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Ernst-Jan Scholte

The entomopathogenic fungus *Metarhizium anisopliae* for mosquito control

Impact on the adult stage of the African malaria vector *Anopheles gambiae* and filariasis vector *Culex quinquefasciatus*

Proefschrift

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Stellingen

1. Ondanks aanvankelijke scepsis kan de insect-pathogene schimmel *Metarhizium anisopliae* worden ingezet bij bestrijding van malaria muggen (dit proefschrift).

2. De ontdekking van *Bacillus thuringiensis israelensis* in 1976 heeft het onderzoek naar, en ontwikkeling van, entomopathogene schimmels voor bestrijding van muggen sterk afgeremd (dit proefschrift).

3. Biologische bestrijding van plagen is veiliger voor de volksgezondheid dan chemische bestrijding (Francis G. Howarth in *Ann. Rev. Entomol.* 1991, 36: 485-509).

4. In etymologische zin kunnen muggen ook worden opgevat als amfibieën.

5. Het feit dat sommige Afrikanen zich grappend afvragen of blanken wel benen hebben geeft aan dat de meeste blanken zich tijdens een bezoek aan Afrika te weinig uit hun auto begeven om zich onder de plaatselijke bevolking te mengen.

6. Het is bizar dat het land met het grootste wapenarsenaal, een land dat in de afgelopen 100 jaar bij 15 oorlogen betrokken is geweest zonder dat het land zelf ooit werd binnengevallen, zich opwerpt als beschermengel van de wereldvrede en democratie.

7. Als de vissers op de Kilombero-rivier visnetten gebruiken die net zulke grote gaten bevatten als de meeste klamboes in dat gebied, zullen ze weinig vis vangen.

8. Kunst die mensen beweegt voldoet niet aan de wet van entropie.

Stellingen behorend bij het proefschrift van: Ernst-Jan Scholte

The entomopathogenic fungus *Metarhizium anisopliae* for mosquito control
-- Impact on the adult stage of the African malaria vector *Anopheles gambiae* and filariasis vector *Culex quinquefasciatus* --

Wageningen, 30 November 2004

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Vampire bugs

Out from the light, the inside is dark
Out in the field, not far from the park
Light beams are dancing, cover all cracks
That's where they're hiding behind our backs

Vampire bugs engorged with warm-served soup
Vampire bugs frightened of our tubes
They bite, they hide
Waiting silently for the night

Smoke tears my eyes, my hope sinks deep
The holes in the nets would make a fisherman weep
Cobwebs entangle my unwashed hair
Tiny angles on walls, people beware

You don't notice your liver is not having fun
You get invaded, overthrown without gun
Gets you down to the bottom line
Out of order for indefinite time

Aan mijn ouders

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

This thesis concerns biological control of African mosquito vectors with an insect-pathogenic fungus. Before addressing the central research question and the objectives, an overview is given of two important African mosquito-borne diseases: malaria and lymphatic filariasis. This is followed by a description of the biology of their vectors, the available methods for mosquito control, including an overview of biological control and the part therein of entomopathogenic fungi.

VECTOR-BORNE DISEASES

Vector-borne diseases are diseases of which the pathogens are transmitted by animals. A large number of human infectious diseases is being transmitted by arthropods, among which onchocerciasis (vectored by black flies), leishmaniasis (sand flies), African trypanosomiasis (tsetse flies), American trypanosomiasis (triatomine bugs), plague (fleas), and Lyme disease (ticks). The largest group of vector-borne diseases is transmitted by mosquitoes, including yellow fever (*Aedes* spp.), dengue(-hemorrhagic) fever (*Aedes* spp.), West Nile disease (*Culex* spp.), Japanese encephalites (*Culex* spp.), Rift Valley virus, as well as malaria (*Anopheles* spp.) and filariasis (*Anopheles* & *Culex* spp.) (Gratz, 1999; Kettle, 1995; Durden & Mullen, 2002). The latter is considered one of the fastest spreading insect-borne diseases of man in the tropical world (WHO, 1992), and malaria the single most important mosquito-borne disease in the world (WHO, 1994; Collins & Paskewitz, 1995; WHO, 2000).

MALARIA

Over 2 billion people (nearly 40% of the world's population) live in malaria-endemic parts of the world, notably Asia, Latin America and Africa, in 100 countries or territories (Collins & Paskewitz, 1995; Kettle, 1995; WHO, 2000; Foster & Walker, 2002). Up to 500 million new clinical cases of the disease are reported annually, causing high morbidity and mortality rates (Gratz, 1999). It is estimated that malaria is responsible for over one million deaths annually, of which 90 % occurs in sub-Saharan Africa (Burkot & Graves, 2000; WHO, 1994; WHO, 2000; Breman, 2001). The disease has been identified as a key contributor to weak economic growth and investment in Africa because it experiences the most intense malaria transmission in the world (Hay *et al.*, 2000). Malaria is resurfacing in areas where it had previously been eradicated and is spreading due to ecological and social changes such as deforestation, global warming, human population migrations, urbanisation and industrialisation (Knudsen *et al.*, 1997; WHO, 2000).

Malaria is caused by protozoan parasites of the genus *Plasmodium*. Of the four existing human malaria species, three belong to the subgenus *Plasmodium*: *Plasmodium vivax*, *P. malariae* and *P. ovale*, whereas the fourth, *P. falciparum*, is in the subgenus *Laverania* (Burkot & Graves, 2000). *P. malaria* and *P.ovale* infections cause relatively little morbidity and hardly any mortality. *P. vivax* infections are more severe but usually not lethal, whereas infection with *P.falciparum* is always life-threatening in non-immune individuals (Collins & Paskewitz, 1995). The latter species is the most prevalent malaria parasite found in sub-Saharan Africa, killing mainly children under the age of 5 and pregnant women (WHO, 2000). More detailed information and references about the four human malaria-parasites,

clinical aspects and epidemiology can be found in Kettle (1995), Burkot & Graves (2000), and Wernsdorfer & McGregor (1988).

All human malaria vectors belong to the genus *Anopheles* (Diptera: Culicidae). Of the 422 *Anopheles* species known, only 68 are recognised as malaria vectors, of which circa 40 are considered important (Service, 1993). Vectors of human malaria often occur as sibling species: closely related taxa that are normally indistinguishable morphologically, in a species complex. Members of the *Anopheles quadrimaculatus* complex were vectors of human malaria in North America. Members of the *An. albimanus* complex are vectors in Central and South America. *An. dirus*, *An. culicifacies*, *An. maculatus* and *An. minimus* complexes transmit human malaria in Asia. The *An. maculipennis* complex includes former vectors in Europe, and members of the *An. punctulatus* complex are responsible for transmission of malaria and filariasis in the south-west Pacific. In Africa, the *An. gambiae* complex and, to a lesser degree, the *An. funestus* complex are responsible for malaria transmission. *An. gambiae sensu lato* consists of seven sibling species (Hunt *et al.*, 1998). They differ in varying degrees from each other in aspects such as geographical distribution, larval habitat type, cytogenetics, vectorial capacity, host preference (highly anthropophilic, opportunistic anthropophilic/zoophilic or strictly zoophilic), biting behaviour (endophagic/exophagic or mixed), resting behaviour (endophilic/exophilic or mixed), circadian rhythms, breeding site selection (fresh/mineral or salt water), and longevity. However, many of these aspects overlap between the siblings. On the whole, concerning the Afrotropical zone, the species *An. gambiae sensu stricto* and *An. arabiensis* are regarded the most important vectors of human malaria, although this may vary in different regions. For detailed information on the life cycle of *Plasmodium* in the human host, see Garnham (1988).

FILARIASIS

The WHO (1992) estimated that in 1990 750 million people were living in areas endemic for lymphatic filariasis and Michael & Bundy (1997) estimated that about 146 million people are actually infected throughout the tropical and sub-tropical regions. Three species of the filariasis parasite cause human lymphatic filariasis: *Brugia malayi*, *Brugia timori*, and *Wuchereria bancrofti* (Kettle, 1995). Of these species, *W. bancrofti* is the most widely distributed, and its infection the most prevalent (Lok *et al.*, 2000).

The lymphatic filariasis parasites are helminths, belonging to the phylum Nematoda, and are often referred to as filarial worms. The most widespread cause of lymphatic filariasis is the nocturnal periodic form of *W. bancrofti* (Lok *et al.*, 2000). Globally, the main vector is *Culex quinquefasciatus* (Kettle, 1995), but in rural areas of Africa, *Anopheles* spp. are also considered important vectors (Kettle, 1995; Bøgh *et al.*, 1998; Mboera, 1999; Lok *et al.*, 2000). Several studies in rural Tanzania have shown that besides *Cx. quinquefasciatus*, especially *Anopheles gambiae*, *An. arabiensis* and *An. funestus* are important vectors (White, 1971; Mboera, 1999; Snow & Michael, 2003). For more information and references on the lifecycle of the parasites, see Kettle (1995) and Foster & Walker (2002).

BIOLOGY OF AFRICAN VECTORS OF MALARIA AND FILARIASIS

Anopheles gambiae

Anopheles gambiae Giles *s.l.* lays single eggs on the water surface or on wet mud at the edge of shallow pools (Lyimo, 1993). *Anopheles* eggs distinguish themselves from other mosquito genera by the presence of floats (Burkot and Graves, 2000). Unlike eggs from the genus *Aedes*, eggs of *Anopheles* spp. cannot withstand dessication. One or two days after oviposition, the eggs hatch into the first larval stage.

The larvae are filter feeders, feeding on organic matter suspended in water. Depending on food availability, larval density and temperature, the development through the four larval stages, including the pupal stage, takes about 7-20 days (Clements, 1992; Service, 1977; Schneider *et al.*, 2000; Koenraadt, 2003). During the pupal stage they do not feed. Larval populations generally suffer mortalities up to 93% (Service, 1977), due to both drying out of larval habitats and factors such as predation, parasitization and diseases (Lyimo, 1993). They colonise a variety of transient, mainly sunlit, rainwater pools, like borrow-pits, drains, cartracks, foot and hoofprints around ponds and water holes (Van Someren *et al.*, 1955; Coene, 1993; Lyimo, 1993; Koenraadt, 2003). Most of these larval habitats are temporary, and in some areas breeding is highly seasonal and follows rain patterns of that specific area. Gillies & Coetzee (1987) mention that in the dry season the breeding sites seem to shift to more permanent habitats like wells, edges of permanent swamps or drying riverbeds. After emerging from the pupal stage, both males and females rest for a short time, after which males and a large proportion of females go in search of plant-sugars (Foster, 1995; Gary and Foster, 2004). Around the time of sunset and sunrise, male adults aggregate to form swarms. Females, receptive to mating cues, fly into these swarms and copulate (Charlwood & Jones, 1980).

Studies on the biting cycle of *An. gambiae s.s.* and *An. arabiensis* in Nigeria (Molineaux and Gramiccia, 1980), Ethiopia (White, 1974), and Kenya (Chandler *et al.*, 1975), showed that females were active around the eaves of houses early in the night, although the actual biting activity was later and started about 3 hours after sunset, slowly increasing to reach a peak around 05.00 hrs in the morning, after which a fast decrease of biting activity was observed. Gillies (1958), however, found very little activity up to 21.00-22.00 hrs, followed by an increase, to a feeding peak activity between midnight and 04.00 hours, after which the activity decreased to zero shortly before dawn.

An. gambiae s.s. is considered largely endophagic (Gillies, 1988), taking bloodmeals mostly indoors, whereas *An. arabiensis* Patton shows a greater tendency to feed outdoors (Gillies, 1988), although also within *An. arabiensis* populations there are different levels of endophagy (Coluzzi, 1979). In situations where man is by far the most available host, the Human Biting Index (HBI: the proportion of freshly found *Anopheles* to contain human blood), in *An. gambiae s.s.* can reach as high as 100% (White, 1974). Even where a wider host choice is available, the endophilic behaviour of *An. gambiae s.s.* tends to keep the HBI around 80-90%. If besides humans, cattle are present, a high proportion of *An. arabiensis* bloodmeals may be taken on animals, resulting in a relatively lower HBI, whereas *An. gambiae s.s.* will still prefer to feed on humans. *An. gambiae s.s.* is therefore considered anthropophilic, and *An. arabiensis* opportunistic/zoophilic (White, 1974).

After feeding, females rest to digest the blood meal. *An. gambiae s.s.* usually rests indoors (Lines *et al.*, 1986; Gillies, 1955; 1988; Bøgh *et al.*, 1998), whereas those *An. arabiensis* that fed indoors usually leave human dwellings to rest outdoors (Lines *et al.*, 1986; Smits *et al.*, 1995; 1996; Mnzava *et al.*, 1995). Gillies (1955) estimated that only 6.3-7% of all bloodfed females left the house during the course of the night but that about 50% of *An. gambiae* would leave the shelter of houses by the second day after a blood meal. There are strong indications however, that the use of residual insecticides shifted feeding and resting behaviour from endophagic and endophilic to somewhat more exophagic and exophilic tendencies, both for *An. gambiae* and *Cx. quinquefasciatus* (Mnzava *et al.*, 1995; Magesa *et al.*, 1991; Bøgh *et al.*, 1998). When the eggs are fully developed, females will search for a suitable breeding site to oviposit. After oviposition, two-thirds or more of the females feed again that same night, or even "nearly all", according to Molineaux and Gramiccia (1980) and Gillies (1955). The first gonotrophic cycle lasts 3-4 days, followed by gonotrophic cycles of 2-3 days (Gillies & Coetzee, 1987; Gillies, 1955).

When taking a blood meal from a malaria infectious human host, gametocytes are ingested which, in the midgut, will exflagellate and form micro and macrogametes. Fertilized macrogametes turn into motile ookinetes (zygotes), which can pass the midgut epithelium of the mosquito to form an oocyst. Mature oocysts will burst to release sporozoites, which will migrate through the haemolymph to the salivary glands, and in turn can infect a new host. Depending on the temperature this process takes approximately 10-15 days for *P. falciparum*. For a detailed description and references on *Plasmodium* life cycles see Garnham (1988). The fact that this process of sporogony takes 10-15 days makes longevity of the mosquito an important factor regarding vectorial capacity. This has long been recognized, but MacDonald (1957) was the first to give a mathematical equation describing the sporozoite rate in the formula $s = (p^n ax) / (ax - \log_e p)$ where p= probability of survival through one day; n= duration in days of the extrinsic cycle of the parasite in the mosquito; a= average number of blood meals taken on man in 1 day; and x= proportion of bites infective to man. This equation shows that small changes in daily survival rate can have an important effect on vector efficiency. This demonstration has been a major factor in estimating the effects on transmission of residual spraying campaigns and stresses the importance of reducing adult female life span in any vector control programme aimed at adult anophelines, including ITNs. On the whole, estimates of mosquito life spans vary between 1-5 weeks, with daily survival rates between 0.75-0.94 (White, 1974), with the rate of increase in mortality declining at advanced ages (Clements & Patterson, 1981). A value for daily survival rate of *An. gambiae* that is often referred to is one estimated by Gillies (1961) of 0.84. About 65% of mosquitoes caught indoors are in their first week, 27% in their 2nd, 8% in their 3rd, 0.9% in their 4th and 0.3 and 0.02% in their 5th and 6th week, respectively (Gillies and Wilkes, 1966).

Culex quinquefasciatus

Cx. quinquefasciatus is a member of the *Culex pipiens* complex (Service, 1986; 1993). The species is widely distributed in tropical and subtropical areas, and is the third most commonly distributed mosquito in the world (Mboera, 1999). In East Africa the species is abundant both in urban areas as well as in rural areas (Subra, 1981; Beier *et al.*, 1990; Mboera, 1999).

Eggs from the genus *Culex* are positioned upright, packed together in floating rafts. The average larval and pupal development times depend on temperature and takes between 6 and 9 days in the tropics (Subra, 1981; Service, 1986). In contrast to larvae of anophelines, which are predominantly found in clean, non-polluted water, larvae of *Cx. quinquefasciatus* are usually found in habitats containing highly polluted water rich in organic matter (Subra, 1981; Coene, 1993; Beehler & Mulla, 1995; Mboera, 1999). Larvae can develop in virtually all types of aquatic habitats, most of which are closely linked to human environments, like latrines, soakage pits and septic tanks. In the absence of polluted water, the species can utilize wells, small containers, gutters, ditches, ponds and remains of empty tins, bottles, broken metal cans and old tyres filled with rain water. Mating occurs 36-72 hrs after emergence (Subra, 1981). Usually, females are fertilized before, but occasionally after the first blood meal. The feeding preference of *Cx. quinquefasciatus* is mixed anthropophilic and zoophilic (White, 1971; Service, 1986; Subra, 1981), with large variations between regions (Subra, 1970; Service, 1986). The antrophophilic females are both endophagic as exophagic and rest indoors or outdoors (Subra, 1981). In East Africa *Cx. quinquefasciatus* seems to be mainly endophagic (Van Someren, 1958). In a study on mosquito longevity, Clements and Patterson used data from Samarawickrema (1967), who found that more than 2% of the females completed 3 gonotrophic cycles, surviving some 14 days, and smaller percentages survived 4 cycles. Mean longevity of females at 30 °C is 30.1 days, and 64.4 days at 25 °C with a maximum of over three months (Oda *et al.*, 2002).

