SUSCEPTIBILITY OF LABORATORY COLONIES OF MEMBERS OF THE *ANOPHELES GAMBIAE* COMPLEX TO ENTOMOPATHOGENIC FUNGI *BEAUVERIA BASSIANA*

By

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DECLARATION

I, Christophe Kikankie, declare that this dissertation is my own independent work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

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Signature

…2nd… Day of…March…2009

ABSTRACT

Control of the major African malaria vectors of the *Anopheles gambiae* complex continues to rely heavily on the application of insecticides either by indoor residual house spraying (IRS) or bednet impregnation (ITNs). However, growing concerns about their negative impact on human health, their potential impact on the environment, and the ever-increasing spread of insecticide resistance across sub-Saharan Africa has diverted attention to alternative strategies that are not based on chemicals. Therefore, alternatives such as vector control with biological agents may be effective. Levels of susceptibility to the entomopathogenic fungus *Beauveria bassiana* were assessed against insecticide-resistant and insecticide-susceptible laboratory colonies as well as a wild-caught sample of the *An. gambiae* complex of which most members are major or localised malaria vectors.

Insecticide susceptibility tests against all four classes of insecticides recommended by WHO for vector control was performed according to the standard WHO bioassay protocol. Fungal susceptibility tests using suspensors dusted with dry conidia of *B. bassiana* isolate IMI 391510 were performed on laboratory-reared colonies of *An. gambiae* complex members as well as on a sample of wild caught, insecticide susceptible *An. arabiensis* from Malawi.

Our data showed that there is no interaction between insecticide susceptibility and fungal susceptibility. Survival of both insecticide susceptible and insecticide resistant colonies as well as wild mosquitoes was significantly affected ($P < 0.001$) by fungal infection. All infected mosquitoes succumbed to fungal infection within 7-20 days post-exposure compared to control groups for which 70-85% of mosquitoes were still alive at that time. The evidence of fungal infection was confirmed with more than 90% of the mosquito cadavers sporulating 3-5 days after incubation.

These results provide hope for alternative control strategies for adult anopheline mosquitoes other than those that rely entirely on insecticides. There was evidence that insecticide susceptible and resistant mosquitoes can be killed by the fungus *B. bassiana* within the 14 day period before they become infective with the malaria *Plasmodium* parasite. However, conidia formulations and concentrations as well as a suitable delivery method for efficient coverage remain the areas of focus for field implementation.

DEDICATION

This thesis is dedicated with love to the memory of Angelique Mendaku Lubamba 1944-2004. I will always be thankful for my mother's advice, encouragement and unconditional love. She was a wonderful daughter (Mwanabuwa), sister, wife, mother, grandmother, and best friend to all who knew her. I miss her dearly.

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CONTENTS

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%	Percentage
µl	Microlitres
bp	Base pair
DDT	Diethyl diphenyl trichloroethane
DNA	Deoxyribonucleic acid
DNTPs	Nucelotide triphosphates
h	Hour(s)
HR	Hazard ratio
IRS	Insecticide residual spraying
ITNs	Insecticide treated bednets
kb	Kilobase
M	Molar
mg	Milligram
min	Minute(s)
ml	Millilitre
mm	Millimetre
mM	Millimole
nm	Nanomole
ºC	Degrees Celsius
P	Probability level
PCR	Polymerase Chain Reaction
rpm	Revolutions per minute
S	Second(s)
T	Time

CHAPTER I
INTRODUCTION

1.1 Malaria overview and impact

Malaria is the most common and devastating parasitic disease in the world and is confined mainly to the tropics. It is postulated that ninety per cent of all cases occur in sub-Saharan Africa, accounting for 300-500 million clinical cases and over one million deaths each year (WHO, 2005).

1.2 Malaria transmission and mosquito vectors

Human malaria is caused by protozoan parasites of the genus *Plasmodium*. There are four species of *Plasmodium* that cause human malaria: *P. falciparum, P. malariae, P. vivax,* and *P. ovale*. Recently a fifth human parasite was discovered, *P. knowlesi*, with an animal reservoir (Singh *et al*., 2004; Cox-Singh *et al*., 2008). Malaria is transmitted by female mosquitoes of the genus *Anopheles* (Diptera: Culicidae), with members of the *An. gambiae* species complex, as well as *An. funestus* of the *An. funestus* species group, being the main vectors (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987).

The *An. gambiae* complex consists of at least seven species which are morphologically similar and can only be distinguished by analysis of polytene chromosome banding patterns (Coluzzi and Sabatini, 1969; Hunt, 1973), allozyme

analysis (Miles, 1978) or molecular methods (Collins *et al.*, 1987; Scott *et al.*, 1993). Members of this complex vary in their ability to transmit malaria. The two principal vectors in the complex are *An. gambiae sensu stricto*, which is the most efficient vector, and *An. arabiensis* (Gillies and Coetzee, 1987).

1.2.1 The *Anopheles gambiae* complex

The *An. gambiae* complex consists of at least seven sibling species (Gillies and Coetzee, 1987; Hunt *et al.*, 1998). It includes the major vectors *An. gambiae* and *An. arabiensis* as well as two saltwater breeding species *An. merus* and *An. melas*. *Anopheles bwambae, An. quadriannulatus* species A and B complete the list.

Members of the *An. gambiae* complex show varying degrees of vectorial capacity. *Anopheles gambiae* and *An. arabiensis* are the most efficient vectors and often occur in sympatry in sub-Saharan Africa (Coluzzi *et al.*, 1979; Gillies and Coetzee, 1987).

1.2.1.1. *Anopheles gambiae sensu stricto*

Distribution records of *An. gambiae s.s.* indicate that the species is more common in West and East Africa and has a patchy distribution in southern Africa (Coetzee *et al.*, 2000). This species is often localised in areas with closed forest canopy. *Anopheles gambiae* breeds in a variety of shallow, open, sunlit pools. The origin of such pools is

legion, and may range from burrow pits, drains, brick pits, ruts, car tracks, hoof prints, ponds and water holes (Gillies and De Meillon, 1968). Although *An. gambiae* thrives under rather cool conditions, it is tolerant of relatively high temperatures. In general terms, seasonal changes in *An. gambiae* populations tend to follow the seasonal pattern of rainfall. Thus in savannah zones, with a single rainy season, numbers start to rise explosively soon after the first main rains, peaking in the middle of the rainy season and declining steadily thereafter as the levels of water become stabilized and vegetation and predators become established (Gillies and De Meillon, 1968).

Anopheles gambiae is regarded as the most efficient malaria vector in sub-Saharan Africa, particularly in West Africa (della Torre *et al.*, 2002). Female *An. gambiae* are highly anthropophilic, feeding preferentially on humans (White 1974; Coluzzi *et al.*, 1979), although in West Africa they are less discriminating and will feed readily on other animals like horses and cattle (Diatta *et al.*, 1998; Bøgh *et al.*, 2001). The endophilic and anthropophagic behaviours of *An. gambiae* create an intimate association between human reservoir and the insect vectors of malaria. Savannah populations, especially in East and South-eastern Africa, tend to show higher levels of zoophily (Gilles and De Meillon, 1968).

1.2.1.2. *Anopheles arabiensis*

Anopheles arabiensis has a wide distribution in Africa ranging from Madagascar to Senegal and Sudan to South Africa (Coetzee *et al*., 2000). The range and relative abundance of *An. arabiensis* tend to be influenced by climatological factors, especially total annual precipitation (Lindsay *et al.,* 1998). This species preferably breeds in fresh, temporary, sunlit, rain water pools (Gilles and Coetzee, 1987). *Anopheles arabiensis* tend to be more tolerant of higher temperatures and is able to survive in drier conditions. This explains why it is found biting during the dry season (Petrarca *et al*., 2000).

Anopheles arabiensis has a number of strategies which allows it to persist in arid conditions: adult females tend to lay their eggs on damp surfaces rather than water, with hatching being delayed in a proportion of eggs (Coluzzi, 1965 cited by Lindsay *et al*., 1998), and females aestivate during periods of prolonged dryness (Omer and Cloudsley-Thomson, 1970). *Anopheles arabiensis* has a more opportunistic feeding behaviour although it can be entirely zoophilic, as studies from Madagascar have shown (Duchemin *et al*., 2001). The species also tends to be more exophagic and exophilic. The variable behaviour of *An*. *arabiensis* females, being anthropophilic and zoophilic as well as endophilic and exophilic (White, 1974), makes them incompletely vulnerable to house-spraying (Gilles and Coetzee, 1987).

Seasonal abundance of *An. arabiensis* with peaks following the onset of rains makes it largely responsible for seasonal malaria transmission in southern Africa (Hargreaves *et al.*, 2003).

1.2.1.3. *Anopheles merus*

Anopheles merus is a saltwater breeding member of the *An. gambiae* complex which has been shown to be involved in low rate malaria transmission (Muirhead-Thomson, 1951 cited by Sharp, 1983). Its distribution is limited to the East and southern Africa and the adjacent coastal islands (Sharp, 1983). Larvae of *An. merus* are found in coastal Avicennia mangrove swamps, salt pans, brackish ponds (Gilles and De Meillon, 1968) and mineral springs (Coetzee and Le Sueur, 1988). In the absence of domestic animals, *An. merus* bites man readily both indoors and outdoors. Gillies and De Meillon, (1968) observed that in the presence of other hosts *An. merus* is more attracted to cattle.

1.2.1.4. *Anopheles quadriannulatus*

The two zoophilic members of the *An. gambiae* complex, *An. quadriannulatus* species A and B have a limited distribution associated perhaps with more subtropical climates than the other members of the complex (Hunt *et al*, 1998). Sibling species A

and B are recognized as allopathic members of the *An. gambiae* complex. Species A represents *An. quadriannulatus sensu stricto*, widespread in southern Africa (Hunt *et al.*, 1998), whereas *An. quadriannulatus* species B occurs in Ethiopia (Fettene *et al.*, 2002). *Anopheles quadriannulatus* is a cattle feeding, outdoor-resting member of the *An. gambiae* complex and is not known to transmit malaria parasites (Coetzee, 1989). Both species A and B are freshwater breeders and are adapted to lower developmental temperatures.

1.2.1.5. *Anopheles melas*

The name *melas* was retained for this West African saltwater-breeder even though the species could not definitely be identified according to the parameters set down by Coluzzi (1964). *Anopheles melas* is of local importance in malaria transmission in West Africa as it breeds in localised coastal areas. Generally, it shows a low sporozoite rate resulting in a minor role in malaria transmission (Gillies and Coetzee, 1987).

1.2.1.6. *Anopheles bwambae*

Anopheles bwambae is known only from the Bwamba region of Uganda where it breeds in warm, mineral spring water in the Semliki forest. It is of local importance in malaria transmission (Gillies and Coetzee, 1987).

1.2.2 The *Anopheles funestus* group

Anopheles funestus is a major malaria vector in Africa, and is the nominal member of the *An. funestus* group. This group consists of nine morphologically similar species: *An. funestus*, *An. aruni*, *An. rivulorum*, *An. vaneedeni*, *An. parensis*, *An. brucei*, *An. confusus*, *An. fuscivenosus* and *An. leesoni* (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). *Anopheles funestus* Giles has a similar distribution over Africa to that of *An. gambiae s.l.*, but prefers to breed in large permanent water bodies. This species is highly anthropophilic and endophilic making it susceptible to control by indoor residual spraying (Gillies and De Meillon, 1968).

1.3 Malaria control

Malaria is a preventable and curable disease and WHO recommends the following interventions: case management, vector control and prevention of the disease by use of anti-malarial drugs (WHO, 2001).

The world's prime choice for malaria vector control still remains the selective application of residual insecticides through either indoor residual house spraying or bednet impregnation (WHO, 2003). These approaches have been highly effective in reducing malaria morbidity and mortality because results can be obtained on a large scale at an affordable cost (WHO, 2003). However, beyond gains in economic and

public health terms their environmental impact, toxicity to humans and the ever increasing development of resistance to insecticides (Hargreaves *et al*., 2000, 2003; Awolola *et al*., 2002; Diabate *et al*., 2004; Matambo *et al*., 2006) is of great concern. Resistance to insecticides has spread to all classes of insecticides currently used in public health and is widespread geographically (Chandre *et al*., 1999; Hemingway and Ranson, 2000; Etang *et al*., 2003; Coetzee *et al*., 2006; Abdalla *et al*., 2007; Munhenga *et al*., 2008).

It is therefore not surprising that interest in alternative non-chemical strategies has increased over the last decades. The use of biological agents such as predatory fish (Legner, 1995), bacteria (Becker and Ascher 1998), protozoa (Chapman, 1974; Legner, 1995), nematodes (Kaya and Gaugler 1993) and entomopathogenic fungi (Scholte *et al*., 2004; Thomas and Read, 2007; Farenhorst *et al*., 2008) have shown promise in malaria vector control.

An interest in using entomopathogenic fungi to control insect pests started in 1879 when the fungus *Metarhizium anisopliae* was used to control wheat beetles. It was later used to control the sugar beetle curculio, *Cleonus punctiventris* (Cloyd, 1999). The use of *M. anisopliae* against mosquitoes started with the targeting of *An. gambiae* populations (Scholte *et al*., 2004).

1.3.1 Vector control

Vector control is based on the use of pesticides and offers the best prospect for malaria control by decreasing contact between humans and vectors. It includes larval control, indoor residual spraying (IRS), use of insecticide treated bednets (ITNs) and biological control.

1.3.1.1. Indoor residual spraying (IRS)

Malaria vector control using insecticides directed at indoor resting mosquitoes began in the 1930's with the use of pyrethrum extracted from flowers (De Meillon, 1936). During the Global Malaria Eradication campaign of 1955-1969, DDT and dieldrin replaced pyrethrum as the insecticides of choice for an indoor-spraying vector control strategy (WHO, 1970). Resistance to DDT and dieldrin by target mosquito populations coupled with concern over their environmental impact led to the total banning of dieldrin and discontinued use of DDT in most regions (Collins and Paskewitz, 1995). The advent of synthetic pyrethroids in the 1980's has seen this class of insecticide replacing DDT for house spraying in malaria control programmes. Pyrethroid-based indoor residual spraying and insecticide treated nets and curtains are currently advocated as standard malaria vector control strategies (WHO, 2001). Despite the long history of malaria control, total eradication has not been achieved. This is partly due to the rapid development of insecticide resistance over the last five decades because of an over-reliance on chemical control strategies.

It is currently recommended that an integrated approach be used. This involves the utilization of all appropriate technological and management techniques to bring about an effective degree of vector suppression in a cost-effective manner (WHO, 2003). This in fact is not a new concept for suppression of vectors. It was developed in the early part of the twentieth century, but was overshadowed by the development of the long-lasting residual insecticides during the 1940s which presented a single method of vector control with an extremely high, but incalculable, benefit/cost ratio and became the major component in many vector control efforts (Collins and Paskewitz, 1995). However, the development of resistance to insecticides in vectors, concern about environmental contamination and human safety, and the increased costs of alternative insecticides led to an emphasis on the development and use of several control techniques simultaneously i.e. integrated vector management. Biological control of vectors, through the use of bacterial agents, fish and other predators and pathogens, is a method which could compliment other control strategies (Curtis, 1990; Collins and Paskewitz, 1995).

1.3.1.2. Insecticide treated bednets (ITNs)

The widespread use of mosquito nets treated with insecticide aims to prevent malaria in areas where the infection is common. ITNs are widely promoted by international agencies and governments to reduce the malaria burden (RBM, 2005).

ITNs have been found to be highly effective in reducing childhood mortality and morbidity from malaria by providing 15-20% protective efficacy compared to no nets and up to 23% protective efficacy compared to untreated nets (Lengeler, 2004; Mathanga *et al.,* 2005). Shifting from conventional nets which require insecticide re-impregnation every 6-12 months, several companies have recently developed long-lasting insecticide treated nets (LLINs) capable of retaining lethal concentrations of insecticide for 3 to 5 years. The WHO is strongly recommending the use of these LLINs for the prevention of malaria in Africa (WHO, 2008). However, despite the fact that mosquitoes can still bite people outdoors or before they get under their nets for sleeping, the cost of an ITN is a major barrier to ownership and usage for that proportion of Africans who are amongst the poorest of the poor and are also the most affected by malaria. Further, after prolonged exposure to an insecticide over several generations, mosquitoes may develop insecticide resistance – basically defined as a capacity to survive contact with an insecticide. Several researchers have reported on the increasing occurrence of insecticide resistance in malaria mosquito populations to commonly used insecticides either for IRS or ITNs (Hargreaves *et al.,* 2000; Awolola *et al*., 2002; Coetzee *et al*., 2006; N'Guessan *et al.* 2007).

