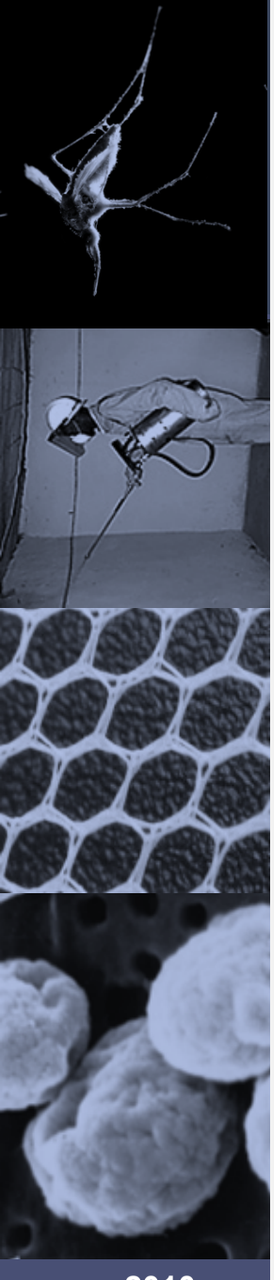

Integrating Fungal Entomopathogens in Malaria Vector Control

Marit Farenhorst

2010

Marit Farenhorst

Integrating Fungal Entomopathogens in Malaria Vector Control

Propositions

1. Fungal entomopathogens provide a necessary alternative control option against insecticide-resistant mosquitoes - this thesis.
2. Biological and chemical insecticides are not mutually exclusive but could be used as complementary technologies to improve the efficiency and sustainability of malaria interventions - this thesis.
3. Only low-tech, low-cost malaria vector control tools that are easy to distribute and use, will be successful in tropical African settings.
4. Albert Einstein's quote: "If you do what you did, you get what you got" is applicable to the current approach in malaria control, where solutions are continually sought in chemical tools in spite of the risk of resistance development.
5. We already have enough knowledge and tools to successfully eliminate malaria.
6. Tomorrow's research question should not hamper the implementation of today's knowledge.
7. Ambitious women do not merely seek to be equal with men.

Propositions accompanying the PhD thesis: "Integrating fungal entomopathogens in malaria vector control", by Marit Farenhorst (Wageningen University, 13th of December, 2010)

Integrating fungal entomopathogens in malaria vector control

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

Integrating fungal entomopathogens in malaria vector control

Marit Farenhorst

Thesis
submittted 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 Monday 13 December 2010
at 4 p.m. in the Aula.

Marit Farenhorst
Integrating fungal entomopathogens in malaria vector control
129 pages.

Thesis Wageningen University, Wageningen, NL (2010)
With references, with summaries in Dutch and English

ISBN 978-90-8585-803-4

Knowing is not enough;
we must apply.
Willing is not enough;
we must do.

– Johann Wolfgang von Goethe

Contents

Abstract

Widespread and long-term pesticide use has caused a selection and spread of resistance in malaria mosquito populations, which endangers the effectiveness of contemporary malaria control strategies that are based on chemical insecticides. The fungal entomopathogens *Metarhizium anisopliae* and *Beauveria bassiana* show potential as alternative and more sustainable malaria vector control agents. These hyphomycetes can effectively infect anophelines and potentially reduce malaria transmission by killing the mosquitoes within several days before they can transmit malaria parasites.

The aim of this thesis was to evaluate the potential of fungal entomopathogens for integration in chemical-based malaria interventions. Its objectives were to evaluate fungal spore application methods, to develop novel field delivery tools with potential for integrated use, to measure fungal efficacy against insecticide-resistant anophelines and to test the compatibility of fungi and chemical insecticides.

The first part of the work (Chapter 3-5) focused on evaluating fungal spore application methods and developing novel delivery systems that could potentially be effective in tropical field settings and integrated into existing malaria control strategies. Spraying, dipping and coating were effective methods for applying an infective layer of fungal spores on mosquito resting surfaces. A coating method, involving the application of uniform spore layers on papers, was developed to enable accurate laboratory evaluations, and a rotating spray apparatus to standardize the application of oil suspensions inside clay pots. The combination of formulation and substrate was shown to have a high impact on spore infectivity, with viscous suspensions being only effective on porous susbstrates. Spore application dose, exposure time and type of mosquito contact were key factors of fungal virulence, as they influenced the number of spores effectively picked up by a resting mosquito.

Two novel delivery methods were developed and tested in the laboratory. Clay pots showed potential for use as indoor and outdoor point-source objects to target resting mosquitoes with fungal spores. Oil-based *Metarhizium* suspensions were effective in infecting and killing mosquitoes after spray application inside clay pots and did not affect their attractiveness to resting male and female anophelines. Fungus-impregnated netting showed potential for use as house screens to target host-seeking mosquitoes. Spores of *Metarhizium* and *Beauveria* were most effective applied by spraying nets with evaporative suspensions. Fungi were infective on small- and large-meshed polyester and cotton nets, with spores being more viable on cotton. These two fungus delivery systems offer multiple deployment options and could potentially be used complementary to chemical-based malaria control measures such as insecticide-treated bednets (ITNs) or indoor residual spraying (IRS).

The second part of the work (Chapter 6 & 7) focused on evaluating the efficacy of fungi against insecticide-resistant *Anopheles* mosquitoes and their compatibility with public health insecticides. *Metarhizium* and *Beauveria* were highly effective against a diverse suite of insecticide-resistant *Anopheles* mosquitoes. Four metabolically resistant anopheline strains were equally susceptible to *B. bassiana* infection as their baseline counterparts. Both fungi were also highly effective in killing a laboratory strain and field population of West African *An. gambiae s.s.* with genetically conferred knockdown resistance (*kdr*) to public health insecticides.

Moreover, fungi and insecticides were highly compatible and enhanced each other's efficacy. Fungal infection increased the sensitivity of resistant mosquitoes to the neurotoxic insecticides permethrin and DDT. Fungus-infected mosquitoes with metabolic resistance mechanisms showed a significant increase in mortality after insecticide exposure compared with uninfected control mosquitoes. Reciprocally, permethrin increased subsequent fungus-induced mortality rates in a laboratory and field population of *kdr*-resistant *An gambiae*. Several combinations of insecticide and fungus were shown to induce synergistic effects on mosquito survival. Synergy was highest after simultaneous co-exposure to both agents. These findings suggest that integrated control tools that induce contact to fungi and insecticides within a single feeding episode would have the highest impact on mosquito survival and may enable control at more moderate levels of coverage. Moreover, the synergistic and resistance breaking properties of fungi show potential for augmenting current malaria interventions and managing the further spread of insecticide resistance.

Several factors still remain to be optimized before fungus-based malaria mosquito control can be realised. Cost-effective field deployment will require the development of high quality, low cost mass-production of mosquito-pathogenic fungi, persistent formulations and efficient delivery systems. The laboratory studies in this thesis provide useful knowledge and tools for future implementation research on these novel biological vector control agents. The potential field delivery systems that were created will, however, still need to be further evaluated in field settings under realistic environmental conditions.

In this thesis it was, for the first time, shown that fungi are effective against insecticide-resistant malaria vectors and induce the highest impact on mosquito survival when used in combination with chemical insecticides. These findings make a compelling case for viewing novel fungus-based and existing chemical-based control measures not as mutually exclusive, but as complementary interventions that would reach the greatest malaria control benefit once successfully integrated.

1 Introduction

1.1 Malaria

Malaria is a worldwide distributed vector-borne disease that is endemic in tropical and subtropical regions. The disease causes an estimated 300 million acute illnesses and more than one million deaths annually (WHO, 2005), of which the majority occur in Africa and affect young children and pregnant women (WHO, 2009). Malaria is considered a major public health challenge that undermines the social and economic development of developing countries (Sachs and Malaney, 2002; WHO, 2003).

The disease is caused by single-celled parasites of the genus *Plasmodium*. The four main species that are parasitic to humans are *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae* (Weller, 2003). Malaria parasites have a complicated life cycle with several different life stages. They are transmitted through the bite of female *Anopheles* mosquitoes, upon which they enter the human bloodstream, infect liver cells and subsequently multiply in red blood cells (Sinden, 1983). In this last stage, large numbers of parasites are produced and released into the blood stream where they continuously infect and destroy other red blood cells in a cyclic manner, which causes the typical periodic fever and anaemia in malaria patients. *P. falciparum* is considered the most dangerous parasite as it can cause severe disease and fatal complications, including liver failure, cerebral disease and coma (Mackintosh *et al.*, 2004).

1.1.1 Malaria transmission

Plasmodium parasites need mosquitoes to complete their life cycle and to infect new human hosts. Human malaria is transmitted only by female mosquitoes of the genus *Anopheles* (Figure 1.1), of which several species prefer to feed on humans (Constantini *et al.*, 1999). *Anopheles gambiae* and *An. funestus* are two well-known species found

throughout sub-Saharan Africa that can efficiently transmit human malaria (Coetzee *et al.*, 2000). The life cycle of an *Anopheles* mosquito starts in aquatic stages, namely egg, larvae and pupae, after which the flying adult mosquito emerges.

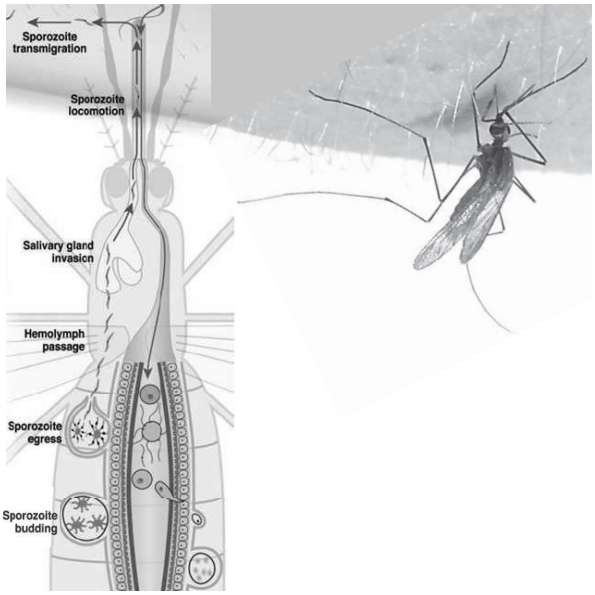

Figure 1.1: *An Anopheles female taking a blood meal (right), courtesy D. Knorre, and a schematic picture of malaria parasite development and movement inside the mosquito (left) (from Matuschewski, 2006).*

All newly emerged mosquitoes are free from malaria parasites, since there is no vertical transmission from adult to egg (Alavi *et al.*, 2003). Only when a mosquito feeds on a malaria-infected human, she can get infected with the parasites and become a vector of this disease. *Plasmodium* parasites undergo sexual reproduction inside the mosquito and need to develop in the insect for several days, generally 10-14 days (Figure 1.1) (Matuschewski, 2006). Because only a small proportion of older female mosquitoes is capable of transmitting malaria, malaria transmission can be blocked by shortening the lifespan of mosquitoes (Coluzzi, 1992).

1.2 Malaria control

Malaria prevention relies primarily on the use of anti-malarial drugs and interventions that block transmission by the mosquito vector. Development of vaccines is underway, although so far none provide full and life-long protection and it may take decades before these are commercially available (Graves and Gelband, 2006). The effectiveness of malaria treatments is threatened by increasing levels of anti-malarial drug resistance in *Plasmodium* parasites (Schellenberg *et al.*, 2006), which was recently also found to occur against drug combination treatments (Denis *et al.*, 2006). In this light, there is an increasing need for other, effective malaria interventions.

1.2.1 Vector control

Malaria vector control focuses on disease prevention through reducing vector–human contact and vector population density and survival. There are several control methods available against malaria mosquitoes, including the use of larvicides (Fillinger *et al.*, 2003), environmental management (Keiser *et al.*, 2005), house screening (Lindsay *et al.*, 2003), bednets (Kilian *et al.*, 2008) and indoor spraying of insecticides (Roberts *et al.*, 2000). Epidemiological models predict that the most effective way to reduce malaria transmission is to target adult female mosquitoes (Macdonald, 1957). Contemporary interventions, therefore, focus on targeting adult mosquitoes through the use of fast-killing chemicals on bednets or in indoor residual spraying (IRS) (Takken and Knols, 2009). Insecticide-treated nets (ITNs) and IRS have been successful in reducing malaria cases and deaths in several countries (WHO, 2009).

There are four classes of insecticide approved for use in public health interventions, of which the two most important are DDT (dichlorodiphenyltrichloroethane) and pyrethroids (Nauen, 2007). These neurotoxic insecticides act rapidly and cause mosquito paralysis and death by blocking neuronal activity in the insect's nerve membranes (Burt and Goodchild, 1974). DDT was the first synthetic organochlorine insecticide to be commercialized and, despite environmental and health risks (Rogan and Chen, 2005), is still extensively used in malaria eradication campaigns especially for IRS (Mandavilli, 2006; Rehwagen, 2006; Knols *et al.*, 2010). Pyrethroids are the only insecticides safe enough to be applied on bednets (ITNs), and their use in malaria control has recently scaled up tremendously (Zaim *et al.*, 2000).

The effectiveness and sustainability of insecticide-based malaria interventions relies on the continuing susceptibility of *Anopheles* mosquitoes to the available chemicals. Widespread and long-term use of insecticides in public health and agriculture has, however, caused a selection and spread of resistance in mosquito populations (Corbel *et al.*, 2007; Nauen, 2007; Protopopoff *et al.*, 2008).

1.2.2 Insecticide resistance

Several studies show high levels of insecticide resistance in various parts of Africa. Increasing incidences of resistance have been reported in the major African malaria vector species *Anopheles gambiae s.s.* (Diabate *et al.*, 2004; Tia *et al.*, 2006; Yadouleton *et al.*, 2010), *An. funestus* (Hargreaves *et al.*, 2000; Brooke *et al.*, 2001) and *An. arabiensis* (Hargreaves *et al.*, 2003; Abdalla *et al.*, 2007; Balkew *et al.*, 2010),

Mosquitoes can become resistant to DDT and pyrethroids through a point-mutation in the voltage-gated sodium channel gene, which disables insecticide attachment to these nerve membrane sites (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000). This so-called target-site resistance also prevents mosquito paralysis and is therefore known as knockdown resistance (*kdr*) (Hemingway *et al.*, 2004). Additionally, resistance can be caused by enzymes that metabolically degrade insecticides. Mosquitoes with elevated levels of monooxygenases, esterases or glutathione S-transferases have shown to be resistant to insecticides (Vulule *et al.*, 1999; Amenya *et al.*, 2008; Ranson *et al.*, 2001). It is not uncommon for mosquitoes to exhibit a combination of resistance mechanisms (Figure 1.2), with both target-site and metabolic resistance playing a role (Corbel *et al.*, 2007; Donnelly *et al.*, 2009).

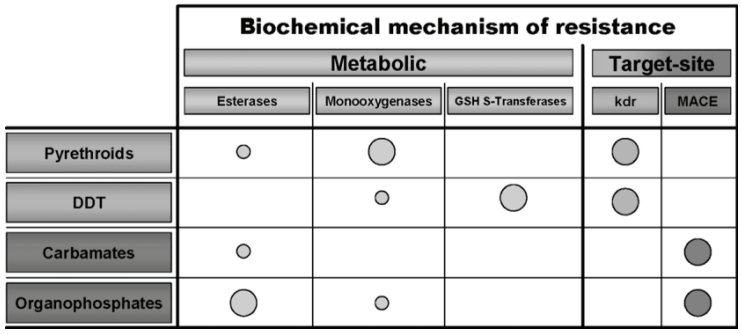

Figure 1.2: *Mechanisms of mosquito resistance to the four classes of WHO approved public health insecticides, with large spots indicating the most important mechanisms (from Nauen, 2007)*

Insecticide resistance in anopheline populations has been shown to severely reduce the effectiveness of malaria control strategies in several parts of Africa (Hargreaves *et al.*, 2000; N'Guessan *et al.*, 2007b; Sharp *et al.*, 2007; Ranson *et al.*, 2009), and is expected to be even more problematic in the future due to the focus on and scaling up of chemical control with IRS and ITNs (Brogdon and McAllister, 1998; Nauen, 2007).

Approaches to manage insecticide resistance include deployment of different insecticides in rotations or mosaics and the development of novel insecticides (Hemingway *et al.*, 2006; Coleman and Hemingway, 2007; Kelly-Hope *et al.*, 2008; Pennetier *et al.*, 2008). However, practical options are few, as there is cross-resistance between the currently approved insecticides and no new public health insecticide has been introduced in the last thirty years (Hemingway and Ranson, 2000; Zaim and Guillet, 2002; Nauen, 2007; Kelly-Hope *et al.*, 2008).

1.2.3 Sustainable vector control

Chemical insecticides are selected on a rapid mode of action; killing insects within a day following exposure (Wright, 1971; Hemingway *et al.*, 2006). By killing mosquitoes early in life, insecticides exert a high selection pressure on the formation and spread of resistance alleles in mosquito populations (Read *et al.*, 2009). Any novel fast-killing insecticide is thus at risk to eventually meet the same fate as existing ones; loss of activity due to resistance. This implies that alternative agents with different modes of action are needed (Read *et al.*, 2009; Knols *et al.*, 2010).

Since *Plasmodium* parasites need 10-14 days to develop inside the mosquito before they can be transmitted to another human, malaria mosquitoes are basically harmless in their first two weeks of life. To prevent malaria transmission, there is thus no need to kill young mosquitoes but only the older, infectious females (Thomas and Read, 2007; Read *et al.*, 2009; Knols *et al.*, 2010). Control agents that shorten the female adult life span with just a few days would still have high impact on malaria transmission, whilst limiting the risk of resistance development (Hancock *et al.*, 2009; Read *et al.*, 2009). Late-life acting agents could, therefore, enable more sustainable malaria vector control (Read *et al.*, 2009; Knols *et al.*, 2010) and slow-killing fungal entomopathogens have been suggested as suitable candidates for this novel approach (Thomas and Read, 2007; Hancock *et al.*, 2009; Read *et al.*, 2009).

1.3 Entomopathogenic fungi

Entomopathogenic fungi comprise approximately 700 species of Zygomycetes, Ascomycetes and Deuteromycetes (Hajek and St. Leger, 1994). Within the group of Deuteromycetes, fungi belonging to the Hyphomycetes have simple, non-sexual life cycles and are typically opportunistic pathogens with a broad insect host range (Goettel and Inglis, 1997). *Metarhizium anisopliae* and *Beauveria bassiana* are two of the most widely used hyphomycete species for insect pest control. They are ubiquitous worldwide and comprise a large number of different strains and isolates of different

geographical origin and host specificity (Roberts and St. Leger, 2004; Goettel and Inglis, 1997). Under natural conditions, *Metarhizium* and *Beauveria* are found in the soil where moist conditions allow filamentous growth and the production of infectious spores, called conidia, which infect soil-dwelling insects upon contact.

1.3.1 Mode of infection

Metarhizium and *Beauveria* spores are small (2-6 μm) hydrophobic propagules that can attach to the insect cuticle through electrostatic interactions (Boucias *et al.*, 1988; Sosa-Gomez *et al.*, 1997). Under suitable (moist) conditions they can germinate and produce germ tubes that penetrate the insect cuticle (Figure 1.3) using mechanical pressure and cuticle-degrading enzymes (Pekrul and Grula, 1979; Smith and Grula, 1981; Pedrini *et al.*, 2007). The fungus can then enter the haemocoel (Figure 1.3) by evading the insect's humoral immune response, which usually requires sufficient numbers of penetrating spores (Chouvenc *et al.*, 2009). Fungal hyphae will start to slowly grow, depleting nutrients, destroying mosquito cells, and eventually killing the insect (Gillespie and Claydon, 1989). This infection process takes several days, with the overall time to death depending mostly on fungal dose and virulence of the fungal isolate (Goettel and Inglis, 1997). When humidity is high enough, the fungus can grow out of the dead insect and produce conidia that can passively infect new insects.

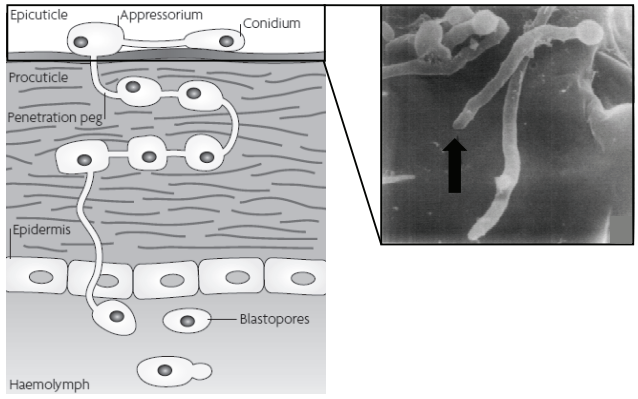

Figure 1.3: *A schematic overview of the fungal infection process (from Thomas & Read 2007), with the inset showing a scanning electron micrograph of a germinated B. bassiana spore penetrating (black arrow) an earworm cuticle (from Pekrul & Grula, 1979).*

1.3.2 Fungi for insect control

Fungi can be deployed against pest insects outside the soil environment through so-called inundative augmentation, which involves applying large amounts of spores for short-term insect control (Shah and Pell, 2003). Hyphomycetous conidia are relatively easy to isolate and mass-produce on artificial culture media and hence suitable for biological control (Goettel and Inglis, 1997). *Metarhizium* and *Beauveria* spores have been successfully used in pesticide formulations against insect pests such as whiteflies, aphids, thrips, termites, locusts and beetles (Zimmermann, 1993; Devi and Rao, 2006; Feng *et al.*, 1994; Thomas and Blanford, 1998). The relative instability of fungal conidia compared to chemicals can, however, pose a practicle obstacle to successful deployment. Fungal activity is, for instance, strongly influenced by humidity and temperature (Fargues and Luz, 2000; Thomas, 1997), and UV-light has a negative impact on spore viability (Fernandes *et al.*, 2007; Inglis *et al.*, 1995). Technological advances have, however, made it possible to overcome these hurdles.

Due to the increasing global interest in reducing environmental pollution with chemical pesticides, there have been several promising developments in fungus-based insect control, particularly since the 1990s. Molecular techniques have enabled the identification of isolates and virulence factors (Ansari *et al.*, 2004; St. Leger *et al.*, 1996). Advances in spore formulations have improved fungal effectiveness under low humidity conditions and UV exposure (Kassa *et al.*, 2004; de Faria and Wraight, 2007; Alves *et al.*, 1998) and increased potential deployment options (Bateman and Alves, 2000). The development of solid-state mass-production systems has made large spore quantities available for field trials (Jenkins *et al.*, 1998; Feng *et al.*, 1994; Ypsilos and Magan, 2005). Advances in quality control, such as optimizations on the substrate, incubation temperature, harvest time and storage conditions (Jenkins and Grzywacz, 2000), have enabled the production of fungus products with standardized quality (Roberts and St. Leger, 2004).

1.3.3 Commercial developments

A recent review showed that there are approximately 129 fungus-based insect control products commercially available, of which the majority is based on *Beauveria* or *Metarhizium* conidia (de Faria and Wraight, 2007). Mycotrol, for example, is a *Beauveria*-based mycoinsecticide that is commercially produced in the USA. Mycotrol formulations can be used on crops for control of grasshoppers, whiteflies, thrips and aphids, and conidia can remain infective for 12 months when stored at 25°C (de Faria and Wraight, 2007). GreenMuscle is a well-known mycoinsecticide that is commercially produced in Africa and contains spores of *M. anisopliae var. acridum*, which

are selectively pathogenic to locusts and grasshoppers. Green Muscle was developed during a 12 year research programme called LUBILOSA (Thomas, 2000), in which substantial advances in mass-production, formulation and application of *Metarhizium* were made (Jenkins *et al.*, 1998; Kassa *et al.*, 2004; Lomer *et al.*, 2001). The product can be sprayed with ultra-low-volume sprayers (Bateman and Alves, 2000), achieve 70-90% effective coverage in the field (Lomer *et al.*, 2001) and can remain infective for $>$12 months when stored at 30°C (Moore *et al.*, 1996; Jenkins *et al.*, 1998).

More than 100 fungus-based insect control products have been registered for commercial use at the USA Environmental Protection Agency since 1995 and undergone the required stringent environmental safety tests (de Faria and Wraight, 2007; Shah and Pell, 2003). Mycotrol, for instance, was fully registered after the product was shown to induce only low, acceptable risks for non-target insects (Roberts and St. Leger, 2004). Green Muscle showed no detrimental effects on non-target organisms (Lomer *et al.*, 2001) and is now registered and recommended for locust and grasshopper control by the United Nations. These and several other environmental risk studies demonstrate that fungal biopesticides are relatively safe for the environment, especially when compared to chemcial pesticides (see Zimmermann 2007a,b for reviews).

The availability of registered products significantly boosted fungal biopesticide deployment (Shah and Pell, 2003). Use of low-tech, labour-intensive mass-production in developing countries has made the sales prices of mycoinsecticides compatible with those of chemical pesticides (Li *et al.*, 2010). Nevertheless, biopesticides still only account for approximately 5% of all agricultural pesticides, and fungus-based products only a very small part of that (the majority of commercial biopesticides are based on microbes) (Federici, 1995; Shah and Pell, 2003). The role of mycoinsecticides in integrated pest management (IPM) is, however, already substantial in some countries and expected to grow in the near future. In Brazil, 40 fungus-based control products were commercially produced in 2007 and approximately one million hectares was treated with *Metarhizium* for spittlebug control in 2008 (Li *et al.*, 2010). In China, half a million hectares of forest land is currently being treated with *Beauveria* annually (Li *et al.*, 2010).

1.4 Fungi for mosquito control

Research on the use of entomopathogenic fungi for mosquito control started already in the 1960s and several fungal species have been tested, especially for the control of mosquito larvae (see Scholte *et al.* 2004a for a review). The genera *Lagenidium*, *Coelomomyces*, and *Culicinomyces* in particular showed good potential for larval control (Federici, 1995). These fungi are, however, difficult to deploy cost-effectively

because they require direct larval contact and high application doses, are difficult to mass-produce, and often have short shelf-lives and low persistence after application (Scholte *et al.*, 2004a). *Lagenidium giganteum* is the only registered and commercially produced aquatic mycoinsecticide for mosquito control (May et al. 2006). The mycelia-based product can remain effective against *Culex* and *Aedes* larvae for a whole season, but is not suitable for *Anopheles* control or applications in organically rich water (Legner, 1995).

From 1977, the focus of larval control shifted to the use of the mosquito-pathogenic bacterium *Bacillus thuringiensis israelensis* (Bti), which was a more selective, persistent and cost-effective biological control agent(Scholte *et al.*, 2004a). The increasing problems with insecticide resistance in mosquito vectors and advances in fungus production and formulation have, however, revived the interest in mycoinsecticdes, and in particular those based on hyphomycetes. *Beauveria* and *Metarhizium*, for instance, were shown to have potential for effective control of *Aedes*, *Culex* and *Anopheles* larvae (Alves *et al.*, 2002; Bukhari *et al.*, 2010).

Most of the recent research efforts have focused on developing a mycoinsecticide against adult mosquitoes. Fungal spores can be deployed against these flying insects by applying them on surfaces with which they make contact. A range of *Metarhizium anisopliae* and *Beauveria bassiana* isolates have been shown successful in infecting and killing *Anopheles*, *Aedes* and *Culex* mosquitoes when applied on several different substrates, (Scholte *et al.*, 2003a; Blanford *et al.*, 2005; Scholte *et al.*, 2005, 2007). Depending on the dose and virulence of the isolate, hyphomycetes can kill mosquitoes within several days, mostly between 4 and 14 days (Scholte *et al.*, 2003b; Bell *et al.*, 2009; Mnyone *et al.*, 2009a,b).

1.4.1 Impact on malaria transmission

Fungi act slower than fast-acting chemical insecticides and do not offer personal protection from mosquito bites. They do, however, have potential to kill mosquitoes before they can transmit parasites (*i.e.* < 10 days) and hence to reduce malaria transmission (Scholte *et al.*, 2005; Hancock *et al.*, 2009). Moreover, fungi have been shown to prevent the development of *Plasmodium* sporozoites, which could block malaria transmission altogether (Blanford *et al.*, 2005; Read *et al.*, 2009). Fungal infection can also cause reductions in blood-feeding frequency (Blanford *et al.*, 2005) and reproductive fitness (Scholte *et al.*, 2006) prior to death, which could further impact on the mosquito's capacity to transmit malaria (Hancock *et al.*, 2009).

1.4.2 Fungus-based vector control

Most research on fungal entomopathogens for mosquito control has relied on laboratory experiments and focused on fundamental aspects of fungus-mosquito interactions (Scholte *et al.*, 2004b; Bell *et al.*, 2009; Blanford *et al.*, 2009). One small-scale field study in Tanzania has shown promising results of fungal spores applied on black ceiling cloths (Scholte *et al.*, 2005). A main challenge for practical use of fungi for malaria control remains the development of delivery systems that maximize mosquito infection rates, enhance spore persistence and that can be integrated into existing control strategies (Knols and Thomas, 2006; Farenhorst and Knols, 2007; Knols *et al.*, 2010), which was therefore a research focus in this thesis.

Optimal delivery of fungal spores should ensure high rates of mosquito infection whilst minimizing application to reduce costs and health risks (Knols and Thomas, 2006; Farenhorst and Knols, 2007). *Beauveria* and *Metarhizium* are usually not infectious to humans, since their spores require insect cuticle-specific cues for germination and generally do not grow at temperatures exceeding 35°C (Zimmermann, 2007a,b). Even though *Beauveria* is found in the soil worldwide, human infections are very rare and occur, for example, much less frequently than infections with brewer's or baker's yeast (Darbro and Thomas, 2009). Furthermore, because it is the innate immune response that fights off a fungal infection, there is no extra danger for immunocompromised humans (Romani, 2004). Large amounts of dry spores can, nevertheless, induce allergenic responses and it is important to consider the acceptability of fungus-based control measures. Development of novel delivery tools should, therefore, aim to minimize human-fungus contact, for instance through the use of formulations that reduce the number of airborne conidia (Darbro and Thomas, 2009).