VECTOR CONTROL

Vector control is an important strategy as a means to control mosquito-borne diseases (Hougard *et al.*, 2002; Trape *et al.*, 2002; Killeen *et al.*, 2002; 2003). The principle objective of vector control is the reduction of morbidity and mortality by reducing the levels of transmission. The approach to achieve this is to reduce and maintain vector densities (both adult as larval populations) at a sufficiently low level, to decrease mosquito longevity, and lessen their anthropophilic tendencies (Knudsen *et al.*, 1997). Table 1 lists five groups of techniques that are used in vector control. For the last half century, vector control in Africa depended almost exclusively on the use of insecticides such as indoor residual spraying (Curtis, 1994; Roberts *et al.*, 2000), insecticide-treated bednets and other materials (Lengeler, 2001; Macintyre *et al.*, 2003). The use of these compounds are of enormous value regarding public health, killing large numbers of mosquitoes and thereby saving thousands of lives each year. However, opposition against chemical use is growing (Hornsby *et al.*, 1996; D'Allessandro & Coosemans, 1997; WWF, 1999; Callaghan *et al.*, 2001; PAN, 2002), and resistance of mosquitoes against these compounds remains an issue of concern (Enserink, 2002; Hemingway *et al.*, 2002; Ranson *et al.*, 2000). Unfortunately, the array of existing vector control techniques and agents that do not depend on synthetic insecticides is limited. The development of new techniques, among which biological control, is encouraged (WHO, 2000), but despite their potential (Guillet *et al.*, 1990; Fillinger *et al.*, 2003; Scholte *et al.*, 2004) they are not considered a key point for current research direction by the major malaria research institutions (Hougard *et al.*, 2002).

BIOLOGICAL CONTROL OF MOSQUITOES: PREDATORS AND PATHOGENS

Biological control is the use of natural enemies for the control of pests (Garcia and Legner, 1999). Both classical as well as inundative and inoculative biological control strategies have been successful in controlling invertebrate pests in diverse environmental settings, including scale insects, white fly, thrips, and mosquitoes (Wawrzynski *et al.*, 2001; Hajek *et al.*, 2003; Loonmans, 2003).

Fish

Fish are the most commonly used predators for larval mosquito control. Various species are used, such as *Cyprinus carpio* (Bellini *et al.*, 1994; Legner, 1995), *Orthodon microlepidotus* (Cech & Linden, 1987), *Lairdina hopletupus* (Laird, 1977), *Aplocheilus latipes* (Laird, 1977), and *Ctenopharygodon idella* (Weiser, 1991; Legner, 1995). The best known, however, are the mosquito fish *Gambusia affinis* and the common guppy, *Poecilia reticulata* (Chapman, 1974; Service, 1983; Lacey & Lacey, 1990; WHO, 1992; WHO, 2000). Both fish are viviparous, small, and have high reproduction rates. The mosquito fish is the most widely disseminated biological control agent of mosquitoes in the world. It has been used successfully in Iran where it was claimed to have played an important role in malaria eradication, as well as in southeastern Turkey where a 50% reduction in malaria cases was observed after *G. affinis* became established (Legner, 1995). This fish has been used extensively for mosquito control in rice cultivation areas in California against *Culex* and *Anopheles* spp. (Kramer *et al.*, 1987). In India they are commonly put in wells in order to control the malaria vector *An. stephensi*, and in Afghanistan they were integrated into antimalaria campaigns. In Italy they are commonly used to control *Cx. pipiens* in the Po-delta (Bellini *et al.*, 1994). The common guppy, *P. reticulata*, is being more widely applied in Asia, where it has been successfully used for the control of wastewater mosquitoes, especially *Cx. quinquefasciatus* (Sunahara *et al.*, 1998; Lardeux *et al.*, 2002). In Sri Lanka, wild fish

populations have been caught and used for the control of mosquitoes in abandoned wells, coconut husks, and other sources rich in organic debris (Sabatinelli *et al.*, 1990). The fish occurs in India, Indonesia and China, has been introduced into Burma for filariasis control, and has shown potential to control *An. gambiae* breeding in cisterns in the Comoros islands (Sabatinelli *et al.*, 1990). In China, the common carp, *Cyprinus carpio*, has been used for both mosquito control and as a human protein source (Weiser, 1991; Legner, 1995; Wang *et al.*, 2000).

A drawback of using *Gambusia* is that the fish also preys on various other (native) fish and invertebrates which may result in algal blooms, causing fish to die and allowing mosquitoes to increase in numbers (Service, 1983). Subra (1981) mentions that several centuries ago the Arabs, who had founded towns and villages along the east coast of Africa, introduced larvivorous fish in tanks ("birikas") where the water was kept for ritual ablutions and where *Cx. quinquefasciatus* develops. Regarding *An. gambiae*: hardly any fish survive in temporary habitats that periodically dry out. This severely limits their usefulness for control of this malaria vector in sub-Saharan Africa. Besides this inability to survive drought, fish are also unable to adapt themselves to highly polluted waters, which are the most productive breeding places of *Cx. quinquefasciatus* (Subra, 1981). These drawbacks drastically limit the use of fish for the control of malaria and filariasis vectors in Africa. There is at least one exception though; populations of *An. arabiensis*, a local vector of malaria in northern Somalia, were much reduced by introducing a local tilapine fish-species of the genus *Oreochromis* into water catchment basins (Alio *et al.*, 1985).

Predaceous Arthropods

Some aquatic Coleoptera, e.g. Dytiscidae, have been shown to prey on mosquito larvae (Bay, 1974). Immature dragonflies are also known to feed on larval mosquitoes, although they are not as well suited for predation on mosquito larvae as other species such as several notonectid Hemiptera (Bay, 1974). Regarding adult mosquito predators, some spiders (Araneae) have been studied (Dabrowdka-Prot *et al.*, 1968; Wesolowska & Jackson, 2003). Also several crustaceans feed on mosquito larvae: In southern California the tadpole shrimp (*Triops longicaudatus*) feeds on *Aedes* and *Psorophora* species, and the cyclopod *Mesocyclops aspericornis* feeds extensively on *Ae. aegypti* and *Ae. polynesiensis* (Legner, 1995). However, none of these arthropods were ever valued highly as biological control agents. Mosquitoes belonging to the genus *Toxorhynchites* (Diptera: Culicidae) can kill large numbers of *Aedes* and *Ochlerotatus* larvae. Several field experiments with *Toxorhynchites* have had small-scale successes (Focks *et al.*, 1979), for example the control of *Ae. aegypti* on St. Maarten (Gerberg & Visser, 1978), and *Ae. albopictus* and *Cx. quinquefasciatus* on a Japanese island (Myagi *et al.*, 1992), but in general their potential for biological control of mosquitoes on a larger scale is considered low (Service, 1983; Focks *et al.*, 1979; Laird, 1977; Muspratt, 1951). The main reasons are that their spatial and temporal distributions do not overlap well with the life-cycle durations of their prey. Also, these mosquitoes have a low fecundity, its eggs cannot withstand desiccation, and the species disperses relatively slowly (Service, 1983; Focks, 1979). Since they are known to prey on larvae of mosquitoes breeding in container habitats, such as *Ae. aegypti*, *Ae. albopictus* and *Ae. polynesiensis*, they can hardly be considered useful for biological control of African anophelines.

Table 1. Overview of mosquito vector control techniques.

Method	Brief description
Environmental management *	Modification of the environment to deprive the target populations of its requirements for survival, emphasis laid on source (breeding site) reduction (e.g. periodic drying of rice-fields, periodic flushing in streams, changing salinity of breeding habitats, floating layers of polystyrene beads or oils on water-surface in breeding places, removal of potential breeding sites, improved housing). Also included are repellents based on odors to reduce the risk of mosquito-human contact
Insecticides	
adults: Indoor residual spraying *	Indoor house spraying using insecticides (e.g. DDT or synthetic pyrethroids).
adults: Space-spraying *	Outdoor spraying, used especially during epidemics in urban areas.
adults: Insecticide-treated materials *	Insecticide-treated bednets, curtains, hammocks, eave strips, papyrus mats and cloths are used to repel or kill mosquitoes.
larvae: Chemical Larvicides *	Synthetic chemicals applied to breeding sites for the control of larval populations, e.g. Temephos
larvae: Insect growth regulators *	Mimics of juvenile hormone that interrupt larval development in such a way that larvae do not survive, e.g. methoprene.
Genetic control	
Sterile Insect Technique*/**	e.g. sterile males: mass reared male mosquitoes are sterilized and mixed in wild populations to produce sterile eggs in the field (population control/elimination).
Transgenic mosquitoes **	Identification and insertion of genes into mosquitoes that code for refractoriness to infection with *Plasmodium* parasites, that move to fixation in the wild population (population replacement).
Odor-baited traps **	Semiochemicals (pheromones and kairomones) are used to attract and trap ovipositing females. Also, techniques are in development to use synthetic host odors serving as decoy for vectors to be placed around human dwellings.
Biological control */** (See Introduction)	predators, parasites and pathogens such *Bacillus thuringiensis israelensis*, *B. sphaericus* and entomopathogenic fungi applied in breeding sites to reduce the population density of vectors

* = in use ** = in development

Flatworms & Coelenterates

Turbellaria are nearly all free-living, non-parasitic worms, several species of which are known as predators of mosquitoes in freshwater habitats. *Mesostoma macroprostatum* offers good potential for the control of some *Aedes* spp.. The flatworm *Dugesia dorotocephala* is an effective predator of mosquito eggs, larvae and pupae, including those of *Cx. quinquefasciatus* and *An. quadrimaculatus* (Service, 1983; Legner, 1977, 1995; Melo & Andrade, 2001). Flatworms have been experimentally shown to devastate mosquito cultures, killing far more larvae than they consume. They are relatively tolerant to varying water qualities, and can be mass-produced. Since the habitats in which flatworms can be used against mosquitoes are mainly rice fields, they are not considered to have high potential as biological control agents against mosquitoes in Africa (Legner, 1977).

Some coelenterates showed promise for consideration and use in selected breeding habitats. *Chlorohydra viridissima* (Pallas) and *Hydra americana* are efficient in suppressing culicine larvae in ponds with dense vegetation (Qureshi & Bay, 1969; Chapman, 1974). However, even though the species can be mass-produced, work on these predators has declined, probably due to the discovery and subsequent popularity of *Bacillus thuringiensis* (Legner, 1995).

Nematodes

Several nematodes are virulent pathogens of mosquitoes (Becnel & Johnson, 1998; Paily & Palaraman, 2000). The best-known nematode for biocontrol of mosquitoes is *Romanomermis culicivorax* (Kaya & Gaugler, 1993; Legner, 1995). It is the only mermithid that has ever been commercialized as a biocontrol agent (Fairfax® "Skeeter Doom"). *R. culicivorax* has been found on at least 16 mosquito species in the field, and over 80 species can be experimentally infected, of which the genus *Anopheles* is the most susceptible (Laird, 1977). Pre-parasites concentrate near the surface and thus have a high degree of contact with mosquito larvae (Service, 1983; Legner, 1995). *R. culicivorax* has the potential to recycle, although poorly and unpredictably. Aquatic fauna like beetles, dragonfly nymphs, ostracods and copepods appear to be predators of the pre- and post-parasitic stages (Service, 1983). Barriers to its use include intolerance to low levels of salinity, polluted water and low oxygen levels, predation by aquatic organisms, and the potential for development of resistance by the host (Brown *et al.*, 1977). *Strelkovimermis spiculatus* has demonstrated tolerance for high levels of organic pollution and was pathogenic to nine mosquito species, although *Cx. quinquefasciatus* showed considerable tolerance to invasion (Becnel & Johnson, 1998). However, from theoretical considerations and results from a large-scale field study in El Salvador (Laird, 1977; Molloy and Jamnback, 1977), it was deduced that mermithids are likely to cause only moderate reductions in mosquito vector populations (Service, 1983; Molloy and Jamnback, 1977).

Parasitic Protozoa

Many protozoa have been isolated from mosquitoes, including flagellates, eugregarines, ciliates, schizogregarines and microsporidia (Weiser, 1991; Legner, 1995; Garcia and Legner, 1999). Especially microsporidia are common protozoans of natural mosquito populations, and have been recorded from over 116 mosquito species (Service, 1983). Best known are *Vavraia culicis*, and *Nosema algerae*, parasites of culicines and anophelines, respectively. Three species of the genus *Nosema* have been reported from anopheline mosquitoes; *Nosema stegomyia, N. anophelis and N. algerae* (Anthony *et al.*, 1972). *Nosema*-infected *An. albimanus* have reduced fecundity and usually do not live long enough to transmit malaria (Anthony *et al.*, 1972). This result was confirmed by Undeen & Alger (1975), who also showed that although nosematosis had little effect on survival of

larvae and pupae, the adult life span of *An. stephensi* was reduced to an extent that malaria transmission would be impaired. *V. culicis* has been introduced as a biocontrol agent against *Cx. quinquefasciatus* on the Pacific Island Nauru in 1967, but the infection rates were very low and the mosquito population appeared unaffected (Reynolds, 1972). In Pakistan, *N. algerae* was introduced as biocontrol agent of anophelines, but, even at infection rates of 40-50%, no lasting control was achieved (Laird, 1977). The endoparasitic ciliate, *Lambornella clarki*, a natural pathogen of tree hole mosquito *Ae. sierrensis* is being studied for its potential to control container breeding mosquitoes (Washburn, 1986; Legner, 1995). Microsporidia of the genus *Amblyospora* have a complex life cycle, including multiple hosts (copepods as well as mosquitolarvae), horizontal transmission and several different types of spores (Becnel & Andreadis, 1998; Sweeney *et al.*, 1990). Besides horizontal transmission, *Edhazardia aedis* involves also transovarial transmission (Johnson *et al.*, 1997). Problems associated with the use of microsporidia to control mosquito larvae include sedimentation of spores, low toleration of ultraviolet radiation, and their relatively low impact on mosquito population densities (Undeen & Alger, 1975). Also, their complex lifecycle and difficulties in mass production do not offer much hope to use them for biological control of mosquito larvae (Service, 1983; Legner, 1995).

Viruses

There are three main groups of viruses that kill mosquitoes, namely Iridescent viruses, Nuclear Polyhedrosis Viruses (NPV) and Cytoplasmic Polyhedrosis Viruses (CPV). Recent developments in research on viruses regarding mosquitoes and vector control are slowly changing the general view of the past, when the potential of viruses for mosquito vector control was not considered high (Lacey & Undeen, 1986; Chapman, 1974). Female *Ae. aegypti* mosquitoes, infected with a covert (sublethal) viral infection of Invertebrate Iridescent Virus 6 (IIV-6) exhibit reduction of adult longevity and a 50% reduction in reproduction (Marina *et al.*, 1999, 2003). High infection rates and accompanying mortality were achieved with the newly discovered baculovirus, CuniNPV (family *Baculoviridae*, genus *Nulclepolyhedrovirus*) in *Cx. pipiens* (83.0-14.4%), *Cx. pipiens molestus* (80.4% infection), and *Cx. salinarius* (48.0-43.1%) (Andreadis *et al.*, 2003). Infection levels were only high when the water contained high levels of magnesium. *Cx. restuans* was also susceptible but infection rates were lower (21.3-12.5%). No infections were obtained with any species of *Aedes, Culiseta* or *Ochlerotatus*. Although much basic research still needs to be done, and the use of viruses to control anophelines remains unclear, some viruses are considered for control of culicines (Becnel *et al.*, 2001; Marina *et al.*, 2003).

Bacteria

Probably the most successful biological control agents for mosquito larval control are the spore forming bacteria *Bacillus thuringiensis israelensis*, sero-type H-14 (*B.t.i.*) and *B. sphaericus* 1593 (Laird, 1977; Service, 1983; WHO, 1995; 1999; Becker & Ascher, 1998; Fillinger *et al.*, 2003). They are commercially available from several companies. The strains of *B. thuringiensis* have a broad spectrum of activity against larvae of many species of Lepidoptera, Coleoptera and aquatic Diptera (Weiser, 1991; Legner, 1995; Garcia and Legner, 1999). One spore-forming strain was isolated by Goldberg and Margalit in 1977, of which the toxin proved to be highly effective as microbial insecticide for mosquitoes and blackflies. The bacteria form spores, each containing a proteinaceous inclusion called a crystal, which is the source of the toxins that cause most larval mortality. Upon ingestion by a larva, the crystal is dissolved in the midgut, releasing δ-endotoxins that are highly specific and lethal to mosquito larvae (WHO, 1992; WHO, 1995). These proteins are protoxins that must be activated by midgut proteases before they can interact with the gut epithelium and

disrupt its integrity. Soon after, the insect dies (Gullan & Cranston, 1994). Among the genera affected by *B.t.i* are *Anopheles*, *Aedes*, *Culex*, *Culiseta*, *Mansonia*, *Coquillettidi*, *Ochlerotatus*, *Psorophora*, and *Uranotaenia*. Also in Africa the use of *B.t.i.* as mosquito larvicide is being studied, for example in Kenya (Fillinger et al., 2003), Nigeria (Obeta, 1998), Liberia (Bolay *et al.*, 1990), and Ethiopia, where both *B.t.i.* and *B. sphaericus* were tested for the control of *An. arabiensis* (Seyoum & Abate, 1997). Although not impossible, and large-scale field studies are yet to be carried out, the use of *Bacillus* against malaria vectors in Africa may prove to be difficult in terms of locating all breeding sites to cover a certain area. A field study in Kenya by Logan and Linthicum (1992) in which the impact of *B.t.i.* on riverine-associated floodwater mosquito populations was studied proved unsuccessful. This was probably due to the high turbidity in the water, which increased the settling rate of the *Bacillus*-product. This sinking of the product is a general drawback with applying these products to control anophelines as these larvae feed predominantly at the water surface and the products remain out of reach. The WHO (1992) claims that *B. sphaericus* is the most promising biocontrol candidate to control filariasis vectors. In general the mosquito genera *Culex* and *Psorophora* are highly susceptible to *B. sphaericus*, followed by *Mansonia*, *Anopheles* and *Aedes* species (WHO, 1992). Especially the susceptibility of *Culex* larvae appears to be high, resulting in efficacy of the bacterium, which can compete with the currently used synthetic chemical larvicides.

A matter of concern, however, are reports of resistance to these bacterial products, observed in mosquito populations both in the laboratory as in the field. *Cx. quinquefasciatus* has developed resistance to *B. sphaericus* (Wirth *et al.*, 2000; Yuan *et al.*, 2000; 2003; Mulla *et al.*, 2003), and, although at very low levels, *Cx. pipiens* is reported to have developed resistance to *B.t.i.* (Saleh *et al.*, 2003).