Although widespread access to ITNs is currently being advocated by the Roll Back Malaria initiative, Lengeler (2004) quoted that universal deployment will require major financial, technical, and operational inputs and, moreover, ITN coverage does not guarantee usage. Advocacy concerning behaviour change is an

intense area of focus to ensuring the protection of those that are most vulnerable to malaria.

1.3.1.3. Environmental vector management

Environmental management was the most effective method for reducing malaria in some regions during the early 1900s. WHO defines environmental management for vector control as the planning, organization, carrying out and monitoring of activities for the modification and/or manipulation of environmental factors or their interaction with humans with a view to preventing or minimizing vector propagation and reducing human-vector-pathogen contact (WHO, 1982). Environmental management for malaria vector control is all about good housekeeping of the environment, ensuring that opportunities for vectors to exploit breeding places are reduced to a minimum. It entails two goals: environmental modification requiring permanent infrastructural changes of a capital-intensive nature and, secondly, temporary activities aimed at achieving unfavourable conditions for vector breeding (WHO, 1988).

Environmental vector management may be achieved using various environmental manipulations that include straightening of rivers and removal of obstructions in the waterway, swamp drainage, house screening**,** control of water levels, stream sluicing or flushing, changes of water salinity, shading of stream banks, use of larvivorous fish, etc. In Malaysia for example, it was demonstrated that

draining and filling swamps in one year led to a sharp reduction in malaria and later to malaria eradication by the selective clearance of tropical forest around settlements, good drainage and the restriction of housing to at least 1 km from the edge of the nearest undrained rainforest (Singh and Tham, 1988). However, the ability of an environmental management strategy to control malaria depends critically on how well it is matched to the ecological requirements (climate conditions and habitat) and behaviour of the target malaria vectors in an area (Lindsay *et al*., 2004).

1.3.1.4. Biological control

Because of insecticide resistance and the environmental impact of spraying insecticides, considerable resources have been devoted to the search for biological control agents which present no risk to either human health or the environment and have shown promise in malaria vector control.

Biological control is based on the use of natural enemies to control pests (Garcia and Legner, 1999). There are two major schemes in which this can be achieved (i) classical biological control which involves the importation of natural enemies to control native pests and (ii) inundative and inoculative biological control which involve releasing biological control agents without the goal of permanent establishment (Eilenberg *et al*., 2001). These methods have been used with great success to control a variety of invertebrate pests, including scale insects, white fly, thrips, and mosquitoes (Wawrzynski *et al.*, 2001; Hajek *et al.*, 2003).

1. Bacteria

The discovery, in the 1970s, of bacterial agents specific to a narrow range of Diptera, including mosquitoes, brought a new dimension to their control using biological means. The spore bacteria *Bacillus thuringiensis israelensis*, serotype H-14 (*Bti*) and *B. sphaericus* 1593 have been used successfully to control various pests of both agricultural and public health importance (Service, 1983; WHO, 1995; Becker and Ascher, 1998; Fillinger *et al.*, 2003). The toxins produced by *B. thuringiensis* are now available commercially and are very effective in the control of mosquito larvae. Among the genera of mosquitoes controlled by *Bti* are *Anopheles, Aedes, Culex, Culiseta, Mansonia* and *Coquillettidia* (Bolay *et al*., 1990; Seyoum and Abate, 1997; Fillinger *et al*., 2003). Culicines are generally more susceptible than anophelines because of their bottom-feeding habits.

Other genetically-modified bacteria have been developed and found effective in controlling malaria vectors. An example is the *Asaia* bacterium which is associated with *An. stephensi*, an important mosquito vector of *P. vivax* in Asia. Researchers found that *Asaia* colonize the gut and salivary glands in females as well as the reproductive tract in males; it was also proven that the bacteria can also spread to mosquito offspring, as they found it in the eggs, ovaries and testes of mosquitoes and also in pupae and larvae (Favia *et al*., 2007). However, safety studies based on scientific facts are still needed to determine the future use of these genetically-modified bacteria as a weapon against malaria.

2. Viruses

Mosquitoes also suffer from viral infections. These include nuclear polyhedrosis virus, cytoplasmic polyhedrosis virus, mosquito iridescent virus, turquoise mosquito iridescent virus, etc. None of these viruses are easy to culture and mass produce. For instance, transmission of the baculovirus CuniNPV has only been reported as successful in mosquitoes of the genus *Culex*. It was found to be highly virulent with the ability to persist and recycle (Becnel *et al.,* 2001, Andreadis *et al.,* 2003). However, no infections have been found with any species of *Aedes, Toxorhynchites, Ochlerotatus, Culiseta* or *Anopheles* exposed in laboratory assays (Becnel *et al*., 2001, Andreadis *et al*., 2003).

3. Predatory fish

Biological control of mosquitoes started with the targeting of the aquatic larval stages. Various species of mosquito fish are the most widely disseminated biological control agent of mosquitoes in the world. They have been used with some success in various countries. In Turkey, the establishment of the larvivorous fish *Gambusia affinis* resulted in a 50% reduction in malaria cases (Legner, 1995). This fish has been used extensively for mosquito control in rice cultivation areas in California and Northern Somalia (Kramer *et al*., 1987; Curtis, 1990). Other fish species such as *Poecilia reticulata* are widely used in Asia to control waste water mosquitoes such as *Culex*

quinquefasciatus (Lardeux *et al.,* 2002). Wild fish are also widely used in areas such as Sri Lanka to control mosquitoes in abandoned wells. This method is however not feasible for *An. gambiae* mosquitoes due to the nature of their preferred larval breading sites (temporary water bodies, rain pools, etc).

4. Protozoa

Several species of protozoa are known to cause infection in mosquito larvae. Jenkins (1964) (cited by Chapman (1974)) stated that 83 protozoan species are known from mosquitoes. Many of these species, especially the ciliates, flagellates, schizogregarines and eugregarines, have been insufficiently studied because of their low pathogenicity, or because they are commensal or facultative parasites. Most studies have concentrated on microsporidia because of their wide distribution, common occurrence in a wide variety of mosquito hosts, and apparent pathogenicity to many species (Chapman, 1974).

Endoparasitic ciliated protozoa have been known to infect mosquito larvae since 1921, when Lamborn first reported the occurrence of *Lambornella stegomyiae* infection in the larvae of the Asian tiger mosquito *Aedes albopictus* (Lamborn, 1921 cited by Bina Pani Das, 2003). Corliss *et al.* (1976) isolated a second species, *Lambornella clarki*, from tree-hole breeding mosquito larvae of *Aedes sirensis*, in California. In India, studies on endoparasitic ciliates of mosquito larvae found *An.*

barbirostris, An. hyrcanus group and *An. philippinensis* infected with a *Lambornella* sp. (Narain *et al.*, 1996). The new ciliated protozoan species *Chilodonella uncinata* was found to cause 25–100% mortality in Japanese encephalitis vector larvae (Bina Pani Das, 2003). The infectious stage of *Ch. uncinata* was found to be resistant to desiccation and can be produced asexually in a variety of media. It also carries the capacity for trans-ovarial transmission through its mosquito host (Bina Pani Das, 2003).

However, although these protozoans were found to cause chronic and fatal infections in natural populations of mosquito larvae in general, anopheline larvae were less susceptible to widely distributed endoparasitic ciliated protozoan infection than culicine larvae.

5. Nematodes

Several nematodes have been found to be virulent pathogens of mosquitoes and have been used as a mosquito control strategy (Legner, 1995; Becnel and Johnson, 1998). One of the most commonly used is *Romanomermis culicivorax,* a mosquito-parasitic nematode that has achieved high rates of mosquito kill (Brown *et al*., 1977; Peterson 1977; Peterson *et al*., 1979; Rojas *et al*., 1987). *Romanomermis culicivorax* is an obligate parasite of Culicidae larvae, and infects no other organisms. It therefore presents no risk to either human health or the environment. However, supplies of

Romanomermis are not commercially available as this agent cannot be cultured artificially in a way that retains its infectivity.

6. Entomopathogenic fungi

Fungal diseases in insects are common and widespread and can decimate populations. Therefore it follows that if careful manipulations are achieved, fungi can effectively regulate pest and disease vector populations. They owe their perpetuity to varied survival strategies ranging from parasitism to saprophytic feeding. Fungi regulate pest populations naturally and therefore lend themselves to vector control.

There are thought to be over 100 000 species of fungi of which about 750 have been identified as pathogenic to insects (Ferron, 1978; Boucias and Pendland, 1991; Glare and Milner, 1991; Hajek and St. Leger, 1994; Bidochka *et al.*, 2000). Only 10 of these have been or are currently being developed for insect control (Lacey *et al.*, 2001). Fungal pathogens such as *Lagenidium*, *Coelomomyces* and *Culicinomyces* are known to affect mosquito populations and have been studied extensively. Lasko and Washino (1983) found that the entomopathogenic fungus *Lagenidium giganteum* can cause up to 95% mortality in mosquitoes and was also found to be effective against other putative disease vectors.

The Hyphomycetes *Beauveria bassiana* and *Metarhizium anisopliae* have been found to be very effective against both the larval and adult stages of mosquitoes

(Clark *et al*., 1968; Roberts, 1970; Scholte *et al*., 2003 a&b; Blanford *et al*., 2005; Farenhorst *et al*., 2008). Species of Hyphomycetes (*Culicinomyces*, *Beauveria*, *Metarhizium* and *Tolypocladium*) are widely used because of their broad range of insect hosts and ease with which they can be cultured. Entomopathogenic Hyphomycetes have been investigated for use against a broad range of insect pests, including whiteflies, aphids, thrips, termites, grasshoppers and locusts, beetles and others (McCoy *et al*., 1988; Ferron *et al*., 1991; Fargues and Maniania, 1992; Khan *et al.,* 1993; Zimmermann, 1993; Devi, 1994; Milner and Prior 1994; Feng *et al*., 1994; Goettel., 1995, Groetel *et al*., 2000).

The fungus *Tolypocladium cylindrosporum* has the property of transtadial transmission, so that an infected larva that hasn't died from the infection will become a carrier as an adult and infect its own eggs with the fungus (Sweeney and Panter, 1977).

Mode of action of entomopathogenic fungi

Entomopathogenic fungi normally invade the hemocoel through the host's cuticle. It is believed that they infect pests by a disease mechanism which proceeds in 5 steps (Fig. 1.1):

(i) Attachment: fungal spores attach to the insect cuticle.

(ii) Germination: the spore germinates on the insect cuticle and forms a germ tube.

(iii) Penetration: the germ tube penetrates directly into the cuticle. This invasion involves both enzymatic and physical processes.

(iv) Growth: the fungus grows in the hemocoel as a mycelium or blastospore. The spreading fungus kills the host by the invasion of organs. However some entomopathogenic fungi kill their host before the extensive invasion of organs takes place, presumably by the production of toxins and competition for food.

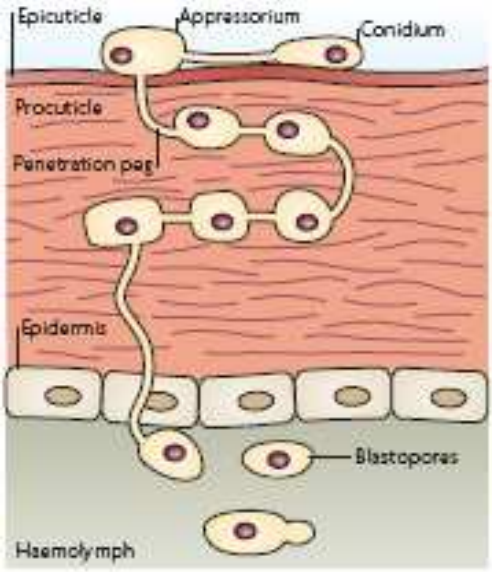

Figure 1.1 Infection process of the insect cuticle (after Thomas and Read, 2007).

(v) Saprophytic Growth: the fungus grows on the outside of the insect and produces aerial conidial spores that can be harvested and recycled. (Eyal *et al*., 1994; Scholte *et al*., 2004)

a) *Metarhizium anisopliae spp.*

Metarhizium anisopliae is an entomopathogenic fungus with a global distribution and wide range of host species. It is characterised by mycelial forms that bear asexual spores termed “conidia”, produced by specialized conidiogenous cells (Bidochka *et al*., 2000). The species is soil-borne, affecting mainly soil dwelling insects.

The first use of *M. anisopliae* as a microbial agent against insects was in 1879, when Elie Metchnikoff used it in experimental tests to control the wheat beetle, *Anisopliae austriaca* (Cloyd, 1999). It was later used to control the sugar beet curculio, *Cleonus punctiventris* (Tulloch, 1976). Since then several studies have demonstrated the potential of entomopathogenic fungi. Laboratory studies by Rath (2000) showed that termites, a major pest of timber and timber products, are highly susceptible to *Metarhizium*. The effects of *Metarhizium* on locusts based on trials from the early 1990s showed a considerable reduction in grasshopper populations after aerial spraying with Green Guard® - a conidial based pesticide (Lawrence, 2006).

Metarhizium is also known to be effective against dipterans such as mosquitoes. Many laboratory studies have shown the potential of *M. anisopliae* as a mosquito control agent. Unformulated conidia of *M. anisopliae* were found effective against larvae of *An. stephensi*, *An. quadrimaculatus*, *Ae. aegypti*, *Ochlerotatus atropalpus*, *Oc. taeniorhynchus*, *Cx. pipiens*, *Cx. restruans* and *Cx. salinarius* (Roberts, 1970). Besides larvae, adult mosquitoes are also known to be susceptible to *M. anisopliae* (Scholte *et al*., 2003 a,b; Blanford *et al*., 2005; Farenhorst *et al*, 2008). Scholte *et al*. (2003a,b) discovered that *Cx. quinquefasciatus* and *An. gambiae* were susceptible to both formulated and unformulated conidia while Scholte *et al*. (2006) and Ondiaka *et al*. (2008) found that fungal infection with *M. anisopliae* reduces

feeding propensity. However, blood meal size and fecundity remained unaffected in female mosquitoes of *An. gambiae* under laboratory conditions (Ondiaka *et al*., 2008).

b) *Beauveria bassiana spp.*

Beauveria bassiana is one of the most frequently isolated entomopathogenic fungal genera and has a cosmopolitan distribution. Under natural conditions, *Beauveria* is rarely associated with mosquitoes with only four cases having been reported: infection of *Cx. tarsalis, Cx. pipiens* and *An. albimanus* by *B. bassiana* and infection of *Oc. sierrensis* by *B. tenella* (Clark *et al*., 1968). Conidia of *B. bassiana* are effective in killing mosquito larvae when applied as a conidial dust to the water surface of breeding sites (Clark *et al.,* 1968). This is mainly because conidia are hydrophobic and thus float on the water surface, coming into contact with mosquito larvae that feed on the surface such as anophelines. This has been demonstrated experimentally in the laboratory where the fungus was found to be virulent against larvae of *Cx. pipiens, Cx. tarsalis, An. albimanus* and *Cx. Tritaeniorhynchus* through contact with the breathing tube (Clark *et al*., 1968; Sandhu *et al*., 1993; Geetha and Balaraman, 1999).

In pilot field trials, reductions of 82%, 95% and 69% of both larvae and pupae were observed when conidia of *Beauveria bassiana* were applied to the water surface to control *Cx. pipiens* (Clark et al., 1968). *Beauveria* is also known to be effective against adult mosquitoes. In a laboratory study 100% mortality was observed in *Cx.*

tarsalis, *Cx. pipiens*, *Ae aegypti*, *Oc. nigromaculis* and *Oc. sierrensis* after an exposure period of only 5 days (Clark *et al*., 1968). In similar studies on *An. gambiae*, it was noted that 82% of the population can be infected within 3.5 days (Scholte *et al*., 2003a,b).

1.4. **Rationale**

In Africa, the use of chemical insecticides is the most widely used method for malaria vector control. However, growing concerns about their negative impact on human health, their potential impact on the environment and the ever increasing spread of insecticide resistance across Sub-Saharan Africa has diverted attention to alternative strategies that are not based on chemicals (e.g. biological control). Most of the biological control methods currently in use target the larval stages of mosquitoes. However, reduction in the survival of adult mosquitoes is considered to have a much higher impact on malaria transmission than a reduction in the number of mosquito larvae. Certain entomopathogenic fungi have been recognized by researchers to be pathogenic to different insects and have been successfully used as biological control agents targeting adult insects. Entomopathogenic fungi are considered to be safe to humans. With such advantages, research on the use of these fungi for malaria vector control is essential.

1.5. **Objectives of the study.**

The general objective of the study was to investigate the susceptibility of laboratory-reared *Anopheles gambiae, An. arabiensis, An. merus* and *An. quadriannulatus* species A to chemical insecticides and to the Hyphomycetes entomopathogenic fungus *Beauveria bassiana*.

