Behavioural effects of fungal infection by *Metarhizium anisopliae* in adult malaria mosquitoes

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Abstract

Malaria remains a major global health problem with the burden of disease greatest in Sub-Saharan Africa. The strategies for malaria control differ throughout the world according to levels of endemicity and the magnitude of disease but the focus remains either to control malaria parasites or vectors. A high degree of drug resistance and the absence of malaria vaccines are a major hindrance to control of the disease. In such circumstances, vector control becomes an alternative and has remained the most effective means to prevent malaria transmission. Contemporary adult mosquito control is almost exclusively based on indoor application of chemical insecticides in the form of indoor residual spraying (IRS) of walls and ceilings and insecticide-impregnated bed nets. However, sustainable use of chemicals is undermined by problems of insecticide resistance in mosquito populations, environmental contamination and risks to human health. Biological control based on fungal pathogens has shown potential to complement existing vector control methods. The entomopathogenic fungi (EPF) *Metarhizium anisopliae* and *Beauveria bassiana* have demonstrated ability to infect, kill and reduce the survival of malaria vectors. However, the effect of EPF on the behaviour of malaria vectors has not been fully addressed.

This thesis was designed to provide baseline information on mosquito-fungus interaction focusing on the efficacy of entomopathogenic fungus *M. anisopliae* ICIPE 30 on the important life-history behaviours of the African malaria vector *Anopheles gambiae* Giles *sensu stricto* under laboratory and semi-field conditions. The information is important to facilitate the further development of malaria vector control based on biological control agents. Host-seeking, sugar-feeding, mating and oviposition were the behaviours investigated. Since mosquito-fungus contact is crucial for infection with EPF, a paper sheet (28.6 × 14.3 cm) lined inside a plastic cylinder (9-cm diameter and 15-cm height) was developed as a cost effective method of infection. Moreover, 0.1 g (approx. 10^{11} conidia/ m^2) of dry conidia and 6 hr exposure time sufficient for *An. gambiae* to pick up large numbers of conidia were established to cause high pathogenicity (Chapter 3). As the impact of EPF on insect behaviour was reported to occur at least three days post-exposure to fungal pathogen (Chapter 2), all experiments were conducted with a special focus on mosquitoes three days post-exposure to fungus. It is, however, important to mention that on average 50% of the mosquitoes died on the third day after fungal exposure (Chapter 3) and only those that survived were used for behavioural assays.

The host-seeking capability of *An. gambiae* mosquitoes is an important parameter in the vectorial capacity equation. At short-range (1 m from host) assessment using a dual-choice olfactometer under semi-field conditions, infection with EPF strongly reduced the host-seeking response of mosquitoes, but did not impair their olfactory-based capability to discriminate between hosts (Chapter 4). At medium-range, using experimental cages (3 x 3 x 2 m) under laboratory conditions, fungal infection reduced the host-seeking response and feeding propensity of female *An. gambiae* mosquitoes (Chapter 7) whereas at long-range (7 m from host) inside a semi-field enclosure, infection with EPF sharply reduced the house-entry response and the hourly human-biting responses of host-seeking mosquitoes indoors and outdoors (Chapter 5). Plant sugar feeding is an important component in the biology of mosquitoes and is the main priority for both sexes at emergence. Infection with fungal pathogen strongly reduced the survival and sugar-feeding propensity of both sexes of the malaria vector *An. gambiae* but did not affect their potential to feed and digest meals (Chapter 6). Mating behaviour plays a key role in population growth. The activity takes place after sugar feeding and thereafter, the females search for their blood meal host. Infection with *M. anisopliae* strongly reduced multiple mating propensity and the mating performance of adult male *An. gambiae* mosquitoes in a large arena such as a screenhouse. Although this resulted in a reduction in the number of females inseminated, it facilitated the transfer of fungal conidia to conspecific healthy females during mating (Chapter 8). Finally, after blood meal intake, the females prepare to

lay eggs. Infection with *M. anisopliae* reduced the oviposition propensity of female *An. gambiae* mosquitoes although the number of eggs laid remained unaffected (Chapter 7).

In conclusion, these findings demonstrate that the entomopathogenic fungus *M. anisopliae* alters the major life history behaviours of *An. gambiae* mosquitoes. This is possible because the fungus strongly impairs flight performance of mosquitoes that makes the insect less able to fly and engage in host-seeking, sugar-feeding, mating and oviposition behaviours. The high mortalities observed in the early days of infection prior to conducting behavioural assays, mortalities observed while conducting behavioural assays and a reduction in behavioural response of *M. anisopliae*-infected mosquitoes collectively are likely to have a significant impact in suppressing a vector population. The susceptibility of male mosquitoes to fungal conidia opens a new strategy for mosquito vector control. Overall, this thesis has demonstrated that EPF may be a good complement to other mosquito vector control tools for the reduction of mosquito bites, and transmission of malaria and other mosquito-borne diseases.

Chapter 1

General Introduction

Malaria burden

Malaria remains a common and life-threatening disease in many tropical and subtropical areas, exerting an unacceptable toll on the health and economic welfare of the world's poorest communities (Breman et al. 2007, WHO 2011). The disease is currently endemic in over 100 countries with the burden of disease being greatest in Africa (Figure 1). The most vulnerable are children under the age of five due to their lower level of malaria immunity (WHO 2006a) and pregnant women due to adherence of *Plasmodium falciparum* to chondroitin sulphate A in the human placenta (Fried and Duffy 1996). Each year, at least 600,000 deaths from the direct effects of the disease occur on the continent (WHO 2011) and it is therefore regarded as the leading cause of morbidity and mortality in the sub-Saharan region (Day 2005). Even though the existence and the use of affordable interventions in malaria-affected areas has resulted in a 25% decline of malaria cases and deaths (WHO 2011), the burden of the disease still persists.

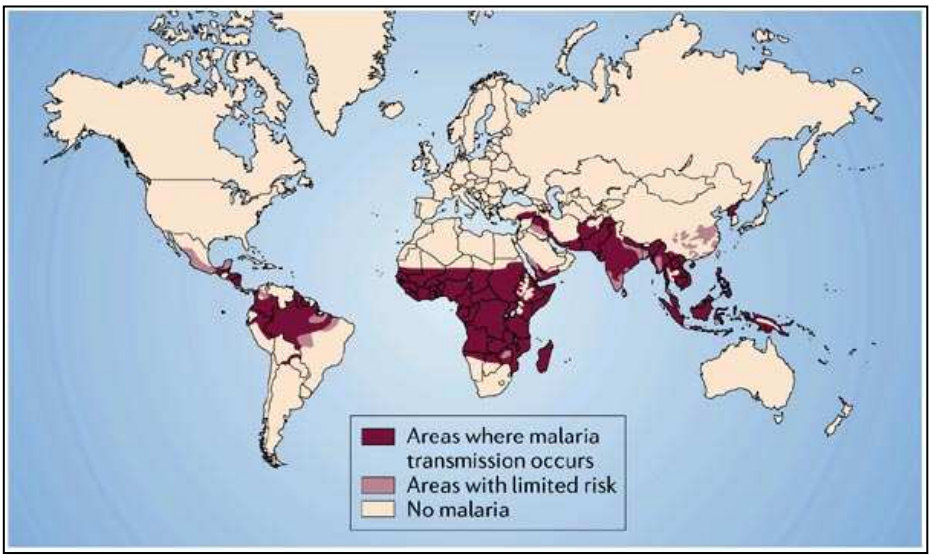

Figure 1. Global distribution of malaria (Bell et al. 2006)

Malaria parasites

Malaria is caused by protozoan parasites of the genus *Plasmodium* and is transmitted through bites of mosquitoes belonging to the genus *Anopheles*. Five species of malaria parasites namely *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* infect people (Bell et al. 2006, Cox-Singh et al. 2008, Krief et al. 2010). Of these, *P. falciparum* is the most common but most dominant in sub-Saharan Africa, clinically severe and life threatening. *Plasmodium vivax* is the second most significant species and is prevalent in Southeast Asia and Latin America. *Plasmodium ovale* is common in Africa but scanty in numerous islands in the western Pacific and on the Asian mainland while *P. malariae* shares geographical coverage with *P. falciparum* although it is less common and irregular in prevalence (Collins and Jeffery 2005). Primarily, *P. knowlesi* causes malaria in monkeys but has recently been reported to infect humans in Southeast Asia (Singh et al. 2004, Luchavez et al. 2008, Figtree et al. 2010). Overall, the *Plasmodium* species are not evenly distributed globally and their relative importance varies between and within different malaria regions (Beier 1998). As a result, the risk of contracting malaria varies from country to country and even between areas in a country. Knowledge on global distribution of malaria parasites and on the biology and ecology of the principal disease vectors is therefore essential to the development of an integrated vector control approach.

Malaria vectors

Malaria parasites are transmitted between humans by female mosquitoes of the genus *Anopheles* (Diptera: Culicidae). Of over 400 different *Anopheles* species described worldwide except in the Antarctica (Figure 2), only about 60 are vectors of malaria (White 1985, Walker 2002, Besansky et al. 2004). Each species has a different behavioural pattern and occurs in a different region of the world. The *Anopheles gambiae* complex comprised of seven sibling species: *Anopheles gambiae sensu stricto*, *An. arabiensis, An. melas*, *An. merus, An. bwambae*, *An. quadriannulatus* species A and *An. quadriannulatus* species B (yet to be assigned a scientific name) are predominant in sub-Saharan Africa and belong to the world's most efficient vectors of human malaria (Mattingly 1977, White 1985, Hunt et al. 1998, Coetzee 2004). Of these, *An. gambiae* and the none *An. gambiae* complex species, *An. funestus*, are the most important since they exhibit anthropophilic (preference for a human blood meal), endophagic (indoor biting) and endophilic (indoor resting) behaviours whose interaction with humans influences the risk of malaria infection and transmission (Costantini et al. 1999, Takken and Knols 1999). In addition, the vectors display high vector competence and high survival rates (Day 2005). The less efficient vectors, such as *An. culicifacies*, *An. stephensi* and *An. minimus* are dominant in Asia, *An. darlingi* in South America and *An. freeborni* in North America.

Malaria control

Strategies for malaria control differ throughout the world according to levels of endemicity, the magnitude of disease, and the malaria vector potential of *Anopheles* mosquitoes (Walker 2002). The World Health Organization (WHO) has prioritised and pioneered the control of malaria since its inception in 1948. In its efforts, WHO characterised four basic approaches to fight the disease: 1) provision of early diagnosis and prompt treatment for the disease, 2) planning and implementation of selective and sustainable preventive measures, including vector control, 3) early detection for the prevention or containment of epidemics and 4) strengthening of local research capacities to promote regular assessment of countries' malaria situations, in particular the ecological, social and economic determinants of the disease (WHO 1993, WHO 2006a). All these approaches are tailored to either control malaria parasites or vectors. However, it remains elusive to single out a successful approach but there is every indication that integrated approaches will be needed for effective and sustainable control. Simply having a new antimalarial drug, an effective malaria vaccine, or a new way to kill mosquitoes is still a long way and many years from the achievement of effective malaria control (Beier 1998). The reasons for this are manifold, but the high degree of drug resistance and resistance against insecticides, together with the environmental variability of malaria epidemiology call for a complex disease situation that requires an integrated approach for its control (Alonso et al. 2011).

Antimalarial drugs

Timely and accurate diagnosis of malaria by use of rapid diagnostic test (RDTs) kits and microscopic examination of the thin and thick smear of finger-prick stained with Giemsa is the key to effective disease management (Cooke et al. 1999, McCutchan et al. 2008, Pöschl et al. 2010). For a long time, several anti-malarial drugs such as quinine and chloroquine have been used as prophylaxis and for the treatment of uncomplicated and severe cases of malaria. However, with emergence of resistance to drugs such as chloroquine, fears of toxicity and decreased efficacy for sulfadoxine/pyrimithamine, several countries have adopted the use of artemisinin-based combination therapy (ACT) in the treatment of uncomplicated malaria since 2001 (WHO 2009) and artesunate derivatives for severe malaria (Dondorp et al. 2005). Although these new combination therapies are proven to provide effective treatment and/or prevention, other new medications have to be developed and the existing ones improved, to be prepared for the emergence of resistance with the continuous use of

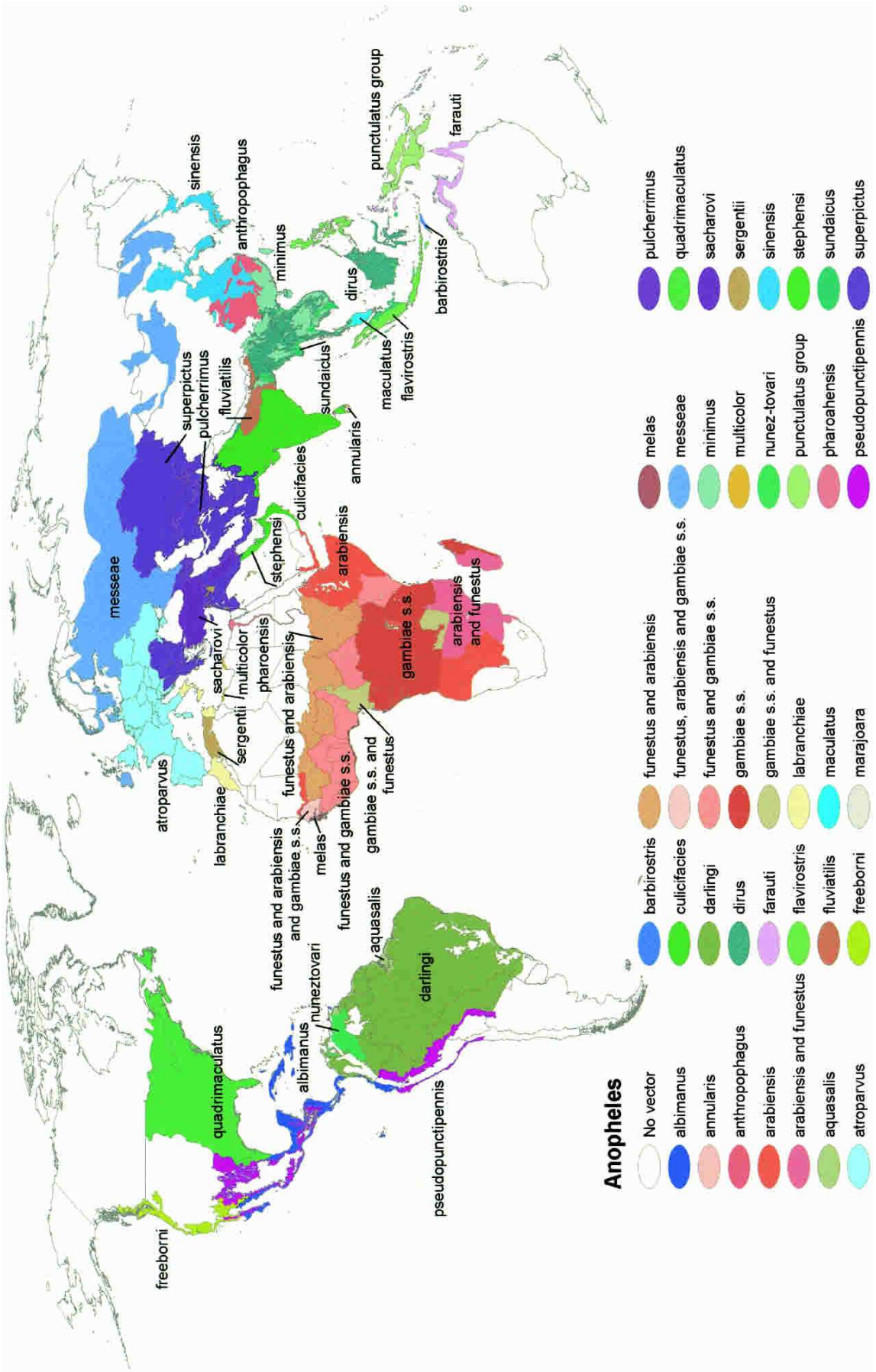

Figure 2. World distribution of predominant malaria vectors (Kiszewski et al. 2004)

prescribed antimalarial drugs. The first case of drug tolerance against artemisinine has already been reported from Southeast Asia (WHO 2008, WHO 2010).

Malaria vaccines

Malaria vaccines could be one of the most cost-effective interventions to reduce the burden of disease, particularly in Africa. For a long time, development of malaria vaccines at global level has been the subject of much research (Cohen 1982). Since then, much research has been devoted in the search and development of a malaria vaccine, of which some have reached the state of clinical trials. Recently, studies with the candidate vaccine RTS,S in Mozambique have provided the first evidence that vaccines can be developed that provide a reasonable degree of protection in infants and small children (Sacarlal et al. 2008). Despite considerable progress with the RTS,S vaccine, which currently undergoes phase III clinical trials in Africa, its full deployment remains a distant goal (Bojang et al. 2001, Richie and Saul 2002, Crompton et al. 2010).

Vector control

In the absence of effective treatment and vaccines against various vector-borne diseases, vector control remains the most generally effective measure to prevent malaria transmission (WHO 2006b). The approach aims to prevent human-vector contact, reduce vector population densities and life span of mosquitoes by targeting the larval or adult stages of *Anopheles*. The current vector control strategies include environmental management, larval control, personal protection measures, indoor spraying of insecticides and biological control.

Various human activities such as agriculture, irrigation and construction can generate larval breeding sites (WHO 1982). Therefore, environmental modification is essential to reduce the burden of malaria over a long term. The interventions focus on preventing the creation of vector breeding areas, changing natural habitats, or improving human habitation to reduce the abundance of mosquitoes while creating minimal adverse environmental and social impacts. This approach is mostly applicable in urban and peri-urban areas and its success requires community participation and to a greater extent intersectoral collaboration (Mukabana et al. 2006).

The larval control approach can be adequate on the condition that breeding sites can be mapped and characterised, especially in urban and peri-urban areas. Therefore, environmental management, community participation and suitable chemical and biological control agents are important for larval control (Walker and Lynch 2007). Chemical larvicides such as Temephos and aquatain (Bukhari et al. 2011b) and insect growth regulators such as methoprene are effective in suppressing larval populations (WHO 1997). Biological control agents such as the bacteria *Bacillus thuringiensis israelensis* (Bti) and *B. sphaericus* (Fillinger et al. 2003, Fillinger and Lindsay 2006, Geissbühler et al. 2009) and entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* (Lacey et al. 1988, Bukhari et al. 2011a) have been used successfully to control mosquito larvae. In addition, predators such as the larvivorous fish *Gambusia affinis* have been used to control mosquitoes for over 100 years (Bay 1967). However, larval control is not extensively used to prevent malaria transmission since the process is considered to be labour intensive and contributes less to reduction of vectorial capacity (Walker 2002).

Vector control in Africa is almost exclusively based on indoor application of chemical insecticides in the form of insecticide treated bed nets (ITNs) or indoor residual spraying (IRS) of walls and ceilings (Maxwell et al. 2002, Lengeler 2004, Mabaso et al. 2004). The use of ITNs is the most promising amongst other personal protection measures in reducing malaria morbidity and mortality at community and individual levels (Lengeler 2004), and also reduces vector populations (Killeen and

Smith 2007). The more effective and desirable long lasting insecticidal nets (LLINs) are widely in use and are being adopted to replace the use of ITNs (WHO 2009). In addition, other personal protection measures include wearing protective clothing, use of repellents and improved housing. Indoor residual spraying (IRS) is the most powerful way to rapidly reduce malaria transmission by reducing the survival of malaria vectors entering houses or sleeping units (WHO 2009).

Although ITNs and IRS are considered to be the most effective vector control interventions, their sustainable use is undermined by problems of insecticide resistance in mosquito populations. Growing concern to these problems has increased interest in the search for alternative approaches that rely less on chemicals to be integrated with the existing ones (Zaim and Guillet 2002). Biological control with the use of entomopathogens presents an alternative. At present, the principal biological control agents that have been successfully used against mosquitoes are predators, particularly fish, and the bacterial pathogens *Bacillus thuringiensis israelensis* (Bti) and *Bacillus sphaericus* (Bs) that attack the larval stages of the mosquito (Das and Amalraj 1997). Strategies under development include bacteria such as *Wolbachia* spp against adult mosquitoes (McMeniman et al. 2009) and entomopathogenic fungi in the genus *Metarhizium* and *Beauveria* against larvae and adult vectors (Blanford et al. 2005, Scholte et al. 2005, Knols and Thomas 2006, Thomas and Read 2007a). Other organisms showing promise include the nematodes *Romanomermis culicivorax* (Lacey and Lacey 1990), viruses, parasitic protozoa and plant extracts (Scholte 2004, Howard 2010). Of the biological control agents, EPF are most likely to be available for use in integrated vector control programs in the near future.

Entomopathogenic fungi

Entomopathogenic fungi (EPF) are common natural enemies of arthropods worldwide. As a result, researchers have focused on their biology and ecology for understanding on their potential in host population regulation (Roy et al. 2006). Globally, there are over 100,000 species of EPF of which about 750 have been recognised as insect pathogens (Hajek and St Leger 1994). These species have been classified into eight taxonomic divisions namely Basidiomycota, Zygomycota, Ascomycota (formerly Deuteromycota), Glomeromycota, Neocallimastigomycota, Microsporidia, Blastocladiomycota and Chytridiomycota based on their morphological and sexual characteristics (Vega et al. 2009). Molecular techniques are being employed to re-assess classification to correct for possible short-comings encountered with classification based on sexual characteristics (Driver et al. 2000). Within the group Ascomycota, fungi belonging to Sordariomycetes (Chapter 2) are typically opportunistic and have the widest host range. Two of their members *Metarhizium anisopliae* and *Beauveria bassiana* are the most used for insect pest control. Both species are soil-borne, cosmopolitan in distribution, host specific and relatively safe for humans and the environment (Roberts and St. Leger 2004, Zimmermann 2007b, a), characteristics that have triggered attention to develop them as biopesticides for the control of arthropod pests. Currently, over 150 fungus-based pest control products have been developed (de Faria and Wraight 2007) and successfully used for the control of spittle bugs in Brazil, forest pests in China (Li et al. 2010b) and locusts in West Africa (De Groote et al. 1999) amongst other pests.

Fungal pathogens invade their host through the integument and cause death by production of toxins, destruction of vital tissues and depletion of host metabolites (Hajek and St Leger 1994). The infection pathway begins by the attachment of the conidium to the insect cuticle (Figure 3). Under humid conditions, the conidium germinates and forms an appresorium that penetrates through the insect cuticle aided by cuticle-degrading enzymes such as chitinases and proteases (St. Leger 1995). The fungus enters the haemocoel by overcoming the host response and immune defence reaction through production of toxins (e.g. destruxins for the case of *M. anisopliae*). The fungus spreads within the host by formation of hyphal bodies (blastospores) causing damage to host tissues besides

depleting nutrients. Eventually the host dies (Ferron 1978, Gillespie and Claydon 1989). The time from infection until death of the host varies depending on the host species, fungal species, fungal virulence and environmental conditions. Under humid conditions, fungal hyphae grow out of the dead insect and will eventually form new conidia.

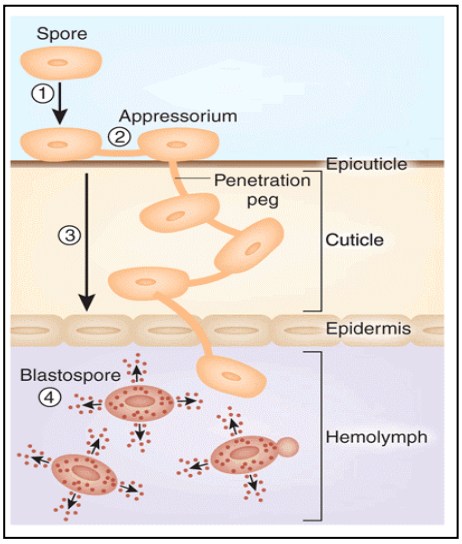

Figure 3. Mode of action of entomopathogenic fungi on contact with the arthropod host (Thomas and Read 2007b)

Because of their mode of action, the relatively slow death of infected target insects, fungal pathogens have been criticised for their potential to control pests and offer protection to humans against insect bites. However, the strategy of using fungal pathogens for malaria vector control poses some advantages over insecticides on the mosquito's capacity to transmit malaria. Despite low virulence, fungi have the potential to kill mosquitoes before they can transmit malaria parasites (i.e. within 10 days) (Scholte et al. 2005). Within this period, the females are likely to be able to mate and reproduce thus limiting risks for evolving resistance in the mosquitoes (Knols and Thomas 2006, Thomas and Read 2007a). Moreover, fungi have demonstrated potential to block malaria parasite transmission inside the mosquito by preventing the development of *Plasmodium* parasites (Blanford et al. 2005).

Despite the challenges facing EPF as biocontrol agents, fungal diseases in insects are common and widespread and virtually all insects are susceptible to fungal infections. A direct impact of EPF in infected pest or vector populations is the reduction of survival rate, which is a major contribution to population suppression (Zimmermann 1993). Indirectly, EPF inflict behavioural changes in the host. A comprehensive review on the impact of EPF on insect behaviour is presented in Chapter 2 of this thesis. Extensive information exists on behavioural changes in plant pests infected with fungus but this is still unexplored in disease vectors, particularly mosquitoes. However, before EPF are fully embraced as a potential method of vector control, further research is needed to assess their specificity, mode of action, and safety in human and environmental health. Methods of mass production procedures and delivery and formulation also need attention (Farenhorst et al. 2008, Farenhorst and Knols 2010, Mnyone et al. 2010a).

Problem definition

So far, our understanding of the dynamics of malaria vector populations in sub-Saharan Africa, their behaviour and ecology, and how these affect transmission of disease is still marginal (Takken and Knols 1999, Ng'habi et al. 2010). Thus, there is a need to exploit the behavioural patterns of malaria mosquitoes with respect to mating, host seeking, sugar feeding and oviposition (Figure 4) in order to reduce contact with human hosts. Therefore, the goal of my study was to investigate the impact of fungal infections on these behaviours. Mating as one of the aspects that characterises mosquito life history is least understood and understudied. But as sexual reproduction for population growth is dependent on it, this aspect should receive the highest attention when seeking new avenues for mosquito control. Mated female *An. gambiae* mosquitoes seek human hosts for a blood meal to complete egg development (Chambers and Klowden 2001). Interrupting mating is therefore likely to reduce host-seeking population of *An. gambiae* females and thereby reduce the chances for malaria

parasite transmission. Studies in which female mosquitoes infected with fungi and allowed to mate with uninfected males, showed successful horizontal transmission of fungus inoculums between the sexes. This may contribute to the spread of fungus within target mosquito populations in the field (Scholte et al. 2004a). It is of interest to know if the same occurs by mating infected males with virgin females and if fungal infection affects the mating performance of males.

Host-seeking is arguably the most important component of mosquito vectorial capacity (Zwiebel and Takken 2004) on which success of the other behaviours depends. Results from a laboratory study in which, female mosquitoes three days post-exposure to fungus *Metarhizium anisopliae* were blood-fed in small cups revealed that fungal infection reduces (but does not eliminate) feeding propensity and fecundity (Scholte et al. 2006). However, it remains unknown whether this is also the case under more realistic conditions, where mosquitoes have to locate their blood host from a distance. Similarly, little is known to what extent the sugar feeding behaviour of mosquitoes is affected by fungal infection. Fungal effects on mosquito activity may lead to a considerable reduction in survival rate and reproduction in the vector population, and thus transmission of disease. Overall, a notable change in behavioural characters in the mosquitoes due to fungal infection is likely to be significant in campaigns aimed for the reduction of malaria infection and transmission.

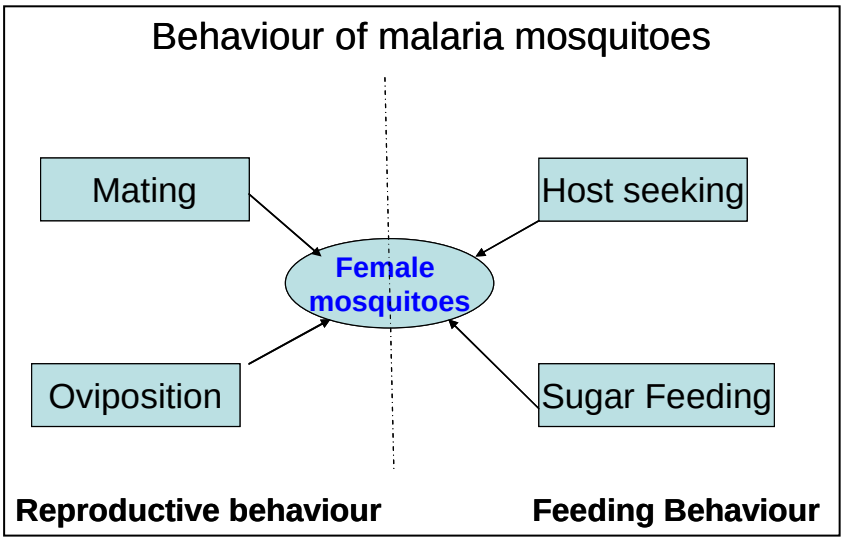

Figure 4. Major life-history behaviours of female mosquitoes (Takken and Knols 1999)

Justification

Malaria as stated above, poses a heavy burden on societies with high morbidity and mortality throughout the tropical world. The transmission rates and risks of the disease can be reduced by vector control which at present is exclusively based on chemical insecticides (Alonso et al. 2011, WHO 2011). However, development of resistance to chemicals is of great concern for sustainable control efforts. Thus, alternative methods for vector control that can be integrated with the existing ones are required. Biological control is one such option and studies in the laboratory and under rural field conditions in Africa have demonstrated the effectiveness of the entomopathogenic fungus (EPF) *Metarhizium anisopliae* ICIPE-30 against adult *An. gambiae* mosquitoes (Scholte et al. 2003a, Scholte et al. 2003b, Scholte et al. 2005). Fungi are slow killing agents, an attribute that gives them an advantage over chemicals in terms of resistance development, and thus impose a reduced risk for

resistance formation in malaria mosquitoes (Thomas and Read 2007a, Read et al. 2009, Knols et al. 2010). Additionally, EPF have demonstrated potential against malaria vectors that have developed resistance to insecticides (Farenhorst et al. 2009, Howard et al. 2010a, Kikankie et al. 2010) and expresses synergistic effects when used in combination with conventional insecticides (Farenhorst et al. 2010). Although biological control has reached an advanced development for many insect pest species, it is still at its infancy for disease vectors particularly mosquitoes. Under development are the mass production procedures and formulation and delivery methods of candidate biocontrol agents. However, the behavioural and ecological consequence of fungal infections in mosquitoes, that is how the fungus alters their behaviour, ecology and fitness, has not been fully addressed. This is important when a large community setting is to be targeted to evaluate the potential public health benefits of the fungus control approach. Therefore, the current thesis research aimed to investigate the efficacy of *M. anisopliae* ICIPE 30 on important life-history behaviours of mosquitoes to facilitate the further development of malaria vector control based on biological control agent. At later stages, the findings may be further incorporated in disease transmission models to gauge their full impact on the Entomological Inoculation Rate (EIR), a measure of malaria risk (Hancock et al. 2009).

Objectives of the study

General objective

The principle goal of the study is to provide baseline information on mosquito-fungus interaction, focusing on the effects of entomopathogenic fungi (EPF) on the major life-history behaviours of the African malaria vector *Anopheles gambiae* under controlled and semi-natural situations.

Specific objectives

The specific objectives were to

1. Establish a method that successfully infects mosquitoes with spores of entomopathogenic fungi in the laboratory;
2. Assess the host-seeking response and olfactory discrimination capability of *Anopheles gambiae* mosquitoes on infection with entomopathogenic fungi;
3. Determine the house entry and the outdoor and the indoor hourly human-biting responses of *An. gambiae* infected with entomopathogenic fungi;
4. Determine the effects of fungal infection on food uptake and survival of *An. gambiae* mosquitoes fed on plant sugars;
5. Assess the host-seeking behaviour and fecundity of the malaria mosquito *An. gambiae* on infection with entomopathogenic fungi;
6. Determine the effects of fungal infection on the mating performance of *An. gambiae* and the probability of horizontal transmission;

The outline of the thesis is as follow:
In **Chapter 2** a review of mosquito-fungus interactions, focusing on behavioural effects of fungal pathogens on insects is presented. The review gathered published information on how various insect behaviours are affected by infection with EPF and highlighted if the observed behavioural changes impose direct effects on the efficacy of EPF as biological control agents.

Chapter 3: Contact between fungal conidia and insects are essential for their infection with EPF. Besides, understanding the factors that affect fungal pathogenicity is necessary in developing them as microbial control agents. This chapter describes experiments that aimed to identify a standard

method of infecting mosquitoes with fungal conidia and establish the conidial formulation, dosage and exposure time to enhance pathogenicity.

Chapter 4: Malaria vectors locate their human hosts through olfactory cues. Olfaction enables mosquitoes to further differentiate odours from different human individuals. This chapter describes experiments conducted to study if fungal infection affects mosquito host-seeking responses.

Chapter 5: The success of malaria vectors in transmitting the disease is influenced by their host-seeking capability to locate and bite human hosts indoors and outdoors. For malaria control, it is essential to interrupt the host-seeking capability and this chapter describes experiments employed to test the potency of EPF on the house-entry and the indoor and the outdoor human-biting responses of *An. gambiae*.

Chapter 6: Prior to host-seeking, survival of both sexes of mosquitoes is linked to the plant community. Therefore control measures sought for at this stage could target both sexes. This chapter describes experiments conducted to study the effects of EPF on feeding capability and survival rates of *An. gambiae* mosquitoes exposed to plant sugars.

Chapter 7: This chapter describes experiments that aimed to examine the effects of fungal infection on the host-seeking behaviour, feeding (blood) propensity and the oviposition rate of *An. gambiae* mosquitoes. The behavioural traits are crucial in disease transmission and vector population build-up.

Chapter 8: Mating behaviour is paramount in enhancing the reproductive success of the mosquito population. The activity often occurs prior to host-seeking, and thus its interference would substantially suppress mosquito populations. This chapter depicts experiments aimed at examining the effects of EPF on the mating performance of male mosquitoes and the likelihood of transferring fungal conidia to conspecific healthy females during mating.

Chapter 9: This chapter is the summarizing discussion of the results from the experimental chapters. It discusses the implications of behavioural changes observed due to fungal infection and the possibility of using EPF for the control of malaria vectors. Suggestions for future work and the conclusions of the current research are highlighted.

Part I

INFECTION METHODOLOGY AND ETHOLOGY OF INSECT-FUNGUS INTERACTIONS

Chapter 2

Behavioural effects of entomopathogenic fungi on insects - a review

To be submitted as: Ondiaka S., Takken W., Koenraadt C. J. M., Mukabana W. R. Behavioural effects of entomopathogenic fungi on insects - a review.

Abstract

Several species of fungi are known to infect and cause mortality in insects. Entomopathogenic fungi (EPF) infect the host through the cuticle and proliferate in the haemolymph. Infection depends on virulence of the pathogen, susceptibility of the host and environmental factors. The infection process can give rise to behavioural changes in the host, which in turn may affect the performance of EPF as biological control agents. This paper presents a review of available information on the effects of EPF on insect behaviour and discusses the implications for biological control.

Introduction

Fungal diseases in insects are common and widespread and can cause spectacular epizootics, resulting in the decimation of insect populations. Many entomopathogenic fungal species may therefore be considered as an important factor regulating natural insect pest populations (Carruthers and Soper 1987, Bidochka et al. 2000). Subsequently, they hold potential as microbial control agents of insect pests and disease vectors. The host range of entomopathogenic fungi (EPF) varies between species and between isolates of the same species. So far, at least 90 genera and more than 700 species of EPF have been identified (Roberts and Humber 1981, Hajek and St Leger 1994, Hajek 1997). Of these, approximately 10 species (Table 1) are currently being utilised for insect control (Hajek and St Leger 1994, Bidochka et al. 2000) and more than 170 fungus-based biopesticides have been developed and commercially available for pest control, although some of them have been withdrawn from the market (de Faria and Wraight 2007).

Entomopathogenic fungal species are distributed in eight phyla namely Basidiomycota, Ascomycota (formerly Deuteromycota), Glomeromycota, Zygomycota, Blastocladiomycota, Neocallimastigomycota, Chytridiomycota and Microsporidia (Vega et al. 2009). The majority of the EPF currently under research either belong to the class Zygomycetes and order Entomopthorales in the Zygomycota or the class Sordariomycetes and order Hypocreales in the Ascomycota (Shah and Pell 2003, Furlong and Pell 2005, Roy et al. 2006, Keller 2008) and are pathogenic to terrestrial and aquatic insects. The important EPF in the order Hypocreales are *Beauveria*, *Metarhizium*, *Nomuraea*, *Cordyceps* and *Lecanicillium* and in the order Entomophthorales *Entomophthora*, *Zoophthora*, *Pandora* and *Entomophaga*. Fungi that exclusively attack aquatic insects generally belong to the Chytridiomycota (Butt and Goettel 2000).

Entomopathogenic fungi attack their host by direct penetration of the cuticle and do not need to be ingested like viruses, bacteria and microsporidia (Gillespie and Claydon 1989, St. Leger 1995). They are therefore amenable for control of piercing-sucking insects (Homoptera and Heteroptera) as opposed to bacteria, viruses or microsporidia. Moreover, conidia of EPF can be transmitted horizontally.

A substantial number of entomopathogenic fungus-based biopesticides have been developed worldwide for the control of insect pests and disease vectors (de Faria and Wraight 2007). In addition to mortality, the infection by EPF can induce changes in the host which include feeding, reproduction potential and survival. For instance, reduction in food consumption, blood meal intake (Moore et al. 1992, Ekesi and Maniania 2000, Scholte et al. 2006, Ondiaka et al. 2008a), mating performance and fecundity (Scholte et al. 2006, Dimbi et al. 2009) has been reported in a number of insects as fungal infection develops. Generally, the survival of fungus-infected insects is reduced in case of virulent isolate or highly susceptible host (Hajek and St Leger 1994). On the other hand, the survival can be longer in case of suboptimal concentration of the inoculum or lack of susceptibility of the host to fungal infection (Zimmermann 1993), which may result in compromising the insect's behaviour. The extent to which the behaviour is affected remains elusive. The purpose of this review is therefore to assemble available information on the most important EPF that impact behavioural changes in insects. These fungi belong to the genera *Pandora (Erynia)*, *Entomophaga*, *Zoophthora*, *Entomophthora*, *Lecanicillium*, *Metarhizium* and *Beauveria* (Table 1). We have highlighted the behavioural changes caused by these fungal pathogens in various insect species with a summary presented in Table 2. The implication of the behavioural changes on the performance of fungal pathogens in reducing pest and vector populations is further discussed.

Table 1. Genera and species of entomopathogenic fungi (after (Bidochka et al. 2000, Roy et al. 2006).

