenson, Simon Blanford and Christian Luz for comments, and the Ifakara Health Institute for scientific and administrative support. We thank Frank van Breukelen (Wageningen University), Nina Jenkins and Mathew Thomas (CSIRO, Australia and Penn State University) for supplying the *M. anisopliae* and *B. bassiana* conidia. We also thank Sander Koenraadt for reviewing the statistical analysis.

Chapter 8

Exploiting the behaviour of wild malaria vectors to achieve high infection with entomopathogenic fungus

To be submitted in a slightly modified form as: Mnyone LL, Lyimo IN, Lwetoijera DW, Mpingwa MW, Nchimbi N, Hancock P, Russell TL, Kirby MJ, Takken W, Koenraadt CJM: Exploiting the behaviour of wild malaria vectors to achieve high infection with entomopathogenic fungus.

Abstract

Malaria still afflicts millions of people every year, especially in sub-Saharan Africa. Control of the mosquitoes that transmit malaria has been the mainstay in the fight against the disease, but alternatives are required in view of emerging insecticide resistance. Entomopathogenic fungi have proven to be such an alternative, but to date, very few trials have translated the use of these agents to field-based evaluations of their actual impact on mosquito survival and malaria risk. Delivery techniques that successfully infect mosquitoes need to be developed and tested under realistic field conditions. We applied mineral oil-formulations of *Metarhizium anisopliae* and *Beauveria bassiana* against wild malaria mosquitoes using five different techniques that each exploited the behaviour of mosquitoes when entering, host-seeking or resting in experimental huts. Techniques employed during five trials were eave netting, eave curtains, cloth panels, eave baffles and strips of cotton cloth hung next to bed nets. We used three experimental huts per trial in which we evaluated one of the five techniques over 9 consecutive nights. Every morning a sub-sample of up to 25 mosquitoes was collected from each hut, placed singly in individual plastic tubes and monitored for daily survival. After death, mosquitoes were processed and examined for fungal growth. With the obtained data on fungal infection rates and virulence, we then estimated the impact of fungal infection on malaria transmission risk by using a gonotrophic cycle model of mosquito-malaria interactions. Few mosquitoes entered huts fitted with eave netting and none of these became infected with fungus (trial 1). Application of fungus on eave curtains and panels did not show any impact on mosquito infection or survival (trials 2 and 3). However, after forcing upward entry of mosquitoes through the eaves (baffle design; trial 4) or after application of fungus treated surfaces directly on a bed net with a host (strip design; trial 5), survival of mosquitoes from the treatment group was significantly reduced (by 6-7 d) relative to that of controls irrespective of the fungal isolate used. Moreover, 67.9 to 75.5% of the treatment mosquitoes showed fungal growth and had thus been in sufficient contact with the treated surfaces. With the effect of fungus on survival alone the EIR is estimated to be reduced by 75-80%, whereas EIR is reduced by at least 96% if bed net coverage is ≥0.4 even if mosquitoes are resistant to insecticides and fungus exerts moderate reduction on blood feeding activity. The design for delivering conidia of entomopathogenic fungi to mosquitoes can largely impact on the proportion of mosquitoes actually infected and depends on how female mosquitoes behave towards and around a host. We achieved a fungus infection rate as high as 75% by means of fungus-treated cotton cloth eave baffles/cotton cloth strips hung around the bed nets. Our model confirms that such coverage rates are sufficiently large to achieve major reductions in malaria transmission risk.

Background

Malaria remains the pre-eminent parasitic disease and one of the top three killers among communicable diseases especially in low-income countries (Sachs and Malaney 2002; Snow et al. 2005). The vast majority of malaria cases (85%) occur in Africa followed by the South-east Asia (10%) and eastern Mediterranean regions (4%) (WHO 2009). Vector control remains the most important component of malaria control as it is cost-effective in terms of health gains per dollar spent (Gallup and Sachs 2001). Currently, the most frequently advocated vector control measures include insecticide treated nets (ITN) and indoor residual spraying (IRS) (Rowland et al. 2000; Mabaso et al. 2004; Sharp et al. 2007). These measures have a proven effectiveness in controlling the disease. However, their continued effectiveness is threatened by the mosquitoes developing resistance to the synthetic insecticides (Kelly-Hope et al. 2008; Ranson et al. 2009). For example, assessment of the effect of ITNs in Benin revealed that in areas with insecticide-resistant populations of *Anopheles gambiae,* ITNs no longer prevent such mosquitoes from blood feeding (N'Guessan et al. 2007) or from dying (Yadouleton et al. 2010). Clearly, there is an urgent need to develop novel malaria control strategies that can reliably and sustainably be used to complement or replace existing control measures. Fungal biocontrol of adult mosquitoes may offer such an alternative approach. Laboratory (Blanford et al. 2005; Farenhorst et al. 2008; Mnyone et al. 2009) and small scale field trials (Scholte et al. 2005; Lwetoijera et al. 2010) have demonstrated that malaria vectors can succumb to, and die of fungus infection. Despite these promising results, little effort has been devoted to evaluating techniques that could be used at larger scale to disseminate fungus to large populations of wild mosquitoes.

Fungal formulations could be used to target either host-seeking and/or resting mosquitoes. Host-seeking mosquitoes could be targeted when entering a house through the eaves (Lines et al. 1987; Njie et al. 2009), or when attacking a host under a bed net (Hancock 2009). Resting mosquitoes could be targeted indoors on walls (Gillies 1954) or both indoors and outdoors by means of point source targets, e.g. resting boxes, clay pots, and black cotton cloth attached to the roof or wall (Scholte et al. 2005; Farenhorst et al. 2008). Outdoor bait-stations (OBS) have a demonstrated potential as dissemination tools (Lwetoijera et al. 2010; Okumu et al. 2010). In reality, the effectiveness of a particular delivery technique will depend on the manner in which it interacts with a mosquito's normal behaviour. For successful control with entomopathogenic fungus it is not only necessary that the host (mosquito) contacts a treated surface, but also receives a sufficient dose of infectious conidia upon this contact (Vandenburg et al. 1998; Hughes et al. 2004). The aim of the present study was to develop techniques that maximize fungus infection rates in wild malaria mosquito populations, evaluate the impact of fungal infection on survival of these mosquitoes, and asses the impact on malaria transmission levels. As African malaria vectors tend to blood feed and rest primarily indoors (Charlwood et al. 1995), fungal delivery techniques were focused on exposing mosquitoes to entomopathogenic fungi inside the house. We examined the effectiveness of five different techniques of fungal exposure that each exploited the behaviour of mosquitoes when individuals were either entering (eave netting, eave curtains, cloth baffles), host-seeking (cloth strips hung next to bednets) or resting (cloth panels) in experimental huts. Also, based on the obtained data on fungal infection rates and virulence, we implemented a gonotrophic cycle model of mosquito-malaria interactions (Hancock 2009) to estimate the impact of fungal infection on the malaria transmission risk expressed as entomological inoculation rate (EIR).

Materials and Methods

Study area

The small-scale field trials were conducted in Lupiro village (8.38° S and 36.67° E) (Ulanga District), a rural hamlet 30 km south of Ifakara, in the Kilombero valley, Tanzania. The village lies on a low plateau of about 10 m above the surrounding area at an elevation of 300 m above sea level. The area borders a permanent swamp (near the Ndolo River) extensively cleared for rice cultivation. Most of the residents are farmers and in addition to rice they cultivate maize and cassava. The majority of the houses are made from mud walls with thatched roofs. There are two rainy seasons: the long rains from April to June and short rains normally in October and November. The annual rainfall is about 1200 – 1800 mm. The temperature ranges between 20°C and 32.6 °C. The present study was conducted during the period between May and December 2009 (trials 1-4) as well as March and April 2010 (trial 5). In 2009, temperature and relative humidity inside the experimental huts ranged from 13.8 – 37.9°C and 30.3 – 100% RH. For the experimental months in 2010, temperature and relative humidity ranged from 22.5 – 37.7°C and 45.0 – 99.8% RH. The population of malaria vectors in the area is largely comprised of members of the *An. gambiae* Giles complex, mainly *An. arabiensis* Patton (98%) and some *An. gambiae* Giles *sensu stricto* (Okumu et al. 2010). Although rare, it is not uncommon to encounter *An. funestus* Giles. Estimates of the entomological inoculation rate indicate that unprotected individuals receive as many as 352 infectious bites per year (Killeen et al. 2007).

Fungal isolates and formulation

Two fungal isolates, *Metarhizium anisopliae* var. *anisopliae* (Metsch.) Sorokin isolate ICIPE-30 and *Beauveria bassiana* (Balsamo) Vuillemin isolate I93-825 (IMI 391510) were used in the trials. Fungal conidia were suspended in a 1:1 mixture of highly refined Enerpar oil (Enerpar M002®, BP Southern Africa Ltd) and Shellsol oil (Shellsol T®, South Africa Chemicals). Except for the netting materials in trial 1 (left to dry under the shade for 5 h), cloth materials were left to dry indoors for 72 h at ambient temperature. Unless stated otherwise in the subsequent sections, treatment of materials was done at the laboratories of the Ifakara Health Institute (30 km from the study site), and the materials were transported to the field site after drying. Origin and source of *M. anisopliae* IP 46 is described in Mnyone et al (2009). *Beauveria bassiana* was kindly provided by Wageningen University, The Netherlands (courtesy M. Jumbe, Wageningen University) and Penn State University, USA (courtesy M. Thomas & N. Jenkins, Penn State University). The viability of conidia we used was confirmed by inoculation on Sabourauds Dextrose Agar (SDA). The germination of conidia used for the first three trials ranged between 80 – 85%; and that of the conidia used for the last two trials ranged between 70 – 75%.

Experimental huts

The experimental huts were designed to represent local housing (Ogoma et al. 2010). The huts have been designed in kit form, for ease of portability, and the entire hut can be flat packed. The structure has a galvanized pipe framework. The roof is corrugated iron lined with thatch. The outer walls were constructed from canvas. Inner walls are removable panels coated with mud, to simulate local mud walls. Dimensions of the huts are 6.5 m long, 3.5 m wide and 2 m high. The height of each structure measured 2.5 m at the roof apex. Experimental huts and exit traps were evaluated along with selected human houses to determine if they could attract a representative sample of mosquitoes (S.J. Moore, unpublished data).Three huts were used in our trials and each hut had 4 windows with window exit traps attached to it (Figure 1). The huts were positioned between the village and a nearby rice field, standing approximately 15 m apart.

Figure 1. *Experimental hut type used for the five trials*

Experimental hut evaluations

Three experimental huts were used in all trials. Unless stated otherwise under the respective trial, a 3 × 3 Latin square design was used to simultaneously evaluate two different designs for delivering entomopathogenic fungi and one control. Treatments were randomly allocated to the three different huts, and were swapped between huts each time after three days. Each individual trial was conducted for nine days. During the trials each hut contained two human volunteers sleeping under untreated bednets. The volunteers rotated between huts daily such that each pair spent three days in each hut in total. The volunteers slept in the experimental huts from 19.00 - 7.00 hrs. Two data loggers in total (TinyTag®) were placed inside the huts to monitor environmental temperature and humidity. Exit traps were installed over all four windows (trap: 55.5 × 45.5 × 55.5 cm; funnel opening: 40 × 3 cm) to catch mosquitoes when they exit, except for trial 1 where two window and six eave traps (trap: 100 × 40 × 40 cm; funnel opening: 80 × 3 cm) were used. Mosquitoes were collected by a mouth aspirator from the exit traps at 7.00 am in the morning. A random sub-sample of maximum 25 female mosquitoes from each hut was collected. Each mosquito was placed individually into 50 ml falcon tubes (115 mm × 28 mm diameter), provided with a wad of sugar water-soaked cotton, and their daily survival was monitored in a field-based insectary. When less than 25 mosquitoes were trapped in the window exit traps, all individuals were used. The mortality of the mosquitoes was recorded daily until all mosquitoes had died (maximum survival was 39 d). Mosquitoes remaining in the traps were killed, identified and stored. Cadavers of mosquitoes that died in the survival experiment were left to dry under open air for 2 d, put onto filter paper in petri dishes covered with their lids and kept inside transparent containers (30 cm diameter and 40 cm high) with wet towels (humid chambers) for 5-6 d. Thereafter, they were examined for fungal sporulation. Containers were tightly closed with lids to maintain humidity.

Trial 1: Eave netting

Fungus-treated polyester netting (9 holes cm^{-2}) was fitted over the eaves of the experimental huts to determine whether wild mosquitoes could be infected during passage through this netting. In the laboratory, 40% of the mosquitoes passed through this type of netting when exposed to human stimulus (Chapter 6). A fungal formulation of *Beauveria bassiana* I93-825 was painted with a brush onto the netting laid on a flat surface at a concentration of 2×10^{10} conidia m^{-2} (Figure 2). Treated netting was left outdoors under the shade to dry for about 5 h, before being fitted over the eaves by Velcro strips. Two controls were used: eaves with oil-treated netting, and eaves without netting.

Trial 2: Eave curtain

Fungus-treated 20 cm high sections of black cotton cloth (eave curtains) were fitted onto eaves. Curtains were fixed from the top by Velcro strips and left hanging from below, leaving a gap of about 3.5 cm between the cloth and the wall. Mosquitoes passing the eaves could fly into the hut through the gap at the bottom of the curtain, expectedly after repeatedly contacting the curtain to locate the gap. One hut had curtain treated with oil alone (control), and the other two huts had curtains treated with *B. bassiana* I93-825 or *M. anisopliae* ICIPE-30, respectively. Both isolates were applied onto curtains at a concentration of 2×10^{10} conidia m^{-2} using procedures described by Mnyone et al (2009). Only the surface of the curtain facing the outside was treated.

Trial 3: Eave curtains and panels

Eave curtains were placed in one of the huts, as in Trial 2, except that a four times higher concentration of fungus (8×10^{10} conidia m^{-2}) was used. In the other hut, two black cotton panels (length 120 cm, width 90 cm), one per bed were used. Panels were treated with fungus on both sides by the use of a simple hand sprayer (Mnyone et al. 2009), and were placed next to the bed facing the feet of a sleeping volunteer (Figure 2). Curtains and panels were both treated with *B. bassiana*. The control hut had neither curtain nor panel and eaves were left open.

Trial 4: Eave baffles

Eave curtains were modified to serve as eave baffles (Smith and Hudson 1972). Unlike in curtains where cloth was parallel to the wall with the entry gap from below, cotton cloth baffles were fitted in a slanting orientation with the entry gap (3 cm) from the top (≈ 20 cm inside the wall) (Figure 2). At the bottom, the cloth material was fixed with pins to the outside wall covering ≈10 cm of the outside wall surface. Two huts had baffles treated with *Beauveria bassiana* I93-825 and *M. anisopliae* IP 46 respectively at a concentration of 4.1×10^{10} conidia m^{-2}. The control hut had eave baffles treated with oil alone.

Trial 5: Strips

Long cotton strips (126 cm, 7 cm wide) and short cotton strips (63 cm, 7 cm wide) were treated with *M. anisopliae* IP 46 and hung next to bed nets to surround the entire bed (Figure 2). The gap between the strips was 1 cm. One hut had short cloth strips, one hut long cloth strips and the third hut did not have any strips (control). Fungal formulation used was of a concentration of 5×10^{10} conidia m^{-2}. Treatment of the strips was done at the field site at 17: 00 h to minimize the effect of intense sun light on conidia when drying. Strips were left outdoors for 3 h to allow initial drying, then hung inside huts and left for an extra 24 h to complete drying.

Data analysis

Kaplan-Meier analysis was applied to obtain the median survival times (MST) for mosquitoes collected from treated and untreated experimental huts. The group of mosquitoes collected from treated huts was categorized as infected and non-infected depending on whether or not they showed fungal growth after dying. Cox' regression was used to determine the relative risk of death (hazard ratios) for each specific treatment group compared with control group and with each other. The risk of death for mosq-

-uitoes collected from treated huts relative to controls was determined for infected and non-infected mosquitoes together; and in separation in case of significantly high risk in mosquitoes from treated huts. SPSS version 17 (SPSS Inc., Chicago, IL) was used. The difference between the compared factors was considered significant at p <0.05.

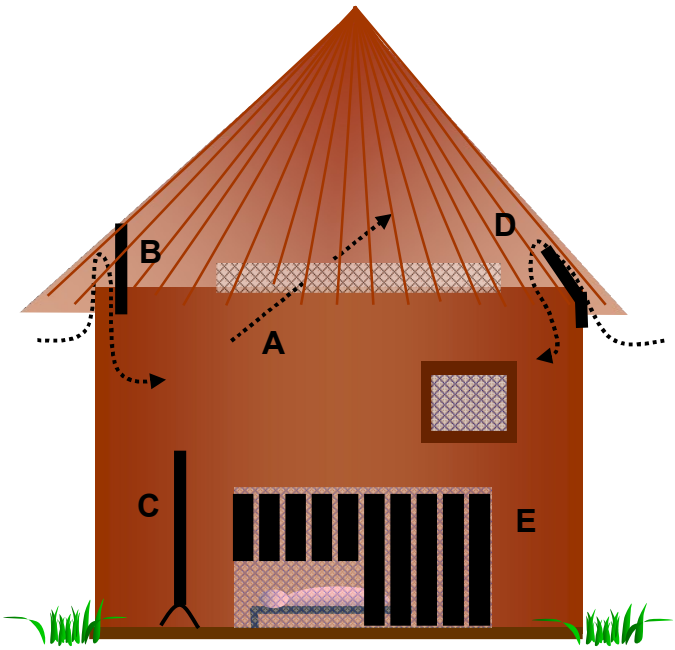


Figure 2: *Different fungus delivery techniques for which efficacy was evaluated: eave netting (A), cotton cloth curtain (B), cotton cloth panels (C) cotton cloth baffles (D) and cotton cloth strips (E).*

Impact of fungal infection on EIR

Based on the data obtained in the present study, the malaria transmission model described by Hancock (2009) was used to estimate the impact of fungal virulence and coverage achieved on the EIR. Virulence of the fungal infection is expressed as the average time until death from fungal infection if all other mortality sources are excluded. The effect of fungus infection on adult mosquito mortality was estimated by fitting Weibull functions to the obtained survival data of the daily-collected mosquitoes from the experimental huts. The coverage of fungus in this case is defined as the daily probability that host-seeking mosquitoes contract the fungal infection. To determine how the impact would vary when fungus is used alongside ITN, the fungal impact was estimated for three different scenarios of ITN coverage: 0%, 40% and 80%.

Ethics approval

The study was conducted after being approved by the Institutional Review Board of the Ifakara Health Institute (IHI) (IHRDC/IRB/No. A-019) and the National Institute of Medical Research (NIMR/HQ/R.8a/Vol. IX/710) in Tanzania.

Results

Trial 1: Eave netting

Very few (range 1-5) and sometimes no *An. gambiae* mosquitoes were collected from the treated huts (fungus-treated & oil control hut) fitted with eave netting (Table 1). No fungus infection was confirmed from the few collected mosquitoes (0/10) in total for the nine nights. By contrast, on average 45 female *An. gambiae* mosquitoes were collected per night from the hut without eave netting (control). None of these were infected with fungus.

Trial 2: Eave curtain

The average number of female *An. gambiae* mosquitoes collected per hut each night ranged from 32.4 to 38.4 (Table 1). The survival of mosquitoes passing curtains treated with *M. anisopliae* ICIPE-30, was not significantly different from that of the controls (Wald = 0.02; df = 1; HR = 0.96; p = 0.89). The median survival time (MST ± SE) was 16 ± 1.0 d and 15 ± 0.4 d for treatment and control groups of mosquitoes, respectively. Similarly, for *B. bassiana*-treated curtains, the survival of the treatment group of mosquitoes was not significantly different from that of the controls (Wald = 0.17; df = 1; HR = 0.89; p = 0.68). The MST was 15 ± 0.5 d. None of the mosquitoes collected from treated and untreated hut was infected with fungus (Table 1).

Trial 3: Eave curtains and panels

The average number of female *An. gambiae* mosquitoes collected per hut each night ranged from 27.9 to 33.9 (Table 1). Neither fungus-treated eave curtains nor panels led to a reduced mosquito survival. With eave curtains, survival of mosquitoes in the treatment was not significantly different from that of mosquitoes in the control (Wald = 0.18; df = 1; HR = 0.88; p = 0.68). The MST was 20 ± 1.3 d and 19 ± 0.7 d for treatment and control mosquitoes, respectively. Similarly, with cloth panels, the survival of the treatment group of mosquitoes was not significantly different from that of the controls (Wald = 0.17; df = 1; HR = 0.89; p = 0.67). The MST was 19 ± 1.8 d. About 18.3% and 10.7% of the cadavers from treated curtain and panels respectively, showed fungal growth while none was observed on control mosquitoes (Table 1).

