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THE USE OF ENTOMOPATHOGENIC FUNGI AGAINST *ANOPHELES FUNESTUS* Giles (Diptera: Culicidae)

By Joel Claude Mouatcho

Thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 2010

DECLARATION

I, **Joel Claude Mouatcho**, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



……..

Signature

…10th…..day of …September…2010…..

ABSTRACT

Malaria vector control relies primarily on the application of chemical insecticides. The increasing incidence of insecticide resistance in target vector populations coupled with the threat of environmental contamination are of major concern in terms of this approach. The use of biological agents to complement existing insecticide based control strategies has been proposed, e.g. *Metarhizium anisopliae* and *Beauveria bassiana.* The efficacy of *M. anisopliae* (Metschnikoff) Sorokin strain ICIPE-30 and *Beauveria bassiana* (Balsamo) Vuillemin isolate I93-825 alone or in combination was assessed against laboratory strains of the major malaria vector *Anopheles funestus.*

Samples of adult females from three laboratory strains of *An. funestus* were exposed to dry conidia of *M. anisopliae* and *B. bassiana* for 3, 6 or 24 hours. Of these *An. funestus* strains, Fang was fully susceptible to all insecticides, Fumoz was partly resistant to pyrethroid insecticides and Fumoz-R has been intensively selected for pyrethroid resistance. Following inoculations, the rate of mortality in all strains was approximately 6-fold higher in fungus infected cohorts compared to their corresponding uninfected control cohorts. Susceptibility to fungal infection in the colonies appeared to follow their pattern of susceptibility to insecticide whereby Fang showed higher rates of mortality following fungus infection than Fumoz and Fumoz-R. Further, Mosquitoes placed in forced proximity of fungal spores for only three hours showed significantly lower rates of mortality than those placed in similar circumstances for 24 hours, showing that the probability of acquiring an infection is a function of time and that a longer potential exposure time leads to the acquisition of greater numbers of infective spores. Approximately 99% of all fungus infected mosquitoes (infection confirmed by follow-up sporulation tests on cadavers) died within 14 days of acquiring their infection. Fourteen days is the maximum time required by malarial parasites to reach the infective sporozoite stage.

Experiments were designed to quantify a possible interaction between susceptibility to the pyrethroid insecticide permethrin and susceptibility to *B. bassiana* or *M. anisopliae* infection, based on the hypothesis that intoxication or infection with one of these agents exerts a synergistic effect on susceptibility to the other. Further, the effect of a non insecticidal substance that inhibits the activity of monooxygenases - piperonyl butoxide (PBO) - on subsequent susceptibility to fungus infection in pyrethroid resistant *An. funestus* was tested. Fumoz-R infected with fungus proved significantly more susceptible to pyrethroid intoxication post fungus infection than uninfected samples from the same cohort. Pre-exposure to PBO did not affect subsequent susceptibility to fungus infection, suggesting that monooxygenases play a negligible role in protection against fungus infection.

Experiments were designed to test for variation in fungus induced mortality rates between blood fed and unfed cohorts of female *An. funestus* as well as to test for differences in fecundity in response to fungal infection. Females blood fed post exposure to fungus showed a slightly higher rate of mortality compared to unfed fungus infected females. Females blood fed prior to fungus infection showed comparable rates of mortality with those of unfed fungus infected cohorts. These results suggest that blood-feeding may affect susceptibility to fungus infection, although the effect is slight at best and of no concern in terms of fungal pathogenicity. Fungus infected females produced significantly fewer eggs than uninfected females. The proportion of progeny from fungus infected females surviving to adulthood was also significantly reduced by comparison to the progeny of uninfected females.

In order to test the effectiveness of entomopathogenic fungi in semi-field conditions it is important to first survey the local malaria vector species composition including their insecticide susceptibility status. Baseline mosquito surveillance was conducted in the Mamfene region of northern KwaZulu-Natal, South Africa, in order to assess the feasibility of using clay pots treated with dry

conidia of entomopathogenic fungi as a delivery/infection system in a field environment. *Anopheles arabiensis*, *An. parensis*, *An. funestus*, *An. merus* and *An. quadriannulatus* were collected inside houses between February and September, 2005. A sample of *An. parensis*, a non-vector, was shown to present false positives for the presence of *P. falciparum* circumsporozoites using the standard ELISA method. This result highlighted the importance of accurate species identification and vector incrimination, and showed how over-reliance on standard methodologies without suitable quality assurance can lead to inaccurate information about malaria transmission dynamics in a given area. Resistance to permethrin (pyrethroid) was detected in *An. arabiensis* and *An. parensis.* Biochemical analysis and insecticide-synergist assays showed monooxygenase elevation leading to monooxygenase based permethrin detoxification in *An. arabiensis.* Preliminary laboratory tests revealed that clay pots treated with dry conidia of *B. bassiana* or *M. anisopliae* are suitable for spore delivery to anopheline mosquitoes resting inside them. However, the efficacy of treated pots, measured in terms of relative infectivity, decreased with increasing time lapse since treatment, so that by 3 months post treatment their efficacy was negligible under standard laboratory conditions.

I conclude that dry conidia of *B. bassiana* and *M. anisopliae* are effective pathogens against *An. funestus*. Infective spores can be delivered using either an attractant such as sucrose or by treating the surfaces of preferred resting sites such as clay pots. Their pathogenicity is not significantly affected by monooxygenase based insecticide resistance, and in almost all cases fungus infected females die within 14 days of acquiring an infection, regardless of blood-feeding status. Further, fungal infection significantly attenuates the expression of insecticide resistance, and also significantly reduces fecundity and fertility in infected *An. funestus* females. These factors enhance the potential of entomopathogenic fungi as biocontrol agents in areas where resistance to insecticide occurs in target vector populations.

This thesis is dedicated to the loving memory of my late brother, Serge D. Mouatcho Leumassi.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Overview of malaria

Malaria is a very old disease. The origin of its name dates back to the ancient Romans. Following the work by Charles Laveran in Algeria who showed that malaria is caused by plasmodium parasites (Garnham, 1966), Sir Ronald Ross working in India demonstrated and confirmed that mosquitoes act as the intermediate hosts of human and bird malaria (Garnham, 1966; Harrison, 1978). With at least 109 countries and territories worldwide at risk of malaria (WHO, 2008), Africa remains the most affected with an estimated 247 million clinical cases amongst 3.3 billion people at risk with nearly 1 million deaths, despite advances in preventive and public health measures (Fig. 1.1) (WHO, 2006, 2008).

1.2 Malaria vectors

Malaria parasites are transmitted to humans by some mosquito species of the genus *Anopheles*. At least 40 anopheline species are important human malaria vectors worldwide. These vector species differ significantly in their transmission potential (Gillies and De Meillon, 1968; Collins and Paskewitz 1995). Their efficiency as malaria vectors is due to their marked preference for human blood and their rapid adaptation to changes in the environment, induced by human habitation and agriculture (Gillies and De Meillon, 1968; Collins and Paskewitz, 1995). Due to

their slower rate of blood digestion which results in higher oocyst infections, malaria vectors are capable of sustaining the development and the transmission of malaria parasites to human hosts (Ponnudurai *et al.,* 1988).

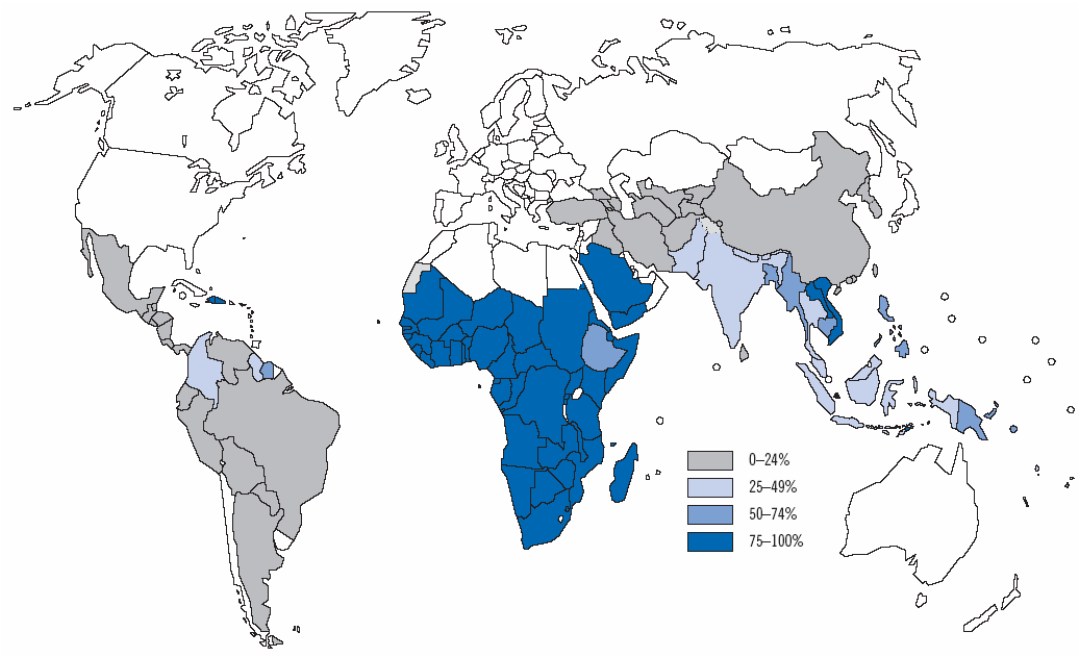


Figure 1.1: Estimated percentages of malaria cases due to *Plasmodium falciparum*, 2006 (WHO, 2008).

Human malaria in Africa is transmitted by three major anopheline species, *Anopheles gambiae* and *An. arabiensis* of the *An. gambiae* complex and *An. funestus* of the *An. funestus* group (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Collins and Paskewitz, 1995). Other species complexes such as *An. nili* and *An. moucheti* contain efficient vectors of malaria in certain areas of West and Central Africa (Fontenille and Carnevale, 2006).

1.2.1 *Anopheles funestus* group

Historically this group consisted of at least 9 morphologically similar species: *Anopheles funestus* Giles, *An. aruni* Sobti*, An. fuscivenosus* Leeson, *An. confusus* Evans and Leeson, *An. leesoni* Evans, *An. brucei* Service, *An. vaneedeni* Gillies and Coetzee, *An. parensis* Gillies, *An. rivulorum* Leeson (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). The members of the group are small dark mosquitoes that show great uniformity in adult characteristics. At specific stages of their development they show slight morphological differences (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). *Anopheles funestus, An. parensis, An. vaneedeni* and *An. aruni* share the same morphology at all life stages (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). *Anopheles rivulorum*, *An. leesoni* and *An. confusus* are easily distinguishable at the larval stage (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). *Anopheles fuscivenosus* can only be differentiated reliably from the rest of the group by chromosomal banding patterns (Green, 1982). *Anopheles parensis*, *An. funestus, An. rivulorum, An. vaneedeni* and *An. leesoni* can be distinguished using a multiplex rDNA-PCR assay (Koekemoer *et al.,* 2002). More recently, Garros *et al.* (2004) developed a single multiplex PCR assay to identify major malaria vectors within the African *Anopheles funestus* and the oriental *An. minimus* groups.

The Afro-tropical *Anopheles funestus* group has recently been re-classified due to similarities with the Southeast Asian *An. minimus* Theobald group (Harbach, 2004; Garros *et al.,* 2005a, b). Despite its Afro-tropical origin, *An. leesoni* is now part of

the *An. minimus* subgroup (Harbach, 2004; Garros *et al.,* 2005b). *Anopheles aruni, An. funestus, An. parensis*, *An. confusus* and *An. vaneedeni* belong to the *An. funestus* subgroup while the *An. rivulorum* subgroup includes *An. brucei, An. fuscivenosus, An. rivulorum,* and *An. rivulorum-like* species (Harbach, 2004; Garros *et al*., 2005b). *Anopheles rivulorum-like* is yet to be attributed a taxonomic name because it has not been fully described. It differs both molecularly and cytogenetically from *An. rivulorum* in South Africa, but no detailed morphological studies have been completed (Hackett *et al.,* 2000; Cohuet *et al.,* 2003; Coetzee and Fontenille, 2004). Similarly, the newest member of the *An. funestus* subgroup *An. funestus-like* discovered in Malawi, awaits formal description and naming (Spillings *et al.,* 2009).

Anopheles funestus is widely distributed throughout sub-Saharan Africa with the remaining members of the group showing more localised distributions (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). *Anopheles aruni* was recorded in Zanzibar. *Anopheles parensis* and *An. confusus* were recorded in eastern Africa and part of South Africa. *Anopheles* v*aneedeni* was found in South Africa while *An. fuscivenosus* and *An. brucei* were reported from Zimbabwe and Nigeria respectively (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). The distributions of *An. aruni, An. brucei* and *An. fuscivenosus* are based on historical data owing to a lack of more recent data. *Anopheles rivulorum-like* is currently found in Cameroon and Burkina Faso (Cohuet *et al.,* 2003). *Anopheles funestus-like* is known only from Malawi at present (Spillings *et al.,* 2009).

Anopheles funestus is the only major vector of human malaria in this group due to its anthropophilic and endophilic behaviours (Gillies and De Meillon, 1968). These characteristics make it susceptible to indoor spraying with residual insecticides. Working in Tanzania, Wilkes *et al.* (1996) showed that *An. rivulorum* can also be involved in malaria transmission, albeit to a far lesser degree than *An. funestus.* *Anopheles vaneedeni* can be infected with *P. falciparum* in the laboratory but apparently does not transmit malaria in nature (De Meillon *et al.*, 1977). Due to their markedly zoophilic status, other members of the group have not been implicated in the transmission of *Plasmodium.* However, the increase in the number of *An. parensis* caught inside houses has been highlighted, but there are no records of malaria transmission by this species (Kamau *et al.,* 2003; Mouatcho *et al.,* 2007). *Anopheles funestus-like* from Malawi, while found resting inside houses, has not yet been shown to be a vector of malaria (Spillings *et al.,* 2009). All members of the group breed in well-vegetated permanent pools, swamps and slow-running streams (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987).

1.3 Malaria in southern Africa

The principal malaria vector species in the region are *An. arabiensis* and *An. funestus* (Gillies and De Meillon, 1968; Coetzee *et al.,* 2000). The intensity of malaria transmission in southern Africa varies considerably and includes malaria-free zones, stable zones and unstable areas (Smith *et al.,* 1977; Coetzee *et al.,* 2000).

In stable areas, malaria transmission is marked throughout the year with little seasonal variation and unlikely epidemics. Unstable malaria is encountered below latitude 20 ºS in areas encompassing South Africa, Botswana, Swaziland, Namibia and Zimbabwe. In these areas transmission is low and varies from year to year with a high potential for epidemics which can result in high levels of morbidity and mortality. Figure 1.2 illustrates the impact of unstable malaria in South Africa with high increases in 1999-2000 (Department of Health, Unpublished Data, 2008). During 1999-2000 the major malaria epidemic that was experienced in South Africa was associated with the return of pyrethroid resistant *An. funestus* (Hargreaves *et al.,* 2000).

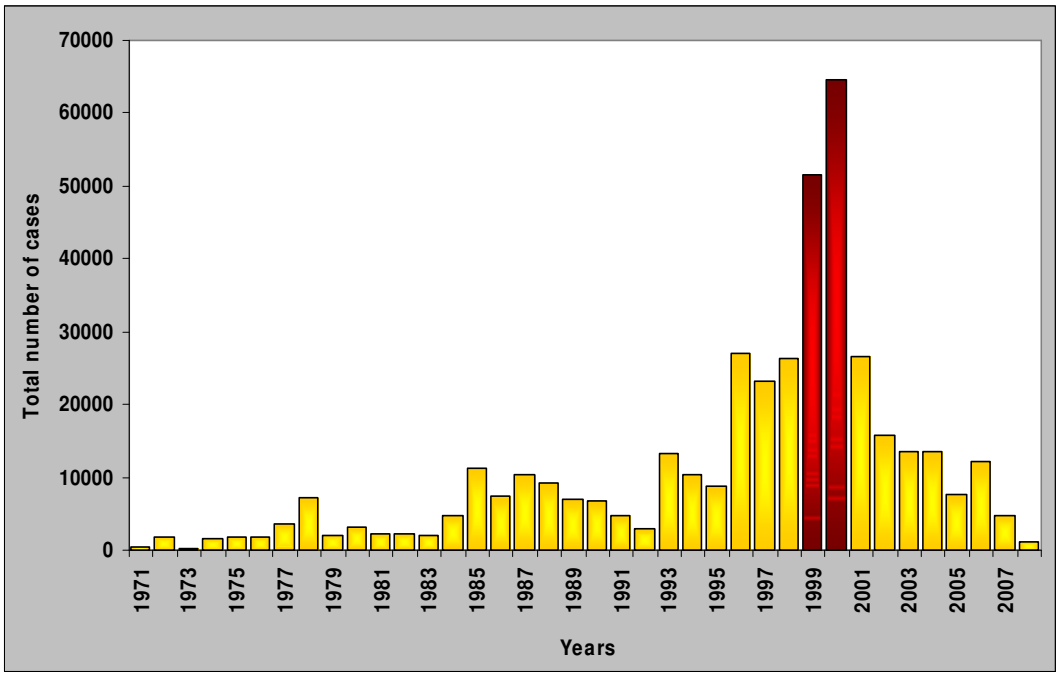


Figure 1.2: Malaria Cases in South Africa: 1971-2008 (Department of Health, Unpublished Data, 2008).

Insecticide resistance is defined by the WHO (WHO, 1957) as:

"The development of an ability in a strain of insects to tolerate doses of toxins that would be lethal for the majority of individuals in a normal population of the same species".

Evidence of insecticide resistance within *An. funestus* has been recorded from several African countries. Resistance to DDT, malathion, fenitrothion (Touré, 1982), dieldrin (Brown, 1986), pyrethroids and carbamates (Hargreaves *et al.,* 2000; Brooke *et al.,* 2001; Casimiro *et al*., 2006) have all been recorded.

The re-introduction of DDT post-2000 resulted in a dramatic reduction of malaria cases in South Africa (Maharaj *et al.,* 2005; Coetzee, 2006). Although DDT remains environmentally unfriendly (WHO, 2000; Zaim and Guillet, 2002), toxicity to humans has not been proven. Increasing public sensitivity to environmental pollution and problems of mosquito resistance to chemical insecticides has led to a global consensus to reduce or wipe out extremely noxious insecticides. This has prompted considerable interest in more benign malaria control strategies including the use of entomopathogenic fungal biocontrol agents (WHO, 2000; Zaim and Guillet, 2002).

1.4 Biological control agents

The selection of candidate biological control agents depends on factors including efficacy, environmental impact, cost consideration and compatibility with other intervention methods (Lacey and Orr, 1994).

1.4.1 Fish

Fish have been used as biological control agents against mosquitoes and other insect pests for many years. They predominantly feed on larvae. A wide variety of fish species have been tested but only a few have proven efficient for mosquito control. *Aphanius discolour* and fish in the *Panchax* group are the most popular mosquito fish in Africa and Asia (Haas and Pal, 1984; Bay, 1985). Although significant reduction in the numbers of mosquito larvae were achieved with the commonly used mosquito fish, *Gambusia affinis*, research showed that temporary water mosquitoes like many anophelines, as opposed to permanent-water breeders such as culicines, are less susceptible to mosquito fish species (Meisch, 1985).

1.4.2 *Toxorhynchites*

A large number of arthropod predators are known to feed on mosquitoes (Collins and Washino, 1985). Mosquitoes of the genus *Toxorhynchites* have been successfully used as biological larval predators in different environments (Collins and Blackwell, 2000).

Species of *Toxorhynchites* are distributed worldwide but are most common in tropical and subtropical African forests with a few species extending to the temperate areas (Trpis, 1973; Steffan and Evenhuis, 1981). The most widespread species in the tropics is the tree hole breeding *Tx. brevipalpis* (Trpis, 1973).

Unlike other species of female mosquitoes, adults of *Toxorhynchites* don't feed on blood but on nectar and other natural occurring derived sugar sources. They are not pests or vectors and are harmless to humans. Their larvae, however, are predators on other mosquito larvae including those breeding in natural and artificial containers (Steffan and Evenhuis, 1981). A further advantage is their ability to rapidly consume large numbers of mosquito larvae for long periods in the absence of other prey (Steffan and Evenhuis, 1981). Under laboratory conditions, it has been shown that the larvivorous activity of *Toxorhynchites* is temperature dependent, with *Tx. brevipalpis* consuming up to 359 larvae of *Aedes aegypti* when reared at 30-32 ºC as against 154 at 20-26 ºC (Trpis, 1972; Carlson and Vigliano, 1985). Taylor (1989) describes the so called "killing behaviour" of *Toxorhynchites* species in which numbers of prey larvae as well as con-specifics are killed but not consumed. The rate of killing is also temperature dependent (Trpis, 1972).

The use of *Toxorhynchites* as a biological control agent for malaria has many drawbacks. Culicines are their preferred prey, and mass rearing requires sufficient quantities of living prey. Further, their larval habitats differ from target anopheline prey species and they resort to cannibalism under crowded conditions (Steffan and Evenhuis, 1981, Jones, 1993).

1.4.3 Bacteria

Although large numbers of bacteria species have been isolated from mosquitoes, only a few are pathogenic. Those currently in use for mosquito control are spore

forming, rod-shaped bacteria of the genus *Bacillus* which commonly occur in soils. They include the most successful of biological control agents, namely, *Bacillus thuringiensis* (*B.t.*) and *B. sphaericus* (*B.s.*) (e.g. Service, 1983; Fuxa, 1987; Lacey and Undeen, 1986). These *Bacilli* have no ill-effects on non target organisms (Karch *et al.,* 1990; Lacey and Mulla, 1990; Lacey and Siegel, 2000) including humans and other vertebrates (Lacey and Siegel, 2000).

Bacteria must be ingested to be effectively pathogenic. They are not contact poisons (Lacey and Undeen, 1986, Lacey and Siegel, 2000). Entomopathogenic bacteria kill their hosts by releasing one or several insecticidal proteins following ingestion (Lacey and Undeen, 1986). These toxins are strain dependent (Lacey and Undeen, 1986). Although the mode of action of these bacterial toxins is not well understood, it is suggested that larvicidal toxins secreted by *B.t.* kill their hosts through a series of cascade events (Höfte and Whiteley, 1989; Schnepf *et al.,* 1998; Lee *et al.,* 2003).

1.4.3.1 *Bacillus thuringiensis*

The gram positive, spore forming *Bacillus thuringiensis* was first discovered in silkworms in 1901 in Japan by Ishawata who called it *Bacillus sotto.* It was only in 1911 in Germany that Ernst Berliner isolated the bacterium from dead flour moths and named it *Bacillus thuringiensis* (Milner, 1994).

Bacillus thuringiensis is the most popular and widely use microbial insecticide because it is broadly pathogenic against larvae of Lepidoptera, Coleoptera and Diptera (Lacey and Undeen, 1986). However, *B.t.* is target specific depending on formulation. *Bacillus thuringiensis* var. *darmstadiensis* and *B. thuringiensis* var. *morrisoni* have almost no effect on Lepidoptera (Padua *et al.,* 1980 and 1984). Generally, formulations are prepared with respect to the area or habitat to be treated (Lacey and Undeen, 1986). The discovery of a single *Bacillus, Bacillus thuringiensis* var. *israelensis* (*B.t.i.*), also called *B.t.* (H-14), with highly insecticidal effects on multiple insect targets, (Goldberg and Margalit, 1977), has revolutionised the use of *B.t.* as a biological control agent. Improvements in formulation and application methods followed the first productions of *B.t.i* against insect targets in the early 1980s. *Bacillus thuringiensis* var. *israelensis* is principally effective against the larvae of mosquitoes (*Aedes, Culex*, *Anopheles* and *Psorophora*), fungus gnats and black flies (Lacey *et al.,* 2001). Field tests using *B.t.i.* against mosquito larvae have proven successful with no reports of resistance (Hembree *et al.,* 1980; Stark and Meish, 1983; Lacey *et al.,* 1984; Sandoski *et al.,* 1985; Karch *et al.,* 1991; Fillinger *et al.,* 2003; Walker and Lynch, 2007). Moreover, adult mosquitoes have also been found to be susceptible to *B.t.i.* (Klowden and Bulla, 1984; Zahiri and Mulla, 1985).

1.4.3.2 *Bacillus sphaericus*

Found in soils and aquatic habitats, *Bacillus sphaericus* was first recognized as a mosquito pathogen in 1965 in California. It is specifically toxic against larvae of

species of *Culex*, *Psorophora* and *Culiseta* (Lacey and Undeen, 1986). Although its spectrum of activity is not as broad as that of *B.t.i*, *B.s.* appears to be particularly effective against the larvae of certain culicine species (Lacey and Singer, 1982; Karch *et al.,* 1990) and is most effective in highly polluted water such as sewer water (Hertlein *et al.,* 1979; Mulla *et al.*, 1984). *Bacillus sphaericus* kills laboratory reared larvae but appears less effective in field tests. There are reports of high levels of resistance in field populations of *An. gambiae* (Karch *et al.,* 1992) and *Culex quinquefasciatus* (Rao *et al.,* 1995; Silva-Filha *et al.,* 1995). Nielsen-Leroux *et al.* (1997) identify at least two different mechanisms by which resistance is conferred. Recently, a high efficacy and efficiency of a new *B.s.* formulation against *An. gambiae* and other Afrotropical anopheline larvae was achieved, but the short life span of *B.s.* in non polluted environments in addition to its narrowed host range as compared to *B.t.i.* makes it a poor candidate for biological control (Fillinger *et al.,* 2003; Fillinger and Lindsay, 2006; Walker and Lynch, 2007; Lacey *et al.,* 2001).

The principal drawback with entomopathogenic bacteria for biological control is the cost of production and delivery which makes them less accessible to developing countries (Lacey *et al.,* 2001).

1.4.4 Nematodes

Nematodes are non-segmented roundworms that occur naturally in soils world-wide. Numerous species may occur in sympatry (Kaya and Gaugler, 1993). Those with pathogenic activities are called entomogenous or entomopathogenic

nematodes. Among the 7 families of entomogenous nematodes (Tetradonematidae, Allantonematidae, Heterorhabditdae, Phaenopsitylenchidae, Sphaerulariidae, and Steinernematidae), the Mermithidae are the largest and attack mosquito larvae (Petersen, 1982; Kaya and Gaugler, 1993; Kaya and Stock, 1997). Entomopathogenic nematodes can be considered to be contact poisons. They invade larvae via the cuticle and/or through body cavities and inject them with endotoxins resulting in the death of the host (Chapman, 1974; Kaya and Gaugler, 1993). They are non-toxic to vertebrates and plants (Kaya and Gaugler, 1993; Becnel and Johnson, 1998).

Nematodes of the family of Mermithidae have been considered for use against several mosquito species (Kaya and Gaugler, 1993). *Anopheles, Aedes, Culex* and *Toxorhynchites* larvae were found to be highly susceptible to the mermithid nematode *Strelkovimermis spiculatus* (Becnel and Johnson, 1998). Two mermithid species, *Romanomermis culicivorax* and *R. iyengari*, proved highly pathogenic to more than 80 species of mosquito larvae including *Anopheles, Aedes* and *Culex* genera (Petersen *et al.,* 1968 and 1969; Santamarina and Perez, 1997). However, mermithid nematodes cannot be produced in vitro and their production is not cost effective, limiting their development as biological control agents (Service, 1983).