Specific objectives were:

1. To establish the baseline susceptibility levels of *An. gambiae s.l.* laboratory colonies to four classes of insecticide.
2. To diagnose the molecular forms of the *An. gambiae* colonies subsequently used for fungal susceptibility tests.
3. To determine the levels of susceptibility of the *An. gambiae s.l.* colonies to the entomopathogenic fungus *Beauveria bassiana*.
4. To compare the levels of susceptibility to the entomopathogenic fungus *Beauveria bassiana* between field-collected mosquitoes and laboratory-reared mosquitoes of the same species.
5. To compare the levels of susceptibility to the entomopathogenic fungus *Beauveria bassiana* between insecticide-resistant and insecticide-susceptible laboratory colonies of the same species.

CHAPTER II
INSECTICIDE SUSCEPTIBILITY

2.1. Introduction

Monitoring insecticide resistance should be part of any malaria vector control program based on indoor residual spraying (IRS) of insecticide and the distribution of insecticide treated bednets (ITNs). Such surveillance aims to provide information on how resistance data can be interpreted with regard to the efficacy of insecticides used in vector control operations (WHO, 1998). The purpose of the WHO insecticide susceptibility test for adult anophelines is to detect the presence of resistant individuals in target mosquito populations or laboratory samples as shown in Fig. 2.1.

Two bioassay methods can be used as diagnostic tests for insecticide resistance on adult mosquitoes. These are either the WHO test kit system using impregnated filter papers with diagnostic concentrations of insecticides or the Centers for Disease Control (CDC) bottle assay (WHO, 1998). Once resistance has been detected in the field and confirmed through susceptibility tests, WHO recommends the following steps be performed: (i) Identify the resistance mechanisms involved and their potential implications in order to avoid cross-resistance patterns, (ii) Evaluate the impact of the development of insecticide resistance on mosquito biting and resting behaviour and on the effectiveness of vector control measures and (iii) Make an

operational decision to change insecticide and implement a resistance management plan in the region (WHO, 1998).

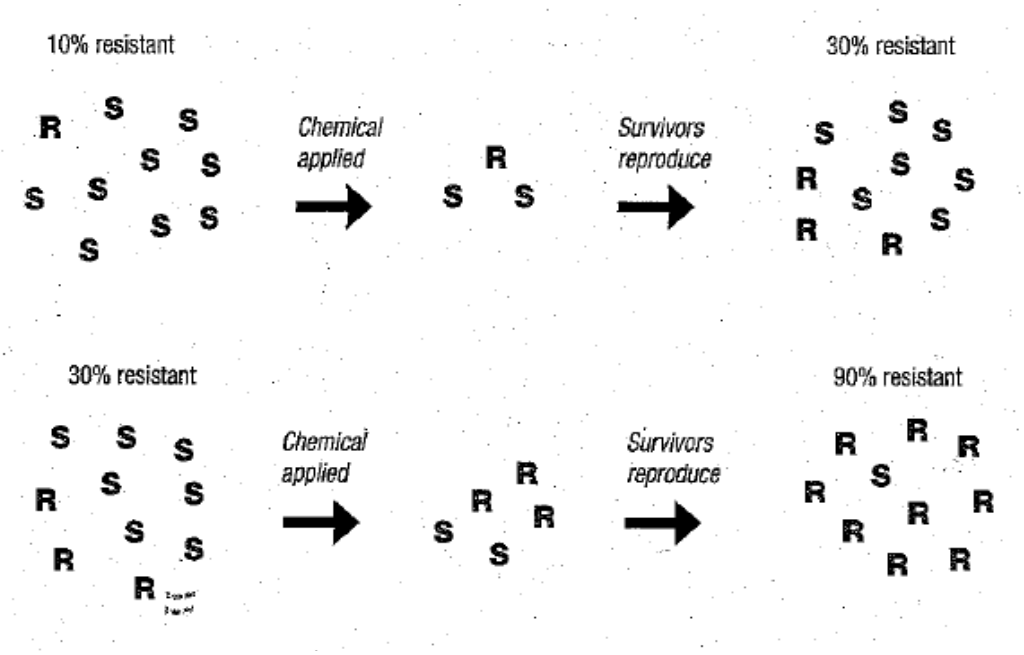

Figure 2.1: Diagrammatic representation of possible scenario for insecticide resistance development, (**R:** resistant population, **S:** susceptible population) as adapted from Scott (1995).

2.2. Materials and Methods

2.2.1. Rearing of laboratory mosquito colonies

Colonies of *Anopheles gambiae*, *An. arabiensis*, *An. merus* and *An. quadriannulatus* housed at the Vector Control Reference Unit of the National Institute for Communicable Diseases, Johannesburg, South Africa, were maintained under

standard insectary conditions of 25 ± 2 ºC, 80% ± 10% relative humidity (RH) and a 12:12 hour day/night cycle with 45 minute dusk/dawn transition photo-periods.

Larvae were fed twice daily (brewer's yeast and finely ground dog biscuits prepared at a ratio of 1:3). Eggs were collected in small petri dishes (Fig. 2.2.A) at least twice a week depending on either the size of the colony or demand for mosquitoes for use in the laboratory. Eggs were then transferred into larger plastic bowls (250 ml) half filled with distilled water (Fig. 2.2.B). The second and third instar larvae were reared in 2 l plastic bowls (Fig. 2.2.C). Fourth instar larvae and pupae were transferred into 5 l plastic bowls covered with gauze nets to prevent emerged adult mosquitoes from escaping (Fig. 2.2.D). Emerged mosquitoes were collected daily from the bowls using an aspirator and transferred to 5 l plastic cages covered with gauze netting (Fig. 2.2.E). Adult mosquitoes were maintained with a 10% sugar solution soaked in cotton pads and were blood-fed thrice a week for egg oviposition and colony propagation.

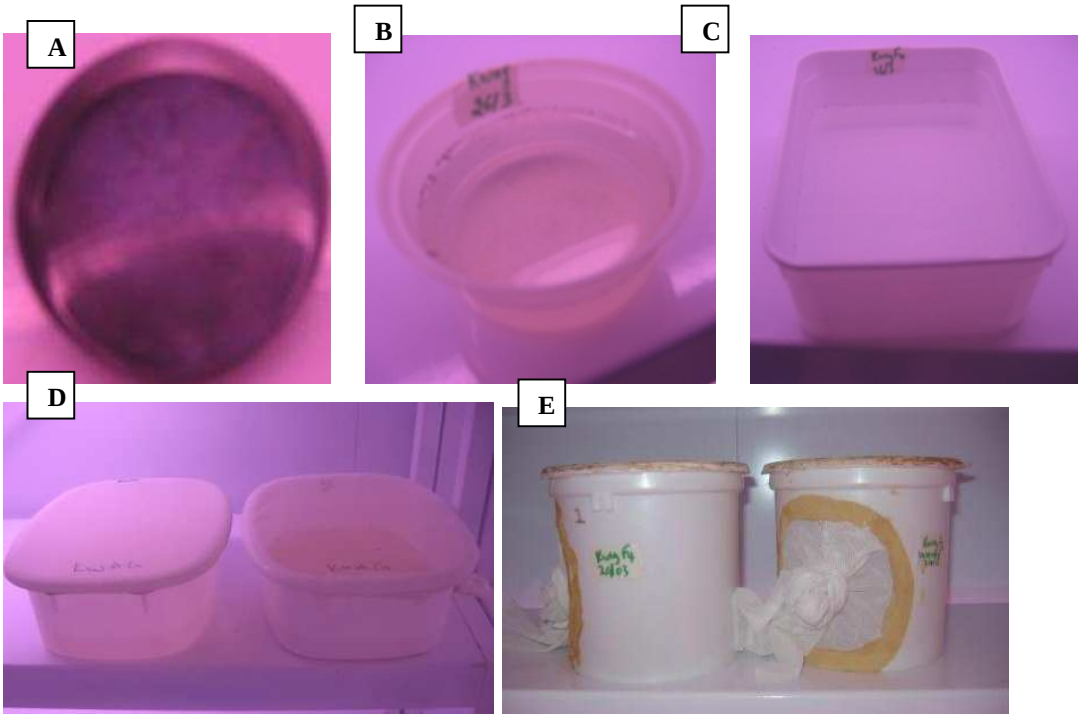

Figure 2.2: Equipment used for mosquito rearing: **A**: Petri dish egg plate, **B**: Plastic container (250 ml) for eggs hatching and 1st instar larvae, **C**: 2 l container for rearing 2nd and 3rd instar larvae, **D**: 5 l container for rearing 4th instar larvae and pupae and for collecting emerging adults, **E**: Plastic cages with gauze netting for adults.

2.2.2. Species-specific identifications

The molecular species identification protocol was done in order to test the species integrity of all colonies used in this project.

The Polymerase Chain Reaction (PCR) method described by Scott *et al.* (1993) was used to identify samples from each colony to *An. gambiae* complex

species level. This assay is based on species-specific sequences in the rDNA intergenic spacer (IGS) region (Collins *et al.*, 1988).

A set of 5 oligonucleotide primers (Inqaba Biotech, South Africa) (Table 2.1) were used in order to identify *An. gambiae, An. arabiensis, An. merus* and *An. quadriannulatus.* The primers consist of one universal primer that is complementary to all 5 species and 4 species-specific primers for the above mentioned species of the complex. A single mosquito leg per specimen was used in a master mix (1.25 µl of 10x PCR reaction buffer, 0.25 mM of each of the four deoxynucleotide triphosphates (dATP, dCTP, dTTP, dGTP)), 1 mM $MgCl_2$, 0.5 units Taq polymerase (Inqaba, South Africa), 0.3 µM each of *An. gambiae*, *An. arabiensis*, *An. merus* and universal primer and 0.15 µM of *An. quadriannulatus* primer and made up to 12.5 µl with distilled water (Table 2.1).

Table 2.1: Species-specific PCR primers with respective DNA sequences and transcript length of the four members of the *An. gambiae* complex (Scott *et al.*, 1993).

Primer name	**DNA sequence (5'to3')**	**Transcript length**
UN (universal)	GTG TGC CCC TTC CTC GAT GT	
GA (gambiae)	CTG GTT TGG TCG GCA CGT TT	390 bp
ME (merus)	TGA CCA ACC CAC TCC CTT GA	464 bp
AR (arabiensis)	AAG TGT CCT TCT CCA TCC TA	315 bp
QD (quadriannulatus)	CAG ACC AAG ATG GTT AGT AT	153 bp

Centrifuge tubes (1.5 ml) containing the master mix and leg were centrifuged at 13,000 rpm for 10 s (Heraeus'-Biofuge-Pico, Kendro, Germany) to release the template DNA from the mosquito leg. The PCR was performed using a thermal cycler (Primus 96, MWG Biotech, South Africa) under the conditions described in Table 2.2.

Table 2.2: PCR conditions for *An. gambiae* complex species-specific diagnostic assay.

Cycle	Conditions
1x	Denaturing at 94ºC for 30 seconds
29x	Denaturing at 94ºC for 30 seconds Annealing at 50ºC for 30 seconds Extension at 72ºC for 30 seconds
1x	Extension at 72ºC for 5 minutes

Loading dye (5 µl) was added to each PCR product. Each sample (10 µl) was loaded on a 2.5% TAE agarose gel stained with ethidium bromide. The gel was electrophoresed in 1x TAE buffer for about 1 h at 100-110 V or until the bromophenol blue markers had migrated 3 cm from the end of the gel. The amplified products were visualized under UV light using a gel documentation system (Syngene G-box, Synoptics group, Cambridge, U.K).

In all amplifications, a negative control, which consists of all the reaction components except template DNA, was used in order detect contamination. Positive controls (DNA from known samples of *An. merus, An. gambiae, An. arabiensis* and *An. quadriannulatus)* were used. Mosquitoes identified as *An. gambiae* sensu stricto were further assigned to the M or S molecular forms using the method described by Favia *et al*. (2001) (see below).

2.2.3. *Anopheles gambiae* forms

Anopheles gambiae has been sub-divided into five chromosomal forms namely: Forest, Savanna, Mopti, Bamako and Bissau (Coluzzi *et al*., 1985). In their study, Bryan *et al.* (1982) reported that the Forest and Bissau chromosomal forms prevail in humid and coastal areas of West Africa and could both belong to a single potentially panmictic unit widely distributed. However, the Bamako, Savanna and Mopti chromosomal forms are better adapted to dry environments and are often sympatric with evidence of assortative mating (Bryan *et al*., 1982; Wondji *et al.,* 2005). Furthermore, the Savanna, Bamako and Mopti chromosomal forms may occur in sympatry in West Africa (Touré *et al.,* 1998; della Torre *et al*., 2001; Taylor *et al*., 2001; Coluzzi *et al*., 2002). Molecular analysis of West African *An. gambiae* identified two molecular "forms", M and S, that did not correspond with the chromosomal forms throughout their distribution (Favia *et al.* 1997). Wondji *et al*. (2002) found a highly significant genetic differentiation between the M and S molecular forms in Cameroon (Central Africa). Therefore, they propose that the M and S molecular forms of *An. gambiae* are distinct species due to the strong assortative mating within forms and general lack of hybrids in areas of sympatry.

The polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method based on a PCR-amplified fragment of 1.3 kilobases in the intergenic spacer (IGS) of ribosomal DNA (rDNA) provides a diagnostic molecular

marker as well as evidence of reproductive isolation between *An. gambiae* M and S forms (Favia *et al.* 1997).

a) DNA extraction

DNA was extracted from twenty mosquitoes from each colony according to the method described by Collins *et al.* (1987). To extract DNA, mosquitoes were individually ground in 200µl of grinding buffer (80 mM NaCl, 160 mM sucrose, 130 mM Tris, 50.8 mM EDTA pH 8, 0.5% sodium laurel sulfate). Homogenate was incubated for 30 min at 70ºC followed by addition of 28 µl of cold 8 M potassium acetate. Each sample was incubated on ice for 30 min. This was followed by centrifugation for 30 min at 13,000 rpm. The supernatant was transferred to a new 1.5 ml micro-centrifuge tube. Ice-cold ethanol (99.8%; 400 µl) was added to the supernatant and centrifuged for 30 min at 13,000 rpm after which the ethanol was pipetted off without disturbing the newly formed pellet. This was followed by the washing of the pellet in 200 µl of 70% ice-cold ethanol and centrifugation for 30 minutes at 13 000 rpm. The ethanol was discarded and the pellet air dried before re-suspension in 200 µl of 1x TAE buffer.

b) M/S PCR identification.

DNA (1 µl) was added to the reaction mixture with specific primers for the M and S molecular forms (Table 2.3). The reaction mixture was prepared on ice by adding 2 µl each of primers R5, R3, Mopti and B/S. The PCR was performed using a thermal cycler (i-cycler, Bio-Rad, South Africa) under the conditions described in Table 2.4

(Favia *et al.* 1997). The amplified products were electrophorezed as above in section 2.2.2. Positive controls using known M and S forms were used.

Table 2.3: Primers used for M and S diagnostic assay with their respective sequences.

Primer name	DNA sequence
R5	5'-GCC AAT CCG AGC TGA TAG CGC-3'
R3	5'-CGA ATT CTA GGG AGC TCC AG-3'
Mopint	5'-GCC CCT TCC TCG ATG GCA T-3'
B/S int	5'-ACC AAG ATG GTT CGT TGC-3'

Table 2.4: M and S PCR conditions

Cycle	Conditions
1x	Denaturing at 94ºC for 10 minutes
25x	Denaturing at 94ºC for 30 seconds Annealing at 63ºC for 30 seconds Extension at 72ºC for 30 seconds
1x	Extension at 72ºC for 7minutes

2.2.4. Insecticide susceptibility tests

Eighteen laboratory-reared colonies belonging to four members of the *An. gambiae* complex (*An. gambiae*, *An. arabiensis*, *An. merus* and *An. quadriannulatus*) as indicated in Table 2.5 were screened. Each colony was exposed to discriminating dosages of insecticide belonging to 4 different insecticide classes recommended by WHO for malaria vector control as shown in Table 2.6, using WHO susceptibility test kits and protocol as described by WHO (1998). Newly emerged adults from each colony were separated by gender and placed into separate cages prior to insecticide exposure. These were fed on a 10% sugar solution for 48 h. Mosquitoes were exposed to WHO insecticide-treated filter paper (Table 2.6) while controls contained untreated filter papers (WHO, 1998). Twenty five non blood-fed mosquitoes were exposed to insecticides for 1 h. After exposure, mosquitoes were transferred to clean holding tubes and provided with cotton pads soaked in a 10% sugar solution. Knock-down was recorded at 60 minutes post-exposure and final mortality 24 h post-exposure. Each test was duplicated for fully susceptible colonies and triplicated for resistant colonies.