Entomopathogenic fungi

There are thought to be over 100,000 species of fungi of which about 750 have been identified to be pathogenic to insects (Ferron, 1978; Boucias & Pendland, 1991; Glare & Milner, 1991; Khachatourians, 1991; Hajek & St. Leger, 1994; Moore-Landecker, 1996; Bidochka *et al.*, 2000), belonging to some 90 genera (Khachatourians, 1991). Entomopathogenic fungi are those fungi that cause the premature death of an insect host (Glare & Milner, 1991). Fungal taxonomy existed before the knowledge of insect diseases, and the species of fungi found on insects have been fitted into the existing classification framework. Traditionally, the major groups of fungi and the relationships between them have been based on comparative morphology and the developmental patterns of the sexual reproductive structures (Glare & Milner, 1991; Deacon, 1997). The latter characteristic creates difficulties in classification because many entomopathogenic fungi belong to the Anamorphici, which have lost the ability to produce or rarely produce sexual spores. This group of fungi was traditionally placed in the former division, Deuteromycota, within the artificial class of Hyphomycetes (Inglis *et al.*, 2001; Burnett, 2003). The concept of "biological species" is therefore difficult to apply, and these fungi are referred to as 'morphological species' instead (Burnett, 2003). The members of this artificial class Hyphomycetes, which includes *Metarhizium anisopliae*, are characterized by mycelial forms that bear asexual spores, termed 'conidia', produced by specialized conidiogenous cells. For entomopathogenic Hyphomycetes, strains/isolates or pathotypes are often more important units of organisms than species. Isolates/strains may be host and/or habit-specific or may be identified by geographical origin (Bidochka *et al.*, 2000). However, entomopathogenic fungi are basically still classified on morphological similarities, although more and more of the classification is being reassessed using molecular techniques (Deacon, 1997; Driver *et al.*, 2000). This strategy sometimes results in reclassification of certain species or groups (Kirk *et*

al., 2001). The taxonomic names of the major groups used in this thesis are based primarily on the names adopted by Alexopoulos *et al.* (1996). The 750 entomopathogenic known species are distributed over several different taxa, including two kingdoms, 5 divisions and a form-division. The kingdom Chromista contains one division with entomopathogenic species: Oomycota, which contains two genera with species that are strongly associated with mosquitoes: *Leptolegnia* and *Lagenidium*. The kingdom Fungi contains the remainder of 4 divisions; Ascomycota, Basidiomycota, Zygomycota, Chytridiomycota, and the form-division Anamorphici/Deuteromycota.

Fungal diseases in insects are common and widespread and epizootics can decimate their populations. Several species have been used, with mixed successes, in insect control (Table 2) (Federici, 1981; Ferron *et al.*, 1991; Glare & Milner, 1991; Legner, 1995; Bidochka *et al.*, 2000; Hajek & St. Leger, 1994; Hajek *et al.*, 2003). Virtually all insect orders are susceptible to fungal diseases, including Dipterans. Fungal pathogens such as *Lagenidium*, *Coelomomyces* and *Culicinomyces* are known to affect mosquito populations, and have been studied extensively. There are, however, many other fungi that infect and kill mosquitoes at the larval and/or adult stage, among which the Hyphomycetes *Beauveria bassiana* and *Metarhizium anisopliae*. A comprehensive review on entomopathogenic fungi related to mosquitoes is presented in Chapter 2 of this thesis. The life cycles and mechanisms involved in infections in each fungal group are also described in Chapter 2. As this thesis concerns the Hyphomycete *M. anisopliae*, a general outline for Hyphomycetes is given below.

Table 2. Examples of some of the entomopathogenic fungi that are commonly used or currently under development for insect pest biocontrol.

Fungus	Taxon	Target insects
Lagenidium gigantum	Oomycota/Pythiales	Mosquito larvae
Coelomomyces spp.	Chytridiomycota/Blastocladiales	Mosquito larvae
Entomophaga maimaiga	Zygomycota/Entomophtorales	Specific for gypsy moth larvae
Entomophaga grylli	Zygomycota/Entomophtorales	Specific for certain grasshopper families
Entomophaga muscae	Zygomycota/Entomophtorales	Specific for certain Diptera
Zoophtora radicans	Zygomycota/Entomophtorales	Aphids, caterpillars, psyllids, leafhoppers
Beauveria bassiana	Deuteromycetes	Many, including corn borers, beetles, thrips, aphids and grasshoppers.
Beauveria brongniartii	Deuteromycetes	Scarab grubs, cockchafers.
Metarhizium anisopliae	Deuteromycetes	Many, including grasshoppers, locusts, termites, cockroaches, weevils, fruit flies, and mosquitoes
Paecilomyces fumosoroseus	Deuteromycetes	Whitefly, thrips, spider mites.
Paecilomyces lilcanus	Deuteromycetes	Planthoppers.
Verticilium lecanii	Deuteromycetes	Whitefly, aphid and thrips.

LIFE CYCLE OF ENTOMOPATHOGENIC HYPHOMYCETES

In general, entomopathogenic fungi infect their hosts by penetrating the host cuticle. The early stages of fungal pathogenesis can be subdivided into conidial attachment, germination and appressorium production, and the invasion of the fungus through the cuticle into the haemocoel. Enzymes such as chitinases and proteases, as well as mechanical forces are involved. The fungus then spreads through the haemocoel. The insect normally dies 3-14 days after spore infection, after which the fungus starts mycelial growth to form spores/conidia (Gillespie & Claydon, 1989).

In some host-fungal pathogen systems, the specificity of attachment of the conidia to the host is established by parasite recognition of specific surface topography and cell wall ultrastructure of the hosts. This is determined through binding of complementary macromolecules on the surfaces of both host and pathogen. These macromolecules include proteins, glycoproteins and carbohydrates (Manocha & Chen, 1990). Conidia of fungi belonging to the Deuteromycota are about 5-10μm in size, and have a hydrophobic surface. The outer surface of a conidium consists of a resilient layer of well-organized interwoven fascicles of rodlets (Boucias & Pendland, 1991). The attachment of conidia of e.g. *Metarhizium anisopliae* to the insect's (cuticulin layer of the) epicuticle however, is passive and non-specific, mediated by strong binding forces (Boucias *et al.*, 1988; Boucias & Pendland, 1991). Besides the hydrophobic nature of the conidial wall, the surface topography and chemical properties of the insect epicuticle appear to mediate the adhesion process (Boucias *et al.*, 1988; Boucias & Pendland, 1991; Bidochka *et al.*, 2000). The conidia of *M. anisopliae* are capable of attaching to all body regions, but prefer cuticle surfaces that contain short cuticular spines (Boucias *et al.*, 1988). Although the conidia are capable of binding over the entire cuticle, they are quite easily removed from smooth exposed sclerite epicuticle, but remain firmly attached to the epicuticle associated with the protected intersegmental folds (Boucias & Pendland, 1991). In addition, Ferron (1981), and Moore-Landecker (1996) mention that the most common sites of entry for the fungus are the host's membranes occurring at the joints and between segments. Various enzymes (chymoelastase protease (Pr1), esterase, and N-acetylglucosaminidase) have been detected in pregerminating conidia of *M. anisopliae* and several other entomopathogenic fungi (Boucias & Pendland, 1991; Ferron *et al.*, 1991; St.Leger *et al.*, 1991). Potentially, the primary function of many of these enzymes is to hydrolyze the epicuticular wax layer and provide nutrients required for germ tube formation (Boucias & Pendland, 1991).

The onset of germination depends on both chemical and topographical signals. For germination, the spores or conidia use cuticular nutrients, like certain long-chain fatty acids, some amino acids and sugars to form a germtube. This germtube differentiates into an appressorium (St. Leger *et al.*, 1991; Bidochka *et al.*, 2000). Where germination fails, it is generally attributed to inhibitory compounds such as short chain fatty acids, quinone and phenols (Hsiao *et al.*, 1992). Data from St Leger *et al.* (1991) suggest that protein phosophorylation events are involved in conidial germination and appressorium formation. It was shown that several membrane-bound cell receptor systems are important in triggering selective activation in cell differentiation and appressorium formation (St. Leger *et al.*, 1994). However, the precise mechanisms and chemicals involved in selective activation of specific signal elements which lead to germination, appressorium formation and finally cuticle invasion are still poorly understood (Bidochka *et al.*, 2000). A penetration peg forms beneath the appressorium and breaches the insect cuticle (Bidochka *et al.*, 2000), using both mechanical force as well as the action of the hydrolytic enzymes (Moore-Landecker, 1996). During passage from the epicuticle to the haemocoel the fungus encounters various cuticular components such as proteins, chitin, lipids, waxes, melanin, diphenols, and carbohydrates (Hsiao *et al.*, 1992). Some of these compounds may be used as nutrition for the fungus, whereas others may inhibit fungal growth (Hsiao *et al.*, 1992). During penetration, appressorial cells of *M. anisopliae* and *B. bassiana* synthesize large amounts of a single subtilisin-like extracellular protease called Pr1 (chymoelastase protease) to degrade the insect cuticle (Gillespie & Claydon, 1989; Boucias & Pendland, 1991; Khachatourians, 1991; St. Leger *et al.*, 1991; St. Leger *et al.*, 1995; Gillespie *et al.*, 1998; Bidochka *et al.*, 2000). Pr1 is encoded by a pathogenicity gene and is produced in large amounts. Since insect cuticles consist of up to 70% protein, this enzyme may have a particularly important role in host penetration (Gillespie *et al.*, 1998). Other proteases produced by *M. anisopliae* seem to be

involved in hydrolyzing cuticular proteins and peptide products. These include trypsins, metalloprotease, aminopeptidases, dipeptidyl peptidase and carboxypeptidases. Bidochka *et al.* (2000) mention that more than 20 different proteases or their isoforms have been described from *M.anisopliae*. Other extracellular enzymes, such as chitinases, N-acetyl-D-glucosaminidases, lipases and esterases also degrade insect cuticular components (Khachatourians, 1991). The chitinases of entomopathogenic Hyphomycetes (like e.g. *M. anisopliae* and *B. bassiana*) are normally secreted well after the emission of proteases (Ferron *et al.*, 1991). These same authors mention that the chitinolytic enzymes of *M. anisopliae* appear to be non-constitutive, and subject to an inducing-repressing system. Some authors however doubt the involvement of these chitinases (N-acetyl-D-glucosaminidases, lipases and esterases) as virulence factors (Bidochka *et al.*, 2000). The whole process of germination and subsequent cuticle penetration generally takes 12-48 hrs (Boucias & Pendland, 1991). For more detailed information on fungal physiology, see Khachatourians (1991), or Boucias & Pendland (1998a,b).

Once the cuticle has been penetrated, yeast-like bodies called hyphal bodies, or blastospores, are produced. These blastospores disperse throughout the insects' haemolymph. Their cell wall composition is different from mycelia, containing more carbohydrate in the glycoproteins. This might be a mechanism of the fungus to escape host immune recognition responses (Bidochka *et al.*, 2000). Blastospores produce cytotoxic compounds, including destruxins and, potentially, a combination of other cyclic depsipeptides and hydrophobins (Kachatourians, 1991). Destruxins are cyclic depsipeptide toxins produced by *M. anisopliae* that cause insect paralysis and death (Ferron, 1981; Khachatourians, 1991). Histopathological studies of tissues infected by *M. anisopliae* suggest that the insect probably dies due to a combination of mechanical damage to internal organs, nutrient depletion and toxicosis (Ferron, 1981; Gillespie & Claydon, 1989).

The insects defend themselves either behaviourally (Ouedraogo *et al.*, 2003) or by activating their immune system (Wilson *et al.*, 2001). The latter process involves both cellular and humoral responses: The cellular responses involve insect haemolytic recognition and encapsulation of a non-self foreign body such as an invading fungus (Boman, 1981; Hajek & St. Leger, 1994; Gillespie *et al.*, 1997; Bidochka, 2000). The humoral responses involve the constitutive production or induction of proteins and peptides most of which are antibacterial (Gillespie *et al.*, 1997). In case the insect succumbs to the infection and dies, the fungus turns to mycelial growth, provided that the ambient humidity is high. When the ambient relative humidity is close to 100%, mycelia will penetrate the cuticle, growing out of the insect to form conidiophores within 24 hrs. Large production of conidia follows one to two days later, (one large scarabeid larva may produce over 5 billion conidia). If the humidity is insufficient for sporulation, the fungus remains inside the host, where it can survive for several months, waiting for favourable conditions to sporulate (Gillespie & Claydon, 1989; Glare & Milner, 1991; Inglis *et al.*, 2001).

PROBLEM DEFINITION AND RESEARCH OBJECTIVES

In the frame-work of vector control in Africa, which is based almost exclusively on chemicals, there are some major problems. The continuous decrease in susceptibility of African mosquito vectors towards insecticides, and the damaging effects of those insecticides on human health and the environment may render the use of insecticides more and more problematic. Thus, alternative methods for vector control that can be integrated with existing ones are urgently required. I believe that, integrated with environmental management, biological control aimed at both the larval and the adult stage of mosquitoes may contribute to such alternatives. There are a few highly efficient biocontrol agents that are able to reduce

larval mosquito populations, but there are none that are aimed at the adult stage of mosquitoes. Without underestimating the role that (biological) larvicides can play in vector control programmes, it is important to realize that it is not without reason that existing vector control in Africa is almost exclusively aimed at the adult stages of mosquito vectors: malaria transmission models show that reduced survival of female mosquitoes has much more impact on transmission than emergence rates from the aquatic stages. Therefore, any method that reduces adult mosquito survival is an extremely valuable asset for vector control. This PhD project was focused on a biological alternative to residual spraying of chemicals to control the adult stage of African mosquito vectors.

The overall objective was to develop a strategy for contaminating wild malaria (*Anopheles* spp.) and filariasis (*Culex quinquefasciatus*) mosquitoes with conidia of the entomopathogenic fungus *M. anisopliae*, based on exploiting our knowledge about their biology. I focused on entomopathogenic fungi because a pilot study on the efficacy of *Metarhizium anisopliae* had shown high virulence of this fungus in adult mosquitoes. The basic idea is based on the methodology used in residual spraying of chemicals inside houses. Indoor resting mosquitoes contact the insecticidal compound and die as a result of this. Instead of using chemicals, conidia of an environmentally friendly insect-pathogenic fungus of low human toxicitiy were used.

Chapter 2 In this chapter a general overview is given of those insect-pathogenic fungi that have been found in the field or tried in the laboratory against mosquitoes. The biology of those fungi are outlined, and their advantages and disadvantages regarding mosquito control, particularly regarding African malaria and lymphatic filariasis, are evaluated.

Chapter 3 In order to find a suitable Hyphomycetous fungus for the proposed mosquito control, several insect-pathogenic fungi should be screened for virulence to *Anopheles gambiae s.s.* In this Chapter several different fungi were isolated, from insects and from soil, and their efficacy on adult *An. gambiae* tested and compared with a fungal isolate that was known to be highly pathogenic to various other insect pests.

Chapter 4 The fungus most virulent to *An. gambiae* from those that were screened in Chapter 3 was used for further studies. In Chapter 4 it was tested also on *Cx. quinquefasciatus*, and a standard laboratory testing procedure to test Hyphomycetous fungi on adult mosquitoes was developed. To assess how much inoculum is needed to significantly reduce mosquito life spans, a dose-response experiment was carried out.

Chapter 5 From other studies it is known that insects, infected with *M. anisopliae*, have reduced feeding propensity. In Chapter 5 I examined whether this was also the case for malaria mosquitoes. This was studied because transmission of the parasite occurs during feeding and a lower number of bloodmeals per mosquito will thus result in a reduced risk of malaria transmission.

Chapter 6 The proposed method to contaminate wild mosquitoes with the fungus is by applying fungus-impregnated sheets inside houses. In order to assess whether the proportion of fungus-infected mosquitoes among the populations can be increased, it was assessed whether horizontal transmission can occur. It was examined whether conidia of *M. anisopliae* can be transferred from a contaminated to an uncontaminated mosquito through body contact during mating.

Chapter 7 The proposed method to contaminate wild mosquitoes implies that mosquitoes will land on materials that are impregnated with conidia of the fungus. It is thus essential that mosquitoes are not repelled by the presence of those conidia. In this Chapter experiments are described in which *An. gambiae* females were exposed to conidia at close range, and had the opportunity to avoid contact with the conidia.

Chapter 8 In field applications it is desirable that the period between applications is as long as possible. To determine for how long impregnated conidia would remain virulent to mosquitoes, pathogenicity tests on *An. gambiae* were carried out at various intervals up to 3 months after impregnation. Also, shelf life under various conditions was determined up to 6 months.

Chapter 9 This Chapter describes a field experiment, carried out in Tanzania, where black cotton sheets were impregnated with conidia of *M. anisopliae* and placed indoors. At daily intervals, mosquitoes were collected from these houses to determine whether the fungus is able to contaminate, infect and kill wild *An. gambiae* and *Cx. quinquefasciatus*.

Chapter 10 This Chapter is the general discussion, based on the results of the other chapters, that integrates and evaluates the potential of *M. anisopliae* for controlling African mosquito vectors. An important part of this chapter is dedicated to implementation of the field-data of Chapter 9 into an epidemiological model that describes malaria transmission intensity. It estimates the impact that implementation of this fungus in a large-scale programme may have on malaria transmission.

CHAPTER 2

ENTOMOPATHOGENIC FUNGI FOR MOSQUITO CONTROL: A REVIEW

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ENTOMOPATHOGENIC FUNGI FOR MOSQUITO CONTROL: A REVIEW

ABSTRACT

Fungal diseases in insects are common and widespread and can decimate their populations in spectacular epizootics. Virtually all insect orders are susceptible to fungal diseases, including Dipterans. Fungal pathogens such as *Lagenidium*, *Coelomomyces* and *Culicinomyces* are known to affect mosquito populations, and have been studied extensively. There are, however, many other fungi that infect and kill mosquitoes at the larval and/or adult stage. The discovery, in 1977, of the selective mosquito-pathogenic bacterium *Bacillus thuringiensis* Berliner *israelensis* (*Bti*) reduced interest in the search for other suitable biological control agents thereafter. In recent years interest in mosquito-killing fungi is reviving, mainly due to continuous and increasing levels of insecticide resistance and increasing global risk of mosquito-borne diseases. This review presents an update of published data on mosquito-pathogenic fungi and mosquito-pathogen interactions, covering 13 different fungal genera. Notwithstanding the potential of many fungi as mosquito control agents, only a handful have been commercialized and are marketed for use in abatement programmes. We argue that entomopathogenic fungi, both new and existing ones with improved efficacies may contribute to an expansion of the limited arsenal of effective mosquito control tools, and that they may contribute in a significant and sustainable manner to the control of vector-borne diseases such as malaria, dengue and filariasis.