Trial 4: Eave baffles

The average number of female *An. gambiae* mosquitoes collected per hut each night ranged from 40.6 to 74.2 (Table 1). Overall, mosquitoes from huts with fungus-treated baffles survived less relative to mosquitoes collected from the control hut, regardless of the fungal species (Figure 3&4). The daily risk of death was approximately two-fold greater in mosquitoes collected from *M. anisopliae*-treated hut relative to the controls (p <0.001), whereas the daily risk of death was over two-fold greater in mosquitoes from *B. bassiana*-treated huts relative to the controls (p <0.001, Table 2). When survival data for only the fungus-infected mosquitoes was run against controls, the daily risk of death was close to four-fold greater in mosquitoes infected with *M. anisopliae* relative to the controls (HR = 3.9 [95% CI = 2.98 – 5.07], p <0.001), whereas the daily risk of death was over four-fold greater in mosquitoes infected with *B. bassiana* relative to the controls (HR = 4.5 [95% CI = 3.47 – 5.85], p <0.001).

The risk of death for non-infected mosquitoes collected from treatment huts was not significantly different from that of the controls (*M. anisopliae*: HR = 1.0 [95% CI = 0.78 – 1.29], *B. bassiana*: *M. anisopliae*: HR = 1.02 [95% CI = 0.76 – 1.37], p >0.05).The median survival times (MST) ranged between 13 – 21 d (Table 2). The confidence intervals for the HR estimates for the two fungal strains overlapped and as such there was no clear difference in efficacy of the two fungal species. Overall, of 178 mosquitoes that were sampled from the *M. anisopliae* IP 46 treated hut, 69.1% showed fungal growth. For the *B. bassiana* treated hut, 68.0 % of the 206 mosquitoes showed fungal growth (Table 1). About 2.0 % of the controls showed fungal growth.

Trial 5: Strips around bed net

The average number of female *An. gambiae* mosquitoes collected per hut each night ranged from 24.3 to 86.2 (Table 1). Overall, mosquitoes from huts with either treated-short or –long strips survived less relative to mosquitoes collected from the control hut (Figure 5). The daily risk of death was over 1.5 to 1.6 times greater in mosquitoes exposed to *M. anisopliae* IP 46 applied on short and long cotton cloth strips relative to the controls (p <0.001). There was no significant difference in survival between mosquitoes exposed to short or to long strips (p = 0.36; Table 3). When survival data for only the fungus-infected mosquitoes was run against controls, the daily risk of death was over four-fold greater in infected mosquitoes relative to the controls (short strips: HR = 4.22 [95% CI = 3.27 – 5.46], long strips: HR = 4.82 [95% CI = 3.77 – 6.32], p <0.001). The risk of death for non-infected mosquitoes collected from treatment huts was not significantly different from that of the controls (HR = 1.0 [95% CI = 0.83 – 1.20], p = 1). Median survival times (MST) ranged between 13 – 21 d (Table 3). Overall, of 155 mosquitoes which were sampled from the hut with treated long strips, 75.5% showed fungal growth. For the hut with treated short strips, 74.6% of the 189 mosquitoes showed fungal growth. About 3.3% of the controls showed *M. anisopliae* growth (Table 1).

Table 2: Median survival time (MST), hazard ratios (HR) plus 95% confidence interval (CI) of wild *Anopheles gambiae* s.l. mosquitoes exposed to *Metarhizium anisopliae* IP 46 and *Beauveria bassiana* I93-825 formulation applied on cotton cloth eave baffles.

Treatment	MST± SE	HR [95% CI]	*p* value
Control	21 ± 0.6		
M. anisopliae	14 ± 0.7	1.86 [1.51 - 2.29]	<0.001
B. bassiana	13 ± 0.7	2.20 [1.79 - 2.70]	<0.001

Table 3: Median survival time (MST), hazard ratios (HR) plus 95% confidence interval (CI) of wild *Anopheles gambiae* s.l. mosquitoes exposed to *Metarhizium anisopliae* IP 46 formulation applied on short and long cotton cloth strips hung next to bed nets.

Treatment	MST± SE	HR [95% CI]	*p* value
Control	21 ± 0.4		
Short strips	14 ± 0.7	1.64 [1.34 - 1.99]	<0.001
Long strips	13 ± 0.7	1.48 [1.20 - 1.83]	<0.001

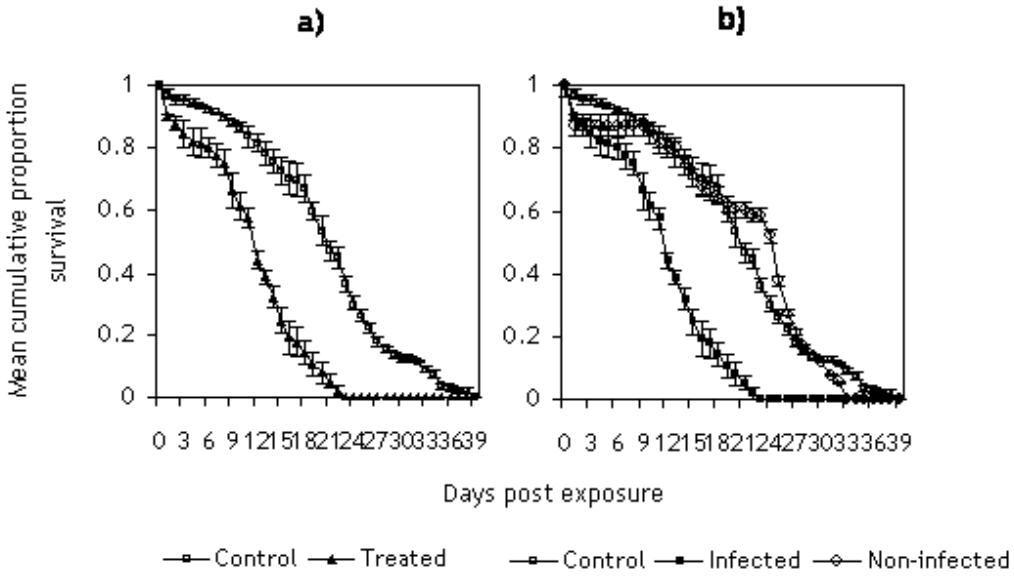


Figure 3: *Survival of wild female Anopheles gambiae s.l. mosquitoes collected from the hut with Metarhizium anisopliae IP 46 formulation applied on eave baffles (trial 4); a) infected- and non-infected mosquitoes pooled together b) infected- and non-infected mosquitoes plotted separately. Error bars show the standard error of the mean cumulative proportion survival.*

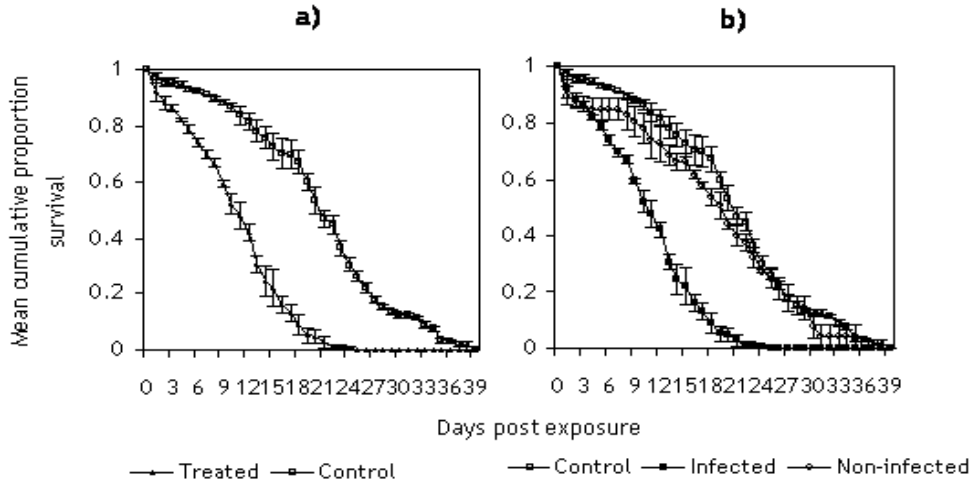


Figure 4: *Survival of wild female Anopheles gambiae s.l. mosquitoes collected from the hut with Beauveria bassiana I93-825 formulation applied on eave baffles (trial 4); a) infected- and non-infected mosquitoes pooled together b) infected- and non-infected mosquitoes plotted separately. Error bars show the standard error of the mean cumulative proportion survival.*

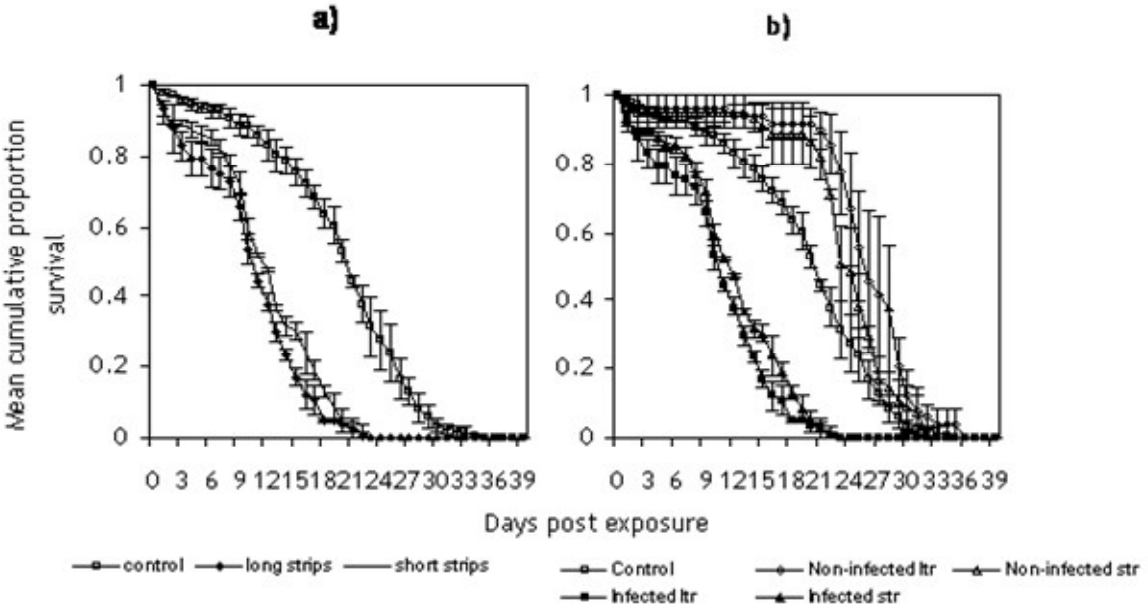
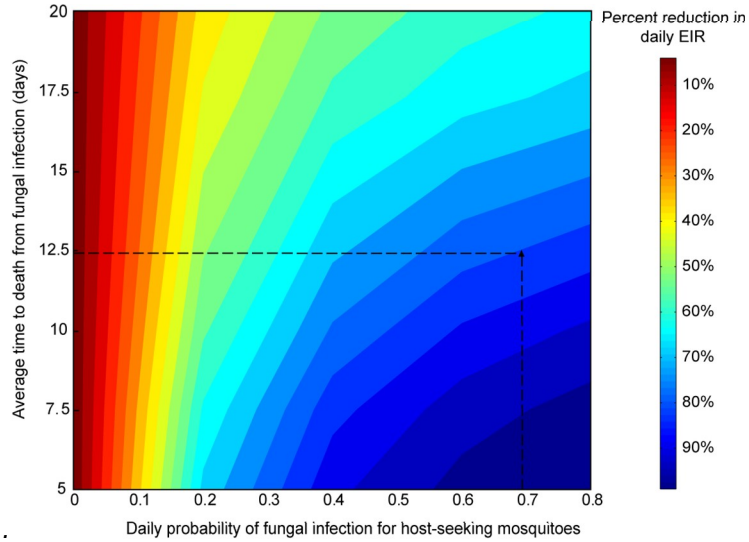


Figure 5: *Survival of wild female Anopheles gambiae s.l. mosquitoes collected from the huts with formulation applied on Metarhizium anisopliae IP 46 formulation applied on short and long strips hung next to bednets (trial 5); a) infected- and non-infected mosquitoes pooled together b) infected- and non-infected mosquitoes plotted separately. Error bars show the standard error of the mean cumulative proportion survival.*

Impact of fungal infection on EIR

Similar impact of *M. anisopliae* and *B. bassiana* on malaria transmission is expected because of the closely related trend for observed survival curves (trial 4 and 5). Therefore, only the survival data of mosquitoes exposed to *B. bassiana* applied on eave baffles was modeled, with a focus on trial 4, in which mosquitoes were exposed to fungus applied from cloth baffles placed in the eaves. The average time to death from fungal infection, or the 'virulence', was estimated to be 12.4 d. The daily probability of fungal infection obtained, or the 'coverage' achieved is 0.68-0.76. In the absence of both ITN's and sublethal effects of fungus infection, the estimated reduction in the daily EIR that could be achieved by our *B. bassiana* application is 75-80% (Figure 6). The model demonstrates that both virulence and coverage have a strong influence on EIR, but the most important requirement is that coverage is not at a low level. For example, when coverage is low (<0.15), reduction in EIR is relatively small, regardless of fungal virulence. Another simulation that shows the impact of ITN coverage rates, sub-lethal effects (reduced blood-feeding activity) and insecticide resistance given the achieved virulence (12.4 d) and coverage (68%), confirms that EIR can be reduced by 88-92% if no ITNs are in use, and at by least 96% if ITN coverage is between 0.4 to 0.8 even if mosquitoes are completely resistant to chemical insecticides and fungus infection only exerts a moderate effect on blood-feeding activity (Figure 7).



.

Figure 6: *The percent reduction in the daily EIR for varying levels of the average time to death from fungal infection ('virulence') and the daily probability of fungal infection ('coverage'). Dashed lines show the coverage and virulence levels reported for Beauveria bassiana I93-825 (Figure 4).*

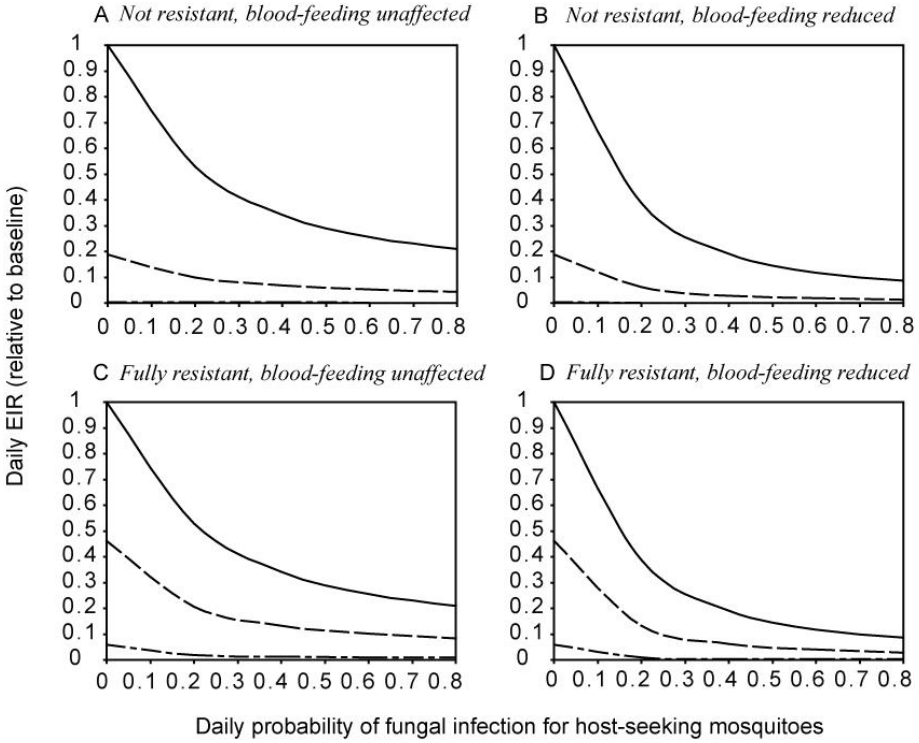


Figure 7: *The effect of the fungal bioinsecticide and ITN interventions on the EIR for varying levels of entomopathogenic fungi coverage and ITN coverage. Solid lines show zero ITN coverage, and dashed lines and dot-dashed lines show ITN coverage of 40% and 80%, respectively. A. No insecticide resistance in the mosquito population and no effect on blood-feeding activity; B. No insecticide resistance in the mosquito population and a moderate effect on blood-feeding activity; C. Complete insecticide resistance in the mosquito population and no effect on blood-feeding activity; D. Complete insecticide resistance in the mosquito population and a moderate effect on blood-feeding activity.*

Table 1: The number of female *Anopheles gambiae* s.l. sampled daily from each treatment of the five independent trials, and the proportion that showed fungal growth after dying and being assessed for fungal sporulation.

Trial No.	Treatment	Daily sub-sample of *Anopheles gambiae sensu lato*										
		Average catch /night	Days	1	2	3	4	5	6	7	8	9	Sporulation (%)
1. Eave netting	*B. bassiana* I93-825	1.1 ± 0.35	No. collected	0	1	1	0	1	2	3	0	2	0
			No. sporulated	0	0	0	0	0	0	0	0	0	
	Oil control	1.0 ± 0.24	No. collected	0	1	1	2	1	0	2	1	1	0
			No. sporulated	0	0	0	0	0	0	0	0	0	
	Open eaves (control)	45.0 ± 7.8	No. collected	25	25	21	21	19	25	21	25	25	0
			No. sporulated	0	0	0	0	0	0	0	0	0	
2. Eave curtain	*M. anisopliae* ICIPE-30	36.4 ± 6.0	No. collected	6	14	20	20	10	25	21	23	16	0
			No. sporulated	0	0	0	0	0	0	0	0	0	
	B. bassiana I93-825	32.4 ± 4.4	No. collected	8	13	11	17	16	13	12	17	16	0
			No. sporulated	0	0	0	0	0	0	0	0	0	
	Oil control	38.4 ± 4.1	No. collected	13	17	15	17	17	21	25	23	19	0
			No. sporulated	0	0	0	0	0	0	0	0	0	
3. Eave curtain & panels	*B. bassiana* I93-825 on curtain	27.9 ± 1.7	No. collected	11	17	13	9	15	12	13	14	11	18.3
			No. sporulated	0	2	2	1	4	3	4	2	3	
	B. bassiana I93-825 on panels	30.2 ± 1.3	No. collected	19	21	25	21	19	17	23	20	21	10.7
			No. sporulated	3	4	2	1	3	0	2	2	3	
	Open eaves (control)	33.9 ± 1.2	No. collected	21	20	25	17	16	25	20	23	21	0
			No. sporulated	0	0	0	0	0	0	0	0	0	
4. Eave baffles	*M. anisopliae* IP 46	40.6 ± 8	No. collected	7	19	25	22	22	23	14	23	23	69.1
			No. sporulated	4	5	16	17	19	17	10	18	17	
	B. bassiana I93-825	56.3 ± 11.4	No. collected	22	10	25	25	25	25	25	24	25	67.9
			No. sporulated	13	9	17	18	17	17	15	17	17	
	Oil treated baffles (control)	74.2 ± 14.5	No. collected	15	25	25	25	25	21	19	25	22	1.98
			No. sporulated	0	1	0	0	0	2	1	0	0	
5. Long & short strips next to bed nets	*M.anisopliae* IP 46 on long strips	24.3 ± 3.2	No. collected	4	13	15	24	20	20	17	25	17	75.5
			No. sporulated	3	9	12	20	14	15	12	17	15	
	M.anisopliae IP 46 on short strips	38.4 ± 6.8	No. collected	1	25	25	25	21	25	23	25	19	74.6
			No. sporulated	1	20	16	20	15	18	17	20	14	
	Open eaves (control)	86.2 ± 12.7	No. collected	16	25	23	25	25	25	25	24	23	3.3
			No. sporulated	0	0	0	0	3	0	0	1	0	

Discussion

As many as 75% of house-entering mosquitoes became infected with fungus when either the eaves were provided with fungus-treated baffles or strips of fungus-treated cotton cloth hung around the bed net. Moreover, the daily risk of dying of these mosquitoes was 3.9 to 4.8-fold higher compared to controls. By contrast, eave netting, eave curtains and cotton panels placed next to the bed net were ineffective in exposing mosquitoes to fungi sufficiently high to affect their survival or even infect them. Few mosquitoes were able to enter the huts via netting. Of the few that were collected none became infected with fungus. It could be that the netting was a poor substrate for disseminating conidia (Howard et al. 2010). Mosquitoes which contacted fungus-treated netting and flew off may have become infected. This, however, could not be confirmed with our experimental design. Eave netting was not the most promising delivery technique for evaluating fungus; especially considering that conidia loose heir potency rapidly when applied on such netting (Chapter 7; M. Farenhorst et al*.,* unpublished data). Our results, however, suggest that netting acted as an effective barrier, preventing mosquitoes from entering the huts. This emphasizes, given other factors, the potential of house screening in reducing malaria transmissions (Kirby et al. 2009).