Table 2.5: Origin and detailed background of mosquito colonies used for WHO insecticide susceptibility tests. KZN = Kwazulu/Natal, KNP = Kruger National Park.

Colony Name	Country of origin	Locality	Date colonized
Anopheles arabiensis			
SENN*	Sudan	Sennar	1980's
SENN-DDT*	Sudan	Sennar	2004
MBN-base*	South Africa	Mamfene, KZN	2001
MBN-DDT*	South Africa	Mamfene, KZN	2001
Anopheles gambiae			
IAN P20	Nigeria	Iworo	1970's
CIG	Cote d'Ivoire	Bouake	1999
BOA	Cote d'Ivoire	Bouake	1997
NAG	Nigeria	Ibadan	2001
GAH*	Ghana	Ahafo	2006
PALA	Burkino Faso	Pala	1970's
G3	The Gambia	Not known	1970's
JS3	Nigeria	Iworo	1970's
SOG*	Ghana	Obuasi	2004
BENROG*	Ghana	Obuasi	2004
SUA*	Liberia	Suakoko	?
Anopheles merus			
MAF*	South Africa	Mafayeni, KNP	1988
ZAM	South Africa	Makani's drift, KZN	1980
Anopheles quadriannulatus			
Sangwe*	Zimbabwe	Sangwe	1998

*Colonies used for fungal exposure studies in Chapter III.

Table2.6: Insecticides used for WHO susceptibility tests.

CLASS	INSECTICIDE	Diagnostic concentration (%)
Pyrethroids	Deltamethrin	0.05
	Permethrin	0.75
Carbamates	Bendiocarb	0.1
	Propoxur	0.1
Organo-phosphates	Malathion	5
	Pirimiphos-methyl	0.9
Organochlorines	DDT	4
	Dieldrin (cyclodine)	4

2.2.4. Statistical analysis

The number of mosquitoes exposed per tube, knockdown after 60 minutes and final mortalities 24 h post-exposure were recorded on a standard WHO form for insecticide diagnostic tests. Data were statistically analyzed using paired sample t-tests. Percentage mortalities were corrected based on control group results (mortality >5%) using Abbott's formula (WHO, 1998):

$$\frac{\text{\% test mortality - \% control mortality}}{\text{100 - \% control mortality}} \times 100$$

2.3. Results

2.3.1. Species-specific identification

The species identity of 10 mosquitoes from each of eighteen laboratory colonies representing various members of the *An. gambiae* complex was confirmed by species-specific PCR assay. The majority (61.1%) were *An. gambiae*, while 22.2% were *An. arabiensis*, 11.1% were *An. merus* and the remaining 5.5% *An. quadriannulatus* (Fig. 2.3 and Table 2.4).

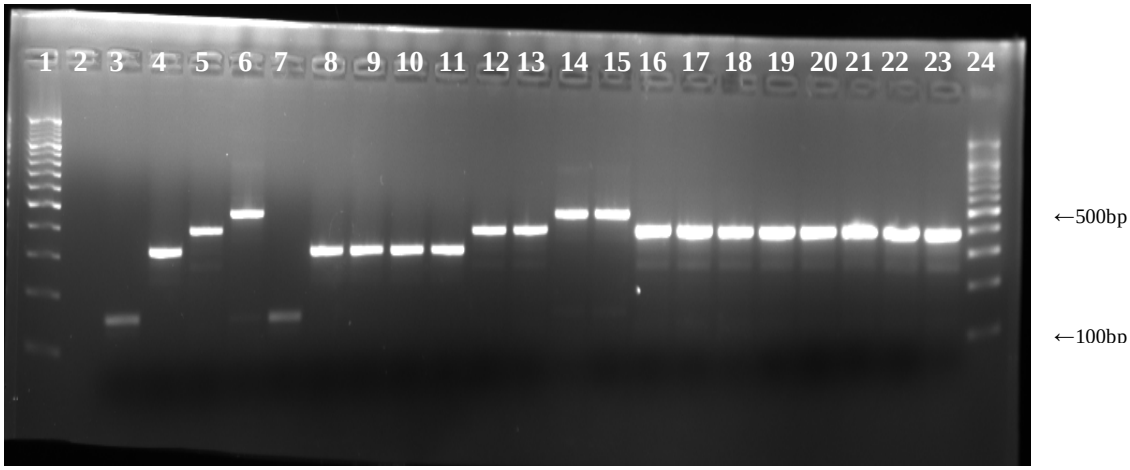

Figure 2.3: *Anopheles gambiae* species-specific diagnostic PRC results. Lane 1 and 24, 1kb molecular marker; Lane 2, negative control; Lane 3, *An. quadriannulatus* positive control; Lane 4, *An. arabiensis* positive control; Lane 5, *An. gambiae* positive control; Lane 6, *An. merus* positive control; Lane 7, *An. quadriannulatus* (Sangwe colony); Lanes 8-11, *An. arabiensis* (MBN, MBN-DDT, SENN and SENN-DDT colonies); Lane 12 and 13, *An. gambiae* (IANP20 and CIG colonies); Lane 14 and 15, *An. merus* (MAF and ZAM colonies) and Lanes 16-23, *An. gambiae* (BOA, NAG, GAH, PALA, G3, JS3, SOG/BENROG and SUA colonies).

2.3.2. PCR identification of the M/S forms

Molecular diagnostic assays for discriminating the M and S forms by PCR showed the presence of either M or S molecular forms in the selected laboratory colonies of *An. gambiae s.s.* used for fungal susceptibility tests. The longest segment (1.3kb) is species-specific for *An. gambiae s.s.,* ensuring that the DNA amplified comes from this species. A product of 475 bp indicates the presence of the S form and a fragment of 727 bp indicates the presence of the M form. These were compared to a 10 kb molecular marker (Fig. 2.4). Three colonies which originate from Ghana (SOG, BENROG and GAH) were found to be S form while the one that originates from Liberia (SUA) was found to be M form.

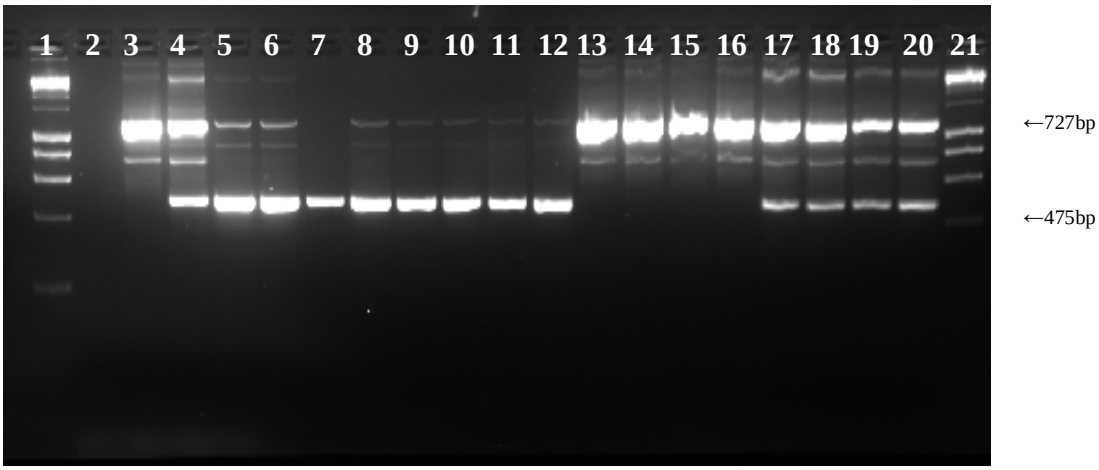

Figure 2.4: *Anopheles gambiae s.s.* molecular forms diagnostic PCR results. Lane 1 and 21 molecular marker; Lane 2, negative control (no DNA); Lane 3, M form positive control; Lane 4, S form positive control; Lanes 5-8, S form result for SOG colony; Lanes 9-12, BENROG colony (S form); Lanes 13-16, SUA colony (M form) and Lanes 17-20, GAH colony (S form).

2.3.3. Insecticide bioassays

Colony insecticide susceptibility status was determined according to WHO criteria: colonies were considered resistant if more than 20% of individuals survived the diagnostic dose 24 h post exposure compared to the susceptible control. A result of 98-100 % mortality indicates full susceptibility while 80-97 % mortality suggests the possibility of resistance that needs further confirmation (WHO, 1998).

The insecticide susceptibility status of mosquito strains to all four classes of insecticides approved for use in malaria control is shown in Table 2.7. Overall mortalities ranged between 1% and 100%. There was no evidence of insecticide resistance in MBN and Sangwe except for the permethrin results. SUA was susceptible to all insecticides except permethrin and malathion. However, there was evidence of resistance to insecticides in SENN, NAG, MAF, ZAM, IANP20, CIG, BOA, PALA, G3, JS3, SOG, SENN-DDT, MBN-DDT and BENROG. GAH showed measurable resistance to all insecticides tested against it. Based on these results, susceptible and resistant colonies were chosen to assess their susceptibility to the entomopathogenic fungus *Beauveria bassiana* isolate IMI 391510.

Comparisons between insecticide susceptibility levels for colonies of *An. arabiensis*, *An. gambiae*, *An. merus* and *An. quadriannulatus* that were subsequently used for fungal susceptibility tests are shown in Fig.s 2.5, 2.6 and 2.7.

Table 2.7: Insecticide susceptibility status based on standard WHO bioassays with 50-75 mosquitoes exposed to insecticide treated papers for 1 hour and final mortality recorded 24 h post-exposure.

	PYRETHROIDS		**CARBAMATES**		**ORGANO-PHOSPHATES**		**ORGANO-CHLORINES**	
COLONY	0.75% Permeth-rin	0.05% Delta-methrin	0.1% Bendio-carb	0.1% Propoxur	0.9% Pirimiphos -methyl	5% Mala-thion	4% DDT	4% Dieldrin
Anopheles arabiensis								
SENN	49.5*****	99	100	99.5	100	100	98.5	88.5
SENN-DDT	8.5*****	81.5	94.5	100	57.5*	39.5*	15.5*	27.5*
MBN	88.5	100	100	100	96.5	100	100	100
MBN-DDT	1*****	14.5*****	3*****	90.5	12*****	17.5*****	6*****	89.5
Anopheles gambiae								
SOG	23.5*****	50*****	100	92	98.5	100	60.5*****	15.5*
BENROG	30*****	92	48*****	62*****	68.5*****	99.5	59.5*****	29*****
IANP20	27.5*****	100	100	100	99	99.5	100	25*****
CIG	54.5*****	82.5	100	85.5	100	99	99	16*****
BOA	33*****	98	100	97.5	96	91.5	90	36*****
PALA	52*****	100	100	98	98.5	100	98.5	23*****
NAG	88	100	100	100	98.5	100	99	6.5*****
G3	32*****	100	100	99.5	95.5	99	95.5	18*****
JS3	30.5*****	88.5	100	97	91	99	91	23*****
SUA	87.5	100	99	96	99.5	87	98	100
GAH	32*****	70*****	70*****	61*****	1.5*****	67.5*****	51*****	8.5*****
Anopheles merus								
MAF	63.5*****	100	100	100	99.5	100	100	100
ZAM	72*****	99	100	98	91	100	99	100
Anopheles quadriannulatus								
SANGWE	87.5	100	100	97	95.5	100	100	100

***** Resistant colony according to WHO criteria.

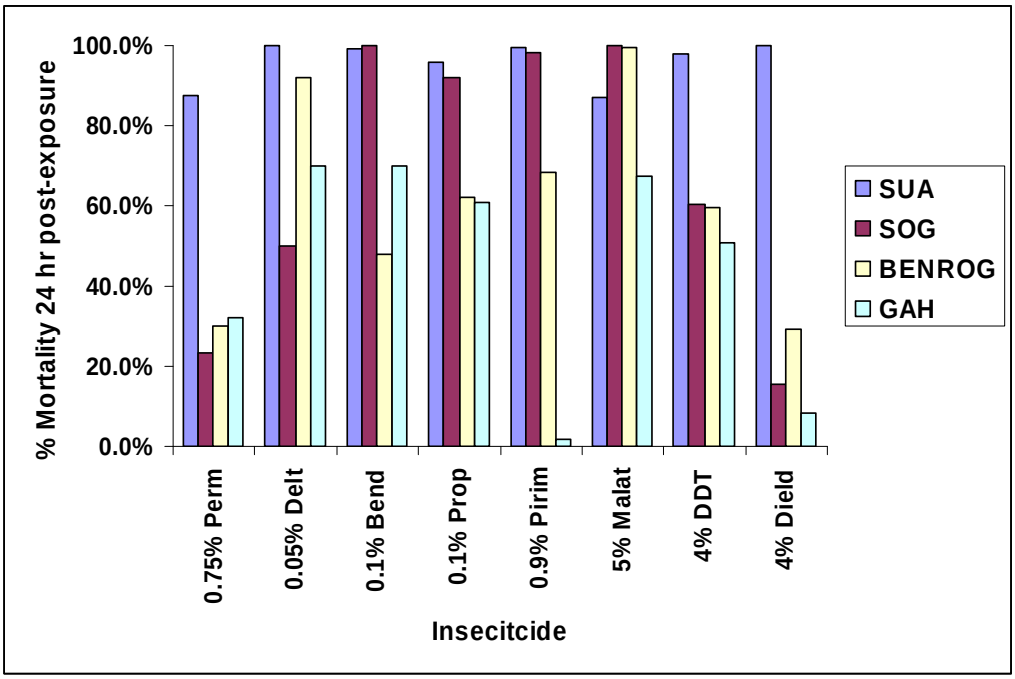

Figure 2.5: Summary of WHO insecticide susceptibility tests for *An. gambiae* colonies subsequently used for fungal exposures.

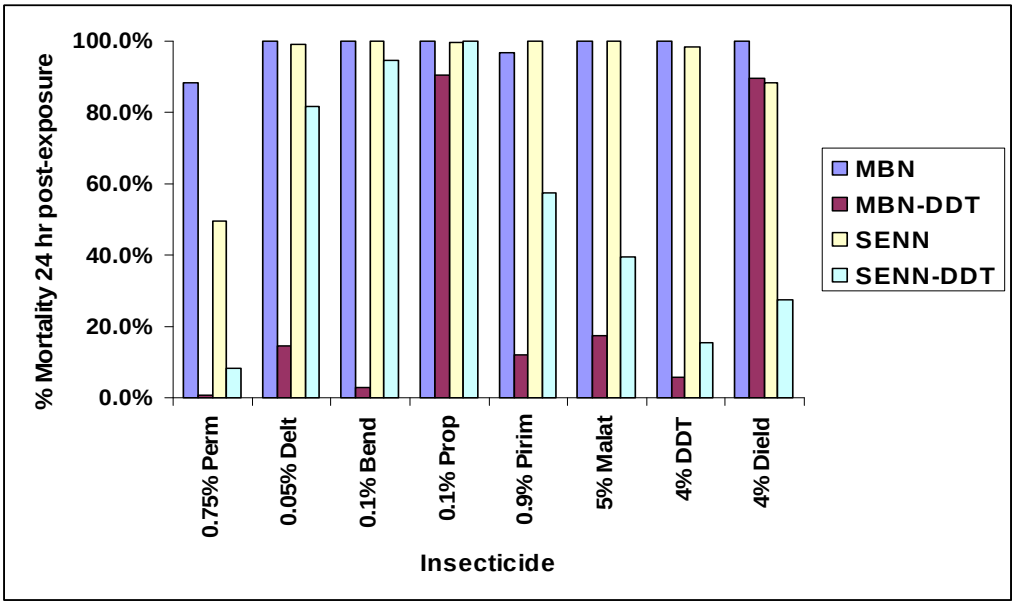

Figure 2.6: Summary of WHO insecticide susceptibility tests for *An. arabiensis* colonies subsequently used for fungal exposures.