INTRODUCTION

The world's prime choice to curb nuisance biting by mosquitoes or their transmission of parasitic or arboviral disease continues to be the selective application of residual synthetic insecticides. The public health benefit delivered by these, both in tropical resource-poor settings, as well as in temperate zones, cannot be over-emphasized – they save thousands of lives each year. Powered by a strong industrial lobby, new and more environmentally friendly compounds replace older, more harmful, ones. However, beyond gains in economic and public health terms, the stark reality of environmental impact and ever-developing resistance remains an issue of grave concern (Hemingway and Ranson 2000; Brooke *et al.*, 2002; Chandre *et al.*, 1999). It is therefore not surprising that interest in alternative non-chemical strategies has increased over the last decades. The use of biological control agents such as predatory fish (Legner, 1995), bacteria (Becker and Ascher 1998), protozoa (Chapman 1974; Legner 1995), and nematodes (Kaya and Gaugler 1993) have all shown promise as a means to control mosquito populations, and progress in these fields has recently been reviewed.

The available literature on entomopathogenic fungi for mosquito control, however, is rather scattered and lacks of recent reviews (Roberts 1974; Ferron *et al.* 1991). The purpose of the present review is to collate and update the available information about the most important entomopathogenic fungi for mosquitoes. Particular focus is on species belonging to the genera *Lagenidium*, *Coelomomyces*, *Entomophthora*, *Culicinomyces*, *Beauveria*, and *Metarhizium* (see Table 1), discussing their potential and drawbacks to be used as biological control agents in reducing mosquito populations. Table 2 contains a comprehensive list of fungi isolated and/or tested on mosquitoes. Except for the anamorphic fungi, we used the taxonomic nomenclature conforming to the 9th edition of the dictionary of the Fungi (Kirk *et al.* 2001).

Table 1. Overview of fungal taxa (Kingdom to genus) discussed in this review (classification and nomenclature partly after Kirk *et al.*, 2001).

1) Kingdom	2) Phylum	3) Class	4) Order	5) Family	6) Genus
CHROMISTA [1]					
	Oomycota [2]				
		Oomycetes [3]			
			Saprolegniales [4]		
				Saprolegniaceae [5]	
					Leptolegnia [6]
			Pythiales		
				Pythiaceae	
					Pythium
					Lagenidium
			Myzocytiopsidales		
				Crypticolaceae	
					Crypticola
FUNGI					
	Chytridiomycota				
		Chytridiomycetes			
			Blastocladiales		
				Coelomomycetaceae	
					Coelomomyces
	Zygomycota				
		Zygomycetes			
			Entomophthorales		
				Ancylistaceae	
					Conidiobolus
				Entomophthoraceae	
					Entomophthora
					Erynia
	Trichomycetes				
			Harpellales		
				Legeriomycetaceae	
					Smittium
	Deuteromycetes (Hyphomycetes)				
					Culicinomyces
					Beauveria
					Metarhizium
					Tolypocladium

1. Oomycota

The phylogenetic relationship of Oomycetes (watermolds) to fungi has been debated for many years (Kerwin and Peterson 1997). The prevailing view is that Oomycetes belong to the kingdom Chromista, which includes diatoms and brown algae (Sleigh, 1989). Watermolds are aquatic organisms, some of which are facultative parasites of mosquito larvae. Some genera, like *Aphanomyces*, appear from time to time in mosquito insectaries and may cause temporally but disruptive epizootics (Seymour and Briggs 1985). Others, like *Leptolegnia*, *Pythium* and *Cryptiloca*, although pathogenic to mosquitoes, have received only limited attention. *Lagenidium giganteum* Couch is an aquatic species that has been studied extensively and is commercially available as a mosquito control agent.

1.1. *Leptolegnia*

In the oomycete genus *Leptolegnia*, only *Leptolegnia caudata* deBary (Bisht *et al.* 1996), and *L. chapmanii* R.L. Seymour (McInnis and Zattau 1982) have been isolated from insects. *L. caudata* was isolated from the malaria vector *Anopheles culicifacies* Giles (Bisht *et al.* 1996). In laboratory bioassays, a zoospore concentration of 7 x 10^3 L^{-1} caused 100% mortality of *An. culicifacies* larvae after 7 days, and the authors suggested inclusion of this fungus in larval control campaigns to reduce malaria transmission. *L. chapmanii* was first reported on *Ochlerotatus triseriatus* (Say) larvae in Louisiana (USA) in 1971 (Seymour 1984). It is a virulent pathogen of first and second instar larvae of *Ae. aegypti* (L.), which suffer 100% mortality within 24 hrs after exposure. Less than 40% of third and fourth instars were infected after 72 hrs (McInnis and Zattau 1982). The authors reported equal susceptibility of *Culex quinquefasciatus* Say, *An. quadrimaculatus* Say and *An. albimanus* Wiedemann to the fungus. Nnakumusana (1986) found 100% mortality of *An. gambiae* Giles larvae after 72 hrs. Lord *et al.* (1988) studied the potential of this fungus against the salt marsh mosquito *Ochlerotatus taeniorhynchus* (Wiedemann) in Florida, USA. Unfortunately the fungus failed to form zoospores and therefore seemed to have little potential to control mosquito populations in saline environments. McInnis and Schimmel (1985) investigated the host range of *L. chapmanii* by testing it on six different aquatic insect orders, reporting no infections. *Leptolegnia* spp. are easy to culture *in vitro*, but tend to lose their larvicidal activity after prolonged culture, although this effect can be reduced by growing the fungus on sterol-rich media (Nnakumusana 1986). For detailed information regarding the *Leptolegnia* life-cycle and infection of mosquito larvae, see Zattau and McInnis (1987) and Seymour (1984).

1.2. *Pythium*

Most species belonging to the genus *Pythium* are pathogens of vascular plants, other fungi, and algae (Van der Plaats-Niterink 1981). Some species however, have been found to be mildly to highly pathogenic to insects. A *Pythium* sp. caused a high level of mortality in a field collection of the treehole mosquito *Ochlerotatus sierrensis* (Ludlow) (Clark *et al.* 1966). In 1988, Saunders *et al.* isolated *P. flevoense* Van der Plaats-Niterink from wild populations of *Oc. sierrensis* in California, occuring in 42% of the sampled treeholes, although this fungus caused infections in only 14% of larvae during 21 weeks of exposure in laboratory bioassays.

Nnakumusana (1985) mentions that in a laboratory bioassay an unidentified *Pythium* species proved pathogenic to early instars of *Ae. aegypti*, *Ae. africanus* (Theobald), *Ae. simpsoni* (Theobald), *Cx. quinquefasciatus*, *Cx. tigripes* Grandpré and Charmoy and *An. gambiae*, reaching mortalities between 50-100%. In other laboratory tests, an unidentified *Pythium* species selectively killed larvae of *An. freeborni* Aitken, *Oc. sierrensis*, *Oc. triseriatus* (Say), *Cx. tarsalis* Coquillet, *Culiseta incidens* (Thomson), *Cs. inornata* (Williston), *Orthopodomyia californica* Bohart, and *Uranotaenia anhydor* Dyar that were mechanically punctured with forceps (Clark *et al.* 1966). The fact that this fungus infected mechanically injured larvae rather than healthy larvae indicates that the fungus is opportunistic rather than strictly entomopathogenic. Even though different mosquito species proved to be susceptible, Clark and colleagues concluded that the conditions under which the infective stage of the fungus could become an important control agent would be hard to achieve, and rather impractical.

Table 2. Overview of fungal species found or tested on mosquito species, either in the laboratory* or in the field**.

Fungal species	Mosquito host	Infected stage	Reference
Leptolegnia sp. (unidentified)	*Ae. albopictus*	larva *	Fukuda *et al.* (1997)
id	*An. gambiae*	larva **	Nnakumusana (1986)
id	*M. titillans*	larva **	Lord & Fukuda (1990)
id	*M. dyari*	larva **	Lord & Fukuda (1990)
Leptolegnia chapmanii	*Ae. aegypti*	larva **	McInnis & Zattau (1982), Lord & Fukuda (1990)
id	*An. albimanus*	larva **	McInnis & Zattau (1982)
id	*An. quadrimaculatus*	larva **	McInnis & Zattau (1982)
id	*Cx.. quinquefasciatus*	larva */**	McInnis & Zattau (1982), Lord & Fukuda (1990)
id	*Oc. taeniorhynchus*	larva **	Lord *et al.* (1988)
id	*Oc. triseriatus*	larva *	Seymour (1984)
Leptolegnia caudata	*An. culicifacies*	larva */**	Bisht *et al.* (1996)
Pythium carolinianum	*Ae. albopictus*	larva **	Su *et al.* (2001)
id	*Cx.. quinquefasciatus*	larva **	id
Pythium sierrensis	*An. freeborni*	larva **	Clark *et al.* (1966)
id	*C. incidens*	larva **	id
id	*Cx.. tarsalis*	larva **	id
id	*O. californica*	larva **	id
id	*Oc. sierrensis*	larva *	id
id	*Oc. triseriatus*	larva **	id
id	*U. anhydor*	larva **	id
Pythium flevoense	*Oc. sierrensis*	larva */**	Washburn *et al.* (1988)
Pythium sp.	*Ae. aegypti*	larva **	Nnakumusana (1985)
id	*Ae. africanus*	larva **	id
id	*Ae. simpsoni*	larva **	id
id	*An. gambiae*	larva **	id
id.	*Cx. tigripes*	larva **	Nnakumusana (1985)
id	*Cx. quinquefasciatus*	larva **	id
Lagenidium giganteum	*Ae.aegypti*	larva **	Rueda *et al.* (1990), Golkar *et al.* (1993)
id	*An.gambiae*	larva **	Golkar *et al.* (1993)
id	*An.freeborni*	larva **	Kerwin *et al.* (1994)
id	*An.quadrimaculatus*	larva **	Rueda *et al.* (1991)
id	*Cx. pipiens*	larva **	Golkar *et al.* (1993), Kerwin *et al.* (1994)
id	*Cx. quinquefasciatus*	larva **	Patel *et al.* (1990), Rueda *et al.* (1990), Orduz & Axtell (1991)
id	*Cx. tarsalis*	larva ***	Woodring *et al.* (1995)
Crypticola clavulifera	*Ae. aegypti*	larva **	Frances *et al.* (1989)
id	*Oc. kochi*	larva *	id
Coelomomyces africanus 1)	*An. nigerrimus*	larva *	Chapman *et al.* (1987)
id	*An. sinensis*	larva *	id
Coelomomyces angolensis	*Cx. guiarti*	larva *	Ribeiro (1992)
Coelomomyces iliensis	*Cx. modestus*	larva *	2)
Coelomomyces indicus	*An. arabiensis*	larva *	Service (1977)
id	*An. culicifacies*	larva **	id
id	*An. gambiae*	larva *	Muspratt (1963)
id	*An. indefinitus*	larva *	Whisler *et al.* (1999)
id	*An. stephensi*	larva *	id
id	*An. vagus*	larva *	id
Coelomomyces irani	*An. maculipennis*	larva *	Weiser *et al.* (1991a)
Coelomomyces lairdi	*An. punctulatus*	larva *	Maffi & Nolan (1977)
Coelomomyces maclaeyae	*Ae. polynesiensis*	larva *	Pillai & Rakai (1970)
Coelomomyces numularius	*An. squamosus*	larva *	Ribeiro & Da Cunha Ramos (2000)
Coelomomyces opifexi	*Op. fuscus*	larva *	Pillai & Smith (1968)
Coelomomyces pentangulatus	*Cx. erraticus*	larva *	Ribeiro & Da Cunha Ramos (2000)
Coelomomyces polynesiensis	*Ae. polynesiensis*	larva *	Pillai & Rakai (1970)
C. psorophorae var. *psorophorae*	*Ae. cinereus*	larva *	Popelkova (1982)
id	*Ae. vexans*	larva */ adults *	Mitchell (1976), Goettel (1987a)
id	*C. inornata*	larva */ **	Shemanchuk (1959), Federici & Roberts (1975)
id	*Oc. taeniorhynchus*	larva **	Federici & Roberts (1975)
C. psorophorae var. *tasmaniensis*	*An .quadrimaculatus*	larva **	Roberts (1974)
id	*C. inornata*	larva */**	Roberts (1974), Chapman (1985)
id	*Oc. australis*	larva *	Buchanan & Pillai (1990)
id	*Oc. taeniorhynchus*	larva **	Roberts (1974)
id	*Op. fuscus*	larva *	Buchanan & Pillai (1990)
id	*Ps. howardii*	larva *	id
Coelomomyces punctatus	*An. crucians*	larva *	Pillai & Rakai (1970)
id	*An. quadrimaculatus*	larva *	id
Coelomomyces solomonis	*An. punctulatus*	larva *	Laird (1956)
id	*U. barnesi*	larva *	id

Su *et al.* (2001) isolated *P. carolinianum* Matthews from Guizhou province, China, in 1994. In outdoor bioassays the authors found infection levels of 13.3-100% in *Cx. quinquefasciatus* larvae, and mentioned that a population of *Ae. albopictus* (Skuse) was 'markedly controlled', but no infection percentages were given. Notwithstanding the pathogenicity of some *Pythium* species to mosquitoes, on the whole they are not considered suitable for biocontrol of mosquitoes. For detailed taxonomic information about the genus *Pythium*, see Van der Plaats-Niterink (1981).

1.3. *Lagenidium*

Only one species of the genus *Lagenidium* is known to be a facultative parasite of mosquito larvae, namely *L. giganteum*. It consists of two stages: oospores (sexual), and zoospores (asexual) (See Fig. 1). Although this fungus has been named *L. culicidum* Umphlett in some publications (Umphlett and Huang 1970; McCray *et al.* 1973), this was later shown to be *L. giganteum* (Couch and Romney 1973).

L. giganteum was first described by Couch (1935) from a combined collection of copepods and mosquito larvae (*Culex* and *Anopheles*) in North Carolina, USA. The geographical distribution is wide: North America, Europe, Africa, Asia, and even Antarctica (Federici, 1981). The fungus has caused high mortalities in mosquito populations in many laboratory, small- and large-scale field studies (California and North Carolina), especially in *Culex* (Merriam and Axtell, 1982; Jaronski and Axtell, 1983), *Mansonia* (Florida) (Cuda *et al.* 1997) and *Anopheles* species (Kerwin and Washino, 1987). Laboratory tests by McCray *et al.* (1973), showed that the fungus could successfully infect and kill larvae of *Ae. aegypti*, *Oc. triseriatus*, *Ae. mediovittatus* (Coquillett), *Oc. taeniorhynchus* Wiedemann, *Oc. sollicitans* (Walker), *Cx. nigripalpus* Theobald, *Cx. quinquefasciatus* and *Cx. restuans* Theobald (Umphlett and Huang, 1970). Anophelines were not found to be susceptible. Also, the fungus is not effective against mosquitoes in brackish or organically rich aquatic habitats (Merriam and Axtell, 1982). The fungus was also ineffective against *Oc. atlanticus* Dyar and Knab, *Oc. tormentor* Dyar and Knab, *An. crucians* Wiedemann, *Cx. peccator* Dyar and Knab, *Psorophora howardii* Coquillett, *Uranotaenia sapphirina* (Osten Sacken) (Glenn and Chapman 1978), and *Ae. albopictus* (Becnel *et al.* 1996). Suh and Axtell (1999) found maximum virulence of *L. giganteum* against *Cx. quinquefasciatus* at concentrations of >150 zoospores ml^{-1} of water, at water temperatures between 20 and 30°C.

Survival of zoospores as indicated by mosquito larval mortality was greatest at 25°C and was similar at 30, 33, and 35°C. No infection occurred at 17°C and less than 20% larval mortality occurred at 19°C with any age of zoospores. Golkar *et al.* (1993) studied this variation in susceptibility between different culicines and anophelines in terms of encysting zoospores and host defense reactions. For *An. gambiae* it was found that, even though a larger number of zoospores attached to its cuticle than would normally be expected in nature, its efficient fast and intense defense (melanization) reaction successfully protected 56% of exposed specimens from death.