With eave curtains made of cotton cloth, mosquitoes from treatment and control huts had an equal survival suggesting no or poor impact of fungal formulations. Possibly, mosquitoes flew directly into the huts without contacting the treated surfaces. Alternatively, if they happened to contact treated surfaces, time spent on the cloth could have been too short to pick up substantial amounts of conidia. Success of fungal pathogens depends on the host contacting treated surfaces and receiving a threshold amount of infective conidia (Vandenburg et al. 1998; Hughes et al. 2004). Short exposure times may result in small infective doses that can be countered by immune responses. Insect responses to entomopathogens involve melanisation, encapsulation and phagocytosis of invading fungal blastospores (Bogus et al. 2007), but it is likely that these responses can be overcome at high doses of conidia. Although the concentration of fungal conidia in the subsequent trial, where curtain was re-evaluated simultaneously, but independently, with cotton cloth panels, was 4 times higher, an impact of the fungus was still not observed. Considering the cotton cloth panels, the host-seeking mosquitoes that we collected from the exit traps may have spent the entire time on bed nets and other alternative surfaces in stead of landing on the panels. Or else, mosquitoes may have spent too short a time on these surfaces (curtain and panel) to pick up sufficient amounts of conidia. These could be the reasons for not observing any significant impact on mosquito survival despite the infection rates of 18.3% and 10.7% for curtains and panels respectively.

Mosquitoes from huts fitted with treated eave baffles survived 6-7 d less relative to the mosquitoes collected from the control huts and at least 68% picked up a fungus infection when entering the hut. This result is in sharp contrast with that of huts fitted with eave curtains (trial 3) hang parallel to the wall with an entry gap from below so that the mosquitoes had to negotiate their way downwards before entering the hut, where only up to 18% (*B. bassiana*) became infected . In huts with baffles, these were fitted in a slanted orientation with the entry gap at the top. It is quite striking that such a small modification of the position of fungal-treated surface may have improved the probability of mosquitoes contacting, and spending time on treated surfaces. The slanted cloth most likely prompted mosquitoes to walk over the cloth before flying into the huts, thereby picking up an infective amount of conidia. A reduced survival on wild malaria vectors due to fungal infection in this same village has been reported previously (Scholte et al. 2005; Lwetoijera et al. 2010). Scholte et al (2005) used cotton cloth placed on the ceilings, and more than 53.6% of the surviving mosquitoes were directly recovered from treated cloth. By contrast, in the present study the sub-sample of mosquitoes that were monitored for survival and fungus-infection status were collected from the window exit traps. Lwetoijera et al (2010) used OBS with treated cotton cloth that covered most of the internal surface. Seemingly, with such set ups, the level at which mosquitoes could have contacted conidia was relatively high. However, still the reduction in survival by 6-7 d in mosquitoes collected from treated huts recorded in the present study compares with that of the 6 d (Scholte et al. 2005) and 8 d (Lwetoijera et al. 2010) from the previous studies.

However, the median survival time of 21 d of mosquitoes in the controls in the present study is two-fold greater than that of 10 d recorded in the other two studies, suggesting that the relative impact of the fungi in the current study was even larger than in the other studies. Besides, application of fungus on eaves targets a large proportion of host-seeking mosquitoes (≥85%) (Njie et al. 2009), and therefore, would potentially cause a large reduction of malaria transmission if more than 50% of them become infected and die within 14 d. Similarly, the majority of host-seeking mosquitoes, after house entering, may be targeted when contacting a bed net, such as cotton cloth strips hung next to bed nets, as their host-seeking drive, stimulated by the hosts under the bed net, leads them to contact with the netting and/or cloth. Interestingly, short and long strips were equally effective; and as such, short strips would be cost effective in terms of the size of cloth materials and amount of conidia required. Arguably, it might be more comfortable to sleep under nets with short strips as long strips might increase the temperature under the bed net and act as a barrier to air currents. In the present study sleepers had a similar experience and seemed to favour the short strips, should they have had an option to choose. However, such observation was not confirmed in this study since we did not directly measure temperatures under the bednets.

Daily survival is the most sensitive component of vectorial capacity (Garrett-Jones 1964; Miller et al. 1973). For fungal tools to be effective in terms of reducing the EIR it has been estimated that a considerable proportion of house entering mosquitoes (≥50%) would need to be infected and their survival reduced (Scholte et al. 2005; Hancock 2009; Takken and Knols 2009). The only other field based study achieved a coverage rate of 23% (Scholte et al. 2005), whereas in the present study we were able to achieve fungal coverage as high as 75% (Trial 5). With this coverage, fungus with relatively low virulence can still produce substantial reductions in the EIR. Therefore, at this coverage level, extra benefit in terms of reducing EIR can be gained by improving fungal virulence rather than increasing the coverage (Figure 6). In the absence of sublethal effects of fungus infection, the estimated reduction in the daily EIR caused by the *B. bassiana* application is 75-80%. Therefore, even in the absence of any sublethal effects of fungal infection on the mosquito lifecycle, the intervention can have a strong impact on the EIR. This is largely due to the high fungal infection rate achieved in the present study. Based on the prevailing threat of malaria mosquito resistance against most of the commonly used insecticides, heightening integrated vector management (IVM) strategies is inescapably important. As part of an integrated strategy, fungi could respond to, or avert emergence of serious levels of insecticide resistance (Farenhorst et al. 2009; Howard et al. 2010); because of the move away from using one strategy to using a combination of strategies which has proven efficacy for preventing resistance (Kelly-Hope et al. 2008). Most striking, our model estimates suggest that with the achieved fungus coverage, the EIR is reduced by 88-92% if no ITNs are in use, and by 96% if ITN coverage is between 0.4 to 0.8 even if mosquitoes are completely resistant to chemical insecticides and fungus infection only exerts a moderate effect on blood-feeding activity. Fungal infection can reduce mosquito blood-feeding activity and fecundity (Scholte et al. 2005, Blanford et al. 2005). Based on the model estimates, depending on situations, both eave baffles and strips could be combined with ITNs. The ITNs either through offering a physical barrier and/or having an insecticide with irritant effect, could force mosquitoes to spend a longer time searching for unprotected hosts or resting on alternative surfaces (Lengeler 2004). This tendency would most likely maximize contact on fungus-treated surfaces. Insecticides could also stress mosquitoes, and thus they would tend to prolong time spent on other surfaces, unless insecticides themselves are repellent for example deltamethrin. If the other surfaces happen to be treated with fungus, the fungal effect would be exacerbated. Efficacy of fungal pathogens can also be enhanced when used with insecticides that cause stress to target pests. Diflubenzuron, an insect growth regulator, was shown to have an additive effect in combination with *B. bassiana*, enhancing the infectivity of the fungus against grasshoppers (Reuter et al. 1995). This however, remains a possibility and whether or not mosquitoes can be stressed by such insecticides remains to be confirmed.

Surprisingly, a small proportion (≤3%) of mosquitoes from control huts showed fungal growth (trials 4 and 5). This could be explained by assuming that few mosquitoes remained undetected in the huts during the removal of mosquitoes prior to switching the treatments. Another explanation may be that

mosquitoes from treatment huts visited control huts during the same night or later. These results suggest that fungal-infected mosquitoes move between houses following an initial infection. In trial 4 and 5, the average number of female anopheline mosquitoes collected from control huts was higher than that collected from treatment huts. As treatments and volunteers were rotated among huts, this cannot have been due to a positional effect or individual variation in attractiveness. This difference could be due though to a behavioural effect of *M. anisopliae* IP 46, causing some repellency or avoidance. However, such effects have not been observed with *M. anisopliae* ICIPE-30 and *B. bassiana* I93-825 in laboratory studies (Scholte et al. 2003; Chapter 6). In trial 5 (figure 5), the non-infected mosquitoes from treatment huts tended to survive slightly longer than the control mosquitoes, but this was not significant. Overall, the behavior of mosquitoes once infected with a fungus and the potential repellency of fungus treated material under field conditions remain of scientific relevance and need to be investigated.

Conclusion

Fungus infection rates as high as 75% can be achieved by targeting mosquitoes entering houses through eaves, or when attacking a sleeping host by means of cotton cloth eave baffles and cotton cloth strips hung next to bednets. With such infection levels, it is estimated that a high reduction in malaria transmission risk can be achieved with fungus alone, more so, when combined with ITNs even in scenarios of insecticides resistance (Farenhorst et al. 2010). Therefore, by means of eave baffles and or strips fungus could further be evaluated or applied against the wild malaria vectors on the realm of integrated vector management. Efforts geared at producing high quality fungal products in terms of virulence however, should be continued as there is an extra benefit to be accrued from such attributes in terms of fungal impact on malaria transmission.

Acknowledgements

Financial support was provided by the Adessium Foundation (Reeuwijk, The Netherlands). We thank all those who voluntarily slept in the huts during the study. We also thank Novatus Lipindi, Haji Kambwili and Petro Komba for technical assistance and Jason Moore for assisting with the construction of the experimental huts.

Chapter 9

Summarizing discussion

Introduction

Major global efforts are currently underway to comprehensively control and even globally eradicate malaria. The global target is to reduce the number of malaria cases and deaths per capita by ≥50% between 2000 and 2010, and ≥75% between 2000 and 2015 (WHO 2009). Many countries are struggling to achieve this goal and some have over the past decade been able to cut down malaria cases by more than 50%. Malaria control today relies on a limited arsenal of tools, in particular artemisinin derivatives, insecticide treated bednets (ITN) and Indoor Residual Spraying (IRS). The development of additional novel tools for vector control and other preventive measures, diagnosis, treatment and surveillance must be a priority if we are to achieve the prospected targets and possibly eliminate malaria. Besides, the available arsenal of malaria parasite and vector control tools is seriously threatened by a number of potential problems, such as the evolution of resistance (Coleman et al. 2006; Corbel et al. 2007; Nauen 2007; Kelly-Hope et al. 2008) and insufficient funding to attain universal coverage (WHO 2009; Snow and Marsh 2010). Resistance to insecticides, especially pyrethroids, is an urgent and growing threat to sustainability of current methods of vector control that need to be addressed. Consequently, there is an increasing focus on resistance management strategies, whereby efforts are made to use existing insecticides in ways that can maximize the time period for which they are useful for disease control; and to develop newer control tools which may suffer less from the problem of resistance. The research described in this thesis is a part of the global efforts to achieve that mission. Here, we centered on the development of entomopathogenic fungi for use as control agents against mosquito vectors, with the main focus on malaria-transmitting mosquitoes. These agents do not cause instant mortalities to mosquitoes, and as such it is empirically suggested that they are less likely to suffer from resistance, or else, if it occurs it would probably take considerably long to evolve (Read et al. 2009). For this purpose, isolates belonging to two genera of Hyphomycetous fungi, *Metarhizium anisopliae and Beauveria bassiana,* were evaluated, with the overall aim of developing efficacious formulations and delivery techniques for applying these agents against the wild population of malaria vectors and possibly other mosquito vectors of infectious diseases. Present and earlier studies have repeatedly emphasized the potential of these agents for use against these important disease vectors (Scholte et al. 2005; Blanford et al. 2005; Farenhorst et al. 2008; Mnyone et al. 2009; Lwetoijera et al. 2010; Howard et al. 2010). But, less has been done to develop formulations and delivery techniques that can effectively be used to evaluate these agents under field settings. This chapter provides a synthesis of the preceding chapters on the optimization of fungal formulations and delivery tools for use against malaria vectors in view of their availability for large scale evaluation to determine their epidemiological impact on malaria transmission, and the possibility of them being integrated into the existing vector control tools. Suggestions for additional research questions that need to be addressed to further improve the technology are also provided.

Overview of the major results

Effect of fungal formulations on mosquito vectors

One way to achieve high fungus infection rates in mosquitoes is by increasing the conidial dose until a certain threshold is reached. Higher doses of *B. bassiana* and *M. anisopliae* resulted in higher levels of mosquito mortality and 2×10^{10} conidia m^{-2} was the lowest concentration that achieved a sufficient reduction in survival time (Chapter 3). Under field settings dose response may vary due to less stable ambient conditions and or the ability of the infected host to thermo-regulate as it has been reported for *Schistocerca gregaria* locusts exposed to *M. anisopliae* var *acridum* (Arthurs and Thomas 2001). Besides such individual behavioural responses, environmental factors such as temperature, relative humidity, UV radiation and water content, will also affect the performance of entomopathogenic fungi (reviewed in Chapter 2). This has important implications for the field use of entomopathogenic fungi because at some ambient conditions lower doses could be applied with minimal effects on efficacy, whereas, in others a drop in dose may render the application completely ineffective.

Formulation of conidia in oil and/or improvement of fungal virulence may allow the use of a standard dose of conidia while still achieving high impacts on the insects' survival (McClatchie et al. 1998; Luz and Batagin 2005). Combining two entomopathogens with different levels of tolerance to sub-optimal ambient conditions would also be an option (Inglis et al. 1999; Malakar et al. 1999), but our study revealed no additive or synergistic effects when *M. anisopliae* ICIPE-30 and *B. bassiana* I93-825 were combined (Chapter 3). This, however, was tested under stable conditions, thus considering re-evaluating the two isolates under less stable field conditions might be beneficial in terms of their efficacy and persistence.

Both fungal isolates were found to reduce mosquito survival even with an exposure of as short as 5 min (M. Farenhorst, unpublished data) and 15 min (Chapter 3). This suggests, therefore, that fungal delivery techniques can exploit both the host-seeking and resting behaviour of mosquitoes to maximize infection with entomopathogenic fungi. Although both host-seeking and resting mosquitoes can be targeted, much focus should probably be on the host-seeking ones (normally non-blood fed) since they are more susceptible to fungal infection than the blood-fed ones (Chapter 5). Host-seeking mosquitoes comprise a considerable proportion of a mosquito population; therefore, targeting it successfully would exert considerable impact on malaria transmissions. Host-seeking mosquitoes can be targeted while entering houses through eaves (Lines et al. 1987; Njie et al. 2009; Chapter 8) and/or when trying to attack a person sleeping under the bednet (Hancock 2009; Chapter 8). Resting mosquitoes can be targeted on resting surfaces such as the internal wall or ceiling of human dwellings (mud walls in most cases for our rural settings) where mosquitoes usually settle after taking a bloodmeal (Gillies 1954; Scholte et al. 2005).

Development of entomopathogenic fungi delivery options

Mud and black cotton cloth present suitable substrates for fungal conidia, and can be used to evaluate and apply fungal formulations in the field (Chapter 7). Polyester netting is not a suitable substrate for application of fungal formulation (Chapter 7). Cotton cloth can be employed in delivery techniques that target either host-seeking or resting mosquitoes both indoors as well as outdoors (Scholte et al. 2005; Lwetoijera et al. 2010; Chapter 8). Mud walls could be used for application of fungal formulations as in chemical insecticides (IRS; Indoor Residual Spraying). The outdoor baited stations described by Okumu et al (2010) could also possibly be made out of mud walls, which as suggested in Chapter 5, would be beneficial in terms of prolonging the lifespan of conidia. By using cotton cloth eave baffles and strips hung around the bed net we were able to achieve infection rates up to 75% (Chapter 8), which is much higher than achieved before (23%) (Scholte et al. 2005). Baffles were used to target mosquitoes entering experimental huts through eaves, whereas strips were used to target mosquitoes when approaching the person sleeping under a bed net. Both, eave baffles and strips, therefore, present techniques that can be used to further field evaluate or apply entomopathogenic fungi against the wild mosquito vectors (Chapter 8). As they are, the two techniques seem to guarantee considerable flexibility for use in an integrated fashion with either IRS or ITNs should the combination prove beneficial in reducing malaria transmission in large scale intervention. Integrated vector management is vital for achieving considerable reductions in disease transmission and may be useful in counteracting the problem of resistance (Read et al. 2009; Farenhorst et al. 2009; Hancock 2009; Howard et al. 2010). Houses could have walls treated with insecticides and eaves fitted with fungus-treated baffles. Insecticide treated bed nets could also be used in combination with eave baffles or strips in a similar fashion. A malaria transmission model based on experimental hut data of the present study (Chapter 8) predict a considerable success in terms of reducing malaria transmission by combining the use of entomopathogenic fungi and ITNs even in situations where mosquitoes are resistant to insecticides. However, since we now have techniques that can be used to apply fungus, a combination strategy between fungus and ITNs needs to be validated with field data.

Logistically, eave baffles and strips seem to provide good options for fungal delivery as low amounts of conidia will be required relative to the amount required in treating the entire internal walls. With eave baffles, disturbance of surfaces in relation to routine indoor activities will be largely limited, thus, fall off of the conidia from treated surfaces will be highly minimized. Importantly, chances of people contacting the treated surfaces will be minimized, and that could probably heighten acceptance of the technology for these people. Although entomopathogenic fungi are safe to use (Strasser et al. 2000; Zimmermann 2007a; 2007b), safety issues should never be underestimated as just a few cases of allerginicity, though rare (Dabro et al. 2009), would compromise the uptake of the technology. The risk of exposure to airborne conidia and thus to allergic reactions, however, is lower when formulated in oil. These conidia stick to the impregnated substrates because of the oil and, and in addition, the oil acts as a protective shell around the fungus particle. Strips of cotton cloth treated with fungus can be used in cases where houses do not have eaves or defined walls. Many people contract *Plasmodium* infections when they shift to the agricultural sites where they sleep in temporary houses, which often do not have walls or have walls that leave multiple entry points for mosquitoes (Charlwood et al. 2000). Interestingly, with short strips a similar impact of the fungus on mosquito survival was achieved as with of fungus applied on long strips. This would be further beneficial in terms of the amount conidia required. Strips might also offer a good strategy for targeting culicine mosquitoes with fungus, since the majority may enter houses through entry points other than eaves (M. Kirby, personal communication).

Integrated vector management (IVM)

Since 2001, WHO has been promoting IVM as the new strategic approach to vector control (WHO 2001). IVM basically aims at strengthening impact through complementary methods of vector control with operational flexibility, while minimizing detrimental effects to the environment. Through IVM, on the long term, costly setbacks such as those that may arise due to behavioural changes of vectors or development of insecticide resistance can be prevented. For years, there have been efforts to develop various malaria vector control options, however, to date it is only IRS and ITN that have practically achieved a large-scale use (Roberts et al. 2000; Lengeler et al. 2004; WHO 2009; Pluess et al. 2010). Some observational evidence indicates that the combination of IRS and long lasting insecticide treated nets (LLIN) is more effective than either intervention alone especially if the combination helps to increase the overall coverage of the interventions (WHO 2009). Certainly, this idea is worthy validating, however, integration efforts should consider other available but less developed control methods (larval control using *Bacillus thuringiensis israelensis* and fish, repellents, zooprophylaxis and environmental management) because there is a limited choice of insecticides that can be employed for IRS and LLIN. Besides, efforts to improve and develop novel options should be strengthened. Novel forms of vector control such as production of males carrying dominant lethal transgenes (Phuc et al. 2007) and generation of disease-refractory mosquitoes (Morelli et al. 2007; Yoshida et al. 2007) although at the infancy stage, should equally receive attention.

There can be different suggestions on how and which methods can be integrated. However, the strategy should ensure that each method to be used is appropriate in the specific physical and social settings (WHO 2001). Larval control is likely to ensure a reduction in the number of emerging adults, whereas cattle for example in or near houses will prevent some of the adults from feeding on humans (Scholte 2004). Repellent odours, natural or synthetic, can be used to reduce the numbers of mosquitoes entering houses even further; and mosquitoes that would still bypass zooprophylaxis or repellents, and enter indoors will encounter a treated bed net, which will further reduce their chances of transmitting malaria parasites (Killeen et al. 2007). Certainly, additional novel tools will open up more options for an integrated strategy of control. Biological control of adult mosquitoes using microbial agents shows a great potential (Read et al. 2009; Hancock 2009).

Microbial agents under development include engineered densoviruses (Carlson et al. 2006), bacteria e.g. *Wolbachia* spp (Cook et al. 2008; McMeniman et al. 2009) protozoa e.g. *Microsporidia* spp (Koella et al. 2009) and entomopathogenic fungi (this thesis). Entomopathogenic fungi, however, are most likely to be available for programmatic use within the short-term future. As described in this thesis, the fungal species *M. anisopliae* and *B. bassiana* further demonstrated their ability to infect and reduce survival of wild malaria vectors (Chapter 8). With the additional behavioural effects these fungi exert on malaria vectors (e.g. reduced blood feeding propensity and fecundity), and their ability to impede malaria parasite development (Blanford et al. 2005; Scholte et al. 2006), we envisage a considerable promise for their use to control malaria. These agents render a possibility of being incorporated into an IVM strategy. As discussed in the preceding sections, the two effective fungal delivery techniques (eave baffles and strips) guarantee a large possibility of being validated along line with ITNs, IRS and other control strategies. This kind of integrated strategy may be beneficial in prolonging the lifespans of "old" and newly developed insecticides (Ffrench et al. 2005). Indeed, the recent finding that these fungi can be effectively integrated with ITNs in areas of high insecticide resistance (Farenhorst et al. 2010) provide further evidence for their incorporation in mosquito control strategies.

Challenges and future directions

Based on evolutionary theories it is hypothesized that development of resistance in mosquitoes against entomopathogenic fungi is not very likely (Ffrench et al. 2005; Read et al. 2009). Such a possibility, however, should not be underestimated (Hutchinson and Cunningham 2005; Michalakis and Renaud 2005); and as in any other control method, its effectiveness should be assessed over time to be able to detect any signs for resistance well in advance. These assessments should probably also go hand in hand with monitoring for unexpected effects on none targeted organisms, and on human health.