Phylum	Class	Order	Family	Genus	Species
Zygomycota	Zygomycetes	Entomophthorales	Entomophthoraceae	*Pandora (=Erynia)*	*neoaphidis*
				Entomophaga	*aulicae*
					maimaiga
					grylii
				Zoophthora	*radicans*
				Entomophthora	*muscae*
					schizophorae
Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	*Lecanicillium* (=*Verticillium*)	*longisporum*
					muscarium[*]
					lecanii[*]
				Metarhizium	*anisopliae*
					flavoviridae
				Beauveria	*bassiana*
					brongniartii
				Nomuraea	*rileyi*
	Eurotiomycetes	Eurotiales	Trichocomaceae	*Isaria (=Paecilomyces)*[*]	*fumosorosea*[*]
					lilacinus[*]

[*]Genera and species groups with no behavioural impact on insects.

1. Zygomycota

Members of this phylum possess a great potential as biological control agents due to their ability to cause epizootics, resulting in the natural regulation of insect populations. Intensive studies have been carried out with the aim of understanding disease distribution, epidemiology and management in crops (Pell et al. 2001). The fungi are obligate pathogens and do not produce toxins for the progression of an infection. They exhibit parasitic relationships with their insect hosts through which they keep the host alive until all resources are exhausted. Further, they are widely distributed and express specificity to the host insects (Roy et al. 2006). Five genera in the order Entomophthorales have been reported to trigger behavioural changes in several insect orders.

1.1 *Pandora (=Erynia)*

Aphids (Homoptera: Aphididae) are insect pests of many crops and important vectors of plant diseases. Despite several EPF being effective against aphids, the fungus *Erynia neoaphidis* (Remaudière and Hennebert), re-classified as *Pandora (Erynia) neoaphidis* (Remaudière and Hennebert) Humber, is the most specific to aphids (Baverstock et al. 2005). The fungus causes spectacular epizootics in pea aphid *Acyrthosiphon pisum* (Harris) populations (Dean and Wilding 1971, Feng et al. 1992, Barta and Cagáň 2006). Infected aphids exhibit modifications in their feeding (perching), mating and oviposition behaviours. Jensen et al. (2001) observed that *P. neoaphidis*-infected pea aphids crawl to the undersides of alfalfa leaves or move off the plants to the surrounding habitat. In contrast, Roy et al. (1999) observed that infected pea aphids settle on the upper surfaces of plant leaves and hardly move away from the plant. When the insects drop off the plant, they take more time to climb back to the plant. On the other hand, the less mobile wheat aphid *Sitobion avenae* (Fabricius) re-locates and remains stationery in upper sections of the plant (Roy et al. 2002). The re-location or further moving off the plant may be a strategy to increase fitness of the infected ones and to prevent conspecifics from fungal infections (Jensen et al. 2001, Roy et al. 2006) or a fungal-induced modification to increase the within-plant dispersal and transmission of the fungus. Aphids are highly sensitive to alarm pheromones since these are important for their movement away from predators. Aphids infected by *P. neoaphidis* become less sensitive to alarm pheromone or, if they do respond, then the ability to detect the pheromone is reduced (Roy et al. 1999, Baverstock et al. 2005). In other instances, production of the alarm pheromone is increased in *P. neoaphidis*–infected individuals although they remain less sensitive to alarm pheromone emitted by other, healthy, conspecifics (Roy et al. 2005). The modified behaviour associated with response to alarm pheromone impacts negatively on reproductive success in aphid populations. *Pandora neoaphidis* has also been reported to reduce the frequency with which the hymenopterous parasitoid *Aphidius rhopalosiphi* (De Stefani-Peres) oviposits in *Metopolophium dirhodum* infected (Walker) nymphs (Brobyn et al. 1988).

1.2 *Entomophaga*

Entomophaga aulicae (Reichardt in Bail) Batko is considered a complex of species but two species namely *E. aulicae* and *E. maimaiga* (Hajek et al. 1990, Hajek et al. 1991) are important pathogens of many species of Lepidoptera (Hajek and Eastburn 2003). Healthy arctiid caterpillars *Chionarctia nivea* (Ménétriés) forage on the ground and feed on leaves of small herbaceous plants. Similarly, gypsy moth *Lymantria dispar* (Linnaeus) larvae forage both belowground and aboveground depending on the larval stage. This foraging behaviour changes whereby *E. maimaiga*-infected larvae (Hajek 1989, Hajek and Soper 1991, Hajek 2001) and *E. aulicae*-infected caterpillars (Yamazaki et al. 2004) crawl up high into grasses, herbs, tree canopies and tree trunks. This is often described as behavioural fever response that allows the host to eliminate the fungus by heat therapy from the sun (Karban 1998). In addition, the response facilitates dispersal and transmission of conidia to other

lepidopteran larvae by wind. In larvae of the spruce budworm, *Choristoneura fumiferana* (Clemens), and of the forest tent caterpillar, *Malacosoma disstria* (Hubner), infection with *E. aulicae* caused reduction in feeding activity during late days of infection (Tyrrell 1990).

1.3 *Zoophthora*

The species *Zoophthora radicans* (Brefeld) Batko is widely distributed and arguably pathogenic to many insect pests, especially lepidopteran and homopteran species (Wraight et al. 2003). Infection by *Z. radicans* has been reported to induce behavioural changes in some lepidopteran larvae. For example, in the diamondback moth, *Plutella xylostella* (Linnaeus), the sexual attractiveness was altered due to a reduction in release of sex pheromones by infected females (Reddy et al. 1998); however, feeding capacity was not affected (Furlong et al. 1997). In advanced stages of infection (three days after infection), the males completely fail to become attracted to the females and die on day four. In this case, reproductive fitness of the insect is compromised although not beneficial to the fungus, which requires three to four days to colonise the host (Furlong et al. 1995).

1.4 *Entomophthora*

The genus *Entomophthora* primarily infects dipteran insects such as adult mosquitoes (Culicidae); midges (Chironomidae); blackflies (Simuliidae); dance flies (Empididae) and others. The fungal infections are host specific and are therefore rarely transmitted from one species to another (Brobyn and Wilding 1983). When transmission occurs, the infection rate is very low (Steinkraus and Kramer 1987). The species is highly pathogenic to dung flies and house flies. During the summer months in Europe and America large numbers of the housefly, *Musca domestica* (Linnaeus), the yellow dungfly, *Scatophaga stercoraria* (Linnaeus), and the carrot flies, *Psila rosae* (Fabricius), generally succumb to infection by *Entomophthora muscae* (Cohn) Fresenius (Mullens et al. 1987). Perching behaviour in fungus-infected houseflies is not affected. In dung flies, however, fungus-infected insects climb and settle on the downwind side of the upper parts of a plant where they perch abnormally in a highly specific manner. In contrast, healthy flies feed on the upper surface of the low canopies (Mullens et al. 1987). In this way, healthy flies are susceptible to infection when fungal conidia are dispersed from the cadavers of infected insects (Maitland 1994). The sexual behaviour of the infected house flies (Moller 1993, Watson and Petersen 1993) and dung flies (Maitland 1994) is not affected but the fungus *E. muscae* enhances its own transmission by causing fungus-killed females to be highly attractive sexually to healthy male flies. The attraction in male flies is a function of visual, chemical and fungus-induced cues (Moller 1993). Although mating and oviposition behaviours were not affected during the early days of infection with *E. muscae*, during late days of infection, mating activity is reduced, fewer females are mated and inseminated, resulting in a reduction of fecundity (Watson and Petersen 1993). Oviposition behaviour in carrot flies *P. rosae* in the early days of infection by *Entomophthora schizophorae* (Keller and Wilding) was not affected but, instead, the females relocated and oviposited in elevated positions away from the food plant. In later days of infection i.e. 5 days, egg-laying behaviour is similar to that of healthy flies (Eilenberg 1987). House flies infected with *E. muscae* frequent areas with high temperatures that range between 35-42° C in the early stages of infection and cooler areas shortly before death. In contrast, healthy flies preferred a temperature range between 26-35° C. These behavioural changes are beneficial for the insect to combat the infection (Watson et al. 1993, Kalsbeek et al. 2001). Manipulating the reproductive capacity can effectively reduce the pest status of insects.

2.0 Ascomycota

Members of the Ascomycota are generally considered to be opportunistic pathogens infecting a wide range of insect orders. Host death is often associated with a combination of mechanical damage to

Table 2. Behavioural alterations in insects infected by entomopathogenic fungi

Infecting fungal species	Insect common name	Insect species	Behavioural effects	References
Pandora neoaphidis	Pea aphid	*Acyrthosiphon pisum*	Migration off the plant and preference to settle on the underside rather than on the upper side of leaves	(Jensen et al. 2001)
			No migration and infected aphids remain on the upper side of leaves	(Roy et al. 1999, Roy et al. 2002)
			Increased production of alarm pheromone and a reduction in response to it	(Roy et al. 1999, Roy et al. 2005)
Entomophaga aulicae	Arctiid caterpillar	*Chionarctia nivea*	Effects migration from the ground to the summit of stems or grasses	(Yamazaki et al. 2004)
,,	Forest tent caterpillar	*Malacosoma disstria*	Reduced larval food consumption	(Tyrrell 1990)
,,	Spruce budworm	*Choristoneura fumiferana*	Reduced larval food consumption	(Tyrrell 1990)
Entomophaga maimaiga	Gypsy moth	*Lymantria dispar*	Migration from the ground to the tree summit and preference to settle on the underside of leaves	(Hajek and Soper 1991, Hajek 2001)
Entomophaga grylli	Grasshopper	*Camnula pellucida*	Basks in the sun to increase body temperatures	(Carruthers et al. 1992)
Zoophthora radicans	Diamondback moth	*Plutella xylostella*	Reduced larval food consumption and oviposition rate	(Furlong et al. 1997)
			Inhibited response to and production of sex pheromones in adults	(Reddy et al. 1998)
Entomophthora muscae	Housefly	*Musca domestica*	Induced behavioural fever with which infected flies migrate to sun-lit areas to curb infection	(Watson et al. 1993, Kalsbeek et al. 2001)
			Increased sexual attraction of healthy males to fungus-killed females over healthy ones	(Moller 1993, Zurek et al. 2002)
			Increased sexual attraction of healthy males to fungus-killed females but perching behaviour is not altered	(Mullens et al. 1987)

			Reduced sexual response of males that cause reduction in insemination rate and fecundity	(Watson and Petersen 1993)
"	Yellow dungfly	*Scatophaga stercoraria*	Altered perching behaviour that cause dungfly to rest on the underside of leaves	(Maitland 1994)
"	Carrotfly	*Psila rosae*	Induced abnormal oviposition behaviour whereby, eggs are laid away from instead of close to food plant	(Eilenberg 1987)
Entomophthora schizophorae	Housefly	*M. domestica*	Induced behavioural fever with which, infected flies migrate to sun-lit areas to curb infection	(Kalsbeek et al. 2001)
Lecanicillium longisporum	Green peach aphid	*Myzus persicae*	Migration to tree summit to curb infection, reduced food consumption and production of fewer offspring	(Roditakis et al. 2008)
Metarhizium anisopliae	Mosquito	*Anopheles gambiae*	Reduced blood feeding and fecundity	(Scholte et al. 2006)
"		*An. gambiae*	Reduced fecundity and healthy males are infected with fungus-infected females during mating	(Scholte et al. 2004a)
"		*An. stephensi*	Reduced blood feeding propensity	(Blanford et al. 2005)
"		*An. stephensi*	Behavioural fever not induced	(Blanford et al. 2009)
"		*Culex quinquefasciatus*	Reduced blood feeding in wild population that is resistant to various insecticides	(Howard et al. 2010b)
"	Desert locust	*Schistocerca gregaria*	Reduced food consumption	(Moore et al. 1992, Roy et al. 2006)
			Reduced fecundity and induced behavioural fever to curb infection	(Blanford and Thomas 2001)
			Reduced food consumption, reduced fecundity and induced behavioural fever to curb infection	(Arthurs and Thomas 2001)
			Reduced food consumption and flight capability	(Seyoum et al. 1994, Seyoum et al. 2002)
"	Senegalese grasshopper	*Oedaleus senegalensis*	Induced behavioural fever with which infected individuals migrate to sun-lit areas to curb infection	(Blanford et al. 1998)
"	Migratory locust	*Locusta migratoria*	Induced behavioural fever with which infected locusts migrate to sun-lit areas to curb infection	(Ouedraogo et al. 2003, Ouedraogo et al. 2004)
"	Variegated grasshopper	*Zonocerus variegatus*	Reduced food consumption	(Thomas et al. 1997)

Table No. 2 continued

Infecting fungal species	Insect common name	Insect species	Behavioural effects	References
"	Brown locust	*Locustana pardalina*	Reduced feeding propensity and fecundity rate was similar to that of healthy individuals	(Arthurs and Thomas 2000)
"	Mediterranean fruit fly	*Ceratitis capitata*	Reduced fecundity and fertility	(Quesada-Moraga et al. 2006)
"	Tephritid fruit fly	*Ceratitis spp.*	Reduced mating performance and competitiveness of the male flies	(Dimbi et al. 2009)
"	Sweetpotato weevil	*Cylas puncticollis*	Reduced food consumption, fecundity and egg fertility	(Ondiaka et al. 2008a)
"	Longhorned beetle	*Anoplophora glabripennis*	Reduced oviposition rate and egg fertility	(Hajek et al. 2006, Hajek et al. 2008)
"	Spotted stem borer	*Chilo partellus*	Reduced larval food consumption	(Tefera and Pringle 2003)
"	Legume flower thrips	*Megalurothrips sjostedti*	Reduced pollen consumption, fecundity and egg fertility	(Ekesi and Maniania 2000)
"	Pea leafminer	*Liriomyza huidobrensis*	Reduced feeding propensity and the oviposition rate	(Migiro et al. 2011)
"	Legume pod borer	*Maruca vitrata*	Reduced fertility	(Ekesi et al. 2002)
"	Pod sucking bug	*Clavigralla tomentosicollis*	Reduced fertility	(Ekesi et al. 2002)
Beauveria bassiana	Mosquito	*Cx. quinquefasciatus*	Reduced blood feeding	(Howard et al. 2010b)
"		*An. stephensi*	Reduced blood feeding propensity and the flight capability	(Blanford et al. 2005, Blanford et al. 2011)
"		*Aedes aegypti*	Increased chance for transfer of fungal conidia from fungus-infected to healthy conspecifics during mating	(Garcia-Munguia et al. 2011)
"	Plant bug	*Lygus hesperus*	Reduced food consumption and the oviposition rate	(Noma and Strickler 2000)
"	Pea aphid	*Acyrthosiphon pisum*	Increased production of alarm pheromone and a reduction in response to it	(Roy et al. 2005)
"	Mediterranean fruit fly	*Ceratitis capitata*	Reduced fecundity and egg fertility	(Quesada-Moraga et al. 2006)
"	Wasp	*Cephalonomia tarsalis*	No effects on the mobility to forage and on the oviposition behaviour	(Lord 2001)

"	Leafhopper	*Cnaphalocrosis medinalis*	Reduced food consumption	(Sivasundaram et al. 2008)
"	Tarnished plant bug	*Lygus lineolaris*	Reduced food consumption	(Sabbahi et al. 2008)
"	Fruit fly	*Anastrepha ludens*	No effects on male mating competitiveness but reduced the quantity of sperm transferred	(Novelo-Rincon et al. 2009)
"	Cowpea leaf beetle	*Ootheca mutabilis*	Reduced feeding propensity	(Ekesi 2001)
"	Spotted stem borer	*Chilo partellus*	Reduced larval food consumption	(Tefera and Pringle 2003)
"	Grain beetle	*Tribolium castaneum*	Reduced fecundity	(Pedrini et al. 2010)
"	Sweet potato weevil	*Cylas puncticollis*	Reduced food consumption, fecundity and egg fertility	(Ondiaka et al. 2008a)
"	Mexican fruit fly	*Anastrepha ludens*	Altered male mating performance, reduced fecundity and enhanced conidial transfer to conspecifics during mating	(Toledo et al. 2007)
"	Colorado potato beetle	*Leptinotarsa decemlineata*	Reduced food consumption and fecundity	(Fargues et al. 1991, Fargues et al. 1994)
Beauveria brongniartii	Longhorned beetle	*Anoplophora glabripennis*	Reduced oviposition rate	(Hajek et al. 2006)
Nomuraea rileyi	Cotton leafworm	*Spodoptera littoralis*	Reduced food consumption	(Fargues and Rodriguez-Rueda 1980)

internal organs, nutrient depletion and toxin production overwhelming host-defence responses (Samuels et al. 1988, Gillespie and Claydon 1989, Shah and Pell 2003, Roy et al. 2006). Unlike Zygomycetes, Ascomycetes are characterised by a well-defined parasitic phase within insect hosts and saprophytic phases upon death of their host. Three genera in the order Hypocreales assert behavioural changes in insects.

2.1 *Lecanicillium*

The genus *Lecanicillium* Zare & Gams (formerly the *Verticillium* species complex) is widely distributed and includes species such as *L. longisporum* and *L. muscarium* that are pathogenic on a wide range of insects (Zare and Gams 2001, Goettel et al. 2008). Besides causing mortality in insect pests, *L. longisporum* induces changes in movement, reproduction and feeding behaviours of the green peach aphid *Myzus persicae* (Sulzer). For instance, reproduction rate and food intake (indicated by a decline in honeydew excretion) are reduced in infected aphid. In the early days of infection, movement is increased in infected aphids and this contributes to the spread of the fungus to other individuals (Roditakis et al. 2008).

2.2 *Metarhizium*

Metarhizium has a worldwide distribution and is found in every habitat type, from arctic to the tropics on insects as well as in the soil (Bidochka et al. 2000, Zimmermann 2007b). *Metarhizium anisopliae* consists of four sub-species (Driver et al. 2000) of which *M. anisopliae* var. *acridum* (formerly *M. flavoviridae*) and *M. anisopliae* (Metschnikoff) Sorokin are most widely used fungus throughout the world for control of insect pests. *Metarhizium anisopliae* occurs on a wide range of invertebrate hosts including insects (Boucias and Pendland 1998). For detailed information on the taxonomy and mycopesticides of *Metarhizium anisopliae* see Roberts and St. Leger (2004) and Zimmermann (2007b).

2.3 *Beauveria*

Beauveria like *Metarhizium* is widely distributed in nature on insects and in soil (Rehner 2005). It has been reported on more than 700 species of insects (Goettel et al. 1990, Inglis et al. 2001). Two species, *B. bassiana* (Balsamo) Vuillemin and *B. brongniartii* (Saccardo) Petch, are commonly used for control of insect pests (Zimmermann 2007a) and has been developed as commercial mycoinsecticides (Shah and Pell 2003). For detailed information on taxonomy and mycopesticides of *Beauveria bassiana* see Zimmermann (2007a).

Infection by *M. anisopliae* and *B. bassiana* results in behavioural changes in the infected host. Behavioural fever is a common host response to many pathogens in some insect species that are able to regulate and maintain body temperatures to a level that suppresses pathogen growth (Inglis et al. 1996, Inglis et al. 1997). Infected insects achieve this by basking in the sun to elevate the temperature. This behaviour occurs particularly in orthopteran insects such as locusts and grasshoppers infected with *M. anisopliae* (Seyoum et al. 1994, Blanford et al. 1998, Blanford and Thomas 2000, Arthurs and Thomas 2001, Blanford and Thomas 2001, Gardner and Thomas 2002, Seyoum et al. 2002, Ouedraogo et al. 2003, Ouedraogo et al. 2004). Behavioural responses have also been observed in the mosquito *Anopheles stephensi* (Liston) infected with *M. anisopliae* or *B. bassiana* (Blanford et al. 2009, Blanford et al. 2011) and in the wasp *Cephalonomia tarsalis* (Ashmead) infected with *B. bassiana* (Lord 2001). Insects with raised temperature i.e. “developed fever” take longer to die than those maintained at temperatures preferred by healthy insects. Despite the extended survival, the insect fitness is compromised as these “fever” insects fail to reproduce (Elliot et al. 2002). The process is inevitably not beneficial to the insect.

The occurrence of behavioural fever affects other processes such as feeding efficiency, reproductive potential and escape from predation. Several studies have reported a reduction in locust feeding associated with *M. anisopliae* infection (Moore et al. 1992, Seyoum et al. 1994, Thomas et al. 1997, Arthurs and Thomas 2000, Arthurs and Thomas 2001, Gardner and Thomas 2002, Roy et al. 2006), which again is an important behavioural consequence of infection with cumulative benefits in terms of overall control. In other insects, *M. anisopliae* infection reduces food consumption in the pea leafminer *Liriomyza huidobrensis* (Blanchard) (Migiro et al. 2011), thrips *Megalurothrips sjostedti* (Trybom) (Ekesi and Maniania 2000), sweet potato weevil *Cylas puncticollis* (Boheman) (Ondiaka et al. 2008a), cowpea leaf beetle *Ootheca mutabilis* (Sahlberg) (Ekesi 2001), stemborer *Chilo partellus* (Swinhoe) (Tefera and Pringle 2003), plant bug *Lygus lineolaris* (Palisot de Beauvois) (Sabbahi et al. 2008), leaffolder *Cnaphalocrosis medinalis* (Guenee) (Sivasundaram et al. 2008), plant bug *Lygus hesperus* (Knight) (Noma and Strickler 2000) and blood feeding in anopheline and culicine mosquitoes infected by *M. anisopliae* and or *B. bassiana* (Blanford et al. 2005, Scholte et al. 2006, Howard et al. 2010b, Blanford et al. 2011). An increase in searching behaviour, in the number of eggs oviposited and in the reproductive capability of locusts is apparent in the early days of infection and that is reduced later as the insects near death (Arthurs and Thomas 2000, Arthurs and Thomas 2001). The oviposition has also been reported to decrease in late stages of infection before death in other groups of insects infected with *M. anisopliae* or *B. bassiana* such as the Asian longhorned beetle *Anoplophora glabripennis* (Motschulsky) (Hajek et al. 2006, Hajek et al. 2008), the fruit fly *Ceratitis capitata* (Wiedemann) (Quesada-Moraga et al. 2006), the mosquito *Anopheles gambiae* (Giles) (Scholte et al. 2006), the plant bug *Lygus hesperus* (Noma and Strickler 2000), the sweet potato weevil *Cylas puncticollis* (Ondiaka et al. 2008a) and the pea leafminer *Liriomyza huidobrensis* (Migiro et al. 2011) However, mating behaviour is not impaired following infection by *M. anisopliae* and *B. bassiana* in many insects such as mosquitoes (Scholte et al. 2004a, Garcia-Munguia et al. 2011), fruit flies (Toledo et al. 2007, Dimbi et al. 2009, Novelo-Rincon et al. 2009), house flies (Zurek et al. 2002), termites (Rath 2000) and beetles (Pedrini et al. 2010).

Discussion

Although many and diverse fungal species infect arthropods, of these the Hypocreales (Ascomycetes) and Entomophthorales (Zygomycetes) have a significant impact on insect behaviour. All the major life history behaviour of the insects that include feeding and reproduction are strongly affected. Furthermore, the interaction between fungi and insects is parasitic whereby the fungi attack the insects and the insects respond with a defence against the fungi.

Many Entomophthorales are host-specific and have minimal or no infectivity to non-target organisms, which is one of the attributes when being considered for biological control of insect pests. The interest is mainly due to their potential to cause epizootics in nature and also offer opportunity for conservation and inoculation biocontrol strategies (Hajek and St Leger 1994). However, one of the constraints of using Entomophthorales in inoculation and inoculative strategy is the difficulty to mass produce them due to their specific nutritional requirements. Hypocreales, by contrast, have a wide range of hosts which may be a concern for the safety of non-target species. However, these fungi are considerably easier to mass produce and have a good shelf-life. They represent almost the totality of the biopesticides commercially available worldwide (Zimmermann 1993, Zimmermann 2007b).

Both Entomophthorales and Hypocreales induce behavioural changes in their hosts. Feeding behaviour is the most studied behavioural trait because of its direct impact on crop damage and disease transmission. The efficacy of a pathogen is generally evaluated in terms of mortality; however, feeding behaviour can offset mortality which occurs much later. A reduction in feeding in insects infected with Entomophthorales occurs at a later stage of infection than with Hypocreales

infections. This delay is fungus-induced to allow the host to feed and grow for as long as possible, ensuring maximum growth and reproduction of the pathogens. The reproductive fitness of an individual is directly dependent on the number of viable offspring produced, and both the pathogen and the host adopt strategies to maximise reproductive output. Therefore, it is not surprising that a number of studies report modifications of host-reproductive behaviour ranging from direct effects on fecundity to changes in the production of and response to alarm and sex pheromones. Both hypocrealean and entomophthorean fungi manipulate the insects by ensuring that infected hosts oviposit normally but produce fewer progeny (Blanford and Thomas 2001). Indirectly, fungal infection reduces the pest population by allowing insects to produce fewer progeny than the uninfected ones. Generally, insects infected with entomophthoralean fungi often climb to the top of plants just prior to death ("summit disease syndrome") where they die firmly clasping the plant. The movement is a fungal strategy that ensures that the spores contact potential hosts within and beneath the plant canopy. This mode of spore dispersal is uncommon in insects infected with hypocrealean fungi. The ability of a pathogen to infect, cycle and disperse is an important factor in the development of natural epizootics in insect population.

The behavioural response of insects to fungal infections is mostly advantageous as highlighted in this review except in the early stages of infection (often referred to as the incubation period) whereby hosts behave normally as their healthy conspecifics. During this period, insects respond to the invading pathogen by triggering the immunological defense mechanism. For instance, some infected pests climb to high positions on the plant where they are exposed to the sun while grasshoppers and locusts migrate and settle in sunlit areas. The aim is to raise their body temperature to a level that suppresses disease progression irrespective of fungal virulence. The incubation period is a prerequisite in fungal pathogenesis. The pathogen undergoes an infectious process which involves attachment of the conidium to the insect cuticle, germination of the conidium on the insect, penetration of the cuticle, growth of the fungus in the haemocoel, production of toxins and death of the host (Ferron 1978, Hajek and St Leger 1994, Rath 2000, Zimmermann 2007b, a). The process may last up to at least three days depending on the host species, fungal species, conidial virulence, fungal dose, exposure time, application method and environmental conditions before it becomes infectious (Gillespie and Claydon 1989, Hajek and St Leger 1994, Inglis et al. 2001, Furlong and Pell 2005). In the advanced stages of infection, however, behaviours are altered and the insects eventually succumb to death when the fungus overwhelms the host defense.

Although the interaction between the fungal pathogen and its insect host may seem complicated, it is perceived as parasitic in favour of the fungus. Behavioural alterations in the equation are often ignored even though they affect parameters essential to pathogen and host evolution such as transmission and longevity (Moore et al. 1992). It is predicted that generalist pathogens (Hypocreales) interact more diffusely whereas specialist pathogens (Entomophthorales) engage in a tight process of coevolution. Changes in host behaviour reflect these diverse relationships and enable us to begin to address whether these are pathogen-induced, host-mediated, or incidental (Roy et al. 2006). Entomophthorales appear to modify the behaviour of their hosts purposely to increase their transmission and thus fitness. This has been exemplified by studies on the response and production of alarm pheromones by *P. neoaphidis*-infected aphids (Roy et al. 1999). Therefore, understanding the biology of EPF (Bidochka et al. 2000) and their interaction with hosts from a behavioural perspective is more practical to the development of novel strategies for increasing pathogen transmission within agroecosystems (Roy et al. 2005). This will support other interventions that have focused more on ways that suppress pest populations by reducing their survival or longevity (Nadeau et al. 1994, Nielsen and Hajek 2005, Vu et al. 2007, Goettel et al. 2008). Furthermore, mechanisms and consequences of insect-behavioural modifications would facilitate more accurate predictions of the pest population dynamics and consequently allow researchers to make better use of fungi as biological control agents (Butt et al. 2001, Deacon 2006).

In summary, among the many EPF, the genera of *Beauveria* and *Metarhizium* strongly manipulate the behaviour of their hosts and have strong potential for the control of insect pests. Manipulation of the insect behaviour aids their fitness, transmission and dispersal in insect populations. Knowledge on alteration of the various insect behaviours may be an opportunity to exploit other infection pathways such as autodissemination besides insects coming directly into contact with fungal spores. This review underpins the behavioural effects of fungal infection to be elusive in insect vectors compared to the vast influence in pest insects. Therefore, understanding the role of fungal pathogens on the behaviour of insect vectors particularly mosquitoes is critical (this thesis) if their full potential as biocontrol agents is to be realised.

Finally, the safety of these fungal pathogens towards humans, the environment and non-target organisms is an important criterion for consideration. Existing research however suggests that, there are minimal effects of EPF on non-target species, and they offer a safer alternative for use in Integrated Pest Management than chemical insecticides (Goettel and Hajek 2001, Thungrabeab and Tongma 2007, Zimmermann 2007a, Zimmermann 2007b). To affirm this, continued research on impact of EPF on non-targets is recommended.

Chapter 3

Infecting *Anopheles gambiae* with spores of *Metarhizium anisopliae* in the laboratory

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Abstract

Mosquitoes are highly susceptible to entomopathogenic fungi (EPF). As a tool for rapid assessment of fungal virulence as well as fungal delivery, we developed a new tool that can be used to infect mosquitoes with EPF in the laboratory. It consists of a transparent plastic cylinder of 9-cm diameter and 15-cm height. Four experiments were conducted to (i) establish preferred resting sites of the malaria mosquito *Anopheles gambiae* inside the cylinder; (ii) evaluate the effect of texture surface and colour on mosquito landing; (iii) determine the effective dosage and exposure time and (iv) compare the efficiency of the cylinder with clay pots and paper sheets that were treated with fungus using a K-bar coating machine. Mosquitoes showed no preference for a particular spot inside an empty cylinder or when lined with paper of varying texture and colour. Therefore, to allow optimal infection of adult *An. gambiae* mosquitoes with fungal spores, all paper surfaces were treated with fungal inoculum. Rough textured papers were more preferred to smooth-textured papers and so were black papers to white ones. Mosquitoes became infected and expressed high mortalities after exposure to fungal spore-treated paper. There was no effect of dose (0.1, 0.2 and 0.3 g) or exposure time (2, 4, 6 and 8 hr) on infection rate and survival of mosquitoes. Thus, each of the spore concentrations and exposure times were sufficient to effect inoculation. With an oil-suspension of spores that were spread on paper using a K-bar coating machine, a concentration of 10^{12} conidia/m^2 was more effective compared to 10^{10} conidia/m^2 but not different from 10^{11} conidia/m^2 (P=0.05). Infection rates of mosquitoes with fungal spores through exposure to a clay pot were not different from that observed with the K-bar coating machine or the plastic cylinder (P = 0.05). Because of its simple use and low cost, the plastic cylinder was recommended as the standard method for infection of mosquitoes with spores of *M. anisopliae* in the laboratory.

Introduction

The search for alternative methods to control malaria is intensifying with increasing insecticide-resistance in the malaria vectors. Entomopathogenic fungi (EPF) in the genera *Beauveria* and *Metarhizium* are primarily known for their potency against agricultural pests (Zimmermann 1993). Laboratory and field studies have demonstrated that *Beauveria bassiana* and *Metarhizium anisopliae* are pathogenic to larval and adult stages of the malaria mosquitoes (Lacey et al. 1988, Scholte et al. 2003a, Blanford et al. 2005, Scholte et al. 2005, Bukhari et al. 2011a). Furthermore, EPF is effective against malaria vectors that have developed resistance to insecticides (Farenhorst et al. 2009, Howard et al. 2010a, Kikankie et al. 2010) and express synergistic effects when used in combination with conventional insecticides (Farenhorst et al. 2010). Unlike resistance to insecticides which appears in the main insect disease vectors from every genus (Brogdon and McAllister 1998), the specific features of fungal infection such as slow-speed of kill, parasite transmission blocking and host behavioural changes, provide opportunities to minimize the risk of resistance evolution (Thomas and Read 2007a).

Since EPF have proven to infect, kill and reduce the survival of mosquitoes, an understanding of factors that affect their pathogenicity is critical to developing them as microbial control agents. Such factors include conidial viability, virulence, persistence, formulation and dosage. Viability of a fungal pathogen is affected by spore quality, formulation, application method, substrate and prevailing environmental conditions (Moore et al. 1995, Darbro and Thomas 2009). Spores can be viable but not virulent hence virulence is the capability of a fungus to cause disease in the infected organism. Persistence describes the length of time conidia remain viable after application. Thus, it dictates effectiveness of entomopathogens in field situations where it is greatly affected by ultra violet light, temperature and relative humidity (Ignoffo 1992). Entomopathogenic fungi can be applied as dry spores or wet spores formulated in oil or other solvents as a carrier (Lacey et al. 1988, Kannan et al. 2008, Howard et al. 2011). Dose is expressed as the quantity of spores sufficient to effect high mortality rates in the infected population in the shortest time possible. With EPF such high mortality has been reported between 3 -7 d post-exposure (Shah and Pell 2003, Schrank and Vainstein 2010).

The interaction between the insects and the EPF influences the outcome of the association. For instance, infection is effected when the malaria vectors come into contact with fungal spores (Chapter 1). Moreover, a successful infection rate depends on the behaviour of mosquitoes at the time of exposure to fungal spores; the fungal delivery tools used such as the texture and colour of the fungus-treated surface and the exposure time during which mosquitoes can pick up fungal conidia. Host-seeking and resting mosquitoes are the main targets for infection. Both behaviours of the Afrotropical malaria mosquito *Anopheles gambiae* Giles are expressed inside human dwellings (Rishikesh et al. 1985, Day 2005, Riehle et al. 2011). The preference of *An. gambiae* to frequent human habitats has contributed to the success of mosquito control with insecticides through indoor residual spraying and use of insecticide treated nets (Pates and Curtis 2005). Such an approach can be adopted and modified for other intervention tools as with EPF. Host-seeking mosquitoes are characterised with short-contact with surfaces prior to reaching the human host for a blood meal as opposed to long-contact in the resting population. Resting behaviour is dominant in blood-fed females compared to un-fed females as they invest time of up to 24 hr for digestion of the blood-meal and egg development. With these differences, exposure time becomes a prerequisite to ensure that the vectors pick up spores on the treated surfaces they come in contact with. Establishing exposure time will also guide in developing realistic field dissemination tools having pre-defined whether the intervention targets the host-seeking or resting population, or both (Mnyone et al. 2009b).

Different application methods have been used to infect mosquitoes with EPF in the laboratory and in the field (Howard et al. 2010a). The texture and colour of the substrates are important as they influence the landing choice of mosquitoes. Choice for texture surface (roughness) is mediated by tactile stimuli whereas visual stimuli dictate the choice for colour (O'Gower 1963). Research conducted to investigate the effect of texture and colour of the substrate colonized by stream invertebrates showed a preference for rough textured over smooth textured with no colour preference between white and dark-brown substrates (Clifford et al. 1989). In anopheline mosquitoes, only *An. gambiae* spp. show a preference for rough textured surfaces over smooth surfaced ones (Hansell 1970). A similar preference is expressed by *Aedes aegypti* L. where rough surfaces were selected for oviposition in favour of the smooth surfaces (O'Gower 1963, Wilton 1968). With colour, *An. gambiae* are attracted to dark areas inside human dwellings and when provided with cloth or fabric of various shades of colour preferred white, yellow, black and green in that order (Mutinga et al. 1995). Generally, researchers have given little attention to studies that involve texture surface and colour.

Although delivery strategy, formulations and dose of EPF have been widely studied and prescribed, none has been singled out as the standard for infecting mosquitoes with fungal pathogens in the laboratory, in semi-field and field situations. Therefore, the present study aimed to establish a method that successfully infects mosquitoes with spores of *M. anisopliae* in the laboratory. The specific objectives were to (i) determine the preferred *An. gambiae* resting sites inside a transparent plastic cylinder when empty and when lined with paper substrates that varied in texture and colour (ii) determine exposure time sufficient for mosquitoes to pick up fungal spores and become infected, (iii) determine the dose that causes optimal infection rate and (iv) compare infection methodologies i.e. clay pots and a K-bar coating machine to a transparent plastic cylinder.

Materials and Methods

Mosquitoes

Experiments were carried out using laboratory-reared female mosquitoes obtained from a colony of *Anopheles gambiae* Giles *sensu stricto* (hereafter termed *An. gambiae*) established from wild gravid females collected in Mbita Point (000 25'S, 340 13'E), western Kenya in 1999 (Menge et al. 2005). All mosquito life stages were maintained under ambient conditions in the mosquito insectary at the Thomas Odhiambo Campus (TOC), Mbita Point of the International Centre of Insect Physiology and Ecology (*icipe*). Larval and adult stages of the mosquitoes were raised using rearing procedures described by Olanga et al. (2010). All the experiments were conducted using 3-5 d-old adult females that had not received a blood meal.

Fungal isolate

The entomopathogenic fungus *Metarhizium anisopliae* isolate ICIPE 30 was used in the study. The isolate has been described earlier as virulent in infecting anopheline mosquitoes (Scholte et al. 2003b). The fungus was originally isolated from the stem borer *Busseola fusca* in Kendu Bay, western Kenya in 1999 (courtesy Dr. N.K. Maniania) and has been maintained at the *icipe*'s Germplasm Centre. Conidia were produced on long rice as substrate following the technique described by Maniania (1998). Harvested spores were dried for 48 h in a desiccator containing active silica gel and stored in a refrigerator (4-6°C) until required. The viability of conidia was determined using the technique described by Goettel and Inglis (1997) before being used in the experiments. Germination rates >85% after 24 h on Sabouraud dextrose agar was considered adequate for use in the experiments.

Fungus delivery tools

In most African homes, clay pots (Figure 1) are used for storage of drinking water because of their cooling effect. When turned side-way, its shape keeps the inside dark and this has been capitalised on for use as sampling tool for outdoor resting mosquito populations (Odiere et al. 2007). Further, it is used as delivery tool to infect mosquitoes with spores of EPF under laboratory conditions (Farenhorst et al. 2008). The K-bar coating machine and spreader (Figure 3) has been tested and it enhances an even distribution of oil formulated spores on an infecting paper substrate (Farenhorst and Knols 2010). The transparent plastic cylinder (Figure 2) is more portable than clay pots and more readily available than a K-bar coating machine but its efficiency as an infecting tool has not been established.