This immune response was much faster than that observed for *Ae. aegypti* and *Cx. pipiens* L.. Although a very small number of zoospores attach to and penetrate the cuticle of *Ae. aegypti* and *Cx. pipiens* (compared to the number attached to *An. gambiae*), approximately 99% of both species succumb to fungal infection. McCray *et al.* (1973) found 100% mortality of several *Aedes* and *Culex* larvae, including *Cx. quinquefasciatus*. Other studies show that 100% mortality occurs when the larvae are very young. Orduz and Axtell (1991) reported high virulence for 1-2 day old larvae, intermediate mortality in 3-day-old larvae and low mortality in 4-5 day old larvae. Kerwin and Washino (1987) supported this

(Table 2, continued)

Fungal species	Mosquito host	Infected stage	Reference
C. stegomyiae var. *stegomyiae*	*Ae. aegypti*	larva */**/adults*/ **	Shoulkamy *et al.* (1997), Lucarotti & Shoulkamy (2000)
id	*Ae. albopictus*	larva */ adults *	Laird *et al.* (1992), Ramos *et al.* (1996)
id	*Ae. multifolium*	larva *	Briggs (1968)
id	*Ae. polynesiensis*	larva */***	Padua *et al.* (1986), Laird (1967)
id	*Ae. quadrispinatus*	larva *	Briggs (1968)
id	*Ae. scutellaris*	larva *	Padua *et al.* (1986), Laird (1967)
C. stegomyiae var. *stegomyiae*	*Ae. variabilis*	larva *	Briggs (1968)
Conidiobolus destruens	*Cx. pipiens*	adults *	Mietkiewski & Van der Geest (1985)
Entomophthora conglomerata	*Cx. pipiens*	adults *	Roberts (1974)
Entomophthora coronata	*Cx. quinquefasciatus*	adults */**	Low *et al.* (1968), Low & Kennel (1972)
id	*Oc. taeniorhynchus*	adults **	Low & Kennel (1972)
Entomophthora culicis	*Ae. aegypti*	adults **	Kramer (1982)
id	*Ae. detritus*	adults *	3)
id	*An. maculipennis*	adults *	id
id	*An. stephensi*	adults **	Kramer (1982)
id	*Cx. pipiens*	adults *	id
id	*Culex* spp.	adults *	Roberts (1974), Roberts & Strand (1977)
Entomophthora destruens	*Cx. pipiens*	adults ***	Cuebas-Incle (1992)
Entomophthora musca	*Ae. aegypti*	adults **	Steinkraus & Kramer (1987)
Entomophthoraceae (unidentified)	*Cx. pipiens*	adults **	Cuebas-Incle (1992)
Eryinia conica	*Ae. aegypti*	adults **	id
id	*Cx. restuans*	adults **	id
Zoophthora radicans	*Ae. aegypti*	adults **	Dumas & Papierok (1989)
Smittium (unidentified)	*An. gambiae*	larva **	4)
Smittium (unidentified)	*Ae. aegypti*	larva **	5)
id	*Cx. pipiens*	larva **	id
Smittium morbosum	*An. hilli*	larva **	Sweeney (1981d)
id	*Ae. albifasciatus*	larva *	Garcia *et al.* (1994)
Smittium morbosum	*Ae. crinifer*	larva *	id
id	*Ae. serratus*	larva *	id
id	*An. annulipalpis*	larva *	id
id	*Cx. dolosus*	larva *	id
id	*Cx. intricatus*	larva *	id
id	*Cx. maxi*	larva *	id
id	*Cx. pipiens*	larva *	id
id	*M. indubitans*	larva *	id
id	*Ps. Ferox*	larva *	id
id	*U. nataliae*	larva *	id
Aspergillus flavus	*An. stephensi*	larva **	Sur *et al.* (1999)
id	*Cx. peus*	larva **	Toscano & Reeves (1973)
Aspergillus niger	*An. stephensi*	larva **	id
Aspergillus ochraceus	*An. stephensi*	larva **	Sur *et al.* (1999)
id	*Cx. pipiens*	larva *	Badran & Aly (1995)
Aspergillus terreus	*An. stephensi*	larva **	id
id	*Cx. pipiens*	larva *	id
Aspergillus parasiticus	*Ae. aegypti*	larva **/adults **	Nnakumusana (1985)
id	*An. gambiae*	larva */**/adults **	id
id	*Cx. quinquefasciatus*	larva **/adults **	id
Beauveria bassiana	*Ae. aegypti*	adults **/larva **	Clark *et al.* (1968);Miranpuri & Khachatourians(1991)
id	*An. albimanus*	adults **/larva **/***	Clark *et al.* (1968)
id	*An. nigromaculis*	adults **/larva ***	id
id	*Cx. pipiens*	adults **/larva **/***	id
id	*Oc. sierrensis*	adults **/larva **	id
Beauveria bassiana	*Cx. tarsalis*	adults **/larva **/***	Clark *et al.* (1968)
Beauveria tenella	*Ae. aegypti*	larva **	Pinnock *et al.* (1973)
id	*Ae. dorsalis*	larva **	id
id	*Ae. hexodontus*	larva **	id
id	*An. stephensi*	larva **	6)
id	*C. incidens*	larva **	id
id	*Cx. pipiens*	larva **	id
id	*Cx. quinquefasciatus*	larva **	id
id	*Cx. tarsalis*	larva **	id
id	*Oc. sierrensis*	larva */**	id
Culicinomyces spp. (unidentified)	*An. amictus hilli*	larva **	Sweeney (1978)
id	*Cx. quinquefasciatus*	larva **	id
Culicinomyces bisporales	*Ae. kochi*	larva *	Sigler *et al.* (1987)

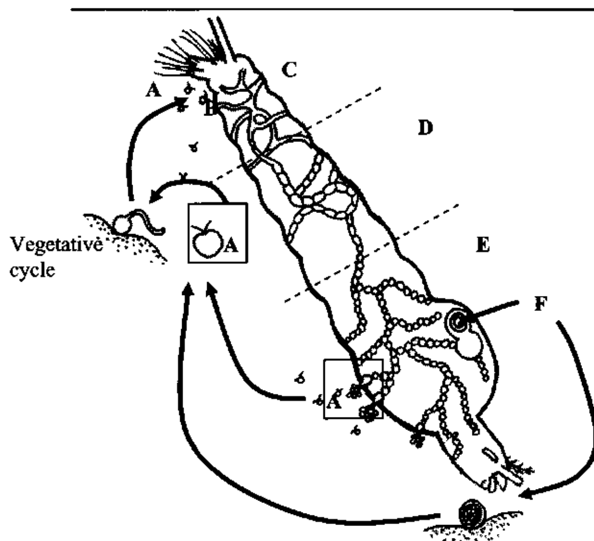

Figure 1. Generalized life cycle of *Lagenidium giganteum*. Motile zoospores (A) attach to, and penetrate (B) cuticle of mosquito larva (lateral view). Hyphae grow (C), septate (D), and start forming sporangia (E). Exit vesicles with zoospores (F) (asexual cycle) or zoospores (G) (sexual cycle) are formed (drawn by Ernst-Jan Scholte, modified after Fetter-Lasko and Washino (1983).

finding and suggested that *L. giganteum* zoospores might not recognise late instars of otherwise susceptible mosquito species.

As a facultative parasite, *L. giganteum* can grow vegetatively both as pathogen on mosquito larvae, or as a saprophyte in aquatic environments (Federici 1981; Service 1983; Legner 1995; Sur *et al.* 2001). Maintenance of the fungus *in vivo*, though labour-intensive, is possible (Kerwin and Petersen 1997), but *in vitro* culture using both defined and complex media (Kerwin *et al.* 1986; Guzman and Axtell 1986; Sur *et al.* 2001) is more commonly practiced (Kerwin and Petersen 1997). For small-scale culture of *L. giganteum*, solid media are used. For large-scale (10-1000 L) production, liquid cultures of yeast extract-based media (Kerwin and Petersen 1997) are utilised.

Fungal reproduction is both asexual (zoospores) and sexual (oospores) (Federici 1981). In order to infect mosquito larvae, zoospores must be formed. These biflagellate, motile zoospores are the asexual stage of the fungus. They do not have a cell wall, and are therefore too fragile to be applied directly for mosquito control (Kerwin *et al.* 1994). A further disadvantage of the asexual stage is its short shelf-life; zoospores survive for only 48 hrs after emerging from an infected larva. Further problems include the need to keep the mycelium completely hydrated, its susceptibility to being overwhelmed by contaminating microorganisms following formulation, lack of stability under extreme temperatures, and special handling required to keep the formulated product from becoming anaerobic (Su *et al.* 1986; Kerwin and Washino 1986; Kerwin *et al.* 1994). Several methods have been tried to overcome these problems, for instance by encapsulating the fungus in several types of calcium alginate beads (Rueda *et al.* 1990, 1991; Patel *et al.* 1990). This proved to increase retention of infectivity against mosquito larvae and more convenience in storing, handling and application (Rueda *et al.* 1990). The level of control of anophelines, for instance, was found to increase when 1% ground cork was added to the capsules (Rueda *et al.* 1991).

(Table 2, contined)

Fungal species	Mosquito host	Infected stage	Reference
Culicinomyces clavisporus	*Ae. aegypti*	larva **	Cooper & Sweeney (1982)
id	*Ae .altropalpus epactius*	larva **	Knight (1980)
id	*Ae. atropalpus epactius*	larva **	Couch *et al.* (1974)
id	*Ae. rubrithorax*	larva *	Frances (1986)
id	*Ae. rupestris*	larva */***	Goettel *et al.* (1984), Sweeney & Panter (1977)
id	*An. amictus hilli*	larva */**	Cooper & Sweeney (1982), Sweeney (1978, 1983)
id	*An. farauti*	larva **	Sweeney (1978)
id	*An. punctipennis*	larva **	Couch *et al.* (1974)
id	*An. quadrimaculatus*	larva */**	id
id	*An. stephensi*	larva **	id
id	*C. inconspicua*	larva *	Frances (1986)
id	*C. inornata*	larva *	Goettel *et al.* (1984)
id	*C. melanura*	larva **	Couch *et al.* (1974)
id	*Cx. erraticus*	larva **	Couch *et al.* (1974)
id	*Cx. quinquefasciatus*	larva **	Couch *et al.* (1974), Cooper & Sweeney (1982, 1986)
id	*Cx. restuans*	larva **	Couch *et al.* (1974)
id	*Cx. territans*	larva **	id
id	*Ps. confinnis*	larva **	id
id	*Uranotaenia* sp.	larva **	id
Metarhizium anisopliae	*Ae. aegypti*	larva **	Ramoska *et al.* (1981); Daost *et al.* (1982)
id	*Ae. albopictus*	larva **	Ravallec *et al.* (1989)
id	*An. albimanus*	larva **	Ramoska *et al.* (1981)
id	*An. stephensi*	larva **	Crisan (1971); Daoust & Roberts (1982)
id	*Cx. pipiens*	larva **	id
id	*Cx. quinquefasciatus*	larva **	Ramoska *et al.* (1981); Lacey *et al.* (1988)
Fusarium culmorum	*Cx. pipiens*	pupae *	Badran & Aly (1995)
Fusarium dimerum	*Cx. pipiens*	pupae *	id
Fusarium oxysporum	*Ae. cantans*	larva **	7)
id	*Ae. detritus*	larva */**	"Husan & Vago (1972); Breaud *et al.* (1980)"
id	*Cx. pipiens*	larva **	id
Fusarium pallidoroseum	*An. stephensi*	larva *	Ravindranath & Kapadnis (1991)
Fusarium semitectum	*Ae. cantans*	larva **	7)
id	*An. stephensi*	larva **	Sur *et al.* (1999)
Geotrichum candidum	*Ae. pionysis*	larva *	8)
id	*An. maculipennis*	larva *	id
id	*An. stephensi*	larva **	Sur *et al.* (1999)
Paecilomyces farinosus	*Cx. pipiens*	larva *	8)
Paecilomyces lilacinus	*Ae. aegypti*	larva **	Agarwalda *et al.* (1999)
Tolypocladium cylindrosporum	*Ae. aegypti*	larva **	Goettel (1988)
id	*Ae. subalbirostris*	larva ***	Gardner & Pillai (1987)
id	*Ae. vexans*	larva ***	Goettel (1987b)
id	*C. inornata*	larva ***	Goettel (1987b)
id	*C. minnesotae*	larva ***	id
id	*Cx. tarsalis*	larva **	Soarés (1982)
id	*Cx. territans*	larva ***	Goettel (1987b)
id	*Oc. australis*	larva */***	Gardner & Pillai (1987)
id	*Oc. sierrensis*	larva */**, adults **	Soarés (1982), Saunders *et al.* (1988)
id	*Oc. triseriatus*	larva **	Nadeau & Boisvert (1994)
Trichophyton ajelloi	*An. stephensi*	larva **	Mohanty & Prakash (2000)
id	*Cx. quinquefasciatus*	larva **	id
Verticillium lecanii	*Oc. triseriatus*	larva *	Ballard & Knapp (1984)

Notes:

"* : natural occurrence; ** : laboratory study; *** : field study"

"*Ae.*: *Aedes*; *An.*: *Anopheles*; *C.*: *Culiseta*; *Cx.*: *Culex*; *M*: *Mansonia*; *O.*: *Orthopodmyia*; *Oc.*: *Ochlerotatus*; *Op.*: *Opifex*; *Ps.*: *Psorophora* *U.*: *Uranotaenia* "

1) A far more extensive list of *Coelomomyces* spp. and their mosquito hosts can be found in Couch & Bland (1985)
2) cited in Apperson *et al.* (1992). Original paper by Deshevykh (1973). Seasonal infection of larval *Culex modestus* by *Coelomomyces* fungi in the Ili river basin.
3) cited in Kramer (1982). Original book by Marshall (1938). The British mosquitoes.
4) cited in Sweeney (1981d). Original paper by Coluzzi (1966). Experimental infections with *Rubetella* fungi in *Anopheles gambiae* and other mosquitoes.
5) cited in Sweeney (1981d). Original paper by Dubitskii (1978). Biological control of blood sucking flies in the USSR.
6) cited in Geetha & Balaraman (1999). Original paper by Balaran *et al.* (1979).
7) cited in Sur *et al.*, (1999). Original paper by Kalvish TK and Kukharchuk IP (1974). Pathogenic mycoflora of blood sucking mosquitoes of western Siberia and the Far East.
8) cited in Sur *et al.* (1999). Original paper by Kalvish & Kukharchuk (1974). Pathogenic mycoflora of bloodsucking mosquitoes of western Siberia and the Far East.

Oospores, the sexual stage of *L. giganteum*, can also be used as inoculum. They are dormant propagules, resistant to dessication and mechanical abrasion and stable for at least seven years, which allows multivoltine persistence of the fungus in some habitats (Kerwin and Washino 1986; Kerwin *et al.* 1994). Unfortunately, mass production yields of oospores remain orders of magnitude below that of the less stable mycelial (asexual, presporangial) stage, and continued problems with spore activation have prevented large-scale field tests (Merriam and Axtell 1982; Kerwin and Washino 1987; Legner 1995, Kerwin and Petersen 1997). Research is continuing to improve oospore yields, which would be much more useful than zoospores in large-scale operational mosquito control programmes.

Zoospores of the fungus appear harmless to most aquatic invertebrates (one or more species in the animal groups of Polychaeta, Ostracoda, Copepoda, Cladocera, Diptera, Coleoptera, Hemiptera, and Odonata) (Nestrud and Anderson 1994), and to vertebrates (mallard ducks, mice, rats, and rabbits) (Kerwin *et al.* 1990). Only *Daphnia* spp. and copepods reported by Couch (1935), three cladoceran species and a chironomid species reported by Nestrud and Anderson (1994) were found susceptible.

In spite of these non-target effects, a *L. giganteum*-based product was commercialised under the name Laginex™ by AgraQuest (California, USA) until 1999. It was claimed to be particularly effective against *Culex* spp., but the kind of spore used was not mentioned (Khetan 2001). The fungus is compatible with the bacterial agents *Bti* and *B. sphaericus* Meyer and Neide when used against *Cx. quinquefasciatus* (Orduz and Axtell 1991), with the fungus having the distinct advantage over *Bti* that it is able to recycle in stagnant water, infecting multiple and overlapping generations of mosquitoes (Legner 1995). In field trials in which Laginex™ 25 was compared with Vectobac™-12AS (*Bti*), Laginex™ reduced *Cx. quinquefasciatus* larvae by 100% for 22 days whereas Vectobac™-12AS required retreatment by the 10th day (Hallmon *et al.* 2000). Results from a small scale field trial in North Carolina indicated that *L. giganteum* recycled for an entire season despite periodic scarcity of hosts and short-term drought, with infections ranging from 0-100% (Jaronski and Axtell, 1983). A large scale field trial in Californian rice fields, using mycelium from either 20 or 30 liters of fermentation liquid per hectare resulted in 40%-90% infection of *Cx. tarsalis* and *An. freeborni* sentinel larvae (Kerwin and Washino, 1987). For details about culture media, see Kerwin and Petersen (1997) and for detailed information about the life-cycle of *L. giganteum*, see Kerwin *et al.* (1994), Kerwin and Peterson (1997), or Woodring *et al.* (1995).

1.4. *Crypticola*

Crypticola clavulifera Humber, Frances and Sweeney has been isolated from the midge *Forcipomyia marksae* Tokunaga (Ceratopogonidae) in Queensland, Australia, in 1984 (Frances *et al.* 1989). Its biology is similar to that of *L. giganteum*. In the laboratory the fungus successfully infected *Ae. notoscriptus* (Skuse), *An. farauti* Laveran, *Cx. annulirostris* Skuse, *Cx. quinquefasciatus* (Frances, 1991), and *Ae. aegypti* (Frances *et al.* 1989, Frances 1991). *Ae. kochi* (Dönitz) was not susceptible (Frances 1991). Despite its pathogenicity to several mosquito species no further studies have been published on this fungus.

2. Chytridiomycota

Members of this phylum are aquatic saprophytes or parasites growing on decaying and living organic material, both in freshwater and soils (Kirk *et al.* 2001). They have flagellate zoospores, and chitinous hyphae. The phylum consists of 5 orders, of which the Blastocladiales contains the only mosquito-pathogenic genus of this group: *Coelomomyces*.

2.1. *Coelomomyces*

The genus *Coelomomyces* consists of more than seventy species of obligatory parasitic aquatic fungi that undergo a complex life-cycle involving alternating sexual (gametophytic) and asexual (sporophytic) generations (Couch and Bland 1985) (See Fig. 2). The genus has been found in all continents except Antarctica (Roberts 1974). Unlike most other entomogenous fungi, which have rather wide host ranges, involving species in several orders of insects, *Coelomomyces* is almost entirely restricted to aquatic Dipteran insects, including Culicidae, Psychodidae, Chironomidae, Simuliidae and Tabanidae (Arêa Leão and Carlota Pedroso 1964; Chapman 1974; Roberts 1970). Susceptibility studies indicate that most *Coelomomyces* spp. are probably not host-specific, but nevertheless have relatively restricted host ranges (Federici 1981).