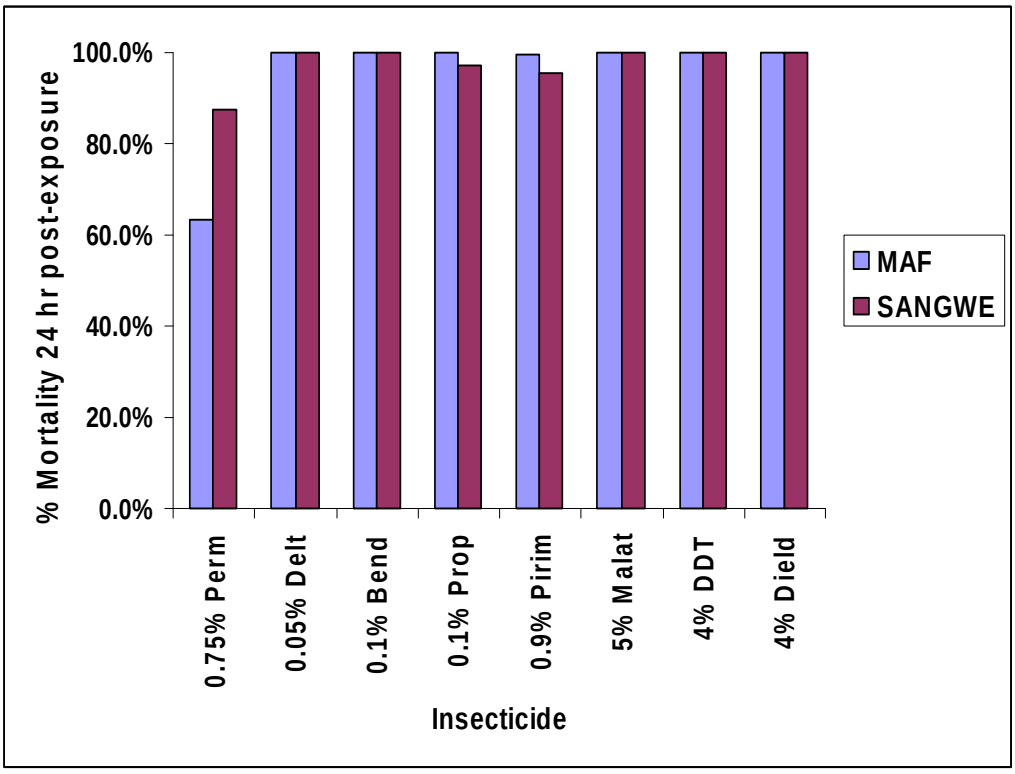

Figure 2.7: Summary of WHO insecticide susceptibility tests for *An. merus* and *An. quadriannulatus* colonies subsequently used for fungal exposures.

2.4. Discussion

This study gives detailed baseline information on the *Anopheles gambiae* complex colonies of the Vector Control Reference Unit of the National Institute for Communicable Diseases in Johannesburg, South Africa. It was necessary to confirm the species status and insecticide susceptibility status as well as to identify the molecular form of each colony in the case of *An. gambiae s.s*. in order to make assessments and comparisons of their relative susceptibilities to entomopathogenic fungal infection. Identification of the molecular forms may be important because of the higher genetic differentiation found between M and S populations than within populations of the same molecular form (Wondji *et al*., 2002). Genetic differentiation between the two forms could be associated with relative differences in susceptibility to insecticide and fungal infection. In this study, three of the mosquito colonies tested are S form and one M form. A high proportion of S form colonies originate from the Obuasi region in Ghana where the *An. gambiae* S form prevails over the M form (Coetzee *et al*., 2006). Comparing the susceptibilities of the two molecular forms when exposed to insecticide, the S form generally showed multiple insecticide resistance (pyrethroids, carbamates and organochlorines) whereas the M form proved susceptible to at least one chemical in all of these insecticide classes. This finding is supported by Coetzee *et al.* (2006) who found wild-caught S form samples from the Obuasi region in Ghana to be resistant to DDT, pyrethroids and carbamates. The SUA colony (M form) however, was colonized many years ago by Prof. Mario Coluzzi's laboratory which may account for its susceptibility status.

In general, resistance to pyrethroids (47.2%) and organochlorines (44.4%) predominated over organophosphate (19.4%) and carbamate (13.9%) resistance. This most likely reflects the pattern of inheritance of insecticide resistance mechanisms from the wild populations from which these colonies are derived. Generally, pyrethroids and organochlorines have been more widely used for agricultural purposes as well as for malaria vector control through IRS and ITN programs in Africa than have carbamates and organophosphates, leading to a bias in terms of insecticide resistance selection.

Resistance to permethrin was recorded in most colonies with even 'susceptible' colonies giving less than 90% mortality. Surprisingly, some of these colonies showed complete susceptibility to deltamethrin, an insecticide from the same chemical class but different sub-class. This may be because some detoxification enzyme systems that recognize permethrin as a substrate do not also recognize deltamethrin as a substrate, negating cross-resistance between the two. Both pyrethroids are commonly used in malaria vector control, especially for bed net impregnation, although permethrin is apparently more widely used for this purpose than is deltamethrin (Lengeler *et al*., 1996; Zaim *et al*., 2000). Alternatively, the permethrin papers used in the tests may have been faulty.

Anopheles arabiensis colonies selected for DDT resistance in the laboratory (MBN-DDT and SENN-DDT) showed high levels of resistance to DDT as expected,

but also showed resistance to other insecticides, especially permethrin. This may be because of genetic linkages between the controlling factors of resistance mechanisms or the possible result of *kdr* cross-resistance between DDT and pyrethroids (Martinez-Torres *et al.*, 1998; Ranson *et al*., 2000; Diabate *et al*., 2002; Fanello *et al*., 2003; Matambo *et al*., 2006). Those colonies that were selected for DDT resistance (SENN-DDT and MBN-DDT) and bendiocarb resistance (BENROG) in the laboratory showed significantly lower levels of mortality compared to their respective baseline colonies (SENN, MBN and SOG). One colony of *An. gambiae* (GAH), which has not been selected in the laboratory for resistance to any insecticide, was found carrying varying levels of resistance to all four insecticide classes. This may be attributable to high insecticide selective pressure in the Ahafo area of Ghana from where the original material was collected.

The high preponderance of insecticide resistance in the *An. gambiae* complex laboratory colonies tested here reflects the wide distribution of insecticide resistance in malaria vector populations in Africa. Resistance to insecticides, coupled to the limited number of insecticides available for use by public health programmes, necessitates the evaluation of the potential efficacy of entomopathogens, such as fungus, as important additions to integrated vector control strategies.

CHAPTER III
SUSCEPTIBILITY TO FUNGAL INFECTION

3.1. Introduction

Entomopathogenic fungi are currently widely used for the control of several insect pests as alternatives or supplements to chemical insecticides. Improvements in virulence and speed of kill can be achieved by understanding the mechanisms of fungal pathogenesis. The use of entomopathogenic fungi is a technology that is still being developed and improvements in production, formulation and field-delivery methods are needed in order to achieve large-scale implementation for malaria vector control. However, many studies that used the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* against malaria and dengue vector species have shown that they present a promising strategy (Scholte *et al*., 2003a,b, 2004; Blanford *et al*., 2005; Knols and Thomas, 2006; Thomas and Read, 2007; Ondiaka *et al*., 2008; Farenhorst *et al.,* 2008).

There are several delivery possibilities when evaluating the potential of the entomopathogenic fungus *B. bassiana* against mosquito vectors in the laboratory. These include the application of oil-formulated conidia on filter papers, spraying of clay pots or use of dry conidia on suspensors. These methods can be used to test the susceptibility of mosquito strains of various origins and insecticide-resistance levels.

3.2. Materials and Methods

3.2.1. Rearing of laboratory mosquito colonies

The origin and date of colonization of the *An. gambiae s.s., An. arabiensis, An. merus* and *An. quadriannulatus* colonies used in this study are listed in table 2.5 marked with *. All colonies were reared and maintained under standard insectary conditions as previously described in section 2.2.1. Wild mosquito collection methods and processing techniques in the laboratory are described in section 4.2.

Newly emerged female mosquitoes were collected with an aspirator and kept in small 2.5 litre plastic cages with access to a 10% sugar solution soaked into cotton wool. After 2-3 days adults were exposed to fungus *Beauveria bassiana*.

3.2.2. Fungal susceptibility tests

3.2.2.1. Fungal production

The fungal strain *Beauveria bassiana* isolate number IMI 391510 used in this study was produced at and obtained from Wageningen University and Research Centre, the Netherlands. Production was performed by culturing mycelia in a suitable medium, harvesting the growing mycelia, treating with a protective agent before drying, grinding and storage at 4^{o}C.

3.2.2.2. Preparation of fungal suspensors

The fungal suspensors were prepared according to the method described by Scholte *et al.* (2003a,b). For each trial, 3 local suspensors (hair-rollers), basically polythene perforated tubes measuring 6.5 cm in height and 2.5 cm in diameter (Fig. 3.1), were lined inside with a sheet of filter paper which also served to hold a plastic vial half filled with 10% sugar solution for moistening the filter paper. The sugar solution was used as a mosquito attractant. Approximately 0.1 g of conidia powder was weighed and dusted onto the suspensor using a small paintbrush under safety conditions and away from UV light in a laminar flow or safety cabinet. Treated suspensors were carefully placed into plastic cages (2.5 l) covered on top with gauze netting and with a sleeve on the side for easy mosquito access (Fig. 3.2) prior to fungal exposures.

An estimation of spore concentration in 0.1 g dry conidia of *Beauveria bassiana* was done according to dry spore weight:

$$1 \text{ spore of } \textit{Beauveria bassiana} \rightarrow 4{,}78 * 10^{-12} \text{g}$$

$$X \rightarrow 0.1\text{g conidia per suspensor}$$

$$X = \frac{0.1\text{g}}{4.78 * 10^{-12}\text{g}} \cong 2 * 10^{11} \text{ spores}$$

The viability of spores was randomly checked one day prior to exposures by mixing 0.05 g of dry conidia of each stock in 10 ml oil (Shell Ondina® oil 917, Shell, The Netherlands). Approximately 0.2 ml of that solution was inoculated onto a Petri-dish containing sabouraud-dextrose agar (SDA) under sterile conditions to avoid contamination. The agar was incubated overnight at 25-28ºC and checked for fungal growth or germination the following day. Only stocks showing high sporulation (≥85%) were used for fungal exposures. Control suspensors were also treated in the same way as for fungal dusted suspensors but without conidia.

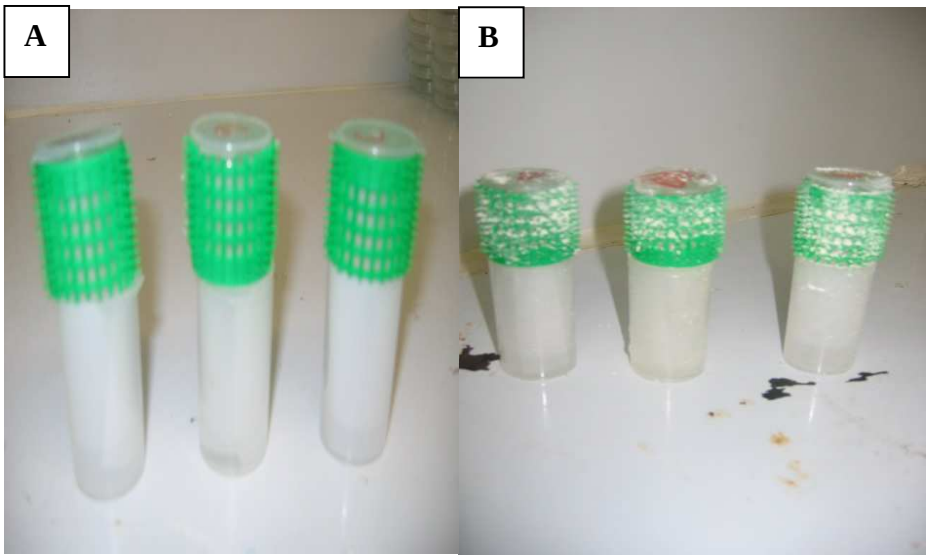

Figure 3.1: Suspensors used for fungal exposures. **A:** control group suspensors lined on the inside with filter paper moistened with 10% sugar solution **B:** treated group suspensors each dusted with 0.1g of dry conidia of *Beauveria bassiana* IMI 391510.

Figure 3.2 Plastic cages (2.5 l) used for fungal exposures; 3 served as controls and 3 as treatments.

3.2.2.3. Bioassays

Member species and individual colonies of the *An. gambiae* complex to be assessed for fungal susceptibility to *B. bassiana* were chosen according to their insecticide susceptibility profile and original geographical location (Table 2.5). Cohorts of 25-35 sugar fed 2-3 day old adult female mosquitoes from each colony were exposed to suspensors dusted with dry conidia for 24 h. A minimum of three trials were conducted per colony, each consisting of three treatment replicates and three controls. After the 24 h exposure mosquitoes were transferred into clean holding cages (2.5 l) using an aspirator and fed on 10% sugar water.

3.2.2.4. Mortality assessment

Mortality in treatment and control samples was recorded daily. All dead mosquitoes were removed from their respective cages using dissecting forceps. Each cadaver was dipped in 70% ethanol in order to remove saprophytic fungi from their cuticles. They were then placed in Petri dishes lined with moistened filter paper and sealed with parafilm to maintain high humidity for fungal sporulation. Petri dishes were incubated at 25±2^{o}C for a period of 2-5 days after which cadavers were screened for external fungal growth under a compound microscope. Any cadaver with visible fungal growth on its body surface was considered to have died as a result of fungal infection. Final fungal infection proportion per sample was recorded.

3.2.2.5. Data recording and analysis

Numbers of mosquitoes which died, numbers of days taken to die and days taken for spores to sporulate were recorded. A comparison was made between the numbers of mosquitoes which died because of fungal infection against the total number of mosquitoes which were exposed in each trial per colony using survival comparisons.

Cox' regression analysis (Cox, 1972) with SPSS 15.0 software was used to compare fungal survival between treatment and controls, as well as between colonies whose susceptibility levels to four classes of insecticides (pyrethroids, carbamates, organophosphates and organochlorines) had already been assessed (Chapter 2). A comparison between *An. gambiae* molecular forms (M and S) and a comparison of laboratory-reared and wild caught *An. arabiensis* was also made. Survival was monitored to the point of death of all mosquitoes in the treatment groups. Survival was measured as a hazard ratio (HR) which is the probability of the event (death) occurring in time t+1.

Differences in the computed survival curves between treated and control mosquitoes, insecticide susceptible and resistant mosquito strains(MBN vs MBN-DDT, SENN vs SENN-DDT, SOG vs BENROG and SUA vs GAH) as well as between laboratory reared *An. arabiensis* and wild *An. arabiensis* (see Chapter IV)

were assessed using the hazard ratio (HR) values (Mean ± standard error) at 95% confidence.

3.3. Results

Survival curves of samples from each colony are shown in Fig.s 3.3-3.12 and their respective P values and hazard ratios (HR) are given in Table 3.1. The curves show the cumulative percentage survival per day throughout the monitoring period. P values and hazard ratio statistics for comparisons made between insecticide susceptible and resistant colonies and M and S *An. gambiae* molecular forms are given in Table 3.2.

Exposure to the fungus resulted in comparatively rapid mortalities of the infected mosquitoes. Complete (100%) mortality was reached 7-12 days post-exposure in the baseline colonies of *An. arabiensis* (MBN and SENN) (Figs 3.3 and 3.5) and *An. gambiae* (SUA, SOG, GAH and BENROG) (Figs 3.7 – 3.10). *Anopheles merus* (MAF) (Fig. 3.11) and *An. quadriannulatus* (SANGWE) (Fig. 3.12) showed the same rapid increase in mortality after fungal exposure. However, mortalities were slower (100% mortalities reached in 15-20 days post-exposure) in DDT-selected *An. arabiensis* colonies (MBN-DDT and SENN-DDT) (Figs 3.4 and 3.6).

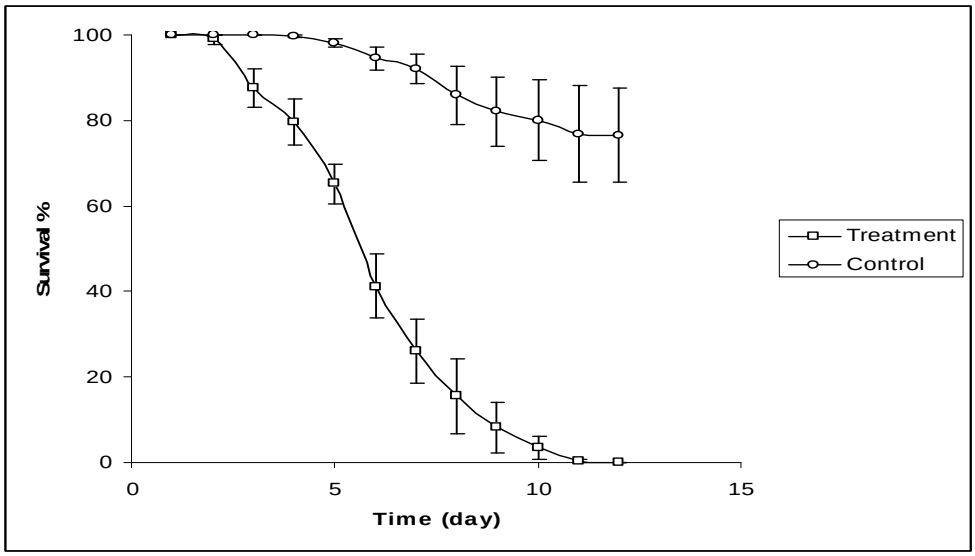

Figure 3.3 Mosquito survival curve of MBN colony (*An. arabiensis* baseline) infected with *B. bassiana* isolate IMI 391510.