Figure 1. Clay pot: width of the opening 24cm; maximum width 39cm

Figure 2. Transparent plastic cylinder: diameter 9 cm; height 15cm

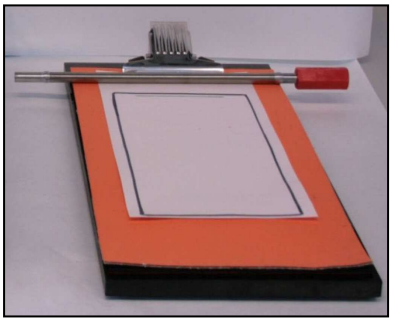

Figure 3. K-bar coating machine and a spreader (Farenhorst and Knols 2010)

Paper substrates

Three paper substrates i.e. (i) white smooth paper (ii) white rough paper and (iii) black smooth paper were used. Black rough paper was excluded because it was not available. The white smooth paper was a special A4 glossy paper that is commonly used in the paint industry. It is an effective substrate in infecting mosquitoes with EPF (Farenhorst and Knols 2010). The white rough paper was represented by the "velvex all purpose towels" commonly used as kitchen product. The paper is strong and rough in texture. These properties make the paper fit firmly inside a transparent plastic cylinder (Figure 2) and also hold dry spores firmly after spreading. Standard A4 printing paper with both surfaces painted black using a photocopying machine was used as black smooth paper. In all the experiments using the three paper substrates separately, the inner and the base surfaces of each cylinder was lined with paper that measured 28.6 × 14.3 cm and 9 cm in diameter respectively.

Determine the preferred resting sites of **An. gambiae** *inside a transparent plastic cylinder*

In this experiment, two equidistantly spaced lines were drawn on the outside surface of a plastic cylinder (Figure 2) using a pencil so as to demarcate it into upper, middle and lower sections. By observing the inner surface of the cylinder, these lines were only visible when the cylinder was empty. Therefore, two lines were also drawn on each paper used to divide it into three equal parts prior to being lined inside the cylinder. A piece of mosquito netting was secured over the mouth of the cylinder using a rubber band and a small hole punched at the centre of the net to serve as an entry/exit point for the mosquitoes. Four tests were conducted when the cylinder i) was not lined with any paper and when lined with; ii) white smooth paper, iii) white rough paper and iv) black smooth paper, respectively. The experiment aimed to establish the effect of the texture and the

colour of paper substrate on the choice of mosquitoes to land on the upper, the middle or the lower section of the cylinder upon release. In each test, three hundred 4 d-old female mosquitoes were assessed each day for two days. To investigate whether time of day had any influence on resting site selection, the 300 mosquitoes used in each of the four tests per day were evaluated at different time periods. One group comprising of 150 mosquitoes was assessed between 10:00-12:00 hr and the other group of 150 mosquitoes between 15:00-17:00hr. During the assessment in each group, a single mosquito was released inside the cylinder using a mouth aspirator; the time taken from release to landing inside the cylinder and the cylinder section where the mosquito landed were recorded. The mosquito was then aspirated out of the cylinder and a second one introduced to record similar data as with the first mosquito. This procedure was repeated until all the 150 mosquitoes were evaluated. The experiments were carried out in the laboratory at *icipe*'s Thomas Odhiambo Campus, Mbita, under ambient conditions with no modifications to shun the light sources. Normal lighting was essential so as to observe with ease the mosquito landing behaviour inside the cylinder especially when the cylinder was lined with a black smooth paper.

Determine the optimal method for mass infection of An. gambiae *with* M. anisopliae

(i) Infecting An. gambiae *with dry conidia inside transparent plastic cylinders*

Five transparent plastic cylinders (Figure 2) were used in the experiment and consisted of the following treatments: i) cylinder not lined with paper ii) cylinder lined with white smooth paper; iii) cylinder lined with white rough paper; iv) cylinder lined with black smooth paper and v) untreated control. Each cylinder was held in a slanting position and 0.1g (approx. 1.0×10^{11} conidia/m^2) of *M. anisopliae* spores was weighed and poured on the paper. Sixty 3-5 d old female mosquitoes were introduced in each cylinder. Control mosquitoes were released in the cylinder free of fungal spores. Mosquitoes were held in the cylinders for 6 hr and were then transferred into separate holding cages (30 × 30 × 30 cm). They were provided with 6% glucose solution on paper wick. The insects were maintained at 28 ± 2˚C and 70 ± 5% r.h. Mortality was recorded daily and dead individuals were placed in a Petri-dish lined with wet filter paper and incubated at 28 ± 2˚C. Cadavers were inspected for fungal growth after three or more days using a compound microscope at 400× magnification. The experiment was replicated four times over several days. Another set of experiments was carried out, whereby 0.2 g (approx. 2.0×10^{11} conidia/m^2) and 0.3 g (approx. 3.0×10^{11} conidia/m^2) of *M. anisopliae* spores were applied following the procedure described above. Following the results, one of the concentrations and one of the paper surfaces was selected for further studies, e.g. survival rates of *An. gambiae* 2, 4, 6 and 8 hr after exposure to *M. anisopliae*. The experimental procedure for infection remained the same as described earlier.

(ii) Infecting An. gambiae *with conidia of* M. anisopliae *formulated in oil inside transparent plastic cylinders*

A mixture of 0.5 g spores and 25 ml of Shellsol T oil was prepared as stock solution and the spore concentration quantified using a bright line haemocytometer. The stock was diluted to concentrations of 10^{10}, 10^{11} and 10^{12} conidia/m^2. One ml of the formulation was drawn from each concentration using a pipette and released on three white smooth papers. A K-bar hand coating machine and a spreader (Figure 3) were used to spread the formulations evenly on the papers. In the control group the paper was spread with one ml of Shellsol T oil only. The coated papers were allowed to dry for 6 hr and later fitted in each of the four transparent cylinders. Sixty 3-5 d old female mosquitoes were then introduced in each cylinder and held for 6 hr after which they were transferred into separate holding cages (30 × 30 × 30 cm). The mosquitoes were then supplied with a 6% glucose solution on paper wick and maintained at 28 ± 2˚C and 80 ± 5% r.h. Mortality was recorded daily and dead individuals were placed in a Petri-dish lined with wet filter paper to confirm

growth of fungus on the insect after three days. The experiment was replicated four times over time. The concentration that resulted in a high infection rate was selected for further study, i.e. comparison of the efficacy of infection methods.

(iii) Infecting An. gambiae *with* M. anisopliae *in clay pots and in transparent plastic cylinders*

The experiment aimed at comparing the efficacy of infecting mosquitoes with fungal conidia through a clay pot (Figure 1) and a transparent plastic cylinder. Three transparent plastic cylinders and one clay pot were used and consisted of the following treatments: i) cylinder not lined with paper and without fungus; ii) cylinder lined with white rough paper spread with 0.1 g (approx. 1.0×10^{11} conidia/m^2) of conidia; iii) cylinder lined with white smooth paper coated with 10^{12} conidia/m^2 and iv) clay pot covered with 2 g (approx. 2.0×10^{12} conidia/m^2) of conidia. The procedure followed to spread dry spores and coat the conidia formulated in oil remained as described in i) and ii) above. Fifty 3-5 d old female mosquitoes were introduced in each of the three cylinders; and 100 females in the clay pot then held for 6 hr. Cylinders and clay pot were covered with mosquito netting. After fungal exposure, the mosquitoes i.e. 50 females from each treatment were transferred into separate holding cages (30 × 30 × 30 cm), provided with 6% glucose and maintained at 28 ± 2˚C and 80 ± 5% r.h.. Mortality was recorded daily and dead individuals were placed in a Petri-dish lined with wet filter paper to confirm growth of fungus on the insect after three days. The experiment was replicated four times over time.

Statistical analysis

Data on resting sites of *An. gambiae* were scored between 10:00-12:00 hr and 15:00-17:00 hr and were calculated separately by expressing the number of mosquitoes that landed on each cylinder part as a percentage of the total number released. The chi-square (χ^2) test (Preacher 2001) was applied using actual scores to establish if time of day had an effect on resting site preference. Mosquitoes that landed within a minute in each section of the cylinder were expressed as a percentage of the total collections in each section. Since time of day had no great impact on mosquito landing preference, collections in the two time-periods were pooled. Further, the numbers that landed on each cylinder section were then expressed as a percentage of the total released. The percent values were subjected to analysis of variance using PROC GLM procedure of SAS (2003). The Student-Newman-Keul's (SNK) test at (P = 0.05) was used to separate the means as a post-ANOVA procedure. Dose response and efficiency of infection methods were calculated by expressing the numbers of mosquitoes killed by fungal infection as a percentage of total mosquitoes exposed to fungus. Percentage mortality data were arcsine-transformed (Gomez and Gomez 1984) to normalize the data after correcting for natural mortality (Abbott 1925). Angular values were then subjected to analysis of variance using the ANOVA procedure of SAS (2003) where means were separated by SNK test at (P = 0.05). Repeated measures logistic regression was used to estimate the lethal time to 50% (LT_{50}) values. LT_{50} values were determined for each replicate and compared among themselves using ANOVA followed by mean separation by SNK. Chi-square (χ^2) test was further used to estimate dose effect relative to paper types. For survival of mosquitoes after time of exposure to fungal spores was varied, difference in survival between the control and *M. anisopliae*-infected groups were estimated using Cox regression analysis in SPSS. Mortality rates, given as Hazard Ratios (HR) estimate risk of dying when infected compared to when not infected with fungus. All analyses were conducted using SAS (version 9.1) or SPSS (version 17.0)

Results

Preferred resting sites of An. gambiae *inside a transparent plastic cylinder*

With all paper surfaces, the numbers of mosquitoes that landed on the cover net, upper, middle or lower sections of the cylinder between 10:00-12:00 hr were not significantly different from those that landed between 15:00-17:00 hr, except with white smooth paper in middle section (P = 0.002) and white rough paper in the lower section (P = 0.047). Therefore, the data were pooled (Table 1) to interpret the effect of paper texture and colour on mosquito resting site preference inside the cylinder. Analysis showed that *An. gambiae* females significantly preferred to settle on the cover net and on the upper section of the cylinder when the cylinder was without paper (F = 18.1, df = 3, P = 0.0004) and when lined with a white smooth paper (F = 12.3, df = 3, P = 0.0016). In both situations, fewer mosquitoes landed in the middle section of the cylinder (Table 1). With white rough paper (F = 10.7, df = 3, P = 0.0025) and black smooth paper (F = 40.7, df = 3, P < 0.0001) inside the cylinder, the distribution shifted with more mosquitoes landing in the upper and lower sections. More mosquitoes in middle section and fewer on the cover net were observed compared to when there was no paper inside the cylinder or when lined with white smooth paper. Generally, more mosquitoes landed on the upper section of the cylinder in all the treatments. Further, the choice of mosquitoes to rest on the cover net was reduced with black smooth paper inside the cylinder; but increased in the middle section compared to other paper surfaces (Table 1). However, irrespective of the different paper texture and colour mosquitoes were exposed to, their choice to land in all of the four resting sections was evident but with variation in numbers. Also, of the mosquitoes that landed in each cylinder section, ≥ 80% (not shown) landed within a minute after introduction into the cylinder.

Table 1. Mean (± S.E) percentage of female *An. gambiae* mosquitoes resting on different sections inside a transparent plastic cylinder. N is the number of replicates, and n the total number of mosquitoes used per treatment.

Cylinder (treatment)	N	n	Distribution (%) of resting mosquitoes over the cylinder			
			Cover net	Upper section	Middle section	Lower section
No paper	4	600	32.0 ± 3.6^{ab}	35.3 ± 3.0^{a}	7.3 ± 0.6^{c}	25.3 ± 2.0^{b}
White smooth paper	4	600	34.7 ± 6.2^{a}	41.0 ± 0.8^{a}	6.3 ± 1.9^{b}	18.0± 4.3^{b}
White rough paper	4	600	16.0 ± 3.6^{b}	33.0 ± 1.9^{a}	17.2 ± 1.8^{b}	33.8± 1.9^{a}
Black smooth paper	4	600	4.5 ± 1.5^{c}	47.2 ± 2.9^{a}	23.7 ± 2.1^{b}	24.7 ± 2.8^{b}

Means followed by the same letters within rows are not significantly different by Student-Newman-Keuls (SNK) test at P = 0.05

Optimal method for mass infection of An. gambiae with M. anisopliae

(i) Infection of An. gambiae *with dry conidia inside transparent plastic cylinders*

The mortality of spore-treated mosquitoes 6 d post-exposure was significantly different from the control with all spore concentrations (0.1 g (F = 11.1, df = 4, P = 0.0005), 0.2 g (F = 115.3, df = 4, P < 0.0001) and 0.3 g (F = 53.9, df = 4, P < 0.0001)). However, the difference was not significant between the types of paper surfaces used to infect mosquitoes in each of the spore concentrations (Table 2).

Similarly, the lethal time to 50 percent mortality (LT_{50}) was not significant between the types of paper surfaces in each concentration but significant when 0.1 g (F = 8.7, df = 4, P < 0.0015), 0.2 g (F = 17.0, df = 4, P < 0.0001) and 0.3 g (F = 50.8, df = 4, P < 0.0001) were compared to that of the control.

Mortality between spore concentrations on the same type of paper was compared using the chi-square (χ^2) test. Significantly, increase in spore concentration did not increase mortality rate when the cylinder was not lined with paper (χ^2 = 5.59, df = 2, P = 0.06) or when lined with white smooth paper (χ^2 = 4.13, df = 2, P = 0.13); white rough paper (χ^2 = 5.64, df = 2, P = 0.06) and black smooth paper (χ^2 = 4.17, df = 2, P = 0.13). Similarly, the difference between the three controls was not significant (χ^2 = 3.84, df = 2, P = 0.15). Based on these results, white rough paper and fungal concentration of 0.1 g were selected for assessment of the survival rate. Moreover, viability of conidia used in each spore concentration varied and ranged from 69 - 86%. This range correlated with the rate of infection in infected insects that was between 55.2 - 87.4% at all concentrations (Table 2).

Table 2. Effects of surface type and spore concentration on mortality of *An. gambiae* mosquitoes infected with spores of *M. anisopliae*. Fungal infection rate, percent mortality and LT_{50} values 6 days post-treatment are shown. n (in parentheses) is the number of mosquitoes infected. Spore viability ranged from 69-86%.

Spore concentration (grams)	Surface type	Percentage of infection (n)	% mortality (± S.E)	LT_{50} (d) (95% Fiducial limits)
0.0	No paper	0	31.2 ± 7.5[b]	9.6 (8.3-11.6)[a]
0.1	No paper	55.2 (117)	84.3 ± 2.9[a]	2.0 (1.8-2.1)[b]
0.1	White smooth paper	61.8 (126)	82.9 ± 6.9[a]	3.2 (3.1-3.3)[b]
0.1	White rough paper	63.7 (120)	87.4 ± 10.0[a]	3.9 (3.8-4.0)[b]
0.1	Black smooth paper	56.6 (100)	81.8 ± 7.4[a]	3.6 (3.5-3.7)[b]
0.0	No paper	0	26.7 ± 3.0[b]	8.6 (7.8-9.9)[a]
0.2	No paper	84.6 (186)	88.7 ± 1.5[a]	2.8 (2.7-2.9)[b]
0.2	White smooth paper	80.6 (179)	89.8 ± 1.9[a]	2.7 (2.6-2.8)[b]
0.2	White rough paper	87.4 (201)	93.3 ± 2.7[a]	3.0 (2.9-3.1)[b]
0.2	Black smooth paper	86.0 (190)	89.6 ± 2.9[a]	3.2 (3.1-3.3)[b]
0.0	No paper	0	23.3 ± 0.7[b]	11.2 (9.4-14.2)[a]
0.3	No paper	70.9 (125)	79.8 ± 5.9[a]	3.0 (2.9-3.1)[b]
0.3	White smooth paper	72.5 (134)	82.3 ± 4.9[a]	3.0 (2.9-3.1)[b]
0.3	White rough paper	77.8 (152)	84.4 ± 5.3[a]	3.2 (3.1-3.3)[b]
0.3	Black smooth paper	71.9 (142)	82.7 ± 5.3[a]	3.2 (3.1-3.3)[b]

Means followed by same letters within column are not statistically different by Student-Newman-Keuls (SNK) test at P = 0.05

The survival of *An. gambiae* exposed to dry spores of *M. anisopliae* for 2, 4, 6 and 8 hr was significantly reduced with 100% mortality observed after five days compared to > 14 days in uninfected mosquitoes (Figure 4). Furthermore, survival of infected mosquitoes in each exposure time was significantly different from its own control. For instance, the daily risk of death was two-

fold greater in mosquitoes exposed for 2 hr (HR = 2.1 [95% Cl= 1.61 - 2.79], P = 0.0001), three fold greater with 4 hr exposure (HR = 3.1 [95% Cl= 2.30 - 4.22], P = 0.0001), close to three-fold greater with 6 hr exposure (HR = 2.8 [95% Cl= 2.05 - 3.69], P = 0.0001) and slightly more than three-fold greater with 8 hr exposure (HR = 3.4 [95% Cl= 2.42 - 4.74], P = 0.0001) relative to their controls. Since there was no difference between the four controls (HR = 1.0 [95% Cl= 0.91 - 1.08], P = 0.874), the data were pooled for a common control. The daily risk (HR^1) of death in each of the four exposure time was greater compared to control (Table 3). The difference between treatments was significant between 8 hr and 2 hr exposure but not between 8 hr and 4 or 6 hr exposures (HR^2).

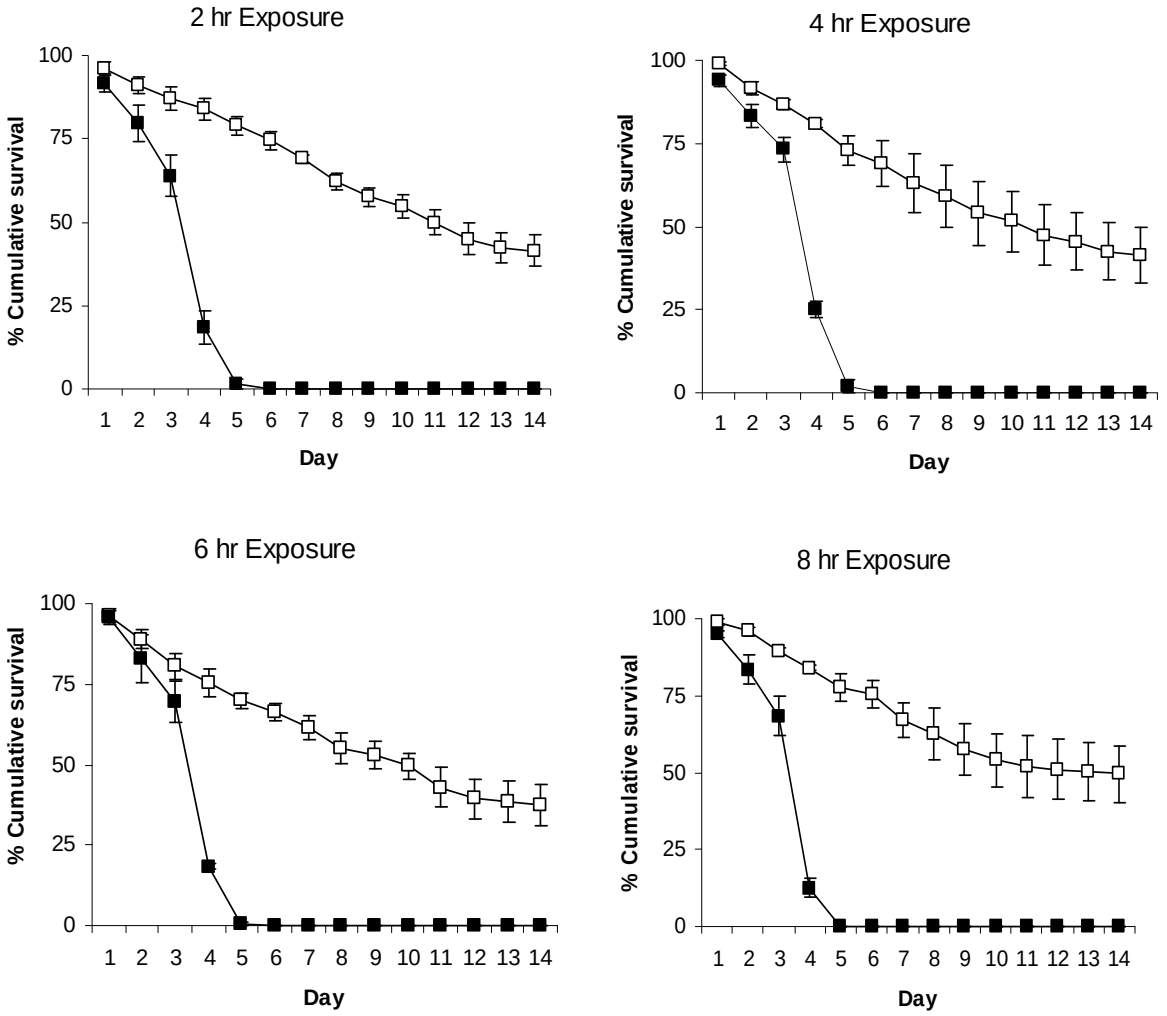

Figure 4. Survival of adult female *An. gambiae* mosquitoes exposed to 0.1 g of *M. anisopliae* for 2, 4, 6 and 8 hr, respectively. Conidia were spread inside transparent plastic cylinders lined with white rough paper. Uninfected and *M. anisopliae*-infected mosquito groups are depicted with open and shaded squares, respectively.

(ii) Infection of An. gambiae *with conidia formulated in oil inside transparent plastic cylinders*

At 10 d post-treatment, the mortality at all spore concentrations was significantly higher (F = 14.4, df = 3, P < 0.001) compared to that of the control mosquitoes. In addition, the highest concentration (10^{12}) caused a significantly higher mortality compared to the lowest concentration (10^{10}) tested. Similarly, the LT_{50} value in the control was significantly (F = 12.3, df = 3, P < 0.002) longer compared

to that of the fungal concentrations. Moreover, LT_{50} was highly significant between the lowest (10^{10}) and the highest concentrations (10^{12}) (Table 4). Viability of the spores used was 69% and this resulted in infection rates of 58.7 - 70.5% in mycosed mosquitoes.

Table 3. Hazard ratios (HR) plus 95% confidence level (Cl) of *An. gambiae* mosquitoes exposed to *M. anisopliae* at different exposure times. HR^1 compares daily risk of death in controls to that in each exposure time. HR^2 compares the risk of death between the 8 hr exposure time to other exposure hrs plus the control.

Treatment	Exposure time (hr)	HR^1 (95% Cl)	P-value	HR^2 (95% Cl)	P-value
Control (pooled)				0.34 (0.28-0.41)	0.0001
M. anisopliae-infected	2	2.14 (1.80-2.53)	0.0001	0.73 (0.60-0.89)	0.002
	4	2.58 (2.15-3.10)	0.0001	0.88 (0.73-1.07)	0.209
	6	2.79 (2.32-3.35)	0.0001	0.95 (0.79-1.15)	0.634
	8	2.92 (2.42-3.53)	0.0001	-	-

Table 4. Mortality of *An. gambiae* mosquitoes exposed to different concentrations of oil formulated *M. anisopliae* spores 10 days post-treatment. Conidia were applied on the infecting surface using a K-bar coating machine. n (in parentheses) is the number of mosquitoes infected. Spore viability was ≥ 70%.

Spore concentration (conidia/m^2)	Percentage infected (n)	Percentage died (± S.E)	LT_{50} (days) (95% fiducial limits)
0 (control)	0	41.2 ± 6.3^{c}	$10.8\ (10.1\text{-}11.7^{a}$
1.0×10^{10}	58.7 (99)	69.3 ± 4.8^{b}	$8.0\ (7.7\text{-}8.2)^{b}$
1.0×10^{11}	61.4 (119)	80.9 ± 3.9^{ab}	$4.9\ (4.7\text{-}5.0)^{bc}$
1.0×10^{12}	70.5 (143)	88.6 ± 6.6^{a}	$2.8\ (2.7\text{-}2.9)^{c}$

Means followed by same letters within column are not statistically different by Student-Newman-Keuls (SNK) test at P = 0.05

(iii) Infection of **An. gambiae** *with* **M. anisopliae** *in clay pots and in transparent plastic cylinders*

There was no significant difference in mortality and LT_{50} values between treatments when mosquitoes were infected with dry conidia in clay pots and transparent plastic cylinders, and transparent plastic cylinders treated with spores formulated in oil (Table 5). However, all fungal treatments were significant (mortality: F = 22.1, df = 3, P = 0.002 and LT_{50}: F = 6.6, df = 3, P = 0.001) compared to control mosquitoes 9 d post-treatment. The surface area (approx. 552 cm^2) of the

transparent plastic cylinder was estimated to be nine times less than that of the clay pot (Figure 1). Thus, the clay pot required 20 times more spores than the cylinder to cause the same mortality on infected mosquitoes (Table 5). Of the dead mosquitoes in all the treatments, > 70% developed mycosis.

Table 5. Effects of infecting surface on infection of *An. gambiae* with *M. anisopliae*. Fungal infection rate, percent mortality and LT_{50} values 13 days post-treatment are shown. n (in parentheses) is the number of mosquitoes infected. Fungus was applied as dry spores (g/m^2) or formulated in Shellsol T-oil ((ml/sheet). Spore viability was ≥ 76%.

Infecting surface (method)	Concentration of *M. anisopliae* per assay	Percentage infected (n)	Percentage died (± S.E)	LT_{50} (days) (95% fiducial limits)
Plastic cylinder not lined with paper	0	0	33.0 ± 5.8[b]	12.6 (11.5-14.1)[a]
Plastic cylinder lined with white rough paper	0.1g / cylinder	75.2 (135)	85.3 ± 4.4[a]	3.1 (3.0-3.2)[b]
Plastic cylinder lined with white smooth paper	1.0×10^{12} / sheet	70.1 (139)	87.4 ± 4.3[a]	3.0 (2.9-3.1)[b]
Clay pot	2g / pot	87.8 (165)	85.2 ± 6.2[a]	3.7 (3.6-3.8)[b]

Means followed by same letters within column are not statistically different by Student-Newman-Keuls (SNK) test at P = 0.05

Discussion

Results from this study show that *An. gambiae* mosquitoes exhibit a varied resting-site selection behaviour that is influenced by the texture and the colour of the surfaces they are exposed to. Although the lower, middle and upper sections of the cylinder were the target areas for evaluation, the mosquitoes expressed an additional preference of landing on the cover net. The preference is more a behavioural than a light-related effect since even in the cylinder lined with black-coloured paper, 4.5% (of 600 mosquitoes) landed on the cover net (Table 1). In the natural environment, *Anopheles* mosquitoes especially *An. gambiae* and *An. stephensi* express a similar behavioural pattern and the majority prefer to land on the apex of the resting-surface (Silver 2008). Moreover, the shape and size of the cylinder used might have influenced the choice for landing site. This has been reported to affect resting-site preference of mosquitoes whether the resting sites are natural or artificial (Burkett-Cadena et al. 2008). From the total collections recorded per cylinder section, 80 - 100% landed within a minute. On close observation, the mosquitoes hovered within the cylinder afterwards. The remaining ≤ 20% kept flying inside the cylinder and landed after 1-3 minutes. The short-contact with a surface layer is characteristic of host-seeking mosquitoes (Mnyone et al. 2009b). With this unpredictable resting-site selection behaviour in mosquitoes, it is recommended that the entire inner surface of the cylinder be treated with spores of *M. anisopliae* to achieve a high infection rate of mosquitoes.

Our finding further demonstrates that *An. gambiae* mosquitoes prefer rough textured surfaces over smooth textured ones. This preference is described to be associated with the leg-waving nature of

the metathoracic legs of the insect (Hansell 1970). This leg-waving motion is only found in *An. gambiae* and is responsible for determining the texture of substrate prior to landing. This might have also contributed to the short-contacts and continuous flight activity within the cylinder while assessing the resting-site preference. Moreover, rough surfaces exhibit some adhesive properties (Brown and Siegmann 2000, Li et al. 2010a) and are thus recommended for use with EPF as they can hold the spores firmly. Therefore, white rough paper was selected for use to infect mosquitoes with the fungus *M. anisopliae* in favour of white smooth and black smooth papers. Future integration of texture surface to improve infectivity of fungal delivery tools is encouraged. It might be a challenge to quantify the degree of roughness of the infecting surfaces, but reasonably easier to select them through observation and touch.

Between black and white coloured substrates, our results show a preference of *An. gambiae* mosquitoes for black smooth paper over white smooth or white rough papers. This concurs with the general understanding that mosquitoes prefer to settle on black areas or on the dark parts of different patterns as a survival strategy (Kennedy 1940, O'Gower 1963). The choice for colour also influences oviposition site selection, with black rough inner vertical walls of the oviposition sites preferred in *An. arabiensis* Patton (Balestrino et al. 2010), *Aedes* spp. (Kaw et al. 2004), *Ae. Triseriatus* (Say) (Wilton 1968) and *Ae. albopictus* (Skuse) (Novak 1992). However, there are exceptions to colour preference. In *An. punctipennis* Say, blood-fed females show a slight preference for black with the unfed females settling for white surfaces (Hecht and Hernandez-Corzo 1963). Other findings in a multi-choice situation have described white-coloured fabrics to be a better attractant of *An. gambiae* mosquitoes compared to yellow, black, red, blue and green coloured fabrics (Mutinga et al. 1995). Since the mosquito's preference for colour appears to be wide (Mutinga et al. 1995), fabric of any colour described above could be used to infect mosquitoes with fungal spores. To increase effectiveness, the fabric treated with fungal spores should be placed in the dark side of the room. Our study used white rough paper since the paper was more readily available than the more preferred black smooth paper.

An increase in spore concentration, from 0.1 g, 0.2 g to 0.3 g, had no effect on the number of mosquitoes infected, irrespective of the type of paper substrate used. Therefore, the lower dose of 0.1 g was considered the most economical for bioassays. A similar concentration of 0.1 g was pathogenic to adults of anopheline and culicine mosquitoes when infected with *Beauveria bassiana* or *M. anisopliae* (Scholte et al. 2003a, Achonduh and Tondje 2008, Kikankie et al. 2010). In other insects, maize weevils *Sitophilus zeamais* (Motsch) and leaf-cutting ants *Atta sexdens rubropilosa* (Forel) were infected and killed by the same concentration of the fungal species (Adane et al. 1996, Jaccoud et al. 1999). Since a standard dose for infecting mosquitoes has so far not been identified, this has to be determined through preliminary studies prior to real bioassays. Fungal impact on high infection rates is affected by the delivery method used and the conidial (spore) viability. For instance, in our study, the infection rate in dead mosquitoes after exposure to fungus increased with increase in spore viability. Nevertheless, the resultant mortality was sufficiently high to predict a strong impact on mosquitoes in the field (Scholte et al. 2005). For future research it is important to remember that retention of high viability and high virulence of spores is a prerequisite for EPF to be effective as microbial control agents (Daoust and Roberts 1983)

An increase in exposure time, from 2 to 8 hr, had no effect on the survival rate of fungus-infected mosquitoes. Hence, within 2 hr mosquitoes picked up a sufficient number of spores to become infected. However, the 8 hr exposure would cause a higher impact compared to 2 hr exposure. In other studies, a high infection rate has been reported in mosquitoes exposed to EPF for as short as 15-30 min (Mnyone et al. 2009b), for 2-8 hr (Paula et al. 2011) and for as long as 24 hr, 48 hr and beyond (Scholte et al. 2003a, Scholte et al. 2007). There are many parameters that may influence the time of exposure. They include fungal formulation i.e. dry spores or wet spores, design of the

infecting tool or surface and host behaviour (host seeking or resting) among others. Just like conidial dosage, standard exposure time for infecting mosquitoes with EPF has so far not been identified. Therefore, our study chose a 6 hr exposure time, which has been adopted by several researchers in evaluating the effects of fungal pathogens on mosquitoes, with which results from different studies can be more readily compared.

A simple and efficient fungal delivery tool is critical in infecting with EPF. A number of tools such as Petri dishes, hair rollers, modified plastic cylinders, clay pots, K-bar coating machine; black cotton cloth attached to the roof or walls, outdoor odour-bait stations (OBS) (Scholte et al. 2003a, Scholte et al. 2005, Farenhorst et al. 2008, Farenhorst and Knols 2010, Lwetoijera et al. 2010, Mnyone et al. 2010a) among other tools have been effective for the infection of mosquitoes in the laboratory and field situations. In the present study we evaluated clay pots, plastic cylinders and paper treated through a k-bar coating machine as infecting tools in the laboratory. However, our results show that the three methods used did not result in differences in infection rate. Therefore, the plastic cylinder was selected as the standard method of exposing mosquitoes on the basis of availability, portability, cost effectiveness and ease of use.

Conclusions

As contact between spores and mosquitoes is crucial for their infection with entomopathogenic fungi, it is important to identify a standard method of infection. Therefore, this study was designed to develop a rapid and cheap bioassay method for infection and for testing the virulence of fungal spores. Mosquito landing behaviour inside a plastic cylinder revealed no preference for a specific resting location while white rough paper emerged as the best substrate for infection. Spore concentration of 0.1 g and a 2 hr exposure time were sufficient to achieve a high infection rate. Lastly, there was no difference between the cylinder assay and other published methods, so the cylinder assay is recommended.

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Part II

FUNGAL INFECTION AND FORAGING BEHAVIOUR OF *ANOPHELES GAMBIAE*

Chapter 4

Metarhizium anisopliae affects the host-seeking response but not the olfactory discrimination of *Anopheles gambiae* mosquitoes

To be submitted as: Ondiaka S., Takken W., Koenraadt C. J. M., Mukabana W. R. *Metarhizium anisopliae* affects the host-seeking response but not the olfactory discrimination of *Anopheles gambiae* mosquitoes.

Abstract

The effect of *Metarhizium anisopliae* infections in the malaria mosquito *Anopheles gambiae* on their host-seeking behaviour at short range was evaluated using a dual-choice olfactometer under semi-field conditions. Two adult men, ranked as highly and poorly attractive to *An. gambiae* mosquitoes, were used as the source of host-seeking stimuli. Three treatment combinations were tested: (i) no odour vs. no odour, (ii) no odour vs. the host stimuli of each person separately and (iii) the odorants of each person against each other. Host odours were tested as complete human emanations, breath only and body odour only. At the start of each test period of 30 min. 100 uninfected mosquitoes and 100 *M. anisopliae*-infected mosquitoes were released in each olfactometer set up. Four sets of data were recorded i.e. numbers of mosquitoes collected in the (i) two trap chambers separately (ii) choice chamber and (iii) release cup. A significant difference in overall response was observed where on average 50% of the mosquitoes died on the third day after fungal exposure. Those that survived were used for behavioural assays. In the absence of human stimuli few mosquitoes responded and there was no significant difference in trap catches between the two tents for uninfected (P = 0.18) and for *M. anisopliae*-infected (P = 0.90) mosquitoes. A significant response of uninfected and *M. anisopliae*-infected mosquitoes to total body emanations, body odour and breath of the highly attractive (HA) and the poorly attractive (PA) person occurred when compared to an empty tent (P = 0.01). Furthermore, the behavioural response of *M. anisopliae*-infected *An. gambiae* mosquitoes when presented with different test stimuli demonstrated that breath harbours inhibitory odorants as opposed to total emanations while compounds present in body odour induce a relatively uniform response of vectors to their hosts. These findings suggest that infection with the entomopathogenic fungus strongly reduces the host-seeking response of mosquitoes, but does not impair their olfactory -based discriminatory capability. The use of the entomopathogenic fungus *M. anisopliae* may, therefore, be a good complement to other mosquito vector control tools for the reduction of mosquito bites, and transmission of malaria as well as for other mosquito-borne diseases.

Introduction

The mosquito *Anopheles gambiae* Giles is the world's most important vector of *Plasmodium falciparum* malaria. Its ability to transmit malaria triggered by its host-seeking behaviour has been and continues to be an important and fascinating area in the field of disease epidemiology and control. This behaviour is mediated by olfactory cues with which the mosquito locates its human hosts (Bowen 1991, Knols et al. 1995, Takken and Knols 1999). For *An. gambiae* these cues are host specific, hence the mosquito's preference for human odours rather than odours emanating from other vertebrates (Besansky et al. 2004, Lefevre et al. 2009). However, even in the presence of human odours, the host-seeking behaviour can be suppressed depending on the physiological status of mosquitoes (Bowen et al. 1988, Klowden 2007). Thus, mosquito host location is largely influenced by its host preference mediated by olfactory cues and its physiological status. This understanding of host-seeking behaviour is relevant when designing measures to better reduce the transmission of the parasite and control the disease.

Furthermore, host-seeking behaviour may be enhanced by the presence of a parasite in the vector's body. For example, infection with *Plasmodium* parasites was associated with increased vector contact with individual hosts (Wekesa et al. 1992) and blood-feeding (Koella et al. 1998). In *Aedes aegypti* L., an infection with dengue virus resulted in reduced feeding behaviour (Platt et al. 1997), while infection with the bacterium *Wolbachia pipientis* caused a significant reduction in blood feeding and even induced early aging (Turley et al. 2009). In other blood-sucking insects such as the phlebotomine sandflies in the genera *Phlebotomus* and *Lutzomyia*, leishmania infections interfered with blood feeding but increased the frequency of biting (Beach et al. 1985, Schlein et al. 1992, Rogers and Bates 2007). However, this is not the case in all parasite-host interactions (Poulin et al. 1994). Although these induced behavioural changes are beneficial to the pathogens, other outcomes are possible such as increased disease transmission.

There is a need to protect humans from blood-sucking arthropods, particularly mosquitoes, and the pathogens they transmit. The current hurdle is to find a cost-effective measure to supplement or replace the use of chemical insecticides which is facing the challenge of vector resistance (Ranson et al. 2009, Alonso et al. 2011). The use of entomopathogenic fungi (EPF) is promising and should be explored for efficacy and impact.

In the recent past, EPF in the genera *Metarhizium* and *Beauveria* have demonstrated potential to infect and reduce the survival of malaria vectors (Blanford et al. 2005, Scholte et al. 2005, Farenhorst et al. 2008, Mnyone et al. 2009b). These fungi do not kill the mosquito instantly but cause sublethal and late-life lethal effects on different stages of the mosquito life cycle. Late-life lethal effects result in reduced blood feeding and fecundity in mosquitoes (Scholte et al. 2006, Howard et al. 2010b). In other insects, the effect was reported in the pea leafminer *Liriomyza huidobrensis* (Blanchard) (Migiro et al. 2011), the Asian long horned beetle *Anoplophora glabripennis* (Motschulsky) (Hajek et al. 2008), the legume flower thrips, *Megalurothrips sjoistedti* Trybom (Ekesi and Maniania 2000) and in the sweet potato weevil *Cylas puncticollis* (Boheman) (Ondiaka et al. 2008a). However, other studies did not report it (Arthurs and Thomas 2000, Ondiaka et al. 2008b). Sublethal effects primarily demonstrate the potential of the infected females to engage in host-seeking and, if gravid, search for, locate and reach suitable oviposition sites (Scholte et al. 2006). Host-seeking is the most important component of mosquito vectorial capacity (Zwiebel and Takken 2004) and is quite distinct from other blood-feeding behaviours such as landing, probing and biting (Bowen 1991). In a laboratory situation, the host-seeking behaviour is reduced in *M. anisopliae*-infected *An. gambiae* females (Scholte et al. 2006, Ondiaka et al. 2008b). However, it is not clear whether similar effects will be observed under more realistic semi-field and field situations.