Because of the high pathogenicity of many *Coelomomyces* species to mosquito larvae, the fungal genus has interested many researchers. Publications on *Coelomomyces* and the Oomycete *L. giganteum* together comprise the majority of studies published on entompathogenic aquatic fungi that affect mosquito larvae. Chapman and Woodard (1966) listed all hosts for *Coelomomyces* found in the USA. They list seventeen species belonging to six mosquito genera (*Culex, Culiseta, Aedes, Anopheles, Psorophora*, and *Uranotaenia*), with fungi being most common in the genus *Anopheles* followed by *Aedes* and *Culex* (Chapman 1974). Roberts and Strand (1977) listed 22 *Coelomomyces* species found on 31 mosquito species and Weiser (1988) listed up to 28 *Coelomomyces* species found on over 60 mosquito species. The basic life-cycle has been determined for 11 of the 60 species and subspecies of *Coelomomyces* recognized by Couch and Bland (1985). Of those 60 species, *C. indicus* Iyengar, a strain with special features and wide distribution has been given much attention. This species has been reported from Africa, the Philippines, India, and Pakistan. The Dipteran hosts include 16 species of anopheline mosquitoes, including several important vectors of malaria, such as *An. gambiae* (Muspratt 1946). It is known to persist and cause periodic epizootics in rice fields in southeast Asia (Whisler *et al.* 1999), Egypt (Gad and Sadek 1968) and Kenya (Service 1977).

The life-cycle of *Coelomomyces* is complex and includes obligatory development in an intermediate microcrustacean host (cyclopoid copepods, harpacticoid copepods, or ostracods) and two mosquito generations for completion (Whisler *et al.* 1974, 1999; Lucarotti and Andreadis 1995). The fungus survives unfavourable environmental conditions, like cold or dry periods, as resting sporangia (RS) (Buchanan and Pillai 1990) that develop from diploid hyphae in infected mosquito larvae. Normally, the larvae die in the 4th stage and RS are released as the cadavers decompose. Meiosis occurs in the RS and the posteriorly uniflagellate meiozoospores that emerge from the RS infect the appropriate microscrustacean host and establish the haploid, gametophytic stage, which develops in the haemocoel (Padua *et al.* 1986; Federici 1995; Lucarotti and Andreadis 1995). At maturation, the gametophyte cleaves forming thousands of uniflagellate gametes. Cleavage results in death of the copepod and escape of the gametes, which fuse forming biflagellate zygotes (zygospores) that seek out and infect another mosquito larva (Lucarotti and Andreadis 1995). The zygotes encyst preferentially on the intersegmental membranes of young and/or recently molted (Lacey and Undeen 1986) mosquito larvae. They enter epithelial cells just below the larval cuticle by means of a penetration tube. From there, the fungus enters the haemocoel where it uses the reserves of the larval fat body to develop into irregularly shaped hyphae without cell walls (Roberts 1974). Sporangia are produced within the hyphae at their tips. These sporangia usually become so numerous that they virtually fill the haemocoel (Roberts 1974). In most cases the larva then dies and forms resting spores (to complete the life-cycle).

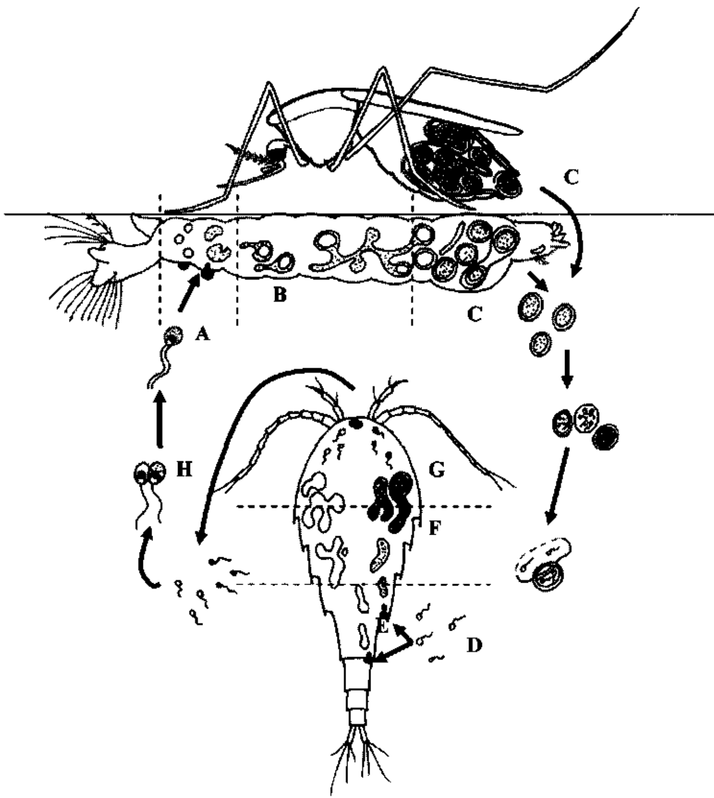

Figure 2. Generalized life cycle for species of *Coelomomyces*. (A) Biflagellate zygote infects haemocoel of mosquito larvae (lateral view) producing hyphagens, which later form hyphae. (B,C) Hyphae grow in the haemocoel and and form resting sporangia. Occasionally, if infected (female) larvae survive to the adult stage, resting spores are formed and dispersed by 'oviposition'. Ultimately, meiospores (D) are released, which infect a copepod and produce gametophytes (E), each one will form gametangia (F) that release gametes of a single mating type (G). Gametes of opposite mating type fuse inside or outside (H) copepod, forming a biflagellate zygote that completes the cycle by infecting another mosquito larva (drawn by Ernst-Jan Scholte, modified after Federici (1981)).

Occasionally, however, the infected larva pupates and ecloses to produce infected adults, an event for which Mitchell (1976) credited the first description to Zharov in 1973 for adult *Ae. vexans* (Meigen) infected with *C. psorophorae* Couch. The same phenomenon was later described for *C. stegomyiae* var. *stegomyiae* Keilin infecting adult *Ae. albopictus* (Laird *et al.* 1992). Later studies by Lucarotti (1992), Shoulkamy *et al.* (1997), and Lucarotti and Shoulkamy (2000), describe this process in more detail for adult *Ae. aegypti* infected with *C. stegomyiae*. In adult female *Ae. aegypti*, the infection is mostly localised in the ovaries (Lucarotti 1992). During the first 72 hrs following eclosion, as the ovaries enlarge under the influence of juvenile hormone, the hyphae in the haemocoel are transferred to the interstitial spaces of the ovaries. The hyphae in the ovaries will mature to RS in response to changes in host ecdysone levels following a blood meal (Lucarotti 1992). Even though *Coelomomyces*-infected females will mate, no eggs will develop in the ovaries. Females do, however, attempt to oviposit, but instead of eggs, resting spores are laid (Lucarotti 1987, 1992). Infected adult female mosquitoes therefore play a role in the transmission of the fungus to new habitats, which is especially useful for those species of *Coelomomyces* that infect mosquitoes

occupying small habitats like tree holes or water containers (Laird *et al.* 1992; Lucarotti and Andreadis 1995; Shoulkamy *et al.* 1997).

In contrast to other types of pathogens reported from mosquitoes, many species of *Coelomomyces* are known to cause significant epizootics, which can persist in larval populations over several years and result in prevalence and mortality rates exceeding 50% and often ranging higher than 90% (Apperson *et al.* 1992). For an extensive overview of the genus *Coelomomyces* and its hosts, its epizootics in mosquito populations and control programmes up to 1985, see Couch and Bland (1985).

In a review of data obtained from mosquito populations in Louisiana, Chapman (1985) noted infection rates of 96% for *C. psorophorae* in *Psorophora howardii* Coquillett and *Cs. inornata* (Williston), 97% for *Cs. macleayae* Laird in *Ae. triseriatus*, 92 % of *C. pentagulatus* in *Cx. erraticus* (Dyar and Knab), and 100% for *C. punctatus* Couch in *An. crucians*. Legner (1995) mentions studies in which infection levels exceeding 95% were reported from *C. inornata* and *P. howardii* by *C. psorophorae* and in *Oc. triseriatus* by *C. macleayae* and 85 percent in *Cx. peccator* Dyar and Knab by *C. pentangulatus*. Infection levels as high as 100% have been reported.

Apperson *et al.* (1992) cite studies by Deshevykh (1973), who found *C. iliensis* Dubitskij for a two-year period in *Cx. modestus* Ficalbi in Kazakstan, and Muspratt (1963), who reported 100% *Coelomomyces* infection in some Zambian *An. gambiae* populations. However, even though many epizootics have been reported, field incidence of mycoses caused by *Coelomomyces* spp. in mosquito larvae is low (Lacey and Undeen 1986). Infection rates of 24-48% were reported in *An. crucians* and *An. quadrimaculatus* in the southeastern USA (Umphlett 1970). The latter infection level was maintained for three years, showing that the fungus did indeed persist, although the infection levels decreased over time to only 10% (Umphlett 1969). This decline in mosquito infection rates over a period of several years may be due to reduced copepod populations resulting from parasitisation by *Coelomomyces* (Apperson *et al.* 1992). In the southern USA, Umphlett (1970) and Chapman and Glenn (1972) reported the persistence of *C. punctatus* for at least 4 years in populations of anopheline larvae, with infection rates ranging from 12-67%. In 1976, *Coelomomyces* was introduced to a small Pacific island to control *Ae. polynesiensis* Marks, a vector of filariasis (Laird 1967). This trial marks one of the few attempts to establish a mosquito pathogen in an area where it did not previously exist. The introduction was successful and the fungus remained active in the new locality for at least seven years. Other successful field trials thus fare have been conducted with *C. iliensis* in the former Soviet Union. High levels of mortality in mosquito larvae over a broad geographical area were reported after inoculation of habitats with infective material (Lacey and Undeen 1986). However, results from such field trials are often less clear-cut, due to large variations in infection levels (Chapman 1974; Federici 1981; Service 1983). For example, following treatment of a rice field in Egypt with sporangia (prior to drying at the end of the growing season) from *An. pharoensis* Theobald, infection levels in larvae collected the following season varied between 0-94% (Roberts 1974). Couch (1972) introduced sporangia of *C. punctatus* into ditches in North Carolina (USA) along with eggs of *An. quadrimaculatus*. Infection rates in larvae collected 10-15 days later varied from 0-100% (mean 60%). Additionally, Federici (1981) mentions that Dzerzhinskij *et al.* (1975) obtained similar fluctuating infection levels with *C. iliensis* against *Cx. modestus* in the former Soviet Union.

These unpredictable infection rates, together with its complicated life-cycle make this fungus unsuitable for the control of mosquito populations, according to Service (1983). In contrast, Federici (1981) and Lacey and Undeen (1986), after reviewing the potential of various *Coelomomyces* species for mosquito control, conclude that the fungus does offer potential. They lay emphasis on the relatively specific host range (mainly mosquitoes) and the

devastating effects of natural epizootics on larval populations. Kerwin and Petersen (1997) add to this the development of resistance by mosquitoes to available insecticides, and the subsequent need to improve knowledge of alternative methodologies, like the use of *Coelomomyces*, to control mosquito populations. The most important reason for the fungus not being used very often for biological control of mosquitoes is the difficult mass-production, because of its complex life-cycle including micro-crustaceans. Even though some progress has been made in culturing mycelia on synthetic media (Castillo and Roberts 1980; Shapiro and Roberts 1976), no species of *Coelomomyces* has been successfully cultured *in vitro* to date. Further application of *Coelomomyces* as a direct mosquito control agent is dependent on the development of easily cultured inocula (Legner 1995). For details on the infection process, see Shoulkamy and Lucarotti (1998), and Couch and Bland (1985).

3. Zygomycota

The phylum Zygomycota exists of the two Classes, the Trichomycetes and Zygomycetes. Zygomycetes are characterised by the presence of a coenocytic mycelium, by the absence of flagellate spores, and by sexual reproduction through the formation of zygospores. The most important entomopathogens in the Zygomycota belong to the order Entomophthorales (Humber 1997). There are presently about 200 known species classified in six genera of entomopathogenic fungi in this order. All but one genus (*Massospora*) are characterised by the production of forcibly discharged spores (Khachatourians 1991) (See Fig. 3). Entomophthorales have been reported from many regions, including Africa, but the majority of the literature is on species from the northern hemisphere. Many epizootics have been reported, most of them in rather cool (1-20°C), moist (44-100% RH) biotopes in middle and northern Europe, the former Soviet Union and North America. They are common, obligate pathogens of a wide range of insects, typically infecting adults (except for *Entomopthora aquatica* J.F. Anderson and Anagnostakis and *E. conglomerata* Sorokin (Keller) that have been found on aquatic stages of insects), and unable to grow saprophytically (Bidochka *et al.* 2000; Eilenberg 2000). Many species have restricted host ranges and some predominantly infect mosquitoes (Humber 1997).

The life-cycle of an Entomophthorales starts with a spore that infects a host by penetration of the integument. This takes place some 12 hrs after initial contact (Low and Kennel 1972). The fungus develops vegetatively in the host haemocoel by producing small, readily circulated, rod-like hyphal bodies and, depending on the temperature, completes the infection-cycle normally within a few days (Humber 1997). After completing the infection, just before the host dies, the insects are sometimes fixed to a substrate (e.g. leaves) by special hyphae (rhyzoids). After death of the host, the fungus produces sporangiophores, which actively discharge new, primary, spores. These primary spores are short-lived. Roberts (1974) reports that infected adults were no longer useful as inoculum eight days after the first spores were produced. Primary spores that do not contact a suitable host upon discharge have the ability to produce secondary spores, which may also be forcibly dispersed (Humber 1997; Eilenberg 2000). Although spores of Entomophthorales are far bigger than conidia of Hyphomycetes, aerial distribution is not uncommon (Hajek and St. Leger 1994). To survive the winter, they develop thick-walled spores, which are usually referred to as resting spores, although in strict mycological terms they are zygospores (Humber 1997). These resting spores form new primary spores in the spring. In reviews on Entomophthorales, their potential for biocontrol is often stressed because of high levels of infection observed in nature, and the theory that they can remain active in a site for several years (Hajek and St. Leger 1994; Eilenberg 2000).

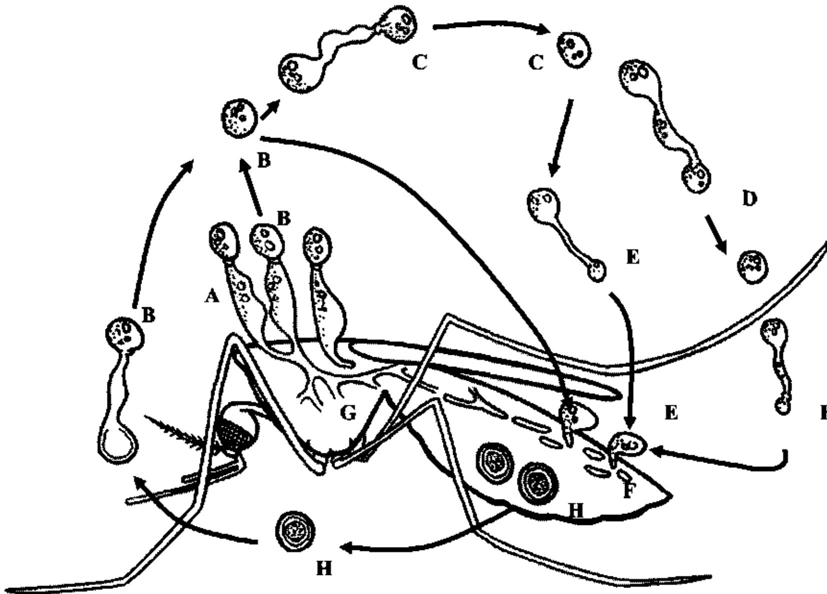

Figure 3. Generalized life cycle of an entomophthorous fungus. Conidiophores (A) on host (in this example a mosquito) forms primary conidium (B), which may form secondary conidium (C), which in turn may form tertiary conidium (D) in absence of a suitable host. Capilliconidia, formed from other conidia, may be formed, infecting a new host. Hyphal bodies (F) are formed and develop into mycelium and stroma (G), which produce resting spores (H) or conidiophores (A). (drawn by Ernst-Jan Scholte, modified after Tanada and Kaya (1993)).

Release-experiments with *E. maimaiga* in Michigan showed that the fungus is able to establish itself easily in new territory and cause epizootics in target species (Smitley *et al.* 1995). Unfortunately, no commercial formulations are yet available. For more detailed information about the biology, infection and host ranges of the order Entomophthorales, see Eilenberg (2002).

Trichomycetes are fungi that live hidden within the digestive tract of many arthropod species within several orders (including larvae of Diptera, Ephemeroptera and Plecoptera, and adults of some isopods, cladocerans, amphipods, copepods, Collembola, Coleoptera, and diplopods (Lichtwardt *et al.* 1999). They may be seen as branched or unbranched fungal bodies (thalli) firmly attached to the gut lining and lying within the gut lumen from which they obtain their nutrients (Lichtwardt 1986). The class contains four orders, only one of which, Harpellales, contains a genus, *Smittium*, with mosquito-pathogenic species. For detailed information on the biology of Trichomycetes, see Lichtwardt (1986).

3.1. *Conidiobolus*

Lowe *et al.* (1968) reported *Conidiobolus coronatus* (Constantin) Batko (formerly *Entomohphtora coronata*) in *Cx. quinquefasciatus* from a colony of adult mosquitoes maintained in large, outdoor screened cages. The fungus has been found in several orders of insects and in two other classes of invertebrates, and is considered to have the widest host range among the Entomophthorales, but it is a rather weak pathogen (Humber 1997). Adult *Oc. taeniorhynchus* and *Cx. quinquefasciatus* treated with *C. coronatus* suffered increased

mortality at 7 days post-treatment (Lowe and Kennel 1972). This increase was greater in *Oc. taeniorhynchus* than in *Cx. quinquefasciatus*. However, besides infecting insects, this species has been reported as causing facial infection in man (Bras *et al.* 1965) and horses (Emmons and Bridges 1961), which therefore excludes its use as a biocontrol agent. Apart from *C. coronatus*, no infections of vertebrates have been reported for insect-parasitising Entomophthorales.