Other potential operational constraints include social and ethical issues. For instance, implementing an intervention that does not provide direct personal protection from mosquito bites may not gain ethical approval. Fungus-based control may, therefore, only be considered acceptable and gain user compliance when deployed in combination with other malaria preventions.

1.4.3 Integrated Vector Management

Modern malaria control strategies highlight the importance of integrated vector management (IVM), which focuses on the combined use of available preventive measures to reach the greatest disease control benefit (WHO, 2008; Kleinschmidt *et al.*, 2009). Currently there is great interest in using combination interventions with distinct modes of action as resistance management strategy, not only to control resistant mosquitoes but to delay the selection of novel resistance (Guillet *et al.*, 2001; Heming-

way *et al.*, 2006). Fungus-based vector control could potentally be a valuable addition to such IVM strategies, but only when fungi are effective against insecticide-resistant mosquitoes.

Field implementation of any novel malaria intervention will have to be embedded in a background of existing control measures and fungus-based mosquito control would, realistically, be used in addition to malaria interventions that are already in place. Regarding ethical considerations, fungal entomopathogens would likely only be used in combination with chemical-based interventions that provide personal protection from infectious mosquito bites. Thus, for fungus-based malaria control to have any real implementation potential, it would need to be successfully integrated in malaria IVM strategies. This can, however, only be achieved when fungi prove compatible with public health insecticides and effective when used in combination with such chemicals.

1.5 Research objectives

The work presented in this thesis aimed to evaluate the potential of fungal entomopathogens for integration in chemical-based malaria interventions. The first part of the research focused on exploring novel delivery systems that could potentially be effective in tropical field settings and suitable for integration into existing malaria vector control strategies. The second part focused on evaluating the efficacy of fungi against insecticide-resistant *Anopheles* mosquitoes and included studies on the compatibility of public health insecticides with the hyphomycetes *Beauveria* and *Metarhizium*.

The specific research objectives were:

- To test the effects of formulation, substrate and application method on fungal infectivity and virulence to mosquitoes
- To develop novel delivery tools for fungal spores that could provide effective mosquito infections in field settings and potentially be used in IVM strategies
- To test the efficacy of fungi against insecticide-resistant mosquitoes
- To test the compatibility of fungi and insecticides and measure their combined impact on mosquito survival

1.5.1 Thesis outline

Chapter 2 provides an overview of materials and methods used throughout the thesis. Specific methodologies are described in each research chapter separately.

Chapter 3 starts with the development of a novel application method for fungal spores through so-called K-bar coating, to provide a tool for accurate laboratory evaluations of fungi against mosquitoes. The coating method was used to test effects of formulation, substrate type, dose and exposure time on *M. anisopliae* and *B. bassiana* efficacy against anophelines.

Two novel delivery systems that could potentially be integrated in existing control measures were developed and evaluated. **Chapter 4** describes the use of clay water storage pots as a delivery tool for fungal spores against resting *Anopheles* mosquitoes. The efficacy of *Metarhizium* after spray application inside clay pots was tested against insecticide-susceptible *Anopheles gambiae s.s.* and insecticide-resistant *An. funestus* mosquitoes. In **Chapter 5**, application of fungal spores on netting was evaluated for potential use as eave curtains against host-seeking anophelines. Experiments tested the impact of *Beauveria* after application on netting substrates with varying fibre types and mesh sizes, and the effect of mosquito passage through an impregnated net.

Chapter 6 describes experiments on fungi against insecticide-resistant mosquitoes that were performed in the laboratory of Johannesburg, South Africa. The impact of *Beauveria* infection was tested in four highly resistant *Anopheles* colonies from South and East Africa and was compared to fungal impact in their insecticide-susceptible counterparts. Effects of a progressing *Beauveria* or *Metarhizium* infection on subsequent mosquito sensitivity to permethrin or DDT were tested in three metabolically resistant anopheline strains.

In **Chapter 7**, fungi were tested against West African anophelines with high levels of resistance to pyrethroids and DDT through the expression of the *kdr* gene. Experiments were performed in The Netherlands and Benin and tested fungal efficacy in a laboratory-reared mosquito colony and a field-collected population from Benin. *Beauveria* and *Metarhizium* were tested in combination with the insecticide permethrin to determine the combined impact of fungi and insecticides on mosquito survival.

Chapter 8 provides a summarizing discussion on the research findings and future perspectives of this novel malaria vector control technology.

2 Materials & Methods

2.1 Mosquitoes

The majority of experiments in this thesis used laboratory-reared *Anopheles gambiae s.s.* mosquitoes originating from Suakoko, Liberia (courtesy of Prof. M. Coluzzi) that were maintained as a laboratory colony since 1989. This strain is fully susceptible to all insecticide classes. Use of other mosquito species or colonies is described in the specific chapters.

2.1.1 Rearing

Mosquitoes were reared in the Laboratory of Entomology, Wageningen, in climate-controlled rooms (27±1°C, 80±10% RH) with artificial 12-hr day/night photoperiods and 45-min dusk/dawn cycles. Mosquito eggs were placed in plastic larval trays of 10×25×8 cm, filled with 1 L tap water and with filter paper on the sides to prevent egg dehydration. The first larval instar stage was fed with 1 drop of Liquifry per tray and approximately 0.1 mg Tetramin® fish food (Tetra, Melle, Germany) per larva per day. The other three larval stages were fed with 0.3 mg Tetramin per larva and were kept at densities of approximately 0.3 larvae/cm^2. Pupae were collected daily, placed in small plastic cups and transferred to holding cages of 30×30×30 cm. Emerging adults were fed ad libitum on a 6% (w/v) glucose/water solution using glass bottles with protruding filter paper rolls. For experiments, 2-7 day old females were used, which were selected using a mouth aspirator. For delicate handling, no more than 20 females were aspirated into the tube at the same time. Experimental mosquitoes were kept in climate-controlled rooms with similar settings as the rearing rooms.

2.2 Fungus

Experiments used spores (called conidia) of the hyphomycetous fungi *Metarhizium anisopliae var. anisopliae*, isolate ICIPE-30 (courtesy Dr. N. Maniania, ICIPE, Kenya) and *Beauveria bassiana* Vuillemin isolate IMI 391510. In previous studies, these two isolates were shown to be effective in infecting and killing *Anopheles* mosquitoes (Scholte *et al.*, 2003a; Blanford *et al.*, 2005). The round, white *Beauveria* spores (Figure 2.1) measured on average a diameter of 2-4 μm and were estimated to weigh approximately 5×10^{-12} gram. The dark green *Metarhizium* spores were elongated in shape (Figure 2.1), measured on average a diameter of 4-6 μm and were estimated to weigh approximately 2.1×10^{-11} gram.

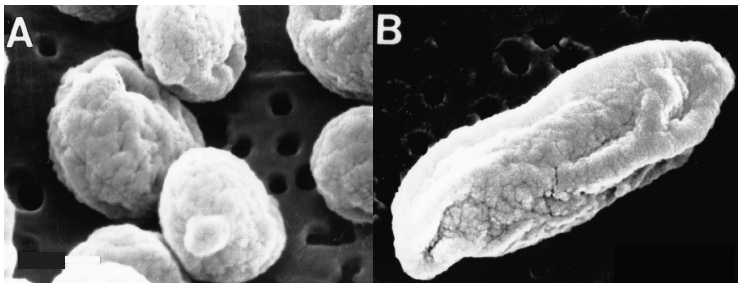

Figure 2.1: *Scanning electron micrographs of Beauveria bassiana spores (A) and a Metarhizium anisopliae spore (B). From Jeffs et al., 1999.*

2.2.1 Production

Conidia of *M. anisopliae* and *B. bassiana* were produced at the Bioprocess Engineering Group of the Wageningen University, The Netherlands (courtesy F. van Breukelen, M. Jumbe and S. Haemers). For production, solid state fermentation in 200 ml aerated packed-bed fermenters was used (Figure 2.2), which is a controlled growth system that provides active aeration and cooling. The growth medium contained 200 g/L of glucose, 25 g/L of yeast extract and 25 g/L bacterial peptone, which was impregnated in hemp particles (Hemparade, Hempflax B.V., The Netherlands) that functioned as a solid support material for fungal growth.

The hemp particles were soaked in the nutrient medium for 24 hrs and fungal spores were added during the last 3 hrs (using 2.5×10^{6} spores/g dry hemp) and mixed through the substrate. The inoculated hemp was transferred to the aerated packed beds, placed

in a temperature-controlled cabinet, in which fungal growth took place for a standard 10 day fermentation period. CO_2 production and O_2 consumption was monitored as a measure of biomass growth. Spores were subsequently harvested by drying the hemp at ambient temperature until moisture content was $<5\%$ and sieving the conidia from the substrate. Dry conidia were stored in 50 ml blue cap tubes in the dark at 4°C until use.

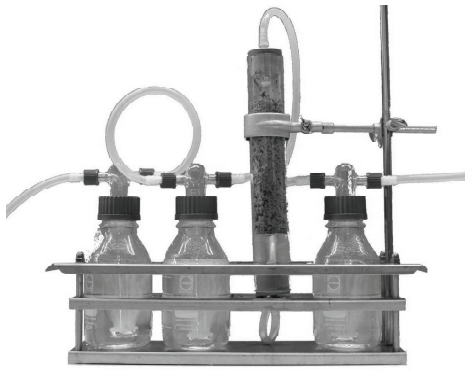

Figure 2.2: *The 200 ml aerated packed-bed fermenter set-up. From F. van Breukelen, Wageningen, The Netherlands.*

2.2.2 Formulation

For application purposes, dry fungal spores were suspended in oil-based solvents. Although water-based formulations can also be used (Bateman *et al.*, 1993), the hydrophobic spores are difficult to keep homogeneously mixed in water. Moreover, oils are generally preferred since they can increase spore adherence to the substrate (Thomas and Blanford, 1998) and provide some protection from dessication (Bateman *et al.*, 1993). Specific amounts of spores were weighed, and mixed in either Ondina oil, which is a highly refined mineral oil (Shell Ondina® Oil 917, Shell, The Netherlands) or Shellsol T solvent, which is a synthetic isoparaffinic hydrocarbon solvent (Shell Shellsol T®, Shell, The Netherlands). Both Ondina and Shellsol have previously been used as spore solvents (Kassa *et al.*, 2004; Blanford *et al.*, 2005) and the choice for either one depended on the application purposes, with Ondina oil being more viscous and Shellsol thinner and evaporative.

Stock solutions were prepared and mixed immediately prior to use through vortexing and sonication at 1000 Hz for 10-15 seconds (Branson sonifier B12, Germany). To

quantify the amount of spores per ml, conidial concentrations of each stock solution were counted using a B*ü*rker-T*ü*rk haemocyte counter with two 16 square chambers of 0.01 cm depth (W. Schreck, Hofheim/TS, Germany). The haemocytometer was filled with a few drops of mixed diluted stock solution and spores counted in all 16 squares of one chamber using a light microscope with 400× magnification. For accurate counts, dilutions providing 20-100 spores per square of the counting chamber were used. The concentration of the stock solution was calculated using the total spore numbers counted times the dilution factor, divided by the volume in the counting chamber.

2.2.3 Viability

Spore quality of each production batch and each solution prepared for application was checked by measuring the viability of the fungal spores. Spore germination (a first and crucial step in the infection process), was used as a measure of viability and tested on a rich agar medium (Jenkins *et al.*, 1998). Germination counts used dry spores suspended in Shellsol solvent and Sabouraud Dextrose Agar enriched with 0.001% Benomyl (Benomyl®, Sigma-Aldrich Chemie B.V., The Netherlands), which is a fungicide that inhibits hyphal growth without affecting spore germination (Milner *et al.*, 1991). Because large and entangled fungal hyphae can make accurate counting difficult (Figure 2.3), Benomyl allowed the use of (longer) standard incubation periods for both fungi.

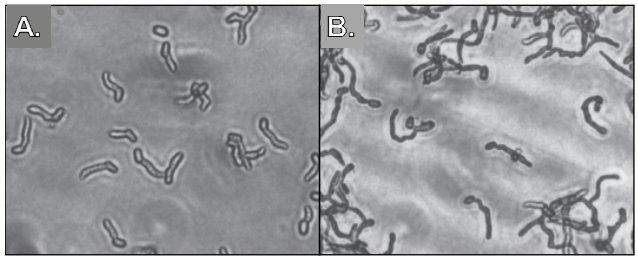

Figure 2.3: *Germinating B. bassiana spores after 40 hrs incubation on SDA agar enriched with 0.001% Benomyl (A) or pure SDA agar (B). From Milner et al., 1991.*

Agar plates were inoculated with one drop ($\pm 25\mu$l) of spore suspension in a laminar flow hood and subsequently sealed with parafilm and incubated at 27$\pm$1°C for 20-28 hours. Using a light microscope at magnification 400×, spores were counted as viable when a protruding germ tube was at least twice the size of the spore. The proportion of germinated spores was determined by counting a minimum of 300 spores per agar

plate in three or more view fields. Unless otherwise stated, experiments used spores that showed 85% or higher germination.

2.2.4 Mosquito infection

Under humid conditions, fungal hyphae emerge from the the dead insect and produce conidia on its exterior (Goettel and Inglis, 1997), which can be used to verify if a mosquito has been infested. Both mosquitoes that were exposed to fungal spores and unexposed control mosquitoes were maintained until death and subsequently checked for emerging fungal hyphae. Dead mosquitoes were removed from the holding cages daily, shortly dipped in 70% ethanol to remove external bacteria and fungi, and subsequently incubated on moist filter paper in sealed Petri dishes at 27$\pm$1°C. Mosquitoes were examined for protruding hyphae using a dissection microscope after 3-5 days of incubation, which was sufficient for sporulation to occur and hence more precise determination of fungus species. Because low infection doses and external factors such as microbiota can affect fungal growth (Roberts and St. Leger, 2004), hyphal growth from cadavers is not a direct indicator of fungal infection and was only used as a positive control observation.

2.3 Insecticide assays

Insecticide assays used standard WHO insecticide bioassays (WHO, 1998) to expose mosquitoes to insecticide and took place in climate rooms (27$\pm$1°C, 80$\pm$10% RH) unless otherwise stated. Colony names and insecticide resistance status of the tested mosquito species are described in the specific chapters.

2.3.1 Insecticide papers

WHO insecticide-treated filter papers were obtained from a producer in Malaysia (Vector Control Reference Unit, Universiti Sains Malaysia, Penang, Malaysia). Papers were impregnated with 0.75% permethrin or 4% DDT, which are the standard doses used to discriminate between insecticide-resistant and susceptible mosquitoes (WHO, 1998). According to the standard WHO protocol, mosquitoes are resistant when 97-100% survive 1 hr exposure to the diagnostic insecticide dose, whereas 80-97% survival is considered possibly resistant but needing further confirmation (WHO, 1998). Experiments used papers from a single WHO production batch that was stored in the refrigerator, to eliminate possible differences in paper efficacy. Papers were re-used for a maximum period of two weeks. Insecticide efficacy was subsequently checked by exposing

insecticide-susceptible mosquitoes to the papers. Experimental data were only used if the insecticide papers still induced 98-100% mortality in susceptible mosquitoes.

2.3.2 Exposure assays

Insecticide exposures used standard WHO test kits (WHO, 1998), and transferred 25-30 female mosquitoes to each holding tube (lined with clean, untreated paper) via an aspirator. After a 10 min settling period, mosquitoes were transferred, through the slide mechanisms in the centre, to the tube lined with insecticide paper via gentle blowing. Mosquitoes were exposed to the insecticide paper for 1 hr, after which they were transferred back to the clean holding tube. Sugar water was provided with cotton bolls on each holding tube and mosquito mortality recorded after 24 hrs (WHO, 1998). Control groups were exposed (as described above) to clean, untreated papers when only insecticides were tested, or to papers treated with Shellsol solvent when fungi were also tested.

2.3.3 Mortality

Insecticide impact was measured using mosquito mortality rates 24 hrs after insecticide exposure. Mosquitoes were counted as dead when unable to fly. When control group mortalities were below 5%, insecticide-exposed mortalities were used as counted. When above 5%, the mortalities of exposed groups were corrected for corresponding control mortalities using the Abbott's formula (WHO, 1998). In experiments that tested the effects of fungal infection on insecticide impact, control groups consisted of mosquitoes exposed to fungus only. In these cases, the mortality induced by the fungus within the 24 hours waiting period (usually $>30\%$) was used to correct the combined fungus-insecticide mortality (with Abbott's formula) in order to show the impact of the insecticide.

2.4 Data analysis

Survival was monitored daily to obtain cumulative daily proportional mosquito survival rates. These were used to plot survival curves that depict mosquito survival over time. Survival analyses were used to measure the impact of fungal infection on mosquito survival and to compare survival rates between different treatment groups. All analyses were performed in SPSS (15.0 or 16.0) and used $P<0.05$ as the significance level.

2.4.1 Survival analyses

Survival analyses provide a means to model the risk until death without parametric assumptions (Hosmer and Lemeshow, 1999) and to measure the impact of a treatment over the whole course of the mosquitoes' lifetime. Especially for slow-killing agents, survival analyses give more comprehensive information on the overall impact than merely comparing mortality rates at a specific time-point. Another benefit of survival analyses is that missing data can be accounted for. For example, data of escaped mosquitoes could still be used, and control groups did not necessarily have to be followed up until the end. These otherwise missing data points were entered as so-called censored data and incorporated in the likelihood function and survival curves (Hosmer and Lemeshow, 1999). Comparisons between survival curves were made with Kaplan-Meier or Cox regression analysis.

2.4.2 Kaplan-Meier

Kaplan-Meier analysis computes survival functions from life-time data and uses a simplistic step approach in which steps are defined by the observed survival and censored times (Kaplan and Meier, 1958).

Analyses in Chapter 4 used Kaplan-Meier pair-wise analysis with the logrank test to compare survival curves of two treatment groups. This nonparametric test compared survival distributions of the two groups. and was used to distinguish significant differences in survival between fungus-infected and control groups and between different fungus exposure treatments. Replicates were pooled when there were no differences between them ($P>0.05$). When no pooling was allowed, each test replicate was compared with the corresponding control replicates separately.

2.4.3 Cox regression

Cox regression computes hazard functions that quantify the instantaneous risk of death at each time-point, while simultaneously adjusting for influential variables (Cox, 1972). The Cox proportional hazards model can be used to compare survival between different treatment groups and assess which factors (covariates) have a significant influence.

Most experiments in this thesis (Chapters 3,5,6 & 7) used Cox regression in SPSS software to analyze and quantify significant differences in survival of fungus-infected and control groups. Mortality data were analyzed separately for each mosquito and fungus species and first checked for proportionality using plots of survivor functions in SPSS (Cox, 1972). Fungal impact was reported in Hazard Ratio (HR) values, which indicated the average daily risk of dying of one group relative to another group. Fungus-

infected groups were compared to control groups such that an HR value of 1 indicated equal mortality rates, an HR$>$1 significantly higher overall mortality rates in fungus-infected mosquitoes and lower when HR's were $<$1. For example, an HR of 5 indicated that the average daily risk of dying was 5 times higher in the fungus-infected group. Note that this quantified relative impact is highly influenced by the survival of the control groups.

To test significant influences of other factors (such as application dose or insecticide resistance status) on fungal impact, Cox regression interaction analyses were performed. These analyses included all test factors and possible interactions as covariates and measured if there were significant interaction effects. For example, to compare fungal impact between an insecticide-resistant and its baseline mosquito colony, the model incorporated the factors fungus, insecticide resistance and their interaction (fungus $\times$ insecticide resistance) as covariates and tested if these had a significant effect on the hazard rate. Although interaction outcomes were also reported in HR values, these do not depict average daily risks of dying, but differences in fungal impact between tested covariates.

3

Application of fungal spore coatings

Abstract

To accurately test fungal efficacy against mosquitoes, methods for uniform and consistent fungal spore application are required. A novel method, using wired K-bars, was tested for coating spore suspensions onto paper substrates. A range of solvents and substrates and two types of coating techniques were evaluated. A standardized bioassay set-up was designed for testing coated *Metarhizium anisopliae* and *Beauveria bassiana* spores against *Anopheles gambiae s.s.* mosquitoes. K-bar coating provided uniform spore applications and the mosquito exposure assay was effective and consistent in measuring fungal impact on mosquito survival. Shellsol T solvent was the most suitable formulation and smooth proofing paper the most effective substrate, resulting in the highest spore infectivity. Manually and mechanically applied spore coatings showed similar and reproducible effects on mosquito survival. The virulence of *M. anisopliae* and *B. bassiana* infections increased step-wise with increasing fungal dose, and with higher doses ($>10^{11}$ spores/m^2) only 5 minute exposure was sufficient to induce lethal infections. Use of this novel application method could help achieve reliable results that are exchangeable between different laboratories.

This Chapter has been published in a slightly different form as:

Farenhorst M. & Knols B.G.J., 2010.
A novel method for standardized application of fungal spore coatings for mosquito exposure bioassays.
Malaria Journal 9 (27).

3.1 Introduction

The potential of fungi to kill anophelines and reduce malaria transmission (Scholte *et al.*, 2005; Blanford *et al.*, 2005; Read *et al.*, 2009) has resulted in a growing interest to develop practical and sustainable mosquito vector control methods based on these biological control agents that can be integrated into the existing arsenal of malaria control tools (Knols and Thomas, 2006; Thomas and Read, 2007). There are multiple methods available for infecting target insects with fungal spores. Dry conidia have been shown to be effective in infecting mosquitoes in the laboratory (Scholte *et al.*, 2003a) but as they become air-borne when handled, the exact exposure dose cannot be determined. Use of fungal suspensions allows for accurate quantifications of spore concentration with microscopy counts and is considered to be more feasible for large-scale experiments and field implementation.

Formulation can be an important determinant of spore infectivity and persistence (Daoust *et al.*, 1983). Solvents that are suitable for applying hydrophobic fungal spores (conidia) include Tween-water mixtures (Prior *et al.*, 1988; Scholte *et al.*, 2007), solvents such as kerosene (Shah *et al.*, 1998) or hydrocarbon isoparaffins (Shellsol T) (Bateman and Alves, 2000), and several oil-based solvents such as vegetable (Consolo *et al.*, 2003) and mineral oils (Ondina) (Bateman and Alves, 2000; Akbar *et al.*, 2005). The choice of solvent depends on its properties, such as colour, odour and viscosity but also on the application method and substrate, which can affect the accessibility of spores to the insect after application. In general, oil formulations are considered to be beneficial for spore persistence in field situations as they can protect spores from desiccation (Alves *et al.*, 1998; Bateman and Alves, 2000).

Fungal entomopathogens infect insects upon contact with the cuticle (§1.3.1). For flightless insects, laboratory evaluations of dose and exposure time can make use of direct spore suspension applications on the insect cuticle (Ansari *et al.*, 2004; Amora *et al.*, 2009). Precise topical applications (with a pipette) are, however, not applicable for flying insects without using sedation, which can have a negative effect on fitness and survival (Nicolas and Sillans, 1989). Applying fungal spores on surfaces on which mosquitoes rest is, therefore, more commonly used to infect anophelines (Blanford *et al.*, 2005; Scholte *et al.*, 2005). It is then the end-concentration of spores per unit surface area that determines the effective exposure dose.

There is currently no conventional application method for testing fungal spores against mosquitoes. Research on mosquitoes has made use of brushing fungal suspensions on cotton cloths (Scholte *et al.*, 2005), dipping (*i.e.* submerging) netting in fungus suspensions, and manual spray applications on various substrates (Blanford *et al.*, 2009; Darbro and Thomas, 2009). Although all effective, none are accurate in

determining the end-concentration of fungal spores. Spraying is considered one of the more feasible application methods for larger scale experiments, but is less accurate due to large spore losses through bounce-off and run-off effects. The effective end-concentration of spores when sprayed onto paper was shown to be only around 10% of the estimated application dose (Bell *et al.*, 2009). To test effects of fungal dose and exposure time accurately, it is important to be able to apply specific amounts of fungal spores per unit surface area in a uniform and reproducible manner. The development of a standardized laboratory assay for testing fungal spores against mosquitoes, therefore, requires a novel and precise application method.

The paint and coatings industry has developed standardized and high precision methods for applying coatings onto substrates. Wired, stainless steel K-bars with specifically sized grooves have been designed to coat solutions, such as paints and coatings, in a uniform layer of equal thickness. Here, the use of K-bars for applying fungus formulations on paper substrates was evaluated using *Metarhizium anisopliae* and *Beauveria bassiana* spores and *Anopheles gambiae s.s.* mosquitoes. Effects of formulation and substrate on fungal infectivity were tested and optimized for use in mosquito exposure assays. Manually and mechanically applied spore coatings were compared and used to evaluate effects of spore dose and mosquito exposure time.

3.2 Materials & Methods

3.2.1 Mosquitoes & Fungus

Experiments used 3-5 days old female *An. gambiae s.s.* that were reared as described in §2.1.1 and spores of *Metarhizium anisopliae* and *Beauveria bassiana* that were produced as decribed in §2.2.1. Exposure time experiments used a production batch of *B. bassiana* from the laboratory of PennState University, USA, which was grown on autoclaved barely flakes in mushroom spawn bags (courtesy Dr. N. Jenkins).

3.2.2 Formulation

The suitability of formulations for coating applications was empirically tested. Ondina oil and Shellsol solvent (§2.2.2) were compared, separately and in a 1:1 mixture. The number of viable spores/ml of each stock suspension was quantified by microscopy counts (§2.2.2) checked for viability as described in §2.2.3.

3.2.3 Substrate

Two different substrates were tested: smooth, gloss-coated proofing paper that was provided with the K-bars, consisting of wood-free Highland chromo paper 5415 (RK Print Coat Instruments Ltd., UK), and cardboard paper from file-folders made of 270 gram chlorine- and acid-fee Colorkraft cardboard (Jalema BV, Reuver, The Netherlands). Total volume and application methods were optimized for both paper types.

3.2.4 Coater

Spore suspensions were applied onto substrates using wired K-bars (K bars®, RK Print Coat Instruments Ltd., United Kingdom), which were made of stainless steel rods with identically shaped grooves that control wet film thickness (Figure 3.1A). Two close wound K-bars were tested, with grooves of 0.15 or 0.31 mm that produced a coating thickness of 12 μm or 24 μm respectively.

The K-Hand Coater and the K-Control Coater Model 202 (RK Print Coat Instruments Ltd., UK) were compared ((Figure 3.1B). The Hand Coater comprised a surface area of 220 $\times$ 340 mm and the Control Coater one of 250 $\times$ 325 mm, with a soft coating bed consisting of three layers (a Melinex, foam and rubber layer on top of each other). Spore suspensions were applied manually onto the paper substrate with a pipette (Figure 3.1C). Using the Hand Coater, film deposits were applied manually by pulling the K-bar over the substrate in one rapid, smooth movement (Figure 3.1D). The K-Control Coater provided motorized applications that exerted a constant pressure between the K-bar and substrate and moved the bar over the substrate at a controlled speed. For experiments, application speed was maintained at 20 cm/sec. K-bars were wedged into the holder of the Control Coater and weights were adjusted for each bar to optimize pressure and horizontal position (using a water level). Spore residues were removed from the K-bar and rubber bed with tissue paper drenched in 70% ethanol.

3.2.5 Bioassays

Coated papers were left to dry overnight in a climate room (27$\pm$1°C, 70$\pm$10% RH) before being placed inside a PVC-tube of 15 cm long and 8 cm diameter (Figure 3.1E). Papers covered the entire inside surface of the tube and were fixed with two small paperclips. Each tube was sealed with plastic microwave foil on either end, on which mosquitoes did not tend to rest. Mosquitoes were released in the tube via an aspirator (Figure 3.1E) and exposed for a fixed time period (see below). After exposure, mosquitoes were transferred to holding buckets of 20 cm diameter and 25 cm height, sealed with sheer nylon socks with the toe part cut off (Figure 3.1E), which

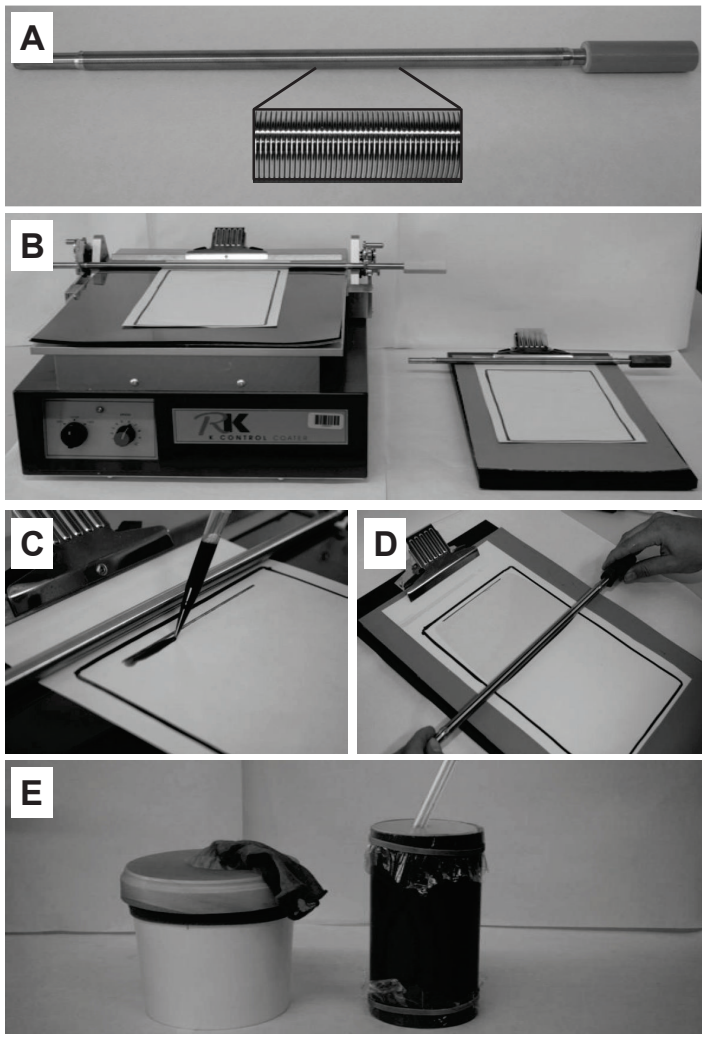

Figure 3.1: *K bar coating of fungal spores. (A) The stainless steel K-bar with wired grooves (inset) that control film thickness. (B) Automated (left) and manual K-Coater (right). (C) Application of fungal spore suspension with a pipette. (D) Coating a paper substrate using a swift top-to-bottom movement of the bar. (E) The mosquito exposure set-up with a coated paper inside a PVC tube (right), sealed with plastic foil. Mosquitoes were transferred into the tube with an aspirator and (after exposure) to the holding buckets (left) via free flight.*

during mosquito transfer were used to enfold one side of the PVC tube to facilitate mosquitoes flying into the holding bucket. Survivors were kept in a climate-controlled room (27$\pm$1°C, 80$\pm$10% RH) and were monitored daily. Mosquitoes were checked for fungal infection as described in §2.2.4. Replicates were performed on separate days with fresh batches of mosquitoes. In the first experiment 6 hrs exposure was used, but 3 hrs gave similar results and was used in all other experiments.