Through olfaction, *An. gambiae* can differentiate the odour of one human individual from the other (Lindsay et al. 1993, Knols et al. 1995, Mukabana et al. 2002). However, it remains uncertain if this ability in mosquitoes will be impaired upon infection with an entomopathogenic fungus.

Therefore, the current study investigated the host-seeking response of *Metarhizium anisopliae*-infected *An. gambiae* female mosquitoes to human odours and established whether *M. anisopliae*-infected mosquitoes can discriminate between odours from different human individuals. The studies were conducted using a dual-choice olfactometer in a semi-field situation.

Materials and Methods

Mosquitoes

Experiments were carried out using laboratory-reared female mosquitoes obtained from a colony of *Anopheles gambiae* Giles *sensu stricto* (hereafter termed *An. gambiae*) established from wild gravid females collected in Mbita Point (000 25'S, 340 13'E), western Kenya in 1999 (Menge et al. 2005). All mosquito life stages were maintained under ambient conditions in the mosquito insectary at the Thomas Odhiambo Campus (TOC), Mbita Point of the International Centre of Insect Physiology and Ecology (*icipe*). Larval and adult stages of the mosquitoes were raised using rearing procedures described by Olanga et al. (2010). All the experiments were conducted using 2-6 d-old adult females that had not received a blood meal. The insects had been starved for eight hr and were provided with water on cotton towels placed on top of the mosquito holding cups to prevent dehydration.

Fungus strain

Metarhizium anisopliae var. *anisopliae* (Metsch.) Sorokin, isolate ICIPE 30 (courtesy of Dr. N.K. Maniania) was used to infect mosquitoes in all experiments. The fungus was originally isolated in 1989 from a stemborer, *Busseola fusca*, at Kendu Bay, western Kenya, and has since been maintained under laboratory conditions at the International Centre of Insects Physiology and Ecology (*icipe*). Mass production of the spores on rice substrate was conducted in the Arthropod Pathology Unit of *icipe* in Nairobi, Kenya using the procedure described by Maniania and colleagues (Maniania et al. 2003, Mburu et al. 2011). Harvested spores were stored dry until they were used for the experiments.

Infection process

Eight transparent plastic cylinders (9cm diameter; 15 cm height) were used. The inside surface and the circular base of each cylinder were lined with white rough-surfaced velvex tissue papers that measured 28.6 × 14.3 cm and 9 cm in diameter respectively. Each cylinder was held in a slanting position and 0.1 g (approx. 1.0×10^{11} conidia/m^2) of *M. anisopliae* spores were weighed and poured on the paper. Using both hands, the cylinders were rolled several times until the papers were covered by the spores. Sixty 2-d-old female mosquitoes were introduced into each cylinder and held for six hr being supplied with 6% glucose solution soaked in cotton pad and placed on top of the netting material covering the cylinder. The mosquitoes were then transferred into one holding cage (30 × 30 × 30 cm) and were supplied with 6% glucose solution on filter paper wicks. The insects were maintained at 28 ± 2˚C and 70 ± 5 % r.h. in a room simulating semi-field conditions. Females used in the dual-choice olfactometer had been exposed to *M. anisopliae* three days prior to the behavioural test. The procedure for uninfected mosquitoes was the same as for those exposed to *M. anisopliae* except that four transparent plastic cylinders were used and no fungal spores were spread on the velvex tissue paper. The number of mosquitoes exposed to fungus was higher than for the

uninfected group to adjust for mortality in the holding cages prior to the start of the experiments on day three post-exposure.

Experimental set-up

The experiments were carried out using a large dual-choice olfactometer which accommodated two humans, each in a separate compartment, as sources of host-seeking stimuli. The structural component of the olfactometer has been described in detail by Mukabana et al. (2002). The modified design described by Olanga et al. (2010) was adopted for use in this study (Figure 1). In order to prevent contamination with fungal spores, two olfactometers were used: set up I was used to conduct experiments with uninfected mosquitoes and set up II with *M. anisopliae*-infected mosquitoes. The two olfactometers were placed inside a screenhouse (13 × 4.7 × 2.3 m).

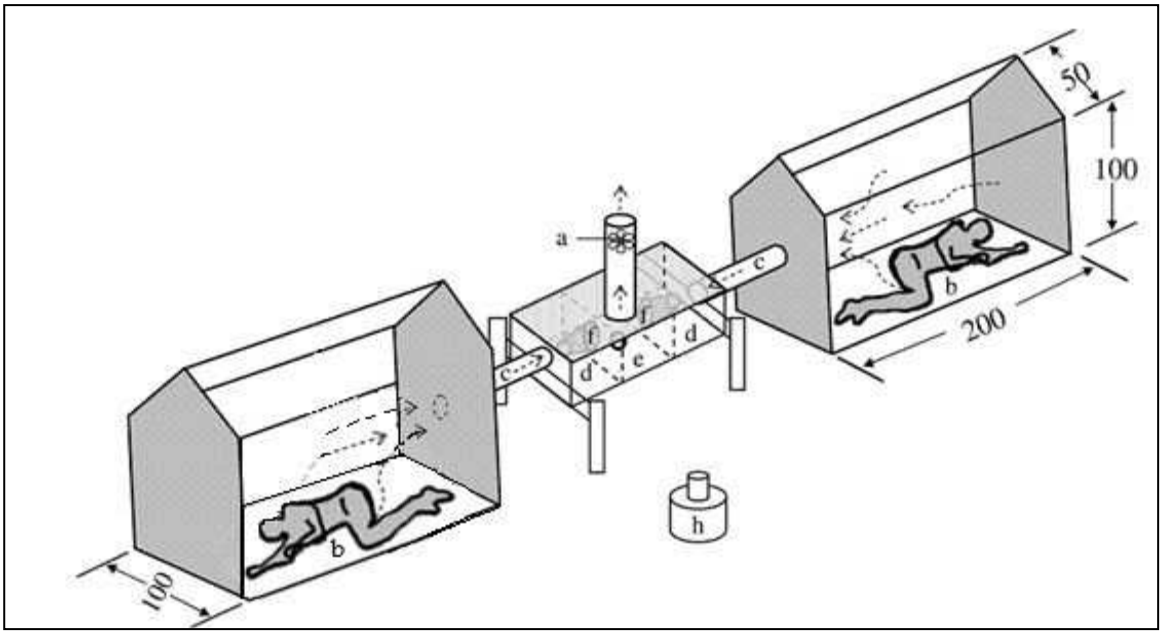

Figure 1. The dual-choice experimental setup. The fan (a) drew air (~130 L/min/tent) from the two tents (b) to the outside environment via PVC pipes (c), trap chambers (d) and central choice chamber (e). An exit trap (f) opened into each trap chamber. The fan pipe and the release cup (h) were fitted on top and on the bottom of the choice chamber, respectively, through circular holes. The trap and choice chamber measured 30 × 15 × 20 and 30 × 20 × 20, respectively. Diagrams are not drawn to scale; all dimensions are in centimeters (Olanga et al. 2010).

Human subjects

Two adult men ranked as highly attractive (HA) and poorly attractive (PA) to *An. gambiae* mosquitoes were selected to participate in the experiments. The men were among the nine Kenyan males ranked on their attractiveness to *An. gambiae* mosquitoes by Mukabana et al. (2002). Their malaria infection status was tested daily during experimental days by microscopic examination of thin and thick smears of a finger-prick stained with Giemsa. Preparations for the men to adhere to prior to the start of experiments followed the procedures described by Mukabana et al. (2002). The presence of the men was rotated between and within tents to assess the responses of uninfected and *M. anisopliae*-infected mosquitoes. Their beddings were not removed during the rotation; instead, two fans were directed in each tent after the end of a night experiments. The tents were air-rated for 24 hr prior to the start of the next experiment to clear residual odours of the two participants. The bed linens were washed after eight d using an odourless soap that was also used by the men to bathe.

Partitioning of human emanations

Three test stimuli, i.e. (i) total body emanations, (ii) breath and (iii) body odour were used for assessment of the response of uninfected and *M. anisopliae*-infected mosquitoes. Here, total body emanations refer to breath plus all volatile discharges of the skin while body odour refers to volatiles discharged from the skin only. Breath and body odour were separated using a one-way breathing valve (Antec Leyden®, the Netherlands). The human subjects wore a breathing valve by the mouth-piece and clipped a sprung nose clip on their nose so that they could inhale and exhale air through the mouth only (Mukabana et al. 2004). Depending on the test stimuli being assessed, breath was either diverted to the tent or directed from the tent to the outside of the screen house.

Baseline studies for dual-choice olfactometer in the absence of human odour stimuli

The experiments were carried out with two experimental set-ups not occupied by human subjects. Only clean bed linens were spread on the bed in each olfactometer tent. The experiments were performed between 19.30-20.00 and 20.30-21.00 hours. During each test period, 100 uninfected mosquitoes or 100 *M. anisopliae*-infected mosquitoes of 5-d-old and starved for eight hr prior to use were released into each olfactometer set-up. Four sets of data were recorded in each test period that included numbers of mosquitoes collected in the (i) two trap chambers separately (ii) choice chamber and (iii) release cup. Each experiment was replicated eight times.

Effect of fungal infection on mosquito response to human odours

In this study, one tent in each of the two experimental setups was occupied by a human subject and the other tent contained only clean bedding. The three test stimuli of the two participants were assessed through six dual-choice assays: (i) an empty tent versus total body emanations of person HA, (ii) an empty tent versus total body emanations of person PA, (iii) an empty tent versus body odour of person HA, (iv) an empty tent versus body odour of person PA, (v) an empty tent versus breath of person HA and (vi) an empty tent versus breath of person PA. The experimental procedure remained as described above under “baseline studies” except that the two human subjects were rotated within and between the two tent set-ups.

Effect of fungal infection on preferential behaviour of mosquitoes

These experiments were designed to ascertain if fungus infection modifies the attraction of host-seeking mosquitoes to human hosts with different odour profiles. Experimental mosquitoes were allowed to make a choice between the following test stimuli: (i) total body emanations of person HA versus total body emanations of person PA (ii) body odour of person HA versus body odour of person PA and (iii) breath of person HA versus breath of person PA. The participants were concealed in the two tents in one of the olfactometer set-ups for two 30 min. test periods (19.30-20.00 and 20.30-21.00 hours) on each experimental night. During each test period, 100 uninfected or 100 *M. anisopliae*-infected mosquitoes of 5-day old and starved for eight hr prior to use were released on separate nights. The numbers of mosquitoes that left the release cup into the choice chamber were caught in trap chambers as they progressed towards the source of stimuli from persons present in the tents. The two persons were rotated within and between the two tent set-ups. The experiments with uninfected and with *M. anisopliae*-infected mosquitoes were each replicated eight times.

Statistical analysis

Mosquitoes that responded to test stimuli of person HA and person PA in favour of an empty tent were expressed as a proportion of the number trapped by each person divided by the sum of the

number trapped by both persons including the collections in the choice chamber. A similar formula was used to compute the proportion of mosquitoes that were collected in trap chambers when both tents were empty. The preferential response of mosquitoes to odours of the two persons was calculated as the number of mosquitoes trapped by the emanations of person HA divided by the sum of the number trapped by person HA and person PA expressed as a percentage. The differences in the number of mosquitoes caught in the absence and in the presence of odours of the two human volunteers in uninfected and in *M. anisopliae*-infected groups was compared using a Generalized Linear Model. This was after the data was transformed to assume a normal distribution using a logarithmic link function. The significance of changes in relative attractiveness between uninfected and *M. anisopliae*-infected mosquitoes was compared by an independent-samples t-test. Data were analysed either by General Statistics analysis software (GenStat® for Windows, 3rd Edition) or Statistical Products and Services Solutions (SPSS, version 17.0).

Ethical clearance

The consent to participate in the study was sought for and confirmed by the two human subjects after explaining to them in detail the objectives and procedures of the experiments. Ethical approval for this study was given by the Kenya National Ethical Review Committee located at the Kenya Medical Research Institute (NON-SSC Protocol number 203).

Results

Baseline studies for dual-choice olfactometer in the absence of human odour stimuli

Eighty three percent (665 of 800) of the uninfected mosquitoes and fifty six percent (448 of 800) of the *M. anisopliae*-infected mosquitoes were collected in the combined area of the choice chamber plus the two trap chambers. The difference between the collections was significant (P = 0.001). Of these collections, the number (expressed as proportion) of uninfected mosquitoes found in trap A (0.010) and in trap B (0.003) were not significantly different (P = 0.18). Similarly, the difference was not significant (P = 0.90) between *M. anisopliae*-infected mosquitoes found in trap A (0.002) to that from trap B (0.000) (Table 1). The overall response of uninfected (0.010 + 0.003) and *M. anisopliae*-infected (0.002 + 0.000) mosquitoes in this situation without odours was very low.

Table 1. Proportion of uninfected and *M. anisopliae*-infected mosquitoes caught per trap in the absence of human emanations in a dual choice assay.

Behaviour stimuli		**Mosquito infection status**	**N**	**Proportion trapped**		**n (percent response)**	**P**	**P[1]**
Tent A	**Tent B**			**Tent A**	**Tent B**			
Empty	Empty	Uninfected	8	0.010	0.003	665 (83)	0.18	0.001
Empty	Empty	*M. anisopliae*-infected	8	0.002	0.000	448 (56)	0.90	

N, the number of replicates. n, the total number of mosquitoes collected in the trap (d in Figure 1) and choice (e in Figure 1) chambers. P, level of statistical difference between catches in tent A versus catches in tent B. P[1], level of statistical difference between n-collections in uninfected and *M. anisopliae*-infected mosquitoes. Levels of significance were generated by two-sample t-test.

Effect of fungal infection on mosquito response to human odours

For the eight hundred mosquitoes that were released in each bioassay, the number of *M. anisopliae*-infected mosquitoes that were recaptured in the choice chamber plus the two trap chambers was significantly (P = 0.001) less than the number of uninfected mosquitoes. Of the total recapture, significantly (P = 0.01) more uninfected and *M. anisopliae*-infected mosquitoes responded to the odour stimuli from the volunteers when (i) total emanations (ii) breath and (iii) body odour of the person HA or person PA were compared to an empty tent (Table 2). The proportion that responded to odours of each person was not much different between treatments. Nevertheless, the response of infected and control mosquitoes to breath of person HA or person PA was less than the response to total emanations or body odour. With person PA, more mosquitoes responded to body odour than to total emanations with both uninfected and *M. anisopliae*-infected mosquitoes. However, with person HA, more uninfected mosquitoes responded to total emanations but the response to body odour was quite close between treatments (Table 2).

Table 2. Proportion of uninfected and *M. anisopliae*-infected mosquitoes caught per trap in presence and in absence of human stimuli in binary assay.

Behaviour stimuli		Mosquito infection status	N	Proportion trapped		n (percent response)	P	P[1]
Person	Empty tent			Person	Empty tent			
PA - TE	Empty tent	Uninfected	8	0.197	0.016	669 (84)	0.01	0.001
PA - TE	Empty tent	*M. anisopliae*-infected	8	0.198	0.000	334 (42)	0.01	
PA - BO	Empty tent	Uninfected	8	0.316	0.019	618 (77)	0.01	0.001
PA - BO	Empty tent	*M. anisopliae*-infected	8	0.327	0.004	275 (34)	0.01	
PA - BR	Empty tent	Uninfected	8	0.046	0.011	630 (79)	0.01	0.001
PA - BR	Empty tent	*M. anisopliae*-infected	8	0.030	0.000	236 (30)	0.01	
HA - TE	Empty tent	Uninfected	8	0.312	0.000	645 (81)	0.01	0.001
HA - TE	Empty tent	*M. anisopliae*-infected	8	0.331	0.000	347 (43)	0.01	
HA - BO	Empty tent	Uninfected	8	0.232	0.017	543 (68)	0.01	0.001
HA - BO	Empty tent	*M. anisopliae*-infected	8	0.422	0.003	294 (37)	0.01	
HA - BR	Empty tent	Uninfected	8	0.086	0.024	593 (74)	0.01	0.001
HA - BR	Empty tent	*M. anisopliae*-infected	8	0.037	0.000	191 (24)	0.01	

Person PA is poorly attractive. Person HA is highly attractive. TE, total emanations, BO, body odour BR, breath. N, number of replicates. Proportion trapped, mosquitoes attracted to different behavioural stimuli (person) relative to contrasting ones (empty tent). n, the total number of mosquitoes collected in the trap and choice chambers. Numbers in parenthesis refer to n expressed as a percentage of the total release. P, statistical difference within treatments i.e. person versus empty tent. P^1, level of statistical difference between treatments i.e. n-collections in uninfected versus *M. anisopliae*-infected mosquitoes. Significance levels were calculated by two-sample t-test

Effect of fungal infection on preferential behaviour of mosquitoes

In the dual-choice test between odours of the HA and PA persons, eight hundred mosquitoes were used in each bioassay. The number of uninfected mosquitoes caught in the choice chamber plus the two trap chambers was significantly (P = 0.001) more than the number of *M. anisopliae*-infected mosquitoes in each of the three test stimuli (Figure 2). Of these collections, the number (expressed as a percentage in Figure 2) that responded to specific odours emanating from the two persons was not much different for the uninfected and infected groups. Besides, when the total number of mosquitoes was considered, significantly fewer mosquitoes infected with *M. anisopliae* responded to odours emanating from total emanations (P = 0.001) and breath (P = 0.001) of the two persons. However, the difference was not significant (P = 0.060) between uninfected and *M. anisopliae*-infected mosquitoes that responded to stimuli from body odour of the two persons (Figure 2). Between the two persons, total emanations of Person HA were significantly more attractive for both uninfected (P = 0.001) and *M. anisopliae*-infected mosquitoes (P = 0.001) compared to those of the poorly attractive person. The attractiveness of body odour of person HA and person PA to uninfected (P = 0.78) and to *M. anisopliae*-infected (P = 0.096) mosquitoes was not different. Breath of person PA attracted more uninfected (P = 0.001) and *M. anisopliae*-infected (P = 0.003) mosquitoes than breath of person HA. Furthermore, the response of both uninfected and *M. anisopliae*-infected mosquitoes to breath from both persons was less than that to body odour and total emanations.

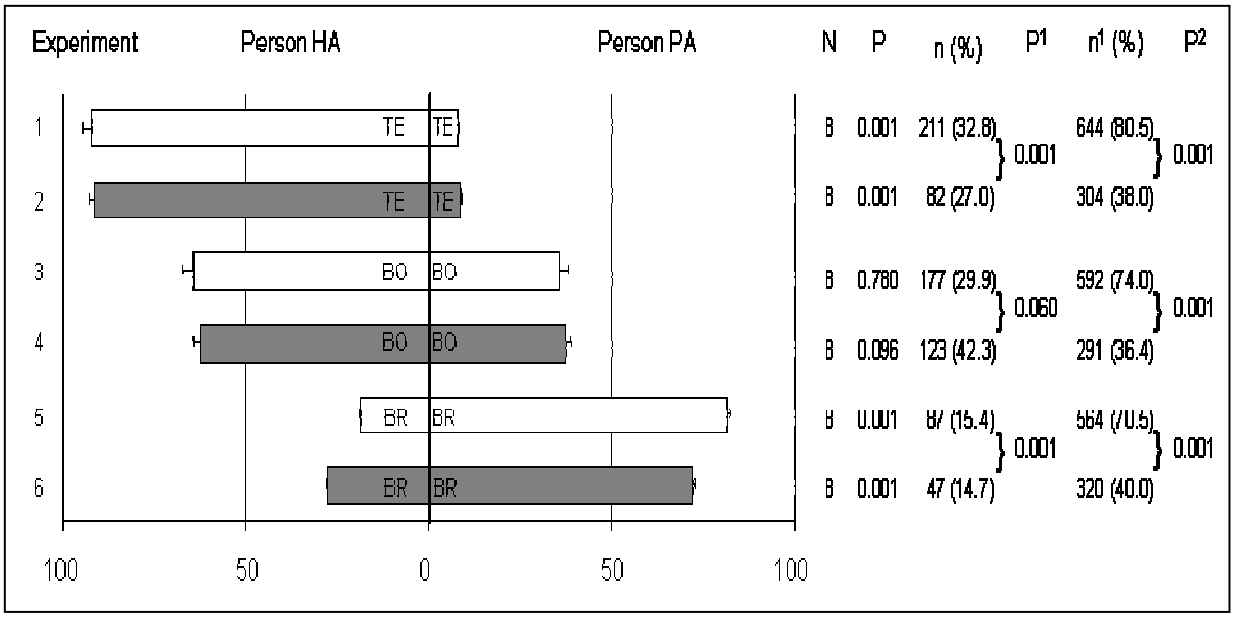

Figure 2. Percent mosquito catches in experiments evaluating mosquito behavioural responses upon infection with *M. anisopliae* to human emanations. Experiments 1 and 2 demonstrate mosquito catches to total emanations (TE) of person highly attractive (person HA) versus TE of person poorly attractive (person PA) to *An. gambiae* mosquitoes. Experiments 3 and 4 describe catches to body odour (BO) of person HA versus BO of person PA. Experiments 5 and 6 illustrate mosquito catches to breath (BR) of person HA versus BR of person PA. Shaded bars refer to *M. anisopliae*-infected mosquitoes and open bars to uninfected mosquitoes. N is the number of replicates. n the total number of mosquitoes attracted to emanations of person HA and person PA (in parenthesis, n presented as a percentage of n^1). P values depicts the level of statistical difference in mosquito catches between emanations of person HA and person PA within treatments. P^1 refers to level of significance between numbers of uninfected and *M. anisopliae*-infected mosquitoes that responded in each of the three odour sources. n^1 is the total number of mosquitoes collected in the choice chamber and the two trap chambers i.e. exclude mosquitoes that remained in the release cup (Figure 1) (in parenthesis, n^1 expressed as percentage of the total mosquitoes released). P^2 refers to level of significance between uninfected and *M. anisopliae* infected mosquitoes in n^1 collections. Levels of significance were generated by two-sample t-test.

Discussion

This study demonstrates that the proportion of *An. gambiae* mosquitoes responding to host odour, three days after infection with the entomopathogenic fungus *Metarhizium anisopliae*, was significantly reduced compared to uninfected mosquitoes under semi-field conditions. However, the ability of the mosquitoes to differentiate between humans based on their odorant emanations was not affected by fungus infection of the mosquitoes.

Throughout the study, the impact of the fungus on mosquitoes was observed right from the start of the infection process. For instance, in order to obtain the same number of mosquitoes of the infected group being alive at the start of the behavioural studies, it was needed to infect twice the number of mosquitoes with *M. anisopliae* compared to the number used for the controls. On average, 50% of the fungus-exposed mosquitoes died on the third day after fungal exposure, while those that survived were used for behavioural assays. This much shortened survival rate in the mosquito population was directly caused by the infection since mortality of uninfected *An. gambiae* mosquitoes during this time was negligible (see also Chapter 3). Other studies have also estimated a similar time frame to impact 50% mortality in *An*. *gambiae* mosquitoes (Scholte et al. 2003a, Scholte et al. 2003b). However, in some studies the lethal time is more than three days (Blanford et al. 2005, Howard et al. 2010a). This variation is affected by dose, the formulation i.e. dry conidia or oil formulation and environmental conditions (Schrank and Vainstein 2010). Moreover, fungus used as dry conidia causes a greater effect compared to conidia formulated in oil or any other solvent (Scholte et al. 2003a). This may have been the case in this study where dry conidia were the mode of infection. Although an entomopathogenic fungus is a “slowly” killing microbial agent even with the most virulent isolates, its instant impact upon contact with host-seeking mosquitoes can be observed quite early after exposure.

Of the mosquitoes that survived for use in our behavioural studies, the effects of the infection continued to affect the insects. For example, in the experiment without host emanations, only 56% of the infected mosquitoes flew out of the release cup compared to 83% of the uninfected mosquitoes during the bioassays. Most likely the non-responders in the infected mosquitoes were too weak to fly. Moreover, in the comparison of no-odour versus the host stimuli of each person separately, between 24% - 43% of the infected mosquitoes and 74% - 84% of the uninfected mosquitoes left the release cup. Furthermore, the response ranged from 36% - 40% in the infected and 71% -81% in the uninfected mosquitoes when presented with odour stimuli of each person against each other. Although infected, these mosquitoes responded nevertheless to human odours, showing a host choice similar to that of uninfected mosquitoes. The infection rate in *M. anisopliae*-infected mosquitoes that were caught in trap chambers was 100%. This was confirmed by growth of fungus on the mosquito cadaver at least three d after performing mycosis tests. Several possibilities are linked to longer survival of mosquitoes after exposure to fungus amongst which is the dose the insect picks up and the immunological reaction of the insect to the pathogen (Lacey et al. 1988). However, over time the insect weakens when the fungus overwhelms its defence mechanism through increased toxin production. Finally, the fungus penetrates into the insect haemolymph and consumes the available nutrients that eventually lead to increased physiological starvation and death of the insect.

Our bioassay findings reveal that few of both uninfected and *M. anisopliae*-infected mosquitoes reached the tent openings when presented with an empty olfactometer. This low response is expected in the absence of human odours. By contrast, a significantly larger proportion of mosquitoes was caught in the trap chambers connected to tents occupied by the two persons ranked as poorly and highly attractive to *An. gambiae* mosquitoes when compared to empty tents. Furthermore, the behavioural responses of *M. anisopliae*-infected *An. gambiae* mosquitoes when

presented with different test stimuli demonstrated that breath harbours inhibitory odorants as opposed to total body emanations while compounds present in body odour induced a relatively uniform response of the mosquitoes to their hosts. Similar results have been reported previously with uninfected mosquitoes (Mukabana et al. 2004), where it was found that human breath contains mosquito-inhibiting compounds. In all these comparisons, infection with the entomopathogenic fungus did not impair the olfactory-based discriminatory capability of mosquitoes but strongly reduced the numbers that responded to human stimuli. The response of *M. anisopliae*-infected mosquitoes is attributed to the impact of fungus on the physiological status of the insects. Through colonization of the haemolymph by the fungus, the insect is depleted of nutrients, which is likely to interfere with its internal metabolism. This directly reduces the primary activity of the mosquito which is flight. For example, in the migratory locust *Locusta migratoria* var. *manilensis* (Meyen), infection with *M. anisopliae* caused depletion of trehalose sugars in the haemolymph (Zhao et al. 2007). Trehalose is the principal sugar circulating in the blood or haemolymph of most insects and is the main source of energy (Thompson 2003). Moreover, the sugar is the potential nutrient source for insect pathogenic fungi like *M. anisopliae* (Xia et al. 2002). The direct consequences of sugar depletion is the reduction of flight energy as observed in the desert locust *Schistocerca gregaria* (Forskal) on infection with *M. anisopliae* var. *acridium* (Seyoum et al. 2002).

Odour-mediated host-seeking has been extensively studied and is utilised by virtually all blood-sucking insects (Lefèvre et al. 2006) to locate their hosts. The results in our current study show that infection with the entomopathogenic fungus *M. anisopliae* had no effect on the olfactory discrimination of the mosquitoes since the person ranked highly attractive (HA) consistently attracted more uninfected and *M. anisopliae*-infected *An. gambiae* mosquitoes compared to the person ranked poorly attractive (PA). This concurs with previous studies where uninfected *An. gambiae s.s* mosquitoes preferred certain individuals despite being presented with total body emanations from other persons at the same time (Lindsay et al. 1993, Knols et al. 1995, Mukabana et al. 2002). This discriminatory capability of mosquitoes to locate their blood hosts is accredited to factors present in an individual's total body emanation such as odour, microbial products, heat and moisture (Braks et al. 1999, Mukabana et al. 2002, Verhulst et al. 2010).

To the best of our knowledge, this study provides the first evidence that the host-seeking ability of *An. gambiae* is reduced upon infection with the entomopathogenic fungus *M. anisopliae* under semi-field conditions, where mosquitoes are exposed to natural environmental conditions. This coincides with other findings in which infection with *M. anisopliae* reduced host-seeking potential of *An. gambiae* mosquitoes under laboratory conditions (Scholte et al. 2006, Ondiaka et al. 2008b). Recently, George et al. (2011) demonstrated a reduced neurosensory response in *An. stephensi* Liston following infection with *Beauveria bassiana* and *M. acridum*. Our study, though suggests that the olfactory discrimination between two human individuals was not affected by the fungal infection. The difference is attributed to the visual observation approach in this study that may be insufficient to verify the findings by George et al. (2011). In non-insect arthropods, the bacterium *Acaricomes phytoseiuli* originally isolated from the predatory mite *Phytoseiulus persimilis* Athias-Henriot (Pukall et al. 2006) was pathogenic to the same mite species. The bacterium induce a non-responding syndrome that renders *A. phytoseiuli* ineffective as a biocontrol agent of the herbivorous spider mites (Schütte et al. 2008).

Our findings suggest that with the use of effective delivery methods, the mosquitoes are likely to pick up the infection early in life. This could further lead to acute mortality and eventually reduce the mosquito population. Although at this stage, the human host may be susceptible to bites, the chance to be infected with the malarial parasite is minimal since the mosquito will die before the parasite fully develops into the infectious sporozoites (Scholte et al. 2005). The bites can be prevented by sleeping under bednets.

We, therefore, conclude that entomopathogenic fungi have the potential to infect, kill and reduce the population of host-seeking mosquitoes as well as the general host-seeking response, but not their capability to discriminate hosts on differences in odorants. Thus, its use can be a good complement to other mosquito vector control tools for reduction of mosquito bites, and transmission of malaria as well as other mosquito-borne diseases.

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Chapter 5

Metarhizium anisopliae infection reduces house entry and human-biting rates of *Anopheles gambiae* mosquitoes

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Abstract

The impact of infection with the entomopathogenic fungus *Metarhizium anisopliae* on the house entry and hourly human-biting rates of the malaria mosquito *Anopheles gambiae* was investigated under natural climatic conditions in a semi-field enclosure, which contained a traditional African hut. House entry behaviour and hourly human-biting responses of uninfected and *M. anisopliae*-infected mosquitoes were studied with a human subject as host. Two experiments, i.e. (i) a control without a human host and (ii) a treatment with human host under a bed net were conducted to examine house entry each with uninfected and fungus-infected mosquitoes separately. Human-biting response experiments were conducted both indoors and outdoors. Of the mosquitoes that were exposed to fungus, fifty percent died within the first three days after exposure. The behaviour of the survivors was compared with that of uninfected mosquitoes. A significantly higher house-entry response of uninfected compared to *M. anisopliae*-infected mosquitoes occurred irrespective of the presence of a human host indoors ($P = 0.001$). Significantly more infected as well as uninfected mosquitoes entered the house in the presence of a human host than in the absence of a human host. Furthermore, the hourly human-biting response was significantly higher in uninfected mosquitoes compared to infected mosquitoes both outdoors and indoors. However, fungal infection did not cause a shift in biting times. These findings indicate that *M. anisopliae* strongly impairs the flight performance of female mosquitoes but not the ability to identify host odours nor did fungal infection cause changes in the nocturnal hourly-biting pattern.

Introduction

Anopheles gambiae Giles *sensu stricto* (henceforth termed *An. gambiae*) is the most efficient vector of human malaria in Africa. The mosquito is both anthropophilic and highly susceptible to *Plasmodium falciparum* (Besansky et al. 2004, Day 2005). Transmission of malaria to humans occurs when parasite-infected mosquitoes make contact, probe and take blood meals. These behaviours are governed by olfaction (Bowen 1991, Costantini et al. 1998b, Takken and Knols 1999, Zwiebel and Takken 2004). Although measures to reduce transmission have increased there is a need to identify ways of improving the outcome of interventions (Baber et al. 2010, Alonso et al. 2011). A more complete understanding of the behaviour of the vector, especially the host seeking component, is implicit (Day 2005, Pates and Curtis 2005).

Host-seeking refers to the in-flight orientation of the avid female towards potential blood hosts. In mosquitoes this involves a chain of responses that includes activation, landing, probing and feeding (Bowen 1991). These responses are independent and initiated by specific stimuli (Hocking 1971, Bowen 1991). Control measures that have impacted greatly on malaria transmission have targeted the activation, in-flight orientation and landing phases. Currently, indoor residual spraying (IRS) and insecticide-treated bed nets (ITNs), including long lasting insecticidal bed nets (LLINs), are the two most important vector control tools (WHO 2006b, Yukich et al. 2008, Zhou et al. 2010, Yakob et al. 2011). The LLINs have further proven to be effective against pyrethroid-resistant *An. gambiae* mosquitoes (Dabiré et al. 2006). These conventional strategies have been used widely with commendable success. However, this has resulted in insecticide resistance in the mosquitoes, which is now widespread in many different parts of Africa (Dabire et al. 2008, Yadouleton et al. 2010, Ranson et al. 2011, Yewhalaw et al. 2011). The development of resistance is a major setback in the global programme for malaria control. Therefore, there is need to develop novel vector control methods that can complement the existing intervention tools. The use of entomopathogenic fungi (EPF) appears a promising alternative (Federici 1995, Scholte et al. 2003a, Lord 2005, Mnyone et al. 2010b).

Several studies have shown that spores of EPF *Metarhizium anisopliae* and *Beauveria bassiana* are pathogenic to *Anopheles* spp. (Scholte et al. 2005, Kanzok and Jacobs-Lorena 2006, Achonduh and Tondje 2008, Kannan et al. 2008, Mnyone et al. 2009a). Similar effects have been reported in *Aedes* spp. (Scholte et al. 2007, de Paula et al. 2008, Paula et al. 2011). Fungal infection is associated with reduction in longevity, blood feeding, fecundity (Scholte et al. 2006), host-seeking potential (Scholte et al. 2006, Ondiaka et al. 2008b) and interferes with the completion of the *Plasmodium* cycle in mosquitoes (Blanford et al. 2005). These effects demonstrate the potential of EPF in reducing transmission of malaria and other mosquito-borne diseases (Thomas and Read 2007a, de Paula et al. 2008).

Host-seeking capability directly influences house entry rate, indoor and outdoor biting rate of *An. gambiae*. Of these, the preference of the vector to blood feed and rest indoors (Takken and Knols 1999, Day 2005) is the most critical in disease transmission and is also a behavioural trait that has contributed to the success of IRS in malaria vector management (Pates and Curtis 2005). The presence of ITNs indoors, however, may repel mosquitoes entering houses leading to low house resting densities and an increase in the proportion of outdoor feeding populations (Russell et al. 2011). This may pose a challenge since control measures that target outdoor populations using chemicals are yet to be designed. Moreover, the density of mosquitoes indoors can be reduced by blocking house entry points i.e. eaves on houses or screening eaves, fitting ceilings and by improving housing (Snow 1987, Lindsay et al. 2002, Kirby et al. 2008, Njie et al. 2009, Ogoma et al. 2010). However, the approach faces similar challenges as the use of chemicals in controlling mosquitoes outdoors. By targeting indoor host-seeking and resting populations, it has been possible to

effectively use EPF to infect, kill and reduce survival of malaria vectors (Scholte et al. 2005, Mnyone et al. 2010a). It is likely that mosquitoes can be infected outdoors by resting on surfaces treated with fungal conidia. Such surfaces include clay pots which were proven to be effective sampling (Odiere et al. 2007) and infecting tools (Farenhorst et al. 2008). Infection with EPF impairs flight performance of mosquitoes (Blanford et al. 2011). Therefore, such occurrence in mosquitoes infected outdoors may have a direct impact on house entry, outdoor biting and indoor biting rates of host-seeking mosquitoes.

In the current study we examined the effect of *M. anisopliae* infection on house-entry responses of female *An. gambiae* mosquitoes. We also evaluated the effect of fungal infection on hourly human-biting response of female *An. gambiae* mosquitoes seeking to find a human host. Evaluations were conducted outdoors and indoors inside a semi-field set-up of a natural malaria mosquito ecosystem (Knols et al. 2002). The aim is to address the significance of EPF in reducing the host-seeking fraction of the mosquito population, human-vector contact and disease risk.

Materials and Methods

Mosquito rearing

The semi-field experiments were conducted using the Mbita strain of the malaria mosquito *Anopheles gambiae* Giles *sensu stricto* (hereafter termed *An. gambiae*). The mosquitoes were reared under ambient climatic conditions in the mosquito insectary at the Thomas Odhiambo Campus (TOC) of the International Centre of Insect Physiology and Ecology (*icipe*) located at Mbita Point, western Kenya. All maintenance and rearing procedures have been described in detail elsewhere (Olanga et al. 2010). The experiments utilised female mosquitoes that were 5-6 days old. The mosquitoes were starved for eight hours and did not receive a blood meal before the start of experiments. To prevent dehydration, the insects were offered water on cotton towels placed on top of the mosquito holding cups.

Fungal isolate

Metarhizium anisopliae var. *anisopliae* ICIPE 30 was used. The fungus was originally isolated in 1989 from the maize stem borer, *Busseola fusca* (Lepidoptera, Noctuidea) near Kendu Bay, Western Kenya. Fungal spores were produced at *icipe*, Nairobi. The viability of the spores was tested with percentage germination rate of conidia on Sabouraud Dextrose Agar (SDA) recorded prior to the start of experiments. At all times, the conidia were stored in the dark at 4°C. Depending on the experiments, 0.05 g and/or 0.1 g of dry conidia were used to infect mosquitoes.

Exposure to Metarhizium anisopliae

Transparent plastic cylinders of 9 cm diameter and 15 cm height were used. The inner and the base surfaces of each cylinder were lined with white rough paper that measured 28.6 × 14.3 cm and 9 cm in diameter respectively (Chapter 3). A piece of mosquito netting material was secured over the mouth of each cylinder using a rubber band. A hole was punched at the centre of the net to serve as an entry point for the mosquitoes. Eight transparent plastic cylinders each lined with white rough paper were used. *Metarhizium anisopliae* spores were weighed and 0.1 g (approx. 1.0×10^{11} conidia/m^2) or 0.05 g (approx. 5.1×10^{10} conidia/m^2) was spread in each cylinder. Sixty 2-d-old female mosquitoes were introduced in each cylinder and held for 6 h being supplied with cotton pads soaked in 6% glucose solution. The pads were placed on top of the netting material that covered the mouth of the cylinders. The mosquitoes were then transferred to a holding cage (30 × 30 × 30 cm)

and supplied with 6% glucose solution on filter paper wicks. The insects were maintained at 28 ± 2˚C and 70 ± 5 % r.h. in a room simulating semi-field conditions. In the control, uninfected mosquitoes were held in four fungus-free cylinders for 6 h prior to release into one holding cage. Twice as many mosquitoes compared to controls were exposed to fungal conidia to adjust for mortality associated with fungal-infection prior to the start of experiments. Fungus-infected mosquitoes used in the experiments were 3-d post fungal exposure.

Experimental set up

The experiments were done under semi-field conditions inside a *Malaria-Sphere* (Figure 1) (Knols et al. 2002). This experimental unit is located at the Thomas Odhiambo Campus of the International Centre of Insect Physiology and Ecology (*icipe*) located near Mbita Point township in western Kenya. It embraces a screen-walled greenhouse (11.4 × 7.1 × 2.8 m, Cambridge Glass House Co. Ltd., UK) in which a traditional African house (3.2 × 2.8 × 1.7 m) is built. Some locally cultivated food crops and naturally established plants that mimic vegetation commonly found in the homesteads were grown inside the set up.