The Steinernematidae and Heterorhabditidae have engendered intense interest as microbial agents for the biological control of pests. Their mutual association with bacteria of the genus *Xenorhabdus,* for Steinernematidae and *Photorhabdus* for

Heterorhabditidae results in a high mortality rate of infected insects in various habitats (Kaya and Gaugler, 1993). These nematodes can be produced on a large scale, and can be used in conjunction with other insecticides (Kaya and Gaugler, 1993; Gaugler *et al.,* 1997).

1.4.5 Viruses

Numerous viruses have been found to be responsible for epizootic death in many insect species, the most affected being dipterans of the suborders Culicidae and Simulidae (Payne, 1982). Viruses, like bacteria, must be ingested to infect their hosts. Kellen *et al.* (1966) described the first pathogenic virus known as cytoplasmic polyhedrosis virus (CPV) from a mosquito species *Culex tarsalis.* Further, viruses including nucleopolyhedrosis viruses (baculoviruses, NPV), mosquito iridescent viruses (iridoviruses, IRV), parvoviruses, granuloviruses (GV), a pox-like virus (entomopoxviruses) and other cytopolyhedrosis viruses (reoviruses, CPV) have also been described in mosquitoes (Chapman, 1974; Clark *et al.,* 1969). The NPV and GV are the most pathogenic viruses against the larvae of many insect species including caterpillars and sandflies (Clark *et al.,* 1969; Chapman, 1974; Lacey *et al.,* 2001) whilst IRV, CPV, entomopoxviruses and parvoviruses are highly pathogenic to culicine mosquitoes (Chapman, 1974). These viruses do not appear to present any safety concerns with respect to non target invertebrates and vertebrates (Gröner, 1990). However, they require live insect hosts for production (Young and Yearian, 1986; Lacey *et al.,* 2001).

1.4.6 Protozoa

Numerous protozoa are found in natural populations of insect species, but only few can play an important role in infecting and reducing mosquito population sizes. Species of microsporida (phylum: Microspora) are among the most commonly observed pathogenic protozoans in mosquito populations (Chapman *et al.,* 1972; Chapman, 1974; Legner, 1995). Entomopathogenic protozoa kill their host by the chronic effects of parasitism (Chapman *et al.*, 1972).

Microsporidia of the genera *Nosema* and *Amblyospora* have received more attention due to their wide occurrence in anopheline mosquito species (Chapman, 1974). Under laboratory conditions it has been shown that although little effect was observed on larvae of *An. stephensi* infected with *N. algerae*, the longevity and fecundity of adult populations was reduced (Undeen and Alger, 1975; Haq *et al.,* 1981). Further, longevity and fecundity of female *An. albimanus* were reduced after infection with *N. algerae,* thereby decreasing malaria transmission (Antony *et al.,* 1978). Horizontal (Sweeney *et al.,* 1990) and transversal (Johnson *et al.,* 1997) transmission of Microsporidia from infected to uninfected hosts has been reported. However, Lacey and Undeen (1986) observed only a moderate level of infection in a field population of anopheline larvae exposed to *N. algerae*.

Infectivity studies of *Amblyospora* species indicate that this genus of Microsporidia requires an intermediate host in its life cycle to infect mosquitoes, limiting their control potential against mosquitoes and other pests (Sweeney *et al.,* 1985).

Although the fecundity and life span of insect hosts are reduced post Microsporidia infection, factors including low levels of infection, high sensitivity to UV-light, sedimentation of spores and production in living hosts, have restricted the development of parasitic protozoa for mosquito control (Lacey *et al.,* 2001).

1.4.7 Entomopathogenic fungi

Entomopathogenic fungi were amongst the first organisms that permeate our environment to be used for the biological control of pests (Hajek, 1997). Mycoinsecticides or mycopesticides have been known since the eighteenth century with 47 species reported against mosquitoes in the early 1960s (Chapman, 1974). Entomopathogenic fungi are common and widespread in almost all classes of insects. To date, approximately 750 species of fungi from about 90 genera have been documented to be pathogenic, but only a few of these species are currently being developed as pathogens against insect pests (Chapman, 1974; McCoy *et al.,* 1988; Roberts and Hajek, 1992; Hajek and Leger, 1994; Fukatzu *et al.,* 1997; Hajek, 1997). Most species are either from the fungal divisions Zygomycota in the order Entomophthorales or Ascomycota of conidial Hyphomycetes which do not reproduce sexually (Hajek, 1997). Unlike Hyphomycetes fungi which infect and colonize insects after death, entomophthorales fungi do not produce major toxins for the progression of an infection and are thus obligate pathogens (Roy *et al.,* 2006). Although these two groups of fungi vary in terms of life history, they both produce asexual spores (commonly called conidia) representing the infective component as well as sexual spores (chlamydospores) enabling survival in the

absence of new hosts (Hajek and Leger, 1994; Roy *et al.,* 2006). These two groups of fungi are also reportedly responsible for epizootic or natural epidemics within susceptible hosts (Hajek and Leger, 1994; Smith *et al.,* 1976; Powell *et al.,* 1986), but this phenomenon is most commonly observed in association with the Hyphomycetes (Pell *et al.,* 2001).

Two types of pathogenic fungi have been studied for use as biological control agents against mosquito species. These are aquatic fungi that comprise species of *Coelomomyces* (chytridiomycetes fungi)*, Lagenidium giganteum* (an oomycete fungus) and *Culicinomyces clavosporus* (a Deuteromycetes fungus), and the terrestrial fungi *Metarhizium anisopliae* and *Beauveria bassiana* (Chapman, 1974; Roberts and Hajek, 1992). Most research has focussed on *Lagenidium giganteum,* followed by *M. anisopliae* and *B. bassiana*. Entomopathogenic fungi are effective against eggs, larvae, intermediate stages and adults of a variety of insects including locusts, grasshoppers, mosquitoes, and others (Chapman, 1974; Hajek and Leger, 1994; Rombach *et al.*, 1986; 1987; Hajek, 1997; Bateman *et al.,* 1998; Scholte *et al.,* 2005; Shi and Feng, 2004; Blanford *et al.,* 2005; Furlong and Pell, 2005; Achonduh and Tondje, 2008).

1.4.7.1 The entomopathogenic Deuteromycetes *Beauveria bassiana* and *Metarhizium anisopliae*

Also known as imperfect entomopathogenic fungi, *B. bassiana* and *M. anisopliae* belong to the division Ascomita, class Deuteromycetes (Hyphomycetes), order

Hypocreales and family Clavicipitaceae (Roberts, 1970; McCoy *et al.,* 1988). For more than 120 years, *B. bassiana* and *M. anisopliae*, in addition to *B. brongniartii,* have been used to control pest insects (Zimmermann, 2007). These fungi are known to infect over 220 insect species (Roberts, 1970; McCoy *et al.,* 1988; Scholte *et al.*, 2003).

Beauveria bassiana (Balsamo-Crivelli) Vuillemin and *M. anisopliae* (Metschnikoff) Sorokin are widely distributed from the artic to the tropics. They can be isolated from insects as well as soils. They are anamorphic fungi (asexual reproduction) and infect various insect species through parasitism (Clark *et al.,* 1968; Robert, 1970; Scholte *et al.*, 2003a, b; Roy *et al.,* 2006).

The biological agents of *B. bassiana* and *M. anisopliae* are very small particles called conidia (singular conidium). The toxic action of conidia is often specific to a single or group of insects (Rombach *et al.*, 1986; 1987; Furlong and Pell, 2005). The infected insect host is killed not by the effect of chronic parasitism but by multiple mechanisms involving mechanical damage of the cuticle, toxins produced by the entomopathogenic fungi, and water loss and food depletion following infection (Chapman, 1974; Clarkson and Charnley, 1996).

Fungal pathogens are currently commercialised and extensively used as biocontrol agents against agricultural pests and public disease-vectors such as flies and cockroaches. Information concerning the commercial used of these fungicides can

be obtained from the U.S. (United States) Environmental Protection Agency www.epa.gov and from the Pesticide Action Network in North America www.pesticideinfo.org . Table 1.1 gives a brief overview on the use of *M. anisopliae* and *B. bassiana* as biological control agents.

Table 1.1: Some examples of *B. bassiana* and *M. anisopliae* developed or being developed for biocontrol of pests (Butt and Copping, 2000).

Fungus	Target	Product (Country)
Beauveria bassiana	Cotton pests including bollworms	**Naturalis-L(**USA**)**
	Army worms	**Proecol (**Venezuela**)**
	Colorado beetle	**Boverin (**former USSR**)** **Boverol (**Czech Republic**)** **Boverosil (**Czech Republic**)**
	Grasshoppers, locusts Whitefly, aphids, thrips	**Mycotrol GH (**USA**)** **Mycotrol WP and BotaniGard (**USA**)**
Metarhizium anisopliae	Spittle bugs	**Metaquino (**Brazil**)**
	Cockroaches	**Bio-Path (**USA**)**
	Termites	**Bio-Blast (**USA**)**
	Sugarcane spittle bug	**Cobican (**Venezuela**)**

1.4.7.1.1 *Beauveria bassiana*

The genus *Beauveria* contains at least 49 species of which approximately 22 are considered pathogenic (Kirk, 2003). *Beauveria bassiana* (Bals.-Criv.) Vuill., a

white muscardine fungus, is the most historically important of the commonly used fungi in this genus. Originally known as *Tritirachium shiotae,* this fungus was renamed after the Italian lawyer and scientist Agostino Bassi who first implicated it as the causative agent of a white (later yellowish or occasionally reddish) muscardine disease in domestic silkworms (*Bombyx mori L.*) (Furlong and Pell, 2005; Zimmermann, 2007). Bassi's work led to innovative vector control strategies which benefited the European silkworm industry.

Beauveria bassiana (Bals.-Criv.) Vuill. is considered to be one of the most effective entomopathogenic fungi for various reasons including: cosmopolitan distribution (found principally in shaded and uncultivated habitats) (Bidochka *et al.,* 1998), ability to infect any life stage of its host, wider host range than the other Deuteromycetes, can infect almost all orders of insects (Clark *et al.,* 1968; Roberts and Hajek, 1992) and can infect certain plant tissues (Bing and Lewis, 1992). *Beauveria bassiana* can easily be isolated from insect cadavers or from soil in forested areas by using sample media (Beilharz *et al.,* 1982), as well as by baiting soil with insects (Zimmermann, 1986). In the laboratory it can be cultured on simple media (Roberts and Hajek, 1992; Goettel and Inglis 1997).

Conidia, which are the infective propagules, measure 2-4µm in diameter and are globose to subglobose or ovoid in shape (Fig.1.3A) (De Hoog, 1972; Butt *et al*, 2001). However, the size of the thin-walled and smooth conidia varies with species (Samson and Evans, 1982). *Beauveria* is characterised by the denticulate and

distinctive zig-zig appearance of conidiogenous rachis which is an extension of sympodial proliferating conidiogenous cell bearing conidia. The conidiospores are generally grouped in clusters (Samson and Evans, 1982).

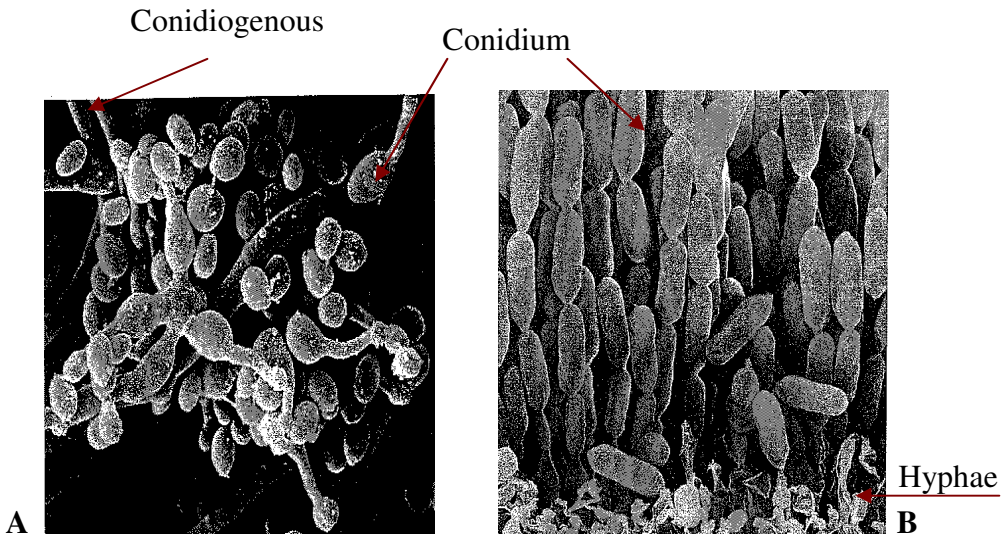


Figure 1.3: Scanning electron micrograph showing conidiogenous cells and conidia of (A) *B. bassiana* (Balsamo-Crivelli) Vuillemin and (B) *M. anisopliae* (Metschnikoff) Sorokin (Pictures adapted from Butt *et al.* 2001).

1.4.7.1.2 *Metarhizium anisopliae*

Metarhizium anisopliae (Metschnikoff) Sorokin (Fig.1.3B), initially known under the name *Entomphthora anisopliae*, was first described near Odessa in Ukraine from infected larvae of the wheat cockchafer *Anisopliae austriaca* in 1879, and later on, *Cleonus punctiventis* by Metschnikoff. It was later renamed as *M. anisopliae* by Sorokin in 1883 (Tulloch, 1976).

Metarhizium causes a disease known as 'green muscardine' in insect hosts because of the green colour of its conidial cells. The genus *Metarhizium* is pathogenic to a large number of insect species, many of which are agricultural and forest insects (Ferron, 1978). In addition, *M. anisopliae* has been found to have potential as a biological control agent of mosquitoes (e.g. Scholte *et al.,* 2005).

The taxonomy of *Metarhizium* is complex. Using the morphological characteristics of conidia and conidiogenous cells, Tulloch (1976) recognised *M. flavoviride* and *M. anisopliae* (Metsch.) of which the latter was further subdivided into two variants namely *majus* (short conidia up to 9µm) and *anisopliae* (long conidia up to 18µm). Driver *et al.* (2000) reassessed the taxonomy of *Metarhizium* using molecular data. The study was based on sequence data from the internal transcribed spacer (ITS) region and the 28S ribosomal DNA (rDNA) region. The Random Amplified Polymorphic DNA (RAPD) technique was also used. The RAPD data identified between 10-15 cryptic species of *Metarhizium* including species initially described by Tulloch (1976). This species range was also suggested by Bidochka *et al* (1994). Currently, *M. anisopliae* consists of 4 varieties or genetic groups (Driver *et al.,* 2000). Two of the most important correspond with *M. anisopliae* var. *acridium* (formerly called *M. flavoviride*) and *M. anisopliae* var. *anisopliae* (Metsch.) Sorok. The other two groups are *M. anisopliae* var. *lepidiotum* and *M. anisopliae* var. *majus.* For the purpose of this thesis, the term *M. anisopliae*, unless specified, will be used to refer to *M. anisopliae* var. *anisopliae.*

Metarhizium anisopliae (Metsch.) Sorok. conidiophores aggregate in dense tufts or palisade-like masses, with repeats that have more or less verticillate branches in parallel arrangement (Butt *et al.,* 2001). Their conidia are usually 5-9µm long with chains that are 1.5-3.5µm wide (Butt *et al.,* 2001).

As opposed to *B. bassiana,* which is preferably isolated from soils in natural, forested habitat, *M. anisopliae* is mainly found in sun exposed habitats or agricultural soils (Vänninen, 1996; Bidochka *et al.,* 1998). Due to these characteristics, *M. anisopliae* is essentially a soil fungus more common in an exposed and regularly distributed soil environment than *B. bassiana*. However, some natural variations can lead to the inhibition of conidial germination and growth (Meyling and Eilenberg, 2006 cited in Meyling and Eilenberg, 2007).

1.4.7.2 Mode of action of entomopathogenic Deuteromycetes fungi

Unlike other biopesticides such as bacteria and viruses, entomopathogenic fungi do not have to be ingested to cause infection, making them valuable as biological control agents. Although some reports suggest a mode of infection through the siphon tips or gut of insect larvae (Lacey *et al.,* 1988; Goettel and Inglis, 1997), entomopathogenic fungi generally infect or penetrate their targets percutaneously (Charnley, 1989). This can occur by adhesion of spores to the insect integument, especially the intersegmental folds, or by simple tarsal contact (St. Leger *et al*., 1986; Hajek and Leger, 1994; Charnley, 1989; Clarkson and Charnley, 1996; Scholte *et al.,* 2003a). Figure 1.4 summarises the general mode of action of fungi

which develop to at least six identifiable stages from the initial infection till the death of the host which occurs between three and seventeen days post infection, depending on the species and size (Clarkson and Charnley, 1996; Scholte *et al.,* 2004a).

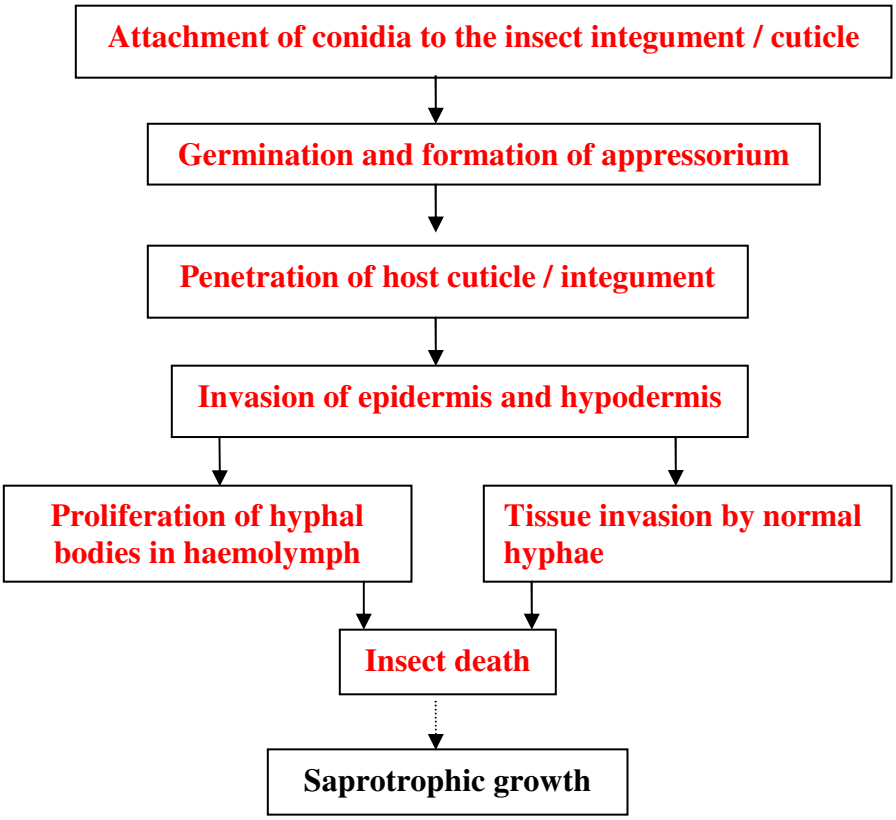


Figure 1.4: Schematic steps of the *in vivo* developmental cycle of fungal pathogens inside the insect host (Clarkson and Charnley, 1996; Narayana, 2004).

Figure 1.5 describes the mode of penetration of fungal conidia into their host. Attachment of the conidia to the host cuticle which produces the germ tube is the first step and most important part of the fungal infection (Charnley, 1989). It is

suggested that contact of conidia to the host integument occurs via non-specific forces exerted by rod-lets (straight and slim cylindrical structure) which are unique to the conidial stage of the infection (Boucias *et al.,* 1988, Bidochka *et al.,* 1995).

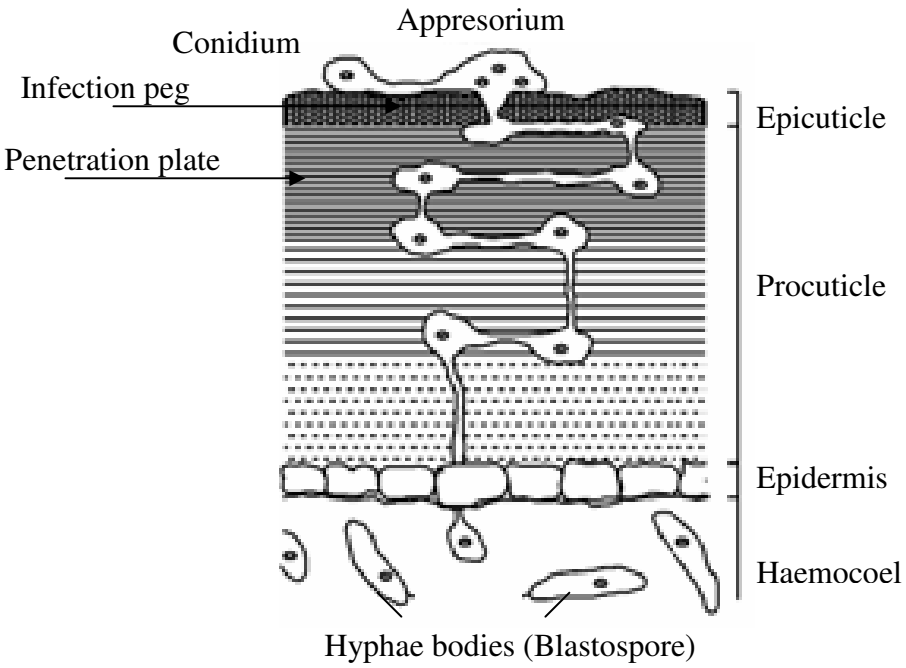


Figure 1.5: Structure of insect integument/cuticle and the mode of penetration of entomopathogenic fungi (Clarkson and Charnley, 1996).

Alternatively, the attachment of conidia to the insect integument may be initiated by hydrophobic interactions between conidial hydrophobins and the waxy surface of the insect cuticle (Bidochka *et al.,* 1995 and 2000). Fungal hydrophobins are a class of hydrophobic-rich proteins that allow the fungus to attach to solid hydrophobic surfaces (Bidochka *et al.,* 1995).

Germination and successful infection depends on a number of factors including, environmental conditions (e.g. temperature, humidity and oxygen concentration), host susceptibility, host stage and age with younger hosts usually more susceptible than older ones, and chemical composition of the host's cuticle. However, attachment and germination can fail, and the failure is generally attributed to inhibitory compounds like fatty acids, phenols and quinine (Ferron, 1978). Activities related to the attachment and germination of conidia and the formation of appressorium have been reported (St. Leger, 1998; St. Leger, *et al.,* 1991; Bidochka *et al.,* 1995). However, literature on the exact mechanism and signal elements responsible for cuticle invasion are still unclear (Bidochka *et al.,* 2000). Generally, germinated conidia produce an appressorium, which then forms an infection peg and a penetration plate (St. Leger *et al.,* 1991). Some researchers reported the absence of the appressoria in certain insects infected with *B. bassiana.* However, these cells, which are apical swellings that function in the attachment of conidia on the host's cuticle, are generally formed after the penetration of germ tube through the host's cuticle (St. Leger *et al.,* 1991; Clarkson and Charnley 1996). The result is the production of a disease in the haemocoel of the insect host (Charnley, 1989). The penetration of conidia through the host's cuticle involves both mechanical pressure and enzymatic degradation (St. Leger *et al*., 1986; Charnley, 1989; Hassan and Charnley, 1989; Hajek and Leger, 1994; Bidochka *et al.,* 1995; Clarkson and Charnley, 1996). Enzymatic degradation involves the production of several and different amounts of cuticle degrading enzymes which vary according to the species and strains of the fungi. These enzymes will exhibit

variable levels of pathogenicity towards their hosts. Following successful penetration of the cuticle, the fungus produces blastospores or hyphae bodies, which are passively distributed in the hemolymph and the fat body (Hajek and Leger, 1994; Clarkson and Charnley, 1996).

In order to kill their host, fungal pathogens release a wide variety of secondary metabolic compounds, commonly called toxins, inside the insect host, particularly in the haemocoel (see Fig. 1.5). A plethora of toxins have been suggested but the most important are destruxins from *M. anisopliae* and beauvericin from *B. bassiana* (Clarkson and Charnley, 1996; Strasser *et al.,* 2000). Dextruxins are cyclic hexadepsipeptides containing five amino acids (ß-alanine, alanine, valine, isoleucine and praline) and a α-hyroxy acid. The ionophore beauvericin is a toxic cyclic hexadepsipeptide and comprises a cyclic repeating sequence of three molecules of *N*-methyl phenylalanine altering with three molecules of 2-hydroxyisovaleric acid (Strasser *et al.,* 2000). During the initial penetration of the host, *M. anisopliae* and *B. bassiana* secrete large amounts of single extracellular protease called chymoelastase protease or Pr1 to degrade the host cuticle (St. Leger *et al.,* 1987, 1992 and 1995). The endomoprotease Pr1, which is the major enzyme secreted by *Metarhizium* during the degradation process of the cuticle, also differs in terms of biochemistry amongst strains. However, the cuticle degrading enzymes are produced in a sequential manner with the proteolytic enzymes and esterases first followed by chitinases, in other words proteins surrounding the cuticle must be

degraded before the actions of chitinases begins (St. Leger *et al.,* 1987, 1992 and 1995).

Time to death of an infected insect varies from 2-15 days post infection depending on the fungal strain and species, but more particularly on the characteristics of the host (Ferron, 1978; Boucia and Pendland, 1998). When the infection process followed by the death of the host is complete, the fungus switches back to its hyphal mode and, under relatively humid conditions, the fungus subsequently grows out of the cadaver surface to produce new, external, infective conidial saprophytic growth (Ekessi *et al.,* 1999; Fargues and Luz, 2000; Soza-Gómez and Alves, 2000; Jianzhong *et al.,* 2003; Mitsuaka, 2004). However under very dry conditions, the fungus may persist in the hyphal stage inside the cadaver where the conidia are produced inside the body (Daoust and Roberts, 1983; Hajek and Leger, 1994; Hedgecock *et al.,* 1995; Hong *et al.,* 1997). Under favourable conditions, sporulated cadavers can infect other individuals from the same target species through horizontal transmission (Meadow *et al.,* 2000; Quesada-Moraga *et al.,* 2004). Horizontal transmission can also occur during the mating process. Scholte *et al.* (2004) demonstrated that male mosquitoes can acquire fungal infection after mating with infected females. In addition, autodissemination of fungal pathogens was proven successful between infected and uninfected adult beetles in the laboratory and under field conditions (Kreutz *et al.,* 2004).

1.4.7.3 Host ranges of fungal species/strains

Although *B. bassiana* and *M. anisopliae* have a wide host range, *M. anisopliae* is more restricted than *B. bassiana.* In addition, certain strains and genotypes are more restricted within species, and even significantly between species (Rombach *et al.*, 1986 and 1987; Bidochka and Small, 2005; Furlong and Pell, 2005). *Metarhizium anisopliae* var. *anisopliae* attacks almost all classes of insects including arachnids whereas *M. anisopliae* var. *majus* is mainly restricted to soil dwelling beetles (e.g. McCoy *et al.,* 1988; Scholte *et al.,* 2003b). LUBILOSA (Lutte Biologique contre les Locustes et les SAuteriaux) identified *M. anisopliae* var. *acridium* as the most effective biological control agent for adult locusts and grasshoppers but not for mosquitoes in environmentally safe areas (Prior and Greathead, 1989; Price *et al.,* 1997; Blanford and Thomas, 2001; Lomer *et al.,* 2001). Furthermore, isolates are also more specific under field conditions compared to laboratory studies (Jaronski *et al.,* 2003) and the geographic occurrence of the fungus seems to play a role in host preference. Rombach *et al.* (1986) and Bidochka and Small (2005) showed that some genetic groups of *Metarhizium* from tropical and subtropical regions were more specific to particular classes of host insect. The virulence of entomopathogenic fungus varies within species and isolates (Prior *et al.,* 1995; Bateman *et al.,* 1996; de Moraes *et al.,* 2003).