To appreciably reduce the malaria burden in most of Africa, vector control tool(s) should attain coverage levels that will ensure direct personal protection and area-wide suppression of the transmission intensity that benefits even the non users (Killeen et al. 2007a). For example in ITNs, coverage of at least 80% is recommended and coverage of the entire population would be even better (Killeen et al. 2007b). High coverage is equally essential for other currently used and newly developed control tools. Therefore, the overall processes of developing entomopathogenic fungi should consider aspects that will heighten people's acceptability and adoption of the technology. Indeed, the benefits of use of fungus-treated materials need to be emphasized to people and their use encouraged in areas considered for control. Interestingly, entomopathogenic fungi have demonstrated the ability to affect other vectors of veterinary and medical importance (Scholte et al. 2003, 2007; Maniania et al. 1998) therefore, considering formulations and or delivery techniques that would equally impact on other vectors would hasten their acceptability. Fore example, fungal delivery techniques that have been developed in the present study can potentially impact on other mosquito vectors e.g. culicine species. Involvement of the local people should be considered as well so as the technology can meet the preferences of the end users. Should the technology prove effective, it would be quite useful to consider community-based small scale production of conidia using as much as possible the locally available growth substrates such as sugarcane bagasse, rice powder and maize bran. Sustainability of the entomopathogenic fungi in terms of efficacy and persistence will also greatly determine the rate of adoption and coverage levels thereof. The results presented in this thesis have on the one hand provided exciting information to aid the development of a sustainable technology, and on the other hand they open up more areas for research to strengthen the technology even further. Below I suggest some of the important questions to be addressed:-

1) Screening other fungal species against mosquitoes. Although *M. anisopliae* and *B. bassiana* were found to be virulent against the targeted mosquitoes, it is possible that other isolates other than these species or another Hyphomycetous species all together will prove to be relatively more virulent/or tolerant to microclimate and macroclimate of the targeted control sites. This can be achieved through testing fungal species that have proven efficacy against other insect species and/or isolating new ones. The extensive review in Chapter 2 can be very useful in identifying candidate isolates and other alternative means to improve fungal isolates and formulations.

2) Studies aimed at determining how and whether the delivery techniques demonstrated in this study can be used in combination with the existing vector control tools such as ITN and IRS to amalgamate reduction in malaria transmissions.

3) Studies aimed at examining the persistence of mineral oil formulations of fungal conidia when applied to eave baffles and strips under realistic field conditions. This should be studied along studies aimed for improvement of such characteristics of conidia as viability, virulence and persistence.

4) Exploration of additional substrates that can be used to deliver fungus (e.g. direct application on ceilings, thatch and or materials used to make traps such as canvas).

5) Studies to develop and improve fungal formulations in terms of efficacy and persistence need to be conducted, and should be done in collaboration with formulation scientists. Microencapsulating conidia to protect them against the adverse environmental conditions is worthy investigating. Other options for improving formulations that can be investigated are reviewed in Chapter 2.

Conclusion

Both *M. anisopliae* and *B. bassiana* significantly reduced the survival of mosquitoes even at lower doses of conidia and exposure times. These fungi did not show any repellency to mosquitoes. Interestingly, application of these fungi by means of cotton cloth eave baffles/or strips hung around the bed net caused high infection rates on wild mosquitoes and significantly reduced their survival. Based on the obtained data, model estimates indicated that fungus can considerably (>75%) reduce EIR. Overall, the results described in this thesis demonstrate the feasibility of the use of entomopathogenic fungi for controlling malaria mosquito vectors. The delivery designs, eave baffles and strips, provide a realistic opportunity for up scaling fungal agents for malaria control, preferably in an integrated fashion with the existing control tools.

References

Abdulla S, Oberholzer R, Juma O, Kubhoja S, Machera F, Membi C, Omari C, Urassa A, Mshinda H, Jumanne A: **Safety and Immunogenicity of RTS, S/AS02D Malaria Vaccine in Infants.** *N Engl J Med* 2008, **359:** 2533.

Abdulla S, Schellenberg R, Nathan R, Mukasa O, Marchant T, Smith T, Tanner M, Lengeler C: **Impact on malaria morbidity of a programme supplying insecticide treated nets in children aged under 2 years in Tanzania: Community cross sectional study.** *BMJ* 2001, **322:** 270-271.

Achonduh OA, Tondje PR: **First report of pathogenicity of *Beauveria bassiana* RBL1034 to the malaria vector, *Anopheles gambiae* s.l (Diptera; Culicidae) in Cameroon**. *Afr J Biotechnol* 2008, **7:** 931-935.

Adam I, Khamis AH, Elbashir MI: **Prevalence and risk factors of *Plasmodium falciparum* malaria in pregnant women of eastern Sudan.** *Malar J* 2005, **4:** 18.

Aitken WB, Niederpruem DJ: **Isotopic studies of carbohydrate metabolism during basidiospore germination in *Schizophyllum commune*.** *Arch Microbiol* 1973, **88:** 331–344.

Albernaz DAS, Tai MHH, Luz C: **Enhanced ovicidal activity of an oil formulation of the fungus *Metarhizium anisopliae* on the mosquito *Aedes aegypti*.** *Med Vet Entomol* 2009, **23:** 141-147.

Alonso-Dıaz MA, Garcı´a L, Galindo-Velasco E, Lezama-Gutierrez R, Angel-Sahagun CA, Rodrı´guez-Vivas RI, Fragoso-Sanchez H: **Evaluation of *Metarhizium anisopliae* (Hyphomycetes) for the control of *Boophilus microplus* (Acari: Ixodidae) on naturally infested cattle in the Mexican tropics.** *Vet Parasitol* 2007, **147:** 336–340.

Alves SB, Pereira RM, Stimac JL, Vieira SA: **Delayed germination of Beauveria bassiana conidia after prolonged storage at low, above freezing temperatures.** *Biocontrol Sci Techn* 1996, **6:** 575-581.

Ansari MA, Vestergaard S, Tirry L, Moens M: **Selection of a highly virulent fungal isolate, *Metarhizium anisopliae* CLO 53, for controlling *Hoplia philanthus*.** *J Invertebr Pathol* 2004, **85:** 89-96.

Arthurs S, Thomas MB: **Effect of dose, pre-mortem host incubation temperature and thermal behaviour on host mortality, mycosis and sporulation of *Metarhizium anisopliae var. acridum* in *Schistocerca gregaria*.** *Biocontrol Sci Techn* 2001, **11:** 411-420.

Barbazan P, Baldet T, Darriet F, Escaffre H, Haman Djoda D, Hougard J-M: **Control of *Culex quinquefasciatus* (Diptera: Culicidae) with Bacillus sphaericus in Maroua, Cameroon.** *J Am Mosq Control Assoc* 1997, **13:** 263-269.

Barson GN, Renn N, Bywater AF**: Laboratory evaluation of six species of entomopathogenic fungi for the control of the house fly (*Musca domestica* L.) a pest of intensive animal units.** *J Invertebr Pathol* 1994, **64:** 107-113.

Beckett EB: **Development and ageing of the salivary glands of adult female *Aedes aegypti* (L.) and *Aedes togoi* (Theobald) mosquitoes (Diptera: Culicidae).** *J Insect Morphol Embryol* 1990, **19:** 277-290.

Begum S, Quiniones AC: **Protection of stored munbean seeds from bean weevil *Callosobrochus chinensis* by vegetable oil application** *Bangladesh J Zool* 1990, **18:** 203-210.

Behle RW, Tamez-Guerra P, Shasha BS, McGuire MR: **Formulating bioinsecticides to improve the residual activity.** *Proceedings of the Formulations Forum 99, March 3-5, Orlando, FL USA, wwwafc-usorg/papers/1999papers/abehlhtm* 1999.

Beier JC: **Malaria parasite development in mosquitoes.** *Annu Rev Entomol* 1998, **43:** 519-543.

Berllini R, Veronesi R, Rizzoli M: **Efficacy of various fish species (*Carassius auratus* (L.), *Cyprinus carpio* (L.), *Gambusia affinis* (Baird and Girard) in the control of rice field mosquitoes in northern Italy.** *Bull Soc Vector Ecol* 1994, 19: 87-99.

Bidochka MJ, Kamp AM, De Croos JNA: **Insect pathogenic fungi: from genes to populations**. Dordrecht: Kluwer Academic Publishers; 2000.

Bittencourt VREP, Souza JE, Peralva SLFS, Reis RCS: **Efficacy of the fungus *Metarhizium anisopliae* in field test with bovines naturally infested with the tick *Boophilus microplus*.** *Rural Braziliensis Med Vet* 1999, **21:** 78–82.

Black RE, Morris SS, Bryce J: **Where and why are 10 million children dying every year?** *Lancet* 2003, **361:** 2226-2234.

Blanford S, Chan BHK, Jenkins N, Sim D, Turner RJ, Read AF, Thomas MB: **Fungal pathogen reduces potential for malaria transmission.** *Science* 2005, **308:**1638-1641.

Bogus MI, Kedra E, Bania J, Szczepanik M, Czygier M, Jablonski P, Pasztaleniec A, Samborski J, Mazgajska J, Polanowski A: **Different defense strategies of *Dendrolimus pini*, *Galleria mellonella*, and *Calliphora vicina* against fungal infection.** *J Insect Physiol* 2007, **53:** 909-922.

Boucias DC, Bradford DL, Barfield CS: **Susceptibility of the velvet bean caterpillar and soybean looper (Lepidoptera: Noctuidae) to *Nomuraea rileyi*: effects of pathotype, dosage, temperature, and host age.** *J Econ Entomol* 1984, **77:** 247–253.

Boucias DR, Pendland JC: **Entomopathogenic fungi; Fungi Imperfecti. In: Principles of insect pathology.** Boucias DR, Pendland JC (Editors). Kluwer Academic Publishers 1988, Doedrecht.

Braga GU, Flint SD, Miller CD, Anderson AJ, Roberts DW: **Variability in response to UV-B among species and strains of Metarhizium isolated from sites at latitudes from 61°N to 54°S**. *J Invertebr Pathol* 2001, **78:** 98-108.

Braga GU, Rangel DE, Flint SD, Anderson AJ, Roberts DW: **Conidial pigmentation is important to tolerance against solar-simulated radiation in the entomopathogenic fungus *Metarhizium anisopliae***. *Photochem photobiol* 2006, **82:** 418-422.

Brei B, Edman JD, Gerade B, Clark JM: **Relative abundance of two cuticular hydrocarbons indicates whether a mosquito is old enough to transmit malaria parasites.** *J Med Entomol* 2004, **41:** 807-809.

Breman J: **The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden.** *Am J Trop Med Hyg* 2001, **64:** 1-11.

Bugeme DM, Knapp M, Boga HI, Wanjoya AK, Maniania NK: **Influence of temperature on virulence of fungal isolates of *Metarhizium anisopliae* and *Beauveria bassiana* to the two-spotted spider mite *Tetranychus urticae***. *Mycopathologia* 2009, **167:** 221-227.

Bukhari T, Middelman A, Koenraadt CJM, Takken W, Knols BGJ: **Factors affecting fungus induced larval mortality in *Anopheles gambiae* and *Anopheles stephensi*.** *Malar J* 2010, **9:** 22.

Bush AO, Fernandez JC, Esch GW, Seed JR. 2001. ***Parasitism:* The diversity and ecology of animal parasites**. Cambridge, UK: Cambridge Univ. Press.

Callaghan A, Hirthe G, Fisher T, Crane M: **Effects of short-term exposure to chlorpyrifos biochemical, behavioural and life-history biomarkers in *Chironomus riparius* Meigen**. *Ecotoxicol Environ Saf* 2001, **50:** 19–24.

Carlson J, Suchman E, Buchatsky L: **Densoviruses for control and genetic manipulation of mosquitoes**. *Adv Virus Res* 2006, **68**: 361.

Carpenter JF, Crowe JH: **Modes of stabilization of a protein by organic solutes during desiccation.** *Cryobiology* 1988, **25:** 459–470.

Carruthers RI, Larkin TS, Firstencel H: **Influence of thermo ecology on the mycosis of a rangeland grasshopper**. *Ecology* 1992, **73:** 190-204.

Chaki PP, Govella NJ, Shoo B, Hemed A, Tanner M, Fillinger U, Killeen GF: **Achieving high coverage of larval-stage mosquito surveillance: challenges for a community-based mosquito control programme in urban Dar es Salaam, Tanzania.** *Malar J* 2009, **8:** 311.

Chappell MA, Whitman DW: **Grasshopper thermoregulation.** Wiley, New York 1990.

Charlwood JD, Smith T, Kihonda J, Heiz B, Billingsley PF, Takken W: **Density independent feeding success of malaria vectors (Diptera: Culicidae) in Tanzania.** *Bull Entomol Res* 1995, **85:** 29-35

Charlwood JD, Vij R, Billingsley PF: **Dry season refugia of malaria-transmitting mosquitoes in a dry savannah zone of east Africa.** *Am J Trop Med Hyg* 2000, ***62*****:** 726-732.

Chen CS, Mulla MS, March RB, Chaney JD: **Cuticular hydrocarbon patterns in *Culex quinquefasciatus* as influenced by age, sex, and geography.** *Bull Soc Vector Ecol* 1990. **15:**129-139.

Chun J, Riehle M, Paskewitz SM: **Effect of mosquito age and reproductive status on melanization of sephadex beads in *Plasmodium*-refractory and -susceptible strains of *Anopheles gambiae.*** *J Invertebr Pathol* 1995, **66:** 11-17.

Clark TB, Kellen W, Fukuda T, Lindegren JE: **Field and laboratory studies on the pathogenicity of the fungus *Beauveria bassiana* to three genera of mosquitoes.** *J Invertebr Pathol* 1968, **11:** 1-7.

Clarkson JM, Charnley AK: **New insights into the mechanism of fungal pathogenesis in insects.** *Trends Microbiol* 1996, **4:**197-203.

Clements AN: *The Biology of mosquitoes: Sensory reception and behaviour, London UK* 1992, **2:**.

Cliquet S, Jackson: **Impact of carbon and nitrogen nutrition on the quality, yield and composition of blastospores of the bioinsecticidal fungus Paecilomyces fumosoroseus.** *J Ind Microbiol Biotechnol* 2005, **32:** 204-210.

Coetzee M, Craig, M, Le Sueur D: **Distribution of African malaria mosquitoes belonging to the *Anopheles gambiae* complex.** *Parasitol Today* 2000, **16:** 74–77.

Colaco C, Sen S, Thangavelu M, Pinder S, Roser B: **Extraordinary stability of enzymes dried in trehalose: simplified molecular biology.** *Bio/Technology* 1992, **10:** 1007–1011.

Coleman M, Sharp B, Seocharan I, Hemingway J: **Developing an evidence-based decision support system for rational insecticide choice in the control of African malaria vectors.** J Med Entomol 2006, **43:** 663–668.

Coluzzi M, Sabatini A, Petrarca V, Di Deco MA: **Chromosomal differentiation and adaptation to human environments in the *Anopheles gambiae* complex.** *Trans R Soc Trop Med Hyg* 1979, **73:** 483-497.

Connick WJJ, Boyette CD, McAlpine JR**: Formulation of mycoherbicides using a pasta-like process.** *Biol Control* 1991, **1:** 281-287.

Cook PE, McMeniman CJ, O'Neill SL: **Modifying insect population age structure to control vector-borne disease**. *Transgenesis and the management of vector–borne disease Landes Bioscience and Springer Science, Brisbane* 2008, 126-140.

Corbel V, N'Guessan R, Brengues C, Chandre F, Djogbenou L, Martin T, Akogbéto M, Hougard JM, Rowland M: **Multiple insecticide resistance mechanisms in *Anopheles gambiae* and *Culex quinquefasciatus* from Benin, West Africa**. *Acta Tropica* 2007, **101:** 207-216.

Couch TL, Ignoffo CM: **Formulation of insect pathogens.** In: Microbial control of pests and plant diseases 1970-1980 (ed. HD, Burges) pp 621-634. Academic Press: New York 1981.

Crowe IH, Crowe LM: **Evidence for direct interaction between disaccharides and dry phospholipids.** *Cryobiology* 1993, **30:** 226-227.

Da Costa MS, Niederpruem D J: **Temporal accumulation of mannitol and arabitol in *Geotrichum candidum*.** *Arch Microbiol* 1980, **126:** 57–64.

Daemen ALH, Van der stege HJ: **The destruction of enzymes and bacteria during the spray drying of milk whey: The effects of the drying conditions.** *Neth Milk Dairy J* 1982, **36:** 211-229.

Daoust RA, Pereira, RM: **Survival of *Beauveria bassiana* (Deuteromycetes: Moniliales) conidia on cadavers of cowpea pests stored outdoors and in a laboratory in Brazil.** *Environ Entomol* 1986, **15:** 642–647.

Daoust RA, Roberts DW: **Studies on the prolonged storage of *Metarhizium anisopliae* conidia: effect of temperature and relative humidity on conidial viability and virulence against mosquitoes**. *J Invertebr Pathol* 1983, **41:** 143-150.

Daoust RA, Roberts DW: **Virulence of natural and insect-passaged strains of *Metarhizium anisopliae* to mosquito larvae.** *J Invertebr Pathol* 1982, **40:** 107-117.

Darbro JM, Thomas MB: **Spore persistence and likelihood of aeroallergenicity of entomopathogenic fungi used for mosquito control.** *Am J Trop Med Hyg* 2009, **80:** 992-997.

Davidson G, Phelps K, Sunderland KD, Pell JK, Ball BV, Shaw KE, Chandler D: **Study of temperature-growth interactions of entomopathogenic fungi with potential for control of *Verroa destructor* (Acari: Mesostigmata) using a nonlinear model of poikilotherm development.** *J Appl Microbiol* 2003, **94:** 816-825.

De La Rosa W, Alatorre R, Barrera JF, Toriello C: **Effect of *Beauveria bassiana* and *Metarhizium anisopliae* (Deuteromycetes) upon the coffee berry borer (Coleoptera: Scolytidae) under field conditions.** *J Econ Entomol 2000*, **93:** 1409-1414.

Deacon JW. **Modern Mycology.** 1997, Blackwell Science Ltd, Oxford.

Devi KU, Sridevi V, Mohan CM, Padmavathi J: **Effect of high temperature and water stress on invitro germination and growth in isolates of the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuillemin**. *J Invertebr Pathol* 2005, **88:** 181-189.

Dimbi S, Maniania NK, Lux SA, Mueke JM: **Effect of constant temperatures on germination, radial growth and virulence of *Metarhizium anisopliae* to three species of African tephritid fruit flies**. *BioControl* 2004, **49:** 83-94.

Dimbi S, Maniania NK, Lux SA, Mueke JM: **Host species, age and sex as factors affecting the susceptibility of the African Tephritid fruit fly species, *Ceratitis capitata*, *C. cosyra* and *C. fasciventris* to infection by *Metarhizium anisopliae*.** *J Pest Science* 2003, **76:** 113–117.

Doberski JW: **Comparative laboratory studies on three fungal pathogens of the elm bark beetle *Scolytus scolytus*: pathogenicity of *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces farinosus* to larvae and adults of *S. scolytus*.** *J Invertebr Pathol* 1981, **37:** 188–194.

Dodoo AN, Fogg C, Asiimwe A, Nartey ET, Kodua A, Tenkorang O, Ofori-Adjei D: **Pattern of drug utilization for treatment of uncomplicated malaria in urban Ghana following national treatment policy change to artemisinin-combination therapy.** *Malar J* 2009, **8:** 2.

Dondorp AM, Day NP: **The treatment of severe malaria.** *Trans R Soc Trop Med Hyg* 2007, **101:** 633–4.

Dondorp AM, Yeung S, White L, Nguon C, Day NPJ, Socheat D, von Seidlein L: **Artemisinin resistance: current status and scenarios for containment.** *Nat Rev Microbiol* 2010, 8**:** 272-280.

Dorta B, Bosch A, Arcas JA, Ertola RJ: **High level of sporulation of *Metarhizium anisopliae* in a medium containing by products**. *Appl Microbiol Biotechnol* 1990, **33:** 712-715.

Driver F, Milner RJ, Trueman JWH: **A taxonomic revision of Metarhizium based on a phylogenetic analysis of rDNA sequence data.** *Mycol Res* 2000, **104:** 134-150.

Drummond J, Heale JB, Gillespie AT: **Germination and effect of reduced humidity on expression of pathogenicity in *Verticillium lecanii* against the glasshouse whitefly Trialeurodes.** *Ann Appl Biol* 1987, **111:** 193–201.

Dutra V, Nakazato L, Broetto L, Schrank IS, Vainstein MH, Schrank A: **Application or representational difference analysis to identify sequence tags expressed by *Metarhizium anisopliae* during the infection process of the tick *Boophilus microplus*.** *Res Microbiol* 2004, **155:** 245–251.

Duval L, Nerrienet E, Rousset D, Mba SAS, Houze S, Fourment M, Le Bras J, Robert V, Ariey F: **Chimpanzee Malaria Parasites Related to *Plasmodium ovale* in Africa.** *PLoS ONE* 2009, **4:** e5520.