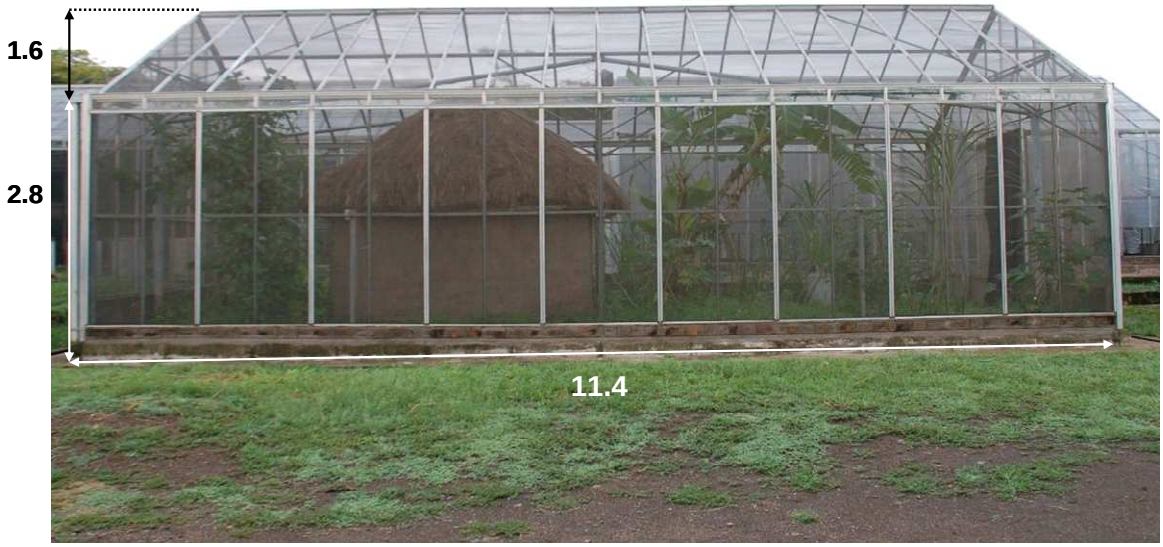

Figure 1. A semi-field set-up in Mbita Point, Kenya (Knols et al. 2002) – photograph: W. Takken

Human subjects

One male African volunteer, ranked as medium attractive (MA) to *An. gambiae* mosquitoes, was used in all the experiments (R.W. Mukabana, unpublished data). The volunteer bathed with odourless bar soap 30 min. prior to the start of experiments. Malaria infection status of the volunteer was tested daily by microscopic examination of thin and thick smears of finger-prick blood stained with Giemsa.

Mosquito collecting devices

Anopheles gambiae mosquitoes that entered the hut in response to human odours after being released inside the *Malaria-Sphere* were collected using two Centers for Disease Control (CDC) light

traps (Costantini et al. 1998a). As *An. gambiae* prefer to bite the feet of their host (De Jong and Knols 1995, Dekker et al. 1998), the traps were positioned at the foot end of the bed near the top of the bed net (Mboera et al. 1998). The human landing technique was later employed to estimate the hourly human-biting rate of female *An. gambiae* mosquitoes outdoors and indoors inside the *Malaria-Sphere*. The technique is the most effective method when studying human-biting rates (Mboera 2005, Silver 2008). Therefore, the human volunteer acted both as bait and as collector using an oral aspirator.

Effect of fungal infection on house entry rate of mosquitoes to find human hosts located indoors

First, control experiments were conducted in the absence of a human host in the hut. An intact untreated mosquito bed net was hung around a bed in the hut. Two hundred 8-h-starved female *An. gambiae* mosquitoes aged 5-d old were then released within the *Malaria-Sphere* at 21:00hr. Experiments were stopped at 06:00hr the next morning when numbers of mosquitoes inside CDC light traps, on the hut walls and on the bed net were collected, counted and recorded. Mosquitoes remaining in the *Malaria-Sphere* were aspirated during day-time before the start of the next night's experiment. Both uninfected mosquitoes and mosquitoes infected with 0.1 or 0.05 g of *M. anisopliae*, released on separate nights, were used. Treatments were conducted using similar procedures except that a human being, sleeping under an untreated bed net, was present inside the hut. The control experiments, in the absence of a human host, were conducted for 16 nights with uninfected mosquitoes and 16 nights with *M. anisopliae*-infected mosquitoes. Similarly, experiments with a human host inside the hut were conducted for 16 nights with uninfected mosquitoes and 16 nights with *M. anisopliae*-infected mosquitoes. In all experimental nights, two data loggers were each suspended inside the hut and within the *Malaria-Sphere* to record temperature and relative humidity.

Effect of infection with **M. anisopliae** *on outdoor and indoor hourly human-biting rates of An. gambiae*

Human-biting rates of 8 hr-starved female *An. gambiae* mosquitoes were assessed outdoors and indoors inside the *Malaria-Sphere*. Two hundred, 5-d old uninfected mosquitoes or mosquitoes infected with 0.05 g of *M. anisopliae* were released in groups of 50 at the four corners of the *Malaria -Sphere* in separate nights. The mosquitoes were released 30 min. prior to the start of landing collections. The human subject performing the collections sat alert on a stool outside the hut for outdoor studies and later inside the hut for indoor studies ready to capture mosquitoes off of his exposed legs and arms using a mouth aspirator. Collected mosquitoes were stored in pre-labeled holding cups. A separate holding cup was used for each hourly collection. Collections were done between 19:00 and 06:00hr the next morning. Holding cups containing mosquitoes were carried from the *Malaria-Sphere* and kept in the refrigerator prior to counting and recording. Mosquitoes remaining in the malariasphere were aspirated at daylight before the start of the next night's experiment. This procedure was conducted for four days outdoors using uninfected mosquitoes, four days outdoors using *M. anisopliae*-infected mosquitoes, four days indoors using uninfected mosquitoes, and four days indoors using *M. anisopliae*-infected mosquitoes.

Statistical analysis

The number of uninfected and *M. anisopliae*-infected mosquitoes that entered the hut included the sum of collections in CDC light traps, on the bed net and on the walls of the hut. These numbers were expressed as means. The counts were subjected to Generalized Linear Model (GLM) for multiple comparison tests to calculate the level of statistical difference between the four treatments. The

hourly human-biting rate of mosquitoes caught was calculated by dividing the number of mosquitoes collected in a given hour by the number of mosquitoes remaining in the *Malaria-Sphere* i.e. excluding the number that had already been recaptured in the elapsed hr(s). This is because the sample size was reduced by a factor "number recaptured" from the start to the end of the experimental time. The difference in hourly biting rate between the uninfected and infected groups was estimated by chi -square (χ^2) test (Preacher 2001). The analysis was conducted using GenStat® (3rd Edition) or SPSS (Version 17.0)

Ethics

Ethical approval for this study was given by the Kenya National Ethical Review Committee located at the Kenya Medical Research Institute (NON-SSC Protocol number 203).

Results

House entry rate of fungus-infected mosquitoes in response to human odours indoors

In the absence of human odours inside the hut, 99% (3180 of 3200) of the uninfected mosquitoes and 96% (3082 of 3200) of mosquitoes infected with 0.1 g of *M. anisopliae* flew out of the release cup. With the hut occupied by a human host, 98% (3134 of 3200) of the uninfected and 84% (2703 of 3200) of mosquitoes infected with 0.1 g of *M. anisopliae* left the release cup. Significantly fewer *M. anisopliae* infected mosquitoes compared to controls left the release cup in the absence (f = 23.8, df = 30, P = 0.001) and in the presence (f = 16.3, df = 30, P = 0.0001) of human odours inside the hut (Table 1). Of the total released mosquitoes, the numbers of infected mosquitoes that entered an empty hut (5.6% versus 9.1%) and the hut occupied by a human subject (16.2% versus 33.7%) were significantly less compared to uninfected mosquitoes. Overall, the response of uninfected and infected mosquitoes under the different experimental conditions differed significantly (P < 0.0001).

Table 1. Mean (± S.E.) number and percent response (in parentheses) of uninfected mosquitoes and mosquitoes infected with 0.1 g of *Metarhizium anisopliae* entering an empty or human-occupied African hut. N is the number of replicates and n the total number of mosquitoes that flew out of the release cup in each experiment. Each treatment tested 3,200 mosquitoes.

Human host in hut?	Mosquito infection status	N	n	Mean ± S.E no. of mosquitoes entering hut (%)
Absent	Uninfected	16	3180	18.06 ± 2.81 (9.1) [a]
Absent	Fungus-infected	16	3082	10.88 ± 1.67 (5.6) [b]
Present	Uninfected	16	3134	66.13 ± 5.36 (33.7) [c]
Present	Fungus-infected	16	2703	27.38 ± 2.15 (16.2) [d]

Means followed by different letters within column (between treatments) are statistically different (P = 0.0001) by multiple comparison tests.

Similarly, a fungal spore concentration of 0.05 g had a significant effect on mosquitoes. With an empty hut, 99% (3158 of 3200) of the uninfected mosquitoes and 92% (2939 of 3200) of *M. anisopliae*-infected mosquitoes left the release cup. In the presence of human odours inside the hut,

99% (3187 of 3200) of the uninfected and 96% (3066 of 3200) of *M. anisopliae*-infected mosquitoes flew out of the release cup. Significantly, more uninfected compared to infected mosquitoes flew out of the release cup with an empty hut (f = 22.6, df = 30, P = 0.0001) and with a human subject inside the hut (f = 12.9, df = 30, P = 0.0001) (Table 2). Of the total mosquitoes released, fewer infected compared to uninfected mosquitoes entered the hut in the absence (2.3% versus 3.7%) and in the presence of human odours (19.1% versus 41.4%). The response of treated and control mosquitoes differed significantly in the absence and in the presence of human host odours (P < 0.0001).

Table 2. Mean (± S.E.) number and percent response (in parentheses) of uninfected mosquitoes and mosquitoes infected with 0.05 g of *Metarhizium anisopliae* entering an empty or human-occupied African hut. N is the number of replicates and n the total number of mosquitoes that flew out of the release cup in each experiment. Each treatment tested 3,200 mosquitoes.

Human host in hut?	Mosquito infection status	N	n	Mean ± S.E no. of mosquitoes entering hut (%)
Absent	Uninfected	16	3158	7.38 ± 1.37 (3.7) [a]
Absent	Fungus-infected	16	2939	4.25 ± 0.65 (2.3) [a]
Present	Uninfected	16	3187	82.56 ± 5.97 (41.4) [b]
Present	Fungus-infected	16	3066	36.56 ± 3.23 (19.1) [c]

Means followed by different letters within column (between treatments) are statistically different (P = 0.0001) by multiple comparison tests..

Outdoor hourly human-biting rates of M. anisopliae*-infected mosquitoes*

In this study, 80% (644 of 800) of the uninfected and 52% (419 of 800) of fungus-infected female *An. gambiae* mosquitoes were recaptured between 19:00 and 06:00 hr (Figure 2). The difference between the catches was significant (χ^2 = 141.89; P = 0.0001) with fewer infected mosquitoes recaptured than uninfected ones. An increase in biting activity was observed from early in the night with the peak occurring between 23:00-24:00 hr in both the uninfected and *M. anisopliae*-infected groups. However, a sharp increase was more pronounced in the uninfected group with a slight decline in infected group between 21:00-22:00 hr. After midnight, the biting rate gradually decreased in both treatments until 03:00 hr and thereafter increased slightly to dawn. Between treatments, significantly (P = 0.0001) fewer *M. anisopliae*-infected mosquitoes responded to odours emanating from the human volunteer throughout the night compared with uninfected mosquitoes except for the periods between 19:00-20:00 hr (χ^2 = 0.68; P = 0.41) and 20:00-21:00 hr (χ^2 = 2.73; P = 0.1) (Figure 2).

Indoor hourly human-biting rates of M. anisopliae*-infected mosquitoes*

When the host was seated indoors, 65% (522 of 800) of the uninfected and 43% (344 of 800) of the *M. anisopliae*-infected mosquitoes were recaptured throughout the night of sampling (Figure 3). Significantly, fewer infected compared to uninfected mosquitoes were collected (χ^2 = 79.75; P = 0.0001). Despite a slight drop in biting activity of mosquitoes between 20:00-21:00 hr, early evening biting was evident with the peak occurring between 21:00-22:00 hr with *M. anisopliae*-infected mosquitoes and from 21:00-23:00 hr with uninfected mosquitoes. Furthermore, the biting rate

decreased gradually up to 03:00 hr in both groups. This was followed by an increase in the activity again in both treatments before a decline at dawn. In this zigzag pattern of response, significantly (P = 0.0001) more uninfected mosquitoes were attracted to the volunteer compared to fungus-infected mosquitoes. However, the difference between the treatments in the number of mosquitoes that responded was not significant for collections between 19:00-20:00 hr (χ^2 = 0.17; P = 0.679), 23:00-24:00 hr (χ^2 = 1.87; P = 0.171) and 02:00-03:00 hr (χ^2 = 1.42; P = 0.233).

Both outdoor and indoor human-biting rates with uninfected and fungus-infected mosquitoes were higher between 19:00 and 01:00 hr than from 01:00-06:00 hr. However, from 19.00-01:00 hr indoor collections were lower than outdoor collections for both treatments. From 01:00 hr until dawn, indoor catches were higher than outdoor ones.

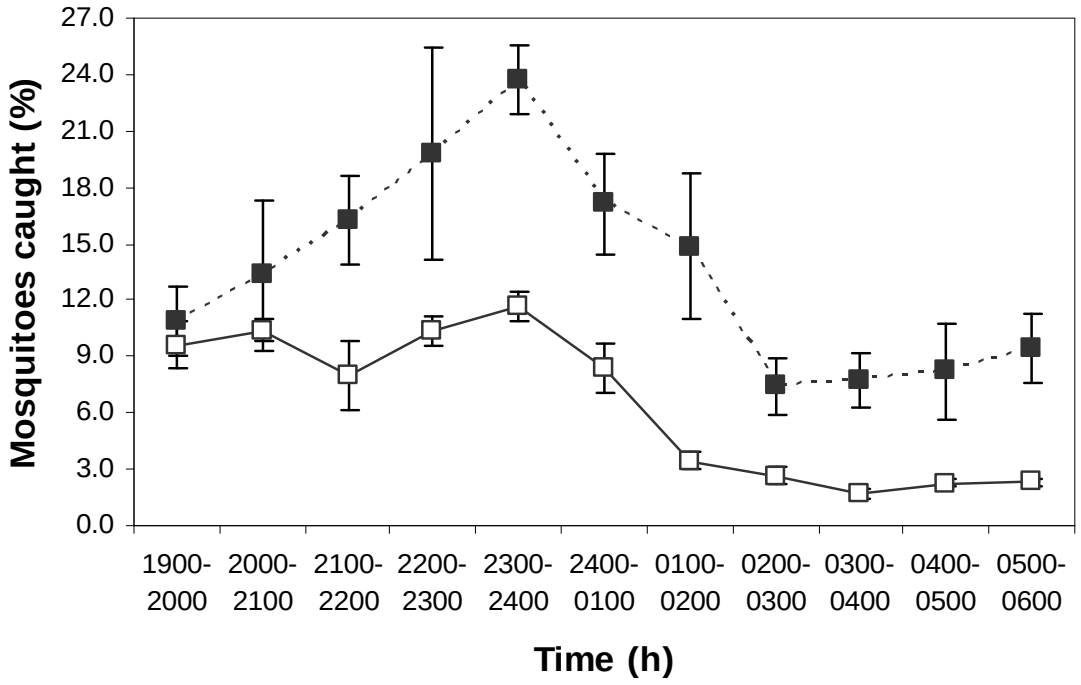

Figure 2. Proportion of female *An. gambiae* mosquitoes that responded hourly to odours emanating from human subject located outdoors throughout the night. Uninfected and *M. anisopliae*-infected mosquito groups are depicted with black and open squares, respectively. Level of statistical difference between treatments was calculated by Chi square (χ^2) test. Each treatment tested 800 mosquitoes.

Discussion

The findings of this study demonstrate that, under semi-field conditions, infection with the entomopathogenic fungus *M. anisopliae* has a strong impact on the mosquito's behaviour, reducing the proportion of *An. gambiae* female mosquitoes that respond to odours emanating from a human host located indoors. Furthermore, the results demonstrate that infection with *M. anisopliae* reduces the proportion of mosquitoes biting people outdoors and indoors, but that the nocturnal hourly biting pattern is not affected by fungal infection.

The mosquitoes for these experiments were utilised three days after infection with *M. anisopliae* based on the understanding that it is around this time after exposure that the fungus expresses a pathologic effect in the host (Shah and Pell 2003, Roy et al. 2006). This can vary, however, depending on the host species, host stage, temperature and virulence of the fungus (Ferron 1978, Zimmermann 2007b). Considering the virulence part, our spore viability of 86% (data not shown) resulted in a high infection rate that significantly reduced the survival of infected mosquitoes by approximately 50% before bioassays. Therefore, we exposed more mosquitoes to the fungus than were held as controls prior to the start of experiments (see Chapter 4). According to (Hajek and St Leger 1994), a reduction in feeding is one of the first signs in an infected insect. Thus, fungal persistence is responsible for the reduction in survival at the early stages of infection. Nevertheless, several factors contribute to the longer survivorship of insects after infection with the entomopathogenic fungi (EPF). They include the number of spores the insects pick up (Moore et al. 1992), the physical condition of the insect at the time of exposure to fungal spores and the immune response of the insect to fungal invasion (Gunnarsson 1988).

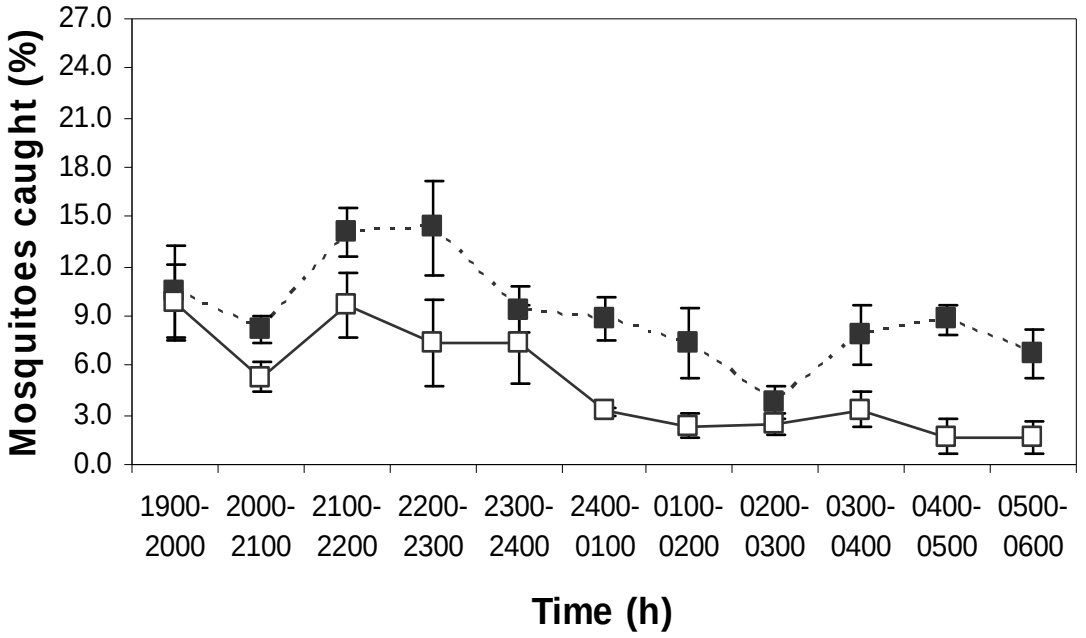

Figure 3. Proportion of female *An. gambiae* mosquitoes that responded hourly to odours emanating from human subject located indoors throughout the night. Uninfected and *M. anisopliae*-infected mosquito groups are depicted with black and open squares, respectively. Level of statistical difference between treatments was calculated by Chi square (χ^2) test. Each treatment tested 800 mosquitoes.

The success of *An. gambiae* to transmit malaria among a human population depends on the availability of blood hosts and the vector's ability to overcome physical barriers and the various host defense mechanisms in order to bite frequently. Therefore, the capability of *An. gambiae* to enter houses and bite people is a critical component of their host-seeking behaviour. The mosquito overcomes barriers by the well-advanced behavioural traits of indoor feeding and resting (Gillies and Coetzee 1987). Further, with the preference to feed on humans, *An. gambiae* also responds to persons located outdoors. Hence, disruption of mosquito house entry and human-biting rates could lead to reduced human-vector contact and subsequent reduced malaria transmission. Disruption of these factors with the use of EPF has been illustrated in this study whereby fungal infection affected

the flight performance of *An. gambiae*. A reduction in flight activity has also been reported in *An. stephensi* infected with *Beauveria bassiana* (Blanford et al. 2011).

In our study, impact of infection on flight performance resulted in a 2-fold reduction in the number of mosquitoes collected indoors in the absence as well as in the presence of human odours, compared to the controls. A reduction with a similar margin between fungus-infected and uninfected mosquitoes has been reported in *An. gambiae* in the laboratory (Scholte et al. 2006, Ondiaka et al. 2008b), in semi-field conditions (see Chapter 4) and in the *An. gambiae* complex under field conditions (Scholte et al. 2005). With other pathogens, costs in terms of flight performance and increased mortality have been reported in *An. gambiae* infected with the malaria parasite *P. falciparum* (Anderson et al. 2000, Charlwood and Tomás 2011) and in *An. stephensi* infested with *P. yoelii* (Rowland and Boersma 1988). However, blood-feeding behaviour which is quite significant in malaria transmission is increased in *Plasmodium*-infected mosquitoes compared to the uninfected ones (Koella et al. 1998, Koella et al. 2002). Nevertheless, disease transmission could be lowered by reducing the vector population at the host-seeking stage before bloodfeeding through conventional and microbial control approaches.

A human volunteer was available outdoors and indoors to simulate the life-style in African villages where household members often stay outdoors conducting businesses or household chores until late into the night. These activities expose humans to high mosquito contact rates and disease risk influenced by the biting rate of the vector. We have demonstrated that although *An. gambiae* bites throughout the night, the outdoor human-biting rate of uninfected and fungus-infected mosquitoes was greater than that indoors. Similar observations with uninfected mosquitoes from the *An. gambiae* complex (Afolabi et al. 2006, Geissbühler et al. 2007, Aldemir et al. 2010) and with the mosquito *Armigeres subalbatus* (Pandian and Chandrashekaran 1980) have been reported under field conditions. The difference is attributed to the presence of a physical barrier i.e. the hut that mosquitoes had to enter in order to reach the human host sleeping indoors. The biting pattern throughout the night was also different from that exhibited by mosquitoes sampled in a field setting. This is because we evaluated a fixed number of mosquitoes as opposed to a field population whose biting habit is influenced by several environmental factors. However, the biting peak observed between 22:00 and 24:00 hr was similar to that reported in *An. gambiae*, *An. arabiensis* (Geissbühler et al. 2007), *An. darlingi* (Fouque et al. 2010) and *An. merus* (Sharp 1983, Mutero et al. 1984). Similarly, the increase in biting rate at dawn has also been reported in *An. gambiae* s.l. (Surtees 1970), *An. bwambae* (Haddow and Ssenkubuge 1973) and *An. arabiensis* (Braack et al. 1994). Therefore, understanding of the human-biting cycle as well as the biting rate of malaria vectors both outdoors and indoors is important in designing control strategies (Rubio-Palis and Curtis 1992, Braack et al. 1994, Faye et al. 1997, Moreno et al. 2007, Fouque et al. 2010, Ghosh et al. 2010).

Although EPF reduced the host-seeking population entering the house or biting indoors and outdoors, the human host is still susceptible to bites from a fraction of infected mosquitoes that responded to host odours throughout the night but that may be overcome by the application of mosquito-repellents and by sleeping under a bed net. The bites may not be a threat in terms of disease transmission considering the mode of action of fungal pathogens that could have advantages over the knock-down effects caused by insecticide usage. First, it is likely that fewer fungus-infected mosquitoes collected indoors or outdoors will blood-feed since infection reduces the feeding propensity (Scholte et al. 2006, Howard et al. 2010b). Secondly, mosquitoes that succeed to ingest a blood meal may further succumb to a reduction in fecundity (Scholte et al. 2006) and a reduction in malaria transmission potential because mosquitoes will die before *Plasmodium* parasites are fully developed (Blanford et al. 2005, Scholte et al. 2005, Read et al. 2009). A reduction in feeding and fecundity due to infection with fungal pathogens has also been reported in other insects (Arthurs and Thomas 2000, Ekesi and Maniania 2000, Tefera and Pringle 2003, Hajek et al. 2008). Third, fungal

pathogens have demonstrated potential to control the vector population indoors and outdoors whereas insecticide usage is more successful with indoor than outdoor species. Furthermore, increased usage of insecticide-treated nets is causing a shift from biting indoors to outdoors (Mathenge et al. 2001, Reddy et al. 2011, Russell et al. 2011). This shift to biting outdoors may be an emerging concern over sustainability of insecticide usage in addition to development of insecticide-resistance by the vectors.

The slow speed of kill, the loss of spore persistence over time and successful infection of mosquitoes outdoors may be a major drawback with fungal pathogen application. However, the resulting direct and indirect effects of fungal infections should not be overlooked. Directly, infection increases mortality in infected insects that lead to reduction in longevity and subsequent reduction in vector population. Indirectly, by the slow-kill nature of fungal pathogen, fungal infection results in a reduction in reproductive fitness (see Chapter 8), feeding propensity and fecundity. Spore persistence could be retained by increasing the dosage, using a suitable carrier that may withstand ultra-violet radiation and by treating entire infecting surfaces with conidia. Indoors, mosquitoes can be infected by resting on surfaces impregnated with fungal conidia (Scholte et al. 2005). Outdoors, mosquitoes have been controlled by combining synthetic insecticides with natural sugar sources as baits (Müller et al. 2010c, Müller et al. 2010b, Müller et al. 2010a). This mixture is either sprayed on the flowering plants in which the insects are killed during feeding or by applying inside stationary traps baited with fermented ripe fruits and flower scent as attractants (Muller et al. 2010). The strategy may be adopted and modified to infect mosquitoes with EPF outdoors. Amongst other alternative pathways, mosquitoes may be infected outdoors using wicker baskets and bait stations impregnated with fungal conidia. However, further evaluation is recommended on the impact of the fungus on non-target species, e.g. pollinators.

Conclusions

Our results clearly show that infection with an entomopathogenic fungus caused a significant impact on the flight performance of *An. gambiae* female mosquitoes. As a result, their house entry rate in response to a human host was sharply reduced. Also significantly reduced was the biting rate of the mosquitoes throughout the night with human volunteers either outdoors or indoors. However, infection did not cause changes in the nocturnal hourly-biting pattern of *An. gambiae* mosquitoes. The findings underscore the potency of fungal pathogens which is critical in the development of the novel tool as microbial control agents against malaria vector.

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Chapter 6

Effects of fungal infection on feeding and survival of *Anopheles gambiae* (Diptera: Culicidae) on plant sugars

To be submitted as: Ondiaka S., Takken W., Koenraadt C. J. M., Masinde E. W., Mukabana W. R. Effects of fungal infection on feeding and survival of *Anopheles gambiae* (Diptera: Culicidae) on plant sugars.

Abstract

The entomopathogenic fungus *Metarhizium anisopliae* has shown great potential for the control of adult malaria vectors. A promising strategy for infection of mosquitoes is supplying the fungus at the feeding site(s). However, the efficacy of fungal infection on plant sugar feeding behaviour of anopheline vectors is unexplored. Therefore, we evaluated the survival of fungus-exposed *Anopheles gambiae* mosquitoes (males and females) on 6% glucose and on plant sugars of *Ricinus communis* (Castor bean) and *Parthenium hysterophorus* (Parthenium weed). Further, we determined the feeding propensity, quantity of sugar ingested and its digestion rate in mosquitoes when fed on *R. communis* for 12 hr, one and three days post-exposure to fungus. The anthrone test was employed to detect the presence of sugar in each mosquito from which the quantity consumed and the digestion rates were estimated. Fungus-exposed mosquitoes lived significantly shorter than uninfected mosquitoes on 6% glucose (7 versus 37 d), *R. communis* (7 versus 18 d) and *P. hysterophorus* (5 versus 7 d), respectively. Significantly fewer male and female mosquitoes, one and three days post-exposure to fungus, fed on *R. communis* compared to their respective controls. Although the quantity of sugar ingested was similar between the treatment groups, fewer fungus-exposed than control mosquitoes ingested small, medium and large meals. The reduction in sugar intake was more prominent in mosquitoes three days than one day post-exposure. Digestion rate was significantly lower in females one day after exposure to *M. anisopliae* compared to controls but remained the same in males. No change in digestion rate between treatments was observed three days after exposure. The results demonstrate that entomopathogenic fungi (EPF) strongly impact survival and sugar-feeding propensity of both sexes of the malaria vector *An. gambiae* but do not affect their potential to feed and digest meals. Moreover, both *R. communis* and *P. hysterophorus* harbour toxins and sugars that are either not easily digestible or inhibit growth compared to 6% glucose to affect longer survivorship of mosquitoes. These findings therefore, suggest that plant sugar sources can be targeted as fungal delivery substrate to infect mosquitoes by EPF. Furthermore, targeting males for population reduction by EPF opens a new strategy for mosquito vector control.

Introduction

Plant sugar acquired from floral and extrafloral nectaries, honeydew, damaged fruits and leaves is essential for the survival of mosquitoes (Nayar and Sauerman Jr 1971, Yuval 1992, Foster 1995). It is the only nutritional source of adult males and an optional dietary supplement to blood for females. However, sugar feeding is an early priority for both sexes as they emerge with little energy reserves (Briegel 1990, Foster and Hancock 1994, Takken et al. 1998, Clements 1999, Gary et al. 2009). Besides survival and building of energy reserves, sugar enhances maturation of ovarian follicles in females and reproductive fitness in males (Gary and Foster 2006). Survival of the malaria mosquito *Anopheles gambiae* is assured with frequent feeding and ingestion of sizeable amounts of sugar meals (Gary and Foster 2004) or by ingestion of small amounts of sugar at a time (Yuval 1992, Foster 1995). Recent studies have shown that mosquitoes feed on a wide variety of plants common in their natural habitats (Impoinvil et al. 2004, Manda et al. 2007b, Gouagna et al. 2010, Muller et al. 2010). Sugars from some of these plants promote longer survival of both sexes, which enhance the vectorial capacity of females (Okech et al. 2003, Gary and Foster 2004). It is clear that plant sugars play a role in regulating the dynamics of mosquito populations. Control interventions that target mosquito sugar feeding may be promising in reducing transmission of malaria parasites and other pathogens (Gu et al. 2011). Therefore, knowledge on the role of plant sugars in the biology of mosquitoes is essential for development and improvement of control strategies against the vector.

Currently, control of the malaria vector *Anopheles gambiae* focuses on the use of synthetic pyrethroids impregnated in bed nets or as indoor residual spray (WHO 2011). The strategy has been effective in reducing malaria morbidity and mortality. However, concern over widespread resistance to chemicals has triggered interest in the search for alternatives such as microbial agents. Entomopathogenic fungi (EPF) are among the microbial agents used against a wide range of insect pests (Lord 2005, Thomas and Read 2007a) including adult mosquitoes (Scholte et al. 2003a). However, fungi often require several days after the initial application to kill their hosts. During these early days of infection, the insects may continue to feed and cause damage to crops or transmit diseases (Thomas et al. 1997, Scholte et al. 2004b). For this reason microbial pathogens have been criticised as alternatives to chemical insecticides. Nevertheless, studies have shown that fungal pathogens reduce survival of *Anopheles* mosquitoes to a level that prevents transmission of malaria parasites (Blanford et al. 2005, Scholte et al. 2005, Blanford et al. 2011). Further, the pathogen reduces blood feeding (Scholte et al. 2006, Howard et al. 2010b) and fecundity and inhibits the development of malaria parasites in the mosquito vector (Blanford et al. 2005). Therefore, the slow speed of kill by the fungal pathogen poses some advantage that impact on the feeding and reproduction behaviours of insects. Most studies, though, have targeted females due to their significant role in malaria transmission with the contribution of males in the whole process often overlooked. Mosquitoes are also associated with plants and male dependence on plants offers opportunities for intervention of the malaria-vector population. However, the potential of this strategy remains un-explored.

Most studies have reported fitness costs associated with infection by EPF in major life history aspects of malaria vectors both in the laboratory using processed sugar as well as in the field (Scholte et al. 2005, Ondiaka et al. 2008b, Bukhari et al. 2011a, Garcia-Munguia et al. 2011). As sugar feeding is central in the biology of adult mosquitoes, we are interested to assess whether infection of mosquitoes with an entomopathogenic fungus impacts sugar feeding.

The present study investigated three aspects of sugar feeding behaviour of both sexes of adult *An. gambiae* mosquitoes under ambient conditions inside a screenhouse. Specifically, we (i) determined the survival of fungus-exposed *An. gambiae* mosquitoes when fed on glucose or plant sugars (ii) established the feeding propensity and the quantity of sugar ingested from plants by the infected

mosquitoes and (iii) assessed the digestion rate of sugar imbibed by fungus-exposed mosquitoes.

Materials and Methods

Mosquitoes

Experiments were carried out using laboratory-reared *Anopheles gambiae* Giles *sensu stricto* (hereafter termed *An. gambiae*) mosquitoes obtained from a colony established from wild gravid females collected at Mbita Point (000 25'S, 340 13'E), western Kenya in 1999 (Menge et al. 2005). All mosquito life stages were maintained under ambient conditions in the mosquito insectary at the Thomas Odhiambo Campus (TOC) of the International Centre of Insect Physiology and Ecology (*icipe*) at Mbita Point. Larval and adult stages of the mosquitoes were raised using procedures described by Olanga et al. (2010). Both sexes were separated at emergence and held under ambient conditions in 30 × 30 × 30 cm cages inside a screenhouse. Before experiments, the insects were maintained either on an aqueous 6% glucose solution or on plant cuttings of *Ricinus communis* (Caster bean) and *Parthenium hysterophorus* (Parthenium weed).

Fungal isolate

The entomopathogenic fungus *Metarhizium anisopliae* isolate ICIPE 30 was used in the study. The fungus was originally isolated from the stem borer *Busseola fusca* in Kendu Bay, western Kenya in 1999 (courtesy Dr. N.K. Maniania) and has been maintained at the *icipe's* Germplasm Centre. Conidia were produced on long rice as substrate following the technique described by Maniania et al. (2003). Harvested spores were dried for 48 hr in a desiccator containing active silica gel and stored in a refrigerator (4-6°C) until required. The viability of conidia was determined before being used in the experiments. Germination rates >85% after 24 hr on Sabouraud dextrose agar was considered adequate for use in the experiments.

Standard sucrose solutions

Standard sucrose solutions of different strength in the series 1, 2, 4, 8, 16, 32, 64, 128, and 256 µg/µl were used and distilled water as the neutral liquid. Initially, 25.6 g of reagent grade sucrose was dissolved in 50 ml of distilled water. More water was added gradually while mixing to make a 100 ml solution, from which eight two-fold serial dilutions were prepared. These solutions were stored at -4° C.

Anthrone reagent

Diluted sulphuric acid was prepared by mixing 380 ml concentrated sulphuric acid with 150 ml distilled water in a fume hood. The hot solution was kept for 5 hr at room temperature to cool and a further 12 hr in the refrigerator at 5°C before use. The anthrone solution was then prepared by mixing 0.15 g of anthrone powder per 100 ml of the diluted sulphuric acid.

Cold anthrone test

This test was used to assess the presence of sugar in the mosquitoes. Three test tube racks were used with each rack holding one hundred 5-ml test tubes. One rack was used to hold 10 test tubes for the standard solutions. The standards were prepared by pipetting 1 µl from each of the nine standard sucrose solutions into the nine separate test tubes. The tenth tube contained 1 µl of distilled water. The other two racks were used to hold both sexes of uninfected and *M. anisopliae*-

exposed *An. gambiae* mosquitoes. Each tube held one mosquito. One drop of chloroform and methanol in the ratio 1:1 was added to each tube containing mosquitoes to dissolve the cuticle. The racks were held in a biological hood where 0.5 ml of anthrone reagent was added to the standards and the mosquitoes. The racks were then transferred into a water bath at room temperature for one hr. In the presence of sugar, the colour of the solutions changes from green to green-blue and further dark-blue depending on the amount of sugar. In absence of sugar, the colour of the sample is transparent yellow. The test is biased towards fructose detection. After one hour, the results were read by comparing the colour change in the mosquitoes and that of the standard solution. The quantity of sugar detected ranged from 1-64 µg. For easy analysis of the data, the mosquitoes were categorised as consumers of small, medium or large meals (Van Handel 1972) if they imbibed 1-4 µg, 8-16 µg or 32-64 µg of sugar, respectively.

Plant species as sugar sources

Two plants, *Ricinus communis* (Castor bean) and *Parthenium hysterophorus* (Parthenium weed) [1], were used. Castor bean is characterised as a plant whose floral component enhances survivorship of *An. gambiae* while Parthenium weed is among the most preferred plants and frequently visited by *An. gambiae* mosquitoes (Manda et al. 2007b)

Infection process

Transparent plastic cylinders of 9 cm diameter and 15 cm height were used to inoculate *An. gambiae* mosquitoes with spores of *M. anisopliae* (Chapter 3). The inner and the base surfaces of the cylinder were lined with white rough paper that measured 28.6 × 14.3 cm and 9 cm in diameter respectively. The cylinder was held in a slanting position and 0.1 g (approx. 1.0×10^{11} conidia/m^2) of *M. anisopliae* spores were weighed and poured on the paper. Using both hands, the cylinder was rolled several times until the spores had distributed evenly over the papers. The inner and the base surfaces of the cylinder used for uninfected mosquitoes were lined with white rough paper without spores.

Four cylinders (two cylinders with fungus and two cylinders without fungus) were used to infect male mosquitoes for survival experiments. Similar numbers of cylinders and their respective treatments were used to infect female mosquitoes. Fifty newly emerged males and females were introduced into each of their respective four cylinders. The insects were held for 6 h being supplied with 6% glucose solution soaked in cotton wool and placed on top of the netting material covering the cylinder. The mosquitoes were then transferred into four separate holding cages (30 × 30 × 30 cm) based on treatment and sex and were supplied with 6% glucose solution on filter paper wicks. The insects were maintained under ambient conditions inside a screenhouse. This procedure was repeated to infect the same number of mosquitoes but the insects were provided with floral parts of *R. communis* and *P. hysterophorus* on separate occasions as source of sugar instead of 6% glucose. The base of the floral parts was hooked on the netting material covering the mouth of the cylinder using a tooth-pick. In this way mosquitoes could feed on the floral parts during the infection period of 6 hr. For sugar quantity and digestion rate experiments eight cylinders i.e. five cylinders with fungus and three cylinders without fungus were used each holding 60 males. The same numbers of cylinders were used to infect females. The number of mosquitoes exposed to fungus was higher than that of the uninfected group to adjust for fungal-induced mortality in the holding cages.