1.4.7.4 Influence of the physiological state of the host

The physiological state of the host insect also plays a role in pathogenicity in the treated population. An increased rate of infection amongst insects already physiologically weakened increases the mortality rate amongst healthy individuals following contact with infected individuals (Scholte *et al.,* 2004). The physiology of insect hosts can be weakened by the absence of sugar or blood (Nayar and Van Handel, 1971; Foster, 1995), or by exposure to chemical insecticides where less resistant hosts will die by intoxication by a low dose of insecticide while the more insecticide resistant individuals will succumb from fungal infection (Delgado *et al.,* 1999; Pachamuthu *et al.,* 1999; Pachamuthu and Kamble, 2000; Irigaray *et al.,* 2003; Ericsson *et al.,* 2007).

1.4.7.5 Influence of temperature, humidity and solar ultraviolet radiation on fungal virulence

Temperature, humidity and light are critical components to the virulence of entomopathogenic fungi. Fungal conidia are very sensitive to high temperatures (over 34 ºC) and ultra violet light, depending on the strains of fungus (Fargues *et al.,* 1997; Jianzhong *et al.,* 2003). Their ideal temperature range is 20-30 ºC (for example, 25 ºC for *B. bassiana* and 27-28 ºC for *M. anisopliae*) (Thomas *et al.,* 1996; Fargues and Luz, 2000; Jianzhong *et al.,* 2003; Mitsuaki, 2004). High and low temperatures as well as humidity affect the speed of insect infection by inhibiting spore germination, which in turn affects the formation of a germ tube and penetration through the insect cuticle (Ekesi *et al.,* 1999; Fargues and Luz,

2000; Soza-Gómez and Alves, 2000; Mitsuaki, 2004). Oscillating temperatures can also slow infection and colonization by fungal pathogens (Inglis *et al.,* 1999). Zimmermann *et al.* (1982) and Morley-Davies *et al.* (1995) demonstrated that conidia can tolerate high temperatures associated with high relative humidity. However, the viability of conidia significantly decreases when exposed to sunlight (Zimmermann, 1982; Morley-Davies *et al.,* 1995).

Temperature and humidity are found to regulate sporulation, with high (over 34 ºC) or low (below 10 ºC) temperatures reducing or preventing sporulation (Ekesi *et al.,* 1999; Soza-Gómez and Alves, 2000; Fargues and Luz, 2000). A relative humidity between 75-100% is favourable for optimal germination of *Beauveria* conidia (Soza- Gómez and Alves, 2000). Under favourable conditions, infection speed and sporulation can be reinstated, followed by a high infection rate where population densities are high (Thomas *et al.*, 1996). However, the delivery and application methods of fungal spores can contribute efficiently to high mortality of hosts in high temperatures (Bateman, 1992).

Although the longevity of conidia is a function of the fungus treated surface (Vänninen *et al.,* 2000; Blanford *et al.,* 2005), it is evident that moisture content of conidia (Hedgecock *et al.,* 1995; Hong *et al.,* 1997), relative humidity and also play an important role in the longevity of conidia (Zimmermann, 1982; Daoust and Roberts, 1983; Hedgecock *et al.,* 1995; Morley-Davies *et al.,* 1995; Hong *et al.,* 1997). The conidia from various fungi and strains within fungus species also

showed varying longevities in relation to relative humidity and temperature (Daoust and Roberts, 1983; Hong *et al.,* 1997). Thus, different formulations of conidia are needed when using fungi against different hosts.

1.5 Rationale

The control of mosquito disease vectors is principally achieved using chemical insecticides inside houses (IRS). However, mosquitoes caught indoors are not necessarily disease vectors. It thus follows that accurate species identification and vector incrimination is required so as to avoid the use of limited resources against non-vectors.

Pyrethroids are the principle insecticides used for public health because of their low mammalian toxicity. However, pyrethroid resistance has been detected in many insect pests including malaria vectors. The occurrence of insecticide resistance to all classes of insecticides available for use in public health as well as mounting criticism concerning the use of chemical insecticides (due to their safety and environmental impacts on non-target beneficial invertebrates and humans), has provided an impetus for the development of alternative forms of vector control. Amongst these is microbiological control of adult mosquitoes (Murphy *et al.,* 1994; WHO, 2000; Zaim and Guillet, 2002).

Biological control is defined as the use of biopesticides to control pest populations. Although disseminated mycoses was observed in severely immuno-compromised

patients with leukaemia, pneumonia, sinusitis or people with a history of injury and surgery (Revankar *et al.,* 1999; Henke *et al.,* 2002; Tucker *et al.,* 2004; Osorio *et al.,* 2007; Bouwman and Kylin, 2009), biopesticides are especially valuable as they occur naturally and are generally considered to be environmentally safe (Genthner *et al.,* 1998; Pevelling *et al.,* 1999; Lomer *et al.,* 2001, Zimmermann, 2007).

Several biological control agents including fungal pathogens have historically been used as larvicides for decades (Lacey and Undeen, 1986; Scholte *et al.,* 2004a). However, targeting only larvae or intermediate developmental stages of malaria vector species is not sufficient to reduce disease transmission because malaria is transmitted by adults even if they only exist in low numbers. The potential of using *M. anisopliae* and *B. bassiana* as biological control agents against adult vector mosquitoes is well described (Clark *et al.,* 1968; Roberts, 1970; Alves *et al.,* 2002; Scholte *et al.,* 2003a, b, 2004a, 2005 and 2006; Blanford *et al.,* 2005; Achonduh and Tondje, 2008).

Under laboratory conditions, Clark *et al.* (1968) described the effectiveness of *B. bassiana* against larvae of *Culex tarsalis, Cx. pipiens, Anopheles albimanus*, *Aedes aegypti, Ae. sirrensis* and *Ae. nigromaculis.* However, the fungus was ineffective against larvae of *Cx. quinquefasciatus* (Alves *et al.,* 2002). Adults of *Culex tarsalis, Cx. pipiens, An. albimanus*, *Aedes aegypti, Ae. sirrensis* and *Ae. nigromaculis* were 100% susceptible to unformulated conidia of *B. bassiana* (Clark *et al.,* 1968). Also under laboratory conditions, Achonduh and Tondje (2008)

reported 80% mortality against *An. gambiae* adults when exposed to dry *B. bassiana* conidia, with less than 10% death in related controls. Further, laboratory *An. gambiae* and *An. arabiensis* were found to be highly susceptible to *B. bassiana* with 80-85% mortality and less than 20% mortality in corresponding control groups (Kikankie, 2009). Blanford *et al.* (2005) reported 80% mortality amongst *An. stephensi* infected with *B. bassiana* by day 14, against 38% mortality from the control group.

Metarhizium anisopliae, like *B. bassiana,* is virulent against larvae and adults of various species of mosquitoes. Under laboratory conditions, larvae of *An. stephensi, An. quadrimaculatus, Ae. aegypti, Cx. pipiens, Cx. restuans* and *Cx. salinarius* were all found to be susceptible to dry conidia of *M. anisopliae* (Roberts, 1970). Adults of *An. gambiae, An. quadrianulatus* and *An. stephensi* proved high susceptibility to unformulated dry and formulated conidia of *M. anisopliae* (Scholte *et al.,* 2003, 2005 and 2006, Blanford *et al.,* 2005). A semi-field study in a rural village in Tanzania revealed that *An. gambiae* can be controlled using formulated conidia of *M. anisopliae* with 100% infected by day 11 (Scholte *et al.,* 2005).

As already mentioned, insecticide resistance is a growing problem in insect control. Blanford *et al.* (2005) reported that it is unlikely that cross resistance between fungal pathogens and insecticides will develop in the same mosquito. Scholte *et al.*

(2006) also showed that the development of fungal resistance will be slowed by the exponential kill of mosquitoes.

An integrated approach can be used to manage insecticide resistance in mosquito disease vectors (Jenkins, 1964). Although a combination of insecticides and fungal pathogens showed mixed results, it was meaningful in terms of raising the mortality of infected insects (Delgado *et al.,* 1999; Pachamuthu *et al.,* 1999; Pachamuthu and Kamble, 2000; Irigaray *et al.,* 2003; Ericsson *et al.,* 2007). Entomopathogenic fungi and chemical insecticides may act synergistically allowing for the use of lower concentrations of either and decreasing the likelihood of resistance to either (Ferron, 1971; Richter and Fuxa, 1984; Quintela and McCoy, 1997; Blanford *et al.,* 2005). Studies revealed high mortality rates in Cockroaches when exposed to boric acid and a low dose of *M. anisopliae* (Zurek *et al.,* 2002). Further, a recent development using entomopathogenic fungi revealed that the susceptibility of greater wax moth caterpillars to fungal pathogens increased following pre- exposure to an insecticide synergist (an enzyme inhibitor) (Serebrov *et al.,* 2006).

There is currently renewed interest in the use of *B. bassiana* and *M. anisopliae* for malaria control (Enserink, 2005; Thomas and Read, 2007). Although *B. bassiana* (Bals.-Criv.) Sorok. and *M. anisopliae* (Metschn.) Sorok. conidia are widely distributed in soil, they have never been reported as a cause of mycoses in laboratory or field *An. funestus* Giles. Rapid identification methods for members of

the *An. funestus* species group (Koekemoer *et al.,* 2002) and the first stable *An. funestus* laboratory colony (Hunt *et al.,* 2005) enables an evaluation of the effectiveness of entomopathogenic fungi against *An. funestus*.

1.6 Hypotheses

1- *Anopheles funestus* infected with *M. anisopliae* and *B. bassiana* will have their physiological activities altered and will die within two weeks post fungus infection.

2- Insecticide resistant and insecticide susceptible *An. funestus* are equally susceptible to fungal infection.

1.7 Aim

The overall aim of this study was to evaluate the effect of *B. bassiana* and *M. anisopliae* var. *anisopliae* against permethrin (pyrethroid) resistant and insecticide susceptible laboratory reared *An. funestus*. A minor aim was to evaluate clay pots as a possible means of fungus delivery in the field

1.8 Objectives

1- To determine the minimum and maximum exposure times required to induce a high mortality in fungal infected *An. funestus* 14 days post infection. Fourteen days is the maximum time required by malarial parasites to reach the infective sporozoite stage.

2- To test for an association between susceptibilities to permethrin (pyrethroid insecticide) intoxication and fungus infection in *An. funestus*. I also determine the effect of exposure to synergist piperonyl butoxide, an inhibitor of cytochrome P450 monooxygenases, on susceptibility to *B. bassiana* or *M. anisopliae* infection in *An. funestus*.

3- To test for an association between blood feeding and relative susceptibility to fungal infection in *An. funestus*.

(a) Effect of a blood meal on susceptibility to *B. bassiana* or *M. anisopliae* infection.

(b) Effect of *B. bassiana* and *M. anisopliae* infection on blood feeding behaviour.

(c) Investigate a correlation between fecundity (egg production, egg hatch and larval survival) and *B. bassiana* or *M. anisopliae* infection.

4- To investigate the use of clay pot as the delivery method for dry conidia of *M. anisopliae* and *B. bassiana* in the field.

CHAPTER 2

MATERIALS AND METHODS

2.1 Mosquito culturing

Mosquito colonies used in this study were cultured in the Botha De Meillon Insectary at the Vector Control Reference Unit (VCRU) of the National Institute for Communicable Diseases (NICD) in Johannesburg, South Africa. All laboratory colonies were reared at 26 ± 2 ºC and 80 ± 2% relative humidity with a 12/12 hours light/dark cycle and a 45 min dusk/dawn regime. Larvae were fed on a mixture of finely ground dog biscuits (BEENO) and brewers yeast (4:1). Adult females were offered guinea pig blood twice a week. Eggs were collected 2-3 days following blood feeding and were transferred to 8 cm diameter plastic bowls containing distilled water for hatching. Third instars larvae were transferred to 20 x 14 cm square plastic bowls covered with gauze in which they pupated and emerged as adults. Adults were transferred to 30 cm diameter plastic cages and were provided with a 10% sucrose solution.

2.2 Mosquito colonies

The Vector Control Reference Unit (VCRU) houses various strains of *Anopheles* mosquitoes including strains of *An. funestus sensu stricto.* The first strain of *An. funestus,* Fumoz, originates from the Belulane region of southern Mozambique and

was colonised in 2000. It is partially resistant to pyrethroids and the carbamate insecticide bendiocarb (Hunt *et al.,* 2005). Fumoz-R is a pyrethroid resistant strain intensively selected from Fumoz using permethrin. Fang is derived from material collected in Calueque, southern Angola, in 2003, and is fully susceptible to all insecticides.

The *An. funestus* Fumoz-R and Fang strains were selected for this study because we were unable to generate an insecticide susceptible Fumoz strain through selection (VCRU, Unpublished data). Fumoz and Fang are closely related genetically (Garros *et al*., 2004; Ntomwa, 2004), validating comparisons between them.

2.3 Entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*

2.3.1 Isolate culture maintenance

Dry conidia of *Metarhizium anisopliae* var *anisopliae* (Metschnikoff) Sorokin isolate ICIPE 30 and *Beauveria bassiana* (Balsamo) Vuillemin isolate I93-825 (courtesy of Dr. N. Maniania and Prof. M. Thomas) were used. These two isolates were chosen because of their well characterised virulence against *An. gambiae* (Scholte *et al.,* 2003b). Upon receipt, conidia were stored in the dark at 4 ºC until used.

2.3.2 Germination tests

Although initial tests were done at the production sites, a second test was carried out on all batches of dry conidia prior to use to make sure that they had not lost viability during transit. Oil formulations were prepared by suspending 0.25 g of dry conidia in 5 ml of mineral oil (Shell Ondina® Oil 917, Shell, The Netherlands) from each stock of *B. bassiana* (≈1.3x10^9 spores/ml) or *M. anisopliae* (≈1x10^9 spores/ml). Each mixture was sonicated in a sonicator bath (Ultrasonic Cleaner, model: UD200SH-6l from Labotec) for 2 minutes in order to break up conidial chains and to separate conidia from any mycelial fragments. Ten µl from each aliquot was aseptically placed in the centre of each Sabouraud-Dextrose Agar (SDA) 9 cm petri dish. The conidial suspension was spread evenly on the agar medium with a sterile glass rod. Each petri dish containing conidial culture was sealed with parafilm and incubated at 27 ± 2 ºC for 20 to 24 hours and then observed for germ tube development (Fig. 2.1).

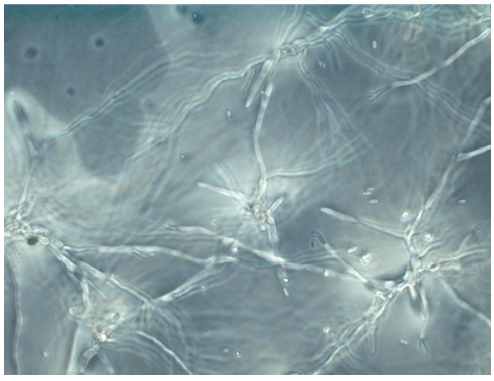
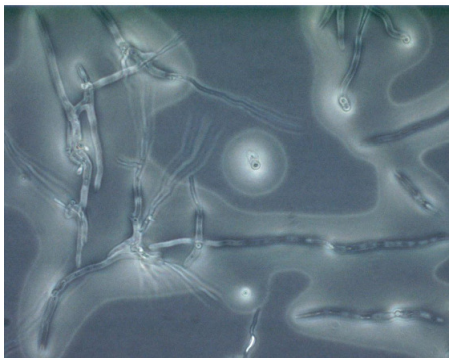
A   **B**

Figure 2.1: Conidia gel tubes on SDA plate (X 400 magnification) after 20 hours incubation at 27 ± 2 ºC (A= *M. anisopliae* and B= *B. bassiana*) (Courtesy Drs. Jennifer Stevenson and Simon Blanford, University of Edinburgh).

Two plates were inoculated per sample batch of conidia. Controls were run concurrently with suspension oil only. These tests were done under sterile conditions inside a biohazard type II safety cabinet (Esco® Airstreams S-Series). Post incubation, conidial viability was determined by quantifying spore germination using a phase contrast microscope at 400X, fitted with a Neubaeur haematocytometer for spore counts. Conidia were deemed to have germinated if germination tubes were longer than half the size of the conidia. Approximately 300 conidia/plate were counted and the average of germinated versus non-germinated spores was calculated in order to determine the percentage germination:

% germination = (total germinated/total number of spores counted) x 100.

Only batches showing 90% or higher conidia germination were subsequently used for bioassays.

2.3.3 Dry spore applications

The technique described in Scholte *et al.* (2003b) was used. Briefly, a suspensor (perforated hair-roller) was lined on the inside with a white filter paper (Whatman #1) which was moistened with a 10% sucrose solution contained in a plastic vial of 5 cm height (Fig. 2.2A). The sucrose acted as a mosquito attractant. Each hair roller (suspensor) designated for dry spore treatment was placed in a Biohazard type II safety cabinet and dusted with 100 mg of dry conidia using an artist's paint brush (Fig. 2.2B). Each fungal dusted hair-roller was then removed from the cabinet and gently placed in an exposure cage for mosquito inoculations (Figs.

2.2C and D). Control suspensors and cages were prepared similarly but without conidia treatment.

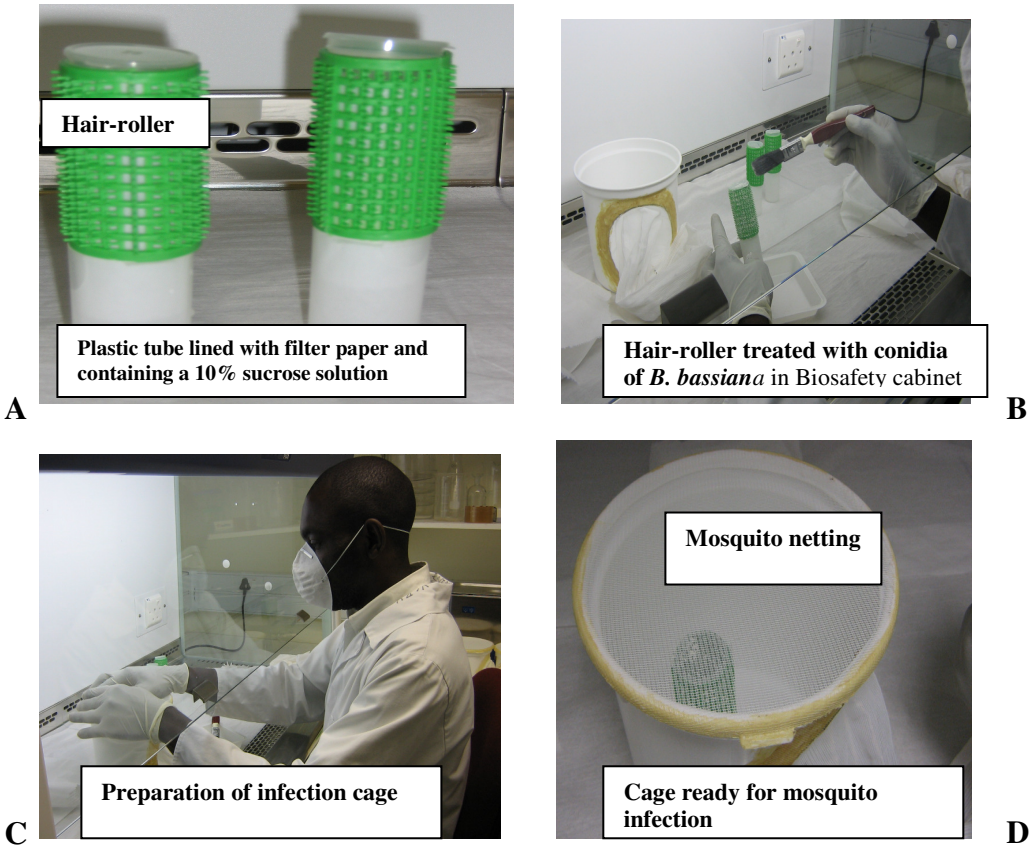


Figure 2.2: Preparatory steps for mosquito fungal infection.

2.3.4 Inoculations

Adult female *An. funestus* between 2-5 days old were used. Each treatment was replicated at least 3 times with at least 30-40 individuals per replicate and the entire experiment was repeated at least 3 times. All infections were carried out in the Hugh Paterson Insectary at the VCRU with Relative Humidity of 80-90% and a

temperature range of 25-28 ºC. During experiments, light was manually switched on at 08.00 hours and off at 18.00 hours daily.

Using an aspirator(sucking tube) mosquitoes were gently introduced into a 30 cm diameter plastic cage covered with white mosquito netting (Fig. 2.3) based on the protocol described by Scholte *et al.* (2003b). Mosquitoes in the experimental cages were infected with fungal spores as they probed the spore treated suspensors lined with sucrose solution. Mosquitoes in the control cages were similarly exposed to untreated suspensors. Mosquitoes were exposed to conidia for different lengths of time depending on the objective of the experiment. After exposure, mosquitoes from the experimental and control cages were transferred to clean 30 cm diameter plastic cages in order to monitor daily survival post exposure. They were maintained on 10% sucrose solution. Separate aspirators for fungal treatments and controls were used to avoid contamination.



Figure 2.3: Mosquitoes are gently transferred into cages containing dry conidia of entomopathogenic fungus.

2.3.5 Mortality assessments

Mortality post exposure was assessed each day for 28 days. Dead mosquitoes (if any) from the experimental and control cohorts were removed from the cages and placed on Whatman #1 filter paper discs (7 cm) lining small glass petri dishes (Ø = 6.5 cm) . Each filter paper was moistened with distilled water to maintain high humidity (~90%) in the petri dishes and to encourage fungal growth. Petri dishes were sealed with parafilm and labelled with the corresponding cage code, date of death and number of cadavers. All petri dishes were kept in the inoculation room and cadavers were monitored for 2- 4 days for evidence of fungal growth on the surface of infected mosquitoes. The species of fungal growth where detected was confirmed morphologically using a dissecting microscope (400X magnification).

2.4 Objective 1: To determine the minimum and maximum exposure times required to induce a high mortality (over 75%) in fungal infected *Anopheles funestus* at 28 days post infection

Three strains of *An. funestus* (2-5 days old females) were used for this experiment: Fumoz, Fumoz-R and Fang. A total of five trials with 30-40 individuals per replicate (n = 3 replicates) were run. Untreated groups were run concurrently under the same conditions as the fungal treated groups. Figure 2.4 describes the general procedures followed to achieve this objective. Lengths of exposure to *M. anisopliae* or *B. bassiana* were set at 3 hours, 6 hours or 24 hours and mortality post exposure was recorded daily. Experiments were monitored for a maximum of 28 days. At the end of the monitoring period, live and dead mosquitoes in each of

the experimental and control cages were counted and the total number of mosquitoes initially exposed in each was calculated. This range of exposure times (3 to 24 hours) was chosen in order to evaluate whether increasing the probability of acquiring an infection actually leads to an increased overall rate of fungus-induced mortality.

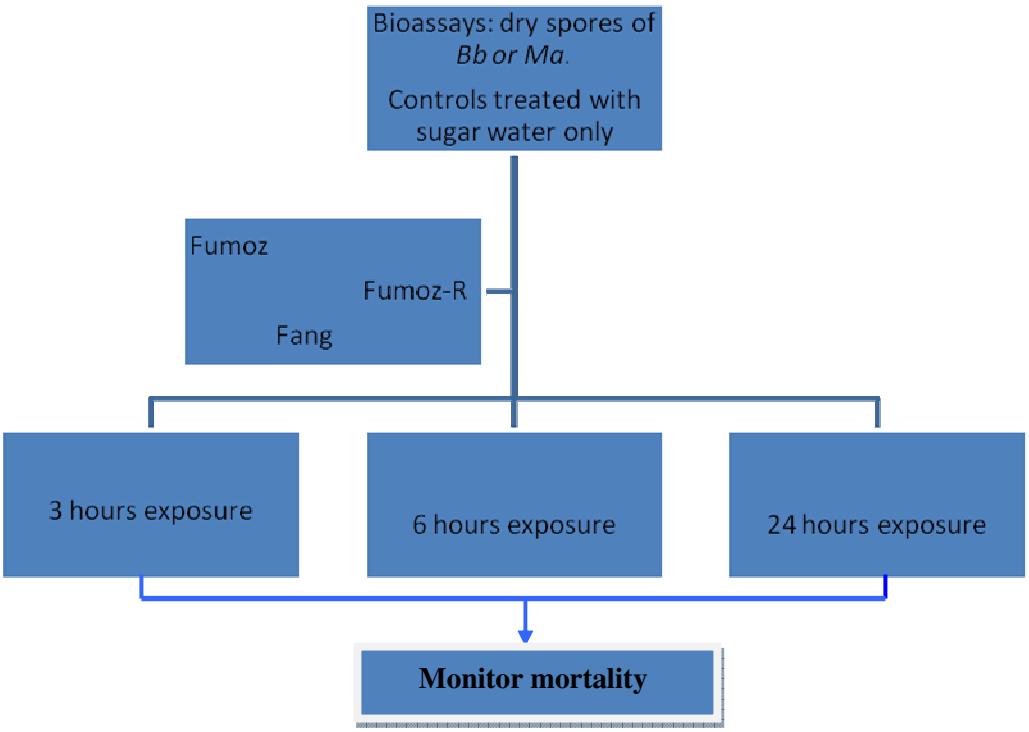


Figure 2.4: Overall experimental procedures for the infection of *An. funestus* with entomopathogenic fungi *B. bassiana* and *M. anisopliae.*

2.4.1 Data analysis

Data collected from the experiments were subjected to analysis using Kaplan-Meier Kruskal-Wallis (KW) ANOVA (*XLSTAT* 2009.2.01 software for Windows), Student's *t*-tests and Chi-square tests using *STATISTIX* 7.0 for Windows. Survival

curves were analysed by Kaplan Meier Krustal-Wallis ANOVA to compare fungal treated versus control groups. The mean survival time and lethal time required to reach 50% mortality (LT_{50}) per replicate was obtained from Kaplan-Meier analysis. The mean survival times of replicates (n = 3) within each trial were compared using 2-sample t-tests or One-Way ANOVA to measure variation between replicates. Replicates showing no significant differences between them were pooled. Outlier replicates showing significant differences were eliminated from the analysis. The LT_{50}-values of treated versus control cohorts were compared using Student's *t*-tests. The means of each of the fungal treated strains of mosquitoes (Fang, Fumoz and Fumoz-R) were compared using one-way ANOVA. The effects of exposure time (the covariate) on mortality or mosquito survival were analysed using the non-parametric Kruskal-Wallis ANOVA (and the parametric one-way ANOVA). Sporulation tests were also assessed. Mortality and sporulation data were corrected (Abbott, 1925) and compared using the Chi-square tests.