Ekesi S, Egwurube EA, Akpa AD, Onu I: **Laboratory evaluation of the entomopathogenic fungus, *Metarhizium anisopliae* for the control of the groundnut bruchid, *Caryedon serratus* on groundnut.** *J Stored Prod Res* 2001, **37:** 313-321.

Ekesi S, Maniania NK, Ampong-Nyarko F: **Effect of temperature on germination, radial growth and virulence of *Metarhizium anisopliae* and *Beauveria bassiana* on *Megalurothrips sjostedti***. *Biocontrol Sci Techn* 1999, **9:** 177-185.

El Damir M: **Effect of Growing Media and Water volume on conidia production of *Beauveria bassiana* and *Metarhizium anisopliae.*** *J Biological Sci* 2006, **6:** 269-274.

Elbein AD, Pan YT, Pastuszak I, Carroll D: **New insights on trehalose: a multifunctional molecule.** *Glycobiology* 2003, **13:** 17–27.

Ellis JJ, Roberson JA: **Viability of fungus cultures preserved by lyophilization.** *Mycologia* 1968, **60:** 296-297.

Etang J, Fondjo E, Chandre F, Morlais I, Brengues C, Nwane P, Chouaïbou M, Amadou D, Simard F: **First report of the kdr mutations in the malaria vector *Anopheles gambiae* from Cameroon.** *Am J Trop Med Hyg* 2006, **74:** 795-797.

Farenhorst M, Farina D, Scholte EJ, Takken W, Hunt RH, Coetzee M, Knols BGJ: **African water storage pots for the delivery of the entomopathogenic fungus *Metarhizium anisopliae* to the malaria vectors *Anopheles gambiae s.s.* and *Anopheles funestus*.** *Am J Trop Med Hyg* 2008, **78:** 910-916.

Farenhorst M, Knols BGJ, Thomas MB, Howard AFV, Takken W, Rowland M, N'Guessan R: **Synergy in efficacy of fungal entomopathogens and permethrin against West African insecticide-resistant *Anopheles gambiae* mosquitoes.** *PloS ONE* 2010, **5:** e12081.

Farenhorst M, Knols BGJ, Thomas MB, Howard AFV, Takken W, Rowland M, N'Guessan R: **Synergy in efficacy of fungal entomopathogens and permethrin against West African insecticide-resistant *Anopheles gambiae* mosquitoes.** *PloS ONE* 2010, **5:** e12081.

Farenhorst M, Mouatcho JC, Kikankie CK, Brooke BD, Hunt RH, Thomas MB, Koekemoer LL, Knols BGJ, Coetzee M: **Fungal infection counters insecticide resistance in African malaria mosquitoes.** *Proc Natl Acad Sci USA* 2009, **106:** 17443-17447.

Fargues J, Goettel MS, Smits N, Ouedraogo A, Rougier M: **Effect of temperature on vegetative growth of *Beauveria bassiana* isolates from different origins** *Mycologia* 1997, **89:** 383-392.

Fargues J, Luz C: **Effects of fluctuating moisture and temperature regimes on the infection of potential of *Beauveria bassiana* for *Rhodnius prolixus***. *J Invertebr Pathol* 2000, **75:** 202-211.

Fargues J, Rodriguez-Rueda D: **Sensibilité des larves de *Spodoptera littoralis* (Lep.: Noctuidae) aux hyphomycètes entomopathogènes *Nomuraea rileyi* et *Paecilomyces fumosoroseus*.** *Entomophaga* 1980, **25:** 43–54.

Feder D, Mello, CB, Garcia ES, Azambuja P: **Immune responses in *Rhodnius prolixus*: influence of nutrition and ecdysone.** *J Insect Physiol* 1997, **43:** 513-519.

Fegan GW, Noor AM, Akhwale WS, Cousens S, Snow RW: **Effect of expanded insecticide-treated bednet coverage on child survival in rural Kenya: a longitudinal study.** *Lancet* 2007, **370:** 1035–39.

Feng Z, Carruthers RI, Roberts DW, Robson DS: **Age-specific dose-mortality effects of *Beauveria bassiana* on the European corn borer, *Ostrinia nubilalis*.** *J Invertebr Pathol* 1985, **46:** 259–264.

Fernandez EKK, Rangel DEN, Moraes AML, Bittencourt VREP, Roberts DW: **Cold activity of *Beauveria bassiana* and *Metarhizium*, and thermotolerance of *Beauveria***. *J Invertebr Pathol* 2008a, **98:** 69–78.

Fernandez EKK, Rangel DEN, Moraes AML, Bittencourt VREP, Roberts DW: **Variability in tolerance to UV-B radiation among *Beauveria spp*. isolates**. *J Invertebr Pathol* 2008b, **96:** 237-243.

Ferron P : **Essais préliminaires de la lutte contre les larves du hanneton commun, *Melolontha melolontha* L. à l'aide de la mycose a *Beauveria tenella* (Delacr.) Siemaszko.** *Phytiatrie-Phytopharmacie* 1967, **16:** 115–123.

Ferron P: **Modification of the development of *Beauveria teella* mycosis in *Melolontha melolontha* larvae by means of reduced doses of organophosphorous insecticides.** *Entomology Experiment et Applicata* 1971, **14:** 457-466.

Ferron P: **Pest control by the fungi *Beauveria* and *Metarhizium*.** In: *In: Microbial control of Pests and Plant Diseases* 1970-1980 Edited by (Editor) BH: Academic Press, London; 1981.

Ffrench-Constant RH, Daborn PJ, Le Goff G: **The genetics and genomics of insecticide resistance**. *Trends Genet* 2004, **20:** 163-170.

Ffrench-Constant RH: **Something old, something transgenic, or something fungal for mosquito control?** *Trends ecol evol (Personal edition)* 2005, **20:** 577-579.

Fillinger U, Knols BGJ, Becker N: **Efficacy and efficiency of new *Bacillus thuringiensis var. israelensis* and *Bacillus sphaericus* formulations against Afro-tropical anophelines in Western Kenya.** *Trop Med Inter Health* 2003, **8:** 37–47.

Fillinger U, Lindsay SW: **Suppression of exposure to malaria vectors by an order of magnitude using microbial larvicides in rural Kenya.** *Trop Med Int Health* 2006, **11:** 1629-1642.

Fontenille D, Lochouarn L, Diagne N, Sokhna C, Lemasson JJ, Diatta M, Knate L, Faye F, Rogier C, Trape JF: **High annual and seasonal variations in malaria transmission by anophelines and vector species composition in Dielmo, a holoendemic area in Senegal.** Am. *J Trop Med Hyg* 1997, **56:** 247-253.

Fontenille D, Simard F: **Unravelling complexities in human malaria transmission dynamics in Africa through a comprehensive knowledge of vectors populations.** *Comp Immun Microbiol Infect Dis* 2004, **27:** 357-375.

Frazzon APG, Junior ISV, Masuda A, Schrank A, Vainstein MH: **In vitro assessment of *Metarhizium anisopliae* isolates to control the cattle tick *Boophilus microplus.*** *Vet Parasitol* 2000, **94:** 117–125.

Friesen TF, Holloway G, Hill GA, Pugsley TS: **Effect of conditions and protectants on the survival of *Penicillium bilaiae* during storage.** *Biocontrol Sci Techn* 2006, **16:** 89-98.

Gallup JL, Sachs JD: **The economic burden of malaria.** *Am J Trop Med Hyg* 2001, **64:** 85-96.

Garrett-Jones C: **Prognosis for interruption of malaria transmission through assessment of the mosquito's vectorial capacity.** *Nature* 1964, **204:** 1173-1175.

Geetha I, Balaraman K: **Effect of entomopathogenic fungus, *Beauveria bassiana* on larvae of three species of mosquitoes.** *Indian J Exp Biol* 1999, **37:** 1148-1150.

Geissbühler Y, Kannady K, Chaki PP, Emidi B, Govella NJ, Mayagaya V, Kiama M, Mtasiwa D, Mshinda H, Lindsay SW, Tanner M, Fillinger U, Caldas de Castro M, Killeen GF: **Microbial larvicide application by a large-scale, community-based program reduces malaria infection prevalence in urban Dar Es Salaam, Tanzania.** *PLoS ONE* 2009, **4:** e5107.

Getsin LW: ***Spicaria rileyi* (Farlow) Charles, an entomogenous fungus of *Trichoplusiani* (Hubner).** *J Invertebr Pathol* 1961, **3:** 2–10.

Gillespie AT, Claydon N: **The use of entomogenous fungi for pest control and the role of toxins in pathogenesis.** *Pest sci* 1989, **27:** 203-215.

Gillespie JP, Bailey AM, Cobb B, Vilcinskas A: **Fungi as elicitors of insect immune responses.** *Arch Insect Biochem Physiol* 2000, **44:** 49-68.

Gillies MT, Coetzee M: **A supplement to the Anophelinae of Africa south of the Sahara.** *South African Institute for Medical Research* 1987, **55**: 79–86.

Gillies MT, Wilkes TJ: **A study of the age-composition of populations of *Anopheles gambiae* Giles and *A. funestus* Giles in North-Eastern Tanzania.** *Bull Entomol Res* 1965, **56:** 237-262.

Gillies MT: **Studies in house leaving and outside resting of *Anopheles gambiae* Giles and *Anopheles funestus* Giles in East Africa. II. The exodus from houses and the house resting population.** *Bull Entomol Res* 1954, **45:** 375-387.

Gimnig, JE, Ombok M, Kamau L, Hawley WA: **Characteristics of larval anopheline (Diptera: Culicidae) habitats in Western Kenya.** *J Med Entomol* 2001, **38:** 282-288.

Glare TR, Milner RJ: **Ecology of entomopathogenic fungi. In: Handbook of Applied Mycology.** Volume 2: Humans, Animals, and Insects. Arora DK Ajello L, Mukerji KG (Editors). 1991, Marcel Dekker, New York.

Global strategic framework for Integrated Vector Management Geneva: *WHO* 2004.

Goettel MS, Roberts DW: **Mass production, formulation and field application of entomopathogenic fungi. In: Lomer, CJ, Prior, C (eds). Biological control of locusts and grasshoppers.** *CAB International & International Institute of Tropical Agriculture*, Ascot, 1992, pp 230.

Gornova IB, Feofilova EP, Tereshina VM, Golovina EA, Krotkova NB, Kholodova VP: **Effect of carbohydrate content of *Aspergillus japonicus* spores on their survival in storage and subsequent germination.** *Mikrobiologiya* 1992, **61:** 549–554.

Greenwood B, Mutabingwa T**: Malaria in 2002.** *Nature* 2002, **415:** 670-672.

Greenwood BM, Bojang K, Whitty CJM, Targett GAT: **Malaria.** *Lancet* 2005, **365:** 1487-1498.

Griffin DH: **Fungal Physiology** (second ed.), *Wiley-Liss, New York*, 1994.

Guerzoni ME, Lanciotti R, Cocconcelli PS: **Alteration in cellular fatty acid composition as a response to salt, acid, oxidative and thermal stresses in *Lactobacillus helveticus*.** *Microbiology* 2001, **147:** 2255–2264.

Hajek AE, Carruthers RI, Soper SR: **Temperature and moisture relations of sporulation and germination by *Entomophaga maimaga* (Zygomycetes: Entomophthoraceae), a fungal pathogen of *Lymantria dispar* (Lepidoptera:Lymantriidae)**. *Environmen Entomol* 1990, **19:** 85-90.

Hallsworth JE, Magan N: **Culture age, temperature, and pH affect the polyol and trehalose contents of fungal propagules.** *Applied and Environmental Microbiology* 1996, **62:** 2435-2442.

Hallsworth JE, Magan N: **Effect of carbohydrate type and concentration on polyhydroxy alcohol and trehalose content of conidia of three entomopathogenic fungi.** *Microbiology* 1994, **140:** 2705-2713.

Hallsworth JE, Magan N: **Manipulation of intracellular glycerol and erythritol enhances germination of conidia at low water availability.** *Microbiology* 1995, **141:** 1109-1115.

Hallsworth JE, Magan N: **Water and temperature relation of growth of the entomogenous fungi *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces farinosus*.** *J Invertebr Pathol* 1999, **74:** 261-266.

Hancock PA, Thomas MB, Godfray HC: **An age-structured model to evaluate the potential of novel malaria-control interventions: a case study of fungal biopesticide sprays.** *Proc R Soc Lond* 2008: 1-10.

Hancock PA: **Combining fungal biopesticides and insecticide-treated bednets to enhance malaria control.** *PLoS Comput Biol* 2009, **5:** e1000525.

Hargreaves K, Koekemoer LL, Brooke BD, Hunt RH, Mthembu JD, Coetzee M: ***Anopheles funestus* resistant to pyrethroid insecticides in South Africa.** *Med Vet Entomol* 2000, **14:**181-189.

Harman GE, Jin X, Stasz TE, Peruzzotti, G, Leopold AC, Taylor AG: **Production of conidial biomass of *Trichoderma harzianum* for biological control.** *Biological Contr* 1991, **1:** 23–28.

Harrington LC, Buonaccorsi JP, Edman JD, Costero A, Kittayapong P, Clark GG, Scott TW: **Analysis of survival of young and old *Aedes aegypti* (Diptera: Culicidae) from Puerto Rico and Thailand.** *J Med Entomol* 2001, **38:** 537-547.

Hay SI, Guerra CA, Tatem AJ, Atkinson PM, Snow RW: **Urbanization, malaria transmission and disease burden in Africa.** *Nat Rev Microbiol* 2005, **3:** 81-90.

Heinrich B: **The Hot-Blooded Insects.** Cambridge: Harvard University Press; 1993.

Hemingway J, Field L, Vontas **J: An Overview of Insecticide Resistance.** *Science* 2002: **298:** 96-97.

Hillyer JF, Schmidt SL, Fuchs JF, Boyle JP, Christensen BM: **Age-associated mortality in immune challenged mosquitoes (*Aedes aegypti)* correlates with a decrease in haemocyte numbers.** *Cell Microbiol* 2005, **7:** 39-51.

Hodjati MH, Curtis CF: **Evaluation of the effect of mosquito age and prior exposure to insecticide on pyrethroid tolerance in *Anopheles* mosquitoes (Diptera: Culicidae).** *Bull Entomol Res* 1999, **89:** 329–337.

Hong TD, Jenkins NE, Ellis RH: **The effects of duration of development and drying regime on the longevity of conidia of *Metarhizium flavoviride*.** *Mycol Res* 2000, **104:** 662-665.

Houston J, Dancer BN, Learner MA: **Control of sewage filter flies using *Bacillus thuringiensis* var. *israelensis* acute toxicity tests and pilot scale trial.** *Water Res* 1993, **23:** 369-78.

Howard AFV, Zhou G, Omlin FX: **Malaria mosquito control using edible fish in western Kenya: Preliminary findings of a controlled study.** *BMC Public Health* 2007, **7:** 199.

Howard AFV, Koenraadt CJM, Farenhorst M, Knols BGJ, Takken W: **Pyrethroid resistance in *Anopheles gambiae* leads to increased susceptibility to entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*.** *Malar J* 2010, **9:** 168.

Hughes WOH, Petersen KS, Ugelvig LV, Pedersen D, Thomsen L, Poulsen M, Boomsma JJ: **Density-dependence and within-host competition in a semelparous parasite of leaf-cutting ants.** *BMC Evol Biol* 2004, **4:** 45.

Hugo LE, Kay BH, Eaglesham GK, Holling N, Ryan PA: **Investigation of cuticular hydrocarbons for determining the age and survivorship of Australasian mosquitoes.** *Am J Trop Med Hyg* 2006, **74:** 462-474.

Huho BJ, Ng'habi KR, Killeen GF, Nkwengulila G, Knols BGJ, Ferguson HM: **Nature beats nurture: a cast study of the physiological fitness of free-living and laboratory-reared male *Anopheles gambiae* s.l.** *J Exp Biol* 2007, **210:** 2939-2947.

Hussy NW, Tinsley TW: **Impression of insect pathology in the people's Republic of China**. *In: Burges, HD (Ed), Microbial control of pests and plant diseases, Academic Press, London* 1981, 785-795.

Hutchinson OC, Cunningham AA: **Benefits and risks in malaria control.** *Science* 2005, **310,** 49-50.

Ignoffo CM: **Environmental factors affecting persistence of entomopathogens** *Florida Entomol* 1992, **75:** 516-266.

Ihara F, Toyama M, Higaki M, Mishiro K, Yaginuma K: **Comparison of pathogenicities of *Beauveria bassiana* and *Metarhizium anisopliae* to chestnut pests.** *Applied Entomology and Zoology* 2009, **44:** 127-132.

Inglis GD, Duke GM, Kawchuk LM, Goettel MS: **Influence of oscillating temperatures on the competitive infection and colonization of the migratory grasshopper by *Beauveria bassiana* and *Metarhizium flavoviridae*.** *Biol Control* 1999, **14:** 111-120.

Itoh T: **Evaluation of long-lasting insecticidal nets after 2 years household use**. *Trop Med Int Health* 2005, **10:** 1321-1326.

Jackson MA, Schisler DA: **Formulation of *Colletotrichum truncatum* microsclerotia for improved bio-control of the weed hemp sesbania (*Sesbania exaltata*)**. *Biological Contr* 1996, **7:** 107-113.

Jenkins NE, Heviefo G, Langewald J, Cherry AJ, Lomer CJ: **Development of mass production technology for aerial conidia for use as mycopesticides** *Biocontrol news and Information* 1998, **19:** 21-31.

Jennings DH**: Understanding tolerance to stress: laboratory culture versus environmental actuality**, pp 1–12. *In* D. H. Jennings (ed.), Stress tolerance of fungi. Marcel Dekker, Inc New York 1993.

Jimenez-Arredondo JI, Rodriguez MH, Loyola EG, Bown DN: **Behaviour of *Anopheles albimanus* in relation to pyrethroid-treated bednets**. *Med Vet Entomol* 1997, **11:** 87-94.

Kaaya GP, Mwangi EN, Ouna EA: **Prospect for biological control of livestock ticks, *Rhipicephalus appendiculatus* and *Amblyomma variegatum*, using the entomogenous fungi *Beauveria bassiana* and *Metarhizium anisopliae*.** *J Invertebr Pathol* 1996, **67:** 15–20.

Karch S, Manzambi ZA, Salaun JJ: **Field trials with Vectolex (*Bacillus sphaericus*) and Vectobac (Bacillus thuringiensis (H-14)) against *Anopheles gambiae* and *Culex quinquefasciatus* breeding in Zaire.** *J Am Mosq Control Assoc* 1991, **7:** 176-179.

Kawamura C, Tsujimoto T, Tsuge T: **Targeted disruption of melanin biosynthesis gene affects conidia development and UV tolerance in the Japanese pear pathotype of *Alternaria alternata*.** *Mol Plant-Microbe Interact* 1999, **12:** 59-63.

Keller, S., Zimmerman, G., 1989. **Mycopathogens of soil insects.** In: Wilding, N., Collins, N.M., Hammond, P.M., Webber, J.F. (Eds.), Insect–Fungus Interactions. Academic Press London, UK 1989, pp. 240–270.

Kelly-Hope L, Ranson H, Hemingway J: **Lessons from the past: managing insecticide resistance in malaria control and eradication programmes.** *Lancet Infect Dis* 2008, **8:** 387–389.

Khachatorians GG: **Physiology and genetics of entomopathogenic fungi**. In: *Handbook of Applied Mycology*, 2001, Volume 2. Humans, animals, and insects.

Kiewnick S: **Effect of temperature on growth, germination, germ-tube extension and survival of *Paecilomyces lilacinus* strain 251**. *Biocontrol Sci Technol* 2006, **16:** 535-546.

Kikankie CK, Brooke BD, Knols BGJ, Koekemoer LL, Farenhorst M, Hunt RH, Thomas MB, Coetzee M: **The infectivity of the entomopathogenic fungus *Beauveria bassiana* to insecticide-resistant and susceptible *Anopheles arabiensis* mosquitoes at two different temperatures.** *Malar J* 2010, **9:** 71.

Kilama WL, Chilengi R, Wanga CL: **Towards an African-driven malaria vaccine development program: history and activities of the African malaria network trust (AMANET).** *Am J Trop Med Hyg* 2007, **77:** 282–288.

Killeen G, Fillinger U, Kiche I, Gouagna L, Knols B: **Eradication of *Anopheles gambiae* from Brazil: lessons for malaria control in Africa?** *Lancet Infect Dis* 2002, **2:** 618–627.

Killeen GF, Mc Kenzie FE, Foy BD, Schieffelin C, Billingsley PF, Beier JC: **A simplified model for predicting malaria entomological inoculation rates based on entomologic and parasitologic parameters relevant to control.** *Am J Trop Med Hyg* 2000, **62:** 535-544.

Killeen GF, Smith TA, Ferguson HM, Mshinda H, Abdulla S, Lenegeler C, Kachur SP: 2007a. **Preventing childhood malaria in Africa by protecting adults from mosquitoes with insecticide-treated nets. *PLoS Medicine***. **4:** e229.