3.2.6 Experiments

Formulation
Cardboard surfaces (15×25 cm) were coated manually with three different formulations of *M. anisopliae* or *B. bassiana*. Ondina suspension (10^{10} spores/ml) was applied in 1 ml with a single movement of the 12 μm K-bar. Due to lower viscosity and higher absorbance, 2 ml of 5×10^9 spores/ml was applied for the Shellsol and Shellsol/Ondina formulations in two bar movements (top to centre and bottom to centre) to give also 3×10^{11} spores/m^2. Control papers were treated with the same volumes of solvents. Per treatment, one group of 50 females was exposed for 6 hrs.

Substrate
On proofing papers, 1 ml of a 10^{10} spores/ml Shellsol suspension was applied with a single movement of the 12 μm K-bar. Cardboard papers were coated with 2 ml of 5×10^9 spores/ml to reach the same end-concentration of 3×10^{11} spores/m^2. For both paper types, three replicates of 50 female mosquitoes were exposed for 3 hrs to *Metarhizium*-coated, *Beauveria*-coated, or control papers (coated with 1 ml Shellsol).

Coater type
Proofing papers were coated with 0.9 ml of 3.4×10^9 *B. bassiana* spores/ml Shellsol (=10^{11} spores/m^2), using the 24 μm K-bar on the K Hand Coater or the K Control Coater. Control papers were treated with 0.9 ml Shellsol. Three replicate groups of 40 females were exposed to each treatment for 3 hrs.

Dose-response
Proofing papers were manually coated, using the 24 μm K-bar, with 10-fold dilutions of the same stock suspensions of *M. anisopliae* and *B. bassiana*, resulting in end-concentrations of 10^9, 10^{10}, 10^{11} and 10^{12} spores/m^2. Control papers were treated with 0.9 ml Shellsol. Per treatment, one group of 40 females was exposed for 3 hrs.

Exposure time
Proofing papers were mechanically coated, using the 24 μm K-bar, with 0.9 ml of a suspension containing 4.2×10^9 or 4.2×10^{10} *B. bassiana* spores/ml to reach end-concentrations of 10^{11} or 10^{12} spores/m^2 respectively. Control papers were treated with 0.9 ml Shellsol. Per treatment, three replicate groups of 40 females were exposed for 5 min, 0.5 hr or 3 hrs.

3.2.7 Data analysis

Differences in mosquito survival between treatment and control groups were analyzed using Cox Regression as described in §2.4.3

3.3 Results

3.3.1 Coating method

Coating applications were optimized for use in mosquito exposure tubes with a surface of 15×25 cm. This 0.0375 m^2 treatment surface was drawn onto an A4 size paper and attached onto the rubber K-coater holding board using the holding clasp (Figure 3.1B). Homogeneously mixed fungal suspensions were applied with a pipette in the centre top part of the treatment surface, not touching the bar prior to pulling (Figure 3.1C) to prevent spread outside the surface boundaries. The K-bar had to be used rapidly (<5 sec) after applying the suspension to prevent absorbance. Substrates such as cotton or netting were not suitable due to too high absorbance and insufficient K-bar contact.

Manual application using the K-Hand Coater required practice to optimize speed, pressure and constancy of the bar pulling movement. Use of both hands on either side and light pressure was most effective (Figure 3.1D). Standardizing the K-Control Coater required only small adjustments of the bar settings and machine speed, which could remain fixed during experiments. Weights on both sides of the bar holder could be adjusted to optimize pressure and levelness (Figure 3.1B). The motorized bar movement was optimized for A4 size papers and maintained at 20 cm/sec for all experiments.

The two K-bars tested, coating a thickness of 12 μm or 24 μm, were equally suitable. Larger grooves (>50 μm) were not effective in spreading liquids homogeneously. Formulation and substrate experiments used the 12 μm K-bar to coat cardboard substrates. For proofing papers, the 24 μm K-bar was most suitable, especially for applying more concentrated formulations, and was chosen as the gold standard. Effective end-concentrations were calculated in spores per m^2 using the concentration of viable spores/ml, the total volume applied and the substrate surface.

3.3.2 Formulation

Ondina oil and Shellsol solvent were both suitable spore carriers. In the viscous Ondina, spores remained mixed homogeneously for >2 hours, whereas in Shellsol only for <10 minutes. Shellsol, however maintained $>10^{11}$spores/ml whilst allowing for use of a pipette, which was not possible with Ondina oil, and was more suitable for spore microscopy counts. Ondina could be applied in small volumes (0.9 ml for 0.00375 m^2)

on various paper substrates, whereas small volumes of Shellsol could only coat less absorbent gloss-coated proofing paper. Odourless Ondina oil and 1:1 Shellsol/Ondina mixtures did not evaporate and lengthy periods of drying (>16 hrs) were required for spores to be infective to resting mosquitoes. Shellsol dried within 1 hr, but more time (>5 hrs) was needed to remove its odour and prevent mosquito knockdown. Drying time was standardized for a minimum of 18 hrs.

Infectivity of *M. anisopliae* and *B. bassiana* spores was tested when suspended in Ondina, Shellsol or 1:1 Shellsol/Ondina mixture and coated on cardboard papers with the K-Hand Coater. Shellsol was the most effective solvent, enabling a 100% kill within 13 days (Figure 3.2). Mosquito survival was significantly reduced compared with controls for both *M. anisopliae* (HR=11.28, P<0.001) and *B. bassiana* (HR=10.02, P<0.001), and respectively 91% and 93% showed fungal infection (emerging hyphae) after death. The Shellsol/Ondina mix also reduced survival when applying spores of *Metarhizium* (HR=3.89, P<0.01) or *Beauveria* (HR=3.06, P<0.01), although less than 40% of mosquitoes were killed within 13 days (Figure 3.2) and only 38% of the *Metarhizium*- and 32% of the *Beauveria*-exposed showed infection after death.

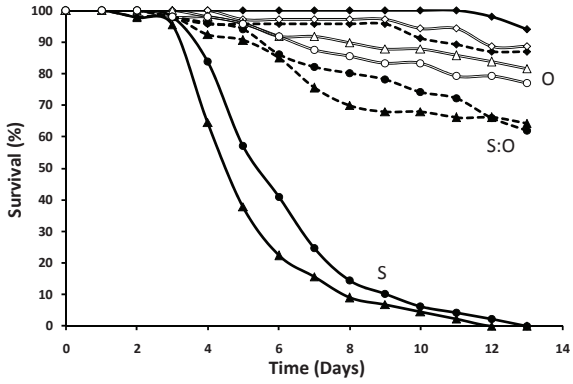

Figure 3.2: *Effect of formulation. Cumulative survival (%) of 50 females exposed for 6 hrs to cardboard papers manually coated with 3×10^{11} spores/m^2 of M. anisopliae (triangles) or B. bassiana (circles) formulated in Shellsol (S), 1:1 Shellsol/Ondina mix (S:O) or Ondina (O). Controls (diamonds) were exposed to the solvents only.*

Hazard Ratio values showed that fungi applied in a Shellsol/Ondina mix induced an approximate three times higher risk of dying in the infected mosquitoes compared with the uninfected groups, whereas with pure Shellsol this was eleven times higher. Pure Ondina oil was the least effective formulation, giving no significant fungus-induced

reductions in mosquito survival for *Metarhizium* (HR=1.49; P=0.18) or *Beauveria* (HR=1.27, P=0.31), and mosquito infection rates of only 12% and 18% respectively. Shellsol was subsequently chosen as the standard coating formulation.

3.3.3 Substrate

The effect of substrate on spore infectivity was tested using gloss-coated proofing paper and more absorbent cardboard papers. *Metarhizium anisopliae* significantly reduced mosquito survival compared to controls when applied on proofing paper (HR=18.92, P<0.001) or cardboard (HR=15.65, P<0.001) (Figure 3.3). *Beauveria bassiana* spores were also highly infective, reducing mosquito survival when coated on proofing paper (HR=17.67, P<0.001) or cardboard (HR=11.84, P<0.001) (Figure 3.3).

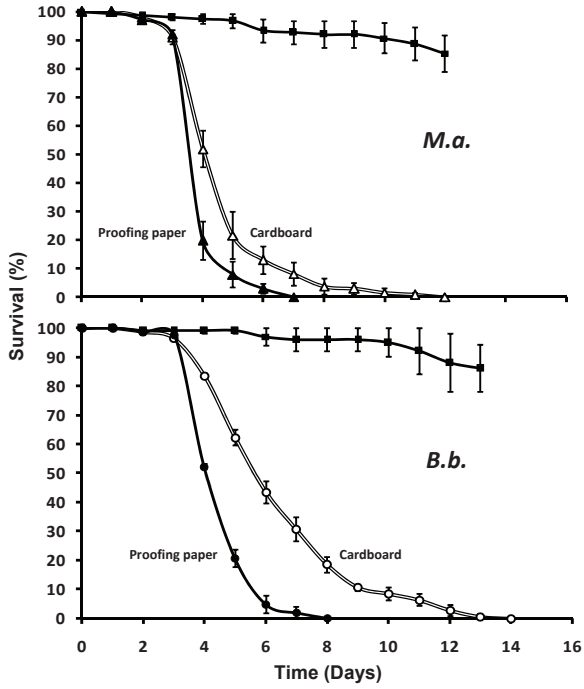

Figure 3.3: *Effect of substrate. Cumulative % survival (mean±SE) after 3 hr exposure to proofing (black) or cardboard papers (white) manually coated with 3×10^{11} viable spores/m^2 Shellsol-formulated M. anisopliae (M.a.) or B. bassiana (B.b.), or Shellsol only (controls, squares).*

Metarhizium-coated proofing papers reduced mosquito survival more than coated cardboards (HR=4.01, P<0.001) and infected 92% of mosquitoes compared with 82% respectively. For the smaller *Beauveria* spores, differences in mortality rates were even larger between proofing papers and cardboard (HR=5.12, P<0.001), infecting 94% and 73% respectively. K-Coater proofing papers were, therefore, used as the standard coating substrate.

3.3.4 Coater type

The efficacy of manually applied spore coatings with the K-Hand Coater was compared with automated applications using the K-Control Coater. For both manually and mechanically applied *B. bassiana* coatings, significant reductions in mosquito survival compared to controls were obtained (HR=15.31, P<0.001 and HR=14.84, P<0.001 respectively) (Figure 3.4). Results were equally consistent and reproducible for both methods and the impact on mosquito survival was not significantly different (HR=0.97, P=0.9).

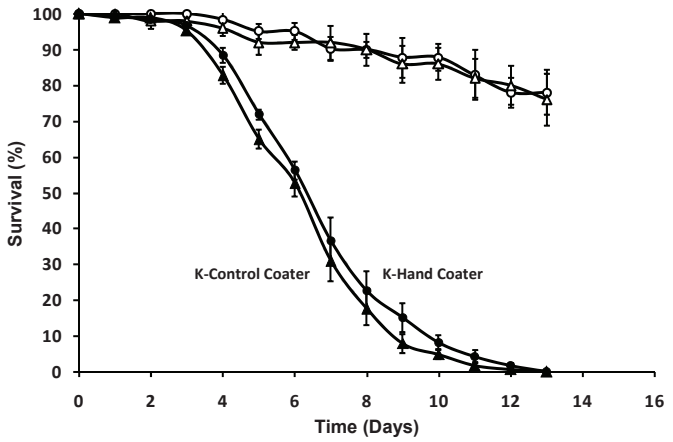

Figure 3.4: *Effect of coater type. Cumulative % survival (mean±SE) of An. gambiae exposed for 3 hrs to controls (open symbols) or 1×10^{11} Beauveria spores/m^2 coated manually with the K-Hand Coater (black circles) or mechanically with the K-Control Coater (black triangles).*

3.3.5 Dose & Exposure time

Dose-response effects of *M. anisopliae* and *B. bassiana* were evaluated by coating 10-fold dilutions of the same stock suspensions with the K-Hand Coater, which gave end-concentrations ranging between 10^9 and 10^{12} viable spores/m^2. For both fungi, all doses reduced survival significantly compared with control mosquitoes ($P<0.001$) (Figure 3.5).

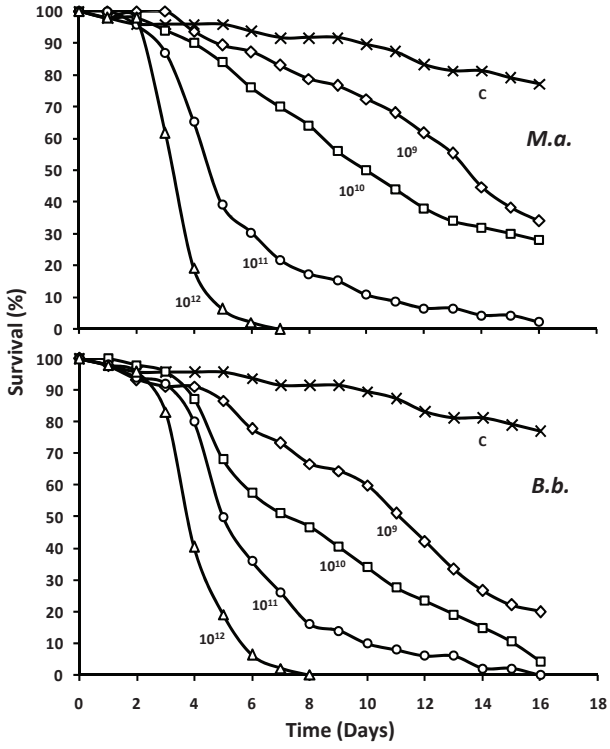

Figure 3.5: *Dose response curves. Cumulative % survival of 40 female An. gambiae s.s. exposed for 3 hrs to control papers (C) or proofing papers manually coated with 10^9-10^{12} spores/m^2 of M. anisopliae (top) or B. bassiana (bottom).*

Mosquito survival data showed a clear and consistent dose-dependent increase in fungal virulence, with 10^9 spores/m^2 causing the smallest reduction in mosquito survival and 10^{12} spores/m^2 the largest (Figure 3.5). Infectivity data also showed a dose-

dependent increase for fungal infection. For *Metarhizium*, 19, 37, 76 and 95% of the mosquitoes showed fungal infection after death when exposed to respectively 10^9, 10^{10}, 10^{11} and 10^{12} spores/m^2. For *Beauveria*, this was 23, 49, 84 and 90%.

The effect of three different exposure times (5 min, 0.5 hr and 3 hrs) was tested using two *B. bassiana* concentrations (10^{11} and 10^{12} spores/m^2). As expected, the lower dose induced smaller reductions in survival than the higher dose (Figure 3.6). Interestingly, exposure time did not cause large differences in fungal virulence. Exposure for only 5 min was sufficient for inducing significant reductions in mosquito survival for both tested concentrations ($P<0.001$).

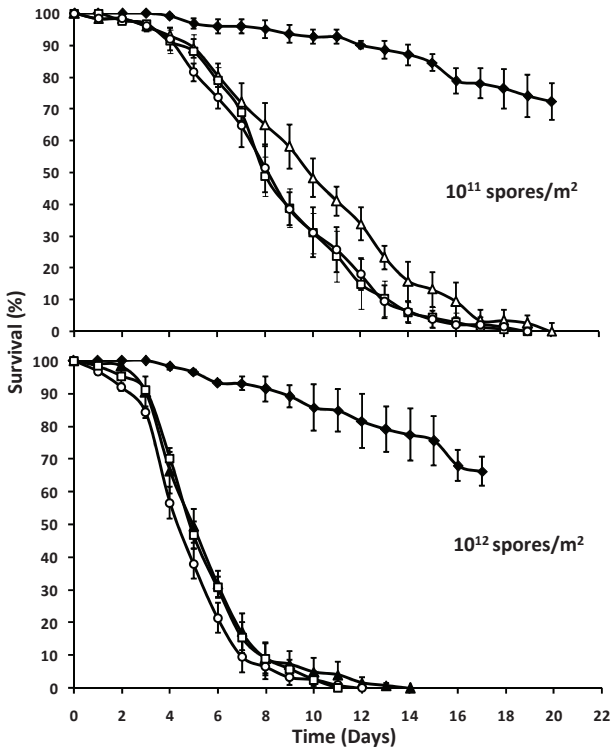

Figure 3.6: *Effect of exposure time. Cumulative % survival (mean±SE) of females exposed for 5 min (triangles), 0.5 hr (squares) or 3 hrs (circles) to control papers (black) or proofing papers mechanically coated with 10^{11} or 10^{12} Beauveria spores/m^2 that were produced in PennState, USA.*

Only for the lower spore concentration did 0.5 hr and 3 hrs exposure have a significantly higher impact on survival (HR=1.36,P=0.016). For the higher dose, all exposure times resulted in similar reductions in mosquito survival (P>0.05). When comparing the survival curves of the 3 hrs exposure time with those of the dose-response experiments for 10^{11} and 10^{12} spores/m^2, the *Beauveria* spores produced using a bag-system in the USA (Figure 3.6) showed to be less virulent than the *Beauveria* spores produced by solid state fermentation in the Netherlands (Figure 3.5).

3.4 Discussion

K-bar coating provided a simple and consistent method for coating layers of fungal spores onto paper substrates. The application of exact suspension volumes onto predetermined substrate surfaces allowed for accurate determinations of the effective end-concentration per unit surface area. The precision of the coating method could be somewhat affected by variations due to the manual use of the pipette and small proportions of formulation remaining on the K-bar as residue, although this could be considered negligent compared to spraying, which has been reported to lose >90% of the total application volume due to vaporization and bounce-off (Bell *et al.*, 2009). For comparison, when applying the same volume per surface area, much more spores ended up on a coated paper compared with a sprayed paper, which is illustrated by its darker colour in Figure 3.7.

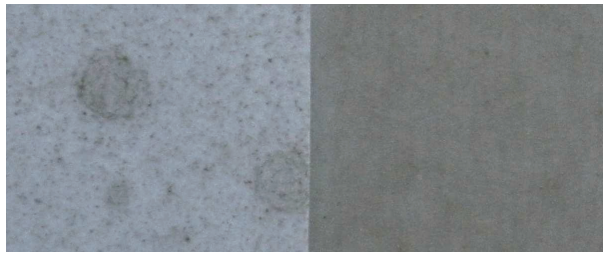

Figure 3.7: *Spore distribution. Photo (zoomed in 4×) of a piece of proofing paper surface sprayed (left), using the method described by Bell et al., 2009, or coated (right) with equal volumes of a 5×10^9 Metarhizium spores/ml formulation (20% Ondina/ 80% Shellsol) .*

The homogeneity of spore layers after application could not be quantified. Although fluorescent dyes may be used to improve visualization of suspended spores (Schading et al. 1995), it was not possible to quantify the number of spores with a

microscope after application onto the paper and coating uniformity could only be determined visually. When using high *Metarhizium* concentrations, the K-bar deposited relatively homogeneous, non-clumping layers, where spraying would result in a more patchy distribution (Figure 3.7). Novel techniques such as quantitative PCR (Bell *et al.*, 2009) may be used to quantify the spore layer of a coated paper and to determine the application efficacy and homogeneity more precisely.

Solvent viscosity showed to be an important determinant of fungal infectivity and virulence. Although oil formulations have shown to be effective when sprayed on crops (Kassa *et al.*, 2004), viscous Ondina oil was not a suitable solvent for spore coatings on papers. Ondina remained in the papers for several weeks, which may have caused strong adherence to the fungal spores and reduced their ability to attach to resting mosquitoes. The evaporative Shellsol solvent dried rapidly and kept spores adhered to the substrate whilst allowing attachment to mosquitoes.

The type of substrate was also an important determinant of fungal infectivity. Gloss-coated proofing paper was the most suitable substrate for Shellsol-formulated spores. Coatings on cardboard were less infective and virulent, especially for the smaller *Beauveria* spores. The higher porosity may have caused spores to end up between the cardboard fibres instead of on the surface, hence being less accessible to mosquitoes. The optimal K-bar coating was a thin layer (12-24 μm) of Shellsol-formulated spores on a proofing paper substrate. Even though other mixtures may be found to be suitable as well, the aim of the formulation experiments was to find an effective solvent for coating applications, which in this case was pure Shellsol. Its relatively short drying time allowed papers to be used shortly after application ($<$6 hrs) and thus limit potential effects of declining spore viability.

K-bar coating was effective in applying both *M. anisopliae* and *B. bassiana* spores and did not require specific adjustments for each fungus. The coating method may also be optimized for other fungus species, target insects and other types of exposure assays. Variables, including bar type, substrate and formulation could be varied to achieve the most appropriate method for customized bioassays. The K-Hand Coater and the K-Control Coater were equally effective and consistent application methods for fungal spore coatings. The Control Coater allowed bar settings and applicator movement to be automated and kept constant between replicates. Between-user differences were not evaluated in this study, but it may be assumed that manual applications are less consistent than automated applications. The K-Hand Coater, on the other hand, does not require electricity and has a smaller size and lower price, which makes it more appropriate for use in field laboratories.

Experiments on spore concentrations showed consistent dose-dependent effects of both *M. anisoplae* and *B. bassiana*. The speed of kill and mosquito numbers showing infection after death increased with increasing fungal dose. Although only one repli-

cate was tested per dose, consistent responses were obtained for both fungi. When using spraying as application method, applying higher doses does not always give a consistent increase in virulence (unpubl. data). Exposure time experiments showed that mosquitoes can pick up a lethal dose of fungal spores within a short period of five minutes. Differences in virulence were only observed when testing lower concentrations, indicating dose-dependent effects of exposure time.

Coating showed to provide consistent fungal spore applications for laboratory assays. Other application methods may, however, prove more effective in terms of spore infectivity or more feasible for field application. For instance, spraying seems to require fewer spores for obtaining similar infectivity and virulence to mosquitoes compared to coated papers (Bell *et al.*, 2009) and is considered more feasible for field implementation. For laboratory assessments, however, the most important requirements are high precision and repeatability. Compared to spraying, K-bar coating can apply more precise volumes in uniform and evenly thick layers, with much less contamination of the work space, and thus provide more precise estimates of effective fungal exposure doses. Coated papers could thus be a valuable tool for laboratory tests on lethal and sub-lethal effects of fungal infection and, for example, to screen for the most persistent fungal isolate.

There are currently no standard conventional methods for fungus application and mosquito exposures. With multiple institutes collaborating to develop fungus-based malaria control tools, the use of a single, standard fungus-mosquito bioassay would be a valuable improvement. Tests on fungal impact and influencing parameters, such as mosquito species, formulation or spore production methods, can only be accurately executed and compared when using a single application method. For instance, using the coating method, it was noted that *Beauveria* produced by Wageningen and PennState differed in virulence, which may otherwise have gone unnoticed or been attributed to different application techniques. Because the K-bar coater is an existing, purchasable applicator, its use could be easily standardized between institutes and could thereby help achieve appropriate and exchangeable results.

4

Clay pots as fungal delivery systems

Abstract

The use of African water storage pots for point-source application of the fungus *Metarhizium anisopliae* and its delivery to malaria vectors was investigated. Clay pots were shown to be attractive resting sites for male and female *Anopheles gambiae s.s.* and were not repellent after impregnation with fungus. *Metarhizium* was highly infective and virulent to both insecticide-susceptible *An. gambiae s.s.* and insecticide-resistant *An. funestus* mosquitoes after spray application inside pots. The two tested fungal doses induced more than 91% infection in both mosquito species. Fungal infection significantly reduced mosquito longevity. *Metarhizium* killed on average 50% of mosquitoes in 4 days and >95% within 7 days, whereas more than 50% of control mosquitoes were still alive after 13 days. Clay pots were thus found to be suitable for fungal spore applications against malaria vectors and provide a novel potential delivery tool that could be used both indoors and outdoors to target resting anophelines with fungi in field settings.

This Chapter has been published in a slightly different form as:

Farenhorst M., Farina D., Scholte E.J., Hunt, R.H., Coetzee M. & Knols B.G.J., 2008. African water storage pots for the delivery of the entomopathogenic fungus *Metarhizium anisopliae* to the malaria vectors *Anopheles gambiae s.s.* and *An. funestus*. *American Journal of Tropical Medicine & Hygiene* 78 (6), 910–916.

4.1 Introduction

Anopheles gambiae s.s. and *An. funestus* are two of the most important malaria mosquito vectors in sub-Saharan Africa as they show high susceptibility to *Plasmodium* infection and high degrees of anthropophily (White, 1974). *Anopheles gambiae s.s.* is highly efficient in transmitting malaria parasites (Coetzee *et al.*, 2000; Rogers *et al.*, 2002) and *An. funestus* extends its activity into the dry season when other malaria mosquito vectors are present in much reduced densities (Mbogo *et al.*, 2003). In the field, populations of both species are showing increasing levels of resistance to one or more of the insecticide classes used in malaria vector control (N'Guessan *et al.*, 2007b; Hargreaves *et al.*, 2000).

Several research institutes are now focussing on the development of novel and sustainable approaches for adult mosquito control based on fungal entomopathogens (Knols and Thomas, 2006). While research on fundamental aspects regarding fungus production and fungus-mosquito interactions is underway, gaps remain between the scientific laboratory discoveries and a successful implementation of fungi in the field. Practical issues concerning the viability, infectivity, formulation and delivery of fungal spores (called conidia) need to be resolved before progress towards field research can be made (Farenhorst and Knols, 2007).

The choice of delivery method is an important determinant of the overall effectiveness of a fungus-based vector control approach. An optimal delivery system requires maximum exposure with minimal application of fungal spores and has to ensure an effective coverage through applying fungus at sites that are both suitable for the fungal spores and attractive for mosquitoes. Several options exist for delivering fungal conidia to adult mosquitoes in the field, including indoor surface application, bednet application and point-source application. Point-source application (on specific target sites) has certain benefits as it reduces human exposure to the fungus and overall costs by decreasing the amount of conidia required. It would also allow for the application of fungi both indoors and outdoors, which could increase the effect on disease transmission through the ability to target both endophilic and exophilic mosquito species.

A recent study in Western-Kenya tested clay water storage pots as a mosquito sampling tool and showed that these pots are highly attractive resting sites for *Anopheles* mosquitoes in the field (Odiere *et al.*, 2007). Three clay pots, placed within 5 m of each sampled house, attracted relatively high numbers of both sexes of *An. gambiae s.s.* and *An. arabiensis* Patton, including females of all physiological stages (unfed, blood fed, gravid). As they are attractive to resting mosquitoes, clay pots may be suitable objects for point-source application of fungal conidia. Moreover, clay pots are widely available, low-cost, portable objects with a dark, cool and humid micro-climate that may

prove an ideal environment for fungal conidia by reducing exposure to UV-light and desiccation.

Research was conducted in the laboratory to test clay pots as delivery systems for *Metarhizium anisopliae* against *An. gambiae s.s.* and *An. funestus*. Assessments were made to determine the attractiveness of a clay pot as a resting site for *An. gambiae* mosquitoes compared to other similar, yet smaller and more portable potential resting sites, *i.e.* a PVC-pipe and a small terracotta pot. The effect of fungus impregnation on the attractiveness of clay pots to resting male and female anophelines was measured. Two different dosages of oil-formulated *M. anisopliae* conidia were tested for infectivity and virulence after spray application inside clay pots against both sexes of insecticide-susceptible *An. gambiae s.s.* and insecticide-resistant *An. funestus*.

4.2 Materials & Methods

4.2.1 Mosquitoes & Fungus

Anopheles gambiae s.s. mosquitoes were reared as described in §2.1.1. *An. funestus* mosquitoes from the FUMOZ strain originated from Mozambique and were selected for pyrethroid resistance (Hunt *et al.*, 2005). Eggs were obtained from the colony at the Vector Control Reference Unit, Johannesburg, South Africa (courtesy Prof. M. Coetzee), shipped to Wageningen, The Netherlands, and reared in a similar manner as *An. gambiae*. Green algae were added to the water trays and finely crushed dog biscuit mixed with brewer's yeast as larval food (Hunt *et al.*, 2005). For experiments, 4-7 day old mosquitoes were used.

Metarhizium anisopliae conidia were produced as described in §2.2.1 and suspended in Ondina oil for application purposes (§2.2.2). Conidial concentrations were counted (§2.2.2) and viability assessed before use in experiments (§2.2.3).