2.5 Objective 2: To evaluate the synergistic effects of two entomopathogenic fungi and permethrin or piperonyl butoxide against *Anopheles funestus*

2.5.1 Does fungal infection affect susceptibility to insecticides?

(This section is published as permethrin resistance assays on page 4 in APPENDIX 4).

Fumoz-R females were used in these tests. Two separate experiments were conducted to in order to test for an association between susceptibilities to the

pyrethroid insecticide permethrin or non-permethrin intoxication and fungal infection.

Figure 2.5 describes the procedure followed to achieve this goal. Briefly, a 3-day waiting period was chosen between fungal exposures and assessments for insecticide resistance to allow for some progression of the fungal infection while not losing large numbers through death. *Anopheles funestus* (Fumoz-R) from the same cohort received either a control, *Beauveria*, or *Metarhizium* treatment, of which 25 females per treatment were exposed 3 days later to a control paper and 25 females to a filter paper treated with 0.75% permethrin for 1 hour according to WHO (1998) (Fig. 2.6). Mosquitoes were then transferred to clean holding tubes and provided with 10% glucose solution by using cotton balls, and the proportion of dead mosquitoes was scored 24 hours after insecticide exposure. Five replicates were performed per mosquito species and for each group. After mortality measurements, mosquitoes were removed from the exposure tubes with an aspirator, killed through drowning in 70% alcohol, and checked for fungal infection as described above. With the same methods, the effect of a more advanced *Beauveria* infection was also tested by measuring the permethrin-resistance levels of control and *Beauveria*-infected mosquitoes 5 days after fungus exposure. Five replicates of 25 mosquitoes each were performed.

Mortality per replicate exposed to permethrin was corrected by using mortality data of counterparts exposed to control papers, according to Abbott's formula (Abbott,

1925). Corrected mosquito mortality was compared by using χ^2 goodness of fit test with GenStat 9.0 software. Data were analysed using *SPSS* 15.0.

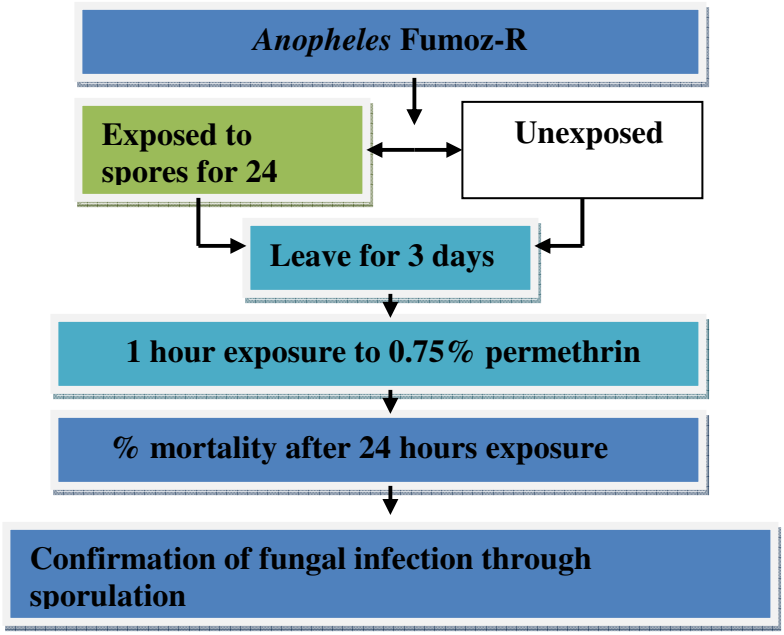


Figure 2.5: Experimental procedures to test the effect of fungal infection on female *An. funestus* Fumoz-R susceptibility to 0.75% permethrin.



Figure 2.6: Bioassay tests of *An. funestus* Fumoz-R exposed to 0.75% permethrin using WHO test kits.

2.5.2 To investigate the effect of the insecticide synergist piperonyl butoxide, an inhibitor of cytochrome P450 monooxygenases, on susceptibility to *Beauveria bassiana* and *Metarhizium anisopliae* infection in *Anopheles funestus*

Female Fumoz-R (2-5 days old) were either exposed for 24 hours to hair-roller infected with fungal conidia of *M. anisopliae* or *B. bassiana* inside a 1.5L cage lined with Wattman number one filter paper impregnated with either 4% or 8% piperonyl butoxide (PBO) or inside PBO free cages. Controls consisted of females exposed to untreated hair-rollers inside PBO treated cages and females exposed to untreated hair-rollers inside untreated cages. PBO treated papers were used to line the side and bottom of the cages. Following exposure, hair-rollers were replaced with a 10% sugar solution. This experiment was repeated 4 times with at least 30 mosquitoes per replicate (n = 3). Mortality was assessed for 28 days as described in section 2.3.6.

2.5.2.1 Synergist preparation

Piperonyl butoxide solutions were prepared by diluting active ingredients (of PBO) with olive oil to desired concentrations (4% and 8%). For a 12.5cm x 15cm Wattman #1 filter paper, 0.7ml of synergist was mixed with 1.2ml of acetone (which acted as a carrier) and evenly sprayed on the paper. Each treated paper was left to dry for 24 hours at room temperature to allow the acetone to evaporate.

2.5.2.2 Data analysis

Survival data was analysed using Kaplan-Meier survival analysis with differences between treatments determined using KW ANOVA in *XLSTAT* version 2009.2.01 software. Assuming there were no variations amongst and between treatments (using one-way ANOVA), the mean cumulative proportion surviving each day and the standard errors were calculated across all replicates (n = 15). Graphs of the percentage cumulative mosquito survival against time after exposure were constructed using Microsoft™ Excel. Significant differences in the treatment combinations would suggest an interaction between PBO and susceptibility to fungus infection and that the effect observed might be either antagonistic or synergistic (Quintela and McCoy, 1997, 1998). LT_{50}-values of PBO treated fungal mosquitoes versus untreated groups obtained from the Kaplan-Meier survival analysis were compared using Student's *t*-tests (in *STATISTIX 7.0*). The effects of PBO concentration on mortality and on fungal growth were assessed using Chi-square (*STATISTIX 7.0*).

2.6 Objective 3: To test for an association between time of blood intake and relative susceptibility to *Beauveria bassiana* or *Metarhizium anisopliae* infection in *Anopheles funestus*

2.6.1 Does blood ingestion post fungus infection affect *Anopheles funestus* susceptibility to fungus infection?

Unfed females Fumoz-R (3-5 days old) were exposed for 24 hours to either *B. bassiana* or *M. anisopliae*. Fungus infected mosquitoes were transferred into

standard mosquito cages and were then offered the opportunity to take blood 3 hours post-fungal exposure. Guinea pigs anaesthetised by the NICD animal unit (Animal Ethics Clearance Certificate no. 1993/047, updated in 2007) were used as a blood source for each experiment. They were placed on top of each cage so as to allow for females to blood feed for 30 minutes in the dark. Control samples were concurrently exposed to hair rollers without conidia and were subsequently offered blood-meals in the same way. The experiment was repeated 5 times with 30-40 cohorts per replicate (n = 3 replicates). Immediately after blood-feeding, mosquitoes were provided with a 10% sugar solution inside the cages. Experiments were analysed as described in section 2.3.6.

2.6.2 Does blood ingestion pre fungus infection affect *Anopheles funestus* susceptibility to fungus infection?

Female Fumoz-R samples (3-5 days old) were offered a blood-meal for 30 minutes as described in experiment 2.6.1., and were then allowed to rest for 1 hour (without access to sugar) before being exposed for 24 hours to fungus treated hair rollers. Control samples were concurrently offered blood-meals and were subsequently exposed to hair rollers without conidia in the same way. Immediately after fungus exposure, cohorts were provided with a 10% sugar solution inside the cages. The experiment was repeated 5 times with 30-40 mosquitoes per replicate and analysed as described in section 2.3.6.

2.6.2.1 Data analysis

The numbers of dead mosquitoes per treatment replicate were recorded daily and the mean cumulative proportion surviving each day of the 3 replicates was calculated. Standard errors were also produced across all replicates. Assuming there were no variations amongst and between treatments (using one way ANOVA), the mean cumulative proportion surviving each day and the standard errors were calculated across all the replicates (n = 15). Graphs of the percentage cumulative mosquito survival against time after exposure were constructed using Microsoft™ Excel. Survival data was analysed using Kaplan-Meier survival analysis with differences between treatments (blood fed plus fungus infected vs. fungus infected only) determined using Kruskal-Wallis ANOVA. The LT_{50}-values of all treatments were calculated using Kaplan-Meier survival analysis and compared using Student's *t*-tests. The means and LT_{50}-values between experiments 2.6.1 and 2.6.2 were compared using Students *t*-tests respectively. The mean proportion of cadavers that sporulated was calculated and compared using Chi-square tests (in *STATISTIX 7*.0).

2.6.3 Investigate a possible correlation between fecundity (egg production, egg hatch and larval survival) and *Beauveria bassiana* or *Metarhizium anisopliae* infection

A total of 120 Fumoz-R females (4-5 days old) were exposed for 24 hours to either *B. bassiana* or *M. anisopliae*. Infected mosquitoes were then transferred into standard cages and provided with 10% sucrose. Prior to blood feeding, mosquitoes

were deprived of the sucrose solution for 3 hours to increase their appetite for blood. Anaesthetised guinea pigs were placed on top of each cage allowing females to feed for 30-45 minutes in the dark. Control groups (30 females Fumoz-R, 4-5 days old) were not exposed to fungal spores but were blood-fed in the same way as the infected mosquitoes. The experiment was repeated three times. Two days after the first blood feed, live individual females were put into separate glass vials (4.5 cm height) with access to a 10% sucrose solution between blood meals. A filter paper disc was used to line the insides of the glass vials which were moistened with distilled water to encourage oviposition. Blood-feeding was repeated on days 4 and 8 following the first feed. The number of eggs laid per female was counted daily and eggs were transferred into small bowls containing distilled water. The hatch rate and life stage progression was monitored and recorded for the progeny of each female.

2.6.3.1 Data analysis

The effect of fungal infection on egg production was assessed by comparing egg production between fungus infected and control groups. Hatch rate and life stage progression was analysed within and between treatments. All analyses were performed using Chi-square tests (*STATISTIX 7.0*).

CHAPTER 3

THE EFFECT OF DURATION OF EXPOSURE OF *ANOPHELES FUNESTUS* FEMALES TO THE ENTOMOPATHOGENIC FUNGI *BEAUVERIA BASSIANA* AND *METARHIZIUM ANISOPLIAE*

3.1 Introduction

Conidia of *M. anisopliae* and *B. bassiana* are easily cultured and can be produced under laboratory conditions (Roberts and Hajek, 1992; Goettel and Inglis, 1997). Currently, there are no reports of fungal infections in adult *Anopheles funestus* using the dry conidia of *M. anisopliae* or *B. bassiana*. Laboratory tests against adult *An. funestus* using oil formulated *M. anisopliae* in clay pots produced 91% mortality within 4 days compared to less than 50% mortality in control groups (Farenhorst *et al.,* 2008). Laboratory experiments against adult mosquitoes have previously shown them to be susceptible to unformulated conidia of *B. bassiana* and *M. anisopliae.* For example, adults of *Culex tarsalis, Cx. pipiens, An. albimanus*, *Aedes aegypti, Ae. sirrensis* and *Ae. nigromaculis* showed 100% mortality within 5 days post-exposure to *B. bassiana* compared to less than 50% mortality in the unexposed control groups (Clark *et al.,* 1968). Scholte *et al.* (2003b) also showed high susceptibility of adult *An. gambiae* and *Cx. quinquefasciatus* to dry conidia of *M. anisopliae* with 100% mortality within 5

days post-exposure. More recently, *B. bassiana* yielded 87% mortality in adults of *An. gambiae* within 8 days post-exposure compared to 10% in the unexposed control groups (Achonduh and Tondje, 2008).

The concentration of conidia in the host is likely to vary with exposure time. It is expected that the longer the host is in contact with conidia the faster or the higher the mortality by mycosis. However, there are conflicting results on the relationship between exposure time and mortality. Early results showed a positive correlation between number of infective conidia and mortality (Ferron, 1978; Clark *et al.,* 1968). On the other hand, Scholte *et al.* (2003b) found no significant variation in terms of mortality between *An. gambiae* exposed for either 24 hours or 48 hours to *M. anisopliae*. It is therefore suggested that prolonged exposure to *M. anisopliae* leads to a decrease in survival of *An. gambiae* and *Cx. quinquefasciatus* adults.

The objective of this chapter was to measure the virulence of dry *B. bassiana* and *M. anisopliae* conidia against female *An. funestus* adults at different periods of exposure (3 hours, 6 hours and 24 hours).

3.2 Materials and Methods

See section 2.4 of Chapter 2.

3.2.1 Mosquitoes

Adult *An. funestus* females (2-5 days old) from the Fumoz, Fumoz-R and Fang colonies were used for all experiments.

3.2.2 Entomopathogenic fungal isolates

Dry conidia (100mg/hair roller) of *M. anisopliae* var *anisopliae* (Metschnikoff) Sorokin isolate ICIPE 30 and *B. bassiana* (Balsamo) Vuillemin isolate I93-825 used for bioassays were kindly provided by Prof. M. Thomas (CSIRO Entomology, Australia).

3.2.3 Standard bioassay procedure

Fugal virulence towards mosquitoes was determined using the protocol described by Scholte *et al.* (2003b). Mosquitoes were exposed for 3, 6 or 24 hours to hair rollers dusted with dry conidia of *M. anisopliae* or *B. bassiana*. Control groups exposed to untreated hair rollers were assayed concurrently. Each experiment included 3 replicates of 30-40 females per replicate, and was repeated 3 times. Mortality was monitored daily for 28 days following fungal exposure. All cadavers were checked for fungal growth as described in section 2.3.5.

3.2.4 Data analysis

See section 2.4.1 in Chapter 2.

3.3 Results

3.3.1 Virulence of *Beauveria bassiana* or *Metarhizium anisopliae* against *Anopheles funestus*

Analysis of survival curves by Kaplan-Meier Kruskal-Wallis ANOVA showed significant differences in the mortality rates between fungal infected and uninfected *An. funestus* ($p < 0.001$) (Figs. 3.1-3.3) females with the exception of Fumoz exposed for 3 hours to *M. anisopliae* ($F=1.76$, $p= 0.18$) (Fig. 3.1). Fang was generally more susceptible to entomopathogenic fungi than Fumoz and Fumoz-R ($p < 0.05$).

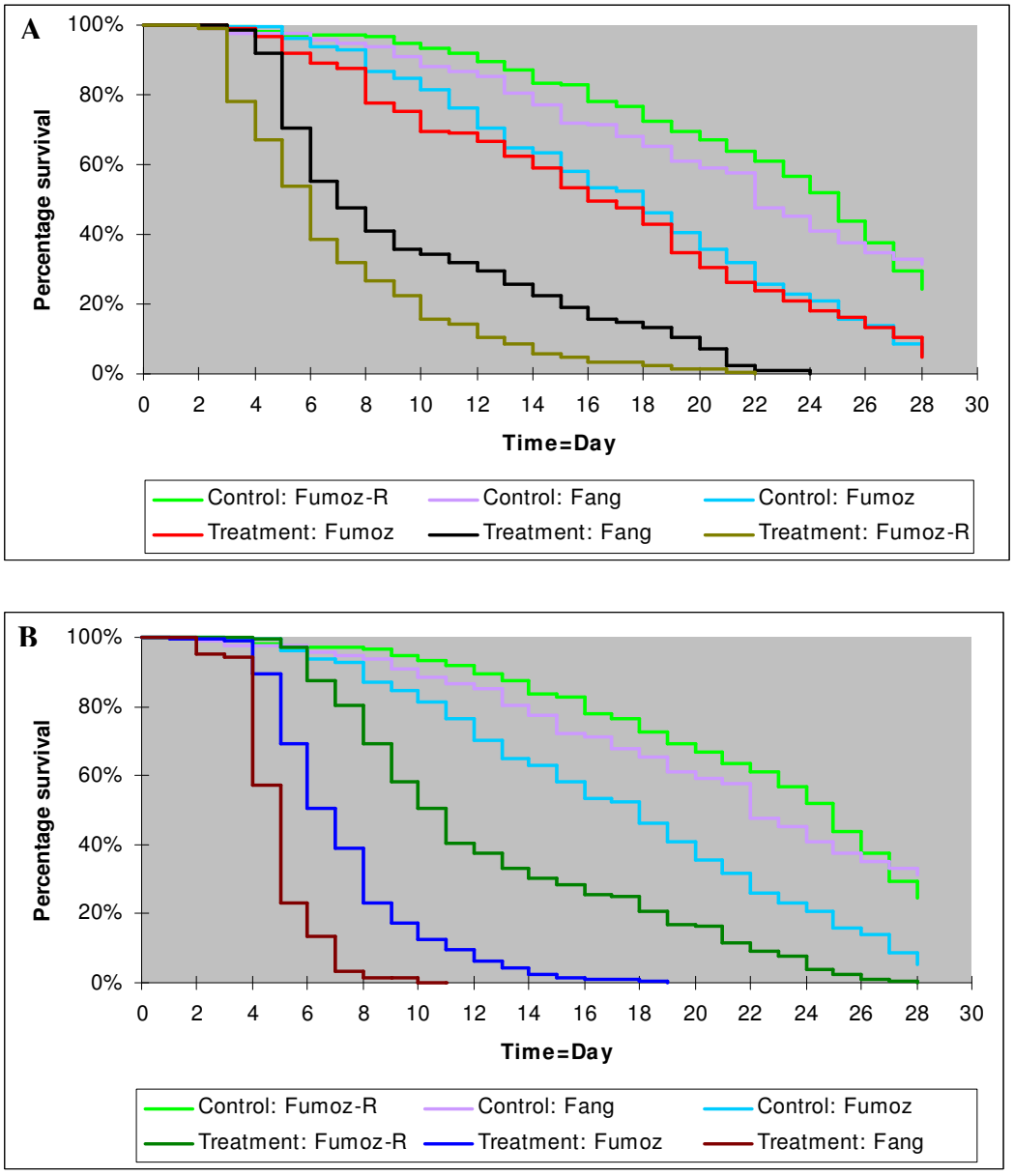
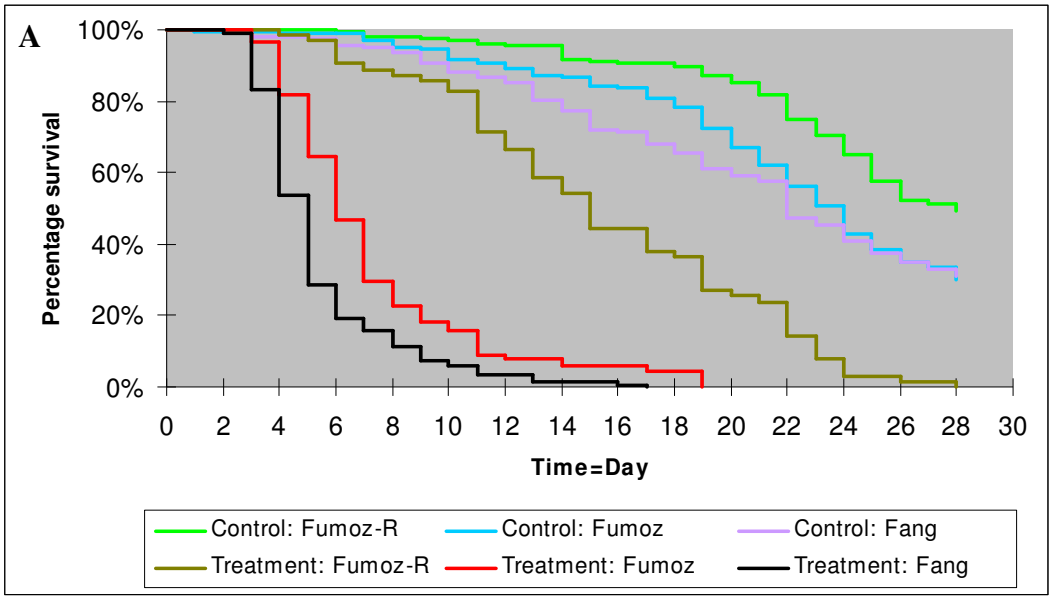


Figure 3.1: Survival curves of fungus infected (treatment) and uninfected (control) *An. funestus* (Fang, Fumoz and Fumoz-R) females following exposure to (A) *M. anisopliae* and (B) *B. bassiana* for 3 hours.



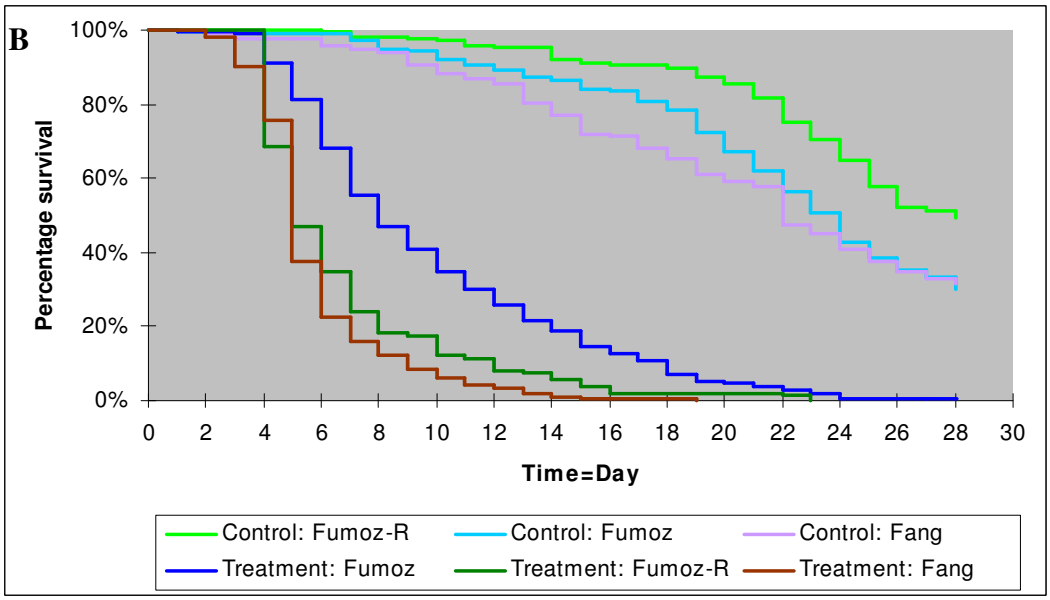


Figure 3.2: Survival curves of fungus infected (treatment) and uninfected (control) *An. funestus* (Fang, Fumoz and Fumoz-R) females following exposure to (A) *M. anisopliae* and (B) *B. bassiana* for 6 hours.

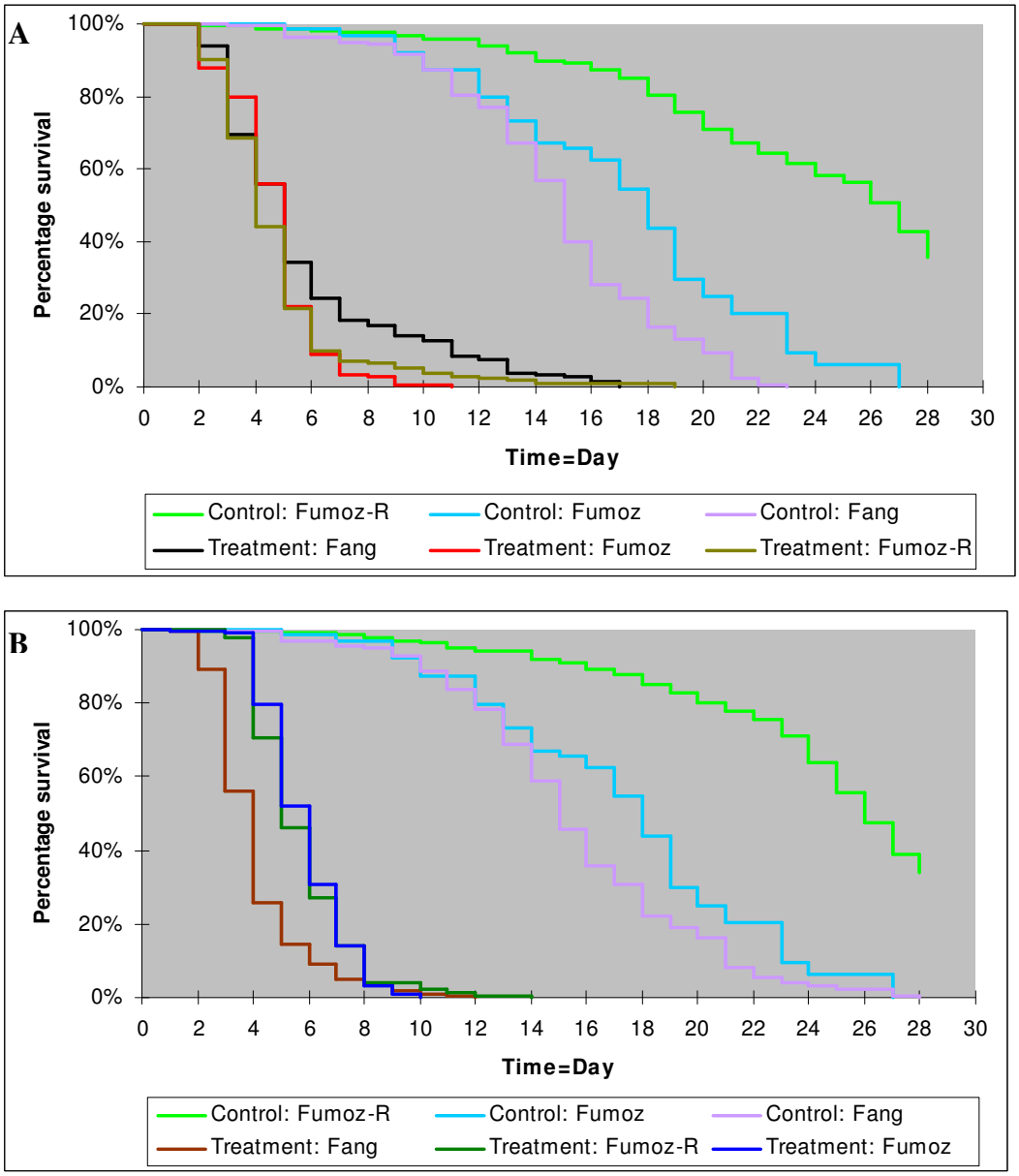


Figure 3.3: Survival curves of fungus infected (treatment) and uninfected (control) *An. funestus* (Fang, Fumoz and Fumoz-R) females following exposure to (A) *M. anisopliae* and (B) *B. bassiana* for 24 hours.

Table 3.1 Percentage mortalities of treated *An. funestus* females at 14 days post-exposure to fungus following different exposure periods.

	Treatment					
	3 hours		6 hours		24 hours	
	Bb	Ma	Bb	Ma	Bb	Ma
Fang Exposed	100	75%	98%	98%	100%	98%
Fang Unexposed	27%		35%		15%	
Fumoz Exposed	98%	47%	92%	80%	100%	100%
Fumoz Unexposed	46%		21%		17%	
Fumoz-R Exposed	70%	45%	95%	90%	100%	99%
Fumoz-R Unexposed	10%		18%		6%	

Mortality rates were approximately 3 to 6-fold higher in the infected samples compared to their corresponding uninfected samples by 14 days post-exposure with the exception of Fumoz 3 hours post exposure to *M. anisopliae* which showed no variation between treatment and control (Table 3.1). Table 3.2 shows the LT_{50} of exposed and non exposed *An. funestus* females 28 days after exposure.