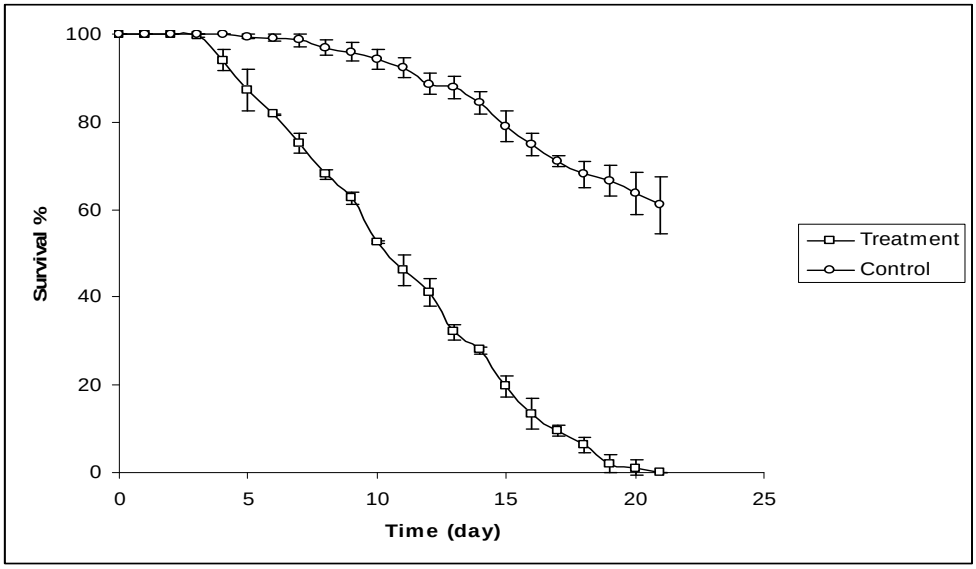

Figure 3.4 Mosquito survival curve of MBN-DDT (*An. arabiensis* selected for DDT resistance) infected with *B. bassiana* isolate IMI 391510.

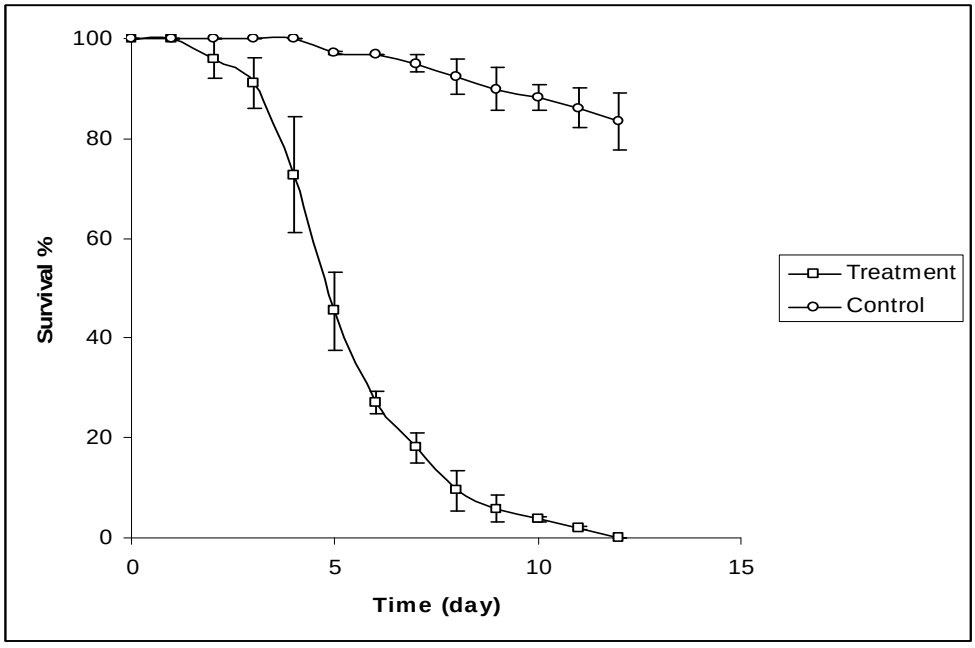

Figure 3.5 Mosquito survival curve of SENN (*An. arabiensis* baseline) infected with *B. bassiana* isolate IMI 391510.

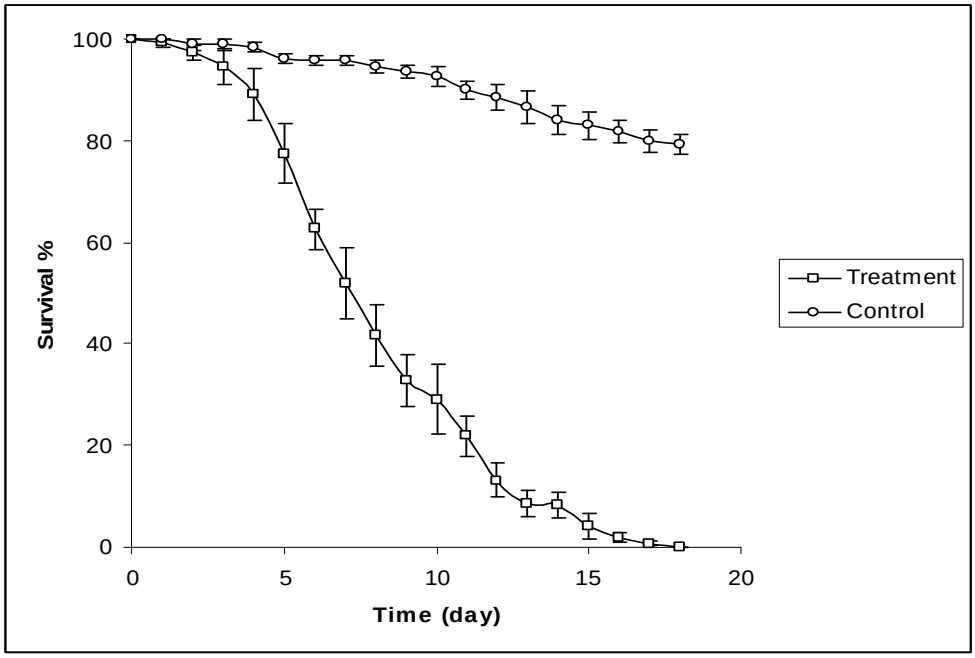

Figure 3.6 Mosquito survival curve of SENN-DDT (*An. arabiensis* selected for DDT resistance) infected with *B. bassiana* isolate IMI 391510.

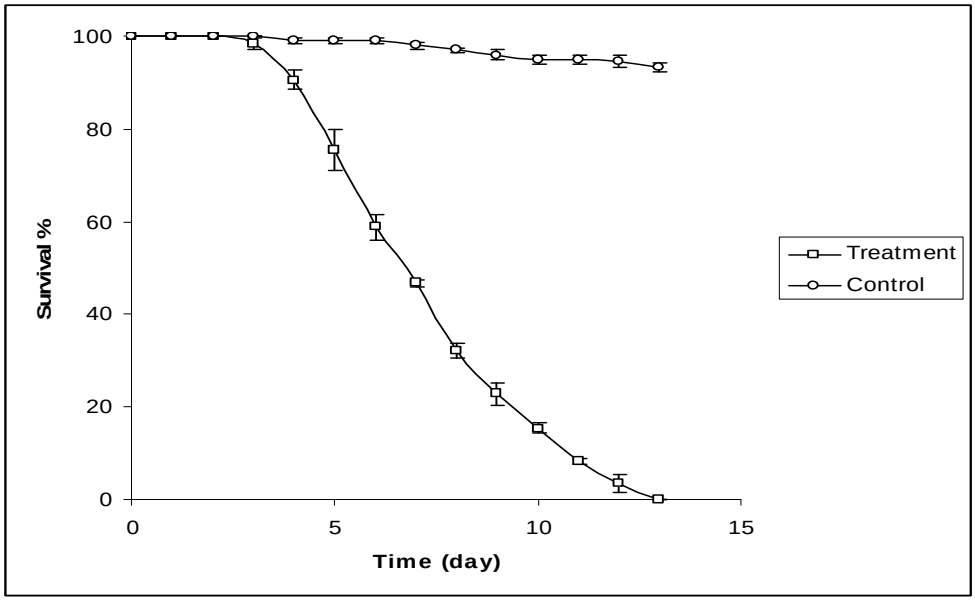

Figure 3.7 Mosquito survival curve of SOG (*An. gambiae* baseline) infected with *B. bassiana* isolate IMI 391510.

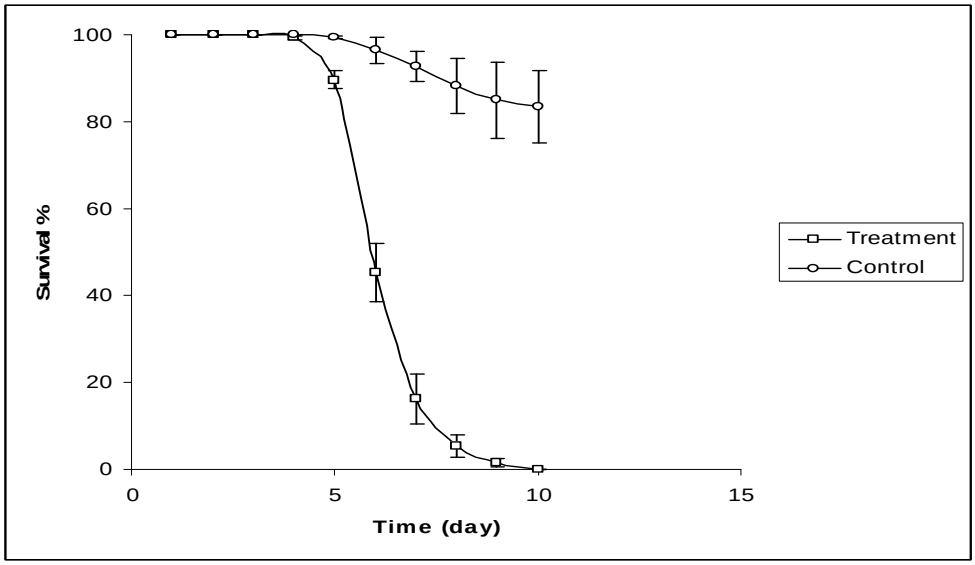

Figure 3.8 Mosquito survival curve of BENROG (*An. gambiae* selected for bendiocarb resistance) infected with *B. bassiana* isolate IMI 391510.

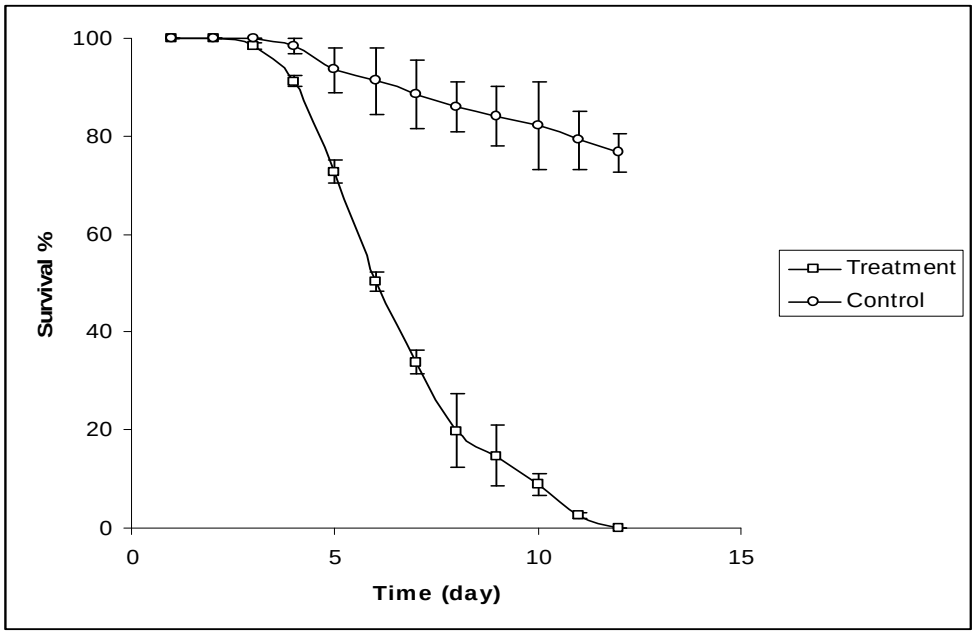

Figure 3.9 Mosquito survival curve of GAH (*An. gambiae* with natural multiple insecticide resistance) infected with *B. bassiana* isolate IMI 391510.

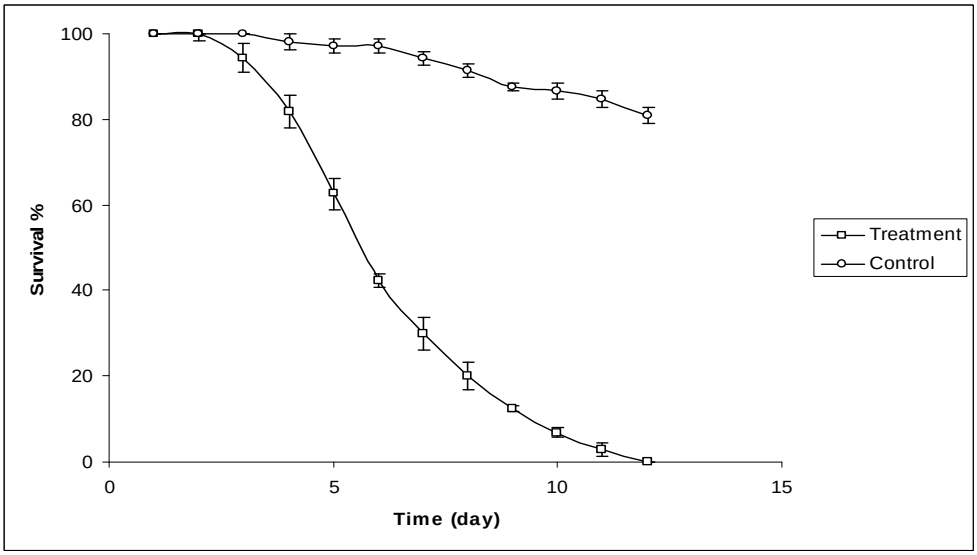

Figure 3.10 Mosquito survival curve of SUA (*An. gambiae* insecticide susceptible) infected with *B. bassiana* isolate IMI 391510.

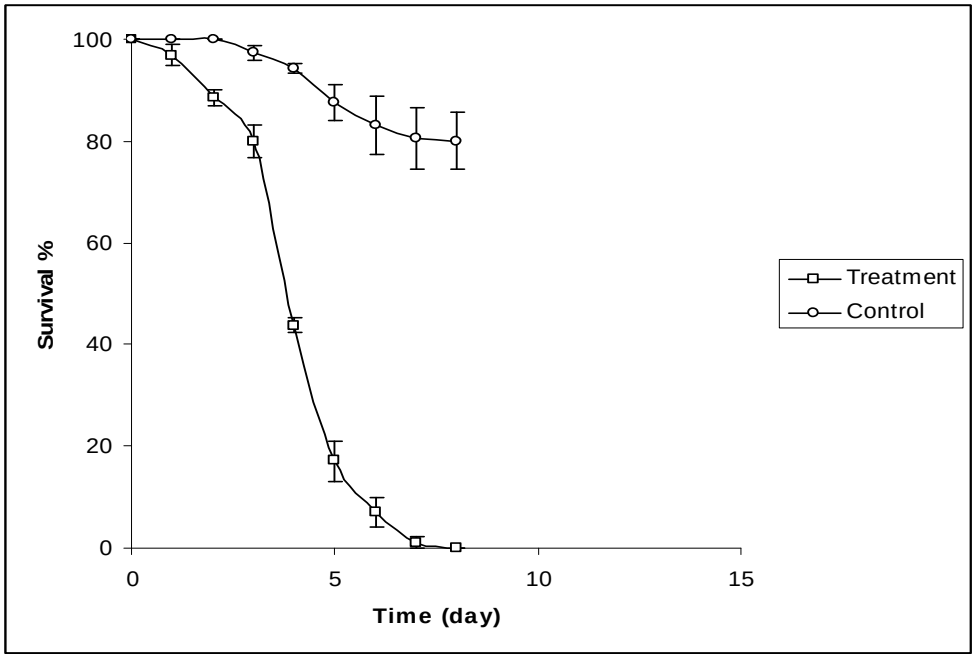

Figure 3.11 Mosquito survival curve of MAF (*An. merus*) infected with *B. bassiana* isolate IMI 391510.