3.2. *Entomophthora*

As in other Entomophthoraleans, *Entomophthora* infections primarily occur in adult rather than in larval mosquitoes. *E. culicis* (Braun) Fresenius was discovered and described from adult *Cx. pipiens* in Germany in 1855 (Braun 1855). Kramer (1982) lists a number of mosquito species on which the fungus occurs in nature, including other *Culex* species, *Oc. detritus* (Haliday), *An. maculipennis* Meigen, *Myzomyia hispaniola* Theobald (synonym for *An. cinereus* Theobald), and an unspecified Asian *Aedes* species. In a laboratory study, 80% infection of adult *Ae. aegypti* and 100% of *An. stephensi* Liston was achieved from diseased adult midges, *Chironomus decorus* Johannsen (Kramer 1982). In a study published a year later by the same author, transmission of *E. culicis* from the donor *Cricotopus similis* Goetghebuer (a Chironomid) to the mosquitoes *An. stephensi* and *Cx. pipiens* was possible, but infection rates differed. 100% of *An. stephensi* succumbed to infection compared to only 20% of *Cx. pipiens* (Kramer 1983). Mortalities of overwintering mosquitoes infected with *Entomophthora* spp. (e.g. *E. destruens* Weiser and Batko and *E. culicis*) in damp, relatively cold biotopes like caves or basements are frequently very high (Chapman 1974; Roberts 1974; Weiser 1988). Mortalities of 85-100% in former Czechoslovakia and 80-90% in the Netherlands have been reported (Teernstra-Eeken and Engel 1967; Mietkiewski and Van der Geest 1985). Roberts (1974) describes a study by Gol'berg (1970), where *Cx. pipiens* was infected with an *Entomophthora* species. Field-collected infected adults were used as inoculum, resulting in 0% infection in 1-3rd instar larvae, 25% in 4th instars, 63-88% in pupae, 33-67% in male adults, and 65-100% in female adults. Roberts (1974) then summarizes several reports of *E. conglomerata*, *E. destruens*, or unclassified *Entomophthora* sp. infecting *Cx. pipiens* in nature (yielding infection rates of 49%, 100% and 97%, respectively) suggesting that the fungal isolates involved may be rather species-specific. *E. muscae* (Cohn) Fresenius, a well-known *Entomophthora* species that causes epizootics among pest flies, proved to be able to infect only 3% of exposed *Ae. aegypti* (Steinkraus and Kramer 1987). Further, *E. conica* (Nowakowski) Remaudiére and Hennebert, *E. culicis, E. destruens, E. gracilis* (Thaxter) Remaudiére and Hennebert, *E. henrici* (Molliard) Humber and Ben-Ze'ev, *E. papilliata*, *E. radicans* (Brefeld) Batko, *E. rhizospora* (Thaxter) Remaudiére and Hennebert, *E. schroeteri*, *E. thaxteriana* Petch, and *E. variabilis* (Thaxter) Remaudiére and Hennebert, have also been found to infect mosquitoes belonging to the genera *Aedes, Culex, Anopheles*, and *Culiseta* (Anderson and Ringo 1969; Roberts 1974; Eilenberg 2000).

There are probably localities where *Entomophthora* spp. could be profitably introduced for control of hibernating mosquito populations, but there are no reports of such trials to date. A drawback of using *Entomophthora* for mosquito control would be that spores are unable to withstand low humidities as they can not survive exposure to humidities below 75% RH (Wildin, 1973). An option could be to use resting spores, which remain viable for long periods, but prolonged dormancy and asynchronous germination have proven to be obstacles for practical application (Hajek and St. Leger 1994). To use Entomophthora for mosquito control, effective *in vitro* growth systems need to be developed, because most species are, as yet, unable to grow under mass-production fermentation conditions (Roberts 1974; Papierok and Hajek 1997; Eilenberg 2000). *E. destruens* has been cultured on several media, but this fungal material was not infective. However, *E. culicis* can be isolated and

grown on several media (Kramer 1982; Papierok and Hajek 1997), but to produce quantities needed for large-scale bioassays and field tests, improvements in culturing methods must be made (Eilenberg 2000).

3.3. *Erynia*

Erynia aquatica (J.F. Anderson and Anagnostakis) Humber is one of the few species within the Entomophthorales that infects aquatic stages of invertebrates. Up to 1981 the species belonged to the genus *Entomophthora* before Humber relocated the species to the genus *Erynia*. Anderson and Ringo found the fungus in 1968 on larvae of *Oc. canadensis* (Theobald), *Cs. morsitans* (Theobald) and *Oc. cantator* (Coquillett) (Andreadis and Magnarelli 1983) as well as on pupae of *Oc. stimulans* (Walker) (Molloy and Wraight 1982). *E. aquatica* is also found on adult mosquitoes, where levels of infection, particularly in overwintering populations of adults, frequently approach 100%. Epizootics have been reported over periods of several years. *E. aquatica*-infected adult *Cx. pipiens* were however capable of flight, taking blood meals, and ovipositing in a study by Anderson and Ringo (1969). The same authors managed to culture *E. aquatica* on an artificial medium, although the fungus grew 'abnormally'. *E. conica* (Nowakowski) Remaudiére and Hennebert is normally found infecting adults of numerous blackfly (*Simulium*) species (Nadeau *et al.* 1996), but it was also able to infect *Ae. aegypti* in a laboratory study (Cuebas-Incle 1992), killing up to 24% of the adult mosquitoes. Two strains of *E. radicans* (Brefeld) Batko (formerly *Zoophthora radicans*) proved to be pathogenic to adult *Ae. aegypti* in a laboratory study (Dumas and Papierok 1989), infecting 100% at the onset of the study, although one strain lost its pathogenicity after several months.

3.4. *Smittium*

Poisson established the genus Smittium in 1936. Nineteen species have been described of which three originate from Culicid larvae: *S. culisetae* Lichtwardt, *S. culicis* Manier and *S. morbosum* Sweeney. The first two species are not generally thought to be detrimental to their hosts as they are confined to the lumen of the gut and are shed with the cuticle during ecdysis (Lichtwardt and Arenas 1996). There are even indications that mosquitoes, infected with *S. culisetae* or *S. culicis*, may be supplied with certain essential nutrients synthesised by the fungus and consequently have a selective advantage over non-infested individuals (Horn and Lichtwardt 1981).

S. morbosum is not shed with gut cuticle at the time of moulting and remains within the host, sometimes persisting through the pupal and adult stages (Sweeney 1981d). In the laboratory, infected *Anopheles* larvae often die because of blockage of the gut by this fungus (Sweeney 1981d). In the same article Sweeney cites two laboratory studies on *Smittium* spp. in which high mortalities were recorded among *An. gambiae* (Coluzzi 1966), *Ae. aegypti* and *Cx. pipiens molestus* (Forskål) (Dubitskii 1978) apparently caused by blockage of the rectum. García *et al.* (1994) found *S. morbosum* infecting three *Aedes*, one *Anopheles*, four *Culex*, a *Mansonia*, *Psorophora*, and one *Uranotaenia* species in Argentina. Despite the records in which *S. morbusum* caused high larval mortalities in the laboratory, they have never been found causing moderate or high mortalities among mosquitoes in the field. . For more detailed information about *Smittium* spp. and their hosts, see Lichtwardt (1986).

4. Deuteromyces

Within the Deuteromycetes a morphological Class of fungi known as Hyphomycetes exists. These are filamentous fungi that reproduce by conidia generally formed aerially on conidiophores arising from the substrate. Many genera of entomopathogenic fungi occur in this group. They have some of the widest of host ranges among entomopathogens, including

several mosquito species. The most common route of host invasion is through the external integument, although infection through the digestive tract is possible (Goettel and Inglis 1997). Conidia attach to the cuticle, germinate, and penetrate the cuticle. Once in the haemocoel, the mycelium grows throughout the host, forming hyphal bodies called blastospores. Death of the insect is often due to a combination of the action of fungal toxins, physical obstruction of blood circulation, nutrient depletion and/or invasion of organs. After the host has died, hyphae usually emerge from the cadaver and, under suitable abiotical conditions, conidia are produced on the exterior of the host. These are then dispersed by wind or water (Goettel and Inglis 1997).

4.1. *Culicinomyces*

In 1972, two separate isolations of mosquito-pathogenic fungi were obtained from laboratory-reared anophelines, one from *An. hilli* Woodhill and Lee in Sydney, Australia (Sweeney *et al.* 1973), and the other from *An. quadrimaculatus* in North Carolina, USA (Couch *et al.* 1974). The Australian fungus was not identified at the time of its discovery but a new taxonomic status was established for the American fungus, which was described as *Culicinomyces clavosporus* Couch, Romney and B. Rao. Both fungi produced similar symptoms in diseased mosquito larvae, but it was not until 1982 when, on the basis of morphological comparison, they were considered strains of the same species (Sweeney *et al.* 1982). In the same year the species name changed from *clavosporus* to *clavisporus* Couch, Romney and B. Rao. A third isolate was found in Canada on larvae of *Cs. inornata* occurring in a permanent pond (Goettel *et al.* 1984) and two years later, in 1984, yet another *Culicinomyces* fungus was found parasitising larvae of *Ae. kochi* in Queensland, Australia. Based on several characteristics described by Sigler *et al.* (1987), it was decided to describe the latter fungus as a new species, namely *C. bisporalis* Sigler, Frances and Panter. Most research to date has focused on *C. clavisporus.*

As in all Deuteromycota, the life-cycle of *Culicinomyces* is asexual. It usually begins with the ingestion of conidia that adhere to and penetrate through the chitinous wall in the fore- or hindgut (Sweeney 1981a, Federici 1981). This invasion is unusual among parasitic fungi that normally invade insects by penetrating the cuticle. Following invasion the fungus colonises the body cavity with mycelium of hyaline, septate, branched hyphae. Larvae may be killed within 2-7 days by growth of hyphae throughout the haemocoel, or within 2 days after ingestion of a high concentration of conidia ($>10^5$ conidia ml^{-1}). In the latter case larvae died before the haemocoel was colonised by mycelium. The reason for this rapid death at high concentrations of inoculum is not known, but might be caused by toxic substances associated with the invading hyphae, which only attain a lethal titer when massive invasion originates from large numbers of conidia (Sweeney 1983). After death of the larva, hyphae penetrate through the external cuticle to form a layer of conidiophores on the outside of the cadaver. These conidiophores produce conidia, most frequently at the posterior abdominal segments, which are infective to healthy larvae. Debenham and Russell (1977) demonstrated that an infection of *C. clavisporus* originating in larvae could be carried over into the adult stage. It has been postulated that infection of the adult mosquito may be important in dispersal of the fungus (Goettel *et al.* 1984).

The fungus is a facultative parasite of a wide range of mosquitoes and related Dipteran larvae (Legner 1995), though in the field only five mosquito species have been found infected, these being *Oc. rupestris* Dobrotworsky (Russell *et al.* 1978), *Oc. rubrithorax* (Macquart) (Frances 1986), *An. quadrimaculatis* (Couch *et al.* 1974), *Cs. inornata* (Goettel *et al.* 1984), and *Cs. inconspicua* (Lee) (Frances 1986). A study on the Australian isolate has shown that within the Nematocera, only members of the Division Culicimorpha were susceptible (families Culicidae, Chironomidae, Ceratopogonidae, and Simuliidae), whereas

species of the Division Psychodomorpha (family Psychodidae) and Tipulimorpha (family Tipulidae) were not susceptible (Sweeney 1979). Both laboratory and field studies have shown that the Australian isolate of the fungus is highly lethal to all larvae of the genera *Anopheles*, *Culex* and *Aedes* (Sweeney 1981b). It proved also lethal to the brackish-water species *An. farauti* and *An. amictus hilli* Woodhill and Lee when the larvae are reared in fresh water (Sweeney 1978b). The host range of the American strain of *C. clavisporus* includes species of Simuliidae, Chaoborinae, Ceratopogonidae, Chironomidae, Ephydridae, and Syrphidae (Knight 1980). No difference was found in virulence between the American and the Australian strains on *An. hilli*, *Cx. quinquefasciatus*, and *Ae. aegypti* (Cooper and Sweeney 1982), but a between-species comparison, with the data pooled for both strains, showed that *Ae. aegypti* was more susceptible to the fungus than *An. hilli* (Cooper and Sweeney 1982).

In the laboratory, sporulation of *C. clavisporus* developed on 87-95% of first instar *Cx. quinquefasciatus* larvae within 5 days after exposure to the fungus. Conidia may remain on the cadavers for more than 3 weeks, after which their viability is still 70-90% (Cooper and Sweeney 1986). In the field however, conidia persist and remain infective for only several days (Sweeney and Panter 1977; Frances *et al.* 1984), up to two weeks (Sweeney 1981b, 1983), suppressing a larval population of *Cx. australicus* Dobrotworsky and Drummond for no more than one generation (Sweeney 1981b). It has been suggested that the application of doses lower than those used in previous tests may increase the proportion of larvae that develop sporulation. Evidence for this was obtained in laboratory observations, which showed that exposure of larvae to 10^3 conidia ml^{-1}, produced greater external sporulation on larval cadavers than exposure to 10^6 conidia ml^{-1} (Cooper and Sweeney 1986). These findings have implications for possible field use of the fungus for biological control, as the latter result would provide more favorable prospects for recycling (Cooper and Sweeney 1986).

The effect of this fungus on mosquito field populations has been shown to be variable and difficult to predict (Service 1983). Several factors influence the level of pathogenicity of *C. clavisporus* to mosquito larvae. Age and species of mosquito larvae have an effect on the degree of susceptibility, with early instar larvae being considerably more susceptible to infection than older instars (Sweeney 1981b, 1983), and anophelines being less susceptible than culicines. Even between different batches of *Anopheles* larvae, considerable differences in susceptibility to *Culicinomyces* have been found (Sweeney 1981b), and amongst culicines, *Ae. aegypti* was found to be far more susceptible than *Cx. quinquefasciatus* (Cooper and Sweeney 1982). Some of these differences may be due to settling of the conidia on the bottom of the assay cups: Conidia of *Culicinomyces* settle out of suspension at a rate of approximately 8 cm/day (Sweeney 1981b). This suggests that the conidia are more accessible to bottom-feeding *Aedes* than to surface-feeding *Anopheles* (Sweeney 1981b; Cooper and Sweeney 1982).

In order to control mosquito populations, conidial suspensions have been effective, but large numbers of conidia are required. In the laboratory, concentrations of 10^4-10^6 conidia ml^{-1} have been found sufficient to cause 100% mortality of mosquito larvae (Sweeney and Panter 1977). In a field experiment, using an aqueous suspension of 10^6 conidia ml^{-1}, 100% of the larvae were killed within 5 days (Sweeney and Panter 1977). Suspensions of 5 x 10^9 and 10^{10} conidia m^{-2} applied to artificial ponds containing caged, laboratory-reared mosquito larvae produced 100% mortality of *Cx. quinquefasciatus* and 68-100% mortality of *An. annulipes* Walker larvae (Sweeney 1981b). A field experiment, using a dose of 10^{10} conidia m^{-2} applied to a 300 m^2 pond produced 90-95% control of late instar *Cx. australicus* during the first week after application. However, the population recovered thereafter, showing no evidence of residual activity of the fungus. Larvae of *An. annulipes*, which were present in low density, were not controlled by the fungus in this trial (Sweeney 1981b).

Experiments with the Australian isolate have outlined its mode of infection, and abiotic requirements for growth and infection of mosquitoes. Optimum temperatures for germination and for growth are 27.5 and 25 °C, respectively. Infection of larvae by this fungus will readily occur at temperatures between 15 and 27.5 °C, but not at 30°C (Sweeney 1978a). The fungus may therefore not be effective against larvae occupying water of which the temperature consistently exceeds 30 °C (Sweeney 1978a). This optimum temperature range is seen in the field, where the majority of natural infections with *C. clavisporus* occur during the cool season (Lacey and Undeen 1986). High salinity and organic pollution inhibits the ability of *C. clavisporus* to infect larvae (Sweeney 1978b).

Large numbers of conidia may be produced by growing the fungus in surface culture on simple and relatively inexpensive artificial media (Lacey and Undeen 1986; Goettel *et al.* 1984) and the complex procedures required for *Lagenidium* and *Coelomomyces* production are unnecessary. Prolonged subculture on artificial media has apparently not diminished the pathogenicity of *C. clavisporus* to mosquitoes (Cooper and Sweeney 1982), although this was contradicted by the same authors (Cooper and Sweeney 1986) showing in comparative bioassays that conidia produced *in vivo* were more potent than those produced *in vitro* in four out of six assays using *Ae. aegypti* larvae as test insects. Submerged culture in 20-750 L fermentors has also produced successful inocula for field trials (Sweeney 1981b). However, problems in storage must be overcome if this fungus is to be widely used (Legner 1995). Aqueous suspensions of conidia lose their infectivity for mosquito larvae after several weeks of storage at 25 °C (Sweeney 1981c) and after 4 months of storage at –20 °C, but not at –70 °C. However, according to Lacey and Undeen (1986), storage at –70 °C is not economically feasible for large-scale field application, which necessitates the development of a suitable protective medium. Another drawback of *C. clavisporus* is the high dosage required (100-1000 L ha^{-1}) for effective control (Service 1983; Lacey and Undeen 1986), and even though conidiogenesis takes place in infected mosquito larvae, persistence of the conidia in the environment is short. Other problems include the relatively high variability and unpredictability of results in the field (Service 1983), the fact that infected larvae are sometimes able to free themselves of the infection when they molt, and the intolerance of the fungus to high salanities (Service 1983). To enhance the potential of this microbial control agent, improvements should be made to develop new culture media, economic storage conditions that can prolong the stability of infective stages, and to develop formulations that increase the likelyhood of contact with target species, such as flotation in *Anopheles* habitats (Lacey and Undeen 1986). In spite of the selectiveness of *C. clavisporus* to kill mosquito larvae, these problems have led to a reduced interest in this species, and research ground to a halt in the mid-1980's. Only recently Campbell *et al.* (2002) focused on this species again by studying a genetic transformation system that forms the basis for genetic manupilation of this fungus for potential use in biocontrol of mosquitoes.