The LT_{50} of Fumoz and Fumoz-R when exposed for 3 hours to *M. anisopliae* was more than 14 days compared to less than 8 days using Fang. Although all the mosquito strains died within 12 days post-exposure to *B. bassiana* (3 hours exposure), their LT_{50} values varied significantly according to the scheme Fang < Fumoz < Fumoz-R (Chi-square: $\chi^2 = 9.50$; $p < 0.05$, df = 2; F= 37.57). The LT_{50} of infected mosquitoes also varied significantly between strains at 6 hours exposure according to the scheme Fang < Fumoz-R < Fumoz for both fungus species (Chi-

square: $\chi^2 = 23.34$; $p < 0.05$; df = 2 for *B. bassiana* and $\chi^2 = 56.80$; $p < 0.05$; F = 182.62; df = 2 for *M. anisopliae*).

Table 3.2: LT_{50} ± SE in days of *An. funestus* unexposed (control) or exposed to dry conidia of *B. bassiana* or *M. anisopliae* at various exposure times.

Anopheles funestus				
Exposure time	**Treatment**	**Fang**	**Fumoz**	Fumoz-R
3 hours	Control	23.7 ± 2.1	18.0 ± 0.6	23.1 ± 1.8
	B. bassiana	4.7 ± 0.2	7.3 ± 0.5	11.6 ± 1.9
	M. anisopliae	7.4 ± 0.6	16.6 ± 1.7	15.0 ± 2.0
6 hours	Control	22.6 ± 1.0	24.1 ± 0.4	24.6 ± 0.3
	B. bassiana	5.1 ± 0.2	8.3 ± 0.4	5.6 ± 0.7
	M. anisopliae	5.0 ± 0.3	6.3 ± 0.3	6.7 ± 0.4
24 hours	Control	15.1 ± 0.2	16.7 ± 0.1	27.2 ± 3.1
	B. bassiana	3.7 ± 0.3	5.4 ± 0.5	5.3 ± 0.1
	M. anisopliae	5.1 ± 0.5	4.5 ± 0.2	4.3 ± 0.3

However, these differences were not reflected in the samples exposed for 24 hours (Chi-square: $\chi^2 = 0.45$; $p = 0.79$; F = 5.37; df = 2 for *B. bassiana* and $\chi^2 = 4.43$; $p = 0.11$; F = 1.37; df = 2 for *M. anisopliae*). The LT_{50} was significantly different between fungal treated and untreated control *An. funestus* ($p < 0.05$) (Table 3.2).

Mean percentages of cadavers from the treatment samples showing fungal growth following culturing were calculated (Fig. 3.4; Table 3.3). Based on these calculations, the pathogenicity of *B. bassiana* (81.4% ± 6.0) appeared higher than that of *M. anisopliae* (72.6% ± 5.7) but the difference was not significant across all infected *An. funestus* strains ($p = 0.32$).

Table 3.3: Fungus infectivity (% mean ± SE) of *An. funestus* exposed to *B. bassiana* *M. anisopliae* at different time periods.

		Anopheles funestus		
Exposure	**Treatment**	**Fang**	**Fumoz**	**Fumoz-R**
3 hours	Control	-	-	-
	B. bassiana	93.1 ± 0.7	49.8 ± 4.9	61.6 ± 5.7
	M. anisopliae	63.3 ± 3.0	47.9± 4.8	62.7 ± 7.3
6 hours	Control	-	-	-
	B. bassiana	93.1 ± 2.2	91.195 ± 2.4	59.2 ± 3.2
	M. anisopliae	90.2 ± 2.6	54.8 ± 8.6	59.2 ±12.4
24 hours	Control	-	-	-
	B. bassiana	91.6 ± 2.2	95.9 ± 2.2	97.0 ± 0.7
	M. anisopliae	83.2 ± 5.9	90.7 ± 2.4	71.9 ± 6.1

-=clean, no fungal growth on control groups

Mean percentages of cadavers from the treatment samples showing fungal growth following culturing were calculated (Fig. 3.4; Table 3.3). Based on these calculations, the pathogenicity of *B. bassiana* (81.4% ± 6.0) appeared higher than that of *M. anisopliae* (72.6% ± 5.7) but the difference was not significant across all infected *An. funestus* strains (p = 0.32). There was no significant difference in mean rate of infection between mosquito strains (ANOVA: p = 0.47; F=0.86; df = 2 for *B. bassiana* and for *M. anisopliae* p = 0.49; df = 2; F = 0.81).

A   **B**

Figure 3.4: *Anopheles funestus* cadavers showing sporulation following infection with (A) white *B. bassiana* and (B) green *M. anisopliae*.

3.3.2 Effect of exposure time period on entomopathogenic infection

Figures 3.1 to 3.3 above also show the mean differences in survival curves of fungus treated *An. funestus* at various time periods. The effect of exposure time on mosquito survival post exposure was determined using the parametric one-way ANOVA and the non parametric Kruskal-Wallis test. Both results showed that mosquito survive longer when exposure for shorter period ($p< 0.05$). Table 3.4 gives a direct comparison of LT_{50} between treatments and mosquito strains at same exposure time using Student *t* tests. Overall results show that the speed of killing of mosquito varied with the entomopatogenic fungus used ($p<0.05$).

Table 3.4: LT_{50} comparisons following exposure to fungus between mosquito strains at respective exposure time periods. P values given following analysis using Student *t* tests.

A-3 hour exposure periods

	Fang		Fumoz		Fumoz-R	
	Bb	***Ma***	***Bb***	***Ma***	***Bb***	***Ma***
Fang	x	x	0.000*	0.000*	0.000*	0.052
Fumoz	0.000*	0.000*	x	x	0.052	0.030*
Fumoz-R	0.000*	0.052	0.027*	0.030*	x	x

B-6 hour exposure periods

	Fang		Fumoz		Fumoz-R	
	Bb	***Ma***	***Bb***	***Ma***	***Bb***	***Ma***
Fang	x	x	0.000*	0.013*	0.511	0.001*
Fumoz	0.000*	0.013*	x	x	0.011*	0.004*
Fumoz-R	0.511	0.001*	0.011*	0.004*	x	x

C-24 hour exposure periods

	Fang		Fumoz		Fumoz-R	
	Bb	***Ma***	***Bb***	***Ma***	***Bb***	***Ma***
Fang	x	x	0.049*	0.458	0.000*	0.146
Fumoz	0.049*	0.458	x	x	0.833	0.262
Fumoz-R	0.000*	0.146	0.833	0.262	x	x

*** indicates a significant difference between strains at 95% confidence.**

The proportion of fungus exposed cadavers that sporulated following culturing was compared with the total number of cadavers counted per exposure time period. The results are given in Table 3.5. Only Fang cadavers exposed for 24 hours showed a significantly lower rate of sporulation as a proportion of total deaths.

Table 3.5: Comparison between infected cadavers and sporulated cadavers using Chi-square tests. df = degree of freedom, p value was calculated at 95% confidence interval.

		Anopheles funestus					
		Fang		**Fumoz**		Fumoz-R	
Time periods		***B.b***	***M.a***	***B.b***	***M.a***	***B.b***	***M.a***
3hours	χ^2	0.60	1.41	14.16	0.77	4.09	15.10
	df	11	11	11	11	11	11
	p	1.000	0.905	0.224	0.681	0.537	0.051
6hours	χ^2	0.8	0.44	8.37	13.14	2.85	2.90
	df	11	11	11	11	11	11
	p	0.999	0.994	0.398	0.022	0.240	0.968
24hours	χ^2	27.10	27.58	0.29	0.32	0.39	14.52
	df	11	11	11	11	11	11
	p	0.002	0.006	0.991	0.997	1.000	0.206

3.4 Discussion

Dry conidia of *M. anisopliae* and *B. bassiana* were highly virulent to adults of *An. funestus* under laboratory conditions. These results support the findings by Clark *et al.* (1968), Scholte *et al.* (2003b) and Achunduh and Tondje (2008). Their modes of exposure of mosquito cohorts to entomopathogenic fungi varied in some cases from the method used here. In this study, all experiments were carried out inside plastic cages containing hair rollers dusted with 100 mg dry conidia, an approach also used by Scholte *et al.* (2003b) to infect *An. gambiae* and *Cx. quinquefasciatu*s. Clark *et al*. (1968) exposed adults of *Cx. tarsalis, Cx. pipiens, An. albimanus*, *Ae. aegypti, Ae. sirrensis* and *Ae. nigromaculis,* in plastic freezer jars with 2 mg of conidia deposited at the bottom of the test container. Achunduh and Tondje (2008) followed the method of Scholte *et al.* (2003b) but with continuous exposure for a maximum of 10 days. They found an average mortality of 80% in the treated groups compared to 10% in the untreated groups. Farenhorst *et al.* (2008) used African clay pots treated with oil formulated spores for the delivery of fungus. Although various techniques were used to infect mosquito hosts, exposed cohorts showed a significantly higher mortality rate than their unexposed counterparts. The mode of infection is likely to be critical (Fernandez *et al.,* 2001) in experiments of this nature. Dry spores are probably more infective than oil-formulated spores, but a potential limitation of the infection technique used here is the reliance on the attractiveness of the sucrose solution in order to produce infections.

Nevertheless, the data given here reveal that *B. bassiana* and *M. anisopliae* are highly pathogenic to *An. funestus* adult females, with 99% mortality achieved within 14 days post infection. Fourteen days represent approximately the time required for *Plasmodium* parasites to produce sporozoites in the salivary glands of female *Anopheles* making them potentially infective to humans. The high mortality rate recorded in fungus infected samples relative to their respective controls is attributed to cuticle damage, fungal invasion of internal organs and tissues as well as fungal toxin release (Clarkson and Charnley, 1996). Entomopathogenic fungi invade their hosts by first penetrating the cuticle. Cuticle thickness is likely to have some effect on the rate of penetration of fungal conidia and therefore represents the first barrier of defence for the host (St. Leger *et al.,* 1991; Clarkson and Charnley 1996). Cuticle thickness varies within *An. funestus* strains and a direct correlation between cuticle thickness and susceptibility to insecticide intoxication has been demonstrated in Fumoz (Wood et al., 2010). Further, it has been shown that sclerotized cuticles vary considerably in stiffness and hardness between insects with low and high resistance to enzymatic or chemical degradation (Hopkins and Kramer, 1992). Variation in cuticle thickness, composition and stiffness could partly explain variation in response to fungal infection between the *An. funestus* strains used in these experiments.

Saprophytic growth on the cadavers drawn from fungus exposed samples was used as confirmation of fungal infection (Clarkson and Charnley, 1996; Roy *et al.,* 2006). The results in Table 3.2 suggest similarly high sporulation rates using *B.*

bassiana (50-97%) and *M. anisopliae* (47-90%). This finding is supported by Walstad *et al.* (1970) who showed that *M. anisopliae* and *B. bassiana* share the same saprophytic requirements whereby maximum growth is achieved in an environment where relative humidity is above 92.5% and within a temperature range of 22-26 ºC. Sporulation is partly regulated by temperature but principally by relative humidity. Soza-Gómez and Alves (2000) demonstrated that relative humidity between 75-100% favours optimal sporulation. Although relative humidity exceeded 75% in all experiments sporulation was not noticed in some cadavers. Random dissections revealed the presence of spores in the haemocoel of non-sporulated individuals suggesting that they died from fungal infection (data not shown). Rizzo (1977) found that *B. bassiana* tends to be more infective and develops faster inside the host than *M. anisopliae*. All cadavers drawn from control samples were conidia free.

The patterns of mortality differed between treatment cohorts by exposure time. Exposure time is likely to be a limiting factor for successful fungus infection under appropriate temperature and relative humidity. The time required to reach 100% mortality decreases with increasing exposure time (Ferron, 1978). The LT_{50}-values for the 6 hours and 24 hours exposure periods were significantly lower than those at 3 hours, suggesting that mosquitoes acquired more spores during 6 hours or 24 hours exposure. Generally, there is a decrease in mosquito survival when exposed continuously to dry conidia of entomopathogenic fungus (Clark *et al.,* 1968; Achunduh and Tondje, 2008). However, exposure time does not always correlate

with subsequent increased mortality rate (St. Leger *et al.*1992). Nevertheless, the mode of exposure of hosts to entomopathogenic fungus might significantly impact on their mortality (Fernandez *et al.,* 2001).

An increased period of exposure is not likely to have negatively affected dry conidia longevity because spore viability over time depends primarily on temperature and humidity (Morley-Davies *et al.,* 1995; Daoust and Robert, 1983; Hedgecock *et al.,* 1995; Hong *et al.,* 1997), the moisture content of conidia (Hedgecock *et al.,* 1995, Hong *et al.,* 1997) and may be fungus species specific (Daoust and Roberts, 1983; Hong *et al.,* 1997). Room conditions were stable (> 75% relative humidity and 25-26 ºC temperature) during the bioassays. In addition, treated hair rollers were not used more than twice after conidial treatment. *Metarhizium anisopliae* gradually loses viability over 3 months at normal room conditions (80-90% relative humidity and 25-26 ºC temperature) with survivorship remaining lower than in the control groups (Scholte *et al.,* 2004).

Variation in mortality rates between *An. funestus* strains may be a function of physiological variation between them and may also associate with their insecticide resistant/susceptible phenotypes. A possible interaction between resistance to insecticides and susceptibility to fungal infection is suggested by these data and is discussed more fully in chapter 4 as well as in Farenhorst *et al*. (2009).

3.5 Conclusion

Beauveria bassiana and *M. anisopliae,* when applied as unformulated conidia, are appropriate agents for inducing a high rate of mortality in adult insecticide resistant and susceptible *An. funestus*. Mortality rates are associated with length of exposure to conidia because increasing length of exposure increases the potential for acquiring a lethal fungal infection. It is therefore suggested that these fungi may be appropriate biological control agents for assisting with the management of insecticide resistance where it occurs in malaria vector populations.

CHAPTER 4

AN ASSOCIATION BETWEEN SUSCEPTIBILITIES TO PIPERONYL BUTOXIDE OR PERMETHRIN INTOXICATION AND ENTOMOPATHOGENIC FUNGUS INFECTION IN *ANOPHELES FUNESTUS*

4.1 Introduction

Pyrethroid resistance is a growing problem facing vector control management. Introduced in the late 1970s for insect control, pyrethroids are a large class of insecticides widely used for malaria control (Elliot *et al.,* 1973; WHO, 1989). Pyrethroid resistance has been identified in several *An. funestus* populations in Mozambique and South Africa (Hargreaves *et al.,* 2000; Brooke *et al.,* 2001; Casimiro *et al*., 2006).

Entomopathogenic Deuteromycetes *B. bassiana* and *M. anisopliae* isolates are potentially important biological insecticides of insect species including mosquitoes (Zimmerman, 1993; Scholte *et al.,* 2005; Blanford *et al.,* 2005). Although conflicting results are found in the literature, combinations of insecticide and entomopathogenic fungi can enhance insect mortality and mycosis compared to fungus alone (Richter and Fuxa, 1984; Anderson *et al.,* 1989; Hassan and

Charnley, 1989; Quintela and McCoy, 1997; Delgado *et al.,* 1999; Pachamuthu *et al.,* 1999; Pachamuthu and Kamble, 2000; Furlong and Groden, 2001). However, this effect has not been tested on mosquitoes including malaria vectors. The combination of chemical and biological insecticides will be particularly useful not only to control resistant mosquitoes but will also reduce the required concentration of each during insecticide spraying operations (Ferron, 1971; Richter and Fuxa, 1984; Quintela and McCoy, 1997) thereby reducing their selective pressure on mosquito populations and delaying the development of resistance. Using caterpillar larvae as a model, Serebrov *et al.* (2006) showed an increase of total esterase and glutathionine S-transferase activities in the hemolymph of insect as a response to fungal infection. Therefore, they suggested a mixture of entomopathogenic fungus and inhibitors of detoxification enzymes for the management of fungus resistant insects, leading to increasing fungal related death in treated hosts.

A possible interaction between exposure to a monooxygenase inhibitor, piperonyl butoxide (PBO), and susceptibility to fungus infection was investigated, based on the hypothesis that monooxygenase inhibition would weaken defence against fungal toxins in fungus infected *An. funestus.* PBO has previously been shown to nullify the expression of pyrethroid resistance in southern African *An. funestus* (Brooke *et al.*, 2001) by attenuating the detoxifying capabilities of monooxygenases.

4.2 Materials and Methods

4.2.1 Mosquitoes: The *Anopheles funestus* Fumoz-R laboratory colony (resistant to pyrethroids) was used for the bioassays. They were reared at 26 ± 2 ºC, 80 ± 2% relative humidity and 12:12 (L:D) cycle with a 45 min dusk/dawn regime (refer to section 2.1 in chapter 2). Only 2-5 day old female adults were used.

4.2.2 Entomopathogenic fungi: unformulated conidia of *B. bassiana* (Balsamo) Vuillemin isolate I93-825 and *M. anisopliae* (Metschnikoff) Sorokin isolate ICIPE 30 were used. The viability of these fungi was validated by germination on Sabouraud-Dextrose Agar (SDA) medium. After incubation at 27 ± 2 ºC for 20 to 24 hours, only batches with 90% or higher viability were used for bioassays (for details refer to section 2.3.2).

4.2.3 Permethrin and piperonyl butoxide: 0.75% permethrin impregnated test papers supplied by WHO were used. PBO treated papers at concentrations of either 4 or 8% (w/v) were prepared in olive oil. Whatman # 1 filter papers (12.5 x 15 cm) were evenly impregnated with a mixture of 0.7 ml PBO solution and 1.2 ml acetone, using a micropipette. The treated papers were left to dry at room temperature for 24 hours.

4.2.4 Bioassays

4.2.4.1 Permethrin- resistant assays

To investigate whether fungal infection affects the expression of insecticide resistance, a series of experiments to examine pre-lethal effects of fungal infection on insecticide sensitivity in resistant mosquitoes was conducted.This section is detailed in APPENDIX 4 page 4. Data were analysed as described in APPENDIX 4 page 5.

4.2.4.2 Piperonyl butoxide- fungus assays

Experiments were performed to allow for comparisons between Fumoz-R samples exposed to permethrin-fungus, PBO-fungus or fungus alone. A total of 350-370 individual mosquitoes were exposed per treatment. Each experiment was repeated 4 times with at least 30 mosquitoes per replicate (n = 3). Details of the experimental designs are given in chapter 2 (section 2.5).

4.2.4.3 Data analysis: In addition to that given in section 2.6, the effect of the synergist PBO was also evaluated by calculating the Synergist Ratio (SR) which is obtained by dividing the mean LT_{50} for assays without synergist by the mean LT_{50} with synergist (Cochran, 1989). The cut-off was set at 1 so that if SR > 1 (low LT_{50} with synergist) then the effect of PBO is additive (i.e. increases mortality rate following exposure to fungus), if SR falls between 1 and 0.5 then the effect is synergistic but does not significantly increase mortality rate and if SR < 0.5 then the synergist exercises no effect on subsequent fungus induced mortality rate

(Cochran, 1989). The Synergist Ratio was statistically analysed using Student *t*-tests (in *STATISTIX 7*).

4.3 Results

4.3.1 Potential interaction between permethrin and entomopathogenic fungus

The effect of *B. bassiana* or *M. anisopliae* infection on the expression of Fumoz-R was assessed. Permethrin induced significantly higher mortality in mosquitoes pre-infected with *B. bassiana* ($p < 0.001$) or *M. anisopliae* ($p < 0.001$) than that in the uninfected groups (Fig. 4.1A). Both fungi induced similar increases in susceptibility to permethrin.

The effects of *B. bassiana* infection on permethrin resistance levels 5 days after fungal exposure were also examined. This revealed a significant ($\chi^2 = 18.38$, $p < 0.001$) increase in permethrin-induced mortality compared with that of the 3-day group (Fig. 4.1B). However, mortality of the uninfected control group was also significantly higher at 5 days ($\chi^2 = 57.22$, $p < 0.001$), which is consistent with age-dependent responses to insecticide exposure found in several mosquito species (Lines and Nassor, 1991; Matambo *et al.,* 2007) and suggests equivalent relative effects of fungus, at least over the initial time course of infection. (Also refer to pages 2-3 in APPENDIX 4).

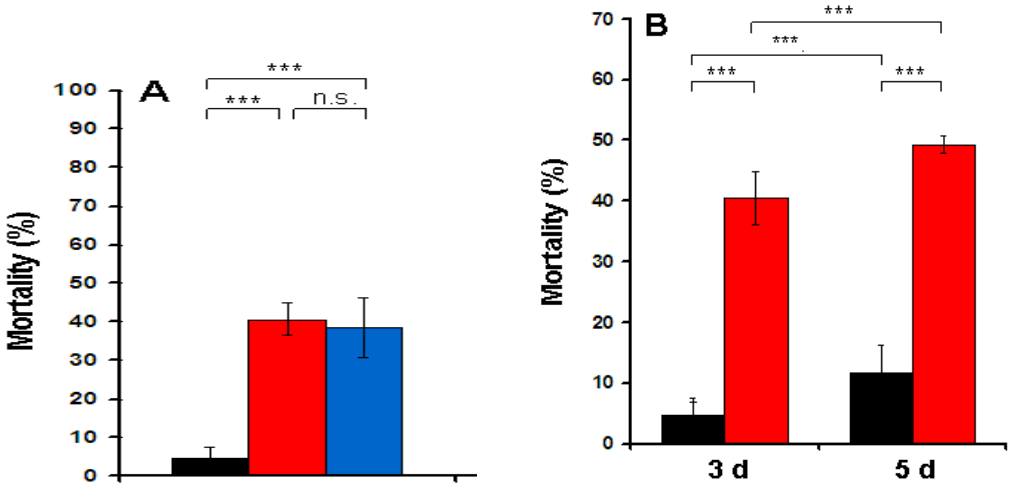


Figure 4.1: Effect of fungus infection on insecticide-resistance levels. (A) Corrected mean (± SEM) percentage mortality of uninfected (black), *Beauveria* - infected (red), and *Metarhizium* - infected (blue), *Anopheles funestus* after exposure to permethrin 3 days after fungal infection. The tested mosquitoes exhibit high baseline levels of permethrin resistance. (B) Effect of permethrin exposure on *Beauveria* – infected (red) and uninfected (black) permethrin – resistant *An. funestus* mosquitoes at 3 days (left) and 5 days (right) after fungal infection. Data represent corrected mean (± SEM) percentage mortality from five replicates, each containing 25 females. Asterisks indicate significant differences determined by χ^2 test. **, $p < 0.01$; ***, $p < 0.001$. (Ref. page 3 in APPENDIX 4).

4.3.2 Test for synergism between exposure to piperonyl butoxide and susceptibility to fungus infection

Figures 4.2 and 4.3 using 4% PBO and 8% PBO respectively show no significant difference in survival curves between exposure to PBO plus fungus vs. exposure to fungus only ($p > 0.05$).

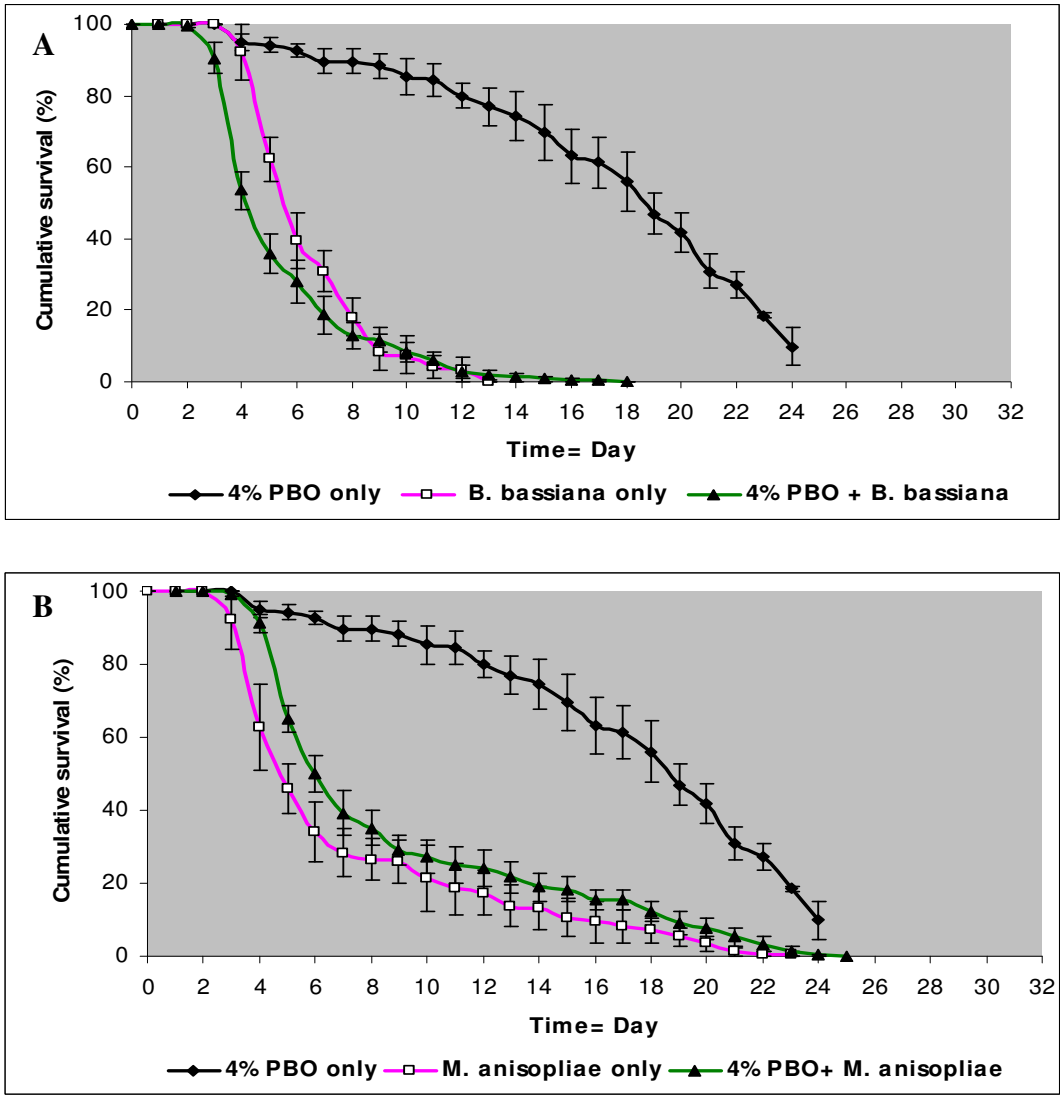


Figure 4.2: Mean survival (% ± SE) of *An. funestus* (Fumoz-R) exposed for 24 hours to either entomopathogenic fungus (A= *B. bassiana,* B= *M. anisopliae*), 4% PBO or to both. There is no significant difference between PBO plus *B. bassiana* and *B. bassiana* alone (p > 0.05).