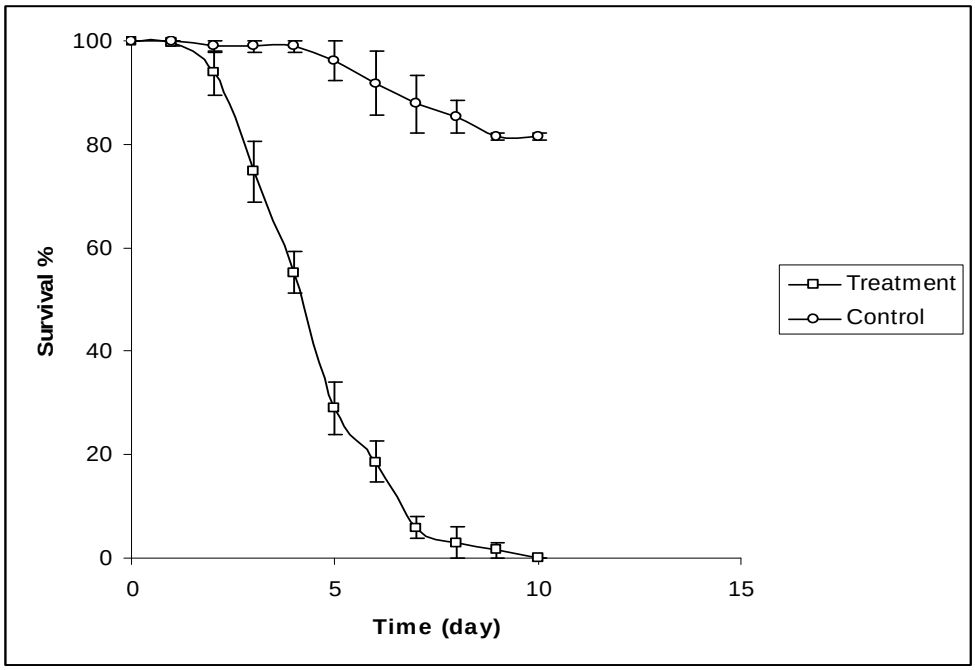

Figure 3.12 Mosquito survival curve of SANGWE (*An. quadriannulatus*) infected with *B. bassiana* isolate IMI 391510.

Table 3.1 Mortality P values and hazard ratios of *An. gambiae* complex colonies. Comparisons were made between fungal infected and control mosquitoes as well as between replicates and trials using Cox-regression full model analysis.

Colony	Total no. exposed mosquitoes	Between treatments and controls		Between replicates/trials	
		P value	Hazard ratio	P value	Hazard ratio
MBN	600	0.001	16.523	0.607	0.986
MBN-DDT	689	0.004	10.557	0.659	0.926
SENN	424	0.001	15.598	0.556	0.953
SENN-DDT	533	0.001	12.045	0.571	1.018
SOG	594	0.001	42.494	0.204	0.961
BENROG	698	0.001	9.625	0.372	1.027
SUA	394	0.001	16.248	0.370	1.105
GAH	391	0.002	14.025	0.743	1.059
MAF	332	0.001	13.700	0.209	1.155
SANGWE	386	0.001	28.569	0.409	0.910

Table 3.2 Summary of P values and hazard ratios comparing baseline and insecticide resistance selected colonies, as well as comparisons within and between species using the Cox-regression full model.

Compared Colonies	P values	Hazard ratio (95% CI)
MBN and MBN-DDT	0.325	0.981 ± 0.019
SENN and SENN-DDT	0.899	1.004 ± 0.029
MBN and SENN	0.237	0.956 ± 0.038
MBN-DDT and SENN-DDT	0.224	0.970 ± 0.025
SOG and BENROG	0.925	1.001± 0.016
SUA and SOG	0.717	0.986 ± 0.038
SUA and GAH	0.285	1.038 ± 0.035
MAF and SANGWE	0.236	0.943 ± 0.050

CI= confidence interval

Cox-regression analyses revealed no significant differences (P>0.05) between replicates/trials within treatment or control groups (Table 3.1). Overall, the hazard ratios were less than or equal to one, indicating that mosquitoes had the same chance of dying across replicates/trials for both infected and control samples. This is evidence that experiments were unaffected by fluctuations of temperature and relative humidity.

Cox-regression analyses also revealed highly significant differences (P<0.001) in survival between control and infected samples. This indicates that the fungus *B.*

bassiana was effective in dramatically reducing the lifespan of infected mosquitoes. Survival curves for fungal infected mosquitoes in all colonies (see Fig.s 3.2-3.12) showed a drastic reduction in survival compared to their respective control samples.

Overall, 93.6 ± 2.1% (mean ± standard error) of infected samples showed fungal growth 3-5 days after death (Fig. 3.13), strongly suggesting that mosquitoes in treatment groups died because of fungal infection.

Figure 3.13 *Anopheles gambiae* mosquito cadaver infected with *B. bassiana* (white muscardine) following exposure to dry conidia.

3.4. Discussion

Fungal infections in adults of malaria vector mosquito species have proved to be effective in reducing mosquito survival to less than 7-14 days post fungal exposure (Scholte *et al*., 2003a,b, 2004; Blanford *et al*., 2005; Farenhorst *et al*., 2008). In this study we compared the levels of fungal susceptibility in samples of insecticide resistant and susceptible members of the *An. gambiae* complex to *Beauveria bassiana* isolate IMI 391510. The results clearly indicate that the fungus affected the longevity of infected mosquito samples of all the *An. gambiae* complex species members tested. This observation is supported by a >90% sporulation rate recorded in dead mosquitoes drawn from the treatment cohorts after 3-5 days of incubation. Similar results were found using spores of *M. anisopliae* (Scholte *et al*., 2003a; Farenhorst *et al*., 2008).

These results also tally with those of Blanford *et al*.(2005) and Achonduh and Tondje (2008). In their studies, they tested the susceptibility of *An. gambiae* mosquitoes to different isolates of *B. bassiana*. They recorded over 80% mortalities 8-10 days post-exposure. The slow killing effect observed with the fungus *B. bassiana* compared to chemical insecticides was expected and is generally due to the mode of action of entomopathogenic fungi. This involves conidia attaching to the insect cuticle soon after coming into contact with the mosquito. This is followed by germination to form a germ tube which penetrates into the hemocoel as a mycelium or blastospore. Insect death occurs by extensive invasion of organs and by the

production of toxins as well as competition for food with the host insect (Eyal *et al*., 1994, Scholte *et al*., 2004). However, this slow killing effect has its own advantages of maintaining self propagation or auto-dissemination between individuals during the period of infection leading up to death (Scholte *et al*., 2004). It has also been shown that fungus-infected mosquitoes have reduced feeding propensity, and this presumably reduces their vectorial capacity (Scholte *et al*., 2006, Ondiaka *et al*., 2008).

Comparison of fungal susceptibility between the samples drawn from colonies of the different species of the *An. gambiae* complex did not reveal any significant difference ($P>0.05$). These results show that relative susceptibility to *B. bassiana* infection is not species-specific within the *An. gambiae* complex. Furthermore, survival analysis between samples of *An. gambiae* M and S molecular forms did not reveal any significant difference ($P>0.05$) in susceptibility to fungal infection. These two forms showed comparable levels of susceptibility to *B. bassiana* infection even though there are differences in their susceptibility levels to insecticide exposure. The SUA colony (diagnosed as M form) showed full susceptibility to all insecticides as compared to the S form colonies (SOG, BENROG and GAH) which show multiple insecticide resistance.

Comparisons using Cox regression showed that *B. bassiana* infection had a significant impact ($P=0.045$, HR=0.576, 95% CI: 0.353-0.938) on the longevity of

selected DDT-resistant *An. arabiensis* MBN-DDT and SENN-DDT compared to their baseline cohorts, MBN and SENN, respectively. The DDT-selected colonies showed longer survival compared to their baseline colonies. A possible reason for this may be associated with higher levels of detoxifying enzyme systems such as glutathione S-transferases (GST) and esterases recorded in the DDT-selected colonies (Matambo *et al.,* 2006). Detoxifying enzymes have been postulated to assist in the degradation of toxins produced by fungi thereby decreasing their susceptibility to fungal infection (Devonshire and Field, 1991; Wilson, 2001; Serebrov *et al.*, 2006). For example, increased GST activity in greater wax moth caterpillars *Galleria mellonella* has been associated with fungal infection with *M. anisopliae* and led to an increased tolerance to the insecticide malathion (Serebrov *et al*., 2006). In contrast, *B. bassiana* infection in insecticide resistant *An. gambiae*, *An. arabiensis* and *An. funestus* mosquitoes increased their levels of susceptibility to the insecticides permethrin and DDT (Farenhorst *et al.,* 2009). However, the role of these enzymes (GST and esterases) in decreasing susceptibility to fungal infection remains to be determined.

It is predicted that a significant reduction in the longevity of adult mosquitoes will have an impact on malaria transmission (Scholte *et al*., 2007), especially if their life spans are reduced to a point which precludes them from delivering *Plasmodium* infective bites. *Beauveria bassiana* isolate IMI 391510 is able to produce such an effect and is therefore a potential biocontrol agent to be used in integrated vector management strategies, particularly in areas where effective vector control is threatened by insecticide resistance.

CHAPTER IV
EFFECTS OF FUNGI ON FIELD-COLLECTED MOSQUITOES

4.1. Malaria in Malawi

Malawi is a land-locked country situated in south-eastern Africa with a total population of about 13.6 million people. It borders Zambia to the West, Tanzania to the North and Mozambique to the East, South and Southwest. Almost 20% of its total area is covered by lake Malawi (formerly Lake Nyasa) occupying most of the country's eastern border. Malawi is one of the poorest countries in the world with more than 80% of its economy heavily dependent on agriculture and a few exploitable mineral resources (www.malawimission.org/malawifacts.htm**)**.

In Malawi, malaria remains one of the most important infectious diseases with almost 100% of the population at risk. The disease mostly affects the poorest people and forces them to spend more than one quarter (28%) of their yearly income on malaria treatment (NMPC, 2007). In a survey conducted by the local Ministry of Health, it was reported that up to 40% of outpatient visits are due to malaria and there are eight million episodes of malaria recorded yearly (MOP, 2008). Children under the age of five, pregnant women and people living with HIV/AIDS represent the most vulnerable populations for malaria related morbidity and mortality. The predominant

malaria parasite species is *P. falciparum* accounting for over 85% of the reported malaria infections (MOH, 2006).

The incidence of malaria in Malawi is seasonal, occurring between December and May, with transmission decreasing in the dry months of June to November when there is little or no rainfall.

The prevention of malaria transmission in Malawi relies on accurate diagnosis and prompt effective treatment of infections with anti-malarial drugs. Artemether-lumefantrin is the first-line drug and a combination of amodiaquine-artesunate acts as the second-line therapy, while quinine is used to treat severe malaria cases and management of malaria during pregnancy (MOH, 2006). The country's health policy recommends the provision of at least 2 doses of sulfadoxine-pyrimethamine (SP) to pregnant women during the second and the third trimester as a way of preventing malaria infection.

Intervention measures to prevent or restrict the transmission of malaria by controlling the vector population form the mainstay of vector control in Malawi. Of the vector approaches available, use of insecticide-treated bednets (ITNs) is a proven method of preventing malaria in the country (MOP, 2008). In partnership with supportive donors and Non-Governmental Organizations (NGOs), the government of Malawi is developing different innovative distribution models to fill the gaps in malaria-ravaged, poor, rural communities without purchasing power. Long-lasting

ITNs are the preferred product for scaling-up coverage throughout the country (NMCP, 2007; MOP, 2008).

However, until recently, IRS had not been considered as a vector control strategy in rural Malawi where the burden of the disease is the highest all year round. According to the country's malaria policy, IRS is only used in a selective manner and is the method of choice for preventing and containing malaria epidemics in well-defined or high risk situations. The first trial on the use of IRS was conducted in the last decade, with the support of the African Development Bank, in two small-scale pilot studies focusing on the operational costs and feasibility of IRS in thirty rural villages using the pyrethroid insecticide lambda-cyhalothrin (NMCP, 2007).

The aim of the this study was to compare the relative levels of susceptibility to the entomopathogenic fungus *B. bassiana* between newly field-collected and laboratory-reared mosquitoes of the same species which have been maintained under controlled climatic conditions where temperature and humidity were kept constant for prolonged periods.

4.2. Materials and Methods

4.2.1. Study area

The study was carried out in Karonga, a district located in the Northern region of Malawi. It borders Tanzania to the North, Rumphi District to the south, Chitipa District and then Zambia to the west, and Lake Malawi to the east (Fig. 4.1). Karonga is a semi-developed rural area covering an area of 3 355 km^2 and has an economy primarily based on subsistence farming of mainly maize and rice.

The total population of Karonga is about 200 000 people of which 100% are at risk of contracting malaria. Mosquito breeding occurs throughout the year because of the easy availability of these breeding sites (Fig 4.2). Breeding sites varied between swamps, tyre tracks, small temporary rain puddles, irrigation pools, shallow wells dug into lake beds and large semi-permanent ponds formed during the rainy season. Mosquitoes in this area have never been under insecticidal selection from IRS vector control strategies. However, insecticidal pressure might come from insecticides used for ITNs and those used for agricultural pest control.

Figure 4.1 Map of Malawi showing Karonga District to the North where the study was carried out (www.maps.com). ➡ indicates the study site.

Figure 4.2 Breeding sites in study area **A**: large semi-permanent pond **B**: rain puddle.

4.2.2. Mosquito collections

Mosquitoes were collected inside traditional houses using an aspirator (Fig. 4.3). Collected mosquitoes were placed into small polystyrene cups (180 ml) covered with gauze netting. Mosquitoes were identified morphologically using hand lenses and the taxonomic keys of Gillies and De Meillon (1968) and Gillies and Coetzee (1987). Only female mosquitoes conclusively identified as members of the *An. gambiae* complex were retained. A cohort of these mosquitoes was maintained with a 10% sugar solution soaked in cotton pads for a few hours after which they were subjected to insecticide susceptibility tests (see below). Another cohort of female mosquitoes was blood-fed for oviposition. These mosquitoes were also maintained with a 10% sugar solution soaked in cotton pads before and during transportation to the

laboratory in Johannesburg, South Africa, for colony rearing and fungal susceptibility tests.

Figure 4.3 Mosquito indoor collections using a torch and aspirator.

4.2.3. Field insecticide susceptibility tests

WHO test kits were used to determine the insecticide susceptibility levels of wild collected mosquitoes. Samples of collected mosquitoes conclusively identified as members of the *An. gambiae* complex were exposed to discriminating dosages of 4% DDT and 4% dieldrin for one hour according to the standard procedure for testing

adult anopheline susceptibility to insecticide (WHO, 1998). These insecticides were chosen because DDT is proposed for future malaria vector control use in the region and dieldrin represents a class of insecticide that targets the GABA receptor in insects. Other insecticides with a similar mode of action may be forwarded for vector control in the future. The susceptibility of this population to other insecticides has previously been described (Coetzee *et al.*, 2007). For each insecticide test, three cylinders, two serving as treatments and one as a control, were used. The treatment cylinders were lined with filter papers impregnated with pertinent insecticide at a standard diagnostic concentration while the control cylinders were lined with untreated filter paper. Twenty five to thirty mosquitoes were assayed per cylinder and were exposed to insecticide for 1 h. The cylinders were kept in an upright position (vertically) for the duration of each test (Fig. 4.4). Mosquitoes were then transferred into clean holding tubes and provided with a 10% sugar solution (soaked in cotton pads). Final mortalities were recorded 24 h post-exposure and the susceptibility status was determined according to WHO criteria. Samples were considered resistant to the standard diagnostic concentration/exposure time if more than 20% of individuals survived 24 h post-exposure. A result of 98-100 % mortality indicates full susceptibility while a result between 80-97 % mortality suggests the possibility of resistance that needs further confirmation (WHO, 1998).

After exposure, all dead mosquitoes were individually kept in 1.5 ml labelled eppendorf tubes with silica gel for desiccation before being transported to the VCRU

laboratory at NICD in Johannesburg, South Africa for further species-specific identification.

Figure 4.4 World Health Organization experimental set-up for insecticide susceptibility tests in Karonga, Malawi.