4.2.2 Clay pot bioassays

Handmade Ghanaian clay pots (Afrikaad, Barendrecht, The Netherlands) of approximately 38 cm in diameter and 35 cm in height were used. The size of the pot opening was on average 14 cm, and the inside surface approximately 0.45 m^2. Conidial formulations were applied inside the pots one day prior to experiments, using a SATA minijet 4 HVLP spray gun (vd Belt, Almere, The Netherlands) at a constant pressure of 1.5 bars. A specially designed apparatus rotated the pot while spraying at a speed of approximately 16 rotations per minute, whilst also automatically moving the spray gun vertically up and down during spraying (Figure 4.1). The pot opening was covered

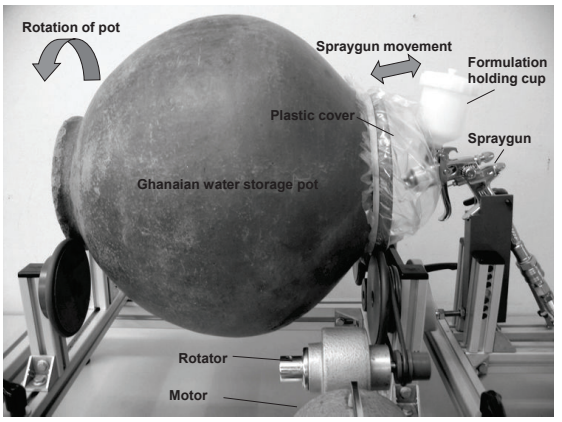

Figure 4.1: *Spray application apparatus for applying oil-formulated conidia of M. anisopliae inside Ghanaian water storage pots.*

during spraying with thin plastic in which a small hole was made in the centre for the spray gun nozzle. Test pots were first sprayed with 30 ml of oil, left to dry for 2 hours and subsequently treated with an additional 15 ml of either 1.2×10^9 or 3×10^8 conidia/ml formulation, reaching an end-concentration of 4×10^{10} or 1×10^{10} conidia/m^2 respectively. Control pots were sprayed with 45 ml of Ondina oil without conidia.

Pots were placed in climate-controlled rooms (27$\pm$2°C, 80$\pm$10% RH) and sealed off with plastic. In each pot, 50 male and 50 female mosquitoes were released through a small hole, drilled in the bottom of each pot, with a mouth aspirator. Mosquitoes were exposed for 17 hrs, having access to a 6% glucose solution through freshly soaked cotton wool pads on the rim of the pot opening, against the plastic. Subsequently, mosquitoes were transferred to a holding cage by securing a holding cage sleeve over the pot opening, removing the plastic and lifting the pot and cage vertically. With gentle blowing most mosquitoes took flight into the holding cage. The remaining individuals were retrieved through aspiration.

Mosquito survival was determined and cadavers checked for sporulating *M. anisopliae* (§2.2.4). For *An. gambiae s.s.*, a total of nine test replicates with 4×10^{10} conidia/m^2 and three control replicates were conducted on three consecutive days, besides three test and control replicates with 1×10^{10} conidia/m^2 on two consecutive days. For *An. funestus* three test replicates with 1×10^{10} conidia/m^2 and two control replicates were conducted on a single day.

4.2.3 Attractiveness tests

The attractiveness of a dry clay pot and a wet clay pot (containing 1.5 litres of water) was compared to a small terracotta pot (22 cm high, 18 cm diameter, 6 cm opening) and a dark-grey PVC-pipe (50 cm long, 15 cm diameter), which were considered as other potential point-source objects. The four objects were placed on the floor in the centre of a large cage of $3\times3\times2.5$ m inside a climate-controlled room ($27\pm2^{\circ}$C, $80\pm10\%$ RH). Subsequently, 80-90 male and 80-90 female *An. gambiae* mosquitoes were released for 3 hrs, after which the number of resting mosquitoes in each object was counted. Three replicate tests were performed in which the position of the four tested objects was randomized.

The effect of fungal formulation on clay pot attractiveness was tested in three replicate tests in which a clean clay pot and a clay pot impregnated (as described above) with 4×10^{10} conidia/m^2 were placed 1 m apart in the centre of the cage and 80 male and 80 female mosquitoes were released for 4 hrs. Resting proportions were determined from the mosquito numbers found resting inside each pot.

4.2.4 Data analysis

Mosquito survival data were fitted to the Gompertz distribution model of which the equations were used to compute LT_{50}-values (Scholte *et al.*, 2003a). Differences in mean LT_{50}'s between infected and control groups were analysed with an Analysis of Variance (ANOVA). Differences in survival of treated and control mosquitoes were analysed using Kaplan-Meier pair-wise comparisons (§2.4.2). Mosquito numbers found resting in the four objects were compared using a χ^2-test, with evenly distributed proportions as expected values.

4.3 Results

4.3.1 Clay pot bioassays

A total of 858 *An. gambiae s.s.* mosquitoes were exposed to a conidial dose of 4×10^{10} conidia/m^2, of which $95.0 \pm 1.2\%$ (mean$\pm$SE) acquired a fungal infection. The lower conidial dose, of 1×10^{10} conidia/m^2, was able to infect $91.5 \pm 0.6\%$ of the 267 exposed *An. gambiae* mosquitoes. For *An. funestus* a total of 233 mosquitoes were exposed to 1×10^{10} conidia/m^2, of which $91.8 \pm 1.2\%$ showed fungal infection after death.

Mosquito survival curves showed a close fit (96%) to the Gompertz distribution model. For both fungal dosages, the computed survival curves of infected male and female *An. gambiae* and *An. funestus* mosquitoes showed a significant reduction in longevity compared with the control mosquitoes (Figure 4.2). Kaplan-Meier pair-wise comparison of the survival curves showed that in all tested groups longevity of fungus-exposed mosquitoes was significantly reduced compared to control mosquitoes ($P<0.0001$). There were no significant differences between male and female mosquito longevity within the control and treatment replicates of *An. gambiae* and *An. funestus* ($P>0.05$).

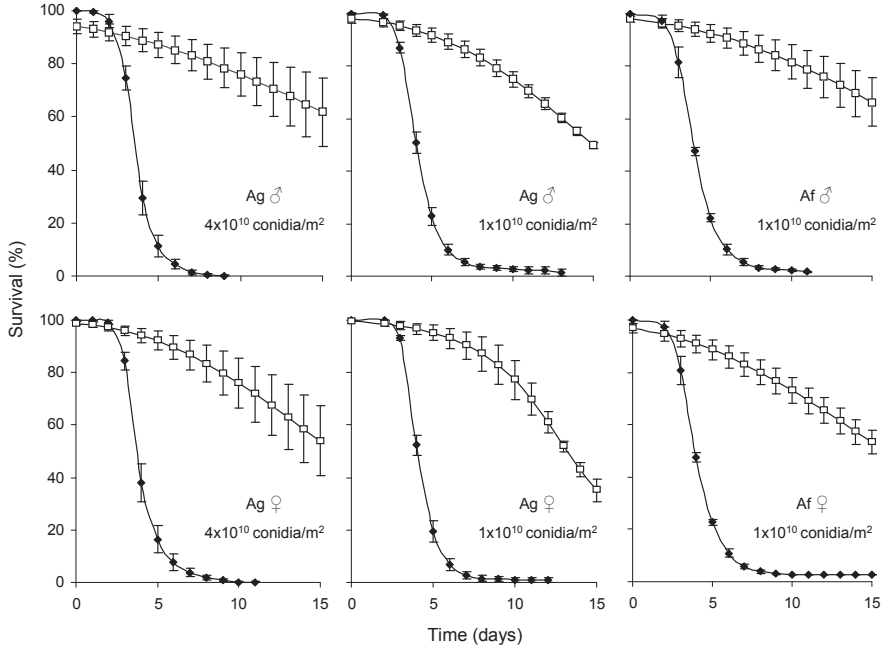

Figure 4.2: *Efficacy of fungus-impregnated clay pots. Survival curves of insecticide-susceptible An. gambiae s.s. (Ag) and resistant An. funestus (Af) after exposure to clay pots sprayed with M. anisopliae formulation (closed symbols) or oil only (open symbols). Data show mean±SE % survival of males (top) and females (bottom) exposed to 4×10^{10} or 1×10^{10} conidia/m^2.*

LT_{50}-values of the control and test treatments were calculated using the parameters of the computed survival functions (Table 4.1). Where control mosquito LT_{50}'s ranged between 13-20 days, this was reduced to only 4 days in *Metarhizium*-infected groups (Table 4.1). For all tested mosquito species and fungal dosages, pair-wise comparisons showed that the differences in LT_{50}-values between the control and infected mosquitoes were highly significant ($P<0.0001$) for all tested mosquito species and fungal dosages (Table 4.1). There were no significant differences between male and female LT_{50}-values for the control and infected groups of *An. gambiae* and *An. funestus* ($P>0.05$).

Table 4.1: LT_{50}-values (mean±SE) of male and female *An. gambiae s.s.* and *An. funestus* after exposure to clay pots treated with oil only (control) or oil-formulated *M. anisopliae* (fungus) doses of 4×10^{10} or 1×10^{10} conidia/m^2.

			Mean LT_{50} ± SE*		
Species	**Dosage**	**Sex**	**Control**	**Fungus**	**P****
A. gambiae s.s.	$4x10^{10}$ conidia/m^2	Male	19.26 ± 4.49	3.54 ± 0.15	<0.001
		Female	16.01 ± 2.82	3.84 ± 0.17	<0.001
A. gambiae s.s.	$1x10^{10}$ conidia/m^2	Male	14.97 ± 0.22	4.03 ± 0.11	<0.001
		Female	13.37 ± 0.34	4.07 ± 0.08	<0.001
A. funestus	$1x10^{10}$ conidia/m^2	Male	19.54 ± 2.56	3.91 ± 0.06	<0.001
		Female	16.01 ± 2.82	3.91 ± 0.07	<0.001

*Standard error of means **ANOVA, pair-wise comparison

4.3.2 Attractiveness

In total, 219 male *An. gambiae* mosquitoes were released and retrieved, of which on average 47.4 ± 7.2% was found resting inside one of the four tested objects. For females (n=245) this was 81.8 ± 2.0%. The remaining mosquitoes were found on the cage netting. Resting male and female mosquitoes showed a high preference for the dry and the wet clay pots compared with the small terracotta pot and the PVC pipe (Figure 4.3). In both the wet and dry large clay pots there were significantly higher numbers of male ($P<0.0001$) and female ($P<0.0001$) mosquitoes found resting than in the other two objects (Figure 4.3). The dry and the wet clay pot were equally attractive, as there was no significant difference between male ($P=0.88$) and female ($P=0.42$) presence in either of these treatments.

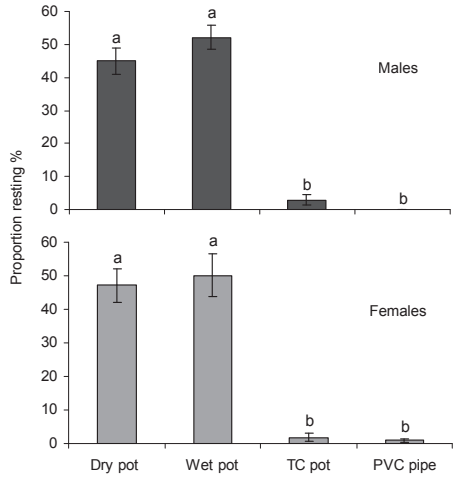

Figure 4.3: *Attractiveness of point-sources. Proportion (%) of resting male (top) and female (bottom) mosquitoes (mean±SE) in four potential resting sites; a dry clay pot (Dry pot), a wet clay pot (Wet pot), a small terracotta pot (TC pot) and a PVC pipe. Significant differences are indicated by non-corresponding letters*

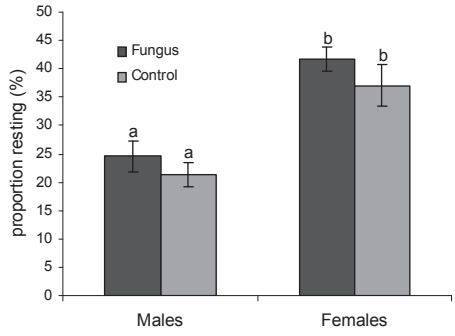

Figure 4.4: *Effect of fungus on attractiveness. Proportion (%) of resting mosquitoes (mean±SE) in a a clean clay pot (Control) and a pot sprayed with Metarhizium formulation (Fungus). Significant differences are indicated by non-corresponding letters*

In a separate comparison, the attractiveness of a fungus-impregnated and a clean clay pot was compared. Both male and female *An. gambiae* mosquitoes were found resting inside the two objects after three hours (Figure 4.4), although significantly more females than males were attracted to both the impregnated pot (P=0.003) and the control pot (P=0.004). There were no significant differences in the numbers of male (P=0.71) and female (P=0.77) mosquitoes between the untreated and fungus-treated pot, implying that the attractiveness of a clay pot as a mosquito resting site was not affected by impregnation with oil-formulated fungal conidia.

4.4 Discussion

Clay water storage pots showed potential as point-source delivery objects for entomopathogenic fungi against adult anophelines. Significantly more mosquitoes were found resting in large clay pots compared with a small terracotta pot and a PVC pipe. Dry clay pots have been shown to be attractive resting sites for both sexes of *Anopheles* mosquitoes in Kenyan field settings (Odiere *et al.*, 2007). Wet clay pots, *i.e.* with a layer of water maintained inside, may prove to be even more attractive to anopheline mosquitoes, especially under more arid environmental conditions, because mosquitoes generally prefer to rest in cool and humid places (Clements, 1992). In this study, the wet and dry pot did not differ in attractiveness to *An. gambiae*, although this could be due to the high humidity in the experimental room.

Fungus impregnation did not affect the attractiveness of clay pots, which is consistent with results from previous studies that showed that formulated *M. anisopliae* conidia were not repellent to *An. gambiae* mosquitoes (Scholte *et al.*, 2004b). For future implementation, the attractiveness of clay pots may be further enhanced through application of additional mosquito attractants inside the pots. Mosquitoes have been shown to be attracted to several host odours, including carbon dioxide (CO_2) and a suite of other kairomones (Zwiebel and Takken, 2004; Njiru *et al.*, 2006). The possibility for adding attractants or compounds that induce landing responses by *An. gambiae*, *e.g.* 2-oxo-pentanoic acid (Healy *et al.*, 2002), has potential for enhancing the number of resting mosquitoes inside the pots and hence the numbers targeted with fungus.

Clay was shown to be suitable material for spray application of oil-formulated conidia. Fungal conidia remained infective and virulent after formulation in mineral oil and spray-application inside clay pots. For field deployment, further application optimizations may be required. For example, although the rotating spray apparatus was highly effective and consistent in applying spores in the laboratory, manual application with hand-held spray guns might prove more feasible for large-scale field implementation. Furthermore, experiments used relatively high spore doses and long exposure times.

Although it may be reasonable to assume that most mosquitoes that choose pots as resting sites will remain inside from at least dusk till dawn, especially after a blood meal (J.D. Charlwood, pers. commun.), the impact of lower fungal doses and shorter exposure times would need to be tested. Preliminary tests showed similar fungal infectivity and virulence after 6 hrs exposure compared to 17 hrs (data not shown).

Fungus-impregnated clay pots were equally infective to male and female *An. gambiae* and *An. funestus* mosquitoes. The ability to target also male anophelines may result in a higher infection coverage of females through horizontal transmission of conidia during mating, which was shown possible under laboratory conditions (Scholte *et al.*, 2004b). The survival curves and LT_{50}-values were consistent with previous studies on the same fungal strain (Scholte *et al.*, 2004b). Furthermore, *M. anisopliae* was for the first time shown to be effective against insecticide-resistant anophelines. Fungal infection induced a significant reduction in survival of the pyrethroid-resistant *An. funestus*. By killing the majority ($>$95%) of mosquitoes within 7 days, regardless of their insecticide resistance status, fungi have high potential for significantly reducing malaria transmission (Hancock *et al.*, 2009).

Pots provide possibilities for both indoor and outdoor application of fungal spores, which could increase the infection coverage and effectiveness of this delivery method. As point-source application requires only small areas to be impregnated, pots may provide a more cost-effective mode of delivery than indoor spraying of large surfaces or cotton ceiling cloths, which were used previously in Tanzania (Scholte *et al.*, 2005). Moreover, the relatively dark, humid and cool environment inside clay pots could be expected to be more beneficial for conidial persistence. Field experiments will, however, be required to test their efficacy under realistic environmental conditions.

Considering that clay pots are potential attractive anopheline resting sites, suitable fungal spore carriers and portable point-source objects, they have potential as effective field delivery tools for fungi. Evidently, the greatest disease control benefit would be reached when novel fungal-based vector control measures are applied in integrated vector management (IVM) strategies. In that sense, clay pots show great potential as they have many features that would allow their incorporation in existing control measures. For instance, fungus-impregnated pots could easily be combined with insecticidal measures such as ITNs or IRS. Moreover, as some public health insecticides exert excito-repellent effects (Roberts *et al.*, 2000), the provision of outdoor lethal resting sites especially may improve the control impact on mosquito populations. The observed effectiveness of fungal entomopathogens against insecticide-resistant *Anopheles* mosquitoes further supports the idea that by complementing existing intervention tools, fungus-based vector control measures may increase the malaria control impact, especially in areas with high levels of insecticide-resistance.

5

Netting for fungal spore delivery

Abstract

The use of netting materials for the delivery of fungal spores to adult malaria mosquitoes was evaluated. Tests were conducted to investigate the efficacy of *Beauveria bassiana* spores after application on netting and effects of formulation, application method, netting material and nature of mosquito contact. *Beauveria* had a twice as high impact on *Anopheles gambiae s.s.* longevity when suspended in Shellsol solvent and applied through spraying (HR=2.12, 95% CI=1.83-2.60, $P<0.001$). Polyester and cotton bednets were the most effective substrates for mosquito infections, with highest spore viability on cotton nets. Fungal impact was highest in mosquitoes that had passed through large-meshed impregnated nets, with <30 min spore contact killing $>90\%$ within 10 days. Results indicate that the use of fungal spores dissolved in Shellsol and sprayed on small-meshed cotton eave curtain nets would be the most promising option for field implementation. Biological control with fungus-impregnated eave curtains could provide a means to target host-seeking mosquitoes upon house entry and could be used in integrated vector management (IVM) strategies, in combination with chemical vector control measures, to supplement malaria control in areas with high levels of insecticide resistance.

This Chapter has been submitted for publication in a slightly different form as:

Farenhorst M., Hilhorst A., Thomas M.B. & Knols B.G.J., 2010.

Development of fungal applications on netting substrates for malaria vector control.

Journal of Medical Entomology 2010, in press.

5.1 Introduction

Deployment of entomopathogenic fungi against malaria vectors requires application and delivery methods that can effectively target adult mosquitoes with fungal spores. Fungal spores can be applied through spraying, dipping, coating or painting suspensions on several substrate types, including cotton cloth, clay and paper (Scholte *et al.*, 2005; Bell *et al.*, 2009; Blanford *et al.*, 2009; Mnyone *et al.*, 2009a). Considering that fungi have potential for integration in existing malaria control strategies (Hancock, 2009), applications need to be compatible with chemical-based interventions such as insecticide-treated bednets (ITNs) and indoor residual spraying (IRS).

The development of delivery systems that maximize mosquito infection rates, enhance spore persistence and can be integrated into existing malaria interventions is one of the remaining challenges for practical use of fungi for malaria control. Exposure to UV-light and high temperatures is known to negatively affect fungal spore viability (Fernandes *et al.*, 2007; Braga *et al.*, 2001) and application in cool and shaded sites could be a means to enhance spore persistence. Two potential delivery methods that consider these requirements are indoor application of spores on ceiling cloths (Scholte *et al.*, 2005) and point-source application in clay water storage pots (Chapter 4). Cool and dark microclimates could not only offer protection to detrimental environmental conditions but also be attractive sites for resting anopheline mosquitoes. A possible downside of targeting resting anophelines, however, is that mosquito resting behaviour can vary greatly between species and environments (Githeko *et al.*, 1996; Mahande *et al.*, 2007). Chemical-based interventions could also influence mosquito behaviour, as excito-repelling insecticides (Roberts *et al.*, 2000; Pates and Curtis, 2005) may shift mosquito preference to resting outdoors. In areas where numerous potential resting sites are available, mosquitoes may not end up in fungus-impregnated locations. A complementary approach, therefore, might be to target host-seeking mosquitoes.

Several important *Anopheles* mosquito species are known to be endophagic and to prefer to feed indoors at night (White, 1974). Doors, windows and eaves are main points of house entry for anophelines and screening of these entry routes can offer successful malaria prevention (Lindsay *et al.*, 2003; Kirby *et al.*, 2009). A possible fungal delivery strategy would thus be to target host-seeking mosquitoes as they enter houses. Fungal application on house screens, such as eave curtains may provide a suitable means to infect anophelines at an early life stage. To enable the development of such novel systems, it is essential to evaluate the efficacy of spores after application on potential house screening materials.

The aim of the current study was to measure the efficacy of fungus-treated netting against malaria vectors, and specifically the infectivity and virulence of *Beauveria*

bassiana against female *An. gambiae s.s.* mosquitoes. Effects of formulation and application method were assessed and used to evaluate different spore doses and exposure times. Fungal viability and infectivity was tested for three different net types made of polyester or cotton fibres. Small and large-meshed polyester nets were compared to evaluate the effects of mesh size and resultant type of contact on fungal efficacy.

5.2 Materials & Methods

5.2.1 Mosquitoes & Fungus

Experiments used 3-6 day old female *An. gambiae s.s.* mosquitoes, which were reared as described in §2.1.1. For contact assays, mosquitoes were selected on a strong response to human odour the previous day and deprived of sugar overnight. Spores of *Beauveria bassiana* were produced as described in §2.2.1and suspended in Ondina oil or Shellsol solvent for application (§2.2.2).

5.2.2 Netting

Three net types, two polyester and one cotton, were tested as substrate for fungal applications (Figure 5.1). Polyester textile netting (PT), produced by Van Heek Textiles

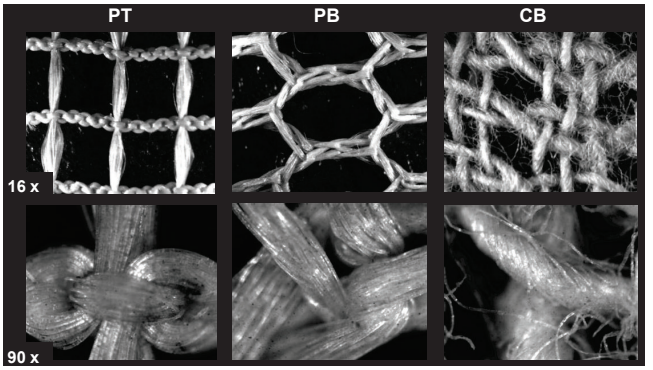

Figure 5.1: *Photos of polyester textile (PT), polyester bednet (PB) and cotton bednet (CB) substrates at 16× (top) and 90× magnification (bottom).*

B.V. (Losser, The Netherlands), consisted of 150 denier square-knitted multifilament polyester fibres. Two PT mesh sizes were tested, a small-meshed net with 56 holes/cm^2

and a large-meshed net with 15 holes/cm^2. Polyester bednet material (PB) from Vestergaard Frandsen (Lausanne, Switzerland) consisted of 3 mm thick 100% multifilament polyester fibres, warp-knitted in a round-meshed 150 denier net. Two PB mesh sizes were tested, a small-meshed net with 28 holes/cm^2 and a large-meshed net with 12 holes/cm^2. Cotton bednet material (CB) from Klamboewinkel B.V. (Groningen, The Netherlands) consisted of 100 denier square-knitted fibres of 100% cotton, with a small mesh of 81 holes/cm^2. Large-meshed cotton netting was not available. For net type experiments, net pieces were hand-washed in 5 L tap water with approx. 5% detergent and 1% bleach and subsequently rinsed five times in tap water.

5.2.3 Application

Pieces of netting (15×25 cm) were treated with 5 or 10 ml *B. bassiana* suspensions (between 10^{10} and 10^{11} spores/ml). Spray applications used a SATA minijet 4 HVLP spray gun (vd Belt, Almere, The Netherlands) at a constant pressure of 1.5 bars. Net samples were attached to a 1 m^2 vertical spray zone within a laminar-flow hood and sprayed evenly at a distance of 30 cm. Dip application was performed by submerging a piece of net in fungal formulation, spreading it manually and subsequently letting it dry horizontally on a wire frame. Net samples were impregnated one day prior to experiments and dried overnight in a climate-controlled room (27±1°C, 80±10% RH). For controls, nets were treated with the solvent only.

5.2.4 Infection assays

Impregnated nets were placed inside a PVC-tube of 15 cm length and 8 cm diameter, covering the entire inside surface. Both ends were sealed with plastic microwave foil prior to releasing 40 *An. gambiae* females in each tube via aspiration (as described in more detail in §3.2.5). After exposure, mosquitoes were transferred to plastic holding buckets of 20 cm diameter and 25 cm height via free flight. Mosquito survival was monitored daily and checked for fungal infection as described in §2.2.4.

5.2.5 Contact assays

A contact assay was constructed to simulate an eave curtain and mimic the type of contact of mosquitoes attempting to pass through. A 20×20 cm net was placed in the centre of two plastic cylinders (25 cm long, 15 cm diameter), which were closed off on each end with gauze. Experiments took place in a climate-controlled room (27±1°C, 80±10% RH) under red light conditions at the simulated time of dawn, as this time-point corresponded with morning hours and was sufficient to give active and responsive

mosquitoes for these laboratory evaluations. Experiments used forty hungry females per replicate, which were released in the left cylinder and attracted to the opposite side by a human hand placed behind the gauze. They were given 30 minutes to try and cross the netting in the centre, after which proportions in both cylinders were recorded, the set-up dismantled and groups in both cylinders transferred to separate holding cages via free flight. Mosquito survival was monitored daily, body size was determined by measuring right wing lengths under the binocular (to the nearest 0.01 mm) (Briegel, 1990) and cadavers verified for fungal infection (§2.2.4).

5.2.6 Experiments

Formulation
Effects of formulation were tested by comparing fungal efficacy of Ondina and Shellsol suspensions on large-meshed (12 holes/cm^2) polyester bednets. Net samples were sprayed with 5 ml of 10^{11} spores/ml *Beauveria* suspension or solvent only (controls) and three replicate groups of 40 females were exposed for 30 min.

Application method
Effects of application method, *i.e.* spraying or dipping, were tested on large-meshed and small-meshed polyester bednet (PB) and polyester textile (PT). Net samples were sprayed with or dipped in 5 ml of 10^{11} spores/ml *Beauveria*-Shellsol suspension or Shellsol only (controls). Survival data were obtained from four replicate groups of 40 females exposed for 30 min.

Net type
Effects of net type on *Beauveria* viability and infectivity were tested using washed pieces of small-meshed polyester textile (PT) polyester bednet (PB) and cotton bednet (CB) material, sprayed with 5 ml of 10^{11} spores/ml *Beauveria*-Shellsol formulation. Spore viability was assessed 1 day, 1, 2 and 4 weeks after application by measuring spore germination rates from 1×1 cm net pieces kept in a climate room (with sprayed papers as positive controls). Spore infectivity was measured 1 day after application by exposing three groups of 40 females for 5 min, to resemble potentially short mosquito contact in the field.

Contact type
Effects of contact type, *i.e.* passage or non-passage through netting in the contact assay set-up, were tested using large (12 holes/cm^2) and small-meshed (28 holes/cm^2) polyester bednet (PB) material sprayed with 5 or 10 ml of 5×10^{10} *Beauveria* spores/ml (or Shellsol only for controls). Three replicate groups of 40 females were released for 30 min and survival rates compared for groups that had passed though the net with groups that had not.

5.2.7 Data analysis

Impact of fungus-treated netting on mosquito survival was analyzed with Cox Regression (§2.4.3). The 95% confidence intervals (95%CI) were provided for each computed Hazard Ratio (HR). Effects of solvent, net type and contact type on fungal impact were assessed by measuring significant interactions between the test factors and fungal impact with a full Cox regression model (§2.4.3).

5.3 Results

5.3.1 Formulation & Application

Both fungal formulations induced significant reductions in mosquito survival after spray application on netting ($P<0.01$) (Figure 5.2). The onset of fungal impact was observed around three days post-exposure, which is consistent with the time-point where these hyphomycetous fungi are known to start proliferating within the insect and to approach their exponential growth phase (Bell *et al.*, 2009).

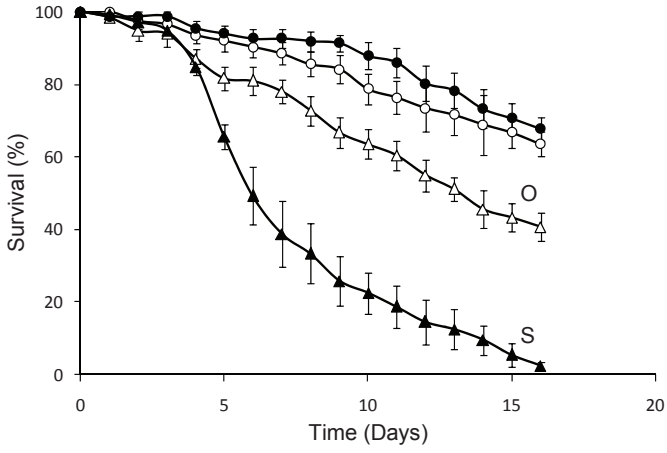

Figure 5.2: *Effect of formulation on fungal efficacy. Impact of B. bassiana (triangles) on An. gambiae survival (n=3) when suspended in Ondina oil (O) or Shellsol solvent (S) and sprayed on PB polyester nets using 5 ml of 10^{11} spores/ml, compared to solvent only treatment (circles).*

Beauveria spores had a twice as high overall impact on mosquito survival when suspended in Shellsol compared with Ondina (HR=2.12, 95%CI=1.83-2.70, P<0.001), suggesting some benefit of a lighter, more evaporative oil. All samples appeared sufficiently dry before use in exposure tests and a longer drying period of 3 days did not affect fungal efficacy (data not shown). Shellsol was, therefore, chosen as the standard formulation for subsequent netting applications.

Spraying resulted in visibly lower concentrations of fungal spores adhering to netting substrates than dipping (Figure 5.3). Interestingly, however, spraying resulted in higher fungus-induced mortality rates (Figure 5.3), with significant interactions in the effect of fungus (compared with corresponding control mortalities) and application method for all net types (P<0.05) except for small-meshed polyester bednet (PB 28 holes/cm^2, P=0.08).