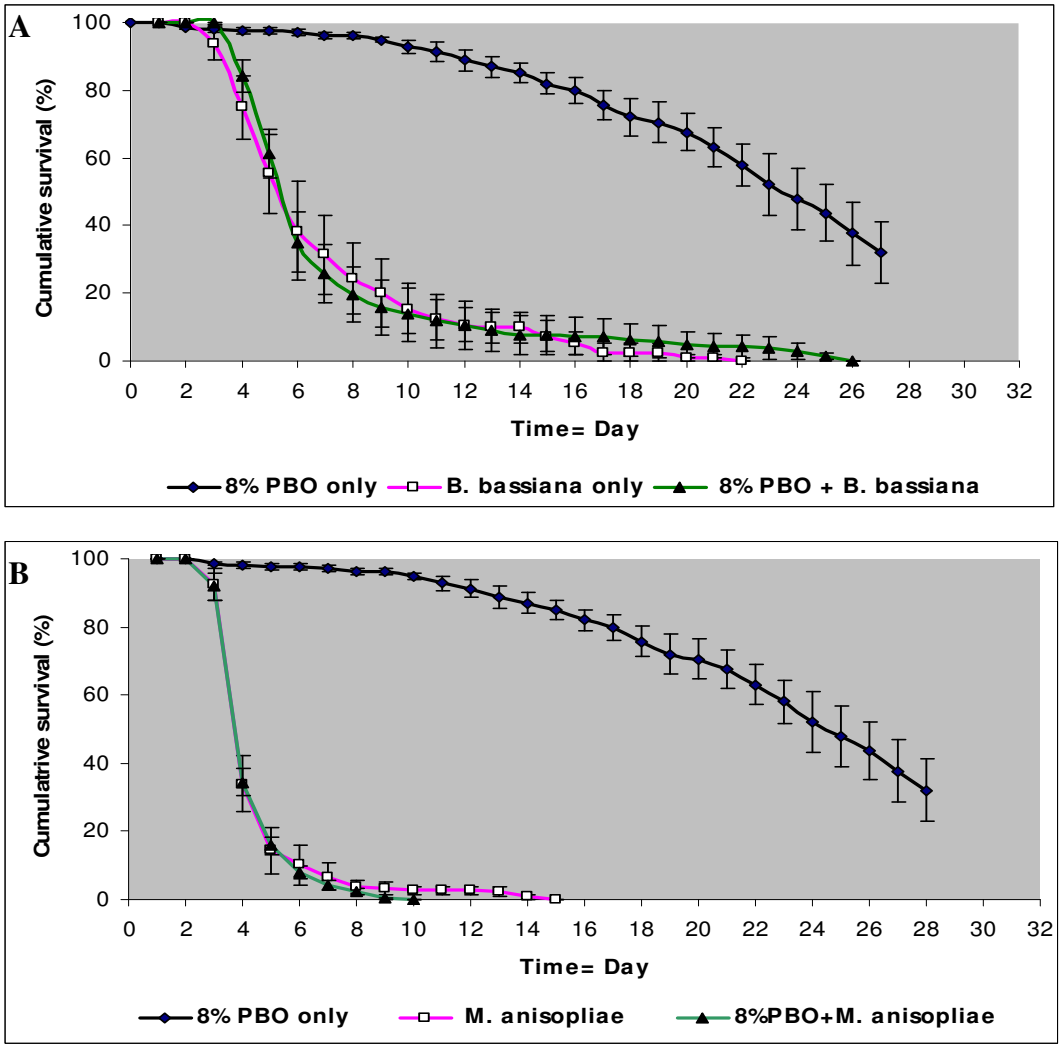


Figure 4.3: Survival curves of *An. funestus* (Fumoz-R) exposed for 24 hours to either entomopathogenic fungus (A= *B. bassiana,* B= *M. anisopliae*) or 8% PBO, or to both. PBO has no effect on the virulence of *M. anisopliae* (p > 0.05).

Table 4.1 shows the mean survival in days and LT_{50} values (number of days) and comparisons between PBO treated and untreated samples following exposure to either *B. bassiana* or *M. anisopliae.* Overall, there was no difference in mean survival of mosquitoes treated with the combination PBO- fungus and fungus alone (Student *t*-test p>0.05).

Doubling the concentration of PBO from 4 to 8% induced no significant difference in susceptibility to *B. bassiana* infection (p=0.84). However, the difference in susceptibility to *M. anisopliae* infection between exposure to 4% and 8% PBO was significant (p=0.001) (Table 4.1 and Figs. 4.2 and 4.3). The LT_{50} in the control groups was at least 3-fold higher as compared to the fungal treatment cohorts. However, mosquitoes in the control groups seemed to survive better at a high concentration of PBO (LT_{50} = 23.0 ± 0.5 and 19.0 ± 0.6 at 8% and 4% PBO respectively).

Synergist ratio (SR) defined as the degree by which the activity of fungus is enhanced by the addition of a synergist (i.e. PBO) was also calculated. It varied between 0.8 (*M. anisopliae*) and 1.3 (*B. bassiana*) (Table 4.1). However, the addition of PBO did not increase mortality in fungal treated mosquitoes (Student's *t*-tests: $p > 0.05$).

Table 4.1: Mean LT_{50} and SR values of *An. funestus* survival after treatment with PBO and entomopathogenic fungus.

	4% PBO +		No PBO		8% PBO+		No PBO	
	B.b	***M.a***	***B.b***	***M.a***	***B.b***	***M.a***	***B.b***	***M.a***
Mean ± SD	5.7 ± 0.6	8.0 ± 0.3	7.1± 0.3	7.2 ± 0.5	5.5 ± 0.1	4.2 ± 0.1	6.6 ± 0.2	3.6 ± 0.1
LT_{50} ± SE	5.0 ± 0.5	5.8 ± 0.4	5.3 ± 0.3	5.0 ± 0.6	4.9 ± 0.3	4.0 ± 0.3	6.3 ± 0.9	3.4 ± 0.2
B.b **SR** M.a	1.1 (p = 0.6) 0.9 (p = 0.3)				1.3 (p = 0.1) 0.9 (p = 0.1)			
	Control 4% PBO only				**Control 8% PBO only**			
Mean ± SD	≈ 17.7 ± 0.6				≈ 21.1 ± 0.3			
LT_{50} ± SE	19.0 ± 0.6				23.0 ± 0.5			

SR= synergist ratio= without PBO/with PBO

4.4 Discussion

In the experiments where mosquitoes were first infected with fungus, mortality was considerably higher when exposed to permethrin than in fungal treatment alone. Our data are supported by Pachamuthu and Kamble (2000) who found a significantly increased rate of mortality in German cockroaches treated with a combination of fungus and insecticides compared to either fungus infection or insecticide exposure alone. They concluded that there is an additive interaction between *M. anisopliae* infection and cyflurin (pyrethroid) intoxication. A synergistic interaction between neuro-active insecticides and entomopathogenic fungi is also apparent in other classes of insects (Quintela and McCoy, 1997, 1998; Furlong and Groden, 2001). The exact mechanism involved in the interaction is

unknown. Biochemical studies in *An. funestus* showed that pyrethroid resistance is metabolically mediated by high levels of monooxygenases (Brooke *et al.,* 2001; Hunt *et al.,* 2005; Casimiro *et al*., 2006).

Direct effects of the neurotoxic insecticides on entomopathogenic fungus and its proliferation inside the infected mosquito were not studied. The fungus was allowed to proliferate for 3 days in the insect before being exposed to permethrin. There were no differences in infection percentages between insecticide-exposed and unexposed groups, but that could be a result of already extensive fungus growth at day 3.

There was no significant increase in the mortality rate of female *An. funestus* when treated with a combination of entomopathogenic fungus and PBO compared to fungus infection alone. This suggests that PBO exposure does not increase the virulence of *B. bassiana* or *M. anisopliae* in *An. funestus.* Furthermore, the mortality rate remained unchanged when the PBO concentration was doubled. The number of days until 50 % of fungal infected mosquitoes are dead is not likely to be reduced by the addition of PBO. The development of mycosis was not affected by the addition of PBO. The fungus growth (2- 4 days post death) varied from 95-97% in fungus treatment alone to 93-98% in mosquitoes infected with fungus and PBO. However, the difference was not significant ($p < 0.05$). The fungus growth on the cadavers was lightly more pronounced with *B. bassiana* than *M. anisopliae.* A study found that *B. bassiana* tends to be more infective and develops faster

inside the host than *M. anisopliae,* but *M. anisopliae* is more stable than *B. bassiana* and therefore has time to cause higher mycosis than *B. bassiana* (Rizzo, 1977). PBO has no intrinsic insecticidal effect, but serves to enhance the toxicity of insecticides that are recognised as metabolic substrates by detoxifying enzymes, particularly monooxygenases. Albeit not in mosquito because this is the first of its kind, the results presented here contrast with those of Serebrov *et al.* (2006) who showed that a detoxification inhibitor (e.g. PBO) sharply increases the mortality rate resulting from fungal infection in the fifth instars larvae of the greater wax moth *Galleria mellonela*.

4.5 Conclusion

The interaction between fungus and permethrin was synergistic which can support the use of entomopathogenic fungus where malaria vectors are resistant to pyrethroids. Also, entomopathogenic fungus infection reducing the expression of permethrin resistance, developing "combination treatments" may enhance the efficacy and effective lifespan of key insecticides (like pyrethroid) where resistance has reached high levels. Overall, entomopathogenic fungi are potential candidates to complement current insecticide based malaria vector strategies. Further research could be designed to analyse the biochemical profiles of fungus infected mosquitoes in terms of detoxoification enzyme levels as well as the effect of exposure to other pyrethroids (such as deltamethrin) on susceptibility to fungal infection. Permethrin and deltamethrin belong to different sub-classes of

pyrethroids which mainly differ in their chemistry (permethrin and deltamethrin are non-cyano and cyano pyrethroids respectively) (Elliot *et al.,* 1973) although they share a similar mode of action on the nervous system of insects (Soderlund and Bloomquist, 1989).

Exposure to PBO has no effect on the virulence of *B. bassiana* or *M. anisopliae*. Further research using other synergist compounds such as triphenyl phosphate (TPP), an inhibitor of esterases (Hemingway and Ranson, 2000; Liu *et al.,* 2006; Mouatcho *et al.,* 2007), may prove valuable because enzymes including esterases are involved in the penetration of fungal conidia through the cuticle of insect hosts (St. Leger *et al.,* 1987, 1992, 1995 and 1996).

CHAPTER 5

EFFECT OF BLOOD FEEDING ON THE PATHOGENICITY OF ENTOMOPATHOGENIC FUNGI AND THE EFFECT OF FUNGUS INFECTION ON FERTILITY AND FECUNDITY IN *ANOPHELES FUNESTUS* FEMALES

5.1 Introduction

Several studies have provided insight into the interaction between blood feeding and susceptibility to entomopathogenic fungus infection in insects. They found that *M. anisopliae* infection reduces blood feeding and fecundity in the beetle *Anaplophora glabripennis* (Hajek *et al.,* 2008), the mosquito *An. gambiae* (Scholte *et al.,* 2006), the German cockroach *Blatella germanica* (Quesada-Moraga *et al.,* 2004) and the legume flower thrip *Megalurothrips sjostedti* (Ekesi and Maniania, 2000). Wang and Knudsen (1993) showed a reduction in fecundity when the Russian wheat aphid was infected with *B. bassiana.* A similar effect was recorded in the Colorado beetle *Leptinotarsa decemlineata* (Fargues *et al.*, 1991).

The effect of blood-feeding on susceptibility to fungus infection was evaluated in female *An. funestus*, based on the hypothesis that blood ingestion induces a suite of enzyme-based physiological responses that may inadvertently affect the progress of

a fungus infection. Further, the effect of a fungus infection on subsequent fecundity and the pre-adult life histories of the progeny of fungus infected females were evaluated.

5.2 Materials and Methods

5.2.1 Mosquito samples: Three to five day old female Fumoz-R (pyrethroid resistant *An. funestus*) were used. A solution of 10% sucrose was made available to them before, during and after treatment with dry conidia of entomopathogenic fungus.

5.2.2 Experiment 1: Effect of blood feeding on *Anopheles funestus* susceptibility to entomopathogenic fungus infection

Two experiments were conducted. Mosquitoes were either blood fed and then exposed to fungus or vice versa. The procedures are explained in chapter 2, section 2.6.1. and 2.6.2.

5.2.3 Experiment 2: Effect of fungal infection on female *Anopheles funestus* fecundity

A total of 120 mosquitoes (4-5 days old) were used per experimental set. The method is explained in chapter 2, section 2.6.3.

5.3 Results

5.3.1 Effect of a blood meal prior to fungus infection

The mortality rate of *An. funestus* females infected with *M. anisopliae* was not affected by a blood meal ingested prior to fungus infection (Fig. 5.1) ($p > 0.05$) as opposed to those infected with *B. bassiana* (Fig. 5.2) ($p < 0.05$) following a Kaplan-Meier Krustal Wallis analysis. The mortality rates of both fungus infected cohorts (blood fed and unfed) were significantly higher than the untreated control cohort ($p < 0.05$).

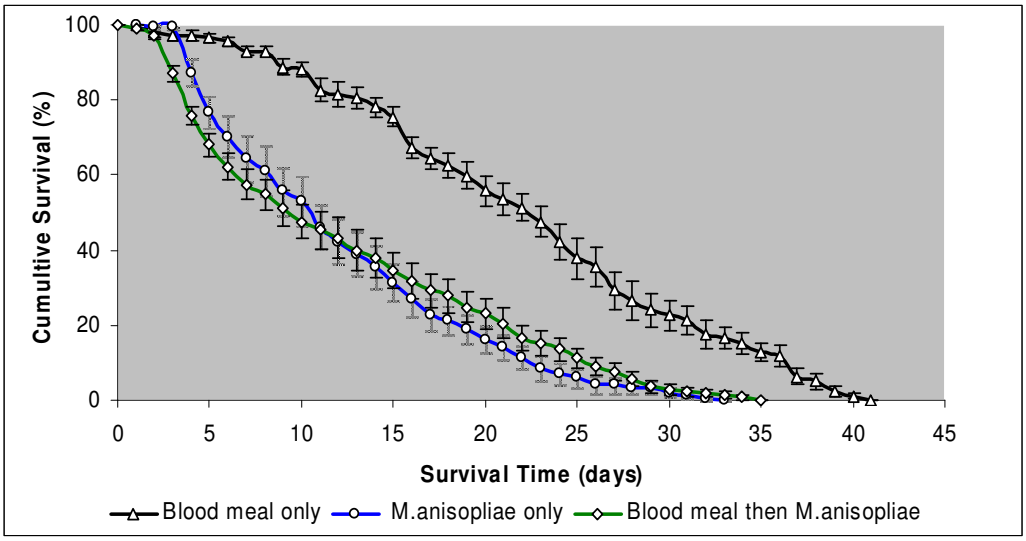


Figure 5.1: Mean cumulative survival distribution of blood fed and unfed *An. funestus* females exposed to *M. anisopliae* for 24 hours.

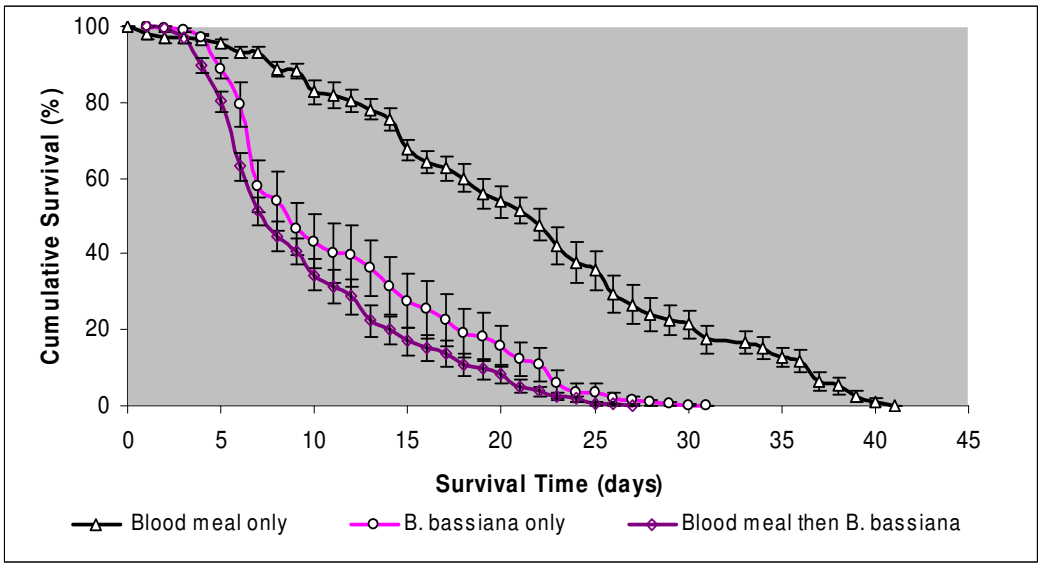


Figure 5.2: Mean cumulative survival distribution of blood fed and unfed *An. funestus* females exposed to *B. bassiana* for 24 hours.

Table 5.1 summarises the mean survival and LT_{50} values for each treatment. The time to kill 50% (LT_{50}) fungus infected mosquitoes was less following *B. bassiana* treatment than treatment with *M. anisopliae* (LT_{50} was 1.2-fold higher in mosquitoes infected only vs. blood fed plus fungus infection). Nevertheless, no difference in survival time was observed between blood fed and unfed *An. funestus* exposed for 24 hours to either entomopathogenic fungus following Krustal-Wallis test ($p > 0.05$). Survival time was significantly higher in the control groups (blood meal only) ($p < 0.05$).

Table 5.1: Mean (± SD) and LT_{50} (± SE) mortality of blood fed *An. funestus* exposed to entomopathogenic fungus for 24 hours.

	Blood meal prior to *M. anisopliae*	*M. anisopliae* only	Blood meal only	*B. bassiana* only	Blood meal prior to *B. bassiana*
Mean ± SD	12.3 ± 0.2	12.3 ± 0.3	21.6 ± 0.5	8.3 ± 0.3	7.4 ± 0.2
LT_{50} ± SE	9.0 ± 1.1	11.0.52	24.0.84	6.0.91	5.0 ± 0.7

SD= Standard Deviation SE= Standard Errors

5.3.2 Effect of post blood feeding on fungal infection

Figures 5.3 and 5.4 show the survival distribution of female *An. funestus* either exposed or unexposed to fungus followed by blood feeding. Using Kaplan-Meier Krustal-Wallis non parametric analysis, cohorts exposed to fungus followed by a blood meal tended to die slightly faster than their unfed counterparts ($p < 0.05$). The mortality rates of both fungus infected cohorts (blood fed and unfed) were significantly higher than the untreated control cohort ($p < 0.05$).

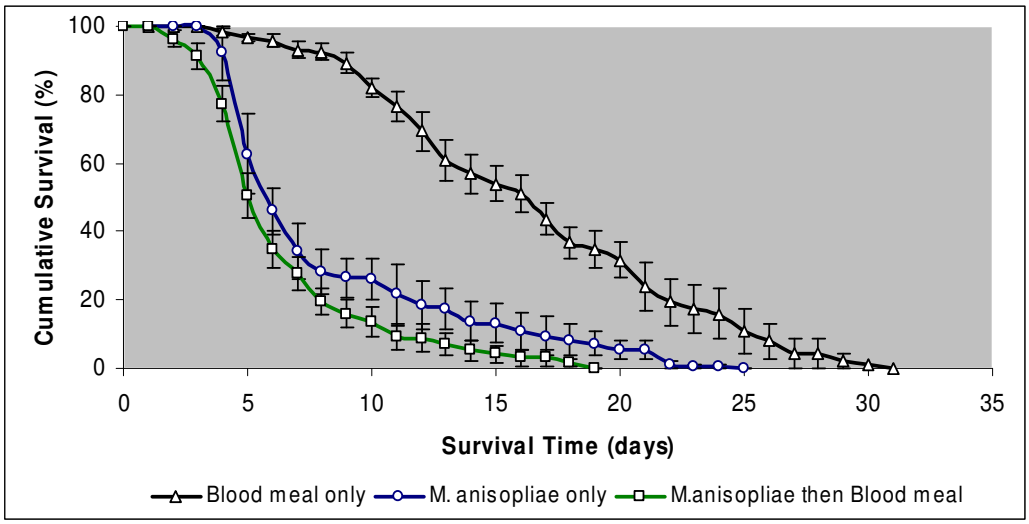


Figure 5.3: Mean cumulative survival distribution of female *An. funestus* either unfed or blood fed post-exposure to *M. anisopliae.*

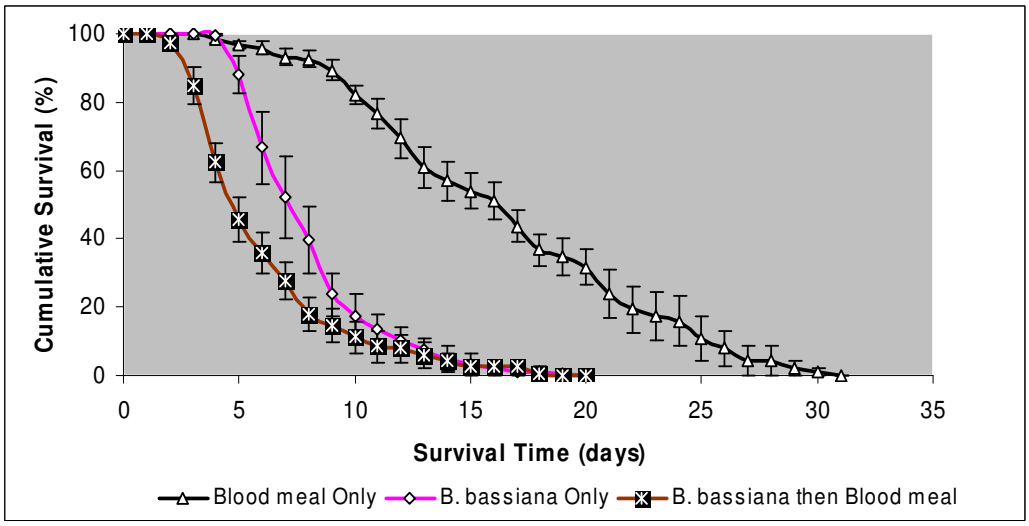


Figure 5.4: Mean cumulative survival distribution of female *An. funestus* either unfed or blood fed post-exposure to *B. bassiana.*

Table 5.2 summarizes the mean survival time and LT_{50} of mosquito's blood fed post fungus infection, blood fed only or exposed to fungus only. The difference between survival means and LT_{50}'s of the blood fed and unfed fungus infected cohorts was highly significant (Student's t-tests: $p < 0.05$). LT_{50} was 1.9 and 1.4

higher in mosquitoes infected with *M. anisopliae* and *B. bassiana* respectively compared to their blood fed counterparts. In addition, Kaplan-Meier analysis showed *B. bassiana* more virulent than did *M. anisopliae* ($p < 0.05$) based on survival time post fungus infection. Survival time was significantly higher in the control group (blood meal only) ($p < 0.05$).

Table 5.2: Mean (± SD) and LT_{50} (± SE) mortality of *An. funestus* infected with entomopathogenic fungus for 24 hours and then blood fed.

	Blood meal post *M. anisopliae*	***M. anisopliae* only**	**Blood meal only**	**Blood meal post *B. bassiana***	***B. bassiana* only**
Mean ± SD	6.5± 0.1	11.8 ± 0.3	14.9 ± 0.5	6.3 ±0.2	10.5 ± 0.3
LT_{50} ± SE	5.8 ± 0.2	11.0 ± 1.0	15.0.84	5.6 ± 0.4	7.8 ± 0.9

5.3.3 Effect of *Beauveria bassiana* and *Metarhizium anisopliae* infection on the fecundity of *Anopheles funestus* females

Table 5.3 summarizes the total number of eggs, larvae, pupae and adults produced by females under different treatments. Of the 120 females used for each set of experiments following exposure to either *B. bassiana* or *M. anisopliae*, 33.3% (n = 40) and 54.17% (n = 65) respectively produced eggs. In the unexposed group, 66.6% (n = 80) produced eggs. The mean (± SE) numbers of eggs produced were 121.8 ± 8.9 for the unexposed control group, 93 ± 9.7 following exposure to *B. bassiana* and 80.7 ± 7.7 following exposure to *M. anisopliae*. Mean egg production

was significantly lower in the fungus infected cohorts compared with the control group (ANOVA: p = 0.005, F = 6.62). There was no difference in mean egg production between *B. bassiana* and *M. anisopliae* exposed cohorts (Student's t-tests: p = 0.29). Figure 5.5 shows numbers of eggs varying from 52-100 (*M. anisopliae*), 60-110 (*B. bassiana*) and 86-185 (control).

Table 5.3: Counted number of eggs, larvae, pupae and adults produced by females exposed and unexposed to *Beauveria bassiana* or *Metarhizium anisopliae*

	Control (n=80)	***B. bassiana* (n=40)**	***M. anisopliae* (n=65)**
Eggs	1218	646	651
Larvae	859	279	352
Pupae	621	188	235
Adults	483	112	151

The time taken by fungus infected and uninfected females to lay eggs varied. The uninfected control group required 1-3 days after their third blood meal to produce a maximum number of eggs compared to 2-8 days required by the fungus treated groups.

The number of eggs that hatched, larvae that pupated and pupae that emerged as adults are given in Table 5.3 and figure 5.5. The number of eggs that hatched was significantly lower in the fungus treated groups compared to the untreated control

(ANOVA: p = 0.0004, F = 11.20). There was no difference in egg hatch between fungus treated groups (Student's t-tests: p = 0.15).

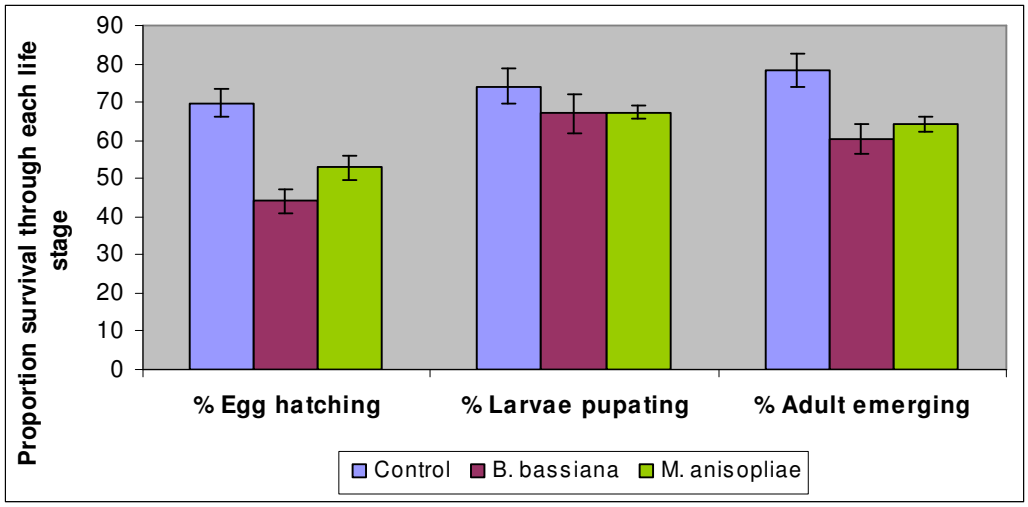


Figure 5.5: Frequency distribution of mean (±SE) egg hatch and mean proportions surviving through each life stage for cohorts of female *An. funestus* treated either with *B. bassiana* or *M. anisopliae.* The control cohort was untreated.

The average proportions of larvae pupating were 74% and 67% in the control and fungus treated groups respectively. The average proportions of adults emerging was slightly lower in the fungus treated groups (60-64%) compared to the untreated control (78%). There was no difference in the mean proportion of adults emerging between fungus treated groups (Student *t*-tests: p = 0.17).