[1] *Parthenium hysterophorus* was recently published as wild quinine (Manda et al 2007), but this is the common name for *Parthenium integrifolium.* In the current paper we use *Parthenium* weed as common name for *Parthenium hysterophorus*.

Effect of infection by M. anisopliae *on survival of* An. gambiae *mosquitoes*

Survival on 6% glucose

One hundred males and 100 females, 6 hr post-exposure to *M. anisopliae* upon emergence, were held in separate holding cages (30 × 30 × 30 cm) and supplied with 6% glucose solution regularly. Mortality was recorded daily and dead individuals were plated in a Petri dish lined with wet filter paper and incubated at 28 ± 2˚C. Fungal growth on the insect was observed under a compound microscope at 400× magnification after at least three days. The experiment was replicated four times over time.

Survival on plant sugars

One hundred males and 100 females, 6 hr post-exposure to *M. anisopliae* upon emergence were held in separate holding cages (30 × 30 × 30 cm). Each cage was supplied with a 250-ml flat bottomed conical flask. The flask contained 200-ml filtered water and at least five stems of *R. communis* with leaves and floral parts intact. The stems were replaced every two days. Mortality was recorded daily and dead insects were plated in a Petri dish lined with wet filter paper and incubated at 28 ± 2˚C. Fungal growth on the cadaver was observed after at least three days under a compound microscope at 400× magnification. The experiment was replicated four times over time. The procedure was repeated by using *P. hysterophorus* in place of *R. communis.*

Effects of infection by M. anisopliae *on amount of sugar ingested by* An. gambiae

Preliminary experiments

These experiments were conducted to determine whether there was a plant species effect on sugar uptake by mosquitoes and to establish the time required for mosquitoes to feed fully. Of the 200 male and 200 female mosquitoes tested on each plant, more males (85% versus 40%) and females (90% versus 37%) ingested sugar on *R. communis* than on *P. hysterophorus*. Moreover, *R. communis* evoked ingestion of small, medium and large amounts of sugar whereas *P. hysterophorous* evoked ingestion of small amounts only. Therefore, *R. communis* was selected for further experiments. Three groups each composed of 50 males and 50 females were established. Mosquitoes in the first, second and third group were fed on the plant for 6, 12 and 24 hrs, respectively. The experiments were replicated four times over time. At 12 hrs, more male (85%) and female (90%) mosquitoes had imbibed sugar compared to males (69%) and females (74%) at 6 h and males (51%) and females (68%) at 24 hrs, respectively. Thus, 12 hrs were selected as the time mosquitoes were exposed to *R. communis* to determine sugar uptake and digestion.

Quantity of sugar imbibed

The amount of sugar ingested by male and female mosquitoes, one and three d post-exposure to *M. anisopliae* and fed on *R. communis* for 12 hr, was evaluated. One day after exposure to fungus, fifty male and female mosquitoes were aspirated, each from their respective uninfected and fungus-exposed cages and released into four separate cages. The insects were starved for 6 hr prior to introduction of a 250-ml conical flask containing stems of *R. communis* in each cage. After 12 hr of feeding, the insects were removed from the cages and held in four separate collection cups. The insects were anaesthetised and their sugar levels quantified. The experiment was replicated four times over time. This procedure was repeated with mosquitoes three days post-infection.

Effects of infection with M. anisopliae *on digestion rate of sugars imbibed from plants*

The digestion rate in *An. gambiae* mosquitoes exposed to *M. anisopliae* was determined by feeding males and females on *R. communis* for 12 hr. One day after exposure to fungus, 50 mosquitoes were aspirated from each cage holding uninfected and fungus-exposed mosquitoes and released into four separate cages. The mosquitoes were starved for 6 hr prior to introduction of the plant in a 250-ml conical flask in each cage. Mosquitoes were allowed to feed on *R. communis* for 12 hr after which the flask containing the plant parts were removed from the cages. Fifty mosquitoes that appeared fully fed were removed and held in separate cages. Ten mosquitoes were removed from the cage at an interval of 8 hr starting from time zero through to 32 hr, anaesthetised and their digestion rate quantified with the anthrone test. The experiment was replicated four times over time. This procedure was repeated with mosquitoes three days post-infection.

Statistics

Survival of uninfected and *M. anisopliae*-infected mosquitoes on glucose (6%), *R. communis* and *P. hysterophorous* was calculated by expressing the number of mosquitoes that succumbed to mortality as a percentage of the total number tested. Difference in survival between uninfected and fungus-infected groups was estimated using Cox regression analysis. Mortality rates, expressed as Hazard Ratio (HR) estimate the risk of dying when infected compared to when not infected with fungus. To evaluate effects of infection on the amount of sugar ingested by infected (one and three days post-exposure) and control mosquitoes, first the number of mosquitoes that had fed on *R. communis* was expressed as a percentage of the total number tested. Further, the number of mosquitoes that imbibed small, medium and large quantities of sugar, respectively, was expressed as the mean percentage of the total number of mosquitoes tested. The difference between control and fungus-infected mosquitoes was calculated with the Chi square (χ^2) test (Preacher 2001). The digestion rate of the sugar ingested by mosquitoes one and three days post-exposure was each calculated by logistic regression. Logistic relationships for uninfected and fungus-exposed mosquitoes were fitted to describe sugar detection success for each time elapsed since feeding. The difference between uninfected and fungus-exposed mosquitoes was estimated by the Chi square (χ^2) test. All analyses were conducted using SPSS (version 17.0)

Results

Survival of M. anisopliae*-infected mosquitoes on different nutritional sources*

Infection with *M. anisopliae* reduced the survival of both sexes of *An. gambiae* with 100% mortality occurring within seven days compared to ≥ seven days with uninfected mosquitoes irrespective of the nutritional source (Figure 1). Survival of infected male and female mosquitoes in each nutritional group was significantly different from their respective controls. For example, the daily risk of death for both sexes was eight-fold greater on 6% glucose; four-fold (males) and eight-fold (females) greater on *R. communis* and two-fold greater for both sexes on *P. hysterophorus* relative to their controls (Table 1). In uninfected mosquitoes, the daily risk of death was three-fold greater for both males (HR = 3.4 [95% CI= 2.91 - 4.21], P = 0.0001) and females (HR = 2.9 [95% CI= 2.45 - 3.55], P = 0.0001) on *R. communis* and 14-fold greater for males (HR = 14.1 [95% CI= 11.33 - 17.6], P = 0.0001) and 13-fold greater for females (HR = 13.4 [95% CI= 10.71 - 16.8], P = 0.0001) on *P. hysterophorus* relative to 6% glucose. Therefore, *P. hysterophorus* caused a drastic reduction in the survival of mosquitoes regardless of fungal infection. Between sexes, survival rate over time in each nutritional regime was not different. Mycosis test results indicated high infection rates (>77%) in fungus-exposed male and female mosquitoes. No fungal conidia were observed on the cadavers of the control mosquitoes.

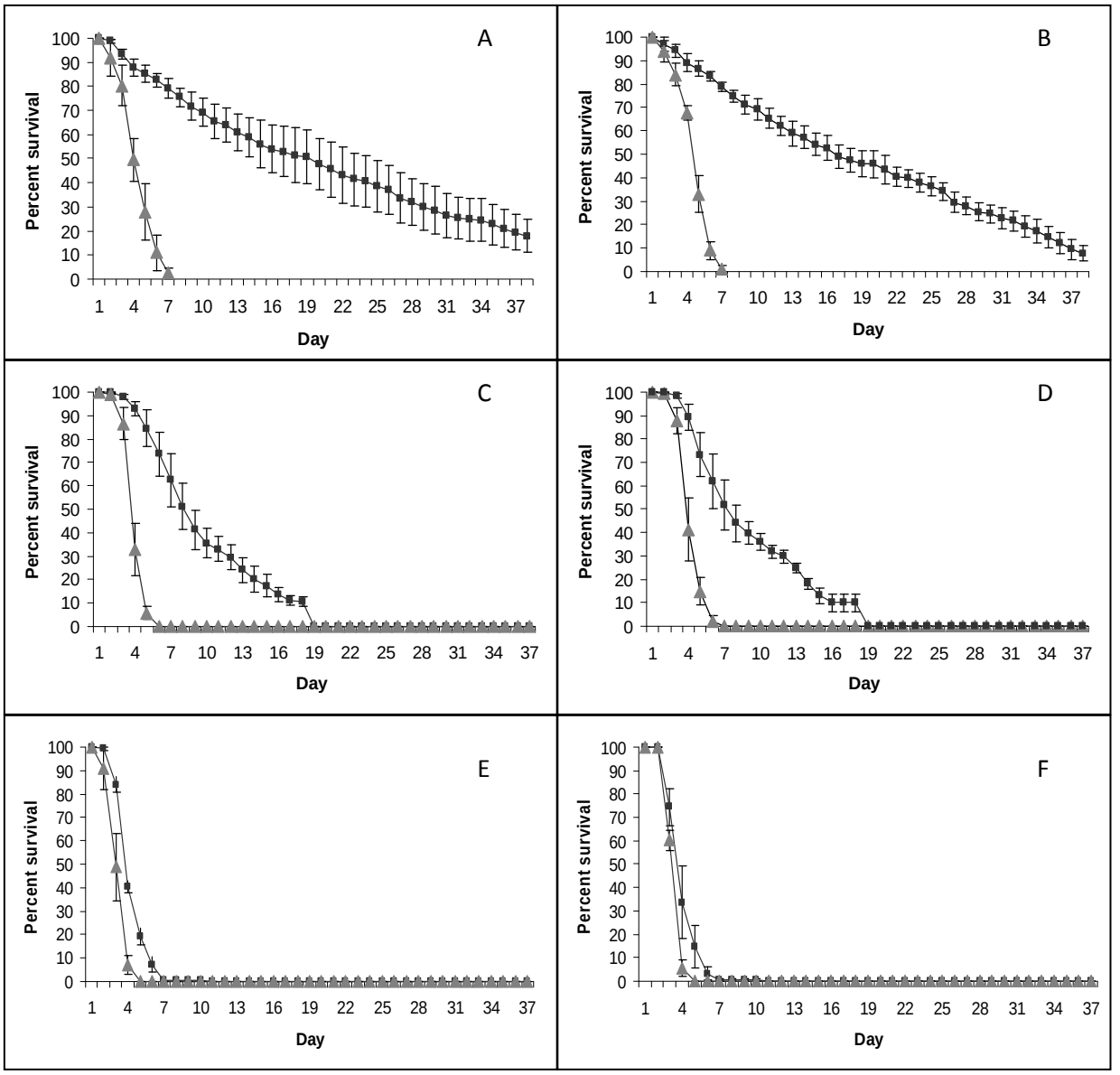

Figure 1. Survival of uninfected and *M. anisopliae*- infected *An. gambiae* females (Panel A, C and E) and males (Panel B, D and F) when fed on: - (i) 6% glucose (panel A and B); (ii) *Ricinus communis* (panel C and D) and (iii) *Parthenium hysterophorus* (Panel E and F). Uninfected and *M. anisopliae*-infected mosquitoes are depicted by closed squares and closed triangles respectively.

Feeding propensity and sugar quantity imbibed by M. anisopliae*-infected mosquitoes*

Significantly fewer male and female mosquitoes exposed to fungus imbibed sugar from *R. communis* compared to mosquitoes not exposed to fungus (Figure 2). Mosquitoes that imbibed sugar were further classified as small, medium or large feeders based on the amount of sugar ingested. In each feeding category, more uninfected males (Table 3) and females, except the large feeders (Table 2) ingested sugar compared to fungus-exposed mosquitoes. Further, fewer mosquitoes (both sexes) three days post-exposure imbibed sugar in each feeding category compared to mosquitoes one day post-exposure. Between treatments, the difference in the amount of sugar ingested was not significant except for medium-feeding females three days post-exposure (Table 2) and small-feeding males, one and three days post-exposure, respectively (Table 3). Results from the mycosis test

demonstrated high infection rates (> 75%) in fungus-exposed males and females. No fungal conidia were observed on the cadavers of control mosquitoes.

Table 1. Survival analysis of *An. gambiae* mosquitoes infected with *M. anisopliae* and fed on different nutritional sources; data show Cox regression Hazard Ratio (HR) outcomes (95% CI), statistical p-values are relative to the relevant control (not exposed to fungus).

Nutritional sources	**HR (95% CI)**			
	Male mosquitoes	**P-value**	**Female mosquitoes**	**P-value**
Glucose (6%)	8.53 (6.68 - 10.89)	0.0001	7.64 (5.99 - 9.75)	0.0001
Ricinus communis	4.33 (3.59 - 5.23)	0.0001	8.21 (6.49 - 10.37)	0.0001
Parthenium hysterophorus	1.62 (1.40 - 1.89)	0.0001	2.15 (1.85 - 2.50)	0.0001

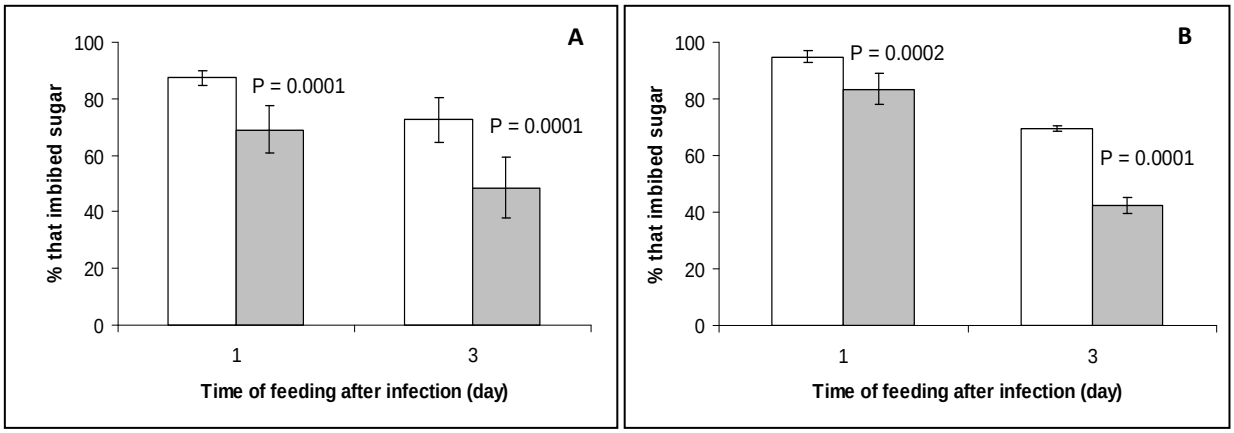

Figure 2. Mean (± S.E) percentage of uninfected and *M. anisopliae*- infected *An. gambiae* males (Panel A) and females (Panel B) that imbibed sugar on exposure to *Ricinus communis* for 12 hr. White and gray shaded bars represent uninfected and *M. anisopliae*-infected mosquitoes respectively. Level of statistical difference between treatments was calculated by Chi square (χ^2) test. Each treatment tested 200 mosquitoes.

Digestion rate of M. anisopliae*-infected mosquitoes*

The proportion of uninfected and *M. anisopliae*-exposed mosquitoes in which sugar could be detected decreased over time (Figure 3). For each time period since feeding, more mosquitoes, one day post-exposure to fungus, tested positive for sugar than uninfected mosquitoes. However, the difference between treatments was not significant except in males at 32 hr (χ^2 = 6.27; df = 1; P= 0.001) and in females at 24 hr (χ^2 = 10.91; df = 1; P= 0.001) and 32 hr (χ^2 = 11.25; df = 1; P= 0.001) of digestion, respectively. Moreover, fewer mosquitoes, three days post-exposure than controls tested positive for sugars until the 16th hr in males and the 24th hr in females after feeding. The difference however between the treatments was at 32 hr of digestion (males: χ^2 = 6.49; df = 1; P= 0.001; females χ^2 = 7.67; df = 1; P= 0.006). Cumulative scores from time zero through to the 32 hr

demonstrate that, more one day post-exposure males (52% versus 45%) and females (58% versus 39%) than controls tested positive for sugar. This was an overall indication that digestion rate was slower in fungus-exposed mosquitoes. The difference was only significant for females, one day post-exposure (Table 4). Further, the proportion of three day post-exposure males (43% versus 43%) and females (53% versus 53%) with sugar was equal to that of the controls. Hence, timing of fungal exposure only had an effect on sugar digestion in females. Results from mycosis tests indicated that, on average 73-81% of males and 78-85% of females were infected with fungus but no spores were observed on the cadaver of the control mosquitoes.

Table 2. Mean (± S.E) percentage of uninfected and fungus-infected *An. gambiae* female mosquitoes (see Figure 1) that imbibed different amounts of sugar when fed on *Ricinus communis* for 12 hr. One and three d post-exposure females were tested.

Sugar quantity	Day after infection	mean % (± S.E) of females that imbibed sugar		χ^2	P
		Uninfected	Fungus-infected		
Small	1	47.5 ± 8.54	38.0 ± 7.96	3.69	0.055
Medium		29.0 ± 4.51	26.0 ± 4.08	0.45	0.502
Large		18.5 ± 8.22	19.5 ± 6.29	0.07	0.799
Small	3	35.0 ± 4.12	30.0 ± 5.6	1.14	0.286
Medium		22.0 ± 2.22	6.0 ± 2.16	22.28	0.001
Large		12.0 ± 4.69	6.5 ± 6.5	3.60	0.058

Statistical significance (P value) between the number of uninfected and fungus-infected mosquitoes in each category of sugar quantity imbibed was calculated by Chi square (χ^2) test. Each treatment tested 200 mosquitoes.

Discussion

Results of this study demonstrate that under ambient conditions, infection with the entomopathogenic fungus *M. anisopliae* reduced the daily survival of *An. gambiae* mosquitoes irrespective of the sugar source. Such a significant reduction in the survival of *An. gambiae* on glucose within 10 d after exposure to *M. anisopliae* has been reported previously under laboratory conditions (Scholte et al. 2003a, Farenhorst et al. 2008, Mnyone et al. 2009b, Mnyone et al. 2009a) Chapter 3. Moreover, both *R. communis* and *P. hysterophorus* had a strong negative effect on survival of healthy mosquitoes. These findings are in agreement with other studies that reported longer survivorship of healthy *An. gambiae* mosquitoes fed on glucose than on plant-derived sugars (Gary and Foster 2004, Impoinvil et al. 2004, Manda et al. 2007a). Recent studies have shown that *An. gambiae* feed from a wide variety of plants and the quantity of sugar affects their survival (Manda et al. 2007a, Gouagna et al. 2010). Moreover, although sugar is present in the leaves, stem and floral parts of the plants, it is in the latter that different sugar types are highly concentrated (Manda et al. 2007b). Therefore, the lower survival on plant sugars relative to 6% glucose may be due to insufficient production of sugar by nectaries of the cut plants, presence of complex sugars

that require more energy than glucose to process into useful products or accumulation of toxic substances due to interruption of nutrient circulation in the plant cuttings.

Table 3. Mean (± S.E) percentage of uninfected and fungus-infected *An. gambiae* male mosquitoes (see Figure 1) that imbibed different amounts of sugar when fed on *Ricinus communis* for 12 hr. One and three d post-exposure males were tested.

Sugar quantity	Day after infection	Mean % (± S.E) of males that imbibed sugar		χ^2	P
		Uninfected	Fungus-infected		
Small	1	56.0 ± 6.16	41.0 ± 9.47	9.01	0.003
Medium		22.0 ± 3.56	21.5 ± 4.19	0.02	0.903
Large		9.5 ± 1.71	6.5 ± 2.63	1.22	0.269
Small	3	52.0 ± 8.49	37.0 ± 6.14	9.11	0.003
Medium		16.5 ± 0.50	10.5 ± 7.37	3.08	0.079
Large		4.0 ± 2.45	1.0 ± 1.0	3.69	0.055

Statistical significance (P value) between the number of uninfected and fungus-infected mosquitoes in each category of sugar quantity imbibed was calculated by Chi square (χ^2) test. Each treatment tested 200 mosquitoes.

The longer survival of uninfected mosquitoes on *R. communis* than *P. hysterophorus* may be attributed to the diversity and the quality of sugars present in the plant. For instance, mosquitoes feed on the leaves, stems and floral parts on *R. communis* compared to feeding on leaves only on *P. hysterophorus* (Manda et al. 2007b). Furthermore, digestible sugars are consumed in larger amounts on *R. communis* than on *P. hysterophorus* (Manda et al. 2007a). The drastic reduction in the survival of mosquitoes on *P. hysterophorus* may be associated with the toxic effects of the chemical compound parthenin (Narasimhan et al. 1984) present in the plant, absence of sugars in the floral parts of the plant and the ingestion of D-allose type of sugar which is a growth inhibitor (Kato-Noguchi et al. 2011). For instance, plant extracts of *P. hysterophorus*, resulted in reduction in fecundity, fertility and behavioural responses in the yellow fever mosquito *Aedes aegypti* (L.) (Kumar et al. 2011). Similar effects on mosquito adult emergence and fecundity have been reported with castor bean extracts (Elimam et al. 2009, Mandal 2010). Interestingly, the negative effects of plant sugars from *R. communis* and *P. hysterophorus* as reported may be entirely overcome when the insects are additionally offered a blood meal and these sugars then are highly beneficial by extending the survivorship (Takken et al. 1998, Gary and Foster 2001, Okech et al. 2003, Stone et al. 2011).

Survivorship is a key feature that defines the vectorial capacity of malaria vectors (Garrett-Jones 1964, Miller et al. 1973). Survival of *An. gambiae* mosquitoes on *R. communis* in this study was longer than the extrinsic incubation period of a pathogen that is as short as 10d for the malaria parasite *Plasmodium falciparum* (Molineaux et al. 1978, Lines et al. 1991, Beier 1998). This concurs with other studies on survival of *An. gambiae* on plant sugars (Gary and Foster 2004, Manda et al. 2007a). As this occurred under semi-field conditions, it is likely that in field situations mosquitoes forage on a wide variety of plants to complete their dietary requirements, sustain longevity and with blood-supplement become efficient as malaria vectors. Therefore, reduction in the life-span of both sexes of *An. gambiae* by EPF as demonstrated in this study could lead to a considerable reduction in malaria transmission.

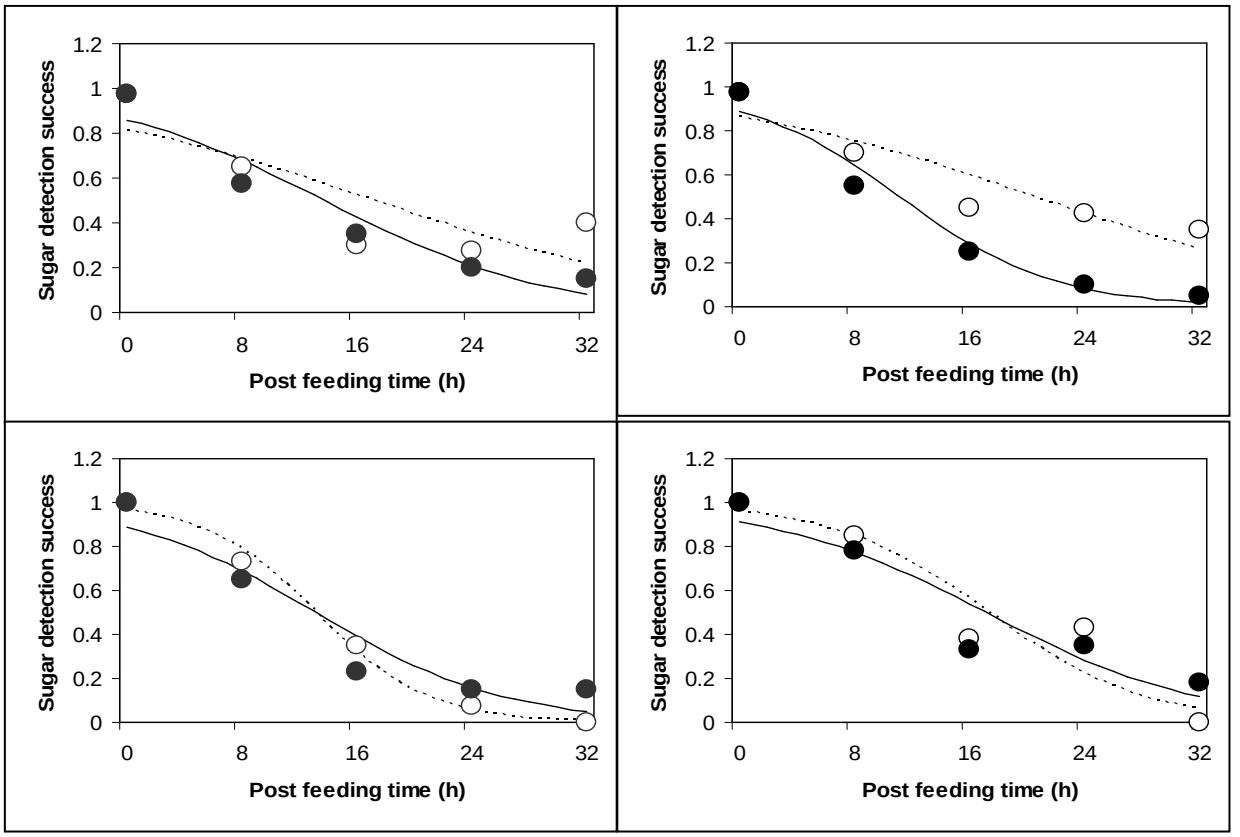

Figure 3. Effect of infection with *M. anisopliae* on sugar detection success in *An. gambiae* mosquitoes. Panels A and B represent sugar detection success in uninfected and in *M. anisopliae*-infected males and females respectively one day post-exposure with Panels C and D represent 3 d post-exposure when fed on *Ricinus communis* for 12 hr. Solid lines representing uninfected mosquitoes and dotted lines representing infected mosquitoes describe the fitted logistic relationships between sugar detection success for each time period since feeding: - Logit (sugar detection success) = $ß_0 + ß_1$ time. Circles denote observed values. Level of statistical difference between treatments was calculated by Chi square (χ^2) test. Each treatment tested 200 mosquitoes.

Infection with fungus strongly reduced the proportion of mosquitoes that ingested sugar from *R. communis* independent of the time since infection. Interestingly, the feeding potential and the quantity of sugar assimilated of the mosquitoes that did feed remained similar between the treatment and the control groups. In other insect species, a significant reduction in feeding in the maize stem borer *Chilo partellus* (Swinhoe) larvae (Tefera and Pringle 2003), adult thrips *Megalurothrips sjostedti* Trybom (Ekesi and Maniania 2000) and the variegated grasshopper, *Zonocerus variegatus* (Linnaeus) (Thomas et al. 1997) occurred as early as one to four days after infection with the entomopathogenic fungus *M. anisopliae*. The normal feeding that we observed has also been reported in corn earworm, *Heliothis zea* (Boddie) larvae (Cheung and Grula 1982) infected with *B. bassiana*. The insects, however, die at a later stage, which may indicate that infection causes starvation due to physiological changes in infected hosts. Reports to-date about mosquitoes have addressed reduction of blood-feeding rather than sugar feeding in fungus-infected females (Blanford et al. 2005, Scholte et al. 2006, Blanford et al. 2011) and these are therefore not comparable with our study. The reduction in sugar-feeding propensity may be attributed to three factors. First, infected mosquitoes may have fed as often as the uninfected ones but the sugar content was too low to be detected. Secondly, the sugar in infected mosquitoes may have already been digested and converted into a metabolic product which the anthrone test (biased towards fructose) could not detect. Lastly, the secondary metabolites produced by the fungus may be responsible for the degradation of tissues including the midgut, thus affecting feeding ability (Vey et al. 1985, Samuels et

al. 1988, Vey and Quiot 1989). The production of these metabolites in combination with utilization of glycogen and lipid reserves and possible mechanical disruption of tissues by mycelial growth may be responsible for the loss of appetite (Thomas et al. 1997). The normal feeding in fungus-infected mosquitoes could be associated with dose (Moore et al. 1992) and the insect defense mechanism that may have resulted in delayed colonization of the insect tissues by the fungus (Seyoum et al. 1994). This is because the immune system of insects responds in defense of fungal attack as early as 12 h after exposure to the pathogen (Gunnarsson 1988).

Table 4. Proportion of uninfected and fungus-exposed *An. gambiae* mosquitoes that tested positive to sugar within 32 hr after feeding on *Ricinus communis* for 12 hr. Males and females were tested one and three d post-exposure.

Sex	Day after infection	% mosquitoes positive to sugar		χ^2	P	Percent (± S.E) infection
		Uninfected	Fungus-exposed			
Male	1	45	52	1.96	0.161	73.0 ± 3.87 (146)
Female		38.5	58	15.29	0.001	78.0 ± 4.16 (156)
Male	3	43.5	43	0.01	0.920	81.0 ± 1.0 (162)
Female		52.5	53	0.01	0.920	85.0 ± 2.08 (170)

Statistical significance (P value) between the number of uninfected and fungus-infected mosquitoes in each category of sugar quantity imbibed was calculated by Chi square (χ^2) test. Each treatment tested 200 mosquitoes.

The study has further shown that infection by *M. anisopliae* had no effect on the digestion rate of sugar except in females, one day post-exposure. However, as the fungal infection progressed, fewer infected than uninfected mosquitoes (both sexes) tested positive to sugars. Digestion of sugar in insects takes place in the crop and midgut and its rate is influenced by the meal size consumed, sugar concentration (Van Handel 1965), metabolic rate (Nayar and Van HandeL 1971b) and the extent of energy reserve, among other factors. The mechanism that affects feeding rate due to pathogen attack may also affect the digestion process. Therefore, the slow digestion rate in early days of fungal infection is likely to be associated with the dose and the mechanical disruption of the midgut tissues by fungal toxins (Vey and Quiot 1989). Furthermore, the increased breakdown of sugar as the infection advances could be associated with the need to replenish the teneral energy reserves depleted by invasive fungal pathogens in the insect haemolymph. These teneral reserves are critical for the survival of insects (Takken et al. 1998, Thompson 2003). In the case where digestion rate between treatments was equal, it is likely that infected mosquitoes imbibed more sugar than controls for two purposes. First, to nourish the storage reserve this is the primary source of nourishment to the fungal pathogen (Xia et al. 2002). Second, to replenish and store sugar in the crop for future use. This is because the accumulation of energy reserves retards digestion (Foster 1995). Between sexes, the proportion of individuals that tested positive to sugars did not differ in spite of their different synthesis of reserves. This concurs with what has been reported by (Van Handel 1965).

The inability of fungus-exposed mosquitoes to sugar feed may pose some advantages. The life-span of both sexes could be reduced to less than five days. During this period, the mating ability of males may be compromised leading to fewer females getting inseminated (Chapter 8). Although females can build their energy reserves from human blood, they may not survive long enough to become

efficient malaria vectors. Therefore, if both sexes become infected early in life, this could lead to population suppression, incomplete development of the malaria parasite in females and reduction in malaria transmission (Scholte et al. 2006, Gary et al. 2009). Moreover, the ability of mosquitoes to feed on and digest sugars may negatively impact on the survival of both sexes and minimize human-mosquito contact. Thus, maintaining the normal rate of food consumption and digestion in fungus-infected insects for as long as possible benefits the fungal pathogen because this maximizes the amount of food available for the entomopathogen (Gary and Foster 2001, Roy et al. 2006). Further research however is needed to determine if a similar impact of fungus can occur in field situations.

The life of male mosquitoes is exclusively tied to the plant community. By focusing on fungal inoculation during plant feeding, therefore, both males and females are likely to become infected. Control strategies that target both sexes may lead to significant reduction in the prevalence and transmission of malaria and other mosquito borne diseases. In recent studies, the efficiency of plant attractants in attractive toxic sugar baits (ATSB) for the control of mosquitoes has been demonstrated (Müller et al. 2008, Müller and Schlein 2008, Muller et al. 2010, Müller et al. 2010c, Müller et al. 2010b, Müller et al. 2010a, Müller et al. 2011). The approach uses odour stationary traps baited with fermented ripe fruits and flower scent as attractants, a sugar solution as feeding stimulant and an oral pesticide (Muller et al. 2010). The strategy can be adopted to infect and kill mosquitoes with EPF during plant sugar feeding in two ways. Firstly, by spraying flowering plants with fungal conidia formulated in a suitable carrier that can withstand ultra-violent effects and retain spore virulence. This strategy however, requires assessment on the impact of fungal pathogens on non-target organisms especially pollinators. Secondly, by spraying fungal conidia in traps baited with fruits and flowers and sugar solution. The first approach may be cost effective since preparation of attractants for the traps may be problematic. Also, more mosquitoes are likely to be targeted and killed by spraying the plants than by being attracted to the baited traps, as these are in competition with flowering plants. Nevertheless, research is needed to demonstrate the possibility of these proposed pathways and other unexplored approaches for infecting wild mosquitoes, particularly males, by entomopathogenic fungi.

Conclusions

This study has demonstrated that the entomopathogenic fungus *M. anisopliae* has the potential to infect, kill or reduce the survival of malaria vectors feeding on plant sugars beyond ages at which they are old enough to transmit malaria. Significantly, infection with fungus reduced the proportion of both sexes of *An. gambiae* that ingested sugar from *R. communis* but not the quantity of sugar imbibed relative to the controls. Moreover, infection by fungal pathogen had no effect on the digestion rate of sugar except in females, one day post-exposure. Fungal infection reduced the proportion of mosquitoes that tested positive to sugars as the fungal infection progressed. The possibility of targeting mosquito males for population reduction by an entomopathogenic fungus opens a new strategy for mosquito vector control.

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Part III

FUNGAL INFECTION AND *ANOPHELES GAMBIAE* REPRODUCTIVE BEHAVIOUR

Chapter 7

Effects of fungal infection on the host-seeking behaviour and fecundity of the malaria mosquito *Anopheles gambiae* Giles

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Abstract

Malaria remains a key hindrance to the improvement of health in Africa. Transmission rates and the risk of the disease can be greatly reduced by vector control. At present, control of adult mosquitoes is almost exclusively based on chemical insecticides. However, development of resistance to chemicals is of great concern for sustainable malaria control. Entomopathogenic fungi are effective against adult vectors and can be used as an alternative to insecticides. As slow-killing agents, fungi are expected to impose limited evolutionary pressure for resistance formation in exposed populations. The host-seeking response, feeding propensity, blood meal size (quantified through haematin analysis), and fecundity was evaluated by exposing mosquitoes infected with the fungus *Metarhizium anisopliae* to human volunteers. It was found that fungal infection reduces feeding propensity but blood meal size and fecundity remained unaffected. The implications of these findings with regard to potential resistance development against fungal infection are discussed.

Introduction

Malaria remains a major global problem, exerting an unacceptable toll on the health and economic welfare of the world's poorest communities (WHO 2005, Breman et al. 2007). The burden of disease is greatest in Africa where children under the age of five and pregnant women are most vulnerable due to their lower level of malaria immunity (WHO 2006a). Each year, over one million deaths from the direct effects of the disease occur in the continent (World-Bank 2007) and it is therefore regarded as the leading cause of morbidity and mortality in the sub-Saharan region. Malaria is caused by protozoan parasites of the genus *Plasmodium* and is transmitted through bites of mosquitoes belonging to the genus *Anopheles*. Females of *An. gambiae s.l.* are the principal vectors of malaria in Africa, besides *An. funestus*. Their dominance as malaria vectors is largely due to a preference for human blood, high vector competence, and high daily survival rates (Besansky et al. 2004, Day 2005).

The transmission rates and risks of the disease can be greatly reduced by vector control (WHO 2006b). Contemporary adult mosquito control is almost exclusively based on indoor application of chemical insecticides in the form of impregnated bed nets or as indoor residual spraying (of walls and ceilings). However, sustainable use of chemicals is undermined by problems of insecticide resistance in mosquito populations, environmental contamination and risks to human health. Growing concern to these problems has increased interest in the search for alternative approaches (Zaim and Guillet 2002). Biological control is one option, and several biological control agents, like *Bacillus thuringiensis israelensis* have been used successfully to control mosquito larvae (Fillinger et al. 2003, Fillinger and Lindsay 2006). Entomopathogenic fungi are effective against adult vectors and are currently being developed as biopesticides (Scholte et al. 2004b, Blanford et al. 2005, Scholte et al. 2005, Knols and Thomas 2006, Thomas and Read 2007a). As slow-killing agents, fungi are expected to impose limited risks for resistance formation in malaria mosquitoes (Thomas and Read 2007a). Fungal resistance is not considered an immediate risk in mosquito populations based on their multiple modes of action. Fungi use an array of weapons to attack the insect, such as chitinases, proteases and release of toxins (Hajek and St Leger 1994). Compared to insecticides, fungi have low virulence as they kill an insect in 6-14 days after infection depending on the fungal species and isolate used. Within this period, the females are likely to be able to mate and reproduce. Therefore, the slow killing mechanism of the fungus imposes a limited selection pressure on the mosquitoes thus reducing the likelihood of anti-fungal resistance (Knols and Thomas 2006).

To curb malaria transmission, understanding behavioural consequences of fungal infections in mosquito populations is vital. The propensity to select humans for blood feeding is arguably the most important component of mosquito vectorial capacity (Zwiebel and Takken 2004). This aspect further determines the success of mating, blood feeding and oviposition. A laboratory study in which infected female mosquitoes were blood fed by arm in small cups revealed that fungal infection reduces (but not eliminates) feeding propensity and fecundity (Scholte et al. 2006). However, it remains unknown if under more realistic conditions, whereby mosquitoes have to perform a host-seeking response, similar results are obtained.

Here we report findings of the impact of progressive fungal infections on the feeding propensity, blood-meal size and subsequent number of eggs laid by female *An. gambiae* one, three and five days after infection with spores of *M. anisopliae*. The experiment was conducted under simulated room conditions in the laboratory where insects had a choice to locate and bite a host instead of making the host directly available to them when placed in cups.

Materials and Methods

Mosquitoes

Anopheles gambiae (Suakoko strain; courtesy Prof. M. Coluzzi) were maintained at 27 ± 1°C, 80 ± 5% relative humidity (RH) and a photoperiod of 12:12 light: dark. Adults were held in 30 cm × 30 cm × 30 cm gauze cages and had *ad libitum* access to a 6% glucose solution on filter paper. They were fed on a human arm twice a week. Eggs were laid on wet filter paper and transferred to water trays. Larvae were reared in tap water in plastic trays and were fed daily on Tetramin® fish food. Pupae were collected daily and placed in adult cages for emergence. A cone of damp white filter paper held in pint-sized cups was introduced in the cages where mated females oviposited following a bloodmeal.