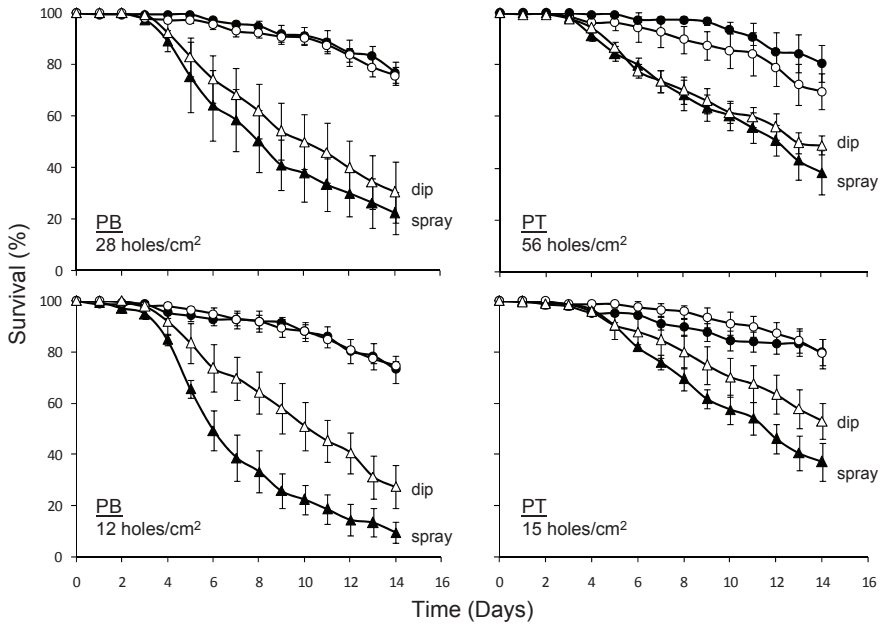

Figure 5.3: *Effects of application method, substrate type and mesh size. Mean (±SE) survival (n=4) after exposure to small- (top) or large-meshed (bottom) polyester bednet (PB) or textile (PT) substrates sprayed with (closed symbols) or dipped in (open symbols) 5 ml Beauveria suspension (10^{11} spores/ml, triangles) or solvent only (circles).*

Netting mesh size did not influence fungal efficacy in the tube exposure assay, as there were no significant interactions between fungal impact and mesh size for either net type. Polyester bednet (PB) induced between 16% and 25% higher average mortality at day 14 compared with polyester textile (PT) (Figure 5.3). Impact of *Beauveria* on PB was higher in sprayed samples, showing significant interactions between fungus and net type for sprayed small-meshed ($P<0.001$) and large-meshed nets ($P=0.003$), which was not observed for dipped nets (Figure 5.3).

5.3.2 Net type

Effects of substrate on fungal efficacy were further explored using three small-meshed net types (Figure 5.1). Spores from the same suspension were less viable after application on polyester compared to cotton or paper (Table 5.1). There was a significant drop (ca. 20%) in spore viability on polyester bednet (PB) and textile (PT) immediately after application. Cotton fibres did not reduce viability compared with the positive control paper substrate (Table 5.1). There were modest differences in the longer-term viability of spores between net types, with germination rates decreasing by 32% on PT, 22% on PB, 21% on CB and 18% on paper within four weeks (Table 5.1).

Table 5.1: Average (n=3) *B. bassiana* spore germination rates measured 1 day, 1, 2 and 4 weeks after spray application on polyester textile, polyester bednet, cotton bednet or paper.

		Spore viability (germination %)			
Net type	Material	1 Day	1 Week	2 Weeks	4 Weeks
Polyester Textile (PT)	polyester	75%	60%	47%	43%
Polyester Bednet (PB)	polyester	71%	63%	50%	49%
Cotton Bednet (CB)	cotton	91%	86%	76%	68%
Positive Control	paper	92%	90%	83%	74%

Exposure tests showed that all three net types were effective *Beauveria* spore carriers, inducing significant reductions in *An. gambiae* longevity after 5 minute exposures (Figure 5.4). Despite differences in viability, the applied spore dose induced similar mosquito mortality on PB and CB substrates ($P>0.05$). Fungal impact on mosquito survival was, however, almost twice as high on bednet material (PB) than on polyester textile (HR=1.88, 95%CI=1.21-2.78, P=0.019), indicating that PT was a less suitable substrate for mosquito-fungus infections.

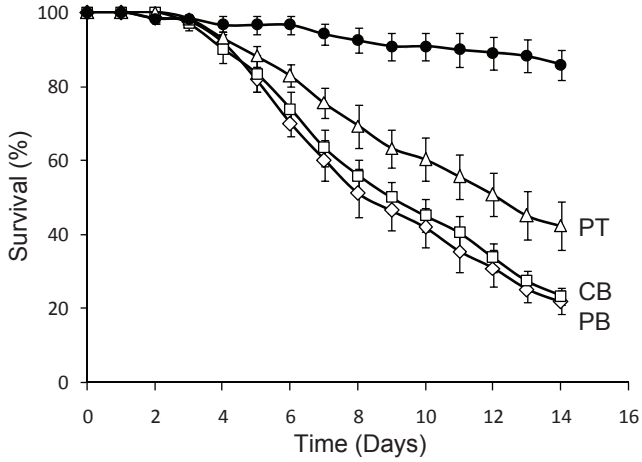

Figure 5.4: *Net type efficacy. Mean (±SE) survival (n=3) after 5 min exposure to small-meshed polyester textile (PT), polyester bednet (PB) or cotton bednet (CB) material sprayed with B. bassiana (5 ml of 10^{11} spores/ml), or PB net treated with solvent only (closed circles).*

5.3.3 Contact type & Mesh size

On average, 48% (range 40-58%) of mosquitoes passed through the 12 holes/cm^2 PB net within half an hour. Wing size sis not differ between groups that passed through the net (2.7±0.5 mm) and those that did not (2.8±0.7 mm) (P=0.319), indicating that body size did not affect the mosquito's capability to cross the net. Duration of contact differed between individuals, although <5% was not enticed to contact the net at least once. Both fungus concentrations induced significant reductions in survival regardless of contact type, killing 100% female *An. gambiae* within 16 days (Figure 5.5), with >85% showing fungal sporulation after death.

The higher spore concentration (5×10^{11} spores) had a significantly greater impact on mosquitoes that did not cross the net compared with the lower concentration (HR=1.83, 95%CI=1.23-2.71 P=0.003). There was no effect of concentration in groups that passed through the netting (HR=1.05, 95%CI= 0.81-1.69, P=0.4). Fungal impact was higher in groups that passed through the net compared with groups that did not, for both the low (HR= 3.73, 95%CI=2.25-6.12, P<0.001) and high spore concentration (HR=1.62, 95%CI=1.13-2.33, P=0.009)(Figure 5.5).

The small-holed PB net (28 holes/cm^2) that blocked all mosquitoes from passage

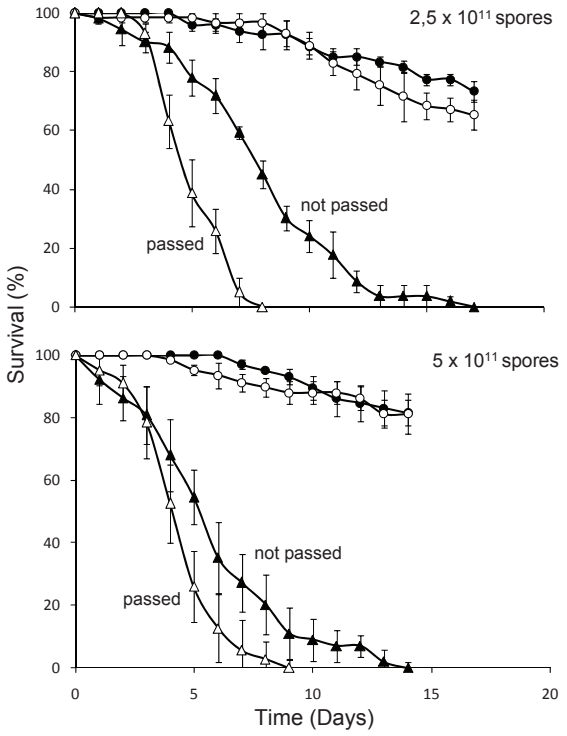

Figure 5.5: *Effects of mosquito passage. Mean (±SE) survival (n=3) after superficial contact with (not passed, closed symbols) or passage through (passed, open symbols) PB netting sprayed with 2.5×10^{11} (top) or 5×10^{11} (bottom) Beauveria (triangles), or solvent only (circles).*

was included in the contact experiments to measure the impact of mesh size on the infection efficacy. Passed and not passed mosquito data from the large-mesh treatment were pooled to compare overall impact on survival. Large- and small-meshed netting were both suitable spore carriers, enabling effective *Beauveria* infections with >90% mortality within 10 days (Figure 5.6). On large-meshed nets, there was no significant effect of spore concentration, whereas on small-meshed nets the high concentration was significantly more effective (HR=1.23, 95%CI= 1.09-1.40, P=0.001)(Figure 5.6).

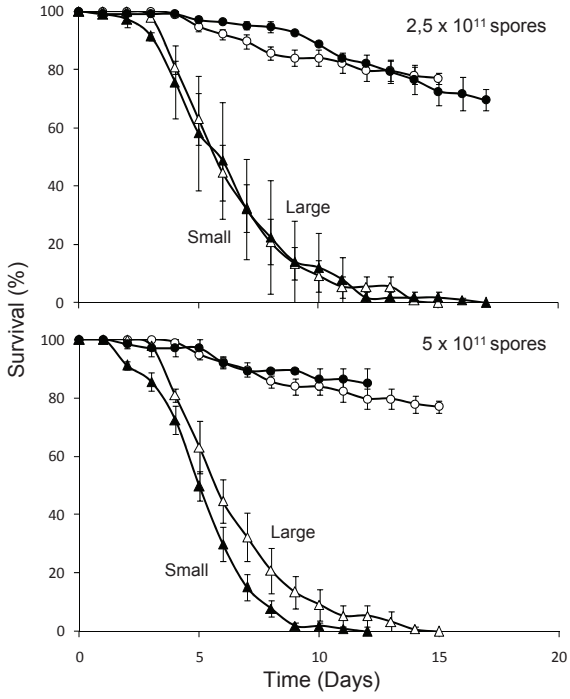

Figure 5.6: *Effects of mesh size. Mean (±SE) survival (n=3) after 30 min contact with large-meshed (Large) or small-meshed (Small) PB netting sprayed with 2.5×10^{11} (top) or 5×10^{11} (bottom) Beauveria spores (triangles), or solvent only (circles).*

5.4 Discussion

Results demonstrated that netting materials could be used to deliver lethal doses of fungal spores to adult mosquitoes. A number of influencing factors were revealed that could be important for further development of the approach. First, the nature of the oil formulation was shown to make a difference, with a light evaporative oil (Shellsol) producing more rapid and extensive mortality than a heavier, viscous oil (Ondina). The reasons for this are unclear, but it is possible that the thicker oil caused stronger adherence of spores to the netting, reducing transfer to mosquitoes. This effect was also observed in the study evaluating coating applications on paper (Chapter 3).

Second, fungal sprays were more effective than dipping applications. This was a slightly surprising result as netting represents a very 'open' target for spraying (*i.e.* there are many gaps through which spray droplets can pass, which is likely why small-meshed nets were more effective) and thus spraying results in a reduced effective spore concentration per unit area. However, it is possible that submerging the netting resulted in spore aggregations and/or high absorbance of spore suspension on or into fibres, whereas spraying may have given a finer distribution of spores on the outer surface that induced better transfer to mosquitoes. Further studies on spore retention and distribution could be informed by methods such as quantitative PCR (Bell *et al.*, 2009).

Third, efficacy varied between the different net types, with polyester textile significantly less effective than cotton in terms of fungal impact on mortality and spore persistence. Because polyester textile has smooth fibres, part of this effect could result from poor spore attachment to the net. However, the accelerated loss in spore viability over time for both polyester materials tested, suggests some sort of chemical effect, either from the polyester itself or from added chemicals such as phthalates, which are used to soften polyester fibres (Pang *et al.*, 2006). Although cotton has been successfully used in bednets and eave curtain nets (Majori *et al.*, 1987), polyester is the most widely used material for bednets and house screens because it's more durable, gives more ventilation and retains insecticides better (Curtis *et al.*, 1996; WHO, 2001). Thus, further research is required on the chemical composition of polyester and potential reactions with fungus or solvent compounds to better understand the effects on spore viability. Such research should also consider including experiments on polyolefin polymers, which are now being used within ITN fibres (Kilian *et al.*, 2008).

Finally, infection rates were highest in mosquitoes that were able to pass through impregnated netting, which was most noticeable for lower spore doses, suggesting greater physical transfer of spores as the mosquitoes traverse the net. However, overall fungal efficacy was not dependant on mesh size or the ability of mosquitoes to penetrate the netting. These results have interesting implications for different application strategies. Use of small-meshed, fungus-impregnated eave or window screens, would infect mosquitoes as they land on the netting and attempt to probe. One concern with eave curtains, however, is the effect they have on airflow and it has been suggested that larger mesh sizes might be more desirable (Majori *et al.*, 1987; Hossain and Curtis, 1989). Large-meshed fungus-impregnated eave nets would allow for more airflow whilst effectively infecting mosquitoes that traverse the net. This would not give immediate personal protection, since fungi take time to kill, but could deliver community-wide benefits by reducing the abundance of old, potentially infectious mosquitoes (Hancock, 2009; Read *et al.*, 2009). For field use, however, such an approach will likely only be acceptable when used in combination with interventions that provide personal protection.

Another possible delivery method would be to follow the ITN model and apply spores directly onto bednets. However, while the insect-pathogenic fungi *B. bassiana* and *M. anisopliae* generally pose negligible risk to human health and the environment (Zimmermann, 2007a,b; Darbro and Thomas, 2009), the safety of fungus-treated bednets would need to be tested before such interventions would be acceptable. Additionally, long-term viability of spores on bednets might be compromised by regular washing (whether this could be overcome by novel formulation or impregnation techniques is not known). Novel techniques in bednet development may, however, facilitate use of fungal spores. For instance, 'two-in-one' combination bednets are being developed, which use a slow-acting, non-irritant chemical insecticide on the apex or roof of the net and fast-killing pyrethroids on the sides (Guillet *et al.*, 2001; Oxborough *et al.*, 2008). Fungal spores may potentially be used as the slow-acting pesticide on the top part of the net, away from human contact, with repellent pyrethroids on the sides preventing blood-feeding and providing personal protection.

Delivery of fungal spores on house screens can, nevertheless, be considered more viable for field implementation in terms of acceptability and long-term efficacy due to less handling, and could provide options to integrate fungal bio-pesticides in contemporary chemical malaria vector control measures. Combined implementations show promise (Hancock, 2009) and could, for instance, be achieved by adding fungi onto insecticide-treated eave curtain nets. The widely used insecticide permethrin, however, acts as a repellent or contact irritant, which may lead to shorter resting times or reduced pick up of fungi if the two were presented together on a combination net. Combination interventions could also be spatially separated by, for example, using fungus-impregnated house screens together with LLINs, which would still allow for co-exposure of mosquitoes to both agents in a single feeding episode. Overall, results indicate that the use of biological control with fungal spores dissolved in Shellsol and sprayed on small-meshed cotton eave curtain nets would be the most promising option for field implementation, with potential for integration into chemical malaria vector control with LLINs or IRS. When used in IVM strategies, fungal bio-pesticides could provide a means to target the resistant fraction of the mosquito population, which could not only help to sustain malaria control but also to slow the spread of insecticide-resistance alleles in anopheline populations.

This laboratory study has explored the potential efficacy of fungal applications on netting and showed that spray applications of fungal suspensions on several netting substrates could be highly effective against anophelines. Although further optimization is required to realise field deployment, fungus-impregnated house screens and barriers could provide a means to target host-seeking mosquitoes and maximize the impact of fungal bio-pesticides on malaria transmission in field settings where insecticide resistance is a growing threat to contemporary malaria interventions.

6

Fungi & insecticide resistance

Abstract

Through their unique mode of action, entomopathogenic fungi provide promising alternatives to chemical malaria vector control. However, potential interactions between fungal infection and insecticide resistance, such as cross-resistance, have not been investigated. Insecticide-resistant *Anopheles* mosquitoes were shown to be susceptible to fungal infection. Four different mosquito strains with high resistance levels against pyrethroids, organochlorines or carbamates were equally susceptible to *Beauveria bassiana* infection as their baseline counterparts, showing significantly reduced mosquito survival. Moreover, fungal infection increased the impact of the key public health insecticides permethrin and DDT in resistant mosquitoes. Mosquitoes with metabolic insectecticide resistance mechanisms that were pre-infected with *B. bassiana* or *Metarhizium anisopliae* showed a significant increase in mortality after insecticide exposure compared with uninfected mosquitoes. These results show a high potential utility of fungal biopesticides for complementing existing vector control measures and provide novel products for use in resistance management strategies.

This Chapter has been published in a slightly different form as:

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

6.1 Introduction

Increasing incidences of insecticide resistance (§1.2.2) are threatening the efficiency of insecticides (N'Guessan *et al.*, 2007b) and new products with unique modes of action are required for sustainable malaria vector control (Knols and Thomas, 2006). Previous work has suggested that entomopathogenic fungi could play this role (Blanford *et al.*, 2005; Scholte *et al.*, 2005; Thomas and Read, 2007). For fungus-based biopesticides to play a prominent role in malaria control, however, an important criterion is that fungal susceptibility will remain unaffected by resistance to insecticides.

The mode of action of entomopathogenic fungi, *i.e.* infection via external contact and proliferation through the haemocoel, makes direct cross-reactions with insecticides unlikely. It has, however, been shown that indirect effects of insecticide resistance mechanisms can reduce pathogen proliferation, and this may also be the case for fungal proliferation. Enhanced concentrations of esterases in organophosphate (OP)-resistant *Culex* mosquitoes have been implicated in limiting growth of filarial worms (McCarroll *et al.*, 2000; McCarroll and Hemingway, 2002), and enhanced monooxygenase levels in pyrethroid-resistant *Anopheles* species to increase oxidative stress to the detriment of *Plasmodium* survival (McCarroll and Hemingway, 2002). Since enzymatic detoxification of insecticides is also an important resistance mechanism in *Anopheles* mosquitoes (Hemingway and Ranson, 2000), enhanced detoxification may interact with fungal metabolites, such as cyclic peptide toxins (Clarkson and Charnley, 1996; Thomas and Read, 2007), and could reduce the effect of these virulence factors.

The compatibility of fungus-based and insecticide-based control methods will also depend on the effect of fungal infection on insecticide-resistant mosquito mortality and resistance levels. Studies on insect hosts other than mosquitoes have indicated fungal infection can act synergistically with insecticides, increasing the impact of otherwise sub-lethal insecticide doses (Pachamuthu and Kamble, 2000; Furlong and Groden, 2001; Ericsson *et al.*, 2007). Mixtures of *Metarhizium anisopliae* and deltamethrin were shown to enhance the virulence of both components when tested against ticks, indicating synergistic effects that would enhance the effectiveness of low fungi and insecticide concentrations (Bahiense *et al.*, 2006). In contradiction to these findings, however, studies on the wax moth *Galleria mellonella* have indicated that the elevation of detoxifying enzymes in response to infection with *M. anisopliae* increases host resistance to organophosphate insecticides (Serebrov *et al.*, 2006). To ensure the compatibility of fungal biopesticides and chemical control tools, such potential adverse effects on resistance levels will have to be excluded for anophelines.

So far, there have been no reports on the effects of insecticide resistance mechanisms on mosquito susceptibility to fungal infection, or the effects of fungal infection

on mosquito insecticide resistance levels. This study investigated the effectiveness of two potential fungal biocontrol pathogens; *Metarhizium anisopliae* and *Beauveria bassiana*, against insecticide-resistant *Anopheles* mosquitoes. Effects of insecticide resistance status on fungal susceptibility and effects of fungal infection on insecticide resistance levels were tested in a diverse suite of resistant *Anopheles* colonies.

6.2 Materials & Methods

6.2.1 Mosquitoes

Experiments took place from January-April 2008 in the insectary of the National Institute for Communicable Diseases, Johannesburg, and used five different mosquito strains of three *Anopheles* species. Table 6.1 shows an overview of the mosquito colony names, abbreviations, resistance selection and colony origins.

Table 6.1: Details of mosquito species used; abbreviations, colony names, resistance selection and origin.

	Abbreviation	Colony name		Selected for	
Species	**(used in text)**	**Baseline**	**Resistant**	**resistance to**	**Origin**
A. funestus	Af^{Perm}	FUMOZ	FUMOZ-R	Permethrin	Southern Mozambique
A. arabiensis	Aa_1^{DDT}	MBN	MBN-DDT	DDT	Mamfene, South-Africa
	Aa_2^{DDT}	SENN	SENN-DDT	DDT	Sennar, Sudan
A. gambiae s.s.	Ag^{Bend}	SOG	BENROG	Bendiocarb	Obuasi, Ghana
	Ag^{MR}	-	GAH	-	Ahafo, Ghana

The *An. funestus* colonies (Af^{Perm}) originated from collections in southern Mozambique. Mosquitoes from the baseline colony (FUMOZ) were selected for permethrin resistance for a period of two years, which resulted in the highly resistant colony FUMOZ-R (Table 6.2) of which adults show 0-1% mortality when exposed to 1% lambda-cyhalothrin for 1hr (Hunt *et al.*, 2005). The two *An. arabiensis* colonies (Aa_1^{DDT} and Aa_2^{DDT}) originated from Mamfene, KwaZulu/Natal, South-Africa (MBN) and from Sennar, south-central Sudan (SENN) respectively. The SENN baseline colony was selected for DDT resistance for 16 generations after which SENN-DDT adults showed 12.1% mortality when exposed to 4% DDT and 0% when exposed to 0.75% permethrin for 1 hr (Matambo *et al.*, 2007). This study, however, used adults of the F_{50}-F_{54} generation, which showed lower baseline resistance levels to DDT and permethrin.

The *Anopheles gambiae s.s.* colony used in survival assays (Ag^{Bend}) originated from Obuasi, Ghana (SOG), of which mosquitoes were selected for high levels of bendiocarb resistance (BENROG) (Table 6.2). The *An. gambiae s.s.* colony used in insecticide resistance assays (Ag^{MR}) originated from Ahafo, Ghana (GAH). This colony was not selected for resistance to insecticides in the laboratory, but carried quantified levels of resistance to all four classes of insecticides (Table 6.2).

A summary of the insecticide susceptibility status of the tested mosquito colonies is given in Table 6.2. The baseline colonies SOG and FUMOZ exhibited low levels of resistance which were increased by orders of magnitude in the selected lines.

Table 6.2: Insecticide susceptibility status of the mosquito colonies and species used.

	Pyrethroid		**Carbamate**		**Organo-phosphate**	**Organo-chlorine**	
Colony	0.75% Permethrin	0.05 % Deltamethrin	0.1% Bendiocarb	0.1% Propoxur	5% Malathion	4% DDT	4% Dieldrin
A. funestus							
FUMOZ	R	R	S	R	S	S	S
FUMOZ-R	R	R	S	R	S	S	S
A. arabiensis							
MBN	S	S	S	S	S	S	S
MBN-DDT	R	R	R	S	R	R	S
SENN	R	S	S	S	S	S	R
SENN-DDT	R	R	S	S	R	R	R
A. gambiae s.s.							
SOG	R	R	S	R	S	R	R
BENROG	R	R	R	R	S	R	R
GAH	R	R	R	R	R	R	R

S = insecticide susceptible and R = insecticide resistant. Colonies were tested for their susceptibility to the insecticides listed according to the WHO standard protocol and labelled susceptible when showing mean percentage mortalities >95%.

Larvae were reared in plastic bowls filled with distilled water (§2.1.1). For *An. funestus*, the water was supplemented with green algae. Larval food contained a mixture of finely crushed dog biscuit and brewer's yeast (Hunt *et al.*, 2005). Adults were collected daily from the bowls and transferred to holding cages in which cotton wool soaked in 10% glucose solution was provided. All species were maintained at 25°C and 80% RH with a 12-hr day/night photoperiod and artificial 45-min dusk/dawn cycles. Experiments used 2-5 day old mosquitoes.

6.2.2 Fungus exposure

Adult mosquitoes were exposed to 100 mg of dry *Metarhizium anisopliae* or *Beauveria bassiana* conidia using the suspensor set-up previously shown to give reliable infections (Scholte *et al.*, 2003a). Females were exposed to one suspensor in a holding cage for 24 hours, after which the suspensor was replaced with clean cotton wool soaked in 10% glucose solution. Control mosquitoes were exposed in the same way, but to a suspensor without fungus.

6.2.3 Survival bioassays

The effect of fungal infection on mosquito survival was tested in baseline colonies and insecticide-resistant colonies of *An. funestus* (Af^{Perm}), *An. arabiensis* from South Africa ($Aa_1{}^{DDT}$), *An. arabiensis* from Sudan ($Aa_2{}^{DDT}$) and *An. gambiae s.s.* from Obuasi, Ghana (Ag^{Bend}). For each colony nine test and nine control replicates were performed on three consecutive days, exposing approximately 30 mosquitoes per replicate to dry spores of *B. bassiana* or control suspensors for 24 hrs. For the baseline *An. funestus* colony, six replicates were performed. Mosquito mortality was recorded daily and cadavers checked for fungal infection as described in §2.2.4.

6.2.4 Permethrin resistance assays

Effects of fungal infection on permethrin resistance were tested in resistant colonies of *An. funestus* (Af^{Perm}), *An. arabiensis* from Sudan ($Aa_2{}^{DDT}$) and *An. gambiae s.s.* from Ahafo, Ghana (Ag^{MR}). A three day waiting period was chosen between fungal exposures and assessments for insecticide resistance, to allow for some progression of the fungal infection whilst not losing large numbers through death. Mosquitoes from the same cohort received either a control, *Beauveria* or *Metarhizium* treatment, of which 25 females per treatment were exposed three days later to a control paper and 25 females to a filter paper treated with 0.75% permethrin for 1 hr, according to the WHO protocol (WHO, 1998) as described in §2.3.2. Mosquitoes were subsequently transferred to clean holding tubes and provided with 10% glucose solution. The proportion of dead mosquitoes was scored 24 hrs post-insecticide exposure (§2.3.3).

Five replicates were performed per mosquito species and for each group, mortality per replicate exposed to insecticide was corrected using mortality data of counterparts exposed to control papers, according to Abbott's formula (WHO, 1998) as described in §2.3.3. After mortality measurements, mosquitoes were removed from the exposure tubes with an aspirator, killed through drowning in 70% alcohol and checked for fungal infection as described in §2.2.4.

Using the same methods, the effect of a more advanced *Beauveria* infection was tested on *An. funestus* (Af ^{Perm}) by measuring the permethrin resistance levels of control and *Beauveria*-infected mosquitoes 5 days after fungus exposure. Five replicates of 25 mosquitoes each were performed and mortality of permethrin-exposed mosquitoes corrected for control mosquito mortality.

6.2.5 DDT resistance assays

The effect of *Beauveria*-infection on DDT resistance was tested in five separate experiments in DDT-resistant *An. arabiensis* from Sudan ($Aa_2{}^{DDT}$) and *An. gambiae s.s.* from Ghana (Ag^{MR}). Three days after *Beauveria* exposure, control and infected mosquitoes (25 females per group) were exposed to 4% DDT papers or untreated papers and mortality measured 24 hrs later, and corrected for control mortality as described for the permethrin assays.

6.2.6 Data analysis

Differences in the computed survival curves of treated and control mosquitoes were analysed using Cox' regression analyses (§2.4.3), for each tested mosquito species. To compare the impact of *B. bassiana* between each baseline and resistant colony, Cox regression interaction analyses were performed, including all main effects and possible interactions in the model (§2.4.3). To assess the impact of fungal infection on permethrin sensitivity, mortality rates of permethrin and DDT-exposed groups were corrected for mortality of their corresponding control groups (not exposed to insecticide) using the Abbott's formula (WHO, 1998) (§2.3.3). Corrected mosquito mortality was compared using a χ^2 Goodness of Fit test with GenStat 9.0 software.

6.3 Results

6.3.1 Susceptibility to fungus infection

All tested mosquito colonies, resistant and baseline *Anopheles* colonies, were susceptible to fungal infection. *Beauveria* caused 100% mortality in all eight colonies within 8-20 days (Figure 6.1), with levels of fungal infection exceeding 95% (confirmed by mosquito cadaver sporulation). Cox regression analyses showed the effect of *Beauveria* infection on mosquito survival to be significant in all tested colonies ($P<0.001$).