The average percentages of adults produced based on the number of eggs produced were 17.8% for the *B. bassiana* treated group (χ^2=11.36, p=0.12, df =7), 23% for the *M. anisopliae* treated group (χ^2=4.32, p=0.60, df =6) and 39.6% for the

untreated control group (χ^2=5.77, p=0.76, df =9). The variation (in terms of standard deviation of the mean) within each treatment for the number of adults produced was 4.52, 6.39 and 10.34 for *M. anisopliae*, *B. bassiana* and control cohort groups respectively. Survivorship to the adult stage was significantly lower in the treated groups compared to the untreated control group (Student *t*-tests: $p < 0.05$) but did not vary between the *B. bassiana vs. M. anisopliae treated* groups (Student *t*-tests: $p = 0.12$).

5.4 Discussion

Blood feeding prior to fungal infection did not affect fungus induced mortality rates whilst blood-feeding post fungal infection induced a slight but significant increase in mortality rate. This difference is possibly attributable to the mode of fungus infection used in these experiments (Fernandez *et al.* 2001). Females acquired an infection during the process of resting on fungus-treated hair rollers (see methods section in chapter 2) in order to feed on the sucrose solution available from the saturated filter paper lining the inside of the hair roller. Blood fed anophelines have a tendency to ignore sugar or nectar (Beier, 1996), hence those that blood fed prior to fungus exposure may have acquired lesser fungus infections as shown by the lower saprophytic growth (20%). This scenario does not present any adverse implications in a field setting because the proposed sources and mode of fungus infection in houses (such as black cloths or clay pots) do not depend on sugar or nectar as attractants. Instead black cloths and clay pots present ideal resting sites for blood fed females, ensuring that a large proportion of this sector of

the mosquito population acquire a fungus infection. Significantly increasing the rate of mortality in blood fed females is likely to translate into to a decrease in malaria transmission (Fernandez *et al.,* 2001).

Exposure to entomopathogenic fungus can alter the cycle of reproduction in female mosquitoes rendering them less attracted to a blood meal source, thus producing unfertilised eggs or fewer eggs compared to their unexposed counterparts (Hajek and Leger, 1994; Ekesi and Maniania, 2000; Scholte *et al.,* 2006). *Anopheles* mosquitoes require multiple blood meals for egg production and egg maturation (Briegel and Hörler, 1995; Beier, 1996). The experiments described here showed a sharp decrease in egg production in fungus infected females, and a general reduction in fecundity in association with fungus infection. Although contradictory results are found in the literature, these data are supported by previous researchers who demonstrated significant impacts on fecundity and fertility in insects following exposure to entomopathogenic fungi (Fargues *et al.*, 1991; Wang and Knudsen, 1993; Quesada-Moraga *et al.,* 2004; Scholte *et al.,* 2006; Hajek *et al.,* 2008). A possible explanation for low fecundity in fungus infected females is related to the production of cuticle degrading enzymes and the suppression of processes that initiate each gonotrophic cycle (St. Leger *et al.,* 1996). Amiri *et al.* (1999) also suggested that hexadepsipeptides enzymes or dextruxins released by entomopathogenic fungus *M. anisopliae* inside their infected host mosquito could reduce the appetite of the infected host for a blood meal, with knock-on effects in terms of egg production. It is also suggested that fungus infected insects have

limited flying ability (Arthurs and Thomas, 2000) which will affect the number of eggs laid if appropriate oviposition sites prove beyond reach (Scholte *et al.,* 2006).

5.5 Conclusion

In general, there was a marked reduction in fecundity and fertility in association with a fungus infection in female *An. funestus* and a blood meal does not affect their susceptibility to fungus infection. The infection method used in this study may partly explain the difference in mortality rate observed in association with the timing of blood-feeding (i.e. pre or post fungus infection). A blood meal can either enhance or reduce the pathogenicity of entomopathogenic fungus in *An. funestus* in the field, depending on how females are attracted to fungus treated surfaces.

CHAPTER 6

BASE-LINE MALARIA VECTOR SURVEILLANCE IN KWAZULU-NATAL AND EFFICACY OF ENTOMOPATHOGENIC FUNGUS TREATED CLAY POTS

6.1 Introduction

In order to test the effectiveness of entomopathogenic fungi in semi-field conditions it is important to first survey the local malaria vector species composition including their insecticide susceptibility status. Mamfene village in northern KwaZulu-Natal, South Africa, is situated in an area that was amongst the worst affected by the malaria epidemic of 1999/2000, largely attributed to the emergence of pyrethroid resistance in *Anopheles funestus* (Hargreaves *et al.,* 2000). Continued entomological surveillance in the Mamfene region subsequently showed the emergence of DDT resistance in the malaria vector *An. arabiensis* as well as in the closely related non-vector *An. quadriannulatus* during 2002 (Hargreaves *et al.,* 2003).

A range of techniques are available for sampling Afro-tropical malaria vectors (Service, 1993), based on their resting and breeding habits. Various mosquito species, including malaria vectors, can be collected from inside clay pots placed either inside or outside houses (Service, 1993; Harbison *et al.,* 2006; Odiere *et al.,*

2007), making pots treated with entomopathogenic fungus an attractive method for infecting adult mosquitoes.

In view of the increasing incidence of insecticide resistance in African malaria vector populations, biological control based on the use of the entomopathogenic fungi is being evaluated and developed as a tool for insecticide resistance management (Scholte *et al.,* 2005; Farenhorst *et al.,* 2009). Laboratory studies using clay pots treated with oil formulated *M. anisopliae* showed promising results for the control of African malaria vectors (Farenhorst *et al.,* 2008).

The effectiveness of clay pots as a delivery system for the dry conidia of entomopathogenic fungi was assayed under laboratory conditions, preceding semi field trials. Baseline surveys of malaria vector species composition and insecticide susceptibility status were also conducted in KwaZulu-Natal, South Africa, in preparation for semi field trials.

6.2 Materials and Methods

6.2.1 Malaria vector surveillance in KwaZulu-Natal

The methodology of this study has been published and is presented in appendices 1 and 2)

6.2.1.1 Study site

Mosquito samples were collected from Mamfene, northern KwaZulu-Natal (KZN), South Africa ($27^{o}23'$ S, $32^{o}12'$ E) between February and September 2005. Mamfene is an undeveloped rural and rice growing area near the Balamhlanga river that forms an extensive marsh during the raining season (October to February). Two types of houses are found in the area: traditional houses which are mud structures and westernised houses built using cement and bricks. During 2005, vector control was conducted by spraying a combination of DDT in traditional houses and permethrin (pyrethroid) in westernised houses.

6.2.1.2 Mosquito collections

Mosquitoes were collected from insecticide sprayed houses using window exit traps (Muirhead-Thomson, 1947). Specimens morphologically identified as *An. funestus* group or *An. gambiae* complex (Gillies and Coetzee, 1987) was transported to the VCRU/NICD, Johannesburg, South Africa. Live females were induced to lay eggs under standard insectary conditions (25 ± 1 ºC, 75-80% relative humidity and 12 hours Light: Dark with dusk/dawn transition lighting). Larvae from each family were reared through to adults for further analysis.

Samples of F1 progeny (1-4 days old) from each family were subjected to standard WHO insecticide susceptibility tests (WHO, 1998). Additional samples of

unexposed males and females (1-4 days old) from various families were frozen at -70 ºC for biochemical analysis (Penilla *et al.,* 1988).

6.2.1.3 Species identification

Females preserved on silica gel were identified at the species level using PCR (Scott *et al.,* 1993; Koekemoer *et al.* 2002).

6.2.1.4 *Plasmodium falciparum* sporozoite detection by ELISA

The heads and thoraces of wild female mosquitoes were separated from the rest of the body and tested for the presence of *Plasmodium falciparum* circumsporozoite protein (CSP) using monoclonal antibodies 2A10 (Wirtz, 1987). ELISA positive specimens were confirmed using PCR (Sambrook *et al.,* 1989; Snounou *et al.,* 1993).

6.2.1.5 Direct ELISA for blood meal source

Half-gravid and fully gravid female mosquitoes that were not used for egg laying were preserved and screened for blood meal source (Beier *et al.,* 1988). Results were analysed using a microtitre plate reader at a wavelength of 405 nm. (APPENDIX 2).

6.2.1.6 Insecticide susceptibility tests

Susceptibility tests were carried according to WHO protocols using insecticide-impregnated and control papers and test kits supplied by WHO (1998). All

exposures lasted 1 hour and knockdown was recorded, and a 10% sucrose solution was made available for survivors. Final mortality was recorded 24 hour post-exposure.

6.2.1.7 Knockdown resistance (kdr) detection

Polymerase chain reaction genotyping to detect the West African Leu-Phe *kdr* allele was performed on samples of *An. arabiensis* resistant to permethrin (Martinez-Torres *et al.,* 1998). PCR conditions were as described by Matambo *et al.* (2007). (APPENDIX 2).

6.2.1.8 Selection for insecticide resistance

Permethrin and carbamate selections were performed on *An. arabiensis* progeny according to the standard WHO protocol (WHO, 1998). The selected lines were named Kwag-Perm and MBN-Carb respectively. Observed differences in susceptibility between the baseline and selected generations were analyzed by comparing LT_{50} values calculated from time-mortality regression analysis. (APPENDIX 2).

6.2.1.9 Synergist assays

These assays were performed using piperonyl butoxide (PBO), an inhibitor of monooxygenases, diethyl maleate (DEM), an inhibitor of GSTs, and triphenyl phosphate (TPP), an inhibitor of esterases, in experiments designed to synergise the bendiocarb and permethrin resistance phenotypes in laboratory *An. arabiensis* from

KZN (Brogdon *et al.,* 1990, 1997; Chareonviriyaphap *et al,* 2003). Three cohorts of adults were used from each resistant strain. One sample was pre-exposed to 4% PBO, 8% DEM or TPP (1% and 6%) for 1 hour and then both samples were immediately exposed to either 0.75% permethrin for 1 hour or 0.1% bendiocarb for 40 min. Control samples were exposed either to insecticide only or to a synergist only (PBO or DEM or TPP). Final mortality after a 24 hours holding period was compared between synergised and unsynergised samples using ANOVA. (APPENDIX 2).

6.2.1.10 Biochemical analysis of detoxifying enzyme systems

Assays designed to quantify relative levels of monooxygenase (Oxy), non-specific esterases and glutathione S-transferase (GST) activity as well as to detect the presence, in individual mosquitoes, of an altered acetylcholinesterase associated with carbamate/organophosphate resistance were performed based on the methods of Penilla *et al.* (1998). The assays used here were designed to test for differences in enzyme activity between the test samples and a baseline insecticide susceptible laboratory reared sample. Details of each enzyme assay is given in APPENDIX 3. Mixed samples of frozen male and female F1 *An. parensis* and *An. arabiensis* progeny of wild-caught females were used. Propoxur was used as a reference carbamate insecticide for the acetylcholinesterase assay. Controls included samples of laboratory reared insecticide susceptible *An. funestus* (Fang) for comparison against the *An. parensis* F1 samples, and laboratory reared insecticide susceptible *An arabiensis* (KGB) for comparison against the F1 *An. arabiensis* samples.

Similar assays were used to quantify exposure levels/activities in the insecticide selected lines (Kwag-perm and MBN-Carb) compared to samples from their respective baseline colonies (Kwag and MBN). Data were adjusted where necessary for total protein content to account for variation in mosquito sizes (section 3.1.6 APPENDIX 3). Data were analysed using Student's t-tests (in *STATISTIX 7.0*) with significance determined at $p < 0.05$.

6.2.2 Efficacy of fungus treated clay pots

6.2.2.1 Mosquitoes

Two to five day old female *An. gambiae* from the SUA laboratory colony (origin: Suakoko, Liberia) were used for all experiments.

6.2.2.2 Preparation of clay pots

The clay pots used in this study was made by a potter in the Mamfene region using traditional moulding and firing techniques. The inside surfaces of the experimental pots were dusted with 200 mg of dry conidia of either *Beauveria bassiana* or *Metarhizium anisopliae* using an artist's hair brush. The pot was rolled over a flat surface to ensure a homogenous distribution of conidia. Control pots were left untreated. A 500 ml white plastic bowl half filled with water was placed at the bottom of each pot to maintain high humidity. The pots were covered with a white net with a hole in the middle to allow for the introduction and removal of test mosquitoes (Fig. 6.1).

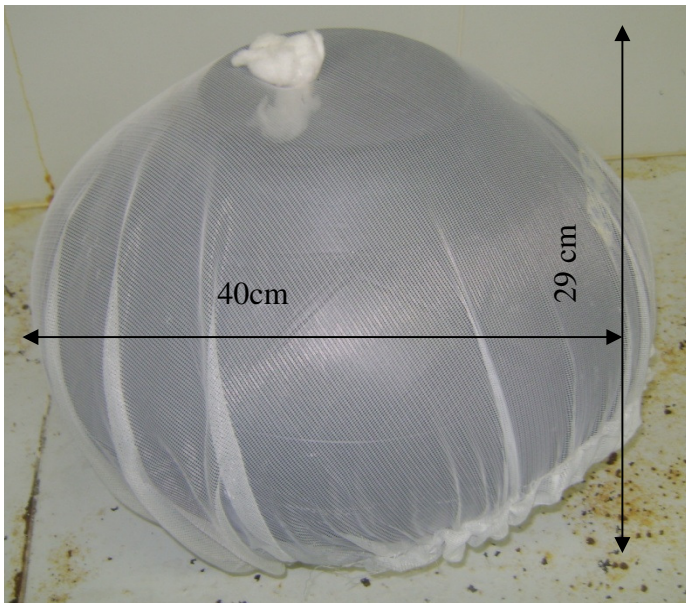


Figure 6.1: Clay pot (volume of 0.036 m^3) treated with 200 mg of dry conidia of entomopathogenic fungus and covered with a clean mosquito net. Cotton wool is used to plug the aspirator aperture through which test mosquitoes were introduced and subsequently removed.

16 cm

6.2.2.3 Bioassays

The efficacy of pots treated with fungus was determined by exposing 40-50 female mosquitoes per pot for 24 hours. Each experiment (one treated pot and one untreated pot) and was repeated three times. Each pot was assessed at the following intervals post fungus treatment: 1 day, 5 days, 6 days, 10 days, 15 days, 21 days and 3 months. After each exposure, mosquitoes were transferred to clean plastic cages using an aspirator. A 10% sugar solution was available to all mosquitoes for the duration of each experiment. Cadavers (if any) were collected daily and were processed as explained in Chapter 2 section 3.6.

Each experiment was terminated 29 days post-exposure. Variation within replicates was analysed using ANOVA and replicates per experiment were pooled for further analysis if not significantly different. The time to 50% mortality (LT_{50}) and mean survival were calculated for all replicates. Mosquito survival was analysed using Kaplan Meier survival analysis. All the tests were carried with *SPSS* statistic software 17.0.

6.3 Results

(Also presented in appendices 1and 2).

6.3.1 Mosquito collections

A total of 269 and 461 mosquitoes were morphologically identified as members of the *An. funestus* group and the *An. gambiae* complex respectively. From the *An. funestus* group, 220 were identified to species level as 218 *An. parensis* and two *An. funestus* (APPENDIX 1). In the *An. gambiae* complex sample 425 *An. arabiensis*, 6 *An. merus* and 6 *An. quadriannulatus* were identified using PCR (APPENDIX 2). The remaining specimens (49 *An. gambiae* complex and 24 *An. funestus* group) could not be identified despite three attempts at PCR amplification.

6.3.2 *Plasmodium falciparum* sporozoite detection

None of the *An. arabiensis* and *An. funestus* specimens tested positive for *Plasmodium falciparum* circumsporozoites. Of the 149 *An. parensis* tested by

ELISA, 20 showed positive. However, they subsequently showed negative for *P. falciparum* amplicons using PCR.

6.3.3 Blood meal identification

Of the 169 blood meal positive samples (167 *An. parensis* and 2 *An. funestus*), > 75% of blood meals were taken from animals. Both *An. funestus* specimens had ingested bovine blood. (APPENDIX 1).

6.3.4 WHO susceptibility assays

Insecticide susceptibility assays on 2-3 day old F1 progeny of *An. arabiensis* revealed 100% susceptibility to 0.1% bendiocarb and suspected resistance to 0.05% deltamethrin (95.91% mortality averaged across all families). However, exposure to 0.75% permethrin showed evidence of resistance in 12/22 families with an average of 62.3% mortality 24 hours post-exposure (Table 6.1). (APPENDIX 2).

Assays carried out on 1-4 day old female F1 *An. parensis* samples by family revealed susceptibility to 4% DDT (100% mortality in all families), 0.1% bendiocarb (98.05% mortality averaged across all families) and 0.75% permethrin (98.31% mortality averaged across all families). Resistance to 0.05% deltamethrin was detected in 4/11 families with an average of 85.29% (Table 6.2). No knock down resistance (kdr) mutations were detected in deltamethrin resistant specimens. (APPENDIX 1).

Table 6.1: Percentage mortalities 24 hour post-exposure to 0.75% permethrin of F1 progeny from *An. arabiensis* families.

Kwag family	No. tested	Mean % mortality
K8	10	83.0
K22	16	58.5
K36	21	36.0
K38	11	50.0
K41	15	83.0
K45	45	67.4
K49	25	62.0
K50	20	33.3
K54	14	57.0
K55	15	86.0
K56	14	44.0
K59	30	87.5

Table 6.2: Percentage mortalities 24 hour post-exposure to 0.05% deltamethrin of F1 progeny from *An. parensis* families. * Resistant family. # Suspected resistance.

Family	Sample size	Mean % mortality
11	18	100.0
13	7	71.4*
18	7	100.0
24	14	100.0
27	31	100.0
28	23	95.0#
29	21	47.5*
34	13	76.9*
37	27	96.4#
45	23	95.8#
52	9	77.8*

6.3.5 Selection for insecticide resistance and synergist assays

The permethrin selection process led to a rapid increase in resistance over successive generations with mean mortalities decreasing from 70% in F0 to 27% in the F1 cohort following a 30 min exposure period (Fig. 6.2). Families 13, 18 and 52 had only small numbers of individuals available at the time of this experiment. Following selection for resistance to bendiocarb, mean mortality decreased from 71% in F0 to 32% in F8 after a 20 min exposure (Fig. 6.3). However, 100% mortality was recorded after 60 min exposure to bendiocarb. Synergist results confirmed the involvement of PBO and DEM in permethrin and carbamate resistance respectively using selected lines (also refer to Table 2 in APPENDIX 2).

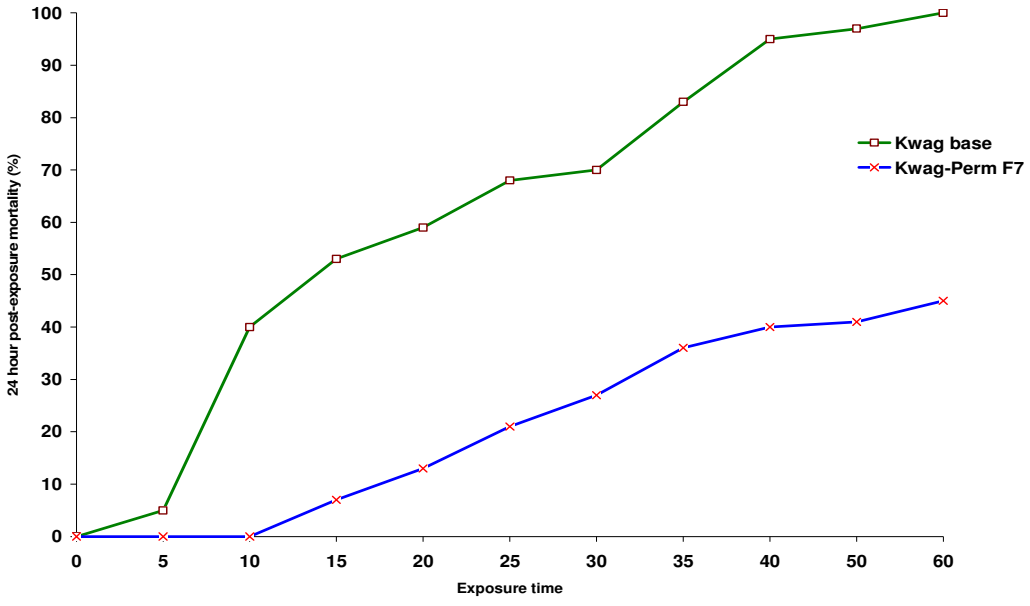


Figure 6.2: Time-mortality curve based on a range of 0.75% permethrin exposure times comparing permethrin resistance selected cohorts (F7) against the baseline colony, Kwag.

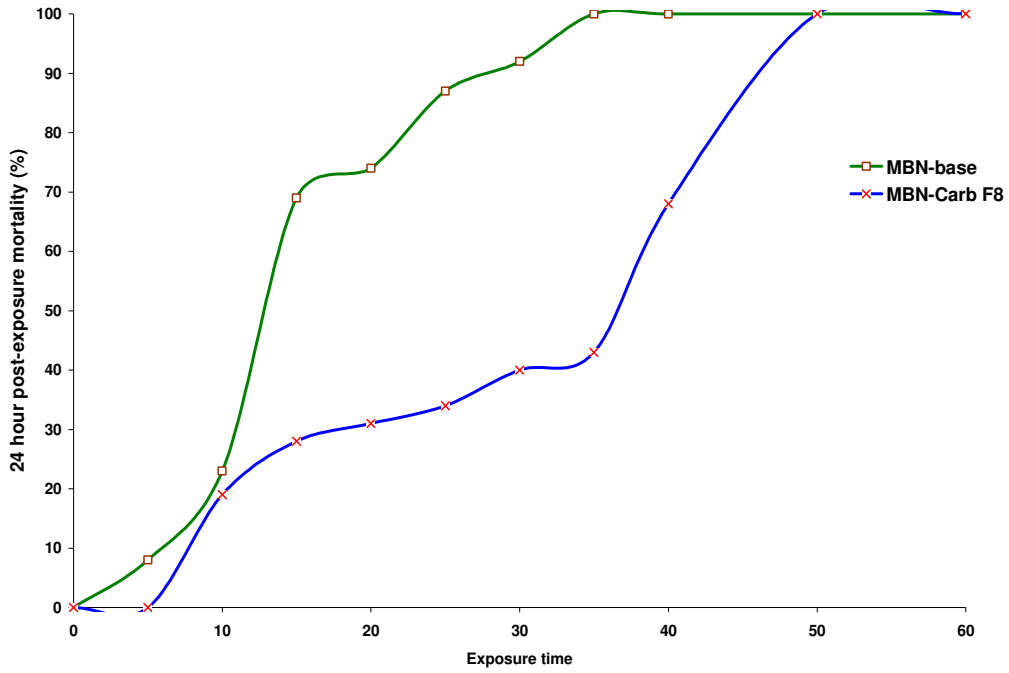


Figure 6.3: Time-mortality curve based on a range of 0.1% bendiocarb exposure times comparing bendiocarb resistance selected cohorts (F8) against the baseline colony, MBN.

6.3.6 Biochemical analysis of detoxifying enzyme systems

Analysis of F7 *An. parensis* showed no elevation in expression levels or activities of any of the detoxifying enzyme systems assayed when compared with an insecticide susceptible *An. funestus* laboratory strain (Also refer to Table 2 page7 on APPENDIX 1). Using F1 *An. arabiensis* samples from wild-caught families, enzyme assays showed high levels of monooxygenases by comparison to respective KGB samples, which correlated with the results of the permethrin bioassays. Elevated levels of non-specific esterases were found in some families (11/12 families using α napthyl acetate and 6/12 using ß napthyl acetate) but no

correlation was found with the permethrin bioassay data (Also refer to Fig.1 page 129 in APPENDIX 2). Non-specific esterase and monooxygenases levels were elevated in the permethrin selected strain (Kwag-perm) whilst glutathione S-transferase and non-specific esterases were elevated in the bendiocarb selected strain (MBN-Carb) (Also refer to Tables 3 and 4 page 130 in APPENDIX 2).

6.3.7 Efficacy of clay pots treated with dry fungal spores

6.3.7.1 Survivorship of fungus infected via clay pots vs. uninfected mosquitoes

There were significant differences in the proportions of female *An. gambiae* surviving daily between treatment and control groups ($p < 0.05$) (Figs. 6.4 and 6.5). An average of 70% of female *An. gambiae* cadavers from the fungus exposed groups showed entomopathogenic fungus sporulation. *Metarhizium anisopliae* and *B. bassiana* infections were confirmed by morphological analysis of hyphae in cadavers.

Survival rates of the infected and control groups were monitored up to 29 days post fungus exposure. All fungus exposed mosquitoes died within 22 days while some uninfected mosquitoes were still alive at day 29 (Figs. 6.4 and 6.5).

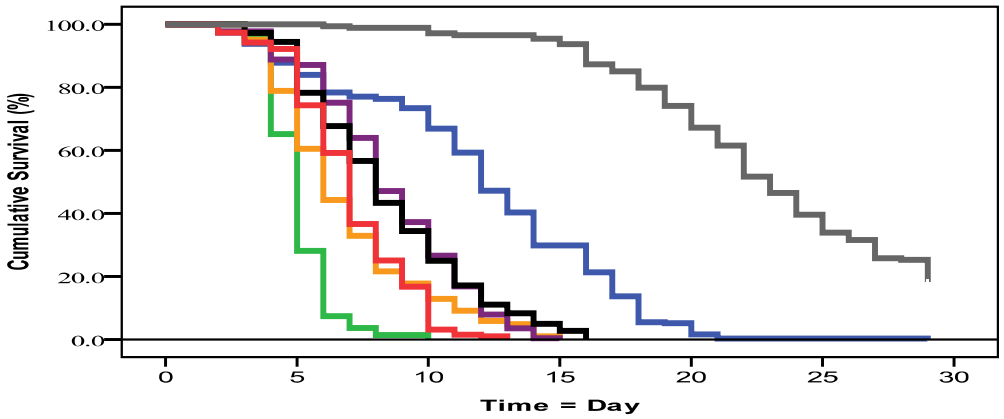


Figure 6.4: Efficacy/virulence of *B. bassiana* dry conidia covering the insides of clay pots at 1 day (green), 6 days (red), 10 days (yellow), 15 days (purple), 21 days (black) and 120 days (blue) post treatment. 3-5 day old laboratory reared female *An. gambiae* (SUA) were exposed to fungus treated pots for 24 hours at each post treatment time interval. Controls included exposure of females from the same cohort to untreated pots (grey).