Fungus application in clay pots

Four Ghanaian clay water storage pots (2 for control and 2 for infection with fungus) were used in the study according to protocols described by (Farenhorst et al. 2008). These pots have previously been shown to be highly attractive resting sites for anopheline mosquitoes in Western Kenya (Odiere et al. 2007). Each of the control pots was sprayed with 50 ml of Ondina oil (Shell, The Netherlands) while each of pots for infection was sprayed with 35 ml Ondina oil. Two hours later, pots for infection were each sprayed with 17 ml of *M. anisopliae* (IC30 isolate) formulated in Ondina oil at a concentration of 4.0×10^{10} spores/m^2. Both control and fungus-treated pots were left to dry for fifteen hours.

Mosquito infection

A wet cotton pad soaked in 6% glucose solution was placed at the mouth of each pot and covered with a cylindrical nylon paper firmly held with a rubber band. Groups of 800 female adults, 3-5 days old, were randomly collected from rearing cages with a mouth aspirator and were introduced in clay pots through a round hole at the base of the pot. The holes were sealed with a stopper to prevent mosquito escape. Each control and fungus-treated pot had 150 and 250 adults, respectively. Six hours later, both infected and control mosquitoes were transferred into separate rearing cages and were provided with 6% glucose solution (supplied on filter paper wicks). For the human volunteer experiments mosquitoes of one, three and five days post infection were used. Approximately 2 hours before each experiment, two groups of 30 mosquitoes, from control and fungus-treated mosquito cages were removed at random and released in a large netting cage (3 m × 3 m × 3 m) fitted in an experimental room maintained at 26 ± 1°C, 75 ± 5% RH. For mosquitoes one and three days post infection, the experiment was replicated twice, for mosquitoes 5 days post infection thrice.

Assessing feeding propensity

Upon release, the insects were given the option to respond, locate and bite a human host and take a blood meal for a fixed period of 30 minutes. The volunteer entered the cage into which the mosquitoes had been released, and laid down on a bed with exposed arms and legs to facilitate biting. Both blood fed and unfed mosquitoes were collected individually into 30 ml cylindrical plastic tubes (9 × 2.5 cm) covered with hollow plastic caps. The tubes were lined with a strip of thin filter paper firmly held with a paper clip for mosquitoes to rest on. The caps had several holes to allow for feeding. Cotton pads soaked in 6% glucose water were placed in each cap. Thereafter, the tubes were assigned numbers (both for blood-fed and non blood-fed). They were then arranged in holding racks and maintained at 27 ± 3°C, 70 ± 10% RH and 12:12 L:D photoperiod for hematin (excreted during the post-diuresis phase) collection. Cotton pads water were replaced daily. After two days, blood-fed mosquitoes were transferred into oviposition tubes fitted with wet filter paper that served as oviposition substrate. The tubes were assigned numbers corresponding to labels on the hematin tubes. Hematin within the tubes was quantified using a standard curve to provide an estimate of

blood meal size (see below). Two days later, filter paper in oviposition tubes containing eggs were removed and the number of eggs per individual recorded by counting under a stereomicroscope. Dead individuals in hematin or oviposition tubes including the non-bloodfed insects were collected and plated on petri dishes containing wet filter paper to allow growth of fungus on the cadaver. Petri dishes were placed in an incubator for three days at 26 ± 2^{o}C to promote fungal growth. Mosquitoes without fungal growth were assumed to belong to the control treatment. The procedure described above was repeated with mosquitoes 3 and 5 days post infection.

Estimation of blood meal size

The amount of hematin excreted was determined by the method of (Briegel 1980). The excreta in holding tubes were dissolved in 1 ml of 1% lithium carbonate ($LiCO_3$) solution. The absorbance of the resulting solution was read at 387 nm and compared to a standard curve made from bovine haematin (Hurd et al. 1995, Hogg and Hurd 1997).

Statistical analysis

Individuals that took a blood meal and died in the hematin or oviposition tubes were excluded from the analysis. Feeding propensity was expressed as mean percentage (± SE) of the total number of mosquitoes that took a blood meal in both the control and infected groups while blood meal size and number of eggs oviposited were expressed as means (± SE) per individual mosquito. These means were compared using χ^2 square analysis.

Results

Reduction in feeding propensity was significant (P = 0.006) for mosquitoes that were three days old after fungus infection when compared against uninfected mosquitoes of that age. However, this difference was not significant between the fungus-infected and control groups one (P = 0.68) and five day(s) (P = 0.33) post infection (Fig. 1A). The amount of blood consumed by fungus-treated mosquitoes was not significantly different from the amounts taken by mosquitoes from the control groups for all periods post infection (Fig. 1B). Unfortunately, a considerable number of mosquitoes in both treatments died before oviposition. Nevertheless, the number of eggs laid by the few surviving individuals was not affected by fungal infection (Fig. 1C).

Discussion

Results from the current study show that feeding propensity decreased in adult female *An. gambiae* mosquitoes that were three days old after infection with fungus *M. anisopliae*, but not one or five days post infection. Such an impact on behaviour will result in a reduction of female lifetime vectorial capacity and hence malaria transmission risk. (Scholte et al. 2006) observed virtually similar effects 2, 3 and 4 days post infection where the 4-day treatment yielded marginal significance (P=0.048) in feeding propensity reduction compared to control mosquitoes. Interestingly, therefore, it appears as if mosquito feeding appetite decreases 2-3 days after infection, but that this effect is no longer apparent one or two days later. From the perspective of resistance developing against fungal infections, this finding is important in the sense that mosquitoes with infections do still engage in host-seeking behaviour and are willing to consume blood meals in similar proportions as their uninfected counterparts.

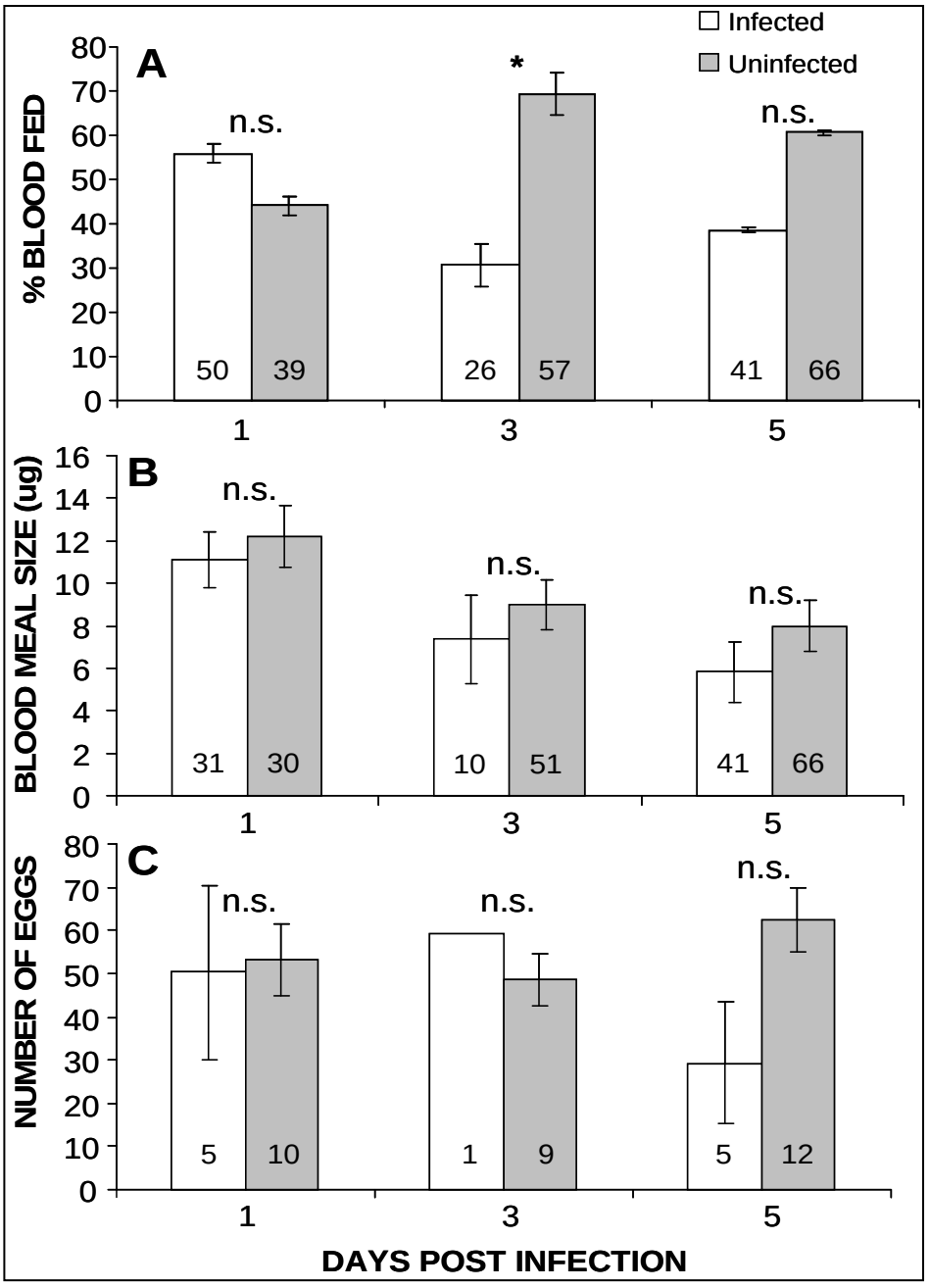

Figure 1. A: Proportion (mean ± SE) of *M. anisopliae*-infected or control female *An. gambiae* blood feeding 1, 3 and 5 days post infection; B: Blood meal size (mean ± SE) of mosquitoes surviving to oviposition, and C: Number of eggs laid by surviving females that blood fed 1, 3 and 5 days post fungus infection. Numbers inside bars indicate number of mosquitoes tested (n).

We further observed that fungal infection had no effect on blood meal size and fecundity. (Scholte et al. 2006) reported a significant reduction in blood-meal size for mosquitoes four, but not two and three days post infection. Our findings differ only for the groups four days post infection, which consumed similar amounts of blood as control mosquitoes. Again, considering that blood meal size remains unaffected by fungal infection in the first five days, it is likely that these mosquitoes will engage in egg development and completion of at least one gonotrophic cycle, thereby enabling reproduction and thus reduction of the potential for resistance development. Lifetime fecundity in

An. gambiae, following infection with *M. anisopliae*, was also reported to reduce significantly (Scholte et al. 2006), though we did not observe this effect in the present study. Such findings have also been reported for other insects infected with fungus. For instance, (Ekesi and Maniania 2000) reported a reduction in fecundity in thrips *Megalurothrips sjoistedti* upon infection with *M. anisopliae.*

According to (Blanford et al. 2005), fungus interferes with blood meal intake in *An. stephensi* 8-14 days after infection. Assuming that this is the same for *An. gambiae* then our focus on insects 1-5 days post infection would not reveal such effect. Generally, upon contact with a mosquito, the fungal spores begin to invade and develop inside the mosquito, after which the fungus multiplies and kills its host within two weeks; the approximate time a malaria parasite takes to develop into its infective form (sporozoites). This slow-kill approach by fungi is an advantage given that mosquitoes cannot transmit sporozoites until about two weeks after an infectious blood feed (Kanzok and Jacobs-Lorena 2006). Besides, (Blanford et al. 2005), when evaluating blood meal intake and using a mouse malaria model system established that fungal infection has a negative effect on *Plasmodium* development in the mosquito. Putting the effects of blood meal intake, which also directly influences fecundity and *Plasmodium* development together, *M. anisopliae* could reduce malaria transmission by approximately 80 times. Nonetheless, mouse malaria may have different characteristics from human malaria and many different factors can come into play when applying research findings in the field. The factors include fungal specificity and the possibility of insects developing resistance to fungi. Mosquitoes might evolve ways to prevent the fungus from entering their body or limit its growth if they become infected but it seems unlikely that they would intensify *Plasmodium* transmission or virulence (Michalakis and Renaud 2005).

The future of using *M. anisopliae* as a novel vector control tool is increasing as pressure mounts on the search for alternative public health insecticides. Studies by Jenny Stevenson (Stevenson *et al.*, unpubl. data) have shown that fungus is effective against a multiple insecticide-resistant strain of *An. stephensi*, fuelling hope to solve problems of insecticide resistance (Knols and Thomas 2006). However, the big challenge towards sustainable use of fungus-based measures for vector control is the possible resistance development by mosquitoes. So far, this has not been reported in mosquitoes or any other insect, and our current findings support the idea that the evolutionary pressure exerted on populations will remain small. Nevertheless, we intend to conduct similar studies under semi-field and field conditions before drawing final conclusions.

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Chapter 8

Effects of Metarhizium anisopliae on the mating performance of the malaria mosquito *Anopheles gambiae* and the probability of horizontal transmission

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Abstract

The entomopathogenic fungus *Metarhizium anisopliae* is a potential biocontrol agent for malaria vectors. The effect of fungal infection on the normal and multiple mating performances were evaluated with *Anopheles gambiae* males 3 d post-exposure under semi-field conditions. Furthermore, horizontal transmission of the fungal conidia during mating was quantified using males 1, 3 and 5 d post-exposure. Two-day old *Anopheles gambiae* virgin males were exposed to fungal conidia for 6 hr. Contaminated males were confined in cages or released in a screenhouse to mate with virgin females in male: female proportions of 1:1, 1:3 or 1:5 for 24 hr. Fungus-free males were used in controls. Overall, survival of infected males was reduced by 50% before the start of experiments. In a screenhouse, *M. anisopliae*-infected males inseminated significantly fewer virgin females compared with the controls. With cage tests, however, the difference in number of females inseminated by infected and uninfected males was not significant. Fungus infection also affected the multiple-insemination capacity, causing fewer inseminations. Horizontal transfer of fungal conidia occurred frequently, but was strongly dependent on exposure time. On average, 62.5, 17 and 6% of virgin females died of mycosis after mating with 1, 3 and 5 d post-exposure males, respectively. These findings suggest that infection with *M. anisopliae* has strong effects on the mating performance of adult male *An. gambiae* mosquitoes. Furthermore, conidial transfer during mating increases the chances of passing on pathogenic fungus to other members of the population. These pathologic effects on the fitness of male mosquitoes may lead to the suppression of mosquito populations and a subsequent reduction in malaria transmission.

Introduction

The mosquito *Anopheles gambiae* Giles is the principal vector of malaria, a disease that affects more than 250 million people and causes more than 600,000 deaths each year (WHO 2011). The disease is transmitted to humans by the bite of a female mosquito infected with the *Plasmodium* parasite. Male mosquitoes do not feed on blood and thus do not transmit disease. However, they play a key role in reproduction and population growth in the field. Therefore, major attempts to limit malaria transmission focus on the control of females by use of insecticide-treated nets and through indoor residual spraying (Pates and Curtis 2005). The approach has been effective but with the emergence of insecticide resistance, alternative methods are needed to control the malaria vector including male mosquitoes. Microbial control agents such as entomopathogenic fungi (EPF) have shown potential for development as an alternative control tool (Lacey and Undeen 1986, Federici 1995)

An understanding of mosquito behaviour has been the key in designing control strategies. However, the behavioural ecology of the males, in particular mating behaviour, remains insufficiently explored (Charlwood and Jones 1979, Ferguson et al. 2005, Takken et al. 2006). Current strategies are based on exploiting sexual competitiveness of the males since that defines their fitness in a population (Takken and Knols 1999). The possible approach to control *An. gambiae* males may be the use of the sterile insect technique (SIT) whereby sterile but sexually competitive males are released to mate with wild females. The application has been widely tested with success in eradicating the screwworm, *Cochliomyia hominivorax* (Coquerel) and other dipterans but its efficacy for the control of mosquitoes remains uncertain (Benedict and Robinson 2003, Townson 2009). The uncertainty with SIT usage confirms the need to search for alternatives. Entomopathogenic fungi have potential to reduce the survival of infected *An. gambiae* males (Scholte et al. 2003a, Scholte et al. 2004a, Scholte et al. 2005, Farenhorst et al. 2008, Mnyone et al. 2009a). The success of the use of the EPF *Metarhizium anisopliae* and *Beauveria bassiana* has been through contact when mosquitoes rest on surfaces impregnated with conidia, i.e. indoor resting targets (Scholte et al. 2005). The authors suggested other non-contact pathways be explored to increase the impact of infection such as autodissemination or horizontal transmission (Scholte et al. 2004a).

Horizontal transmission has been described as one of the strategies that can be adopted to improve the effectiveness of entomopathogens (Ignoffo 1978). The technique entails the use of insects to introduce and spread an entomopathogen in the ecosystem. Some studies utilised the technique and reported its efficiency in management of plant pests in field trials. In all cases, traps either unbaited or baited with a pheromone lure were used as fungal delivery tools. The fungus *Zoophthora radicans* was spread by males of the diamond-back moth *Plutella xylostella* (Linnaeus) to a field population (Pell et al. 1993, Furlong et al. 1995). Similarly both sexes of the Japanese beetle *Popillia japonica* Newman (Klein and Lacey 1999) spread *M. anisopliae* while *B. bassiana* was dispersed by the sap beetle *Carpophilus lugubris* Murray (Dowd and Vega 2003) and the green bug, *Plautia crossota stali* Scott (Tsutsumi et al. 2003), respectively. In non-plant pests, field populations of tsetse were suppressed by *M. anisopliae* spread by the tsetse fly *Glossina fuscipes fuscipes* Newstead infected using an inoculative device (Maniania et al. 2006). These practical examples are encouraging for further studies to develop methods for scaling up of the technique.

In the recent past, research has focused on exploiting mating behaviour as a means to spread EPF. Several laboratory based studies have reported that fungus can be spread from infected to healthy insects during mating. For example, healthy fruit fly females infected by *M. anisopliae* (Quesada-Moraga et al. 2006) and *B. bassiana* (Toledo et al. 2007) during mating experienced shortened survival and reduced fecundity. Some studies reported a reduction in mating success in infected males (Dimbi et al. 2009) while other studies have not reported it (Toledo et al. 2007, Novelo-Rincon et al. 2009). The only two studies on horizontal transfer of EPF in mosquitoes showed that infected

individuals of *An. gambiae* (Scholte et al. 2004a) and *Aedes aegypti* (L.) (Garcia-Munguia et al. 2011) infected their conspecific untreated mates. These studies were done in laboratory cages, where males and females were within close range.

The studies mentioned above demonstrate that EPF can be disseminated in insect populations directly when insects come into contact with conidia. Moreover, the pathogen can be acquired indirectly through mating between uninfected and infected individuals. This suggests that fungus application could pose an advantage over pesticide usage whose mode of action is by direct contact only. Nevertheless, further research is still required to highlight the impact of EPF on mating behaviour in mosquitoes. Studies in females demonstrate a strong impact of infection on fecundity (Scholte et al. 2006) but it is not clear whether the mating process is implicated. The ability of male mosquitoes to inseminate females is the most important component of male biology on which reproductive success in mosquito populations depends (Voordouw and Koella 2007). However, it is uncertain if mating success in *An. gambiae*, which characterises male fitness, can be compromised on invasion with a fungal pathogen. Besides, it is unclear if *An. gambiae* males can transmit fungal conidia through mating.

The current study aimed to investigate, under semi-field conditions, the effects of infection by the entomopathogenic fungus *M. anisopliae* on mating performance of *An. gambiae* males. Specific objectives were (i) to determine the capability of infected males to inseminate virgin females; (ii) to investigate the ability of infected males to inseminate more than one virgin female and (iii) to establish through mycosis tests whether females can acquire infectious conidia from infected males during mating.

Materials and Methods

Mosquitoes

Laboratory-reared *Anopheles gambiae* Giles *senso stricto* (Mbita strain) maintained at the International Centre of Insect Physiology and Ecology (*icipe*) in Mbita Point, western Kenya, since 1999 were utilised. The rearing procedures at ambient conditions have been described in detail elsewhere (Olanga et al. 2010). Experimental males were separated from females at emergence to preserve virginity of both sexes. Mosquitoes were held in separate holding cages (30 × 30 × 30 cm) under semi-natural conditions inside a screenhouse (13 × 4.7 × 2.3 m). They were maintained on 6% glucose solution supplied on paper wicks and were used in the experiments when five days old.

Fungus

The entomopathogenic fungus *Metarhizium anisopliae* ICIPE 30 was used in this study. The fungus has been maintained at the *icipe*'s Arthropod Germplasm, Nairobi, Kenya. It was isolated from the stem borer *Busseola fusca* near Kendu Bay, western Kenya in 1989 (courtesy Dr. N.K. Maniania). Conidia were produced on long rice as substrate following the technique described by Maniania et al. (2003). The viability of conidia was determined before experiments by spread-plating 0.1 ml of suspension at 3×10^6 conidia/ml on 9-cm Petri dishes containing Sabouraud dextrose agar (SDA). The plates (N=3) were sealed with Parafilm® and incubated at 28 ± 2°C for 18 h. A sterile microscope cover slip was placed on each plate and observed under a compound microscope. The percentage germination of conidia was established from 100 spore counts under the cover slip at 400× magnification. The average viability of conidia used in the tests was 86%.

Infecting males with fungus

Six transparent plastic cylinders (9 cm diameter; 15 cm height) were used. The inner and the base surfaces of each cylinder were lined with white rough paper that measured 28.6 × 14.3 cm and 9 cm in diameter respectively. Each cylinder was dusted with 0.1 g (approx. 1.0×10^{11} conidia/m^2) conidia of *M. anisopliae* prior to introduction of 50, two-day old virgin male mosquitoes. The males were held for 6 h and thereafter transferred into one holding cage (30 × 30 × 30 cm). They were offered 6% glucose solution and maintained at ambient conditions inside a screenhouse. In the control, uninfected virgin males were maintained in four fungus-free cylinders for 6 h prior to release into one holding cage. Twice as many males compared to controls were exposed to fungal conidia to adjust for mortalities associated with fungal-infection prior to the start of experiments. All males used in the experiments were 3-d post fungal exposure (i.e. five days since emergence).

Effect of **M. anisopliae** *infection on mating performance of caged male mosquitoes*

Two cages (30 × 30 × 30 cm) were used for the treatment and for the control experiments. In the treatment cage, 100 *M. anisopliae*-infected virgin males and 100 virgin females were introduced at the same time. Similarly, 100 uninfected virgin males and 100 virgin females were introduced in the control cage. They were allowed to mate for 24 h between 12:30hr and 12:30hr the next day. The females were then removed, put in separate collection cups and held inside a refrigerator at 4° C for 30 min. Thereafter, each female was dissected in 0.95% physiological saline solution under a dissecting microscope to remove the spermatheca (Figure 1A). The spermatheca was then placed on a sterile microscope slide moistened with the saline solution. The slide was observed under a compound microscope at 1000× magnification to examine the presence of sperm in the spermatheca. Females with sperms present (Figure 1B) were classified as inseminated while females with an empty spermatheca (Figure 1C) were not inseminated. Males were left to die and the dead individuals were surface-sterilised in 70% ethanol, rinsed in distilled water, plated on moistened filter paper and incubated at 28 ± 2° C. Cadavers were inspected for mycelial growth after three or more days under a compound microscope at 400× magnification. The experiments were replicated four times over time.

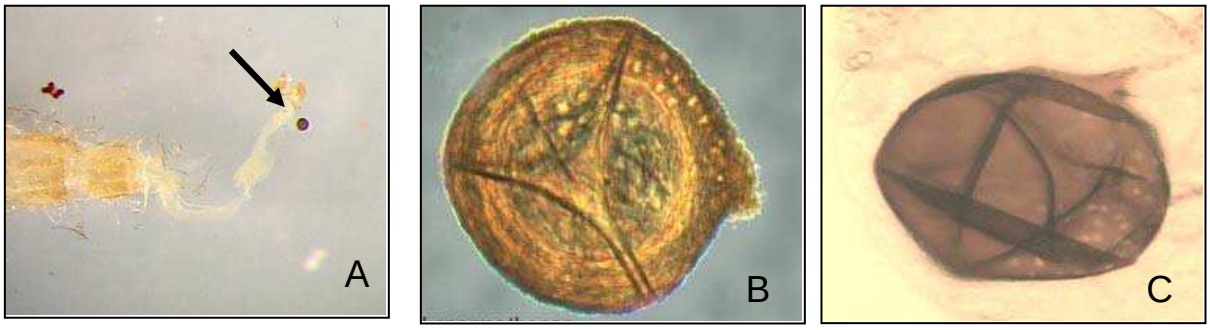

Figure 1. Dissection and insemination status of female spermatheca in mosquitoes. A) Dark-brown ball-shaped spermatheca; B) Spermatheca engorged with sperm (100× magnification); C) Spermatheca without sperm. Courtesy: Staff at the Malaria Research and Reference Reagent Centre (MR4).

Effect of **M. anisopliae** *infection on mating behaviour in free-flying mosquitoes*

The experimental procedure is similar to the caged one described above, except that 100 males and 100 females in separate holding cups were released inside a screenhouse (13 × 4.7 × 2.3 m) at the

same time each night. Virgin females were allowed to mate with *M. anisopliae*-infected virgin males and with uninfected virgin males on separate nights from 18:00 hr to 06:00 hr. After 12 hr, the mosquitoes were recaptured with males and females placed in separate collecting cups. The females were held inside a refrigerator at 4° C for 30 min. Thereafter, each female was dissected under a dissecting microscope and observed under a compound microscope to determine the presence of sperm in the spermatheca. The males in the collecting cup were supplied with 6% glucose until death. Mycosis tests were performed on the dead males to confirm growth of the fungus on the cadaver. The experiments were repeated for four nights with fungus-infected mosquitoes and four nights with uninfected mosquitoes.

Effects of M. anisopliae *infection on multiple mating potential of An. gambiae males*

This study was conducted in cages (15 × 15 × 15 cm) using *An. gambiae* in the ratio of 1:1; 1:3 and 1:5 (male: female). One, three and five virgin females were placed in three separate cages. A single *M. anisopliae*-infected virgin male was then added to each of the cages. Three other cages were set up to hold the same number of females and uninfected male mosquitoes as controls. The mosquitoes were left together for 24 hr from 12:30 hr to 12:30 hr the following day after which the females were removed and held in a collection cup. Other virgin females were then introduced into the cages containing males in the same ratio and allowed to mate for 24 hr. The removal of mated females and introduction of virgin females to the same males was conducted everyday at 12:30 hr until the male died. The mosquitoes were sustained on 6% glucose solution on paper wicks at all times. The sex ratio was varied to ascertain if that had any effect on the mating potential of the males. The collected females were anaesthetised and dissected in the laboratory to observe the spermatheca for the presence of sperm. Dead males on the other hand were surface-sterilised in 70% alcohol, plated on moistened filter paper and incubated at 28 ± 2° C to confirm fungal growth on the cadaver. The experiments with fungus-infected and uninfected males were each replicated four times.

Horizontal transmission of conidia during mating

Three sets of cage experiments were carried out to determine whether males can transfer inoculum to females during mating. The first experiment was conducted by holding 50, 1-d post-exposure males and 50 uninfected virgin females in a cage (30 × 30 × 30 cm). They were allowed to mate for 24 hr between 12:30 hr and 12:30 hr the next day. Females were then removed and kept in a separate cage. Both the males and the females were deprived of sugar water, but had free access to water only. When all individuals had died, they were plated for mycosis tests. The experiment was replicated four times. This experimental procedure was repeated to conduct the second and the third experiments using three and five day post-exposure males respectively.

Statistical analysis

Mating potential of the fungus-exposed and the uninfected males was separately quantified by expressing the number of females inseminated as a percentage of the total number dissected. The difference in insemination rates between the treatments was compared using the chi square (χ^2) test (Preacher 2001). The effect of fungus on multiple mating ability while controlling for time (days) was estimated by a regression linear model in SAS (SAS 2003). The number of females infected upon mating with males one, three and five d post-exposure, respectively, was expressed as a percentage of the total number of females exposed to males. Statistical differences between the numbers of females inseminated were compared using the chi square (χ^2) test. Furthermore, the infection status of the males used in each experiment was calculated by expressing the number that developed fungal growth as a percentage of the total in cages or recaptured in screenhouse. Significant differences in one, three and five d post-exposure males were estimated by the chi square (χ^2) test.

Results

Effect of M. anisopliae *infection on mating performance of caged male mosquitoes*

After dissection, the number of females that had successfully mated and was inseminated by fungus-exposed males did not differ (χ^2 = 0.43, df = 1, P = 0.51) from the controls (Table 1). While 77.8% of 400 fungus-exposed males developed mycosis none of the 400 males used as controls developed mycosis after death.

Table 1. Proportion of *An. gambiae* females inseminated by fungus-infected males after a 24 hr period in test cages. The mean number of females dissected and the percentage of males infected is illustrated. N is the number of replicates and n the number of mosquitoes tested.

Infection status of the mating individuals		N	Mean ± S.E no. of females dissected (n)	Percentage ± S.E of females inseminated (n)	Percentage ± S.E of males infected (n)
Males	**Females**				
Uninfected	Uninfected	4	93.5 ± 2.22 **(**374) [a]	86.6 ± 1.12 (324) [a]	0 (400)
Fungus-exposed	Uninfected	4	93.0 ± 1.22 **(**372) [a]	84.9 ± 3.05 (316) [a]	77.8 ± 3.45 (311)

Effect of M. anisopliae *infection on mating behaviour in free-flying mosquitoes*

Thirty-seven percent (147 out of 400) of the females that had an opportunity to mate with control males and 39% (154 out of 400) of the females that had an opportunity to mate with fungus-treated males were recaptured. The difference between the collections was not significant (χ^2 = 0.26, df = 1, P = 0.61). Of these, significantly (χ^2 = 12.99, df = 1, P = 0.0003) more females that mated with control males were inseminated compared with the treated males (Table 2). Mycosis was observed on 73.4% of the recaptured males that were exposed to *M. anisopliae* and none in control males.

Effects of M. anisopliae *infection on multiple mating potential of* An. gambiae *males*

In this study, 24 male mosquitoes (12 uninfected and 12 fungus-exposed) and 616 females were used over 10 days. Of these females 298 became inseminated i.e. 226 by uninfected males and 72 by fungus-exposed males. The effect of treatment with respect to day and ratio was modeled and indicates a decreasing incidence of insemination as the ratio increases, although not significantly so (P = 0.2703) (data not shown). The model was refitted controlling for days only and the results are presented in Table 3. The parameter estimate for treatment indicates that for a given day, for males treated with fungus, the odds that a female is inseminated is exp (-3.32) = 0.036 times the odds for those not treated with fungus i.e. the odds of insemination is lower among those treated with fungus. This effect of treatment is highly significant (P <0.0001). The results also indicate a highly significant day (time) effect (P <0.0001): given the treatment, the odds of insemination multiply by exp (-0.635) = 0.53 for a one-day increment; that is, there is a 47% decrease. Further, the probability that a female mosquito is inseminated on any given day *x* by a male infected by fungus is presented in Figure 2. Also presented for each day is the sample proportion of females inseminated separately

for each treatment group. Both the sample proportions and estimated probabilities show a decreasing trend as days go by in both the treatment and control groups, although the drop is sharper in the early days in the treatment group.

Table 2. Proportion of *An. gambiae* females inseminated by *M. anisopliae*-infected males after a 12 h period inside a screenhouse. The mean number of females recaptured and the percentage infection of the recaptured males is shown. N is the number of replicates and n the total number of mosquitoes assessed.

Infection status of the mating individuals		N	Mean ± S.E no. of females recaptured (n)	Percentage ± S.E of females inseminated (n)	Percentage ± S.E of infected males (no. recaptured)
Males	**Females**				
Uninfected	Uninfected	4	36.8 ± 4.97 (147) [a]	81.6 ± 1.36 (120) [a]	0 (113)
Fungus-exposed	Uninfected	4	38.5 ± 8.86 (154) [a]	63.0 ± 5.2 (97) [b]	73.4 ± 4.0 (113)

Horizontal transmission of conidia during mating

Fungal inoculum was passed from males to females during mating. On average, 62.5, 17 and 6% of females were infected by one, three and five day post-exposure males, respectively (Table 4). One-day old infected males have, therefore, a higher transfer rate of the conidia than three and five-day old infected males. Mycosis was observed for 77, 72 and 82.5% of one, three and five-day post-infected males, respectively. The infection rate was significantly higher in males that were 5 d after exposure compared with males that had been exposed three days previously (χ^2 = 6.27, df = 1, P = 0.01).

Discussion

Our results demonstrate that the entomopathogenic fungus *M. anisopliae* reduces the proportion of *An. gambiae* female mosquitoes inseminated by infected males in a screenhouse. In cage studies, however, the proportion of females inseminated was not affected by the fungal infection of the male mosquitoes. Moreover, fungal infection is likely to reduce the propensity of multiple mating because infected males are physiologically compromised. Our findings further illustrate that fungal conidia can be transferred to healthy *An. gambiae* female mosquitoes through mating.

In nature, male mosquitoes require a large space for swarming, which is an important component of their mating behaviour (Charlwood and Jones 1980). As a consequence, they may not swarm properly when confined in small cages (Fraccaro et al. 1977) and that could eventually lead to low insemination rates if mating occurred. The results of our cage studies have shown that the males mated successfully and many females became inseminated while confined. Besides swarming, male mosquitoes are guided by sound produced by the females and vision to find a mate (Roth 1948, Charlwood et al. 2002, Gibson et al. 2010) in addition to contact pheromones present on the cuticle (Takken and Knols 1999). Furthermore, any factor that triggers both sexes to fly increases the frequency of mating, leading to a higher proportion of females getting inseminated (Roth 1948). Therefore, the size of the cage we used was not an obstacle in the mating behaviour of males.

Instead, the cages promoted close-range and forced mating that may have resulted in the absence of a difference in insemination rates between fungus-infected and uninfected males.

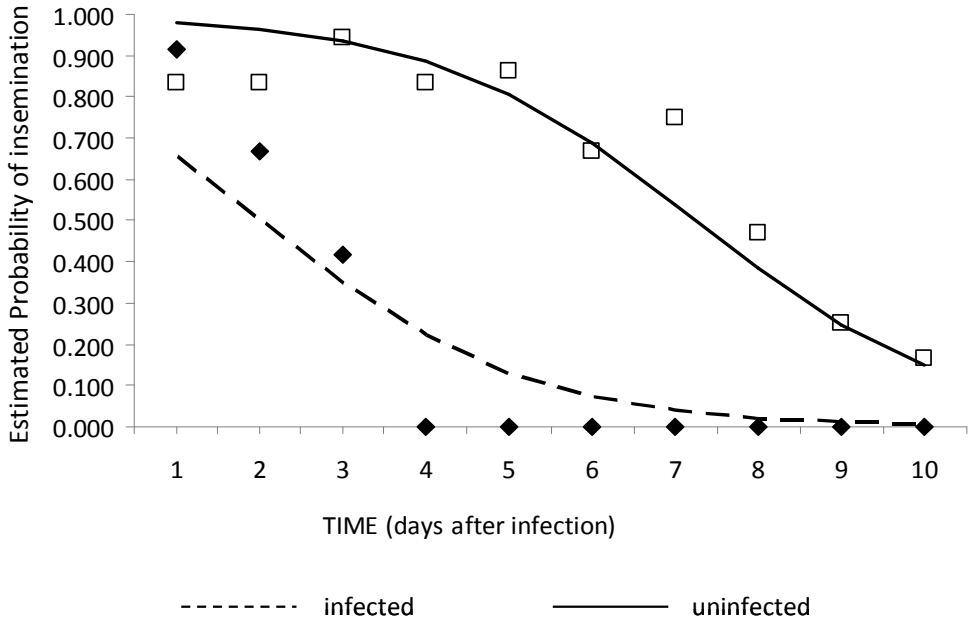

Figure 2. The estimated probability of insemination when a female mosquito is inseminated on any given day *x* by an uninfected and a *M. anisopliae*-infected male

Table 3. Parameter estimates and standard errors from the logistic regression model comparing effect of treatment (uninfected and *M. anisopliae*-infected *An. gambiae* mosquitoes) on multiple-mating ability over time (day)

Parameter	Estimate	Standard error	P-value
Intercept	4.600	0.398	<0.0001
treatment	-3.320	0.285	<0.0001
day	-0.635	0.059	<0.0001

The situation in a screenhouse is different and the significant reduction in mating performance of fungus-infected males observed may be associated with the large space, the absence of nutritional sources and the effect of the fungal pathogen. It is reported that mating in mosquitoes is an energy-dependent activity (Stone et al. 2009, Gouagna et al. 2010) and most males get depleted of energy when mating is completed but replenish this quickly with frequent visits to plant sugar sources (Foster and Takken 2004). In this study, after the mating period had elapsed, both uninfected and infected recaptured males were less active in their respective collection cups (S.O. personal observation) possibly due to depletion of energy as there were no sugar sources in the screenhouse during the experimental period. When provided with sugar water soaked in cotton wool, the control

males engaged in flight to reach the sugar source while their infected counterparts hardly moved and died soon afterwards. We can, therefore, conclude that infected males succumbed to the pathologic effects of the fungus and that this affected their ability to fly and engage in swarming for mating.

The fitness of male mosquitoes is not only defined by their potential to mate and inseminate females, but also by their ability to mate multiple times. The latter is critical since males are assumed to remain in a constant "mating state" after the onset of mating (Roth 1948, Boyer et al. 2011). Interruption of the multiple-mating propensity of male mosquitoes may therefore affect the reproductive capacity and hence population growth rate of mosquitoes. Our cage study results show that multiple mating was not affected, but the frequency of occurrence over time was reduced with a *M. anisopliae*-infection. This is because infected males rarely survived longer than three days (6 d post-exposure) after being in contact with virgin females daily whereas the uninfected ones mated continuously up to the tenth day. The scenario may be worse in a field situation since infected males with reduced fitness may fail to compete and inseminate females. Therefore, the effect caused by an entomopathogenic fungus can enhance early elimination of males in the population and a further decline in reproduction success in the mosquito population.

Table 4. Proportion of females infected after a 24 h mating period with males 1, 3 and 5 d post exposure to *M. anisopliae* in test cages. Percent infection rate in males is shown. N is the number of replicates, n the number of mosquitoes infected.