Killeen GF, Tami A, Kihonda J, Oketch-Okumu FR, Kotas ME, Grundmann H, Kasigudi N, Ngonyani H, Mayagaya V, Nathan R, Abdulla S, Charlwood JD, Smith TA, Lengeler C: **Cost-sharing strategies combining targeted public subsidies with private-sector delivery achieve high bed net coverage and reduced malaria transmission in Kilombero valley, southern Tanzania.** *BMC Infect Dis* 2007b, **7:** 7-121.

Kirby MJ, Ameh D, Bottomley C, Green C, Jawara M, Milligan PJ, Snell PC, Conway DJ, Lindsay SW: **Effect of two different house screening interventions on exposure to malaria vectors and on anaemia in children in The Gambia: a randomised controlled trial.** *Lancet* 2009, **374:** 998-1009.

Koella JC, Lorenz L, Bargielowski I: **Microsporidians as evolution-proof agents of malaria control?** *Adv parasitol* 2009, **68:** 315.

Koella JC, Lynch PA, Thomas MB, Read AF: **Towards evolution-proof malaria control with insecticides.** *Evol Appl* 2009, 9999(9999).

Kooyman C, Godonou I: **Infection of *Schistocerca gregaria* Forskål (Orthoptera: Acrididae) hoppers by *Metarhizium flavoviride* (Deuteromycotina: Hyphomycetes) conidia in an oil formulation applied under desert conditions.** *Bull Entomol Res* 1997, **87:** 105-107.

Kramer VL, Garcia R, Colwell A: **An evaluation of the mosquitofish, *Gambusia affinis*, and the inland silverside, *Menidia beryllina*, as mosquito control agents in California wild rice fields.** *J Am Mosq Control Assoc* 1987, **3:** 626-632.

Kramer VL, Garcia R, Colwell AE: **An evaluation of *Gambusia affinis* and *Bacillus thuringiensis* var. *israelensis* as mosquito control agents in California wild rice.** *J Am Mosq Control Assoc* 1988, **4:** 470478.

Kumar A, Sharma VP, Sumodan PK, Thavaselvam D**: Field trials of biolarvicide *Bacillus thuringiensis* var. *israelensis* strain 164 and the larvivorous fish *Aplocheilus blocki* against *Anopheles stephensi* for malaria control in Goa, India.** *J Am Mosq Control Assoc* 1998, **14:** 457-462.

Lacey LA: ***Bacillus thuringiensis* sero variety *israelensis* and *Bacillus sphaericus* for mosquito control.** *J Am Mosq Control Assoc* 2007, **25:** (supplement to No. 2).

Latch GCM, Kain WM: **Control of Porina caterpillar (*Wiseana* spp.) in pasture by the fungus, *Metarhizium anisopliae*.** *New Zeal J Exp Agr* 1983, **11:** 351-354.

Lawn JE, Cousens S, Zupan J: **4 million neonatal deaths: When? Where? Why?** *Lancet* 2005, **365:** 891-900.

Lazzarini GMJ, Rocha LFN, Luz CI: **Impact of moisture on in vitro germination of *Metarhizium anisopliae* and *Beauveria bassiana* and their activity on *Triatoma infestans.*** *Mycol Res* 2006, **110:** 485-492.

Leal-Bertioli SCM, Butt TM, Peberdy JF, Bertioli DJ: **Genetic exchange in *Metarhizium anisopliae* strains co-infecting *Phaedon cochleariae* is revealed by molecular markers.** *Mycol Res* 2000, **104:** 409-414.

Lecuona RE, Edelstein JD, Berretta M, La Rossa FR, Arcas JA: **Evaluation of *Beauveria bassiana* (Bals.) Vuill. (Hyphomycetes) strains as a potential agent for control of *Triatoma infestans* Klug (Hemiptera:Reduviidae).** *J Med Entomol* 2001, **38:** 172-179.

Legner EF: **Biological control of Diptera of medical and veterinary importance.** *J Vector Ecol* 1995, 20: 59-120.

Lemasson JJ, Fontenille D, Lochouarn L, Dia I, Simard F, Ba K, Diop A, Diatta M, Molez JF: **Comparison of behavior and vector efficiency of *Anopheles gambiae* and *Anopheles arabiensis* (Diptera: Culicidae) in Barkedji, a Sahelian area of Senegal.** *J Med Entomol* 1997, **34:** 396-403.

Lengeler C: **Insecticide-treated bednets and curtains for preventing malaria.** *Cochrane Database Syst Rev* 2004, (2)**:** CD000363.

Li J, Tracy JW, Christensen BM: **Relationship of hemolymph phenol oxidase and mosquito age in *Aedes aegypti*.** *J Invertebr Pathol* 1992, **60:** 188-191.

Lindsay SW, Bødker R, Malima R, Msangeni HA, Kisinza W: **Effect of 1997-98 El Niño on highland malaria in Tanzania.** *Lancet* 2000, **355:** 989-990.

Lindsay SW, Martens WJ: **Malaria in the African highlands: past, present and future.** *Bull World Health Organ* 1998, **76:** 33-45.

Lines JD, Myamba J, Curtis CF: **Experimental hut trials of permethrin-impregnated mosquito nets and eave curtains against malaria vectors in Tanzania.** *Med Vet Entomol* 1987, **1:** 37-51.

Liu HP, Skinner M, Brownbridge M, Parker BL: **Characterization of *Beauveria bassiana* and *Metarhizium anisopliae* isolates for management of tarnished plant bug, *Lygus lineolari* (Hemiptera:Miridae)**. *J Invertebr Pathol* 2003, **82:** 139-147.

Liu JW, Yap W, Thanabalu T, Porter AG: **Efficient synthesis of mosquitocidal toxins in *Asticcacaulis excentricus* demonstrates potential of gram-negative bacteria in mosquito control.** *Nature Biotechnol* 1996, **14:** 343-347.

Liu WH, Xin K, Chao CZ, Feng SZ, Yan L, He RZ: **New irrigation methods sustain malaria control in Sichuan Province, China.** *Acta Trop* 2004, **89:** 241–247.

Luchavez J, Espino F, Curameng P, Espina R, Bell D, Chiodini P, Nolder D, Sutherland C, Lee KS, Singh B: **Human Infections with *Plasmodium knowlesi*, the Philippines.** *Emerg Infect Dis* 2008, **14:** 811-813.

Luz C, Batagin I. **Potential of oil-based formulations of *Beauveria bassiana* to control *Triatoma infestans***. *Mycopathologia* 2005, **160:** 51-62.

Luz C, Fargues J, Romana CA, Moreno J, Goujet R, Rougier M, Grunewald J: **Potential of entomopathogenic hyphomycetes for the control of the triatomine vectors of Chagas' disease**. *Proceedings VIth Int Coll Invertebrate Pathology and Microbiological Control* 1994, **1:** 272-276.

Luz C, Fargues J: **Factors affecting conidial production of *Beauveria bassiana* from fungus-killed cadavers of *Rhodnius prolixus.*** *J Invertebr Pathol* 1998, **72:** 97-103.

Luz C, Fargues J: **Temperature and moisture requirements for conidial germination of an isolate of the hyphomycete *Beauveria bassiana* (Bals.) Vuill., pathogenic to *Rhodnius prolixus* (Stahl) (Hem., Reduviidae)**. *Mycopathologia* 1997, **138:** 117-125.

Luz C, Tai MHH, Santos AH, Silva HHG: **Impact of moisture on survival of *Aedes aegypti* eggs and ovicidal activity of *Metarhizium anisopliae* under laboratory conditions.** *Mem Inst Oswaldo Cruz* 2008, **103:** 214-215.

Lwetoijera DW, Sumaye RD, Madumla EP, Kavishe DR, Mnyone LL, Russell TL, Okumu, FO: **An extra-domiciliary method for delivering entomopathogenic fungi, *Metarhizium anisopliae* IP 46 against malaria vectors, *Anopheles arabiensis*.** *Parasites & Vectors* 2010, **3:** 18.

Mabaso ML, Sharp B, Lengeler C: **Historical review of malarial control in southern African emphasis on the use of indoor residual house-spraying.** *Trop Med Inter Health* 2004, **9:** 846-856.

Magalhães BP, Boucias DG**: Effects of drying on the survival of conidiosphores of *Metarhizium anisopliae* var. *acridum* Driver& Milner.** *J Orthoptera Res* 2004, **13:** 155-159.

Magalhães BP, Frazão HS**: Effects of temperature, water content and substrate on conidial production of *Metarhizium flavoviride*.** *Rev Microbiol* 1996, **27:** 242–246.

Mahande A, Mosha F, Mahande J, Kweka E: **Feeding and resting behaviour of malaria vector, *Anopheles arabiensis* with reference to zooprophylaxis.** *Malar J* 2007, **6:** 100.

Malakar R, Hajek AE, Burand JP: **Within-host interaction of *Lymantria dispar* (Lepidoptera: Lymantriidae) Nucleopolyhedrosis virus and *Entomophaga maimaga* (Zygomycetes:Entomophorales).** *J Invertebr Pathol* 1999, **73:** 91-100.

Maniania NK, Odulaja A: **Effect of species, age and sex of tsetse on response to infection by *Metarhizium anisopliae*.** *BioControl* 1998, **43:** 311–383.

Maniania NK. **Evaluation of three formulations of *Beauveria bassiana* (Bals.) Vuill. for control of the stem borer *Chilo partelus* (Swinhole) (Lep., Pyralidae).** *J Appl Entomol* 1993, **115:** 266-272.

Maniania NK: **A laboratory technique for infecting adult tsetse with a fungal agent.** *Insect Sci Applic* 1994, **15:** 421-426.

Maranga RO, Kaaya GP, Mueke JM, Hassanali A: **Effects of combining the fungi *Beauveria bassiana* and *Metarhizium anisopliae* on the mortality of the tick *Amblyoma variegatum* (ixodae) in relation to seasonal changes.** *Mycopathologia* 2005, **159:** 527-532.

Marrelli MT, Li C, Rasgon JL, Jacobs-Lorena M: **Transgenic malaria-resistant mosquitoes have a fitness advantage when feeding on *Plasmodium*-infected blood.** *Proc Natl Acad Sci U.S.A* 2007, **104:** 5580-5583.

Masanja H, De Savigny D, Smithson P, Schellenberg J, John T, Mbuya C: **Child survival gains in Tanzania: analysis of data from demographic health surveys.** *Lancet* 2008, **371:** 1276-1283.

Matawele P, Trinci APJ, Gillespie AT: **Mutants of entomopathogenic fungi that germinate and grow at reduced water activities and reduced relative humidities are more virulent to *Neophotellix virescens* (green leafhopper) than the parental strains.** *Mycol Res* 1994, **98:** 1329–1333.

Mathenge EM, Gimnig JE, Kolczak M, Ombok M, Irungu LW, Hawley WA: **Effect of permethrin-impregnated nets on exiting behavior, blood feeding success, and time of feeding of malaria mosquitoes (Diptera: Culicidae) in western Kenya.** *J Med Entomol* 2001, **38:** 531-536.

Maxwell CA, Msuya E, Sudi M, Njunwa KJ, Carneiro IA, Curtis CF: **Effect of community-wide use of insecticide-treated nets for 3–4 years on malaria morbidity in Tanzania.** *Trop Med Int Health* 2002, **7:** 1003–1008.

McClatchie G, Moore D, Bateman RP, Prior C: **Effect of temperature on the viability of the conidia of *Metarhizium flavoviride* in oil formulations.** *Mycol Res* 1994, **98:** 749–756.

McGinnis MR, Padhye AA, Ajello L: **Storage of stock cultures of filamentous fungi, yeast and some aerobic actinomycetes in sterile distilled water.** *Appl Microbiol* 1974, **28:** 218-222.

McGuire MR, Tamez-Guerra P, Behle RW, Streett DA: **Comparative field stability of selected entomopathogenic virus formulations** *Biol Microbiol Contr* 2001, **94:** 1037-1044.

McMeniman CJ, Lane RV, Cass BN, Fong AWC, Sidhu M, Wang YF, O'Neill SL: **Stable introduction of a life-shortening Wolbachia infection into the mosquito *Aedes aegypti***. *Science* 2009, **323:** 141.

Meyling NV, Pell JK: **Detection and avoidance of an entomopathogenic fungus by a generalist insect predator.** *Ecol Entomol* 2006, **31:** 162–171.

Michalakis Y, Renaud F: **Malaria - Fungal allies enlisted.** *Nature* 2005, **435:** 891–892.

Miller DR, Weidhaas DE, Hall RC: **Parameter sensitivity in insect population modeling.** *J Theor Biol* 1973, **42:** 263-274.

Millstein JA, Brown GC, Nordin GL: **Microclimatic humidity influence on conidial discharge in *Erynia* sp. (Entomophthorales: Entomophthoraceae), and entomopathogenic fungus of the alfalfa weevil (Coleoptera: Curculionidae).** *Environ Entomol* 1982, **11:** 1166–1169.

Milner RJ, Staples JA, Lutton GG: **The selection of an isolate of the hyphomycete fungus, *Metarhizium anisopliae*, for control of termites in Australia** *Biol Control* 1998, **11:** 240-247.

Mnyone LL, Kirby MJ, Lwetoijera DW, Mpingwa MW, Knols BGJ, Takken W, Russell TL: **Infection of the malaria mosquito, *Anopheles gambiae* with two species of entomopathogenic fungi: effects of concentration, co-formulation, exposure time and persistence.** *Malar J* 2009, **8:** 309.

Mnyone LL, Russell TL, Lyimo NI, Lwetoijera DW, Kirby MJ, Luz C: **First report of *Metarhizium anisopliae* IP 46 pathogenicity in adult *Anopheles gambiae s.s.* and *An. arabiensis* (Diptera; Culicidae).** *Parasite & Vectors* 2009, **2:** 59.

Mohamed AKA, Sikorowski P, Bell JV: **The susceptibility of *Heliothis zea* larvae to *Nomuraea rileyi* at various temperatures.** *J Invertebr Pathol* 1977, **12:** 444–459.

Moore D, Bateman RP, Carey M, Prior C: **Long term storage of *Metarhizium flavoviride* conidia in oil formulations for the control of grasshoppers.** *Biocontrol Sci Techn* 1995, **5:** 193–199.

Moore D, Bridge PD, Higgins PM, Bateman RP, Prior C: **Ultra-violet radiation damage to *Metarhizium flavoviridae* conidia and protection given by vegetable and mineral oils and chemical sunscreens** *Ann Appl Biol* 1993, **122:** 605-616.

Moore GE: **Pathogenicity of three entomogenous fungi to the southern pine beetle at various temperatures and humidities.** *Environ Entomol* 1972, **2:** 54–57.

Moore-Lendecker E: **Fundamentals of the fungi.** Prentice Hall, New Jersey; 1996.

Moreno M, Vicente JL, Cano J, Berzosa PJ, de Lucio A, Nzambo S, Bobuakasi L, Buatiche JN, Ondo M, Micha F: **Knockdown resistance mutations (kdr) and insecticide susceptibility to DDT and pyrethroids in *Anopheles gambiae* from Equatorial Guinea**. *Trop Med Int Health* 2008, **13:** 430.

Mourya DT, Hemingway J, Leake CJ: **Changes in enzyme titres with age in four geographical strains of *Aedes aegypti* and their association with insecticide resistance.** *Med Vet Entomol* 1993, **7:** 11-16.

Mullens BA: **Host, age and sex and pathogen exposure levels as factors in the susceptibility of *Musca domestica* to *Entomophthora muscae*.** *Ent Exp et Appl* 1985, **37:** 330–339.

Mullins DE**: Chemistry and physiology of the haemolymph,** In: *Comprehensive Insect Physiology, Biochemistry and Pharmacology* Edited by *G*A, Kerkut & Gilbert, LI. Oxford: Pergamon Press.1985, 355-392.

Mwangi EN, Kaaya GP, Essuman S: **Experimental infections of the tick *Rhipicephalus appendiculatus* with entomopathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae*, and natural infections of some ticks with bacteria and fungi.** *J Afr Zool* 1995, **109:** 151–160.

N'Guessan R, Corbel V, Akogbéto M, Rowland M: **Reduced efficacy of insecticide-treated nets and indoor residual spraying for malaria control in pyrethroid resistance area, Benin.** *Emerg Infect Dis* 2007, **13:** 199-206.

Nauen R: **Insecticide resistance in disease vectors of public health importance**. *Pest Manag Sci* 2007, **63:** 628–633.

Nayar JK, Sauerman DM Jr**: A comparative study of flight performance and fuel utilization as a function of age in females of Florida mosquitoes.** *J Insect Physiol* 1973, **19:** 1977-1988.

Nelson TL, Low T, Glare TR: **Large scale production of New Zealand strains of *Beauveria bassiana* and *Metarhizium*** *49th Conference proceedings The New Zealand plant protection society incorporated* 1996, 257-261.

Njie M, Dilger E, Lindsay SW, Kirby MJ: **Importance of eaves to house entry by anopheline, but not culicine, mosquitoes**. *J Med Entomol* 2009, **46:** 505-510.

Ogoma SB, Lwetoijera DW, Ngonyani H, Furer B, Russell TL, Mukabana RW, Killeen GF, Moore SJ: **Screening of mosquito house entry points as a potential method for integrated control of endophagic malaria filariasis and arbovirus vectors.** *PLoS Negl Trop Dis* 2010, In Press.

Okara RM, Sinka ME, Minakawa N, Mbogo CM, Hay SI, Snow RW: **Distribution of the main malaria vectors in Kenya.** *Malar J* 2010, **9:** 69.

Okech, BA, Gouagna LC, Killeen GF, Knols BG, Kabiru EW, Beier JC, Yan G, Githure JI: **Influence of sugar availability and indoor microclimate on survival of *Anopheles gambiae* (Diptera: Culicidae) under semifield conditions in western Kenya.** *J Med Entomol* 2003, **40:** 657-663.

Okumu FO, Killeen GF, Ogoma S, Biswaro L, Smallegange RC, Mbeyela E, Titus E, Munk C, Ngonyani H, Takken W, Mshinda H, Mukabana WR, Moore SJ: **Development and field evaluation of a synthetic mosquito lure that is more attractive than humans.** *PLoS ONE* 2010, **5:** e8951.

Okumu FO, Madumla EP, John A, Lwetoijera DW, Sumaye RD: **Attracting, trapping and killing disease-transmitting mosquitoes using odour-baited stations -The Ifakara Odour-Baited Stations.** *Parasites & Vectors* 2010, **3**:12.

Omond E: **The overwintering interactions of the seven spot ladybird (Coccinella septempunctata) and the entomopathogenic fungus *Beauveria bassiana*.** *PhD thesis*, Anglia Ruskin University, Cambridge 2007.

Ondiaka S, Bukhari T, Farenhorst M, Takken W, Knols BG: **Effects of fungal infection on the host-seeking behaviour and fecundity of the malaria mosquito *Anopheles gambiae* Giles.** *Proceedings of Netherlands Entomological Society Meeting* (N.E.V) 2008, **9:** 121-128.

Onofre SB, Miniuk CM, de Barros NM, Azevedo JL: **Pathogenicity of four strains of entomopathogenic fungi against the bovine tick *Boophilus microplus*.** *Am J Vet Res* 2001, **62:** 1478–1480.

Onyabe DY, Conn JE: **The distribution of two major malaria vectors, *Anopheles gambiae* and *Anopheles arabiensis*, in Nigeria.** *Mem Inst Oswaldo Cruz* 2001, **96:** 1081-1084.

Pates H, Curtis C: **Mosquito behaviour and vector control**. *Ann Rev Entomol* 2005, **50:** 53-70.

Pates HV, Takken W, Stuke K, Curtis CF: **Differential behaviour of *Anopheles gambiae* sensu stricto (Diptera: Culicidae) to human and cow odours in the laboratory.** *Bull Entomol Res* 2001, **91:** 289–296.

Phuc HK, Andreasen MH, Burton RS, Vass C, Epton MJ, Pape G, Fu G, Condon KC, Scaife S, Donnelly CA, Coleman PG, White-Cooper H, Alphey L: **Late-acting dominant lethal genetic systems and mosquito control.** *BMC Biol* **5:** 11.

Pluess B, Tanser FC, Lengeler C, Sharp BL: **Indoor residual spraying for preventing malaria (Review).** *Cochrane Database of Systematic Reviews* 2010, 4: Art. No.: CD006657. DOI: 10.1002/14651858.CD006657.pub2.

Pupin AM, Messias CL, Piedrabuena AE, Roberts DW: **Total lipids and fatty acids of strains of *Metarhizium anisopliae*.** *Braz J Microbiol* 2000, **31:** 121–128.

Qiangqiang Z, Jiajun W, Li L: **Storage of fungi using sterile distilled water or lyophilization: comparison after 12 years.** *Mycoses* 1998, **41:** 255-257.

Quedraogo A, Fargues J, Goettel MS, Lomer CJ: **Effect of temperature on vegetative growth among isolates of *Metarhizium anisopliae* and *M. flavoviride*.** *Mycopathologia* 1997, **137:** 37-43.