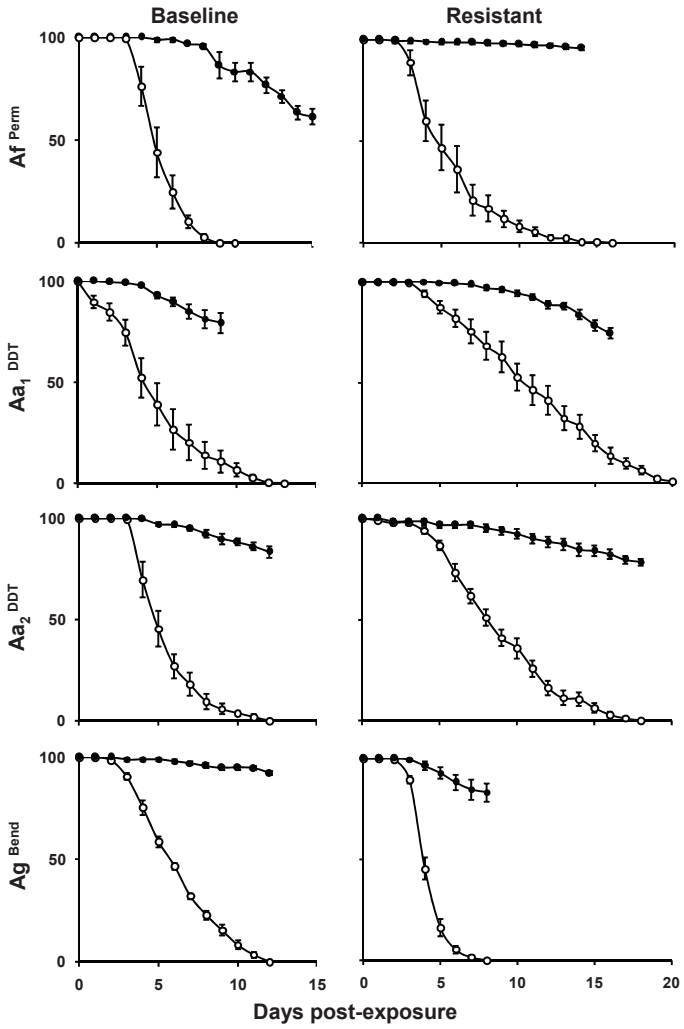

Figure 6.1: *Effect of fungus infection on baseline and insecticide-resistant mosquito survival. Mean (±SE) cumulative survival (%) of Beauveria-infected (open symbols) and uninfected mosquitoes (closed symbols) of four strains; An. funestus (Af), An. arabiensis (Aa_1 and Aa_2), and An. gambiae s.s. (Ag). Survival of baseline colonies is shown on the left and colonies selected for resistance to permethrin (Perm), DDT (DDT) or bendiocarb (Bend) on the right.*

Additionally, a highly significant interaction between fungus treatment and insecticide resistance status was found in *An. funestus* (P=0.002) and *An. gambiae s.s.* (P<0.001), indicating quantitative differences in fungal impact on mosquito survival between the baseline and insecticide-resistant colonies. *Beauveria* reduced survival more strongly in permethrin-resistant *An. funestus* mosquitoes (Hazard Ratio (HR)=47241.42; P<0.001) than in its baseline colony (HR=28.22; P<0.001), but less strongly in bendiocarb-resistant *An. gambiae s.s.* (HR=5.70; P<0.001) than in the baseline mosquitoes (HR=71.70; P<0.001). However, similar differences in Hazard Ratio's were observed in the corresponding uninfected control mosquitoes; a significantly lower daily mortality rate in permethrin-resistant *An. funestus* mosquitoes (HR=0.003; P=0.005) and higher mortality rate in bendiocarb-selected *An. gambiae s.s.* mosquitoes (HR=28.20; P<0.001) compared with their baseline colonies. This suggests that the quantitative differences in the relative impact of fungus infection on survival were caused by inherent differences in laboratory colony longevity rather than any effect of insecticide resistance status on fungal susceptibility.

6.3.2 Impact on insecticide resistance

To investigate whether fungal infection affects the expression of insecticide resistance, a series of experiments was conducted to examine pre-lethal effects of fungal infection on insecticide sensitivity in resistant mosquitoes. First, effects of *B. bassiana* or *M. anisopliae* infection were tested on the expression of permethrin resistance in three mosquito strains with known levels of resistance to this insecticide; permethrin-resistant *An. funestus* (Af^{Perm}), DDT resistant *An. arabiensis* ($Aa_2{}^{DDT}$) and a recently established, unselected, multiple resistant strain of *An. gambiae s.s.* (Ag^{MR}).

Day 3-4 mortality rates of *Beauveria*- and or *Metarhizium*-infected control groups (not exposed to insecticide) ranged between 6 and 19% for all three tested mosquito colonies, and were used to correct fungus-infected mosquito mortality rates after insecticide exposure. Insecticide-induced mortalities of uninfected mosquito groups were not corrected, since uninfected control mortalities were below 5%.

Consistent with previous observations, permethrin had the lowest impact on *An. funestus* survival and the highest impact on the *An. gambiae s.s.* colony (Figure 6.2A). Permethrin induced significantly higher mortality in mosquitoes pre-infected with *Beauveria* (P<0.001) or *Metarhizium* (P<0.001) than in the uninfected groups, in all three mosquito strains (Figure 6.2A). This enhanced impact of permethrin on mosquito survival implies that fungal infection increased the sensitivity of resistant anophelines to this public health insecticide.

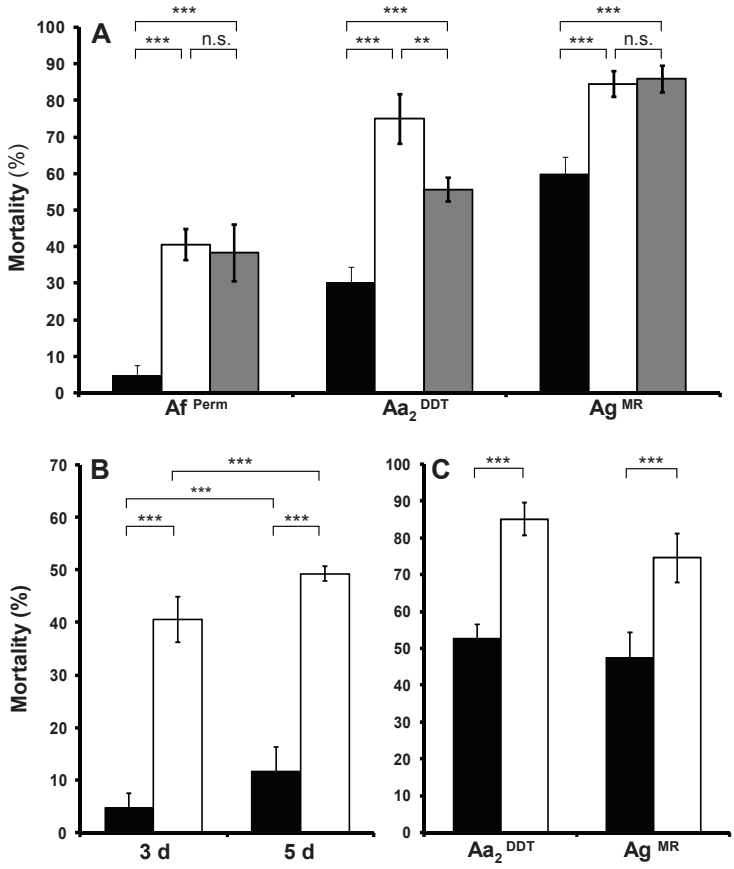

Figure 6.2: *Effect of fungus infection on mosquito insecticide resistance levels. (**A**) Corrected mean (±SE) percent mortality (n=5) 24 hrs after permethrin exposure of permethrin-resistant An. funestus (Af^{Perm}), An. arabiensis (Aa_2^{DDT}) and An. gambiae (Ag^{MR}) that were uninfected (black), or infected 3 days prior with Beauveria (white) or Metarhizium (grey). (**B**) Effect of permethrin exposure on Beauveria-infected (white) and uninfected (black) permethrin-resistant Af^{Perm} mosquitoes (n=5) at 3 days (left) and 5 days (right) post fungal infection. (**C**) Corrected mean (±SE) percent mortality 24 hrs after DDT exposure of Beauveria-infected (white) and uninfected (black) DDT-resistant Aa_2^{DDT} and Ag^{MR}) mosquitoes (n=5). Asterisks indicate significant differences **:χ^2-test P<0.01, ***:χ^2-test P<0.001.*

Both fungi induced similar increases in the impact of permethrin exposure on mosquito survival. On average, a 3-day proliferating infection increased permethrin-induced mortality rates with more than 24% (Figure 6.2A). There were no significant differences between the impact of *Beauveria* and *Metharhizium* in the *An. funestus* and *An. gambiae* colony (Figure 6.2A). In *An. arabiensis*, however, *Beauveria* infection induced significantly higher mortality levels than *Metarhizium* infection (χ^2=36.04, P<0.001), which may be caused by differences in pathogenicity of the two fungal species or by a different insecticide resistance mechanism in this mosquito species.

Using the Af^{Perm} line, the effect of a longer (5 day) proliferating *B. bassiana*-infection on permethrin resistance levels was also tested. There was a significant (χ^2=18.38, P<0.001) increase of 9% in the average permethrin-induced mortality five days after fungal exposure compared to the permethrin impact after three days (Figure 6.2B). However, mortality of the uninfected control group was also significantly higher at five days (χ^2=57.22, P<0.001), which is consistent with age-dependent responses to insecticide exposure found in several mosquito species (Lines and Nassor, 1991; Matambo *et al.*, 2007). The relative impact of fungus did not increase between three and five days after infection, and permethrin sensitivity was equivalent over this initial time course of infection (Figure 6.2B).

The effect of fungus infection on DDT resistance was assessed using DDT-resistant colonies of *An. arabiensis* (Aa_2^{DDT}) and *An. gambiae s.s.* (Ag^{MR}). Replicate samples of mosquitoes were pre-infected with *B. bassiana* spores and susceptibility to DDT was compared between the infected and uninfected group three days later. The two tested mosquito colonies showed similar levels of resistance to DDT (Figure 6.2C). In both colonies, the impact of DDT on mosquito survival was significantly higher (>25%) in *Beauveria*-infected groups compared to uninfected control groups (P<0.001) (Figure 6.2C). This increase in DDT efficacy implies that fungal infection increases the sensitivity of resistant anophelines to DDT.

6.4 Discussion

Results showed that resistance against three of the four classes of public health insecticides does not confer enhanced resistance to infection by *B. bassiana*. The fungus was highly infective and virulent to a diverse suite of resistant *Anopheles* mosquito strains. Furthermore, infection with either *B. bassiana* or *M. anisopliae* pre-lethally interfered with the expression of permethrin and DDT resistance in resistant mosquitoes, increasing their susceptibility to these insecticides.

The exact mechanisms involved in the interactions between insecticide resistance and fungal infection remain unclear. In the tested *An. funestus* (Af^{Perm}) colony, resis-

tance is mainly mediated by elevated levels of mono-oxygenases (Brooke *et al.*, 2001; Amenya *et al.*, 2005; Hunt *et al.*, 2005). In the *An. arabiensis* (Aa_2^{DDT}) colony, the West African *kdr* target-site mutation is present but does not correlate with the resistance phenotype (Abdalla *et al.*, 2007; Matambo *et al.*, 2007) and resistance is conferred metabolically through elevated levels of glutathione-S-transferase and esterases (Matambo *et al.*, 2007). Resistance mechanisms in the *An. gambiae s.s.* (Ag^{MR}) colony have not been identified and could include the West-African *kdr* target site mutation as well as metabolic detoxification.

Since metabolic resistance mechanisms were present in all tested species, it is possible that a re-allocation of insecticide-detoxifying enzymes towards fungal toxins reduced the quantity of enzymes available to target insecticides and resulted in the observed post-fungus infection decrease in resistance. However, as is the case for wild-type resistant mosquitoes (Djouaka *et al.*, 2008), there are diverse, potentially interacting mechanisms conferring resistance in our tested mosquito species, and the lack of a clear correlation between resistance genotype and phenotype complicates assessing the exact interactions between insecticide resistance mechanisms and fungal infection.

Direct effects of the neurotoxic insecticides on the fungus and its proliferation inside the mosquito were not studied. The fungus was allowed to proliferate for three days in the insect before being exposed to the insecticide. There were no differences in infection percentages between insecticide-exposed and non-exposed groups, but that could be a result of an already extensive fungus growth at day three. Especially for testing the efficacy of fungus-insecticide combinations, it would be interesting to test the effect of neurotoxic and other classes of insecticides on fungal infectivity and virulence.

Increased knowledge on fungus-insect interactions would augment options for improving fungus-based applications against mosquitoes. For example, modification of fungal spores to enhance their virulence could be used to improve the commercial effectiveness of fungus-based control methods. Genetic alterations that caused overproduction of a cuticle-degrading protease were shown to effectively increase the speed of kill of the fungus (St. Leger *et al.*, 1996). Furthermore, exploring the effectiveness of fungi against mosquito strains with other resistance mechanisms, such as resistance to microbial agents like *Bacillus thuringiensis var. israelensis* (B.t.i.) or insect growth regulators such as methoprene, would further substantiate the usefulness of fungus-based biological control tools against mosquitoes where other current control measures are failing.

Overall, the significant reductions in mosquito survival and insecticide resistance levels induced by fungal infection support the potential use of fungal biopesticides against mosquito vectors in areas where insecticide resistance is spreading, adding new product options to the very limited selection of chemicals currently available. With

their relatively slow speed of kill, considered to dramatically reduce the selection pressure for resistance development whilst killing mosquitoes before being able to transmit the malaria parasite (Read *et al.*, 2009), fungal biopesticides may provide a novel and sustainable vector control tool.

Like protozoan (Hogg and Hurd, 1995) or nematode infections (Gassmann *et al.*, 2006), fungal pathogen infection exerts an additional fitness cost for the insect. Since these costs are associated with a slower spread of resistance (Gassmann *et al.*, 2009), the additional burden of a fungal infection may reduce the speed of insecticide resistance formation in anopheline vectors. The susceptibility of resistant mosquitoes to fungal pathogens adds weight to the possibility of using biopesticides within insecticide-resistance management strategies, such as rotations or mosaics (Hemingway and Ranson, 2000), to slow the spread of resistance (Thomas and Read, 2007; Read *et al.*, 2009). The use of oil-formulated spores in point-sources such as black cotton cloths (Scholte *et al.*, 2005) and African water storage pots (Chapter 4), or on eave curtains (Chapter 5) has potential for field implementation and would allow for the integration of fungi in existing control measures.

With fungal infection reducing the expression of permethrin and DDT resistance, developing novel combination treatments may help enhance the efficacy and effective lifespan of key insecticides where resistance has reached high levels. Together, these findings provide a compelling case for viewing biopesticides and chemical insecticides not as mutually exclusive, but as complementary technologies that may improve the efficiency and sustainability of integrated malaria vector control programmes.

7

Combining fungi & insecticides

Abstract

The compatibility of the pyrethroid insecticide permethrin and two insect-pathogenic fungi, *Beauveria bassiana* and *Metarhizium anisopliae* for use in integrated mosquito control was assessed using a range of fungus-insecticide combinations against a laboratory colony and field population of resistant (*kdr*) *Anopheles gambiae s.s.* mosquitoes from West Africa. Both mosquito populations were highly resistant to permethrin but susceptible to *B. bassiana* and *M. anisopliae* infection. Combinations of insecticide and fungus showed synergistic effects on mosquito survival. Fungal infection increased permethrin-induced mortality rates in wild mosquitoes and reciprocally, exposure to permethrin increased subsequent fungal impact in both colonies. Simultaneous co-exposure induced the highest mortality; up to 70.3 $\pm$ 2% within 4 days for a combined *Beauveria* and permethrin exposure. The observed synergism in efficacy shows the potential for integrated fungus-insecticide control measures to dramatically reduce malaria transmission and enable vector control in areas where insecticide resistance has rendered pyrethroids essentially ineffective.

This Chapter has been published in a slightly different form as:

Farenhorst M., Knols B.G.J., Thomas M.B. , Howard A.F.V., Takken W., Rowland M. & N'Guessan R. 2010.

Synergy in efficacy of fungal entomopathogens and permethrin against West African insecticide-resistant *Anopheles gambiae* mosquitoes.

PLoS ONE 5(8).

7.1 Introduction

The increasing spread of insecticide resistance in the primary malaria vector species is a major threat to contemporary control efforts, which rely heavily on insecticide-based interventions such as Long-lasting Insecticide Nets (LLINs) and Indoor Residual Spraying (IRS) (Chandre *et al.*, 1999; Vulule *et al.*, 1999; Corbel *et al.*, 2007; N'Guessan *et al.*, 2007b; Nauen, 2007; Ranson *et al.*, 2009). In this regard, there is increasing emphasis on the development of novel integrated vector control strategies, and a growing body of empirical and theoretical studies suggests a potential role for insect-pathogenic fungi.

Fungal pathogens appear equally effective in infecting and killing metabolically resistant anophelines as their susceptible counterparts (Kikankie *et al.*, 2010) (Chapter 6). A recent study showed that a pyrethroid-resistant colony of *An gambiae s.s.* was even slightly more susceptible to fungal infection than an insecticide-susceptible colony (Howard *et al.*, 2010). Moreover, infection with *Metarhizium* or *Beauveria* increased permethrin and DDT sensitivity in highly resistant laboratory-reared *Anopheles* mosquitoes originating from Southern and East Africa, which was suggested to have been caused by a reallocation of insecticide-detoxifying enzymes toward fungal toxins (Chapter 6). These findings suggest potential for novel integrated vector management strategies that combine conventional and bio-pesticidal tools. Further support for this idea is provided by a recent theoretical study, which demonstrated that control strategies using both fungi and insecticide treated bednets could have greater impact on malaria transmission than control measures based on either intervention alone (Hancock, 2009). Such approaches could be of particular use in countries like Benin, where high levels of pyrethroid resistance are already threatening the impact of conventional vector control tools (N'Guessan *et al.*, 2007b; Yadouleton *et al.*, 2010).

Pyrethroid-treated LLINs are currently the primary malaria prevention intervention in Africa and, realistically, fungal-based measures will far more likely be implemented in combination with LLINs than used as a substitute. For such combination interventions to be successful, neurotoxic insecticides and fungal entomopathogens need to be compatible. The current study, therefore, explored the interactions between pyrethroids and fungi when used in combination. Several combinations of *M. anisopliae*, *B. bassiana* and permethrin were tested against laboratory-reared and field-collected West African *An. gambiae s.s.* mosquitoes, which were highly resistant to pyrethroids and DDT through the expression of the *kdr* gene. For optimum design of integrated fungus-insecticide field delivery formats, effects of timing and sequence of exposure were tested. Considering that potential combination interventions such as LLINs with indoor fungal applications could result in mosquito contact to both agents

during the same or separate feeding cycles, experiments included simultaneous and sequential exposure combinations of fungus and permethrin.

7.2 Materials & Methods

7.2.1 Mosquitoes

Experiments used 3-5 day old female mosquitoes. The laboratory mosquito colony (VKPer) was obtained from the insectary of the CREC institute in Cotonou, Benin, and consisted of *An. gambiae s.s.* (S-form) originating from the Kou Valley in Burkina Faso that were homozygously fixed for the *kdr* gene (Martinez-Torres *et al.*, 1998). Eggs of this colony were shipped to the Laboratory of Entomology, Wageningen, The Netherlands and a colony was reared as described in §2.1.1. Experiments on VKPer mosquitoes were performed in climate-controlled rooms (27$\pm$1°C, 80$\pm$10% RH).

The field colony consisted of adult mosquitoes reared from field-collected larvae and pupae obtained from breeding sites near Ladji, Benin (623'23N, 225'56E) in April 2009. Previous studies showed that in this location the anopheline population consists of resistant (*kdr*) *An. gambiae s.s.* (M-form) mosquitoes (Corbel *et al.*, 2007). *Anopheles gambiae* larvae were separated from the field collections and reared in plastic trays filled with tap water and fed on locally purchased cat food. Adults were maintained in the CREC insectary (26$\pm$1°C, >80% RH) and fed ad libitum on honey-water mixtures. Bioassays on this field population were performed in the CREC laboratory, in which temperature was maintained at approximately 20$\pm$2°C during the day, and at 26$\pm$1°C during observation periods (6 pm-8 am) with humidity >80% RH.

7.2.2 Fungus

Spores of *Metarhizium anisopliae* and *Beauveria bassiana* were produced as described in §2.2.1 and formulated and mixed in Shellsol for application (§2.2.2). Stock solutions were counted and checked for viability as described in §2.2.3.

7.2.3 Baseline fungal bioassays

The effect of fungus infection on mosquito survival was tested using the standardized exposure bioassay with fungus-coated papers that was developed in Chapter 3. The K-Control Coater was used to coat exposure papers with 10^{11} spores/m^2 of *B. bassiana* or *M. anisopliae*, or with the solvent only (for control groups). This exposure dose was also used for fungus-insecticide exposure experiments and optimized to cause high

levels of infection whilst not causing too rapid mortality, to monitor possible interaction effects over time. Papers were left to dry overnight and used to line PVC exposure tubes (for details see §3.2.5).

For each replicate, approximately 30 mosquitoes were exposed to the papers for 1 hr and subsequently transferred to clean holding buckets via free flight. Daily mosquito mortality was recorded and cadavers checked for fungal infection (§2.2.4). Tests comprised four treated and control replicates for the VKPer strain and three replicates for the field-collected mosquitoes, all from different mosquito batches.

7.2.4 Fungus-insecticide combination assays

The combined effects of fungus and insecticide on mosquito mortality was tested using a range of combination exposures and sequences, designed to mimic the sequence and timing of insecticide and fungal exposures that might occur under different scenarios of deployment in the field. Table 7.1 provides an overview of the various treatment combinations, and group numbers indicated in this table are used subsequently to describe treatments in the results. Mosquitoes were exposed to control papers (Control) insecticide (Perm), *B. bassiana*-coated (Bb) or *Metarhizium*-coated (Ma) papers, using standard WHO bioassay procedures (WHO, 1998) as described in §2.3.2. A three day interval was chosen between the two exposure rounds to represent the average duration of the gonotrophic cycle of *An. gambiae* and hence the period between consecutive blood meals. This time-point was used in previous assessments on fungal impact on insecticide sensitivity (§6.2.4) and corresponded to the start of fungal proliferation and the first noticeable impact on mosquito survival, thus allowing for measurements on fungal impact whilst not losing too many insects through death.

Exposure 1

In the first exposure round, cohorts of ca. 28 females were transferred to WHO bioassay tubes with an aspirator and exposed for 1 hr to insecticide papers (Perm), *Beauveria*-coated papers (Bb) or *Metarhizium*-coated papers (Ma), as indicated in Table 7.1. Control groups were exposed to untreated papers (§2.3.2). Insecticide exposures used papers treated with 0.75% permethrin from one single WHO production batch (§2.3.1). Fungal spores (10^{11} spores/m^2) were coated onto papers the previous day. Effects of co-exposure were tested by exposing mosquitoes first for 1 hr to fungus-impregnated papers and immediately afterwards for 1 hr to permethrin papers (Bb+Perm & Ma+Perm). After exposure, mosquitoes were transferred to clean holding buckets via free flight and mortality was measured 24 hrs and 3 days after exposure.

Table 7.1: Overview of insecticide and fungus exposure treatments.

	Group	Exposure 1 (Day 0)	Exposure 2 (Day 3)
Controls	1	Control	Control
	2	Control	Perm
	3	Perm	Control
	4	Perm	Perm
Beauveria	5	Bb	Control
	6	Bb	Perm
	7	Bb + Perm	Control
	8	Bb + Perm	Perm
Metarhizium	9	Ma	Control
	10	Ma	Perm
	11	Ma + Perm	Control
	12	Ma + Perm	Perm

Exposure 2

Three days after the first exposure, surviving mosquitoes were once more transferred from the holding buckets to WHO bioassay tubes and exposed either to permethrin papers or to control papers as indicated in Table 7.1. Exposures were performed as described above, for 1 hr, after which mosquitoes were transferred back to holding buckets. Mortality was scored after 24 hrs (Day 4) and 3 days after the second exposure round (Day 7). Dead mosquitoes were removed checked for fungal infection (§2.2.4). Mosquitoes that were still alive on Day 7 were removed from the buckets with an aspirator and killed by drowning in 70% alcohol before verifying fungal infection.

Permethrin-impregnated papers were re-used for a maximum period of two weeks and checked for efficacy after use by exposing insecticide-susceptible mosquitoes to the papers (§2.3.1). In Wageningen, two groups of 25 female *An. gambiae s.s.* of the Suakoko strain were exposed (originating from Liberia, reared in Wageningen). In Cotonou, two groups of 25 female *An. gambiae s.s.* of the Kisumu strain were exposed (originating from Kenya, reared in Cotonou). Experimental data were only used if the insecticide papers induced 100% mortality in these susceptible strains.

7.2.5 Data analysis

Differences in mosquito survival between fungus-infected and control groups were analyzed using Cox Regression as described in §2.4.3. Permethrin-induced mortality was computed from mortality rates 24 hrs after permethrin exposure corrected for corresponding control mortalities (exposed to blank papers) as described in §2.3.3. The group mean differences were analyzed for each mosquito population separately using mortality proportions that were arcsine $\sqrt{}$ transformed prior to analysis, and compared using a one-way ANOVA (SPSS 16.0) and a Tukey post-hoc test. Comparisons between the different exposure groups (insecticide, fungus or both) used a two-way ANOVA.

Synergy between the two species of fungus and permethrin was analysed by comparing mortality rates induced by combinations of both agents (observed) with the sum of mortalities induced by each agent separately (expected). This expected mortality was calculated using the formula $M_e = M_f + M_i (1 - M_f/100)$, where M_f and M_i were the observed percent mortalities caused by the fungus and the insecticide alone (Morales-Rodriguez and Peck, 2009). For all fungus-insecticide combinations, these calculated expected mortality percentages were compared with their corresponding observed mortality percentages (M_{fi}) using a Paired Samples T-Test in SPSS 16.0, which allowed for pair-wise comparisons between each of the replicate measurements and to exclude potential replicate variations such as differences between mosquito rearing batches, fungus applications and insecticide paper efficacy. Positive M_{fi}-M_e values were considered synergistic (Koppenhofer and Kaya, 1998). A significance level of <0.05 was used in all analyses.

7.3 Results

7.3.1 Fungal susceptibility

Both laboratory-reared and field-collected insecticide-resistant *An. gambiae s.s.* were susceptible to *M. anisopliae* and *B. bassiana*, with the moderate spore dose of 10^{11} spores/m^2 inducing 100% mortality within nine days after exposure (Figure 7.1) and $>70\%$ sporulation of cadavers (controls showing 0% sporulation). Survival analysis showed no significant differences in virulence between *Beauveria* and *Metarhizium* in the laboratory colony (HR=1.29, P=0.09) or the field-collected mosquitoes (HR=1.35, P=0.07). There was no significant interaction between fungus treatment and mosquito colony (HR=0.83, P=0.16), indicating that fungal infection had a similar impact on *kdr* mosquito longevity in the laboratory and field populations.

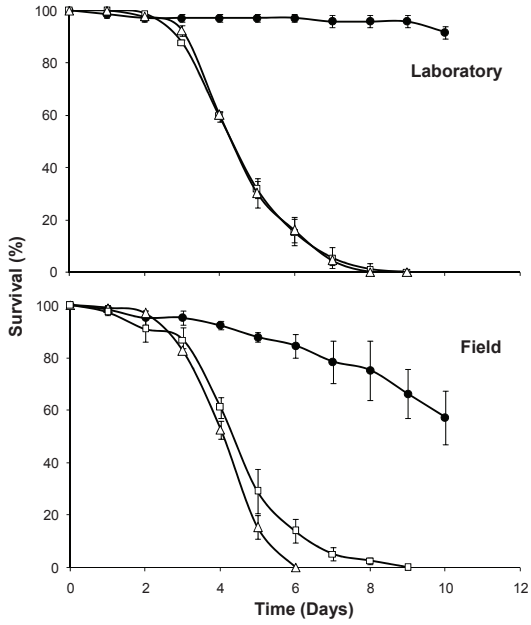

Figure 7.1: *Effect of fungal infection on mosquito survival. Mean (±SE) cumulative % survival of B. bassiana-infected (triangles), M. anisopliae-infected (squares) and uninfected control mosquitoes (black circles), of laboratory-reared (top, n=4) and field-collected (bottom, n=3) insecticide-resistant An. gambiae s.s.*

7.3.2 Permethrin resistance

Permethrin-induced mortality rates were compared between groups that were exposed to permethrin on day 0 (Group 3), on day 3 (Group 2), or on day 0 + day 3 (Group 4, see Table 7.1). Control mortalities (unexposed groups) were below 5% and were, therefore, not used to correct the insecticide-induced mortality rates. Both the laboratory VKPer colony and the colony collected in the field were highly resistant to permethrin, exhibiting only 10-20% mortality following single or repeat exposures (Figure 7.2). Equivalent single exposure of the susceptible mosquito strains resulted in 100% mortality. Statistical analyses on the group means showed that there were no significant differences in sensitivity to permethrin between the laboratory and field mosquitoes (Figure 7.2). Moreover, permethrin resistance levels did not increase in the three day test period and were not significantly affected by repeat exposure (Figure 7.2).

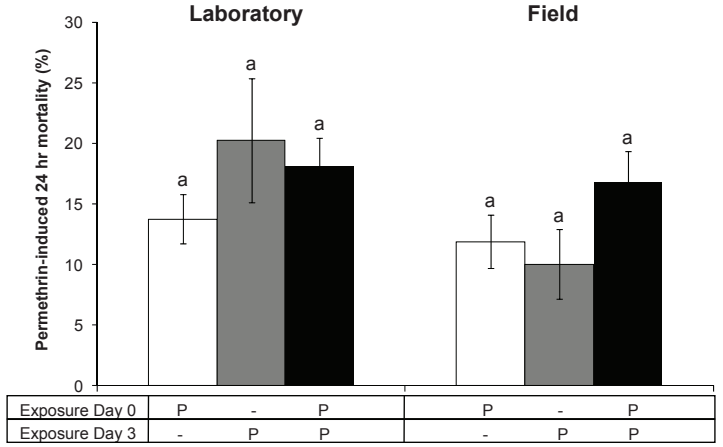

Figure 7.2: *Permethrin sensitivity. Mean(±SE) % mortality of uninfected kdr mosquitoes from the laboratory (left) and field population (right) 24 hrs after permethrin exposure. Data show mortality of 3-day old mosquitoes exposed once on day 0 (white), 6-day old mosquitoes exposed once on day 3 (grey), or 6-day old mosquito survivors exposed a second time on day 3 (black). From left to right, data depict 10, 5, 5, 8, 4, and 4 replicates, with significant differences in non-corresponding letters.*

7.3.3 Fungus-insecticide combinations

To determine the effect of fungal infection on permethrin efficacy, mortality following permethrin exposure was compared between *Beauveria*-infected, *Metarhizium*-infected and equivalent uninfected groups. Mortality rates of fungus-infected groups exposed to permethrin on day 3 (Groups 6 & 10) were corrected for mortalities of corresponding (fungus-infected) control groups (Groups 5 & 9), as explained in §2.3.3, whereas there were no corrections made for the other treatment goups since their control mortalities did not exceed the 5% level.