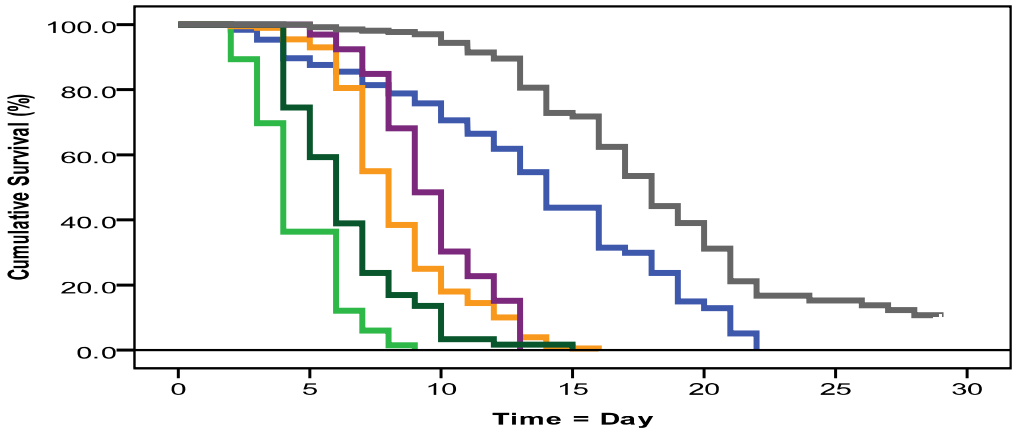


Figure 6.5: Efficacy/virulence of *M. anisopliae* dry conidia, covering the insides of clay pots at 1 day (green), 5 days (dark- green), 10 days (yellow), 15 days (purple) and 120 days (blue) post treatment. 3-5 day old laboratory reared female *An. gambiae* (SUA) were exposed to fungus treated pots for 24 hours at each post treatment time interval. Controls included exposure of females from the same cohort to untreated pots (grey).

Log rank tests showed no difference in mortality rates between mosquitoes exposed at 6 and 10 days post treatment of pots with *B. bassiana* (χ^2 = 0.000 p = 0.991) as well as between 15 and 21 days post treatment of pots with *B. bassiana* (χ^2 = 0.144; p = 0.704) (Table 6.3). In general, the mean mortality rate in the fungus exposed cohorts decreased with increasing time lag between pot treatment with fungal spores and mosquito exposure (Table 6.4). Based on LT50's , this trend is significant with very close correlations (Fig. 6.6). This correlation is based on a linear model which supports the causal relationship between exposure time and mortality.

Table 6.3: Log rank (Mantel Cox) analysis of fungus ((A) = *M. anisopliae* and (B) = *B. bassiana*) treated pot efficacy over time using mortality as the dependent variable.

(A) Lag time post fungus treatment	**5 days**	**10 days**	**15 days**	**120 days**
1 day	21.4			
5 days		26.5		
10 days			7.1	
15 days				61.9

(B) Lag time post fungus treatment	**6 days**	**10 days**	**15 days**	**120 days**
1 day	102.0			
10 days	0.0*			
15 days		22.3		
21 days			0.2**	102.9

***p = 0.991 (> 0.05, ns)** **** p = 0.704 (> 0.05, ns).**

Table 6.4: Efficacy at various time intervals of clay pots treated with the entomopathogenic fungi (A) *M. anisopliae* and (B) *B. bassiana* on female laboratory reared *An. gambiae* expressed in terms of Mean ±SE and LT_{50} ±SE for survival time using SPSS 17.0.

(A)

Lag time post fungus treatment	Mean ± SE (in days)	LT_{50} ± SE (in days)
1 day	4.5 ± 0.2	4.0 ± 0.2
5 days	6.4 ± 0.3	6.0.312
10 days	8.3 ± 0.2	8.0.209
15 days	9.6 ± 0.3	9.0.325
120 days	13.5 ± 0.4	14.0 ± 0.4
Control	19.2± 0.4	18.0.557

(B)

Lag time post fungus treatment	Mean ± SE (in days)	LT_{50} ± SE (in days)
1 day	5.0.102	5.0.105
6 days	7.1 ± 0.0	7.0.0.0
10 days	6.8 ± 0.2	6.0.225
15 days	8.5 ± 0.2	8.0.225
21 days	8.4± 0.2	8.0 ± 0.3
120 days	11.9 ± 0.3	12.0± 0.0
Control	22.8 ± 0.5	24.0.643

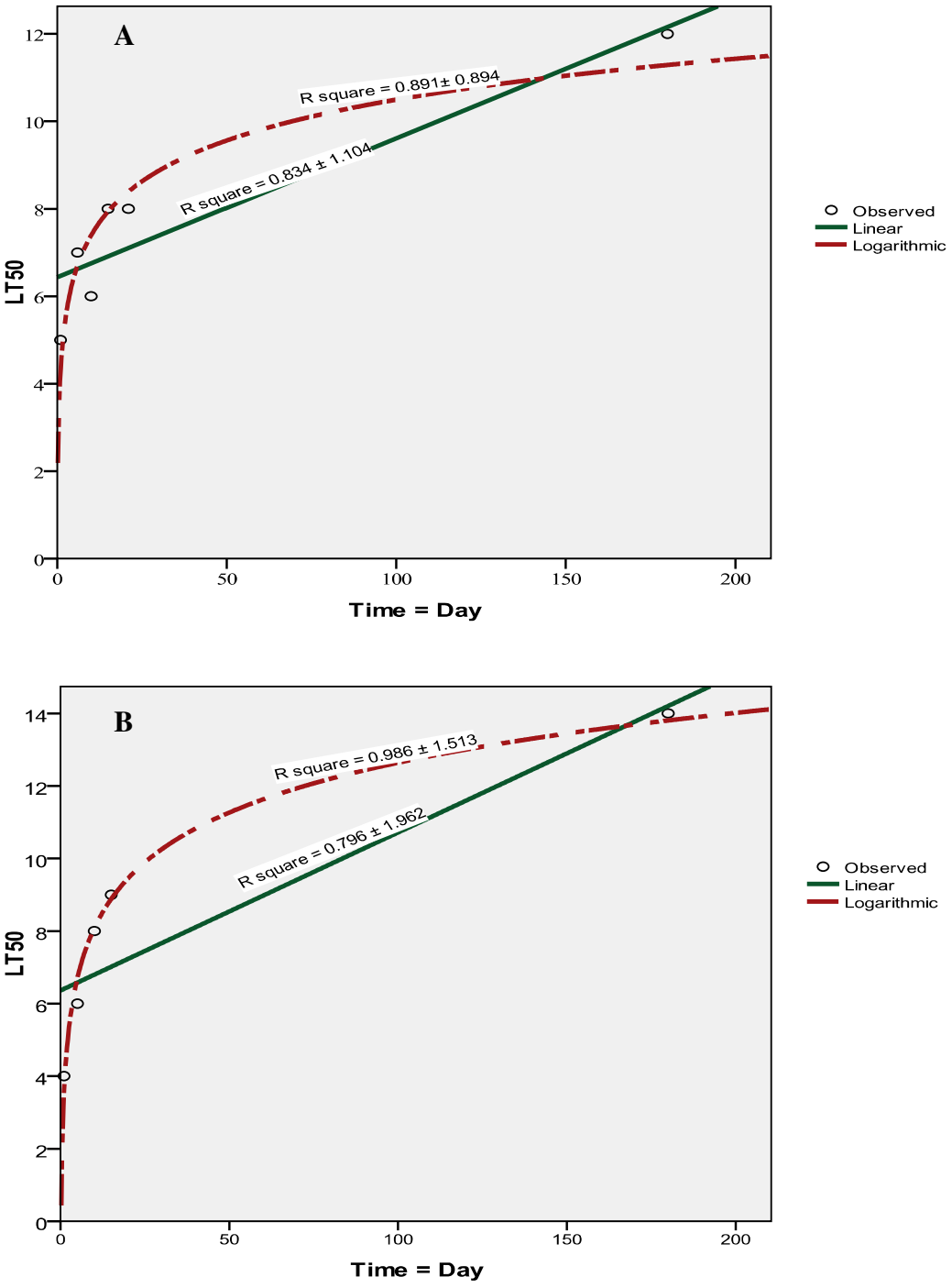


Figure 6.6: Correlation between LT_{50} (time to 50% mortality) and time lag between pot treatment with fungal spores and mosquito exposure. Data represent trends in LT_{50} of *An. gambiae* (SUA) females exposed to (a) *B. bassiana* or (B) *M. anisopliae*. R square ± SE represented on the curves.

6.4 Discussion

6.4.1 Baseline malaria vector surveillance in KwaZulu-Natal

(The study has been published and is presented in appendices 1 and 2).

Based on the use of F1 progeny from wild *An. parensis* females, appreciable levels of resistance were recorded against deltamethrin only. Biochemical analysis did not give an indication of the underlying mechanism of resistance. False *P. falciparum* infections were detected in wild female *An. parensis.* These false positives could be due to traces of animal blood accumulating in the haemolymph (Somboon *et al.,* 1993). However, it is unclear as to why a South African sample of *An. parensis* should give a high number of false positives by the ELISA method when *An. parensis* samples from Kenya did not (Kamau *et al.,* 2003). *Anopheles parensis* is found in eastern Africa from Kenya and Tanzania in the north to KZN in South Africa (Gillies and Coetzee, 1987; Gillies and De Meillon, 1968).

F1 progeny from wild *An. arabiensis* females also showed slightly reduced susceptibility to deltamethrin based on the WHO (1998) criteria which states that 98-100% mortality is susceptible, 80-98% requires investigation and <80% confirms resistance, comparable to the results of an earlier survey in the Mamfene region (Maharaj *et al.,* 2005). The current results also show resistance to permethrin at higher levels. Pyrethroid toxicity is highly dependent on their stereochemical structure (Milan *et al.,* 2000). Permethrin and deltamethrin are Class I and Class II pyrethroid insecticides respectively, which could explain the observed variation in susceptibility. There was a correlation between enzyme levels

and resistance to permethrin across families reared from wild-caught mosquitoes. Bioassay analysis of the permethrin selected strain (Kwag-perm) strongly suggests that pyrethroid resistance in *An. arabiensis* from South Africa is mediated by monooxygenases detoxification, because synergism with PBO was complete. The selection for carbamate resistance was unsuccessful, suggesting that *An. arabiensis* in northern KZN does not possess carbamate resistance genes.

This base-line survey underlines the importance of routine surveillance for malaria vector incrimination and insecticide susceptibility status by species in wild anopheline populations.

6.4.2 Efficacy of fungus treated clay pots

Adult laboratory reared *An. gambiae* (SUA) exposed to fungus treated clay pots acquired fungus infections inducing significantly higher rates of mortality than their uninfected counterparts. However, the efficacy of treated pots, measured in terms of relative infectivity, decreased with increasing time lapse since treatment, so that by 3 months post treatment their efficacy was negligible under standard laboratory conditions.

There was no variation in efficacy between 6 and 10 day old *B. bassiana* pot treatments or between 15 and 21 day old *B. bassiana* pot treatments. Variation with time since treatment was more consistent in the *M. anisopliae* experimental batch. It has previously been shown that the effectiveness or virulence of conidia varies

between fungus species (Daoust and Roberts, 1983, Hong *et al.,* 1997), which may explain why observable fungal sporulation in *An. gambiae* cadavers was higher in the *B. bassiana* treated cohorts (average 75%) than in the *M. anisopliae* treated cohorts (average 65%).

Factors that regulate speed of infection and sporulation of entomopathogenic fungi include temperature and humidity (Ekessi *et al.,* 1999, Soza-Gómez and Alves, 2000; Fargues and Luz, 2000). All these experiments were conducted under consistent conditions of 25 ± 2 ºC temperature and 85 ± 5% relative humidity. It has previously been shown that low (below 10 ºC) and high temperatures (over 34 ºC) reduce or prevent fungus infection (Ekessi *et al.,* 1999, Soza-Gómez and Alves, 2000; Fargues and Luz, 2000) while relative humidity in the range of 75-100% favours optimal germination and longevity of fungal conidia (Daoust and Roberts, 1983, Hong *et al.,* 1997; Soza-Gómez and Alves, 2000). The optimal conditions provided for in these experiments (current chapter) preclude making direct comparisons or assumptions about efficacy of fungus treated pots under variable field conditions. Nevertheless, these experiments give an indication of the best case scenario in terms of efficacy which should prove useful for the design of field trials.

CHAPTER 7

DISCUSSION

Considering the global prevalence of mosquito-borne diseases such as malaria (WHO, 2008), the increasing incidence of resistance to chemical insecticides in target mosquito populations and problems associated with environmental contamination by these compounds (Osorio *et al.,* 2007; Bouwman and Kylin, 2009), alternative methods of mosquito control are becoming increasingly important (Murphy *et al.,* 1994; Zaim and Guillet, 2002). The entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* are regarded as candidate biopesticides for managing insecticide resistance in malaria vector control. Infection with entomopathogenic fungi should significantly lower the rate of *Plasmodium* transmission not only by reducing mosquito longevity, but also by reducing mosquito blood feeding, fecundity and vector competence. Previous studies on *An. gambiae*, *An. arabiensis* (Scholte *et al.,* 2003a, b; 2004b; 2005; Achonduh and Tondje, 2008; Kikankie, 2009) and *An. stephensi* (Blanford *et al.,* 2005; Stevenson, 2008) have showed that *M. anisopliae* and *B. bassiana* are effective biopesticides against adult mosquitoes. A semi-field trial carried out in Tanzania by Scholte *et al.* (2005) showed that *An. gambiae* adults can be infected with *M. anisopliae* spores using spore treated black cloths strategically placed inside human dwellings. Farenhorst *et al*. (2008) demonstrated in the laboratory

that clay pots treated with oil formulated fungal conidia can be used to deliver *M. anisopliae* spores to adult anopheline mosquitoes.

7.1 Pathogenicity of *Beauveria bassiana* and *Metarhizium anisopliae* against insecticide resistant and susceptible *Anopheles funestus* (Chapter 3)

All laboratory strains of *An. funestus* tested were susceptible to infection by unformulated conidia of *B. bassiana* and *M. anisopliae.* These fungi induced significantly higher mortality rates compared to those of concurrent uninfected samples. This difference in mortality between fungus treated and untreated groups must be attributed to the presence of fungal conidia which overpower the immune response of the infected host, and proliferate by penetrating the cuticle and invading the internal organs ultimately causing the death of the host (St. Leger *et al.,* 1991; Clarkson and Charnley, 1996). Susceptibility to fungal infection in the *An. funestus* colonies tested appears to follow their pattern of susceptibility to insecticide. Fang, the colony most susceptible to insecticide, showed higher rates of mortality following fungus infection than Fumoz and Fumoz-R. However, this difference in mortality rate may also be associated with the general biological characteristics of each colony which do not share the same genetic background. Variation in susceptibility to fungus infection within and between colonies is also likely to be attributable to variation in physiological state at the time of testing as well as other factors such as cuticle thickness and structure. Further, variation in the number of infective spores acquired on the integument should also affect the rate of disease progression. Mosquitoes placed in forced proximity of fungal spores

for only three hours showed significantly lower rates of mortality than those placed in similar circumstances for 24 hours, showing that the probability of acquiring an infection is a function of time and that a longer potential exposure time leads to the acquisition of greater numbers of infective spores. Clark *et al.* (1968) demonstrated that continuous exposure in a field environment should induce an exponentially increasing mortality rate in a relatively short period.

Approximately 99% of fungus infected mosquitoes died within 14 days of acquiring their infection, which is encouraging in terms of *Plasmodium* transmission because of the time required for Plasmodia to mature from acquired gametocytes to infective sporozoites presenting in the mosquito salivary glands. Death within 14 days post fungus infection should lead to a significantly reduced potential for malaria transmission, an effect that should be further enhanced by indications that the lifespan of a mosquito infected with both entomopathogenic fungus and *P. falciparum* is likely to be even shorter than one infected with fungus alone (Blanford *et al.,* 2005).

7.2 Synergistic effects of permethrin (pyrethroid), piperonyl butoxide and entomopathogenic fungus (Chapter 4)

Samples of female pyrethroid resistant Fumoz-R infected with fungus proved significantly more susceptible to pyrethroid intoxication post fungus infection than uninfected samples from the same cohort (see Farenhorst *et al.* (2009) in APPENDIX 4). It is concluded that *B. bassiana* or *M. anisopliae* infection at least

partially attenuates the expression of pyrethroid resistance in *An. funestus*, enhancing the potential use of these fungi as an insecticide resistance management tool. The combined use of synthetic insecticides with biopesticides should also carry the added advantage of delaying the development of resistance to either.

The monooxygenase inhibitor piperonyl butoxide (PBO) completely synergises the expression of pyrethroid resistance in southern African *An. funestus* (Brooke *et al.*, 2001). However, pre-exposure to PBO did not affect subsequent susceptibility to fungus infection in *An. funestus*, suggesting that monooxygenases play a negligible role in protection against fungus infection. This result contrasts with that of Serebrov *et al.* (2006) who showed that PBO exposure exponentially increased the rate of fungus induced mortality in larvae of the wax moth.

7.3 Effect of a fungus infection on fecundity and fertility in *Anopheles funestus* (Chapter 5)

Anopheles funestus females blood fed post exposure to fungus showed a slightly higher rate of mortality compared to unfed fungus infected females. Females blood fed prior to fungus infection showed comparable rates of mortality with those of unfed fungus infected cohorts. These results suggest that blood-feeding may affect susceptibility to fungus infection, although the effect is slight at best and of no concern in terms of fungal pathogenicity.

Fungus infected *An. funestus* females produced significantly fewer eggs than uninfected females. The proportion of progeny from fungus infected females

surviving to adulthood was also significantly reduced by comparison to the progeny of uninfected females. Lowered egg production and reduced fertility in association with a fungal infection may be the effect of reduced blood intake as well as an outcome of fungus disease progression (Roy *et al.,* 2006; Scholte *et al.,* 2006; Hajek *et al.,* 2008). These results showed that in addition to direct implication of the adult density fungus might also an indirect effect on pre-adult stages of *An. funestus* infected with *B. bassiana* or *M. anisopliae.* These findings imply a vertical transmission of the infection may play a significant role in malaria vector control.

7.4 Base-line malaria vector surveillance in KwaZulu-Natal and efficacy of entomopathogenic fungus treated clay pots (Chapter 6)

As a precursor to field trials using entomopathogenic fungi, it is important to first conduct baseline surveys of malaria vector species composition including their insecticide susceptibility status. The results of these surveys in northern KwaZulu-Natal, South Africa, are given in full in Mouatcho *et al.* (2007 and 2009) – see APPENDICES 1 and 2. *Anopheles arabiensis*, *An. parensis*, *An. funestus*, *An. merus* and *An. quadriannulatus* were collected inside houses between February and September, 2005. A sample of *An. parensis*, a non-vector, was shown to present false positives for the presence of *P. falciparum* circumsporozoites using the standard ELISA (Wirtz *et al.,* 1987) method. This result highlighted the importance of accurate species identification and vector incrimination, and showed how over-

reliance on standard methodologies without suitable quality assurance can lead to inaccurate information about malaria transmission dynamics in a given area.

Permethrin resistance was detected in *An. arabiensis* and *An. parensis* (Mouatcho *et al.,* 2007 and 2009), confirming the insecticide susceptibility status of *An. arabiensis* from an earlier survey conducted in 2003 (Maharaj *et al.,* 2005). Biochemical analysis and insecticide-synergist assays showed monooxygenase elevation leading to monooxygenase based permethrin detoxification in *An. arabiensis* (Mouatcho *et al.*, 2009).

Preliminary laboratory tests revealed that clay pots treated with dry conidia of *B. bassiana* or *M. anisopliae* are suitable for spore delivery to anopheline mosquitoes resting inside them. Untreated clay pots have previously been shown to be suitable resting sites for adult mosquitoes both inside and outside houses (Service, 1993; Harbison *et al.,* 2006 Odiere *et al.,* 2007). Water inside clay pots has also been shown to serve as a breeding site for anopheline mosquitoes (Harbison *et al.,* 2006; Odiere *et al.,* 2007).

7.5 Conclusion

Dry conidia of *B. bassiana* and *M. anisopliae* are effective pathogens against *An. funestus*. Infective spores can be delivered using either an attractant such as sucrose or by treating the surfaces of preferred resting sites such as clay pots. Their pathogenicity is not significantly affected by monooxygenase based insecticide resistance, and in almost all cases fungus infected females die within 14 days of

acquiring an infection, regardless of blood-feeding status. Further, fungal infection significantly attenuates the expression of insecticide resistance, and also significantly reduces fecundity and fertility in infected *An. funestus* females. These factors enhance the potential of entomopathogenic fungi as biocontrol agents in areas where resistance to insecticide occurs in target vector populations.

APPENDIX 1

PUBLICATION

Joel C. Mouatcho, Keith Hargreaves, Lizette L. Koekemoer, Basil D. Brooke, Shüne V. Oliver, Richard H. Hunt and Maureen Coetzee. 2007. **Indoor collections of the *Anopheles funestus* group (Diptera: Culicidae) in sprayed houses in northern KwaZulu-Natal, South Africa.** *Malaria Journal*, **6**:30.

Contribution:

I carried out the rearing of mosquitoes, conducted the insecticide susceptibility bioassays, ELISA assays and molecular studies, and wrote the first and subsequent drafts of the manuscript.

APPENDIX 2

PUBLICATION

Joel C. Mouatcho, Givemore Munhenga, Keith Hargreaves, Basil D. Brooke, Maureen Coetzee and Lizette L. Koekemoer. 2009. **Pyrethroid resistance in a major African malaria vector *Anopheles arabiensis* from Mamfene, northern KwaZulu-Natal, South Africa.** *South African Journal of Science*, **105:** 127-131.

Contribution:

I carried out all the work on the wild-caught material and wrote the first and subsequent drafts of the manuscript.

APPENDIX 3

BIOCHEMICAL ASSAY

Biochemical assays were carried out on samples of F_1 progeny from wild caught females as well as the F_4 and F_3 generations of MBN-Carb and Kwag-Perm respectively. Glutathione S-transferase, general esterases activity, monooxygenases P450 and altered acetylcholine esterase (AChE) were assayed according to the method described by Penilla *et al.* (1998). For each microtitre plate, a batch of 47 mosquitoes (6 families with 6 mosquitoes each and 11 mosquitoes from KGB colony for comparison) was used. Mean enzyme activity absorbance was compared between the familial mosquitoes and the susceptible *An. arabiensis* (KGB) colony using a two sample t-test of means (Statistix 7.0, Analytical Software).

3.1.1 Homogenate preparation

Individual mosquitoes were transferred individually into microtitre wells using fine forceps and 50 µl of distilled water was added. The mosquitoes were thoroughly ground using a Teflon pestle. The crude homogenate was diluted to a volume of 200µl per well using distilled water and used for enzyme assays as outlined below.

3.1.2 Glutathione-S-Transferase (GST) assay

The activity of GST was assayed using the method described by Lee, (1990) and Penilla *et al.* (1998). Using duplicates of each homogenate of a family line, 10 µl of the homogenate was placed in each well of a 96 well microtitre plate. This was followed by addition of 200 µl of: 10mM reduced glutathione prepared in 0.1M sodium phosphate buffer pH 6.5 and 65mM of 1-chloro-2, 4-dinitrobenzene (CDNB) dissolved in methanol. Ten microlitres homogenate of a susceptible *An. arabiensis* laboratory colony originating from Kanyemba, Zimbabwe (KGB) was placed in separate wells of the same microtitre plate for comparative purposes. A well containing 10 µl of distilled water with 200 µl of CDNB was placed in a separate well to act as a negative control. The assay was then read kinetically on a plate reader (Labsystems Multiskan RC, Genesis software version 3.03) as a change in optical density for 5 minutes at 340 nm.

3.1.3 General esterase assay

The titration level of general esterases was determined. Two substrates: alpha and beta naphthyl acetate which are usually active in resistant insects, were used. Using the prepared homogenate, 2 x 20 µl replicates were pipetted into separate wells of a microtitre plate, 200 µl of alpha-naphthyl acetate solution [130 µl of 30mM alpha-naphthyl acetate (NA) dissolved in 13 ml of 0.02M sodium phosphate buffer (pH 7.2)] was added to one duplicate and 200 µl of beta naphthyl acetate solution [130 µl of 30mM beta-naphthyl acetate also dissolved in 13 ml of 0.02M sodium phosphate buffer (pH 7.2)] was added to the other. After incubating on ice for 30

minutes, 50 µl of: 0.023g fast blue salt dissolved in 2.25 ml distilled water plus 5.25 ml 5% sodium lauryl sulphate (SDS) dissolved in phosphate buffer (pH 7.0) were added to each well. A blank containing 20 µl distilled water, 200 µl of alpha-NA or beta-NA solution and 50 µl of stain were also placed in separate wells. Optical density was read at 570 nm as an end point.

3.1.4 Monooxygenases titration assay

The titration level of monooxygenases was determined per mosquito according to method described by Brogdon *et al*. (1988), Lee, (1990) and Penilla *et al.* (1998). Using homogenate of individual mosquitoes 2 x 20 µl replicates of homogenate was transferred into separate wells of a microtitre plate, following which 80 µl of 0.0625M potassium phosphate buffer (pH 7.2) was added to each replicate. This was followed by 200 µl of: 0.01g of 3, 3', 5, 5'- Tetramethyl Benzidine dissolved in 5 ml of methanol plus 15 ml of 0.25M sodium acetate buffer (pH 5.0) added to each of the replicates. Finally 25 µl of 3% hydrogen peroxide was added to each well. Each plate was incubated at room temperature (23-26 ºC) for 2 hours before optical density was read at 650 nm using a plate reader (Labsystems Multiskan RC, Genesis software version 3.03). Negative controls were run with 20 µl of buffer instead of the insect homogenate.

3.1.5 Insensitive Acetylcholinesterase assay

The purpose of this assay is to detect the presence of an altered acetylcholinesterase following inhibition by the carbamate propoxur as described by Hemingway *et al.* (1986) and Penilla *et al.* (1998) with slight modifications.

2 x 25 µl replicates of crude homogenate per mosquito were placed in separate wells: 145 µl of Triton phosphate buffer (1% Triton buffer in 0,1M phosphate buffer pH 7.8) was then added to each replicate and gently mixed to avoid bubble formation. A solution of 10µl of: [5, 5'-Dithio-bis (2-nitrobenzoic acid) DTNB in 2 ml of 0.1 phosphate buffer pH 7.0)] was added to each replicate. Finally, 25 µl of acetylthiocholine iodide (ASCHI) was added to one replicate while 25 µl of: ASCHI + 5 µl propoxur was added to the other replicate. A control was set up with blanks without any insect homogenate. For comparison a parallel run was done on the same plate using a susceptible laboratory colony (KGB) originating from Zimbabwe. The optical density was read using the Multiskan plate reader at 570nm after 5 minutes incubation.

3.1.6 Protein assay

The purpose of this assay is to measure the amount of total protein in each mosquito. A comparison between the means of each familial sample and the susceptible sample was then done inorder to establish a correction factor that could be used to adjust for the other assays.

Using a microtitre plate 2 x 10 µl replicates per mosquito were placed in separate wells and 300 µl of a BIO-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories GmbH, Cat no. 500-0006) prepared as 1:4 dilution in distilled water was added. One blank was prepared with 10 µl of distilled water and 300µl of BIO-Rad solution. The reaction was read on a plate reader (Labsystems Multiskan RC, Genesis software version 3.03) at 570 nm after incubation for 5 minutes.

APPENDIX 4

PUBLICATION

Marit Farenhorst, **Joel C. Mouatcho,** Christophe K. Kikankie, Basil D. Brooke, Richard H. Hunt, Mathew B. Thomas, Lizette L. Koekemoer, Bart G. J. Knols and Maureen Coetzee. 2009. **Fungal infection counters insecticide resistance in African malaria mosquitoes.** *Proceedings of the National Academy of Sciences*, **106:** 17443-17447.

Contribution:

Marit Farenhorst and I contributed equally to this article. I did all the fungal susceptibility tests involving *Anopheles funestus* and assisted with the insecticide susceptibility bioassays, the subsequent analysis of data and the drafting of the manuscript.

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