Quesada-Moraga E, Carrasco-Daaz JA, Santiago-Ãlvarez C: **Insecticidal and antifeedant activities of proteins secreted by entomopathogenic fungi against *Spodoptera littoralis* (Lep., Noctuidae).** *J Appl Entomol* 2006, **130:**442-453.

Quesada-Moraga E, Ruiz-Garcia A, Santiago-Álvarez C: **Laboratory evaluation of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* against puparia and adults of *Ceratitis capitata* (Diptera: Tephritidae).** *J Econ Entomol* 2006, **99:** 1955-1966.

Quimby PCJ, Zidack NK, Boyette CD, Grey WE: **A simple method for stabilizing and granulating fungi.** *Biocontrol Sci Techn* 1999, **9:** 5-8.

Quintela ED, Lord JC, Wraight SP, Alves SB, Roberts DW: **Pathogenicity of *Beauveria bassiana* (Hyphomycetes: Moniliales) to larval and adult *Chalcodermus bimaculatus* (Coleoptera: Curculionidae).** *J Econ Entomol* 1990, **83:** 1276–1279.

Rangel DEN, Anderson AJ, Roberts DW: **Growth of *Metarhizium anisopliae* on non-preferred carbon sources yields conidia with increased UV-B tolerance**. *J Invertebr Pathol* 2006, **93:** 127-134.

Rangel DEN, Braga GUL, Anderson AJ, Roberts DW: **Variability in conidial thermotolerance of *Metarhizium anisopliae* isolates from different geographical origins** *J Invertebr Pathol* 2005, **88:** 116-125.

Ranson H, Abdallah H, Badolo A, Guelbeogo WM, Kerah-Hinzoumbe C, Yangalbe-Kalnone E, Sagnon NF, Simard F, Coetzee M: **Insecticide resistance in *Anopheles gambiae*: data from the first year of a multi-country study highlight the extent of the problem.** *Malar J* 2009, **8:** 299.

Ranson H, Claudianos C, Ortelli F, Abgrall C, Hemingway J, Sharakhova MV, Unger MF, Collins FH, Feyereisen R: **Evolution of supergene families associated with insecticide resistance.** *Science* 2002, **298:** 179–181.

Rao CUM, Devi KU, Khan PAA: **Effect of combination treatment with entomopathogenic fungi *Beauveria bassiana* and *Nomuraea rileyi* (Hypocreales) on *Spodoptera litura* (Lepidoptera: Noctuidaeae).** *Biocontr Sci Tech* 2006, **16:** 221-232.

Rath AC, Koen TB, Yip HY: **The influence of abiotic factors on the distribution and abundance of *Metarhizium anisopliae* in Tasmanian pasture soils.** *Mycol Res* 1992, **96:** 378-384.

Read AF, Lynch PA, Thomas MB: **How to Make Evolution-Proof Insecticides for Malaria Control**. *PLoS Biol* 2009, **7:** e1000058.

Read AF, Taylor LH: **The ecology of genetically diverse infections.** *Science* 2001, **292:**1099.

Read AF, Thomas MB: **Microbiology: Mosquitoes Cut Short**. *Science* 2009, **323**(5910)**:** 51.

Reuter KC, Foster RN, Black L, Britton J: **Laboratory evaluation of *Beauveria bassiana* with an added chemical stressor.** *Arthr Manage Tests* 1995, **21:** 415–416.

Rhodes DJ: **Formulations of biological control agents. In: Exploitation of microorganisms,** edited by Jones DG, (London: Chapman and Hall), 1993, pp 411-439.

Rizzo DC: **Age of three dipteran hosts as a factor governing the pathogenicity of *Beauveria bassiana* and *Metarhizium anisopliae*.** *J Invertebr Pathol* 1977, **30:** 127-130.

Roberts DR, Manguin S, Mouchet J: **DDT house-spraying and re-emerging malaria.** *Lancet* 2000, 356: 330-332.

Roberts DW, Campbell AS: **Stability of entomopathogenic fungi.** *Miscellaneous publications of the Entomological Society of America* 1977, **10:** 19–76.

Roberts DW, Panter C: **Fungi other than *Coelomomyces* and *Lagenidium***. In Biological Control of Mosquitoes (ed. Chapman, H. C.), pp 99-109. *American Mosquito Control Association* 1985.

Romana CA, Fargues J: **Relative susceptibility of different stages of *Rhodnius prolixus* (Triatominae) aux hyphomyc tes entomopathogenes**. *Memorial Institute of Oswaldo Cruz* 1992, **87:** 363-368.

Rossi IH, Monteiro AC, Machado JO, Barbosa JC: **Supplementation of sugarcane bagasse with rice bran and sugarcane molasses for shiitake (*Lentinula edodes*) spawn production.** *Braz J Microbiol* 2003, **34:** 61-65.

Rowland M, Mahmood P, Iqbal J, Carneiro I, Chavasse D: **Indoor residual spraying with alphacypermethrin controls malaria in Pakistan: a community randomized trial**. *Trop Med Inter Health* 2000, **5:** 472-481.

Rowland M, Mahmood P, Iqbal J, Carneiro I, Chavasse D: **Indoor residual spraying with alphacypermethrin controls malaria in Pakistan: a community randomized trial.** *Trop Med Int Health* 2000, **5:** 472-481.

Rowley WA, Graham CL: **The effect of age on the flight performance of female *Aedes aegypti* mosquitos.** *J Insect Physiol* 1968, **14:** 719-728.

Roy HE, Steinkraus DC, Eilenberg J, Hajek AE, Pell JK: **Bizarre interactions and endgames: Entomopathogenic fungi and their arthropod hosts** *Ann Rev Entomol* 2006, **51:** 331-357.

Ruel JJ, Ayers MP. **Jensen's inequality predicts effects of environmental variation**. *Tree* 1999, **14:** 361–365.

Sachs J, Malaney P: **The economic and social burden of malaria. Nature** 2002, **415:** 680-685.

Samish M, Ginsberg H, Glazer I: **Biological control of ticks.** *Parasitology* 2004, **129:** 389–403.

Samson RA, Evans HC, Latge JP: **Atlas of entomopathogenic fungi.** *Springer Verlag* 1988, Berlin.

Samuels KDZ, Heale JB, Llewllyn M. **Characteristics relating to the pathogenicity of *Metarhizium anisopliae* toward *Nilaparvata lugens*.** *J Invertebr Pathol* 1989, **53:** 25–31.

Santos AH, Tai MH, Rocha LF, Silva HH, Luz C: **Dependence of *Metarhizium anisopliae* on high humidity for ovicidal activity on *Aedes aegypti*.** *Biological Control* 2009, **50:** 37-42.

Saul A: **Zooprophylaxis or zoopotentiation: the outcome of introducing animals on vector transmission is highly dependent on the mosquito mortality while searching**. *Malar J* 2003, **2:** 32.

Scholte EJ, Knols BG, Takken W: **Infection of the malaria mosquito *Anopheles gambiae* with the entomopathogenic fungus *Metarhizium anisopliae* reduces blood feeding and fecundity**. *J InvertebrPathol* 2006, **91:** 43-49.

Scholte EJ, N'gabi K, Kihonda J, Takken W, Paaijmans K, Abdulla S, Killeen GF, Knols BGJ: **An entomopathogenic fungus for control of adult malaria mosquitoes**. *Science* 2005, **308:** 1641-1642.

Scholte EJ, Njiru BN, Smallegange RC, Takken W, Knols BG: **Infection of malaria (*Anopheles gambiae s.s.*) and filariasis (*Culex quinquefasciatus*) vectors with the entomopathogenic fungus *Metarhizium anisopliae*.** *Malar J* 2003, **2:** 29.

Scholte EJ, Takken W, Knols BGJ: **Infection of adult *Aedes aegypti* and *Ae. albopictus* mosquitoes with entomopathogenic fungus *Metarhizium anisopliae*.** *Acta Trop* 2007, **102:** 151–158.

Scholte E-J, Takken W, Knols BGJ: **Pathogenicity of six East African entomopathogenic fungi to adult *Anopheles gambiae s.s.* (Diptera: Culicidae) mosquitoes.** *Proc Exp Appl Entomol NEV, Amsterdam* 2003, **14:** 25-29.

Scholte EJ: **The entomopathogenic fungus *Metarhizium anisopliae* for mosquito control: Impact on the adult stage of the African malaria vector *Anopheles gambiae* and filariasis vector *Culex quinquefasciatus.*** *PhD Thesis* 2004. Entomology Department, Wageningen University.

Schwartz A, Koella JC: **Melanization of *Plasmodium falciparum* and C-25 sephadex beads by field-caught *Anopheles gambiae* (Diptera: Culicidae) from southern Tanzania.** *J Med Entomol* 2002, 39**:** 84-88.

Scott TW, Chow E, Strickman D, Kittayapong P, Wirtz RA, Lorenz LH, Edman JD: **Blood-feeding patterns of *Aedes aegypti* (Diptera: Culicidae) collected in a rural Thai village.** *J Med Entomol* 1993, **30:** 922-927.

Scott TW, Naksathit A, Day JF, Kittayapong P, Edman JD: **A fitness advantage for Aedes aegypti and the viruses it transmits when females feed only on human blood.** *Am J Trop Med Hyg* 1997, **57:** 235-239.

Selman BJ, Said Dayer M, Hassan, M**: Pathogenicity of *Beauveria bassiana* (Bals.) Vuill. to the larvae of diamond back moth, *Plutella xylostella* L. (Lep. Plutellidae).** *J Appl Entomol* 1997, **121:** 47-49.

Service MW: **Community participation in vector-borne disease control.** *Ann Trop Med Parasitol* 1993, **87:** 223-234.

Sharma VP: **Community-based malaria control in India.** *Parasitol Today* 1987, **3:** 222–226.

Sharp BL, Ridl FC, Govender D, Kuklinski J, Kleinschmidt I: **Malaria vector control by indoor residual insecticide spraying on the tropical island of Bioko, Equatorial Guinea.** *Malar J* 2007, **6:** 52.

Shashi S, Gupta RBL, Yadava CPR: **Mass multiplication and formulation of entomopathogenic fungi and efficacy against white grubs**. *J Mycol Plant Pathol* 1999, **29:** 299-305.

Sandhu SS, Rajak RC, Sharma M: **Bioactivity of *Beauveria bassiana* and *Metarhizium anisopliae* as pathogens of *Culex tritaeniorhynchus* and *Aedes aegypti*: effect of instar, dosages and time.** *Indian J Microbiol 1993*, **33:** 191-194.

Shi W-B, Zhang L, Feng M-G: **Time-concentration-mortality responses of carmine spider mite (Acari: Tetranychidae) females to three hypocrealean fungi as biocontrol agents.** *Biological Contr* 2008, **46:** 495–501.

Shililu JI, Tewolde GM, Brantly E, Githure JI, Mbogo CM, Beier JC, Fusco R, Novak RJ: **Efficacy of *Bacillus thuringiensis israelensis*, *Bacillus sphaericus* and temephos for managing Anopheles larvae in Eritrea.** *J Am Mosq Control Assoc* 2003, **19:** 251-258.

Shimazu M: **Effects of temperature on growth of *Beauveria bassiana* F-263, a strain highly virulent to the Japanese pine sawyer, *Monochamus alternatus*, especially tolerance to high temperatures**. *Appl Entomol Zool* 2004, **39:** 469-475.

Silman RW, Bothast RJ, Schisler DA: **Production of *Colletotrichum truncatum* for use as a mycoherbicide: effect of culture, drying and storage on recovery and efficacy**. *Biotechnol Adv* 1993, **11:** 561-575.

Sivasankaran P, Eswaramoorthy S, David H: **Influence of temperature and relative humidity on the growth, sporulation and pathogenicity of *Beauveria bassiana*.** *J Biological Contr* 1998, **12:** 71-75.

Slininger PJ, Schisler DA, Kleinkopf G: **Combinations of dry rot antagonistic bacteria enhance biological control consistency in stored potatoes.** *Phytopathology* 2001, **91:** S83.

Smith A, Hudson JE: **A modification to an experimental hut to reduce mosquito eaves-egress.** WHO/Mal/72.775. In. *WHO* 1972, p. 6.

Smith T, Leuenberger R, Lengeler C: **Child mortality and malaria transmission intensity in Africa.** *Trends Parasitol* 2001, **17:** 145–149.

Snow RW, Eckert E, Teklehaimanot A: **Estimating the needs for artesunate based combination therapy for malaria case-management in Africa.** *Trends in Parasitol* 2005, **19:** 363-369.

Snow RW, Gilles HM: **"The Epidemiology of Malaria."** In *Bruce-Chwatt's Essential Malariology,* 4th ed., ed. DA. Warrell and H. M. Gilles, 2002, 85–106. London: Arnold Publishers.

Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI: **The global distribution of clinical episodes of *Plasmodium falciparum* Malaria.** *Science* 2005, **434:** 214-217.

Snow RW, Marsh K: **Malaria in Africa: progress and prospects in the decade since the Abuja declaration.** *Lancet* 2010, **376:** 137-139.

Soares GG, Marchal M, Ferron P: **Susceptibility of *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) larvae to *Metarhizium anisopliae* and *Metarhizium flavoviride* (Deuteromycotina: Hyphomycetes) at two different temperatures.** *Environ Entomol* 1983, **12:** 1886-1890.

Soper RS, Ward MG: **Production, formulation, and application of fungi for insect control. In "Biological Control in Crop Protection" (G. C. Papavizas, ed.),** *Allanheld & Osmun Publication. Co., Montclair*, NJ. 1981, 161-180.

Stevenson JC: **The use of fungi against adult malaria mosquitoes, PhD Thesis.** *London School of Hygiene and Tropical Medicine, London* 2008.

Stover-Muller D, Sauerborn J: **A commercial iron fertilizer increases the survival of *Fusarium oxysporum* f. sp. *orthoceras* propagules in a wheat flour-kaolin formulation.** *Biocontrol Sci Techn* 2007, **17:** 597-604.

Strasser H, Vey A, Butt TM: **Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the bioactive metabolites of *Metarhizium, Tolypocladium* and *Beauveria* species?** *Biocontrol Sci Technol* 2000, **10:** 717–735.

Styer LM, Carey JR, Wang JL, Scott TW: Mosquitoes do senesce: departure from the paradigm of constant mortality. *Am J Trop Med Hyg* 2007, **76:** 111-117.

Sun J, Fuxa JR, Henderson G: **Effects of virulence, sporulation, and temperature on *Metarhizium anisopliae* and *Beauveria bassiana* laboratory transmission in *Coptotermes formosanus*.** *J Invertebr Pathol* 2003, **84:**38-46.

Tadayyon A, Hill GA, Ingledew WM, Sokhonsanj S: **Contact-sorption drying of *Penicillium bilaii* in a fluidized bed dryer.** *J Chem Tech Biot* 1997, **68:** 277-282.

Takken W, Knols BGJ: **Malaria vector control: current and future strategies**. *Trends Parasitol* 2009, **25:** 101–104.

Talisuna AO, Bloland P, D'Allessandro U: **History, dynamics, and public health importance of malaria parasite resistance.** *Clin Microbiol Review* 2004, **17:** 235-254.

Talman AM, Domarle O, McKenzie FE, Ariey F, Robert V: **Gametocytogenesis: the puberty of *Plasmodium falciparum*.** *Malar J* 2004, **3:** 24-38.

Thomas MB, Jenkins NE: **Effects of temperature on growth of *Metarhizium flavoviridae* and virulence to the variegated grasshopper, *Zonocerus variegatus*.** *Mycol Res* 1997, **101:** 1469-1474.

Thomas MB, Read AF: **Can fungal biopesticides control malaria?** *Nature* 2007, **5:** 377-383.

Thomas MB, Watson EL, Valverde-Garcia P: **Mixed infections and insect-pathogen interactions.** *Ecology Letters* 2003, **6:** 183-188.

Thomas MB, Wood SN, Lomer CJ: **Biological control of locusts and grasshoppers using a fungal pathogen - the importance of secondary cycling**. *Proceedings of the Royal Society of London Series B-Biological Sciences* 1995, **259:** 265-270.

Tirados I, Costantini C, Gibson G, Torr SJ: **Blood-feeding behaviour of the malarial mosquito *Anopheles arabiensis*: implications for vector control.** *Med Vet Entomol* 2006, **20:** 425–437.

Uttam JR, Pandey ND, Verma RA, Singh DR: **Efficacy of different indigenous oils as grain protectant against *Sitophilus oryzae* on barley**. *Indian J Entomol* 2002, **64:** 447-450.

Utzinger J, Tozan Y, Singer B: **Efficacy and cost-effectiveness of environmental management for malaria control.** *Trop Med Int Health* 2001, **6:** 677–687.

Vandenburg JD, Ramos M, & Altre JA: **Dose –response and age- and temperature-related susceptibility of the diamondback moth (Lepidoptera: Plutellidae) to two isolates of *Beauveria bassiana* (Hyphomycetes: Moniliaceae).** *Environ Entomol* 1998, **27:** 1017–1021.

Varela A, Morales E: **Characterization of some *Beauveria bassiana* isolates and their virulence toward the coffee berry borer *Hypothenemus hampei***. *J Invertebr Pathol* 1996, **67:** 147–152.

Vatandoost H, Shamspour S, Abai MR: **Relative efficacy of different synthetic pyrethroids impregnated fabrics (ITNs) against *Anopheles stephensi* in Iran**. *Pak J Biol Sci* 2006, **9:** 503-506.

Walstad JD, Anderson RF, Stambaugh WJ: **Effects of environmental conditions on two species of muscardine fungi (*Beauveria bassiana* and *Metarhizium anisopliae*).** *J Invertebr Pathol* 1970, **16:** 221–226.

Wang C, Skrobek A, Butt TM: **Investigations on the destruxin production of the entomopathogenic fungus *Metarhizium anisopliae*.** *J Invertebr Pathol* 2004, **85:** 168-174.

Wang CS, Li ZZ, Butt TM: **Molecular studies of co-formulated strains of the entomopathogenic fungus, *Beauveria bassiana*.** *J Invertebr Pathol* 2002, **80:** 29-34.

Watson DW, Mullens BA, Petersen JJ: **Behavioral fever response of *Musca domestica* (Diptera: Muscidae) to infection by *Entomophora muscae* (Zygomycetes: Entomopthorales).** *J Invertebr Pathol* 1993, **61:** 10-16.

WHO 2001: **Report of a workshop on a framework for development and implementation of vector control interventions in the WHO African region Harare:** *WHO regional Office for Africa;* 2001.

WHO 2009: **World Malaria Report 2009**. Geneva: World Health Organization.

Winder RS, Wheeler JJ, Conder N, Otvos IS, Nevill R, Duan L: **Microencapsulation: A strategy for formulation of inoculum.** *Biocontrol Sci Techn* 2003, **13:** 155-169.

Winzeler EA: **Malaria research in the post-genomic era.** *Nature* 2008, **455:** 751-756.

Wosten HAB, de Vocht ML: **Hydrophobins, the fungal coat unravelled**. *Biochimica et Biophysica Acta* 2000, **1469:** 79–86.

Yadouleton AW, Padonou G, Asidi A, Moiroux N, Bio-Banganna S, Corbel V, N'guessan R, Gbenou D, Yacoubou I, Gazard K, Akogbeto MC: **Insecticide resistance status in *Anopheles gambiae* in southern Benin.** *Malar J* 2010, **9:** 83.

Yanagawa A, Shimizu S: **Resistance of the termite *Coptotermes formosanus* Shiraki to *Metarhizium anisopliae* due to grooming.** *BioControl* 2007, **52:** 72-85.

Yanagawa A, Yokohari F, Shimizu S: **The role of antennae in removing entomopathogenic fungi from cuticle of the termite, *Coptotermes formosanus*.** *J Insect Sci* 2009, **9:** 9pp.

Yanagawa A, Yokohari F, Shimizu S: **Defense mechanism of the termite, *Coptotermes formosanus* Shiraki, to entomopathogenic fungi.** *J Invertebr Pathol* 2008, **97:** 165-170.

Yeo H, Pell JK, Alderson PG, Clark SJ, Pye BJ: **Laboratory evaluation of temperature effects on the germination and growth of entomopathogenic fungi and on their pathogenicity to two aphid species.** *Pest Manag Sci* 2003, **59:** 156–165

Yoshida S, Shimada Y, Kondoh D, Kouzuma Y, Ghosh AK, Jacobs-Lorena M, Sinden RE: **Hemolytic C-Type lectin CEL-lll from sea cucumber expressed in transgenic mosquitoes impairs malaria parasite development.** *PLoS Pathog* 2007, **3:** e192.

Zhao S, Shamoun SF: **Effects of culture media, temperature, pH, and light on growth, sporulation, germination, and bioherbicidal efficacy of *Phoma exigua*, a potential biological control agent for salal (*Gaultheria shallon*)**. *Biological Sci Techn* 2006, **16:** 1043-1055.

Zhou G, Githeko AK, Minakawa N, Yan G: **Community-wide benefits of targeted indoor residual spray for malaria control in the western Kenya highland.** *Malar J* 2010, **9:** 67.

Zimmermann G: **Effect of high temperature and artificial sunlight on the viability of conidia of *Metarhizium anisopliae***. *J Invertebr Pathol* 1982, **40:** 36-40.