Permethrin-induced mortality measured on day one was not higher in groups co-exposed to fungus compared with groups exposed to only permethrin in either mosquito colony (Figure 7.3), indicating no interactions at the very early stages of fungal infection. However, once fungal infection had proliferated for three days, exposure to permethrin induced significantly higher mortality in *Beauveria*-infected (Group 6; P=0.02) and *Metarhizium*-infected (Group 10; P=0.009) mosquitoes from the field population (Figure 7.3). These differences in permethrin-induced mortality were not observed in

the VKPer laboratory colony (Figure 7.3) even though the fungus-induced mortality rates used to correct co-exposed mortalities were similar for both colonies.

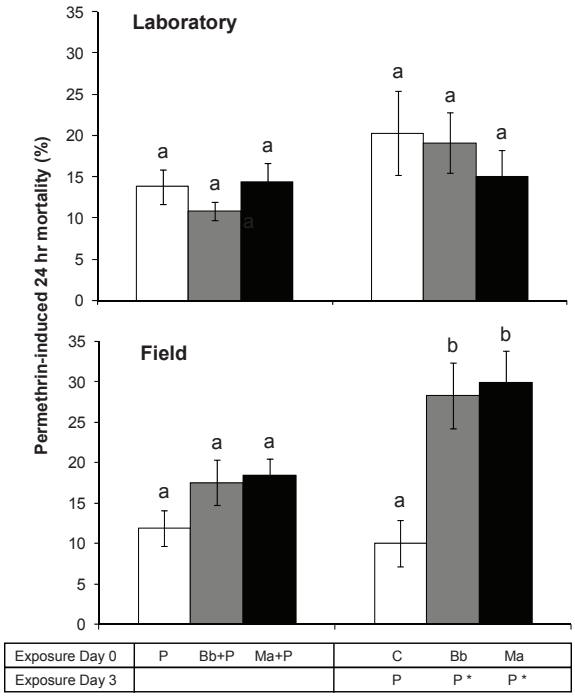

Figure 7.3: *Effect of fungus on permethrin sensitivity. Mean (±SE) permethrin-induced mortality (%) of uninfected (white), Beauveria-infected (grey) and Metarhizium-infected (black) mosquitoes from the laboratory (top, n=10) and field population (bottom, n=8) measured 24 hrs after exposure on day 0 (left) and day 3 (right). * indicate corrected mortalities.*

Reciprocal effects of neurotoxic insecticide on subsequent fungal efficacy were evaluated by comparing uncorrected mortality rates between day 3 and 4 for mosquito groups exposed on day 0 to insecticide (Group 3), fungus (Groups 5 & 9), or both (Groups 7 & 11). Exposure to permethrin alone (P) showed minimal impact on mortality rates among mosquito survivors three days later (Figure 7.4). Consistent with mortality trajectories in Figure 7.1, exposure to fungus alone (F) resulted in a significantly greater day 3-4 mortality rates compared with uninfected controls (Figure 7.4).

Fungus-induced mortality rates were significantly higher in the fungus and insecticide co-exposure treatments (F+P) (Figure 7.4), indicating that permethrin augmented the proliferation of *B. bassiana* and *M. anisopliae* in both the laboratory colony and field-collected mosquitoes. All co-exposure treatments were found to interact synergistically, such that day 3-4 mortality rates were significantly higher ($P<0.05$) than expected from the single treatment effects combined. Further effects on daily mortality rates at the time when wild mosquitoes would be expected to take a second blood meal (*e.g.* on day 7) could not be analyzed as mortality of mosquitoes exposed to the various fungus- permethrin combination treatments was 80-90% by day 7, and not suitable for comparing synergistic effects of different exposures.

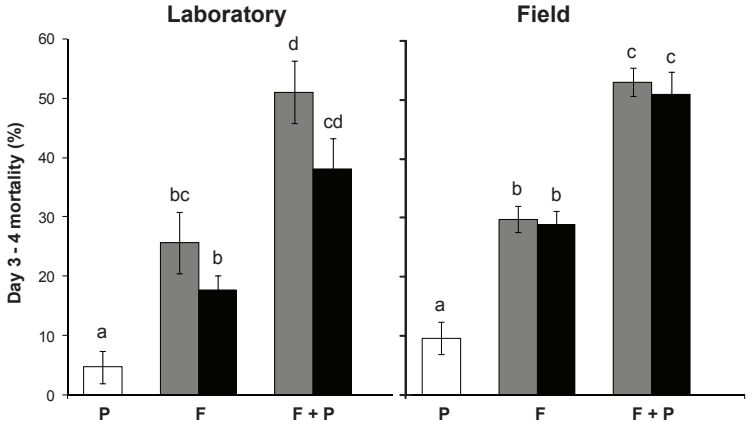

Figure 7.4: *Effect of permethrin on fungal virulence. Mean (±SE) day 3-4 mortality (%) of uninfected (white), Beauveria-infected (grey) and Metarhizium-infected (black) kdr mosquitoes from the laboratory (left, n=5) and field population (right, n=4) exposed to permethrin (P), fungus (F) or both (F+P) on day 0.*

The overall impact of fungus-insecticide combinations was analyzed using uncorrected cumulative day 4 mortality rates, highlighting the extent of more immediate impact over the timeframe of 1-2 mosquito gonotrophic cycles. In the laboratory colony, a single permethrin exposure caused a significant increase in mortality relative to controls, although this was not increased further by a second exposure (Figure 7.5). In the field population, only the double permethrin exposure was significantly different to the controls.

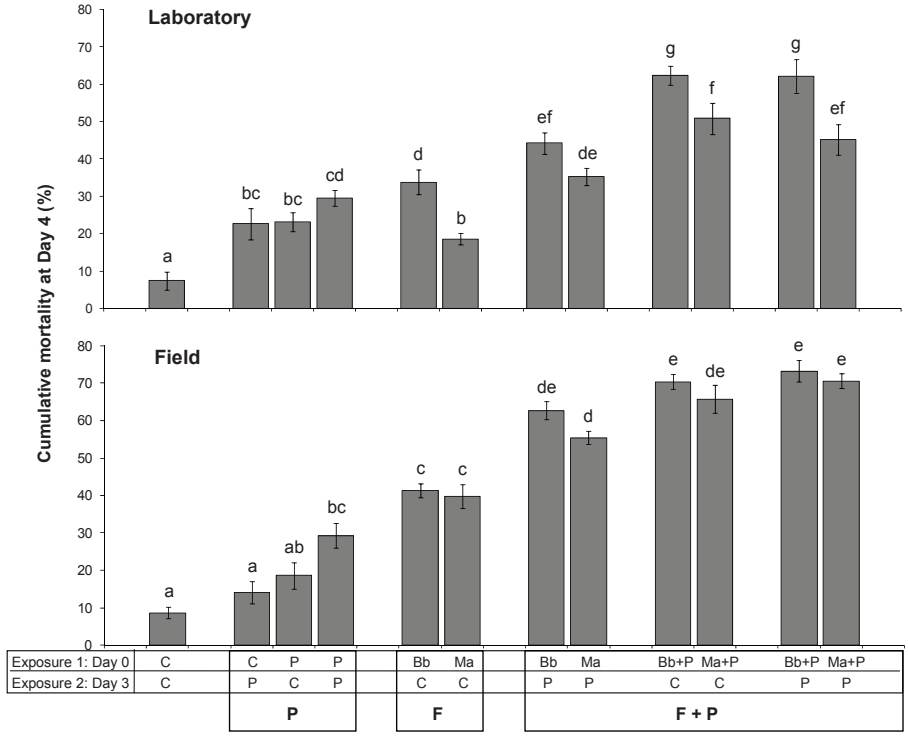

Figure 7.5: *Impact of co-exposure on mosquito survival. Cumulative day 4 mortality (mean % ±SE) of 28 laboratory-reared (top, n=5) and field-collected An. gambiae (bottom, n=4) exposed to permethrin (P), fungus (F) or combinations of both (F+P) in two subsequent rounds on day 0 and day 3 as indicated on the X-axis. Non-corresponding letters show significant differences.*

The maximum overall mortality induced by permethrin was approximately 20-30% compared with 10% in the controls (Figure 7.5), indicating that permethrin did not have a substantial impact on *kdr An. gambiae s.s.* survival. Effects of fungal infection four days after exposure, though still moderate, were slightly higher, inducing 19-41% mortality (Figure 7.5). Impact of fungus tended to be marginally higher in the field population, with no marked differences in the effects of *B. bassiana* and *M. anisopliae*. In the laboratory colony, the effect of *Beauveria* on mosquito survival was significantly greater than *Metarhizium* in most treatments (Figure 7.5).

Both fungus species had a higher impact on mosquito mortality when combined with permethrin. All tested fungus and permethrin combinations (F+P) resulted in higher cumulative mortality compared with the use of permethrin-only (P) ($P<0.001$) or fungus-only (F) ($P<0.001$) treatments in both mosquito strains. Co-exposure to both agents on day 0 induced highest overall mortality (in the order of 60-70%), with no additional mortality from a second permethrin exposure(Figure 7.5).

In the field population, simultaneous co-exposure to *B. bassiana* or *M. anisopliae* and permethrin (Groups 7,8,11,12), as well as sequential exposure to *Beauveria* and then permethrin (Group 6), induced significant synergistic increases in the cumulative mortality rates at day 4. The observed mortality rates from these combination treatments were significantly higher than the expected rates, *i.e.* the added mortality rates of fungus only and insecticide only treatments (Table 7.2). In the laboratory colony, significant synergy between fungus and permethrin was observed only in the single co-exposure treatments (Groups 7 & 11), *i.e.* when fungus and insecticide was applied together on Day 0 (Table 7.2). The other combination treatments showed an additive effect on mosquito mortality.

Table 7.2: Synergistic interactions between fungus and permethrin on laboratory (df=4) and field (df=3) *kdr* mosquito survival.

Exposure		**Laboratory**				**Field**			
Day 0	**Day 3**	**Observed**	**Expected***	**T-test**	**P**	**Observed**	**Expected***	**T-test**	**P**
Bb	Perm	44.3 ± 2.9	49.0 ± 3.2	1.13	0.323	62.7 ± 2.4	49.8 ± 0.6	4.34	**0.023**
Bb+Perm	Control	62.4 ± 2.5	49.3 ± 2.6	4.63	**0.010**	70.3 ± 2.0	52.5 ± 1.1	9.81	**0.002**
Bb+Perm	Perm	62.2 ± 4.5	56.2 ± 3.2	1.44	0.223	73.2 ± 2.9	58.6 ± 1.5	9.95	**0.009**
Ma	Perm	35.3 ± 2.3	37.1 ± 3.5	0.38	0.726	55.4 ± 1.7	49.1 ± 3.7	1.67	0.194
Ma+Perm	Control	50.9 ± 4.2	37.5 ± 2.1	3.46	**0.026**	65.8 ± 3.7	50.7 ± 4.5	11.4	**0.001**
Ma+Perm	Perm	45.2 ± 4.2	42.6 ± 2.1	0.66	0.547	70,6 ± 2.0	57.3 ± 3.4	3.84	**0.031**

*Expected mortality (M_e) = M_f + M_i (1 - M_f/100), with M_f and M_i being observed percent mortalities caused by the fungus and the insecticide alone respectively.

7.4 Discussion

The laboratory colony (VKPer) and field population of *An. gambiae s.s* from West Africa showed limited sensitivity to permethrin following single or multiple exposures across the duration of a gonotrophic cycle. These results are consistent with known high levels of *kdr* expression in these populations. While size or other fitness parameters were not measured and might be more variable in the adults reared from field-collected larvae and pupae, their baseline insecticide sensitivity was similar to the laboratory-reared colony and consistent between the different experiments.

Both populations of *kdr* mosquitoes were highly susceptible to two candidate isolates of *B. bassiana* and *M. anisopliae*. Exposure to an intermediate dose of fungus using a standard WHO bioassay caused 100% mortality within nine days. This treatment mortality was substantially greater than the controls, even in the Cotonou laboratory where survival rates of the field-collected mosquitoes were slightly reduced. Sporulation of fungal cadavers tended to be lower in the *Metarhizium*-infected field mosquitoes, which is consistent with findings that this fungus is not a strong competitor of other microbiota and that hyphal growth can be affected by environmental factors (Roberts and St. Leger, 2004). Mortality data, however, indicated high fungal infectivity of both isolates in both mosquito populations. These results confirmed findings from Chapter 6 and recent studies on the same (Howard *et al.*, 2010) and other resistant mosquito strains (Kikankie *et al.*, 2010), and demonstrated for the first time that also wild populations of West African pyrethroid-resistant *An. gambiae* do not confer resistance to insect-pathogenic fungi. Given the growing problems of pyrethroid resistance and issues of cross-resistance to DDT among malaria vectors, these results highlight an important strength of fungal entomopathogens.

Impact on survival was broadly similar for both fungal isolates, although some results suggested slightly reduced efficacy of *M. anisopliae*, which is likely linked to a lower quality of the production batch available for those tests that showed lower viability on agar than the *B. bassiana* spores (70% vs 92%). Other findings indicated a higher persistence of *Beauveria* spores (Darbro and Thomas, 2009), which implies that this fungus may be more suitable for field implementation. Spore virulence and persistence can differ greatly between different fungal strains within and between hyphomycetous species, and could be optimized through production methods and formulation (Roberts and St. Leger, 2004). Ultimately, the choice of fungal strain would require evaluations of the long-term effectiveness of different species and isolates after application under realistic field conditions, together with evaluation of other operational criteria such as mass production efficiency, long-term storage viability, toxicology and ecotoxicology (Thomas and Read, 2007).

Beyond the ability to infect insecticide-resistant mosquitoes, this study identified the potential for synergistic interactions between fungi and pyrethroids. Firstly, pre-infection with fungus led to an increase in permethrin-induced mortality levels, *i.e.* the 'instantaneous' mortality resulting from exposure to permethrin. This effect was restricted to the field mosquito population and was not apparent in the laboratory colony. The mechanism for this effect is unclear. Previous work suggested that fungal metabolites interfere with enzymatic insecticide resistance mechanisms (Chapter 6) and so it is possible that the observed effects in *An. gambiae* from Ladji result from an effective increase in permethrin sensitivity in the presence of a proliferating fungal infection. While both *An. gambiae* populations are known to express *kdr* and such effects would not be expected where resistance is conferred by target-site insensitivity alone, the VKPer laboratory colony has been fixed for *kdr* resistance through repeated selection and maintained in the laboratory for many years (Martinez-Torres *et al.*, 1998), whereas elevated levels of oxidases and esterases have been reported for the wild *An. gambiae* population at Ladji, Benin (Corbel *et al.*, 2007). Thus, the differences in response to permethrin could be indicative of more complex multiple resistance mechanisms operating in the field population. The slightly more variable environmental conditions in the Cotonou laboratory might, however, also have affected fungal efficacy and survival rates of field-collected mosquitoes.

Secondly, simultaneous exposure to fungus and permethrin increased the daily mortality rate of mosquitoes at the point where fungus starts to proliferate within the insect and approaches its exponential growth phase (see (Bell *et al.*, 2009)). This higher fungal virulence three days post-exposure is most likely caused by indirect effects of the neurotoxic insecticide, since pyrethroids are usually rapidly detoxified by metabolization processes (Burt and Goodchild, 1974) and so would no longer be present inside the insect body at that time-point. Although the exact mechanisms for this effect are unclear, insecticides may affect the insect cuticle and facilitate fungal penetration, or may inhibit cellular and humoral immune responses and facilitate fungal infection inside the body as shown in other insect species (Pachamuthu and Kamble, 2000; Hiromori and Nishigaki, 2001).

Finally, in several combination treatments, and particularly simultaneous exposures, synergistic interactions between fungus and permethrin on overall mosquito mortality were observed. These synergistic effects resulted in approximately 50-70% mortality after four days in most co-exposed groups, compared with 15-40% for permethrin or fungus alone. The higher impact after simultaneous co-exposure compared with subsequent exposure implies that the effects of insecticide on fungal proliferation contribute significantly to the overall impact. There was no additional mortality after a repeat exposure to insecticide, which suggests that a single insecticide exposure at the start is sufficient to induce synergy.

Several theoretical studies have demonstrated that the relatively slow speed of kill of entomopathogenic fungi can be sufficient to impact on malaria transmission since the extrinsic incubation period of the malaria parasite within the mosquito (typically 10-14 days in high transmission settings) creates a window of several days for the fungus to act (Scholte *et al.*, 2005; Hancock *et al.*, 2009; Koella *et al.*, 2009; Read *et al.*, 2009). There may even be evolutionary benefits in slow speed of kill (Thomas and Read, 2007; Read *et al.*, 2009). However, for a slow acting product to be effective, coverage needs to be sufficiently high to ensure contact with mosquitoes early in adult life, otherwise they might escape the negative effects of fungal infection long enough to transmit malaria (Hancock, 2009). The synergy between fungus and permethrin (50-70% mortality within 4 days) has potential to dramatically reduce malaria transmission across the duration of 1-2 gonotrophic cycles and enable control at more moderate levels of coverage even in areas with high levels of insecticide resistance.

Operational deployment of fungal bio-pestticides for mosquito control requires further research, including development of feasible field delivery methods that are compatible with the chemical controls tools already in place (Thomas and Read, 2007; Knols *et al.*, 2010). In this study, co-exposure to fungus and insecticide produced the strongest synergistic effects, thus it would be interesting to explore delivery systems that promote exposure to both products during a single feeding episode, such as combining indoor fungal sprays or fungus impregnated-resting targets (Chapter 4) with LLINs. Results from other studies show good compatibility of fungus-insecticide mixtures (Sanyang *et al.*, 2000; Neves *et al.*, 2001; Bahiense *et al.*, 2006), implying potential for direct combination applications of fungal spores and (non-repellent) insecticides, for example on walls or eave curtains. Although consistent with standard WHO methods, the exposure assays used did not directly simulate fungal exposures that might be expected to occur in the field. Further research would be required to determine the robustness of fungal effects across different environmental conditions and to explore dose-dependent effects to see whether fungal infection can enhance the efficacy of sublethal insecticide doses, which has been shown to be the case in other insect species (Quintela and McCoy, 1998; Pachamuthu and Kamble, 2000; Jaramillo *et al.*, 2005).

Currently there is great interest in using combination interventions with distinct modes of action as management strategy, not only to control resistant mosquitoes but to delay the selection of novel resistance, which indicates a potential role for fungi with other categories of insecticide. Such research could enable the development of novel integrated vector management (IVM) strategies that would sustain the useful lifespan of current insecticide-based interventions and maximize control in the face of emerging insecticide resistance.

8 Summarizing Discussion

8.1 Research findings

The aim of this thesis was to evaluate novel potential methods for integrated fungus-based malaria vector control and to test the efficacy of fungal entomopathogens against insecticide-resistant mosquitoes.

8.1.1 Application methods

The first objective of this research was to test effects of formulation, substrate and application method on fungal infectivity and virulence to mosquitoes. Spraying, dipping and coating were effective methods for applying an infective layer of fungal spores on mosquito resting surfaces. The coating method designed in **Chapter 3** provided a means to apply uniform spore layers on paper substrates and thus a potential method for accurate laboratory evaluations. A rotating spray apparatus was developed to standardize the application of fungal sprays inside clay pots (**Chapter 4**). Even though spraying resulted in lower and more variable end-concentrations of fungal spores (**Chapter 3**), this mode of application gave more infective spore layers on netting substrates compared with dipping (**Chapter 5**), possibly due to less spore adherence or higher doses being picked up from spore clumps.

The combination of formulation and substrate had a high impact the infectivity of fungal spores, with viscous suspensions being only effective on porous susbstrates. Non-absorbent proofing papers (**Chapter 3**) and polyester nets (**Chapter 5**) were most effectively treated with low viscosity Shellsol formulations, whereas porous clay material was successfully impregnated with more viscous Ondina oil suspensions (**Chapter 4**). These findings indicate that the porosity of the substrate and spore adherence to the application layer influence the accessibility of fungal spores to mosquitoes.

Results showed that the virulence of both *Metarhiziun anisopliae* and *Beauveria bassiana* infection increased step-wise with increasing fungal dose (**Chapter 3**), consistent with other reports (Scholte *et al.*, 2003a; Mnyone *et al.*, 2009a), implying that increasing the application dose may be used to compensate losses in spore viability. It remains unclear exactly how many spores are effectively picked up by a mosquito upon contact with a treated surface, but there seems to be a threshold level required for an effective infection (Devi and Rao, 2006; Chouvenc *et al.*, 2009). Novel techniques such as qPCR can be used to elucidate spore dose requirements (Bell *et al.*, 2009). Mosquito exposure time (**Chapter 3**) and type of contact with the substrate (**Chapter 5**) also affected fungal impact, with only 5 minute exposure being sufficient for a lethal infection with high enough spore concentrations ($>10^{11}$ spores/m^2), which implies that these factors determine the effective infection dose picked up by a resting mosquito.

8.1.2 Delivery systems

The second objective was to evaluate novel delivery tools that have potential for field implementation and use in IVM strategies. Clay pots showed potential for use as indoor and outdoor point-source objects to target resting anophelines (**Chapter 4**), and netting for use as house screens to target host-seeking mosquitoes (**Chapter 5**). Both systems were effective in the laboratory and offer multiple options for field deployment. Fungal delivery indoors with nets or inside pots would provide spores with relatively cool microclimates and protection from damaging UV-light. Compared to other indoor delivery options such as ceiling cloths (Scholte *et al.*, 2005) or indoor spraying, the treatment surfaces of pots and eave curtains are relatively small and could minimize human contact and deployment costs.

Clay pots were attractive mosquito resting sites in the laboratory (**Chapter 4**) and Western Kenya (Odiere *et al.*, 2007) but showed not to attract large numbers of indoor resting mosquitoes in northern Tanzania (Van den Bijlaardt *et al.*, 2009), although that may have been influenced by the experimental design (Knols and Farenhorst, 2009). To make sure that sufficient numbers of mosquitoes can be targeted with a novel system, its delivery efficacy would need to be evaluated in a range of tropical field locations. Pots could be used to target also outdoor resting mosquitoes, which may especially be useful in areas where excito-repelling insecticides are deployed. The point-source delivery concept could be further adjusted and optimized for specific field settings by using other objects, such as tent traps (Govella *et al.*, 2009), wicker baskets, resting boxes (Harbison *et al.*, 2006) or odour-baited stations (Lwetoijera *et al.*, 2010).

Fungus-impregnated nets were only tested in the laboratory, but with set-ups that mimicked a realistic type of mosquito contact (**Chapter 5**). The renewed interest in

house screening for malaria vector control (Kirby *et al.*, 2009; Lindsay *et al.*, 2003) may provide opportunities for testing fungus-impregnated eave curtains or window screens. With cotton nets showing highest spore persistence and several mesh sizes showing equal effectiveness, it may be especially interesting to evaluate the efficacy of fungi on small-meshed cotton eave curtains that would also block mosquito house entry. Their effective coverage would need to be tested in the field, and the type of netting, mesh size and formulation could be further optimized to meet specific deployment criteria.

Clay pots and eave curtains could potentially be combined to target both host-seeking and resting mosquitoes, which may improve overall fungal coverage. Moreover, clay pots and eave curtains could be used complementary to existing chemical-based control tools such as ITNs and IRS and thus provide a means to integrate fungus-based into chemical-based mosquito control. Such combination interventions, with the biological and chemical control agents spatially separated, would prevent insecticide repellency to negatively affect mosquito-fungus contact whilst still enabling mosquitoes to make contact with both fungi and insecticides within a single feeding episode.

8.1.3 Impact on resistant vectors

The third objective was to measure the efficacy of entomopathogenic fungi against insecticide-resistant mosquitoes. Fungi were shown to be highly effective against a diverse suite of insecticide-resistant *Anopheles* mosquito strains. **Chapter 4** showed that *Metarhizium* was highly infectious and virulent to pyrethroid-resistant *An. funestus* mosquitoes. **Chapter 6** made direct comparisons of fungal efficacy in insecticide-resistant and susceptible mosquitoes. Fungal impact was equally high in resistant *Anopheles* colonies (with resistance fixed through repeated selection in the laboratory) and their baseline (susceptible) colonies from which they originated, showing that insecticide resistance does not to confer resistance to fungal infection.

In **Chapter 7**, *Metarhizium* and *Beauveria* were shown to also be effective against a laboratory and wild population of West African *An. gambiae s.s.* with *kdr* target-site insensitivity to pyrethroids and DDT. Although in another study fungal impact was shown to be higher in this *kdr* laboratory colony than in a suceptible laboratory colony from Liberia (Howard *et al.*, 2010), there were no direct comparisons included (between baseline and corresponding resistant colonies) and no direct link between fungal susceptibility and target-site resistance can thus be made. The high efficacy of fungi against a field population of genetically resistant vectors, however, implies that fungus-based control could especially be useful in areas with high levels of target-site resistance in mosquito poplulations.

8.1.4 Combining fungi & insecticides

The fourth objective was to test the compatibility of fungi and insecticides and to measure their combined impact on mosquito survival. It was shown that a progressing fungal infection increased the impact of insecticides in resistant anophelines (**Chapter 6 & 7**). Reciprocally, the neurotoxic insecticide permethrin increased subsequent fungal impact (**Chapter 7**). The exact mechanisms involved in the interactions between insecticide resistance and fungal infection remain unclear and require further biochemical studies. The fact that fungal infection increased the impact of permethrin in mosquitoes with metabolic resistance mechanisms (**Chapter 6**) but not in mosquitoes with only target-site resistance (**Chapter 7**) implies that fungal metabolites may interfere with effective enzymatic insecticide degradation.

The synergistic impact of fungus-insecticide combinations showed that fungi and insecticides can increase each other's efficacy (**Chapter 7**), which supports suggestions that integrated control strategies with fungi and insecticide-treated bednets would have greater impact on malaria transmission than either intervention alone (Hancock, 2009). The highest synergy was observed in simultaneously co-exposed mosquitoes (**Chapter 7**), which indicates that control tools that induce contact to both agents within a single feeding episode may be the most effective field implementation options. The faster speed of kill induced by fungus-insecticide combinations could improve the impact on malaria transmission by killing mosquitoes that escaped exposure during their first feeding cycle before they become infectious. Considering also that chemical vector control still plays a vital role in malaria control and that fungi do not offer personal protection from mosquito bites, these findings suggest that biological control with fungal entomopathogens may be used more effectively as an additional control measure than as a replacement of chemical tools.

8.2 Future perspectives

The synergistic and resistance breaking properties of fungi could potentially create new opportunities to augment current malaria interventions, enhance the effective lifespan of our key public health insecticides and manage the further spread of insecticide resistance. Although biological insect control with fungal entomopathogens is a well-founded technology, it is so far only being deployed on a wide scale against agricultural pest insects and not yet against disease vectors such as mosquitoes. In Brazil and China, more than one million hectares is treated with fungal biopesticides annually (Li *et al.*, 2010), and a locust control product based on *Metarhizium* spores is now registered throughout Africa and commercially produced in South Africa and Senegal

(Douthwaite *et al.*, 2001; Lomer *et al.*, 2001). There is thus ample knowledge available on the implementation and production of fungal biopesticides, which can be used for the future deployment of fungi as malaria vector control agents.

8.2.1 Spore persistence

A key factor for successful field implementation is the long-term effectiveness of fungal spores, as a high persistence would minimize the required application frequency and improve cost-effectiveness. Several factors, such as UV-light and high temperature can negatively affect spore viability and persistence (Alves *et al.*, 1998; Fernandes *et al.*, 2007; Braga *et al.*, 2001). The *Beauveria* strain used in this thesis was shown to retain 30% viability after 200 days under laboratory conditions (Darbro and Thomas, 2009), which is promising considering that dose-response experiments showed that even hundred-fold reductions in fungal dose can significantly reduce mosquito survival (**Chapter 3**). Spore viability is, however, only one aspect of fungal persistence. For instance, spores showing no or $<5\%$ viability on agar were still able to infect mosquitoes and reduce their survival (unpublished data). Accurate evaluations of spore persistence would, therefore, need to include tests on the target insect.

A pilot test showed that *Beauveria* spores coated onto papers and kept under controlled laboratory conditions were effective in killing mosquitoes up to seven months after application (unpublished data). *Metarhizium* spores were only persistent for one month, which is consistent with previous reports on this strain in the same laboratory (Scholte, 2004) and in a Tanzanian laboratory (Mnyone *et al.*, 2009a). Field persistence of fungal mosquito control applications is not yet known and would have to be further evaluated. One study in Tanzania with *Metarhizium* applied on cotton sheets showed a 63% decrease in viability in three weeks, but did not measure the impact on mosquito survival over time (Scholte *et al.*, 2005).

Spore persistence can vary greatly between fungal species and even between fungal isolates of the same species (Fernandes *et al.*, 2007). The strains used in this thesis were selected for high virulence to mosquitoes (Scholte *et al.*, 2003b; Blanford *et al.*, 2005) and screening for more persistent fungal strains may be a worthwhile investment to enhance their long-term efficacy (Darbro and Thomas, 2009). The tested application and delivery methods showed exchangeable results for the *Beauveria* and *Metarhizium* strain and could be expected to be equally suitable for other strains. Ultimately, the choice of fungal strain for field implementation would not only depend on its persistence and virulence to mosquitoes, but also on its potential to meet operational criteria, such as mass production efficiency and ecotoxicology (Thomas and Read, 2007; Roberts and St. Leger, 2004).

8.2.2 Optimizing fungal efficacy

To achieve successful field implementation, there are several influencing factors to consider that could be used to optimize fungal efficacy. Firstly, spore production methods could be modified to improve spore quality (Roberts and St. Leger, 2004; Ibrahim *et al.*, 2002). Optimizing culture conditions and medium components could enhance spore yield and improve spore quality (Shah *et al.*, 2005; Ibrahim *et al.*, 2002; Ying and Feng, 2006). Further options to enhance fungal virulence include adding insecticidal genes that overexpress cuticle-degrading enzymes (Fang *et al.*, 2009; St. Leger *et al.*, 1996; Fan *et al.*, 2006) and repeatedly passing the fungus through the target insect during production (Goettel and Inglis, 1997). There are several methods for mass-production of hyphomycetous fungi with multiple options for quality control (Jenkins *et al.*, 1998; Jenkins and Grzywacz, 2000), which can be optimized for each specific fungal strain (Cherry *et al.*, 1999; Ypsilos and Magan, 2005).