4.2.4. Mosquito processing in the laboratory

Blood fed and gravid female mosquitoes collected in the field were transported to the Botha De Meillon insectary at the Vector Control Reference Unit of the National Institute for Communicable Diseases, Johannesburg, South Africa, under standard

insectary conditions of 25±2^{0}C, 80±10% relative humidity and a 12:12 hour light/darkness regime.

A laboratory code MALAG (Malawi *An. gambiae*) was given to each sample in order to differentiate them from other colonies in the same insectary. Each gravid female mosquito was placed in a glass vial lined with a moistened filter paper to allow for the laying of eggs. Individual egg batches (families) were transferred into larger plastic bowls (250ml) half filled with distilled water. F1 larvae were reared in the same bowl until they pupated. All larvae were fed twice daily with a mixture of brewer's yeast and finely ground dog biscuits. Pupae were transferred daily into plastic vials (50ml) half filled with distilled water and covered with gauze netting. Emerged F1 adult mosquitoes were collected using an aspirator and kept in small cups (180ml) covered with gauze netting or in small plastic cages (2.5 litres) with sleeves for easy access. F1 adult progeny were maintained with a 10% sugar solution soaked in cotton pads for 2-3 days before being subjected to fungal susceptibility tests using the *B. bassiana* isolate IMI 391510.

4.2.5. Species-specific identification

Dead mosquitoes preserved after insecticide exposures in the field as well as the mothers of F1 progeny were used for species-specific identification as previously described in Chapter 2.2.2.

4.2.6. Fungal susceptibility tests

Fungal susceptibility bioassays of F1 progeny from wild mosquitoes were performed according to the method described by Scholte *et al.* (2003a,b) as previously described in Chapter 3.2.2. Exposures were performed in two different environmental temperature settings of 21 $\pm 1^{o}$C and 25 $\pm$ 2^{o}C both at 75$\pm$15% relative humidity.

For each family line, three suspensors, two serving as treatments and one as control, were used. The control suspensor was not dusted with fungus conidia. Sugar fed mixed female and male mosquitoes (20-35) were used for each replicate.

4.2.7. Statistical analysis

The number of mosquitoes knocked down after 60 minutes and final mortalities 24 h post-exposure to insecticides was recorded and interpreted according to WHO insecticide bioassay criteria (WHO, 1998). Since mortalities in the control tubes were less than 5% throughout, no corrections using Abbot's formula were required. Survival curves for the fungal susceptibility tests were analyzed by Cox-regression analysis using SPSS 15.0 software (Cox, 1972).

4.3. Results

4.3.1. Mosquito collections

A total of 386 *An. gambiae* complex mosquitoes were collected in Karonga North. They were all identified as *An. arabiensis* by polymerase chain reaction (Fig. 4.5).

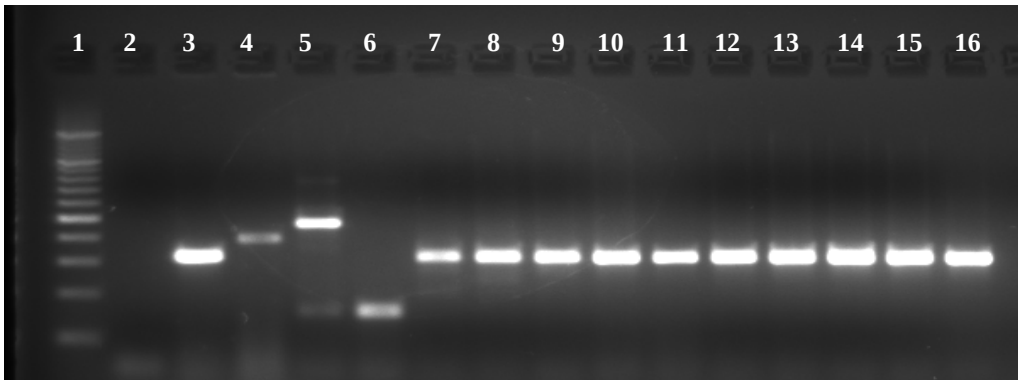

Figure 4.5 *Anopheles gambiae* species-specific PCR results on Malawi samples. Lane 1, 1kb molecular marker; Lane 2, negative control; Lane 3, *An. arabiensis* positive control (315 bp); Lane 4, *An. gambiae* positive control (390 bp); Lane 5, *An. merus* positive control (464 bp); Lane 6, *An. quadriannulatus* positive control (153 bp); Lanes 7-16 wild *An. arabiensis*.

4.3.2. Field insecticide susceptibility tests

Following insecticide susceptibility tests performed on wild caught *An. arabiensis* mosquitoes using 4% DDT and 4% dieldrin, complete susceptibility (100% mortality) to both insecticides was recorded.

4.3.3. Fungal susceptibility tests

Dry conidia of the entomopathogenic fungus *Beauveria bassiana* isolate IMI 391510 were found to be effective in killing the F1 progeny of wild *An. arabiensis* collected in Malawi. Both females and males succumbed to fungal infection but males died faster than females. Cox-regression analyses revealed no significant difference ($P>0.05$) between the two exposure temperatures of 21 ± 1^0C and 25 ± 2^0C (Figs 4.6 & 4.7). However, survival appeared lower at the higher temperature compared to the lower temperature.

A similar analysis comparing mortality rates between F1 *An. arabiensis* progeny of wild-caught females and the MBN and SENN *An. arabiensis* colonies (see Chapter 3) revealed no significant differences (Table 4.1).

Table 4.1 Summary of P values and hazard ratios comparing mortality rates following fungus infection of F1 *An arabiensis* (MALAG) against MBN and SENN laboratory colonies using the Cox-regression full model.

Compared Samples	P values	Hazard ratio
MBN and MALAG	0.517	1.023 ± 0.034
SENN and MALAG	0.935	0.997 ± 0.043

CI= confidence interval

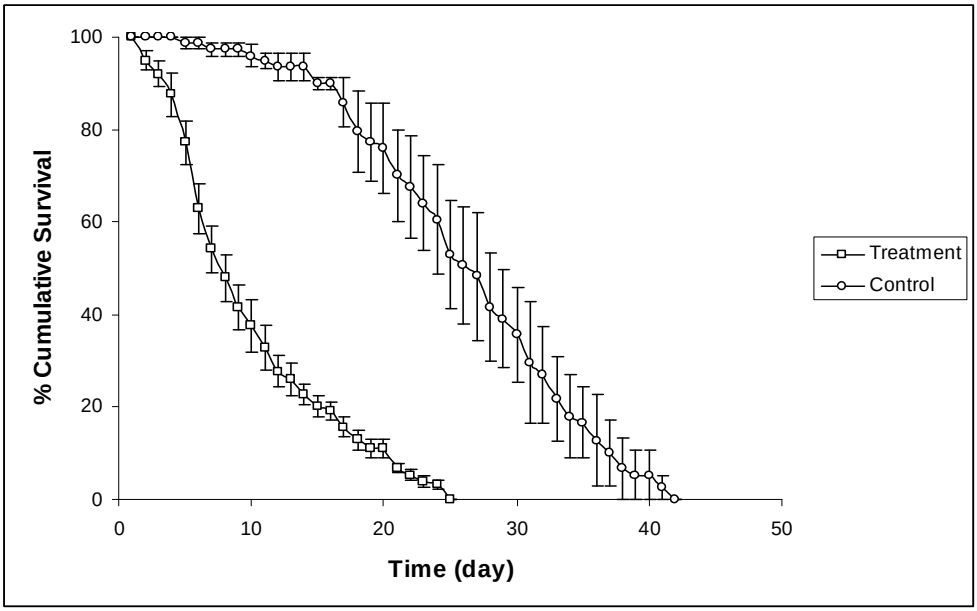

Figure 4.6 Mosquito survival curves of F1 *An. arabiensis* infected with *B. bassiana* at 21±1^{0}C.

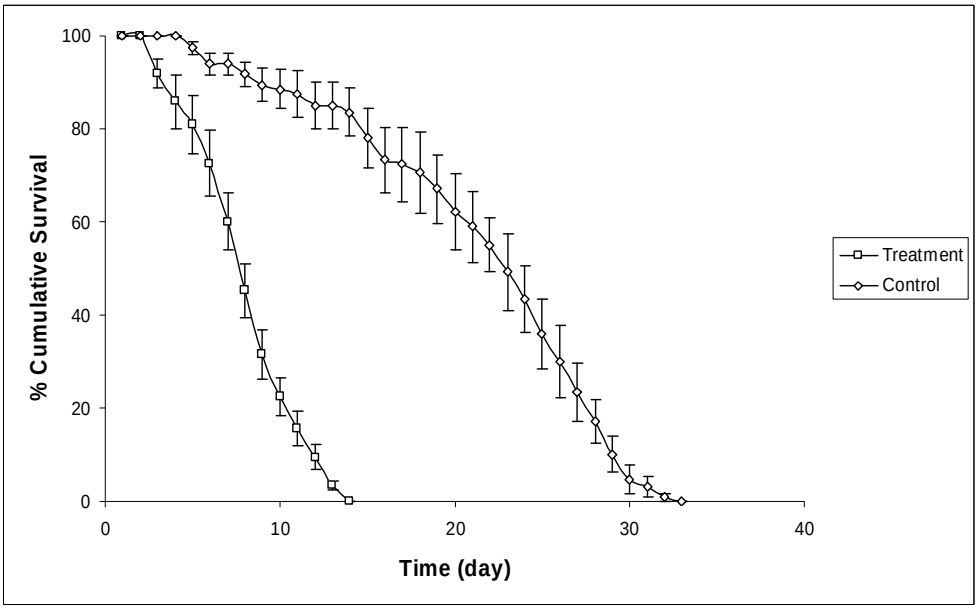

Figure 4.7

Mosquito survival curves of F1 *An*. *arabiensis* infected with *B. bassiana* at 25±2^{0}C.

4.4. Discussion

Anopheles arabiensis was the only species of the *An*. *gambiae* complex caught resting indoors in one of the villages north of Karonga city where sampling was done. Mosquito collections were carried out at the beginning of the rainy season (early December). The weather during that time was dry and hot. The fact that *An*. *arabiensis* was the only species found under these conditions is not surprising as it is known to be tolerant to dry and hot climatic conditions (Gillies and De Meillon, 1968; Gilles and Coetzee, 1987; Lindsay *et al*., 1998). Our results also tally with those of

Petrarca *et al.* (2000). In their studies, they found *An. arabiensis* biting during dry seasons.

All the tested *An. arabiensis* mosquitoes showed complete susceptibility to organochlorines (4% DDT and 4% dieldrin). This is probably because the sampled area has no history of use of this class of insecticide, according to villagers.

Numerous studies have demonstrated the susceptibility of adult anopheline mosquitoes to fungi (Scholte *et al*. 2003a,b; Blanford *et al*., 2005; Farenhorst *et al*, 2008; Achonduh and Tondje, 2008). Ondiaka *et al* (2008) highlighted the decrease of mosquito feeding propensity after fungal infection. In a recent study, Farenhorst *et al*. (2009) demonstrated the effectiveness of *Beauveria bassiana* and *Metarhizium anisopliae* against permethrin and DDT resistant colonies of *An. gambiae*, *An. arabiensis* and *An. funestus,* all major malaria vector species in sub-Saharan Africa. It was also shown in the same study that fungal infection increases mosquito susceptibility to insecticides recommended for malaria vector control.

In this chapter we presented results of fungal infection with *B. bassiana* on wild mosquitoes from Malawi. There was a highly significant difference ($P<0.001$) between control and treatment groups in both trials (21 ± 1^0C and 25 ± 2^0C). This resulted in 100% mortality observed in infected mosquitoes within 8-20 days post-exposure. During this time, 70-80% of mosquitoes were still alive in the control

groups. Similar results with dry conidia of *Metarhizium anisopliae* were found by Scholte *et al*. (2003a). However, Achonduh and Tondje (2008) used dry conidia of *B. bassiana* isolate RBL1034 against *An. gambiae s.l.* and reported that this isolate killed over 80% of treated mosquitoes in less than 10 days post-exposure.

Fungal infectivity was confirmed by over 90% sporulation in treatment cadavers after incubation. A similar study by Scholte *et al*. (2003a) and Achonduh and Tondje (2008) showed 95% and 87 % infectivity respectively.

Cox-regression analyses did not reveal any significant difference ($P>0.05$) between the two temperatures under which mosquitoes were exposed. However, the hazard ratio (HR) (probability of the event occurring in time t+1) was greater at the higher temperatures of 25 ± 2^0C (HR=14.65) than at the lower temperatures of 21 ± 1^0C (HR=7.55). This suggests that the probability of a mosquito dying was greater at higher temperatures than at lower temperatures. This finding suggests that the fungus *B. bassiana* kills mosquitoes faster at temperatures above 25^0C. Further, comparisons of survival following fungal exposure between wild-caught *An. arabiensis* (MALAG) and laboratory reared *An. arabiensis* (MBN and SENN) did not reveal any significant difference ($P>0.05$) in susceptibility to fungal infection

In conclusion, these results show that wild adult *An. arabiensis* are fully susceptible to infection by dry conidia of the entomopathogenic fungus *Beauveria*

bassiana isolate IMI 391510 under laboratory conditions. This fungus could be considered a potential biocontrol agent to supplement current malaria vector control strategies against adult anopheline mosquitoes. The fungus was found effective at killing mosquitoes in presumed mean temperatures for both the winter and summer seasons.

CHAPTER V

CONCLUSIONS

Malaria prevention and control programmes aim to reduce human exposure to the infective bites of mosquito vectors and hence to reduce the burden of malarial disease.

Despite various control measures available, malaria remains a major cause of illness and death in tropical countries. This situation is partly due to ecological factors such as high temperatures, high rain fall and high humidity – all of which affect mosquito survival and development favourably. The presence of mosquito breeding sites, vegetation, land-use, house construction and the use of preventive measures mainly determine the parameters of vector-host contact. In addition to *An. funestus, An. gambiae* and *An. arabiensis* are widely reported to be Africa's most important malaria vectors.

This study summarizes the current insecticide susceptibility status of all mosquito colonies housed at the National Institute for Communicable Diseases in Johannesburg, South Africa. Some colonies were being selected for resistance to particular insecticides at the time they were assayed, and many of these were shown to have developed cross resistance to other insecticides belonging to

different insecticide classes. This was especially true of DDT and permethrin selected colonies.

Fungal infection assays demonstrated that insecticide susceptible and resistant *An. gambiae, An. merus* and *An. quadriannulatus* are highly susceptible to *B. bassiana* infection with 100% mortality being achieved within fourteen days post-infection. Susceptibility to *B. bassiana* infection was greater in insecticide susceptible samples (as determined by mortality rate) as compared to insecticide resistant samples drawn from the *An. arabiensis* colonies. However, there was no correlation between insecticide resistance levels and susceptibility to *B. bassiana* infection. In addition, relative susceptibility to fungal infection was species-dependent with *An. gambiae, An. merus* and *An. quadriannulatus* being more susceptible to fungal infection than *An. arabiensis.*

Wild-caught *An. arabiensis* mosquitoes were completely susceptible to DDT and dieldrin. Susceptibility assays against other classes of insecticide were not carried out because of sample size limitations. Susceptibility to fungal infection amongst F1 progeny reared from wild caught *An. arabiensis* was high and comparable to that of insecticide susceptible *An. arabiensis* laboratory-reared specimens.

Based on the above findings, it can be concluded that the entomopathogenic fungus *B. bassiana* isolate IMI 391510 may be of significant use in integrated

vector control programmes, particularly in areas where effective vector control is threatened by insecticide resistance. This is because individual mosquitoes carrying resistance to one or more classes of insecticide would still be susceptible to fungal infection, were entomopathogenic fungi used in combination with insecticide based interventions. The presence of such fungal strains may alter malaria risk by reducing mosquito feeding propensity, fecundity and vectorial capacity because of a small blood intake when infected with fungus. This scenario could lead to a significant reduction of vector populations, followed by a reduction in the intensity of malaria transmission. The presence of entomopathogenic fungi could also play a role in limiting the development of insecticide resistance in vector populations.

Given that the entomopathogenic fungus *B. bassiana* isolate IMI 391510 has potential for use as an alternative vector control tool, it is recommended that:

1) Further studies be carried out to identify a specific fungal formulation and concentration that gives 100% mortality within the desired time frame (i.e. within 14 days following exposure to fungus), as well as suitable delivery methods.

2). Optimal temperature giving highest mortality and the effect of temperature on fungal exposures, infectivity, and persistence be investigated.

3) Small-scale field trials designed to evaluate the effectiveness and feasibility of this entomopathogenic fungus for malaria control in endemic settings be set up.

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APPENDIX

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. 2008. Fungal infection counters insecticide resistance in African malaria mosquitoes. (submitted to PNAS). My contribution to this work was the baseline work reported in Chapters II and III.