Zimmermann G: **Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii***. *Biocontrol Sci Technol* 2007b, **17:** 553 – 596.

Zimmermann G: **Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*.** *Biocontrol Sci Technol* 2007a, **17:** 879 – 920.

Zimmermann G: **The entomopathogenic fungus *Metarhizium anisopliae* and its potential bio-control agent**. *Pest Sci* 1993, **37:** 375-379.

Samenvatting

Vector bestrijding is een van de effectiefste manieren voor het bestrijden van vectorziekten zoals malaria. Het overkoepelende doel van deze strategie is om personen te beschermen tegen infectieuze beten van (malaria)muggen en, op bevolkingsniveau, de intensiteit van de transmissie van ziekteverwekkers te verlagen. Enkele landen, met name in laag-endemische gebieden, hebben onlangs een daling van het aantal malariapatienten met 50% gemeld over de afgelopen 10 jaar. Dit succes is bereikt door grootschalige toepassing van met insecticide-behandelde klamboes (ITNs), het bespuiten van huizen met residuele insecticiden (IRS) en het verstrekken van effectieve medicatie. Om vaart te houden in deze ontwikkelingen en effectieve malariabestrijding verder uit te breiden, zoals aanbevolen in het wereldwijde programma voor de bestrijding van malaria, moeten ITN en IRS gecomplementeerd worden met alternatieve bestrijdingsmethoden. De “long-lasting” ITNs en IRS zijn, indien als enige methode toegepast, onvoldoende om onderbreking van transmissie te bereiken in holo- en hyperendemische malariagebieden. Bovendien wordt de duurzaamheid van ITN en IRS bedreigd door de ontwikkeling van resistentie in de muggen. Wetenschappelijk onderzoek heeft aangetoond dat biologische bestrijding gebaseerd op entomopathogene schimmels de potentie heeft om bestaande vectorbestrijdingsmethoden te complementeren. Twee soorten entomopathogene schimmels, *Metarhizium anisopliae* en *Beauveria bassiana* hebben een bewezen potentie om volwassen malariamuggen te infecteren en doden.

Dit proefschrift beschrijft de resultaten van laboratoriumonderzoek aangevuld met kleinschalig veldonderzoek in Tanzania in een gebied dat hoog endemisch is voor malaria, en waar the malariamug *Anopheles gambiae* sensu lato in grote aantallen aanwezig is. Het centrale doel van het onderzoek was a) om formuleringen van entomopathogene schimmels te optimaliseren, b) om technieken te ontwikkelen die de toepassing van de schimmels om muggen in hun natuurlijke omgeving te infecteren bevordert en c) om het effect van de schimmels op het overleven van muggen en malaria transmissie te evalueren. Het gedrag van een aantal variabelen dat mogelijk de effectiviteit en persistentie van de schimmels kan beïnvloeden, is onderzocht bij de schimmelisolaten *Metarhizium anisopliae* ICIPE-30, *M. anisopliae* IP46 en *Beauveria bassiana* I93-825 toegepast op volwassen muggen van *An. gambiae*. Dit betrof a) een concentratie van conidia (1×10^{7} - 4×10^{10} conidia m^{-2}), b) de tijdsduur van blootstelling (15 min - 6 uur), c) de materialen waarop de schimmelsporen zijn aangebracht (polyester muggengaas, katoenen doek, gedroogde klei), d) leeftijd van de mug (2 - 12 d), e) de tijdsduur verlopen sinds een vorige bloedmaaltijd (3 - 72 uur) en f) het gedrag van de mug (afstoting door een formulering van conidia). Meng-formuleringen van *M. anisopliae* ICIPE-30 en *B. bassiana* I93-825 in verhoudingen van 4:1, 2:1 & 1:1 zijn ook onderzocht. *Anopheles gambiae s.s.* en *An. arabiensis* zijn blootgesteld aan *M. anisopliae* IP46 zodat de pathogeniteit van de schimmels op deze muggensoorten kon worden bepaald voordat deze werden gebruikt in het veld. De muggen werden blootgesteld aan schimmelsporen die aangebracht waren op papier in een cylindrische tube, behalve wanneer verschillende toepassingsmaterialen werden onderzocht. Bij het gelijktijdig onderzoek aan toepassingsmaterialen zijn stukken muggengaas en zwart katoenen doek aan elkaar gezet met behulp van Velcro zodat zij over een 3D metalen frame van 20 × 20 × 20 cm pasten; ook zijn houten panelen, afgesmeerd met een laagje klei, op een vergelijkbare manier geplaatst in muggenkooien van 20 × 20 × 20 cm. De laboratoriumexperimenten werden uitgevoerd met in het laboratorium gekweekte muggen op het Ifakara Health Institute, Ifakara, Tanzania. Daarna zijn veldexperimenten uitgevoerd in Lupiro (Ulanga district, Tanzania), een plattelandsdorpje 30 km ten zuiden van Ifakara. Hier zijn vijf verschillende methoden van schimmeltoepassing onderzocht in experimentele huizen, zo dat elke methode maximaal effectief was als de mug deze huizen binnenvloog (muggengaas in de dakopeningen; gordijnen voor de dakopeningen, barrières in de dakopeningen), bij het gastheer zoeken (stroken van doek rondom de klamboe) of tijdens het rusten (panelen van doek naast het bed).

De mate waarin de overleving van de mug afneemt was afhankelijk van de concentratie van de conidieen; 2×10^{10} conidia per m^2 was de optimale concentratie waarboven geen verdere afname in overleving werd vastgesteld. Meng-formuleringen van twee schimmelsoorten veroorzaakten geen synergistisch of additief effect betreffende een verlaagde overleving van de muggen. Blootstelling van muggen aan schimmels gedurende perioden van 15 – 30 min. was voldoende om een sterfte van 100% te bereiken binnen 14 d na contact met schimmelsporen. Een langere duur van blootstelling leidde niet tot een snellere dood. Conidia op papier aangebracht bleven tot 28 d na blootstelling infectieus, en dit effect bleek niet afhankelijk te zijn van de gebruikte concentratie van conidia. Muggen van de leeftijd tussen 2 en 12 d waren even gevoelig voor schimmelinfectie, maar ze waren meer gevoelig als ze niet gevoerd waren met bloed. Er was geen afstotend effect van schimmelsporen die in olie waren gesuspendeerd. *Metarhizium anisopliae* IP46 veroorzaakte sterfte in zowel *An. gambiae s.s*. als in *An. arabiensis*. Conidia waren meer effectief als ze aangebracht waren op panelen van gedroogde klei of katoenen doek vergeleken bij polyester muggengaas. Katoenen doeken en gedroogde klei lijken dus een goed substraat voor toepassing van schimmelsporen in het veld.

Twee manieren van het beschikbaar stellen van de sporen voor de mug, 1) barrières in de dakopeningen en 2) stroken van doek rondom de klamboe, bleken zeer effectief om muggen te kunnen infecteren met een entomopathogeen schimmel. Ongeveer 75% van de muggen die het huis binnenvlogen raakte geinfecteerd via een van beide applicatiemanieren. Daarentegen waren muggengaas in de dakopeningen, de katoenen gordijnen in die openingen en de panelen naast het bed niet effectief als infectiebron van muggen. Kennelijk raakte slechts een klein deel van de muggenpopulatie door deze toepassingen geinfecteerd. Een modelvoorspelling uitgevoerd met de gegevens van de twee effectieve infectiemethoden toont aan dat het risico op malariatransmissie met meer dan 75% verlaagd werd.

Geconcludeerd wordt dat de gegevens van het onderzoek aantonen dat met een geoptimalizeerde formulering van schimmelsporen en een gerichte toepassingstechniek, waarbij een zo hoog mogelijk percentage muggen geinfecteerd raakt, een groot aantal muggen binnenshuis met schimmels geinfecteerd kan worden zodat hun overleving sterk verkort wordt en, zo mogelijk, ook de overdracht van malaria sterk afneemt. Deze resultaten leveren basisgegevens op die relevant zijn voor het ontwerp en de uitvoering van grootschalige veldproeven om de voorspelde impact van schimmelinfecties onder reeele endemische situaties te kunnen valideren.

Aknowledgements

At the completion of my PhD thesis, I am very happy that this has been accomplished, having started along a long road to its goal many years ago. This would not have been possible, however, without the support from other people, who have all assisted me in achieving this goal.

I am delighted to acknowledge the immeasurable support from my promoters, Prof. Willem Takken and Prof. Marcel Dicke. I felt so lucky having the opportunity to work with, and learn from these world class scientists. In addition, I was also lucky to at different stages work with, and learn from several other world class scientists. These include Tanya L Russell, Gerry Killeen, Bart GJ Knols, Matthew Kirby, Christian Luz and Sander Koenraadt. I am abundantly grateful to them all for useful advices, criticisms recommendations and discussions. Through the mentorship of them all, my state of knowledge, independence and rigorousness as a scientist within my field has improved quite remarkably. Their collective advice has taught me to become a scientist in my own right, which after all is the purpose of PHD training

I thank the administration of Ifakara Health Institute (IHI) for creating a conducive and supportive environment for executing my research work. I am grateful to my colleagues at IHI, Issa Lyimo, Fredros Okumu and Dickson Lwetoijera for their advices and recommendations which were very useful in fine-tuning my experiments.

I extend my hearty felt gratitude to technicians whose hard work and commitment enabled me to successfully address the research questions I intended to answer. May I recognize the following people for playing such an important role: Monika Mpingwa, Emmanuel Simfukwe, Novatus Lipindi, Edgar Mbeyela, Peter Pazia and Hassan Mtambala.

Back to Wageningen University; I am indebted to thank my fellow PhD students, Tullu Bukhari, Marit Farenhorst, Mgeni Jumbe and Frank van Breukelen for their constant support and encouragement. Many thanks also go to all members of the vector control group for their useful discussions. Mrs Margriet Huisman deserves my hearty felt gratitude for her outstanding administrative support, and showing me around when I first arrived at Wageningen for writing my thesis.

My PHD study and the accompanying research work were funded by the Adessium Foundation. I am so grateful to the Foundation for the offer.

Many thanks also go to my dad, Laurent M. Mnyone and my mum, Leah T. Mfanga for their very credible and tireless support throughout the process of my education. Indeed, the two laid the foundation of my education. Mum was the best English language primary teacher I ever had. Being good at that has had, and will ever have, a considerable contribution on what I am today. Dear mum, remember you had to forgo the intended use of the 700,000/= Tshs you borrowed from the Bank and used it to pay for my schools fees when a financial sponsor for my MSc degree was lacking. Because of that, you and dad had to survive with very little money for so long. That support made a very big break through in developing my career; and now then I have gone even far and finished my PhD. Dear dad, should I witness that you were all right when you always insisted that I should balance between playing “soka” and schooling. The efforts both of you invested in the little Ladslaus (by then, in the past 30 years) have yielded a dependable scientist you can be proud of.

Thanks also to my lovely brother Michael and sisters Lillian and Beatrice. Many thanks for your love, care and loyalty. I will always be proud of you for enabling me to meet my dreams.

Finally, very special thanks go to my lovely and everlasting wife Catherine G. Mkindi. Dear wife, you always gave me support and encouragement when I needed them most. You outstandingly met all the family responsibilities in my absence without any complaint.

To my lovely children Mbike, Nambua and little Nankondo; although I was away from home for so long, you still had much trust in your dad and always welcomed me home with lovely hugs. Each of you, remind me of the copy of my PhD book when you are older. This is the evidence of the hard work your dad has been doing; and hopefully it will form a good miniature for your future go through. Remember “The doors we open and close each day decide the lives we live” ***Flora Whittemore***.

Curriculum Vitae (CV)

On 31st October 1976, Ladslaus Laurent Mnyone was born in Kilimanjaro region, northern Tanzania. He pursued his primary education at Ishinde Primary School, Kilimanjaro, Tanzania. After completion, he joined Shighatini Secondary School, at Mwanga district, Kilimanjaro, and obtained a certificate in ordinary secondary education. Then, he joined Mkwawa High School, Iringa region, Tanzania where he obtained a certificate in advanced secondary education. Afterwards, he joined Sokoine University of Agriculture (SUA), Morogoro Tanzania and studied for a degree in Veterinary Medicine (BVM) from August 1999 to June 2004. In September 2004, Ladslaus was granted an opportunity at the same university to study for a master degree in Preventive Veterinary Medicine (MPVM), which he completed successfully. He received a convocation price on the occasion of obtaining his MSc degree in November 2005. At the MSc level, Ladslaus conducted a study on “Improvement of growth rates in calves through strategic control of tick-borne diseases.” Here is where he started to develop interest and was persuaded to become a vector control biologist. In November 2006, Ladslaus secured an academic post at Sokoine University as an Assistant Lecturer/Research Fellow in the discipline of Entomology. In that post, he was actively involved in research activities and training of undergraduate students. His research activities hinged on the control of human and livestock disease vectors. In 2007, he was seconded from Sokoine University to the Ifakara Health Research and Development Centre (IHRDC), now Ifakara Health Institute (IHI); and later registered for a PhD at Wageningen University (WU), The Netherlands. As a PhD student his main role has been to optimize formulation and delivery technology of entomopathogenic fungi for malaria vectors control. Ladslaus successfully completed the work and submitted his PhD thesis to Wageningen University in July 2010. During the period of 3 years he has enormously reinforced his understanding on vector control majoring on the use of biological control agents. Ladslaus is eager to continue working in this field and become a part of the global efforts to develop and possibly upscale entomopathogenic fungi/other biological control agents against human and livestock disease vectors.

List of publications

Mnyone LL, Kirby MJ, Lwetoijera DW, Mpingwa MW, Simfukwe ET, Knols BGJ, Takken W, Russell TL: Tools for delivering entomopathogenic fungi to malaria mosquitoes: effects of delivery surfaces on fungal efficacy and persistence. *Malaria Journal* 2010, **9:** 246.

Mnyone LL, Koenraadt CJM, Lyimo IN, Mpingwa MW, Takken W, Russell TL: Anopheline and culicine mosquitoes are not repelled by surfaces treated with the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*. *Parasites & Vectors* 2010, **3:** 80.

Mnyone LL, Kirby MJ, Mpingwa MW, Lwetoijera DW, Knols Bart GJ, Takken Willem, Koenraadt Constantianus JM, Russell Tanya L: Infection of *Anopheles gambiae* mosquitoes with entomopathogenic fungi: effect of host age and blood-feeding status. *Parasitology Research* 2010, DOI 10.1007/s00436-010-2064-y.

Mnyone LL, Kirby MJ, Lwetoijera DW, Mpingwa MW, Knols BGJ, Takken W, Russell TL: Infection of the malaria mosquito, *Anopheles gambiae* s.s., with two species of entomopathogenic fungi: effects of concentration, co-formulation, exposure time and persistence. *Malaria Journal* 2009, **8:** 309.

Mnyone LL, Russell TL, Lyimo IN, Lwetoijera DW, Kirby MJ, Luz C: First report of *Metarhizium anisopliae* IP 46 pathogenicity in adult *Anopheles gambiae s.s.* and *An. arabiensis* (Diptera; Culicidae). *Parasites & Vectors*, **2:** 59.

Luz C, **Mnyone LL,** Sangusangu R, Lyimo IN, Rocha LFN, Humber RA, Russell TL**:** A new resting trap to sample fungus-infected mosquitoes, and the pathogenicity of *Lecanicillium muscarium* to culicid adults. *Acta Tropica* 2010, **116:** 105-107.

Lwetoijera DW, Sumaye RD, Madumla E, Kavishe DR, **Mnyone LL,** Russell TL, Okumu FO: An extra domiciliary method for delivering entomopathogenic fungi against wild malaria vectors. *Parasites & Vectors* 2010, **3:** 18.

Mazigo HD, Okumu FO, Kweka E, **Mnyone LL:** Retrospective analysis of suspected rabies cases reported at Bugando referral hospital, Mwanza, Tanzania. *Global Infectious Diseases* 2010, **2:** (3).

Mahande AM, Mwang'onde BJ, Msangi S, Kimaro E, **Mnyone LL,** Mazigo HD, Mahande MJ, Kweka EJ: Is aging raw cattle urine efficient for sampling *Anopheles arabiensis* Patton? *BMC Infectious Diseases* 2010, **10:** 172.

Mazigo HD, Waihenya R, Lwambo NJS, **Mnyone LL**, Mahande AM, Seni J, Zinga M, Kapesa A, Kweka EJ, Mshana SE, Heukelbach J, Mkoji GM: Co-infections with *Plasmodium falciparum*, *Schistosoma mansoni* and intestinal helminthes among schoolchildren in endemic areas of northwestern Tanzania. *Parasite & Vectors* 2010, **3:** 44.

Mbassa GK, Mgongo FOK., Mellau LSB, **Ladslaus L,** Silayo RS, Kimbita EN, Hayghaimo AA, Mlangwa JED, Mbiha ER: Strategic control of ticks with synthetic pyrethroids in *Theileria parva* endemic areas in Tanzania increases calf growth and survival rates. *Tanzania Veterinary Journal* 2008, **25:** (2).

Mazigo HD, Obasy E, Mauka W, Manyiri P, Zinga M, Kweka EJ, **Mnyone LL,** Heukelbach J: Knowledge, attitudes, and practices about malaria and its control in rural northwest Tanzania. *Malaria Research and Treatment* 2010, doi:10.4061/2010/794261.

Mazigo HD, Ambrose EE, Zinga M, Bahemana E, **Mnyone LL,** Kweka EJ, Heukelbach J: Prevalence of intestinal parasitic infections among patients attending Bugando Medical Centre in Mwanza, north-western Tanzania: a retrospective study. *Tanzania Journal of Health Research* 2010, 12 (3).

Mnyone LL, Mbassa GK, Mellau LSB, Mgongo FOK, Silayo RS, Kimbita EN, Gwakisa PS, Mbiha ER, Mlangwa JED, Hayghaimo HH: Improvement of growth rates in calves through strategic control of tick borne diseases in selected herds of Kambala village, Tanzania. Proceedings of the Tanzania Scientific Conference, Arusha Tanzania; December 2005.

To be submitted

Mnyone LL, Koenraadt CJM, Takken W and Kirby, MJ: Environmental factors affecting the use of entomopathogenic fungi as bio-control agents for mosquitoes: A review.

Mnyone LL, Lyimo IN**,** Lwetoijera DW, Mpingwa MW, Nchimbi N, Hancock P, Russell TL, Kirby MJ, Takken W, Koenraadt CJM: Exploiting the behaviour of wild malaria vectors to achieve high infection with entomopathogenic fungus.

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (5 ECTS)

- The potential of entomophathogenic fungi as biocontrol agents for mosquitoes

Writing of project proposal (7 ECTS)

- Optimization of formulation and delivery technology of entomopathogenic fungi for malaria vector control

Post-graduate courses (4.2 ECTS)

- Introduction to R for statistical analysis (2009)
- Bayesian statistics (2009)
- Design of experiments (2009)
- Consumer resource interaction: danger, disease and density dependency (2009)

Laboratory training and working visits (3 ECTS)

- Bioassay techniques to evaluate entomopathogenic fungi; University of Edinburgh, UK (2007)

Invited review of (unpublished) journal (1 ECTS)

- Parasites and vectors: possible control of a filariasis vector by fungus *Fusarium oxysporum* (2010)

Deficiency, refresh, brush-up courses (1.5 ECTS)

- Basic statistics (2009)

Competence strengthening / skills courses (2.7 ECTS)

- Techniques for writing and presenting a scientific paper (2009)
- Project and time management (2010)

PE&RC Annual meetings, seminars and the PE&RC weekend (0.9 ECTS)

- PE&RC Weekend (2010)

Discussion groups / local seminars / other scientific meetings (10.5 ECTS)

- The Walter Reed Project/GEIS Training Course on the Identification of Mosquitoes from Africa; Kisumu, Kenya (2007)
- Local seminars and discussion groups; Ifakara Health Institute (IHI) (2007-1010)

- Weekly vector group presentations and discussions; Entomology, Wageningen (2008-2010)
- Seminar in tropical and travel medicine; Tanzanian Training Centre for International Health (TTCIH); Ifakara, Tanzania (2009)

International symposia, workshops and conferences (9 ECTS)

- Annual Consortium meeting for the Fungus project (2008-2009)
- Multilateral Malaria Initiative meeting; Nairobi, Kenya (2009)
- Annual Joint Scientific Conference of the National Institute for Medical Research; Tanzania (2010)

Lecturing / supervision of practicals / tutorials (1.2 ECTS)

- Bioassay techniques of insecticides against flea vectors of Bubonic plague; 4 days (2009)

Supervision of 1 MSc student; 3 months (3 ECTS)

- Application of entomopathogenic fungus, *Metarhizium anisopliae* IP 46 against African malaria vectors using extra-domicilliary odour baited stations

Funding for the project: This project was funded by Adessium Foundation, The Netherlands.

Photo credits: Mycelia growing across mosquito wing ×1000, background of the cover page (J. Stevenson)

Cover design: Hans Smid

Thesis layout: Ladslaus L. Mnyone

Printed by: Ipskamp Drukkers, The Netherlands