Day post-exposure	N	Percentage ± S.E of females infected (n)	Percentage ± S.E of infected males (n)
1	4	62.5 ± 5.06 (125) [a]	77.0 ± 6.03 (154) [ab]
3	4	17.0 ± 2.08 (34) [b]	72.0 ± 4.32 (144) [b]
5	4	6.0 ± 1.41 (12) [c]	82.5 ± 2.63 (165) [a]

The effect of entomopathogenic fungus on the reproductive performance of males may impose direct and indirect consequences on the mosquito population. Directly, mated but uninseminated females may derive a blood meal from the host, undergo successive gonotrophic development, oviposit but fail to produce progency, a common feature of uninseminated *An. gambiae* (Thailayil et al. 2011). As a result, reproduction success will be reduced in the entire population to a level that can significantly contribute to malaria vector control. Moreover, a successful mating might imply an increased chance of passing on the fungus to other members of the population through horizontal transfer of fungal inoculum. However, considering that males were used at a stage (3 d post-exposure) when the infection process is taking place in the mosquito, the probability of transmission to females at this time is likely to be low (this chapter). Consequently, there is a possibility for indirect sub-lethal effects to occur which is beyond the scope of this study. For instance, in *An. gambiae* females, sub-lethal effects of fungus result in reduced blood-feeding and fecundity (Scholte et al. 2006, Blanford et al. 2011). Therefore, in the males it is likely that infection may cause a reduction in sperm load transferred to the females and a subsequent reduction in sperm viability. This remains to be investigated; however, if this can indeed occur, it may give rise to fewer offspring in the population. One case study shows a reduced fecundity in uninfected *Musca domestica* Linnaeus females mated with males infected with *Entomophthora muscae* (Watson and Petersen 1993). This occurrence could be an advantage since *An. gambiae* females are considered to be

monogamous, becoming refractory to re-insemination and re-mating is rare (Roth 1948, Klowden and Russell 2004, Howell and Knols 2009).

Our study has further demonstrated that the probability of horizontal transfer of fungal conidia is highest after males have been exposed to the fungus for 24 hr. In mosquitoes, there are only two reports that address the aspect of horizontal transmission. One study found that transfer of the fungus *B. bassiana* from *Ae. aegypti* males allowed to mate for 48 hr caused 80-90% mortality in the recipient females (Garcia-Munguia et al. 2011). The second report describes a 34% mortality in *An. gambiae* males that acquired infection during 1 hr of mating with *M. anisopliae*-infected females (Scholte et al. 2004a). These findings and our observation demonstrates that conidial transfer rate is higher in the early days of infection, possibly due to the fact that at that time conidia are still loosely attached to the insect cuticle (Hajek and St Leger 1994, Scholte et al. 2004b, Zimmermann 2007b, a). Transmission is possible even after 3 d post-exposure (Zimmermann 2007b, a) as we also observed, but the effect may be too low to cause a significant impact. Therefore, this approach can be more beneficial when males mate soon after exposure to fungal pathogen. In other insects, transfer of the entomopathogenic fungus *B. bassiana* or *M. anisopliae* from males to females has been reported in the Mexican fruit fly *Anastrepha ludens* Loew (Toledo et al. 2007, Novelo-Rincon et al. 2009), in the African fruit flies *Ceratitis capitata* Wiedemann*, C. cosyra* (Walker) and *C. fasciventris* Bezzi (Dimbi et al. 2009) and in the tsetse fly *Glossina morsitans morsitans* Wiedemann (Kaaya and Okech 1990). The findings in these studies were shortened survival and reduced fecundity. In insects, any activity that enhances contact with fungal spores increases the chance of spreading the fungal pathogen through a host population. Such activities include mating, swarming, tactile communication, grooming and temporary aggression, among others (Billen 2006). Besides, the morphology of the insect plays a role as in mosquitoes where fungal spores were spotted on the lower parts of the first and second pair of legs, the hairs on the wings and the mouth parts of *An. gambiae* males (Scholte et al. 2004a). These parts in males are critical in initiating mating and their invasion with fungal conidia is an advantage for efficient transmission. This could have contributed to the higher conidial transfer to the females as observed in this study.

The efficacy of the entomopathogenic fungus was expressed quite early in the mosquito population. We were required to expose twice as many males to the pathogen compared to the numbers held as controls because on average 50% of individuals in the exposed group succumbed to mortality prior to the start of experiments on day three post-exposure. If a similar impact of the fungus occurs in field situations with wild mosquitoes, then the fungus contributes positively to the reduction of malaria vectors by shortening their survival, and hence removing a fraction of the male mosquito population. Individuals that survived longer and used in the experiments have demonstrated a high mating ability including sperm transfer. This may be explained because they received a lower dose of the fungus or their immune system was stronger to suppress the invasion initially (Gunnarsson 1988, Moore et al. 1992, Seyoum et al. 1994). Moreover, the infection may have induced rapid synthesis of juvenile hormone (Blanford and Thomas 2001) for early maturation of accessory glands to sustain the insemination process. Nevertheless, at a later stage, the insects become overwhelmed and die from the infection. The combined effect of early and delayed mortality in fungus-infected male mosquitoes is significant and results in a low proportion of males present in the population available for mating successfully with the females.

In general, transfer of conidia to the population through the males poses some advantages. Foremost a greater percentage of the population is likely to be affected due to the ability of males to mate with several females in succession. On rare occasions, it is likely that newly emerged males will be infected when approached by mature males in an attempt to copulate as observed by Charlwood and Jones (1979). Males have been found to rest indoors (Charlwood et al. 2003, Scholte et al. 2005, Howell and Knols 2009) and this increases the chance to pick up conidia while resting on fungus-

impregnated surfaces. In turn, infected males will transfer conidia to females during mating outdoors. However, the major challenge remains to be finding a means of infecting males outdoors where they spend most of the time feeding and searching for mates. The possible approach to infect males by fungal pathogens may be by targeting their sugar sources and it is evident that males are inseparable from plant communities (Yuval 1992, Foster 1995). We have demonstrated that males can be infected and killed with entomopathogenic fungus during plant sugar feeding (Chapter 6). Strategies for mosquito control are reported that combine synthetic insecticides with natural sugar sources as baits (Müller and Schlein 2008, Müller et al. 2010c, Müller et al. 2010b, Müller et al. 2010a). Alternatively, flowering plants frequented by mosquitoes can be sprayed with insecticides. Therefore, the approach can be adopted to infect males by spraying flowering plants with infectious fungal conidia formulated in a suitable carrier that can withstand detrimental effects of the UV-light. A more costly approach in terms of bait preparation could be by spraying fungus in stationary traps baited with plant sugars. Besides targeting plants, emergence traps impregnated with fungal conidia may be used in larval habitats to infect both sexes on emergence (Bukhari et al. 2011a).

Further research is still needed on the effect of fungal pathogens on other aspects of male mating behaviour. These include the quantity and quality of sperm transferred, mating competitiveness and possible transfer of conidia to other males in swarms. The information will be vital to underscore the reproductive fitness costs associated with fungal infection in male mating biology and its significance in reduction of malaria vector populations.

Conclusions

Our study has demonstrated that male mosquitoes succumb quickly to infections with entomopathogenic fungi, similar to female mosquitoes. The first three days after exposure, males mate normally when infected with the entomopathogenic fungus *M. anisopliae* in laboratory cages, but significantly less in large outdoor enclosures suggesting a strong impact of the fungus on natural mating behaviour. Horizontal transfer of fungus occurs rapidly during mating, providing a means of infecting female mosquitoes. Furthermore, the frequency of the males to engage in multiple mating and the rate at which females become inseminated is reduced in the advanced stages of infection.

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Chapter 9

Summarizing discussion

Introduction

Malaria remains a major global hindrance to health improvement and economic development in the world's poorest countries where more than 225 million cases and more than 600,000 deaths occur each year (WHO 2011). Ninety percent of malaria-related deaths occur in sub-Saharan Africa, with the majority of deaths being young children. The transmission rates and risks of malaria disease can be greatly reduced by vector control. Widespread insecticide resistance is reducing the efficacy of current tools that target adult mosquitoes (N'Guessan et al. 2007, Ranson et al. 2009). Entomopathogenic fungi (EPF) have demonstrated potential as microbial control agents to complement synthetic chemicals for malaria vector control. Several studies have demonstrated the efficacy of EPF to infect, kill and reduce the survival of insects including mosquitoes (Hajek and St Leger 1994, Scholte et al. 2003a, Lord 2005, Achonduh and Tondje 2008, de Paula et al. 2008). The reduced survival directly affects the transmission potential of malaria parasites by mosquitoes, as a smaller fraction of the population will reach the age when transmission can occur (>10 days after infection). Infection with EPF not only affects survival, but also inflicts behavioural changes in pest insects with little attention given to disease vectors (Chapter 2). Understanding the relationship between pathogens and their hosts from a behavioural perspective is more practical to the development of strategies for increasing pathogen transmission in the ecosystem. Such a relationship is, until now, only marginally explored as is with understanding the dynamics of malaria vector populations, the latter's behaviour and ecology, and how these affect disease transmission. The aim of this thesis was to determine whether fungal infection causes behavioural effects in adult malaria mosquitoes under laboratory and/or semi-field conditions. Host-seeking, plant sugar-feeding, mating and oviposition are the major life-history behaviours studied. This information is important when mosquitoes are to be targeted in their natural environment to evaluate the potential public health benefits of the fungus control approach. In this thesis the entomopathogenic fungus *Metarhizium anisopliae* ICIPE 30, virulent on the malaria vector *Anopheles gambiae* Giles *sensu stricto* (Scholte et al. 2003b), was tested to obtain insights in the potential effects of fungal infection on the major life-history behaviours. The six objectives of the thesis and the findings are discussed in a broader perspective and suggestions for future research are highlighted.

Fungus formulation and application method

Prior to conducting behavioural studies, it was important to identify a tool for infecting mosquitoes in the laboratory under ambient conditions. There were options to choose from amongst several tools that have been tested such as the use of clay pots (Farenhorst et al. 2008) and PVC-tubes (Farenhorst and Knols 2010) but clay pots are not simple to use while PVC-tubes lined with smooth, gloss proofing paper coated with a conidial suspension using a hand-coating machine are not cost effective. I therefore decided to develop a new tool, a paper sheet placed inside a plastic cylinder that was simple to use and of low cost (Chapter 3). Several studies have utilised conidia formulated in mineral oil or other suitable carriers as the search for a standard formulation continues but in this study I used dry conidia that are described to be more virulent than when formulated in a carrier (Lacey et al. 1988, Scholte et al. 2003a). The effective dosage and exposure time established to achieve high infectivity concurs with what has been reported in mosquitoes previously (Scholte et al. 2003a, Paula et al. 2011) and in other pest insects (Jaccoud et al. 1999). These findings indicate that fungus delivery tools are arguably important in facilitating the accessibility of infectious fungal conidia to mosquitoes. Besides, conidial formulation, dosage and exposure time sufficient for pest insects and vectors to pick up large numbers of conidia are important to enhance fungal pathogenicity (Chapter 3).

Impact of infection with *M. anisopliae* on host-seeking response, blood feeding and fecundity of *An. gambiae* mosquitoes

Host-seeking is an important component of mosquito vectorial capacity on which the success of the other behavioural determinants depends. Host-seeking is mediated by olfactory cues (Zwiebel and Takken 2004), which are responsible for the differential attractiveness of humans to the malaria vector *An. gambiae*. Therefore, manipulating the behaviour and the olfactory response in order to reduce human-vector contact may greatly lead to reduction of malaria transmission. (Scholte et al. 2006) showed that fewer caged female mosquitoes made contact with their blood meal host on infection by the entomopathogenic fungus *M. anisopliae* under laboratory conditions. My study aimed to evaluate the ability of fungus-infected mosquitoes to respond to cues from their blood host at close-range, medium-range and long-range. The close range (1 m from host) evaluation, using a dual-choice olfactometer, included human subjects ranked as highly attractive and poorly attractive to *An. gambiae* mosquitoes (Mukabana et al. 2002) to ascertain the impact of fungal infection on the mosquito's discriminatory capability (Chapter 4). The medium-range studies were executed using experimental cages (3 x 3 x 2 m) under laboratory conditions (Chapter 7). At long range (7 m from host), house-entry rate and the outdoor and indoor human-biting rates of malaria vectors were assessed under natural climatic conditions in a semi-field enclosure referred to as *Malaria Sphere* (Chapter 5). It is important to note that in all these studies the survival of mosquitoes was reduced by 50% in the first three days after fungal infection (incubation period), an indication that a fraction of the mosquito population is eliminated before external signs of infection are noticeable.

Infection further affected flight performance in a fraction of the remaining fungus-infected individuals causing a reduction in the host-seeking population (Chapters 4 and 7) and a reduction in the house-entry rate, indoor human-biting rate and outdoor human-biting rate (Chapter 5). Impact of fungus on flight performance and how this directly affects behavioural activities has also been reported for other insects (Seyoum et al. 2002, Blanford et al. 2011). It is therefore likely that behavioural changes observed in insects as described in Chapter 2 are primarily triggered by impaired flight performance. Besides, host-seeking behaviour, in particular in fungus-infected mosquitoes, may be further affected due to an impaired olfactory sensitivity (George et al. 2011) although the visual observation approach in my study was not sufficient to verify this. However, it is evident that where the host was present or absent, the number of infected mosquitoes entering the house was only half of the uninfected mosquitoes. In other words, those that did respond were not impaired (Chapters 4 and 5), but those that did not respond were either physically impaired ('too sick') or olfaction impaired. The differences between the laboratory cage studies by (George et al. 2011) and this study call for further research, especially as the first study was done with *Beauveria bassiana* and the latter with *M. anisopliae*.

Although effective against adult mosquitoes, EPF take time to evoke pathogenicity and that exposes the human host to mosquito nuisance and bites. For example, female mosquitoes ingested a blood meal at five days post-exposure to fungus *M. anisopliae* although blood feeding propensity was reduced (Chapter 7). In other studies, a reduction in blood feeding propensity was reported in *An. gambiae* (Scholte et al. 2006), *An. stephensi* (Blanford et al. 2005, Blanford et al. 2011) and *Culex quinquefasciatus* (Howard et al. 2010b) infected with *M. anisopliae* or *B. bassiana*. Moreover, fungus -infected mosquitoes imbibed equal amounts of blood as the uninfected ones and their fecundity remained unaffected. This however, is in contrast to a reduction in the quantity of blood meal ingested and in fecundity as reported by (Scholte et al. 2006). The difference may be because I assessed an unequal number of uninfected and infected mosquitoes since a fraction of the infected mosquitoes died from infection before oviposition (Chapter 7). Although the mosquitoes took a blood meal, it is unlikely that these mosquitoes can transmit malaria parasites. This is because it takes at least 10 days for the parasite to mature in the mosquito yet the mosquito is killed from

fungal infection in less than 10 days (Chapter 3) depending on the fungal species and dosage used. In addition, fungi negatively impact on *Plasmodium* development preventing the parasite to develop into sporozoites (Blanford et al. 2005). The general effects of fungal infections on mosquitoes are therefore likely to have a strong impact on the transmission potential of the mosquito-borne parasites if mosquitoes are infected early in life.

Impact of infection on feeding and survival of *An. gambiae* mosquitoes on plant sugars

Sugar feeding is central in the biology of both sexes of *An. gambiae*. At emergence, mosquitoes search for sugar to rebuild their energy reserves without which they are less able to mate, blood feed, develop eggs, or lay them (Foster and Takken 2004). Interference of the sugar-feeding activity may therefore impose a direct impact on survival, reproduction in the vector population, and therefore, on malaria transmission. My data have shown that the survival and sugar-feeding propensity of the malaria vector *An. gambiae* is reduced by fungal infection but their potential to ingest and digest meals remains unaffected (Chapter 6). The lack of difference between feeding and digestion rate of uninfected and fungus-infected mosquitoes may be due to their confinement in test cages. However, by considering the observations with blood meal intake (Chapter 7), then there is no doubt that the difference is likely not to occur. The fact that infected mosquitoes succeed to feed is an indication that they have a chance to sustain their physiological requirements including reproduction. This may delay mosquitoes from succumbing to infection quickly but may facilitate the occurrence of sub-lethal effects that can lead to reduction in fecundity as reported in other studies and mating performance in males (Chapter 8).

Impact of fungal infection on the mating performance and the probability of horizontal transfer of fungus in the malaria mosquito *Anopheles gambiae*

Successful mating propels females to seek a human host for a blood meal to complete egg development (Chambers and Klowden 2001) and in the process increases chances of transmitting malaria parasites from one individual to another. This study examined the impact of infection with EPF fungi on the mating performance and probability of horizontal transmission in malaria vectors. The number of female mosquitoes inseminated by fungus-infected males was reduced when mating takes place in a large arena such as a screenhouse. Moreover, infected males were able to transfer conidia to uninfected females during mating increasing the chance of passing on pathogenic fungus to other members of the population (Chapter 8). These pathologic effects on the mating performance of male mosquitoes may lead to the suppression of mosquito populations and a subsequent reduction in malaria transmission.

Implication of fungal infection on mosquito behaviour

Entomopathogenic fungi infect and kill their arthropod hosts without the need for ingestion (Gillespie and Claydon 1989), a characteristic shared with insecticides; and an advantage over bacteria or viruses that infect their hosts through the gut wall. Besides, sub-lethal effects of EPF strongly affect the major life-history behaviours of *An. gambiae* mosquitoes (Table 1), an important advantage over insecticides that only result in the death of the insects. Although I have demonstrated behavioural changes in fungus-infected mosquitoes, the high mortalities observed in the early days of infection cannot be overlooked as both occurrences collectively have a significant impact in suppressing a vector population. For instance, up to 75% of mosquitoes that I exposed to the fungus *M. anisopliae* did not survive beyond 6 d after emergence (3 d post-infection). In addition, behavioural activities in 50% of the remaining fungus-infected mosquitoes were impaired. This indeed is a major contribution of EPF to the reduction in vector populations and subsequent malaria cases in a proportion that approaches the global target of ≥ 75% set to be achieved by 2015 (WHO

2009). Understanding the mechanisms employed by EPF to cause the various behavioural changes in insects is important in emphasising the need to develop the tool as a microbial control agent.

Table 1. Summary of behavioural effects of fungal infection with *Metarhizium anisopliae* on adults of the malaria mosquito *Anopheles gambiae* as observed in this thesis

Behavioural category	Behavioural activity	Effects of fungal infection on the behaviour/traits
Feeding	Host-seeking	- Reduction of host-seeking propensity - Reduction of the house-entry response of the host-seeking population - Reduction of the outdoor and the indoor biting responses of the host-seeking population - No effect on the olfactory discrimination capability of the host-seeking population
	Blood-feeding	- Reduction of blood-feeding propensity - No effect on quantity of blood-meal ingested
	Sugar-feeding	- Reduction of plant sugar-feeding propensity - No effect on amount of sugar imbibed - No effect on digestion rate of sugars consumed
Reproduction	Mating	- Reduction of insemination rate when females mate with infected males in a large arena - Reduction of propensity of multiple mating in males - Enhancement of conidial transfer from infected to healthy individual during mating.
	Oviposition	- Reduction in oviposition propensity - No effect on the number of eggs laid

It is evident that through fungal parasitism, insects exhibit behavioural alterations (Roy et al. 2006). Moreover, through parasitism, arthropod host invasion is made easy by the release of well-characterised enzymes affecting the non-permeable cuticle while host death is often associated with toxin production overwhelming host-defence responses. As slow-killing agents in addition to their invasion and colonization of the host tissues that result in multiple modes of attack on various insect behaviours as demonstrated in this thesis, fungi are expected to impose limited risks for resistance development in malaria mosquitoes (Thomas and Read 2007a, Knols et al. 2010). If resistance occurs, which should be considered (Michalakis and Renaud 2005), then it would probably take a longer time to evolve (Read et al. 2009). Such an occurrence would be advantageous over insecticides to which the insect pests and vectors are prone to develop resistance (Hemingway and Ranson 2000, Hemingway 2004, Ranson et al. 2009, Ranson et al. 2011). Therefore, the outcome of direct (lethal) and indirect (sub-lethal) effects of fungal pathogens envisage their potential as microbial control agents for the control of malaria vector.

The direct effect of EPF on mosquito populations is the potential to infect, kill and reduce the survival of mosquitoes. Diet plays a major role in regulating the life-span of mosquitoes through which reproduction, fecundity and migration/movement are enhanced (Nayar and Sauerman Jr 1971). Teneral reserves of trehalose (sugar carried over from the larval stage), glycogen (sugar stored in the fat body) and sugar (diverted and stored in the crop) are the three possible sources of energy in mosquitoes to support different activities. Teneral energy is used for basal maintenance functions such as respiration, excretion, digestion, resting etc (Clements 1955, Nayar and Van Handel 1971a, Foster 1995, Takken et al. 1998). Glycogen and sugar are primarily used as energy source for flight. During fungal invasion, trehalose is depleted and fungal toxins disrupt enzymatic activities in the midgut that interfere with mosquito feeding. As a consequence, the insects die from starvation. Dosage and immune response of the insects determine when a mosquito dies following exposure to fungal conidia. With the continuous depletion of trehalose by fungus in the insect, more sugar and glycogen are converted to replenish the reserves at the expense of facilitating flight. As a result, the flight performance is strongly compromised with which, sub-lethal effects are expressed in the various behavioural activities of the insect. For instance, with increasing fungal infection, mosquitoes become less able to fly and engage in host-seeking (Chapters 4, 5 and 7), foraging for plant sugars (Chapter 6) and mating (Chapter 8). Although the behaviours are distinct, they interact at so many levels such that foraging for sugar and blood meal presents a challenge to the female mosquito since each feeding decision has a major impact on reproductive success (Stone et al. 2011). To overcome the challenge, females adopt a certain pathway that starts with sugar priority at emergence (Foster and Takken 2004), mutual inhibition of feeding preferences during feeding and digestion, sugar feeding late in the gonotrophic cycle and even permanent dominance of one food or the other in the diet (Foster 1995, Gary and Foster 2006). Frequency of feeding (on blood or sugar) however, is regulated by the nutritional requirement of the insect while the rate of digestion by flight demand but the sustainability of the activities to enhance longer survivorship are jeopardized by fungal infection.

In this thesis, I have demonstrated and now emphasise that in-depth understanding of the insect-pathogen interaction is a guide in determining the extent to which fungal pathogens may be utilised as biocontrol agents. Further knowledge on the biology and ecology of the fungus is the core on how best to explore its pathogenicity (Bidochka et al. 2000). I worked with a fungal species and isolate that is highly virulent against mosquitoes (Scholte et al. 2003b) and identified the most lethal dose that was used throughout the study as a standard dose (Chapter 3). Dry spores in favour of spores formulated in any suitable carrier were used for two reasons. One, it provides a reliable infection method for controlled laboratory-based assays. Secondly, on the basis that formulating fungal conidia slightly lowers its virulence unless a very high dose of up to 10^{11} and 10^{12} conidia/m^2 is used (Blanford et al. 2005, Mnyone et al. 2009a, Farenhorst and Knols 2010, Howard et al. 2010a). All these factors were a prerequisite for the behavioural outcomes observed. It is possible that these findings may be observed when wild mosquitoes are being targeted. However, that will require a modification of fungus delivery tools and dosage to enable the fungus to withstand the fluctuating environmental conditions and retain efficacy and persistence (Knols and Thomas 2006, Blanford et al. 2009). With a control approach aimed at reducing the chance for transmission of malaria and other vector-borne diseases, the advocacy would be maintaining higher doses. Lower doses, however, are still effective in reducing the survival of malaria vectors and allow for expression of sub-lethal effects to lower the chance for resistance development, but instead increase the chance for disease transmission.

Among the behavioural activities and traits studied, extensive research has focused on the host-seeking population because of its relationship of certain parameters in the vectorial capacity equation (MacDonald 1957). Targeting males for population reduction by EPF also opens a new strategy for mosquito vector control (Chapters 6 and 8). Male mosquitoes spend most of the time

outdoors although a fraction of them have been found to rest indoors (Scholte et al. 2005, Howell and Knols 2009). In contrast, malaria vectors *An. gambiae* and *An. funestus* females blood feed and rest indoors while *An. arabiensis* blood feed and rest outdoors. Although not the scope of this study, it would be ideal to identify suitable pathways to infect both sexes of malaria vector indoors and outdoors in order to reap the benefits of EPF in vector control. My results however, add new dimensions to the direct and indirect effects, indicating the impact of fungal infections on insect behaviours that could be considered in designing or modifying control strategies under development. Given the present state of fungal research, very few trials have translated the use of EPF to field-based evaluations of their actual impact on mosquito survival and malaria risk (C.J.M. Koenraadt, personal communication). Thus, it is only possible to interpret these findings in the context of the potential for future malaria control.

Suggestions for future research

The findings in this thesis are encouraging particularly in new areas of plant sugar-feeding and mating performances in *An. gambiae* mosquitoes. For a complete overview of the impact of infection on these behaviours, further research is recommended on:

1. Pathogenicity of EPF in terms of effects on reproductive success and male mating competitiveness: I have shown that infected males can inseminate females but it remains to be investigated whether the quantity and the quality of the sperm transferred are compromised. It should furthermore be established if the fecundity of females inseminated by infected males is affected. The ability of infected males to compete with healthy males for females as occurs in nature is an important element in male mating biology. The findings will underscore the significance of EPF in the behaviour of males and their reproductive ability.
2. Plant sugar feeding: My findings are based on studies with caged mosquitoes and it will be interesting to investigate how infected mosquitoes forage for nectar or honeydew in open spaces that simulate conditions in nature. A more modern and accurate technology is recommended for use to detect lipid and glycogen levels in mosquitoes as a direct indicator of digestion to complement the use of cold anthrone test that is biased towards fructose only.
3. Formulation and delivery methods: I utilised dry conidia and a delivery tool that could infect a small number of insects. To evaluate the impact of EPF in semi-field and field situations, alternative fungal formulations and delivery tools to target large population of mosquitoes are a prerequisite. Further research is also recommended on methods to infect male mosquitoes outdoors where they spend most of the time to sugar-feed and search for mates.

Conclusions

The entomopathogenic fungus *M. anisopliae* significantly reduced the survival of the mosquito *An. gambiae s.s*. Flight performance of mosquitoes was negatively affected by fungal invasion that strongly reduced the host-seeking response, house-entry rate, outdoor and indoor human-biting rate, plant-sugar feeding potential and mating performance of *An. gambiae* mosquitoes. The multiple modes of attack of the fungal pathogen may reduce the likelihood of resistance development. The susceptibility of male mosquitoes to fungal conidia opens a new strategy for mosquito vector control. The findings may further be incorporated in malaria transmission models to gauge their full impact on the Entomological Inoculation Rate (EIR). Overall, this thesis has demonstrated that EPF are a suitable novel tool to complement the use of insecticides in malaria vector control.

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Samenvatting

Samenvatting

Malaria is nog steeds een groot wereldgezondheidsprobleem met de grootste impact in Afrika, met name ten zuiden van de Sahara. Strategieën om malaria te bestrijden verschillen over de wereld al naar gelang de mate van endemiciteit en de omvang van de ziekte. De focus blijft echter om de malaria parasieten of de vectoren te bestrijden. Een hoge mate van resistentie tegen medicijnen en het ontbreken van malaria vaccins zijn belangrijke belemmeringen om de ziekte onder controle te krijgen. In dergelijke omstandigheden is bestrijding van vectoren een goed alternatief en tot nu toe de meest effectieve manier om malaria overdracht te voorkomen. De huidige bestrijding van volwassen muggen is vrijwel uitsluitend gebaseerd op insecticide-geïmpregneerde klamboes en het toepassen van chemische insecticiden binnenshuis in de vorm van 'indoor residual spraying' (IRS) van muren en plafonds. Echter, duurzaam gebruik van chemische middelen wordt ondermijnd door resistentie van muggenpopulaties tegen insecticiden, vervuiling van de leefomgeving en risico's voor de menselijke gezondheid. Biologische bestrijding die is gebaseerd op schimmels als pathogenen heeft reeds veel potentie getoond om de huidige vectorbestrijdingsmethoden aan te vullen. De entomopathogene schimmels *Metarhizium anisopliae* en *Beauveria bassiana* kunnen malaria vectoren infecteren, doden en de overleving beïnvloeden. Echter, het mogelijke effect van entomopathogene schimmels op het gedrag van malaria vectoren is onvolledig bestudeerd.

Het doel van dit proefschrift was het vergaren van basis informatie over mug-schimmel interacties met daarbij de focus op de effectiviteit van de entomopathogene schimmel *M. anisopliae* ICIPE 30 op belangrijke gedragsaspecten van de Afrikaanse malariavector *Anopheles gambiae* Giles *sensu stricto* onder laboratorium en semi-veld omstandigheden. Deze informatie is belangrijk om een verdere ontwikkeling van biologische bestrijdingsmiddelen mogelijk te maken gericht op malariavectoren. Het gastheerzoekgedrag, voeden op suikers, paargedrag en eileggedrag zijn de gedragsaspecten die zijn onderzocht. Omdat mug-schimmel contact cruciaal is voor infectie met entomopathogene schimmels, is een bio-toets ontwikkeld bestaande uit een papieren vel (28.6 x 14.3 cm) in een plastic cylinder (met een diameter van 9 cm en hoogte van 15 cm) als kosteneffectieve manier om muggen te infecteren. Ongeveer 0.1 gram droge sporen ($\approx 10^{11}$ sporen per m^2) en zes uur blootstellingsduur waren voldoende voor *An. gambiae* om grote aantallen sporen op te pikken en hoge pathogeniciteit te veroorzaken (Hoofdstuk 3). Omdat eerder beschreven is dat de impact van entomopathogene schimmels op het gedrag van insecten ten minste drie dagen na blootstelling aan een schimmelpathogeen plaatsvindt (Hoofdstuk 2), zijn alle experimenten uitgevoerd met een speciale focus op muggen drie dagen na blootstelling. Het is echter belangrijk te vermelden dat gemiddeld 50% van de muggen dood was drie dagen na blootstelling aan de schimmel (Hoofdstuk 3). Alleen de muggen die overleefden zijn gebruikt voor de gedragstoetsen.

De bekwaamheid van *An. gambiae* muggen om gastheren te zoeken is een belangrijke parameter in de zogenaamde vector capaciteit vergelijking. Gebruik makende van een tweekeuze olfactometer onder semi-veld omstandigheden, reduceerde een infectie met entomopathogene schimmels de gastheerzoekrespons van muggen op korte afstand (1 meter van de gastheer), maar verhinderde niet dat muggen in staat waren om een op geur gebaseerd onderscheid te maken tussen gastheren (Hoofdstuk 4). Op middellange afstand en gebruik makende van experimentele kooien van 3 x 3 x 2 m in het laboratorium, reduceerde een schimmelinfectie de gastheerzoekrespons en de neiging tot voeding van vrouwelijke *An. gambiae* muggen (Hoofdstuk 7), terwijl op lange afstand (7 m van de gastheer) in een semi-veld opzet een infectie met entomopathogene schimmels de mate verminderde waarmee muggen een huis binnentraden als ook de respons per uur van gastheerzoekende muggen om mensen zowel binnen als buiten te bijten (Hoofdstuk 5). Het voeden op suikers afkomstig van planten is een belangrijke component in de biologie van muggen en heeft de hoogste prioriteit voor beide geslachten na het uitkomen van de poppen. Infectie met schimmelpathogenen verminderde de overleving en neiging om op suiker te voeden van beide

geslachten van *An. gambiae,* maar had geen effect op hun mogelijkheden om maaltijden te verteren (Hoofstuk 6). Het paargedrag speelt een sleutelrol in de groei van populaties. Deze activiteit vindt plaats na het voeden op suikers en daarna zoeken de vrouwtjes een gastheer om bloed te verkrijgen. Infectie met *M. anisopliae* verminderde zowel de neiging om meerdere malen te paren sterk, als ook de paarprestatie van volwassen *An. gambiae* mannetjesmuggen in een grote arena (zoals een kas van gaas). Dit resulteerde in een reductie van het aantal geïnsemineerde vrouwtjes en faciliteerde de overdracht van schimmelsporen naar gezonde, vrouwelijke soortgenoten tijdens het paren (Hoofdstuk 8). Tenslotte, na het opnemen van een bloedmaaltijd, bereiden vrouwtjes zich voor om eieren te leggen. Infectie met *M. anisopliae* verminderde de neiging om eitjes te leggen van vrouwelijke *An. gambiae* muggen, alhoewel het aantal eieren gelijk bleef (Hoofdstuk 7).

Deze bevindingen tonen aan dat de entomopathogene schimmel *M. anisopliae* belangrijke gedragsaspecten van *An. gambiae* verandert. Dit is mogelijk omdat de schimmel het vliegvermogen van muggen sterk beïnvloedt wat er voor zorgt dat de insecten minder in staat zijn om te vliegen, gastheren te zoeken, suiker op te nemen, te paren en eieren te leggen. De grote sterfte die in de eerste dagen van infectie vóór het uitvoeren van de gedragstoetsen wordt waargenomen, de sterfte die wordt waargenomen terwijl de gedragstoetsen worden uitgevoerd en de vermindering van de gedragsrespons van muggen geïnfecteerd met *M. anisopliae* hebben allemaal een significante impact op het onderdrukken van vectorpopulaties. De gevoeligheid van mannetjesmuggen voor schimmelsporen baant de weg voor een nieuwe strategie om muggen te bestrijden. Dit proefschrift heeft aangetoond dat entomopathogene schimmels een goede toevoeging zijn aan andere muggenbestrijdingsmethoden voor de vermindering van muggenbeten, en de overdracht van malaria en andere door muggen overgedragen ziekten.

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Every great success is a joy to the family. Mum and dad, you always emphasised that "education is the key to success" and that "Harry has no blessings but patience pays". I have achieved what you always believed I will so long as I stayed focused. Sadly, you did not live long to witness this-Rest in peace!. My brothers Nathaniel, Nicholas, Shem, Donald, Stanley, Hosea and Micah, sisters Abigael, Mary and Keren, nephews David and Dennis and nieces Mercy and Faith, you have been my pillar. Your love, support, encouragement is enough to make the impossible possible.

Curriculum Vitae

Sopher N. Ondiaka was born on 18th January, 1975 in Kakamega, Kenya. She completed her ordinary secondary education at Kaimosi Girls High School, Kakamega Kenya and went on to the University of Nairobi to study biological sciences. She graduated with a Bachelor of Science Degree in Zoology and Botany in 1999. She then worked as a Research Assistant attached to Professor Canute Khamala and Dr. Gideon Nyamasyo at the same University, Department of Zoology, from 1999-2002. With funding from the European Union, she studied tropical rainforest ecology and conservation in Tanzania (2001) and invertebrate species diversity and conservation in South Africa (2003). In 2002, she was granted the opportunity to pursue a Master of Science Degree in Agricultural Entomology at the same University, Department of Zoology, through a scholarship from The Rockefeller Foundation. Her MSc thesis research focused on "Pathogenicity of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* on sweetpotato weevils, *Cylas puncticollis*". In 2006, she worked on the biological control of stored product pests under Dr. Moshe Kostyukovsky at the Agricultural Research Organization, Department of Food Science, in Israel on a MASHAV-fellowship. In 2007, she was awarded a sandwich PhD fellowship from Wageningen University to pursue a PhD study at the Laboratory of Entomology at this university. The study was part of an international project on the development of entomopathogenic fungi for malaria vector control in Africa (FMCA). The PhD research project focused on "behavioural effects of fungal infection by *Metarhizium anisopliae* in adult malaria mosquitoes". The study was carried out at the Laboratory of Entomology in Wageningen and at the International Centre of Insect Physiology and Ecology (*icipe*), Kenya. The results of this PhD-study are presented in this dissertation submitted to Wageningen University in 2012. In the future, Sopher is eager to continue working on entomopathogenic fungi and contribute to their development as effective biocontrol agents against insect vectors and pests. Starting in June 2012, Sopher has been appointed as a post doctoral fellow in Integrated Plant Protection at the Swedish University of Agricultural Sciences (SLU), Division of Plant Protection Biology under Professor Birgitta Rämert.

List of Publications

Ondiaka S., Bukhari T., Farenhorst M., Takken W., Knols B. G. J. 2008. Effects of fungal infection on the host-seeking behaviour and fecundity of the malaria mosquito *Anopheles gambiae* Giles. *Proceedings of the Netherlands Entomological Society Meeting* 19: 121-128

Ondiaka S., Maniania N. K., Nyamasyo G. H. N., Nderitu J. H. 2008. Virulence of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* to Sweet potato weevil *Cylas puncticollis* and effects on fecundity and egg viability. *Annals of Applied Biology* 153: 41-48

To be submitted

Ondiaka S., Takken W., Koenraadt C. J. M., Mukabana W. R. Behavioural effects of entomopathogenic fungi on insects: A review.

Ondiaka S., Takken W., Koenraadt C. J. M., Maniania N. K., Mukabana W. R. Infecting *Anopheles gambiae* with *Metarhizium anisopliae* in the laboratory.

Ondiaka S., Takken W., Koenraadt C. J. M., Mukabana W. R. *Metarhizium anisopliae* affects the host-seeking response but not the olfactory discrimination of *Anopheles gambiae* mosquitoes.

Ondiaka S., Takken W., Koenraadt C. J. M., Masinde E. W., Mukabana W. R. *Metarhizium anisopliae* infection reduces house entry and human-biting rates of *Anopheles gambiae* mosquitoes

Ondiaka S., Takken W., Koenraadt C. J. M., Masinde E. W., Mukabana W. R. Effects of fungal infection on feeding and survival of *Anopheles gambiae* (Diptera: Culicidae) on plant sugars.

Ondiaka S., Takken W., Koenraadt C. J. M., Masinde E. W., Mukabana W. R. Effects of *Metarhizium anisopliae* on the mating performance of the malaria mosquito *Anopheles gambiae* and the probability of horizontal transmission.

PE&RC PhD Education Certificate

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

Review of literature (6 ECTS)

Behavioural effects of entomopathogenic fungi on insects

Writing of project proposal (4 ECTS)

Behavioural effects of fungal infection by *Metarhizium anisopliae* in adult malaria mosquitoes

Post-graduate courses (5.7 ECTS)

Information literacy, including Endnote; WGS (2007)
Advanced statistics; PE&RC (2008)
Medical mycology; CBS, Utrecht (2008)
Introduction to R for statistical analysis; ICIPE, Kenya (2010)

Laboratory training and working visits (3.6 ECTS)

Mosquito ecology, species identification and control; NIMR, Tanga, Tanzania (2009)

Deficiency, refresh, brush-up courses (4.5 ECTS)

Basic statistics (2007)
Analysis and prevention of health risks in the tropics (2008)

Competence strengthening / skills courses (3 ECTS)

Techniques for writing and presenting a scientific paper; WGS (2007)
Project and time management; WGS (2007)
PhD Competence assessment; WGS (2007)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.5 ECTS)

PE&RC Day (2007 and 2011)
PE&RC Weekend (2008)

Discussion groups / local seminars / other scientific meetings (6.6 ECTS)

Annual Meeting of the Netherlands Entomological Society, the Netherlands (2007)
PhD Students discussion group; Entomology, the Netherlands (2007-2011)
Local seminars and meetings on mosquito vector control, Kenya (2008-2011)

International symposia, workshops and conferences (6.3 ECTS)

Annual Conference of the Society for Invertebrate Pathology; oral presentation; UK (2008)
Annual Consortium Meeting for control of malaria vectors with entomopathogenic fungi; oral presentation each year, the Netherlands (2008-2009)

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Photo credits: Mating mosquitoes in bottom left of cover - Yanis Thailayil and fungus-infected mosquito at the centre of cover.

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