Secondly, the long-term effectiveness of fungi in field settings could be enhanced with the use of long-lasting formulations. A range of different solvents has been shown to improve spore tolerance to dessication and UV (Faria and Wraight, 2007). Protective formulations include oil-based suspensions (Jenkins and Thomas, 1996; Kassa *et al.*, 2004), foam formulations (Dunlap *et al.*, 2007), silicone surfactants (Gatarayiha *et al.*, 2010) or solvents that include protective additives such as sunscreen adjuvants (Shah *et al.*, 1998; Inglis *et al.*, 1995) or photoprotective anionic dyes (Cohen and Joseph, 2008). Microencapsulation has been successfully used to improve insecticide persistence (N'Guessan *et al.*, 2008) and could also be a means to prolong fungal spore viability (Liu and Liu, 2009).

8.2.3 Field evaluation

A crucial step towards successful malaria vector control with fungi would be to evaluate their efficacy in field settings under realistic environmental conditions. Because fungal growth inside exothermic insects is highly dependent on outside temperature, fluctuating conditions could have a significant impact on fungal infection (Kikankie *et al.*, 2010; Thomas and Jenkins, 1997; Blanford *et al.*, 2009). Furthermore, the effective coverage of each potential field delivery system would largely depend on mosquito behaviour, which is known to differ between species and different climates. It may thus be that different delivery methods may be optimal in different settings.

Because field experiments can take long, be costly and include factors that cannot be controlled for, semi-field experiments could provide a good starting point for optimizing fungus-based control tools. Semi-field systems are controlled settings with realistic environmental conditions (Knols *et al.*, 2002) that have proven to be very use-

ful for malaria mosquito research (Njiru *et al.*, 2006). In such experimental settings, released mosquitoe can be effectively retrieved, which would be ideal to compare the efficacy of different fungal delivery systems.

The next, indispensable, step would be to measure the impact of fungi in real field settings, where deployment is faced with variable factors such as climate, malaria endemicity, mosquito species, mosquito age and insecticide resistance levels. Large-scale field trials would be required to evaluate the cost-effectiveness, the safety and long-term sustainability of fungus-based malaria vector control. It would be most informative to measure the environmental risks of fungal entomopathogens relative to that of chemical insecticides (Pevelling *et al.*, 1999). In view of their high potential for use in integrated (IVM) strategies, it would be important to incorporate field evaluations on integrated fungus-insecticide interventions and to test their combined impact. Ultimately, to show the actual malaria control benefit of (integrated) fungus-based interventions it would be necessary to not only demonstrate their impact on mosquito survival but also on disease transmission. Considering that the use of bednets only became widely accepted after several large-scale trials in various African countries unequivocally demonstrated their ability to save lives (Knols *et al.*, 2010), support for fungus-based malaria interventions can only be expected after similar results are obtained.

8.2.4 Improving malaria control sustainability

Improving the sustainablility of control agents is considered an important means to ensure the future effectiveness of malaria interventions. A promising development is the search for new chemicals to replace the available public health insecticides (Hemingway *et al.*, 2006). Indoxacarb (N'Guessan *et al.*, 2007c) and chlorpyrifos methyl (N'Guessan *et al.*, 2010), for instance, show promise as alternative insecticides, and their use could help evade problems with insecticide resistance. Such fast-killing chemicals have the benefit of providing personal protection from mosquito bites, but may not provide long-term sustainable solutions because their rapid activity would induce high selection pressure for resistance development (Knols *et al.*, 2010; Read *et al.*, 2009). Effective slower acting chemicals, such as chlorfenapyr, could potentially provide more sustainable insecticides (N'Guessan *et al.*, 2009, 2007a), although their effects on mosquito reproductive success remains to be evaluated.

Fungal entomopathogens, as slow-killing biological agents that still allow some reproductive success (Ondiaka *et al.*, 2008; Scholte *et al.*, 2006), could provide potentially sustainable control options (Hancock *et al.*, 2009; Read *et al.*, 2009). Whereas resistance to chemicals can be conferred through only a single genetic modification, anti-fungal resistance would require the formation of multiple mechanisms against all infection stages. It is known that other insects can to some extent resist fungal in-

fection through cellular and humoral immune responses (Chouvenc *et al.*, 2009), but fungi have several means to overcome these (Hajek and St. Leger, 1994). In such a co-evolving system, resistance development would likely proceed in very different way than in systems with a one-sided selection pressure from a non-evolving chemical. Studies on *Drosophila* suggested that anti-fungal resistance would not evolve easily (Kraaijeveld and Godfray, 2008), and it has so far not been observed in *Anopheles* mosquito species. It would, however, be important to evaluate the potential for fungal resistance development in anophelines by, for instance, measuring genetic variation in fungal susceptibility and potential fitness costs associated with fungal resistance mechanisms (Kraaijeveld and Godfray, 2008).

The synergism between fungi and insecticides may have implications for the long-term sustainability of integrated approaches, as a faster speed of kill by combination interventions may exert a higher selection pressure for resistance. However, resistance to combination treatments would require cross-resistance to two agents with very different modes of action, which can be considered much less probable (Blanford *et al.*, 2005; Read *et al.*, 2009). Secondly, the additional burden of a fungal infection in insecticide-resistant mosquitoes has potential to increase the insecticide impact and thereby potentially reduce the speed of insecticide resistance formation and spread. Combination interventions may thus lower resistance levels compared to interventions based on insecticides only. And thirdly, the observed synergy between fungi and insecticides may allow the use of more moderate quantities of both agents in field settings whilst still achieving high impact on mosquito survival and particularly malaria parasite transmission, which could potentially reduce the overall selection pressure for resistance.

With their new mode of kill and insecticide resistance breaking properties, fungal entomopathogens could provide a valuable addition to current and novel chemical insecticides. Integrated vector management (IVM) strategies recognise that combining multiple interventions with differents modes of action would be the most efficient and sustainabale malaria control approach (Beier *et al.*, 2008). There may be great opportunities to combine fungi not only with chemical-based control measures but also with other tools, such as larviciding (Fillinger *et al.*, 2003; Bukhari *et al.*, 2010), house screening (Lindsay *et al.*, 2003) and odour traps (Njiru *et al.*, 2006), which could enable the use of several different rotation schedules and help improve the long-term sustainability of our malaria vector control arsenal.

8.3 Conclusions

A range of formulations and spore application methods was shown successful in infecting malaria mosquitoes, thus providing multiple options for laboratory evaluation and field implementation. The development of fungal spray applications inside clay pots and on netting substrates resulted in delivery systems with potential to target resting and host-seeking anophelines in field settings and potential for use in combination with other malaria interventions. These delivery tools could be further optimized for specific implementation purposes and deployed for future field evaluations of fungal entomopathogens for malaria control.

Fungal spores were shown to kill insecticide-resistant anophelines as effectively as susceptible mosquitoes and thus provide a novel control tool for resistant malaria vectors. Fungi and insecticides were not only compatible and effective when used in combination, but also induced synergistic effects on mosquito survival. Co-exposure enhanced the subsequent fungal virulence and insecticide impact. With fungi and insecticides potentially increasing each other's efficacy, they can be viewed as complementary control tools that would reach the highest disease control benefit when successfully integrated.

Currently available knowledge and technologies provide multiple options for optimizing the use of fungal biopesticides in field settings. Future developments of novel integrated vector management strategies based on both biological and chemical control could provide a new malaria vector control approach with potential to be successful even in areas where insecticide resistance levels are increasing and hampering the effectiveness of existing malaria interventions.

Epilogue

When I started working on this project, my main aim was to deploy fungi as an alternative biological control tool against malaria mosquitoes. It was not until my work visit to our collaborating colleagues in South Africa when I ventured out of these boundaries and included experiments on insecticides. This was an unexpected addition and quite a novelty in the field of malaria research, where biological and chemical control approaches are often treated as separate entities and viewed as mutually exclusive.

As a biologist I see the benefits of biological control products in terms of environmental impact and sustainability, but from a public health perspective I also see the important role of chemical-based measures and recognize that successful malaria prevention will require the use of all available interventions. To me, it became quite clear that, realistically, fungus-based interventions will never be used as a stand-alone method, not in the least because fungi do not offer personal protection to humans, but also because fungi, like any novel disease control measure, will have to be embedded in a background of existing interventions. Because malaria is a public health issue, I believe our aim should not be to replace adequate malaria control tools that are already being used, even when their efficacy may be declining, but to strengthen and augment them.

Moreover, I think it is important to focus on creating malaria control tools that can actually be implemented in tropical settings and thus to incorporate a realistic approach already from the start. Developing successful novel mosquito control tools will, in my opinion, also require a more flexible approach to their eventual deployment. Our current knowledge on parasite transmission and vector biology in the tropics teaches us not to expect a single malaria intervention to be equally effective throughout different environments. We should thus be prepared to carefully select and adjust control tools and optimize interventions for each specific environmental setting. I, therefore, think that is worthwile to develop several delivery options for fungus-based applications that can be customized and deployed flexibly and hence successfully in the full range of (tropical) field settings.

I incorporated this more pragmatic point of view in my thesis and chose to emphasize the benefits and importance of integrated use. I was able to show that fungi do not only provide a novel tool to kill adult mosquitoes in a novel way, but also a means to strengthen and prolong the activity of our current malaria vector control arsenal. I am, therefore, convinced that fungi will achieve their highest malaria control benefit when integrated in existing chemical-based interventions. In this light, I see the need to shift our focus towards testing integrated fungus-based control measures in the field instead of evaluating fungi only as a single malaria intervention.

There are many research efforts on several fronts of malaria control, not only on mosquito control but also on medical and social aspects of malaria, and the development of a successful integrated control strategy will need collaboration from all these research fronts. It is, however, evident that malaria control is not hampered simply by a lack of knowledge or research, since we have already been successful in controlling malaria in several parts of the world. Malaria interventions face problems with successful deployment in developing countries mostly because of financial, ethical and political issues. Because all malaria control measures, including existing ones, face these difficulties it is in my opinion not just to use operational arguments against novel, not yet optimised control methods such as fungal entomopathogens.

I strongly believe in the potential of this novel vector control approach and see ample opportunities for its deployment. Our current knowledge and technology provide many options to optimize the use of fungal biopesticides in field settings and to meet operational criteria. This will require successful collaborations with not only research institutes but also entrepreneurial entities that can help fungus-based mosquito control products to reach the market. Adoption of fungal biopesticides in the WHO pesticide evaluation scheme will also be an essential step forward. I am convinced that once we can unequivocally demonstrate the effectiveness of fungi in field settings and achieve a substantial impact on malaria transmission, we will obtain the necessary clearance for wider use and application of this novel technology and, thereby, a means to combat malaria in a more sustainable way.

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Samenvatting

Grootschalig en langdurig gebruik van insecticiden heeft geleid tot het ontstaan en het verspreiden van resistentie in muggenpopulaties. Resistentie tegen chemische middelen vormt een groot probleem voor de effectieve bestrijding van malariamuggen aangezien deze voornamelijk gebaseerd is op het gebruik van geïmpregneerde klamboes en het sprayen van insecticiden binnenshuis. Onderzoek laat zien dat de insectendodende schimmels *Metarhizium anisopliae* en *Beauveria bassiana* potentie hebben als een alternatief, biologisch bestrijdingsmiddel. De sporen van deze schimmels kunnen muggen via contact met de huid infecteren en binnen enkele dagen doden. Door deze relatief langzame werking bieden schimmels geen directe bescherming tegen muggenbeten, maar kunnen ze wel de overdracht van malaria voorkomen door de muggen te doden voordat deze infectieus worden en malaria-parasieten kunnen overdragen.

Dit proefschrift had als doel te onderzoeken hoe schimmels succesvol toegepast zouden kunnen worden in tropische veldomstandigheden en hoe deze methoden te integreren zijn in de huidige malariabestrijding. Hiertoe werd onderzocht hoe schimmelsporen het meest effectief aangebracht kunnen worden en werden twee nieuwe toepassingen ontwikkeld waarmee schimmels in de tropen en in combinatie met al bestaande middelen (zoals klamboes) gebruikt zouden kunnen worden. Daarnaast werd getest hoe effectief schimmels insecticidenresistente malariamuggen kunnen doden en gekeken of schimmelsporen gecombineerd zouden kunnen worden met chemische insecticiden.

In het eerste deel van het proefschrift (Hoofdstuk 3-5) werden methoden voor het aanbrengen en toepassen van schimmelsporen ontwikkeld en geoptimaliseerd. Het spuiten, dompelen en coaten van sporen bleken effectieve manieren om een infectieuze laag schimmels op oppervlakten aan te brengen en daarop rustende muggen te infecteren. Voor nauwkeurige evaluaties in het laboratorium werd een nieuwe aanbrengmethode ontwikkeld, waarmee een homogene laag schimmelsporen (geautomatiseerd) op papier gecoat kan worden. Voor het aanbrengen van een laag schimmels in aardewerken potten werd een roterend apparaat ontwikkeld waarmee sporen op een gestandaardiseerde wijze in de potten gesprayed kunnen worden. Resultaten lieten zien dat het type oplosmiddel in combinatie met het type substraat invloed had op de infectiviteit van de schimmelsporen. Visceuze formuleringen, zoals oliën, waren bijvoorbeeld alleen effectief op poreuze oppervlakten zoals klei. Daarnaast bleek dat de totale impact van een schimmelbehandeling afhangt van de dosering sporen, maar ook het type contact en duur van blootstelling, aangezien deze factoren allen effect hebben op de hoeveelheid sporen die door de mug opgepikt worden en en zodoende de virulentie van de infectie beïnvloeden.

In het Wageningen laboratorium werden twee nieuwe methoden ontwikkeld en getest die mogelijkheden bieden om schimmels praktisch en geïntegreerd in in veldsituaties toe te passen. Als eerste werd gekeken naar het gebruik van schimmels in aardewerken potten, waarvan de donkere, koele binnenkant een aantrekkelijke rustplaats voor muggen en een geschikte plaats voor schimmelsporen zou kunnen zijn. Olie-oplossingen met *Metarhizium* sporen konden efficiënt in de potten worden aangebracht door middel van sprayen en waren effectief in het infecteren en doden van malariamuggen. De schimmelbehandeling had geen invloed op de

aantrekkelijkheid van de potten als rustplaats voor mannetjes en vrouwtjesmuggen. In veldomstandigheden zouden met schimmel-behandelde potten zowel binnen als buitenshuis gebruikt kunnen worden om rustende muggen te infecteren. Als tweede optie werd gekeken naar het gebruik van sporen op gaas voor binnenshuis toepassen van schimmels. Sporen van de schimmels *Beauveria* en *Metarhizium* konden het meest effectief op gaasmateriaal aangebracht worden in een vluchtig oplosmiddel en door middel van sprayen. De schimmelsporen waren infectief voor muggen op zowel polyester als katoenen gaas, maar bleven langer goed op katoen. Het type contact wat muggen maakten wanneer ze door gaas met grote gaatjes kropen, had een positief effect op de infectiviteit. De totale impact van de schimmelspray was echter even groot op gaas met een kleine maaswijdte (waar muggen niet doorheen konden). Met schimmel-behandeld gaas zou gebruikt kunnen worden voor het afschermen van huizen om muggen te infecteren op het moment dat deze op zoek zijn naar een bloedmaaltijd. Zowel potten als gaas bieden meerdere en flexibele opties voor het gebruik van schimmels tezamen met al bestaande bestrijdingsmethoden.

In het tweede deel (Hoofdstuk 6 & 7) werden schimmelsporen tegen insecticidenresistente *Anopheles* muggen getest en werd gekeken naar de impact van schimmels in combinatie met chemische insecticiden. *Metarhizium* en *Beauveria* bleken uiterst effectief tegen meerdere soorten resistente malariamuggen. Allereerst werden in het laboratorium in Zuid-Afrika vier *Anopheles* muggenkolonies met enzymatische resistentie tegen de insecticiden permethrine en DDT getest. Deze resistente muggen bleken even vatbaar voor een infectie met *Beauveria* te zijn als hun verwante, niet-resistente soortgenoten. Daarnaast werd de effectiviteit van schimmels ook getest in West-Afrikaanse *An. gambiae* muggen, die resistent zijn tegen insecticiden door de genetische "*kdr*" mutatie. *Metarhizium* en *Beauveria* konden zowel de wild-gevangen muggen als de muggen gekweekt in het laboratorium effectief infecteren en binnen 10 dagen doden. Deze resultaten tonen aan dat resistentie tegen chemicaliën geen resistentie tegen schimmels veroorzaakt.

Het onderzoek richtte zich ook op de effectiviteit van combinaties van schimmelsporen en chemische insecticiden. Schimmels en insecticiden bleken geen negatief effect op elkaar te hebben, maar juist elkaars werking te versterken. Een schimmelinfectie verhoogde de gevoeligheid van resistente muggen voor de neurotoxische insecticiden permethrine en DDT. Muggen met enzymatische resistentie-mechanismen toonden een veel hogere mortaliteit na insecticideblootstelling wanneer ze geïnfecteerd waren met schimmel. In omgekeerde volgorde, bleek blootstelling aan permethrine ook de virulentie van de schimmelinfectie te kunnen versterken in de West-Afrikaanse muggen. Blootstelling aan zowel schimmels als insecticiden had in meerder combinaties een synergistisch (groter dan verwacht) effect op de overleving van malariamuggen. De totale impact was het grootst in muggen die op dezelfde dag aan beide bestrijdingsmiddelen waren blootgesteld. Dit impliceert dat het het gunstigst zou zijn om schimmels en insecticiden zodanig geïntegreerd toe te passen dat muggen tegelijk of tijdens eenzelfde nacht met beide middelen in contact komen. Doordat ze elkaars werking versterken, zou er minder van beide middelen gebruikt hoeven worden in combinatie-applicaties. Deze synergy en de potentie tot het overwinnen van chemische resistentie duidt aan dat schimmels aanvullend gebruikt zouden kunnen worden om de huidige malariabestrijding te versterken en verdere verspreiding van insecticidenresistentie te beperken.

Er zijn nog verscheidene factoren te optimaliseren om een efficiënte, lang houdbare en kosteneffectieve bestrijdingsmethode op basis van schimmels te kunnen realiseren. Het zal onder andere nodig zijn om goedkope massa-produktie van schimmelsporen te bewerkstelligen en om lang-houdbare formuleringen en effectieve toepassingsmethoden te ontwikkelen. De huidige bevindingen zijn gebaseerd op laboratorium-onderzoek en zouden daarom allereerst ook geëvalueerd moeten worden in realistische, tropische omstandigheden. Dit proefschrift biedt bruikbare kennis en middelen voor toekomstig onderzoek aan nieuwe malaria-bestrijdingsmiddelen op basis van schimmelsporen. Een van de belangrijkste uitkomsten is dat schimmels een optie bieden voor de bestrijding van insecticidenresistente muggenpopulaties en de grootste impact zouden kunnen hebben in combinatie met insecticiden. Biologische en chemische muggenbestrijding hoeft elkaar dus niet uit te sluiten, maar zou juist complementair kunnen werken en in combinatie-toepassingen elkaars werking kunnen versterken. Het gebruik van nieuwe malariabestrijdingsmethoden op basis van schimmels zou zich daarom moeten richten op integratie in al bestaande interventies, om zodoende een zo groot mogelijke impact op malaria te kunnen realiseren.

Acknowledgements

Even though this thesis has been produced in a relatively short period of time, it has (like any PhD) not been an effortless process but one with its ups and downs. It has taken hard work, perseverence and the support of several people to create this end-result I now proudly present. In these next pages, I will do my best to acknowledge all who have contributed to this work.

I would first like to express my sincere gratitutude to my promotor and copromotor; Louise Vet and Matt Thomas. Louise, we have only worked together for a short period of time, but I have come to know you as a very motivating supervisor and an inspiring strong woman. It has been wonderful to work with someone so open, direct and decisive. I am thankful for your positive energy and your support during the last stages of my PhD. Matt, it has been a great privilege to work you and I am grateful for all your contributions and support. You have always been wonderfully timely in your responses and your suggestions have really improved the content of this work and helped me to think critically and in a broader perspective.

The majority of the work has taken place at the Laboratory of Entomology of Wageningen University and I would like to thank all my colleagues there. A special thanks to the Vector group, for all the fruitful scientific discussions. It has been a great experience to work in such a diverse, multinational group and to be exposed to so many research disciplines. I have enjoyed working in Wageningen and have fond memories of the "good times" in the Entomology lab.

This particular work on fungus-based mosquito control was part of a Consortium of research groups, and I am grateful for all the scientific contributions from my colleagues at the Vector Control Reference Unit in Johannesburg, the Bioprocess Engineering group of Wageningen University, the Entomology department of Pennsylvania State University, the Pasteur Insitute in Paris, the Ifakara Health Institute in Tanzania and the International Center of Insect Physiology and Ecology in Kenya. In particular, I would like to thank Maureen Coetzee, Lizette Koekemoer, Basil Brooke, Joel Mouatcho and all others at the VCRU for a wonderful time in South Africa, during which I learned a lot about insecticide resistance. It was a pleasure to work with you and obtain successful results together. Also a special thanks to Arjen Rinzema, Frank van Breukelen, Mgeni Jumbe and Sebastiaan Haemers of the BPE group in Wageningen for consistently providing us with fungal spores for our experiments.

I consider myself lucky to have had the opportunity to collaborate with the Entomology Research Center in Cotonou and the London School of Hygiene and Tropical Medicine during a short but productive work visit to Benin, and I would like to thank Raphael N'Guessan and Mark Rowland for enabling this. Furthermore, I am very thankful to Daniela Farina and Anne Hilhorst, who have contributed to several experiments and were a pleasure to work with. I am also grateful for the collaboration with Anne Osinga and Johan Deschietere and look forward to our future work together.

I would like to thank all who have contributed to the funding and materials required for this work. First and foremost, I sincerely thank my sponsors, the Adessium Foundation, who have enabled me to pursue this PhD research. It has been great to collaborate with you and experi-

ence your genuine interest in malaria prevention. I would also like to express my appreciation to the companies who have provided us with the necessary research equipment. These include Afrikaad (Barendrecht, The Netherlands), who provided the handmade Ghanaian clay pots, RK Print Coat Instruments Ltd (London, UK) who provided the coating instruments and Van Heek (Losser, The Netherlands) and Vestergaard Frandssen (Lausanne, Switzerland), who provided the netting samples. I would like to thank the rearing group of Entomology in Wageningen, including Leo, Frans, Adré, Leon and Dennis for the continuous provision of mosquitoes for our experiments.

There are many friends I would like to thank for indirectly assisting in this work. First of all, I warmly thank my (former) colleagues who are now my friends: Tullu, Martine, Remco, Niels, Fedor, Krijn, Maaike, Patrick, Ties, Roland and Janett, and my squash-ladies Anneke and Joke. You have made Wageningen an enjoyable place for me, which is a difficult thing to achieve for an Amsterdam girl. Thank you all for the wonderful parties we had together and your support through thick and thin. A special thanks to my paranymphs Martine and Tullu, with whom I enjoyed many dinner parties and shopping sprees. Tullu, my hamshira, I am so grateful to have had you as a munafec-support and look forward to continue our friendship and work together.

A big thank you also to my dear friends Marloes, Serge, Inge, Marloes, Ineke, Kirsten, Joki, Matthieu and Marijke for the much needed relaxation during these last years. Even though the parties, grape-picking holidays, city trips etc. were actually not so relaxing but quite exhausting at times, they were a lot of fun and helped me to remember that there is so much more to life than just work. Thank you all for sometimes literally helping me to escape to the real world!

My family deserves a lot of credit and appreciation. My parents have not only made am important genetic contribution but have also been a consistent source of love and support. Thank you so much for everything mum and dad. I hope you'll be able to manage all these doctors in the family. I would like to thank my big brother Rik for his advice and support, in particular on all computer-related matters and text editing with LaTeX. This thesis would not have looked as nice as this without his help. I am also grateful for his lovely family; my sister-in-law Taoying and my nephew Michael.

Last but not least, I decided to dedicate this last paragraph to the person who has been the real center of this research. Professor Bart Knols, thank you, this work is for you! You were a key founder of the mosquito-fungus research and my most important supervisor and motivator. With your never-fading creative energy you inspired me to think out of the box, be inventive, and to have a broad and realistic perspective on research. I am very grateful for your selfless support, especially in publishing, which enabled me to produce a thesis consisting of published chapters. We've been on an interesting journey together, where I started as your student, became your (P)AIO, friend and now even businesspartner. It has always been a real joy to work with you and I am grateful and proud that we, together with Remco, will continue collaborating and pursue science for real impact through IN^{2}CARE.

Curriculum Vitae

Marit Farenhorst (1983) studied Biology at the VU University in Amsterdam during which she took part in research projects on tundra ecology (Svalbard, 2005), *Leishmania* parasitology (Royal Tropical Institute (KIT), 2006) and malariology (Wageningen University, 2007). She obtained her Masters degree in 2007 and started her doctoral research on biological malaria mosquito control with entomopathogenic fungi at the Laboratory of Entomology of the Wageningen University in 2008. Her research was part of a large, international Consortium consisting of research institutes from developed and developing countries. She pioneered the use of fungi against insecticide-resistant mosquitoes in South Africa (2008) and Benin (2009), which received wide community interest and media attention. Marit published all her thesis chapters in peer-reviewed scientific journals, inlcuding one in the *Proceedings of the National Academy of Sciences* (*PNAS*), and is an author on several other publications. She was, furthermore, involved in the training and supervision of students and joined several international conferences and meetings. During her PhD research, she obtained extensive experience in the rearing and handling of mosquitoes and the use of (bio)insecticides, as well as in-depth knowledge on experimental designs and statistical analyses of bioassay data. In her current position as entrepreneur, Marit aims to translate scientific research into applicable technology and to develop effective tools against insect vectors.

Publications

Peer-reviewed publications

- Van der Meide, M.F., Guerra, J., Schoone, G., **Farenhorst, M.**, Coelho, L., Faber, W., Peekel, I. & Schallig, H.D.F.H., **2007**. Comparison between QT-NASBA, real-time PCR and real-time Reverse-Transcriptase PCR for quantification of *Leishmania* parasites. *J. Clin. Microbiol.* 46 (1):73-78.
- **Farenhorst M**., Farina D., Scholte E.J., Hunt, R.H., Coetzee M. & Knols B.G.J., **2008**. African water storage pots for the delivery of the entomopathogenic fungus *Metarhizium anisopliae* to the malaria vectors *Anopheles gambiae s.s.* and *An. funestus*. *Am. J. Trop. Med. Hyg.* 78 (6), 910-916.
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- Howard A.F.V., Koenraadt C.J.M., **Farenhorst M.**, Knols B.G.J. & Takken W., **2010**. Pyrethroid resistance in *Anopheles gambiae* leads to increased susceptibility to the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*. *Malaria Journal* 9 (168).
- **Farenhorst, M.**, Knols B.G.J., Thomas M.B., Howard A.F., Takken W., Rowland, M. & N'Guessan R.K. **2010**. Synergy in efficacy of fungal entomopathogens and permethrin against West African insecticide-resistant *Anopheles gambiae* mosquitoes. *PLoS ONE* 5 (8).

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- **Farenhorst, M.**, Hilhorst A., Thomas M.B. & Knols B.G.J. **2010**. Development of fungal applications on netting substrates for malaria vector control. *J. Med. Entomol.* in press.

Other publications

- **Farenhorst M.** & Knols, B.G.J., **2007**. Fungal entomopathogens for the control of adult mosquitoes: a look at the issues. *Proc. Neth. Entomol. Soc. Meet.* 18: 51-59.
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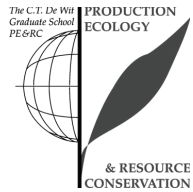
PE&RC PhD Education Certificate



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 (5.6 ECTS)

- Fungal entomopathogens for the control of adult mosquitoes: a look at the issues (2008)

Writing of project proposal (7 ECTS)

- Valorisation research on fungi for the control of African malaria mosquitoes (2008)

Post-graduate courses (5 ECTS)

- Organization & leadership for PhD students; USG, Utrecht (2009)
- Survival analysis; SENSE, WIMEK (2009)

Laboratory training and working visits (4.3 ECTS)

- Fungi against insecticide-resistant mosquitoes; VCRU, NICD, Johannesburg, South Africa (2008)

Invited review of (unpublished) journal (1 ECTS)

- Insecticide resistance in anophelines; Parasites & Vectors (2010)

Competence strengthening / skills courses (1.5 ECTS)

- PhD Competence assessment; WGS (2008)
- Techniques for writing and presenting a scientific paper; WGS (2008)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.8 ECTS)

- PE&RC Day (2008-2010)
- PE&RC Weekend (2009)

Discussion groups / local seminars / other scientific meetings (3.8 ECTS)

- PhD Students discussion group; Entomology (2008-2010)
- Annual meeting of the Netherlands Entomological Society (2008-2010)
- Discussion group on fungus production methods for malaria control(2008-2010)

International symposia, workshops and conferences (4.4 ECTS)

- Annual Conference of the Consortium for Entomopathogens against Malaria Vectors; 1 presentation each year (2008-2010)
- MIM Pan-African Malaria Conference, Nairobi; poster presentation (2009)

Lecturing / supervision of practicals / tutorials (1.2 ECTS

- Ecology (practical); 3 days (2009)
- Magical Mushrooms (guest lecture); 1 day (2010)

Supervision of a MSc student (3 ECTS)

- Impregnation of mosquito netting with fungal spores (6 months)

This project was funded by the Adessium foundation, The Netherlands

Typeset in LaTeXby the author
Cover illustration from i5starsolutions inc.
Printed by Wöhrmann Print Service, Zutphen