4.2. *Beauveria*

Beauveria is one of the most frequently isolated entomopathogenic fungal genera and has a cosmopolitan distribution. Although the genus has a very broad host range (Roberts 1974), the natural occurrence of *Beauveria* on mosquitoes has been reported only four times, three of which were *B. bassiana* (Balsamo) Vuillemin: Two cases were mentioned by Clark *et al.* (1968) infecting *Cx. tarsalis*, *Cx. pipiens* and *An. albimanus*, and one by Pinnock *et al.* (1973), infecting *Oc. sierrensis* (the original paper used the synonym *B. tenella* (Delacroix)). The other species was *B. brongniartii* (Saccardo) Petch, causing the only known epizootic of this genus in mosquitoes in a field population of *Oc. sierrensis* (Pinnock *et al.* 1973). Conidia of *B. bassiana* are effective in killing mosquito larvae when applied as a conidial dust to the

water surface of breeding sites (Clark *et al.* 1968). Conidia are hydrophobic, thus they float on the water surface, and contact mosquito larvae that feed below the surface mainly at the tip of the siphon, although Miranpuri and Khachatourians (1991) found the head to be an equally important infection site.

In laboratory tests the fungus proved virulent against larvae of *Cx. pipiens*, *Cx. tarsalis*, *Cx. tritaeniorhynchus* and *An. albimanus*, but was ineffective against *Ae. aegypti*, *Oc. sierrensis* (Clark *et al.* 1968; Sandhu *et al.* 1993; Geetha and Balaraman 1999), and *Cx. quinquefasciatus* (Alves *et al.* 2002). Susceptible species were prone to infection only shortly after moulting. If infection occurred shortly before moulting, the mycelium was lost within the molt. In three small-scale outdoor tests with conidia of *B. bassiana*, reductions of 82, 95 and 69% were found on *Cx. pipiens* larvae and pupae after two weeks (Clark *et al.* 1968). Studies on treeholes treated with blastoconidia (5 x 10^3 or 5 x 10^5 conidia ml^{-1} water) of *B. brongniartii* delivered reductions between 53 and 71% of emerging adult *Oc. sierrensis* (Pinnock *et al.* 1973). Laboratory studies showed that besides *Oc. sierrensis*, the fungus is pathogenic for larvae of *Ae. aegypti*, *Oc. dorsalis* (Meigen), *Oc. hexodontus* Dyar, *Cx. pipiens*, *Cx. tarsalis* and *Culiseta incidens* (Thomson) (Pinnock *et al.* 1973). Besides infecting larvae, the fungus proved to be virulent to adult mosquitoes as well, although no *Beauveria*-infected adult mosquitoes have been reported from the field. In laboratory tests against adult *Cx. tarsalis*, *Cx. pipiens*, *Ae. aegypti*, *Oc. sierrensis*, *Oc. nigromaculis* (Ludlow), and *An. albimanus*, conidia of *B. bassiana* produced 100% mortality within 5 days after exposure, while less than 50% occurred in corresponding controls (Clark *et al.* 1968). In laboratory tests 82% of adult *An. gambiae* were infected, with an LT_{50} of 3.5 days, against 8.8 days from the control group (Scholte *et al.* 2003). Outdoor tests against adult *Oc. nigromaculis* in screen cages were less successful, yielding only 58% mortality (Clark *et al.* 1968). In this case, the fact that adults rested on the screen walls of their test cages, rather than in the dusted grass that had been provided, may explain the low mortality. Sections of infected adults, fixed and embedded in paraffin immediately after death, revealed some mycelium in regions surrounding the main tracheal trunks. It appeared that the conidia entered through the spiracles, germinated, invaded the walls of the tracheae, and subsequently were thought to release a toxin, which killed the adults (Clark *et al.* 1968). Later studies confirmed the existence of toxins produced by species of this genus; Beauvericin, Bassianin, Bassianolide, Beauverolides, and Tenellin from *B. bassiana*, and Oosporein from *B. brongniartii* (Ferron 1981; Grove and Pople 1980; Strasser et al., 2000). A dichloromethane extract from mycelium showed activity when assayed against *Ae. aegypti* larvae at 100 ppm (Gupta *et al.* 1995). This extract contained Beauvericin and two analogues (Beauvericin A and B). The larvicidal properties of Beauvericin had already been reported by Grove and Pople (1980), who found 86% mortality with *Ae. aegypti* larvae after 48 hrs at 20 μg ml^{-1}, but only 39% when 10 μg ml^{-1} was used. These destruxins pose no obvious risk to humans, and the use of this fungus does not result in toxin levels harmful to the environment (Strasser *et al.*, 2000).

Humidity is considered as one of the critical factors affecting the outcome of both laboratory and field tests with *B. bassiana*. Clark *et al.* (1968) mention a study by Hart and MacLeod (1955), who found optimal germination of *Beauveria* conidia occurring at relative humidities above 94%. Infection does not appear to be dependent on temperature (Schaerffenberg 1964; Ferron 1981). For conidia however, high temperatures may be harmful, especially in combination with high humidity conditions. The effective stages of the fungus against larvae are conidia and blastoconidia, the latter stage being far more pathogenic (Miranpuri and Khachatourians 1991). Although growing blastoconidia is relatively easy, production has been abandoned because of the difficulties of storing this type of conidium (Ferron 1981). To produce conidiospores, a two-stage technique for mass production can be used. A pilot factory in Krasnodar (former Soviet Union) was able to produce 22 tons of

Boverin annually (*B. bassiana* conidia plus an inert carrier, standardized at 6 x 10^9 conidia g^{-1}) (Ferron 1981). Other products from *B. bassiana* include Mycotrol™, ESC 170 GH™, and Naturalis-O™, targeted against whitefly, aphids, thrips, mealy bugs, leaf hoppers and leaf-eating insects, but not mosquitoes or other Dipterans (Khetan 2001). Ago Biocontrol® provides a *B. brongniartii*-product in Colombia that among others also targets Diptera, but fails to specify if Culicidae are susceptible. A problem associated with using conidia is that they have no residual effect. They germinate in mosquito habitats even when not in contact with larvae. This limitation, along with the high dosage needed are serious drawbacks for mosquito larval control. Formulation with oil may overcome some of the problems. *B. brongniartii* was considered as potential biological control agent since this species is pathogenic for larvae of several tree-hole breeding mosquito species, including *Ae. aegypti*, *Oc. dorsalis*, *Oc. hexodontus*, and *Oc. sierrensis*. However, the large doses required for significant mortality rates of mosquito larvae was not considered practical (Pinnock *et al.* 1973; Chapman 1974). Moreover, another problem concerning the use of *Beauveria* would be its safety to vertebrates. Sensitivity has been reported by persons repeatedly exposed to massive amounts of *B. bassiana* conidia preparations (Ignoffo 1967). However, long-term rodent tests with *B. bassiana* conidia, including inhalation tests, subcutaneous injection, and oral toxicity tests, did not show adverse health effects.

4.3. *Metarhizium*

Metarhizium, like *Beauveria*, is one of the most common entomopathogenic fungi, with a worldwide distribution. The species is soil-borne and infects predominantly soil-dwelling insects. Taxonomy of *Metarhizium* is not straight forward. The current classification of the taxon is mainly based on the morphology of conidia and conidiogenous cells. Some authors combine these with biochemical and molecular characteristics (Riba *et al.*, 1986), and/or host pathogenicity, cold-activity and sporulation color (Yip *et al.*, 1992; Rath *et al.*, 1995). Driver *et al.* (2000) used 10 different 'Clades', based primarily on molecular data, although this leaves room for debate. *M. anisopliae* consists of 4 varieties (Driver *et al.*, 2000), two of which are considered important, these being *M. anisopliae* var. *acridum* (previously designated *M. flavoviride)* found mainly on Homoptera and *M. anisopliae* var. *anisopliae* (Metschnikoff) Sorokin, the latter being the best known of the two species. *M. anisopliae* has a large host-range, including arachnids and five Orders of insects (Boucias and Pendland 1998), comprising over 200 species. Mosquitoes are not listed as natural hosts for *M. anisopliae* (Veen 1968) but some strains have been shown to be virulent against mosquito larvae (Roberts 1967, 1970, 1974; Ramoska 1982; Agudelo-Silva and Wassink 1984; Daoust and Roberts 1983a,b; Ravallec *et al.* 1989; Sandhu *et al.* 1993; Alves *et al.* 2002).

On terrestrial insects, the life-cycle begins with a conidium attaching to the host cuticle, forming an appressorium, followed by a penetration peg to enter the cuticle. After entering the haemocoel, hyphae are formed that produce and release toxins, killing the host 4-16 days (depending mainly on the host species) after contamination (Ferron 1981; Khachatourians 1991; Boucias and Pendland 1998). These toxins include Destruxins, Swainsinone, and Cytochalasin C (Strasser *et al.* 2000). Histopathological studies of elaterid tissues infected by *M. anisopliae* suggest that toxins (destruxins) kill the host by inciting degeneration of the host tissues due to loss of the structural integrity of membranes and then dehydration of cells by fluid loss (Ferron 1981). If the conditions are warm and moist, conidiophores will grow through the cuticle to cover the insect with conidia. The cycle in mosquito larvae varies from the above. If floating conidia are applied, larvae will contact them when they break the water surface with their perispiracular valves for air intake. The fungus germinates and penetrates into the respiratory siphon, blocking the breathing mechanism (Daoust *et al.* 1982; Lacey *et al.* 1988). Plugging of the spiracles usually leads to

death before significant invasion of the haemocoel has occurred, so hyphal body formation is minimal. Cadavers in the aquatic environment are overrun with bacteria rather than mycelium, and no new conidia are formed. Although much less frequently observed, larvae can also ingest dry conidia (Crisan 1971; Roberts 1974), where they, apparently without germination, release lethal substances into the gut (Crisan 1971; Roberts 1970, 1974). A mixture of these toxins (70% destruxin A and 30% B) proved toxic to mosquito larvae, with LD_{50} values of 10 to 100 ppm (Roberts 1974).

Many laboratory studies have shown the potential of *M. anisopliae* as a mosquito control agent. Roberts (1970) observed effects on larvae of *An. stephensi*, *An. quadimaculatus*, *Ae. aegypti*, *Oc. atropalpus*, *Oc. taeniorhynchus*, *Cx. pipiens*, *Cx. restuans*, and *Cx. salinarius*, and found all species susceptible to (unformulated) conidia. In a laboratory experiment reported by Ramoska (1982), the fungus suppressed *Cx. quinquefasciatus* larval populations for nearly a month. On the other hand, the strain used by Alves *et al.* (2002) had lost its effect on the same mosquito species after only 3 days. Daoust and Roberts (1982), found that over half of 52 strains from a variety of hosts taken from nine countries caused more than 50% mortality of *Cx. pipiens* larvae treated with 1 mg dry conidia per 16 cm^2. The strains most virulent to *Cx. pipiens* proved to be highly pathogenic to larvae of *Ae. aegypti* and *An. stephensi* as well. In the same study it was shown that virulence of strains towards mosquitoes could increase 1.6 - 2.5 times by passage through mosquito larvae. In small-scale outdoor tests, using 300 or 600 mg of conidia m^{-2} in small artificial ponds reduced *Cx. pipiens* by 91% and 94% and *Oc. sollicitans* by 85% and 98% within three days (Roberts 1974). Besides larvae, also adult mosquitoes proved to be susceptible to the fungus. Recently, adult *Cx. quinquefasciatus* and *An. gambiae s.s.* were infected in a laboratory study. Both species proved susceptible and succumbed to infection with unformulated dry, and oil-formulated conidia, with LT_{50} values ranging from 4-6 days (Scholte *et al.* 2003a,b). A small-scale field study showed that the fungus also infects and kills wild *An. gambiae s.l.* (See Chapter 9).

The optimal growth temperature for most strains is 27-28°C (Ferron 1981), although some exceptions of cold-resistant and heat-resistant strains have been reported (Bidochka *et al.* 1998; Boucias and Pendland 1998). Conidia normally require a relative humidity of at least 92% to germinate (Ferron 1981). Conidia that are stored under dry conditions show higher germination rates (initially 96%, dropping to 80% after 60 days) than conidia formulated in paraffin oil (from 93% to 73%) (Morley-Davies *et al.* 1995). Conidia are found to survive longest at a combination of moderate temperatures and high RH (26 °C-97 % RH or 19°C-97 % RH) or low temperature and low RH (4°C-0 % RH) (Daoust and Roberts 1983a). The fungus can easily be grown *in vitro* (Goettel and Inglis, 1997), and storage conditions are more critical to spore survival and virulence than the substrate upon which conidia are produced (Daoust and Roberts 1983b). When relative humidity is high, conidia can be quite tolerant to high temperatures (Zimmerman 1982; Morley-Davies *et al.* 1995). However, conidial viability decreases rapidly when exposed to UV light (Zimmermann 1982; Morley-Davies *et al.* 1995). In a conidial viability experiment using a sunlight simulator at 40°C, germination ranged between 10 and 50% after 24 hrs of exposure to UV (Morley-Davies *et al.* 1995).

Several types of conidial formulations have been developed and tested for mosquito larval control including granulars, dusts, and wettable suspensions (Daoust *et al.* 1982). Ramoska *et al.* (1981) compared two formulations, floating and sand, and found that mortality occurred sooner using the sand formulation with mosquitoes that fed below surface while the floating preparation worked faster on surface feeders. When suspended in an aqueous suspension containing a surfactant, or when formulated with granular carriers or dust diluents, *M. anisopliae* conidia tend to lose virulence compared to unformulated conidia against *Cx.*

pipiens larvae. In contrast, a diluent derived from dried castor oil significantly enhanced conidial virulence (Daoust *et al.* 1982).

M. anisopliae has several characteristics which make it promising as a microbial mosquito control agent. It causes high mortality of mosquito larvae in laboratory populations, the fungus can be grown in massive amounts on inexpensive artificial media, and conidia can be stored easily. Moreover, its failure to germinate in the mosquito environment until actual exposure to a host and its resulting persistence in the environment, as well as the fact that its effect is not limited to periods of host moulting (like for *B. bassiana*), make this fungus a very promising control agent (Roberts 1970). The fungus is commercially produced by Biocare, Australia, BCP, South Africa, Bayer, Germany (BIO 1020®), and several Brazilian companies, as control agents for German cochroaches and termites, black vine weevil, citrus root weevil, sugarcane pests and *Aeneolamia varia* saccharina Dist., but not mosquitoes (Khetan 2001).

One drawback in the use of *M. anisopliae* for mosquito control is that conidia are not produced on submerged, fungus-killed larvae, and it is presumed that inundative releases would have to be repeated frequently. This would be less convenient than a single introduction that maintains itself, like *Coelomomyces* and *Lagenidium*. Another disadvantage, as with all fungal agents, is that the conidia must contact the larvae physically. Since these are particles rather than solutions, such contact may be difficult in some situations. Zimmermann (1993) claims that because of absence of toxicological or pathological symptoms in birds, fish, mice, rats, and guinea pigs after exposure to conidia of the fungus, *M. anisopliae* was safe. Also Strasser *et al.* (2000) conclude from a risk-assessment study that the fungus poses no obvious risk to humans, or the environment. However, it has been reported that the fungus may cause human keratitis (DeGarcia *et al.* 1997). Other work has shown that the fungus can cause significant mortality of shrimp, frog and fish embryos exposed to conidia (Genthner *et al.* 1994, 1998). The latter effects, however, were not revealed in earlier studies where guppies, exposed to massive amounts of conidia, were not affected for periods up to two months (Roberts 1974). Finally, regarding its broad host spectrum, use of conidia for mosquito control should be done with caution to keep the risk of contaminating non-target insects as low as possible.

4.4. *Tolypocladium*

Tolypocladium cylindrosporum W.Gams was first isolated from *Oc. sierrensis* in California in 1971 (Soarés 1982), and later from *Oc. australis* in New Zealand (Weiser and Pillai 1981), and is a pathogen of mosquito larvae. In one study it appeared that also adult *Oc. sierrensis* were susceptible to infection (Soarés 1982), killing 50% within 5, and 100% of exposed mosquitoes in 9 days. The fungus caused over 90% larval mortality of *Oc. sierrensis* and 67% in *Cx. tarsalis* at 5 x 10^6 conidia ml^{-1} in a laboratory study (Soarés 1982). Both blastoconidia and aerial conidia are infectious to mosquito larvae, but blastoconidia are more virulent (Goettel 1988; Nadeau and Boisvert 1994). They invade the host predominantly through the exterior cuticle, but also through ingestion (Soares *et al.* 1979; Weiser 1988). Nineteen mosquito species were found susceptible to the fungus, including ten *Aedes* spp., six *Culex* spp., two *Culiseta* spp., and one *Anopheles* sp. (Goettel 1987b). Weiser *et al.* (1991b) demonstrated >80% mortality in mosquitoes from *T. terricola* Weiser, Matha and Jegorov after 8 days. Soarés (1982) suggested that mosquitoes may die of toxic metabolites excreted by the fungus. The existence of such a metabolite, named tolypin, was described by Weiser and Matha (1988), who found symptoms of intoxication (knock-down) of *Cx. pipiens* larvae during the first hour of exposure to concentrations of 0.026-0.4 mg ml^{-1} in water.

It was suggested that *T. cylindrosporum* was a candidate for control of mosquito and blackfly larvae in aquatic habitats in temperate areas (Goettel 1987a,b). Soarés (1982),

however, suspected that replication of laboratory results with this fungus in the field could be expected only under a narrow range of conditions, which would not make the fungus practical for mosquito control. Field studies in New Zealand (Gardner and Pillai 1987) and in Alberta, Canada (Goettel 1987b), did indeed show evidence that the fungus was not very effective. Moreover, apparent lack of residual activity (Gardner *et al.* 1986) decreased the interest in this fungus for biocontrol of larval mosquito populations.

Other Anamorphic fungi that have been found on mosquitoes include some species of the genera *Aspergillus*, *Fusarium*, *Paecilomyces*, *Penicilium*, and *Verticillium* (Hasan and Vago 1972; Roberts and Strand 1977; Ballard and Knapp 1984; Sur *et al.* 1999; Agarwala *et al.* 1999; Scholte *et al.* 2003a). *Fusarium oxisporum* Schlecht has been isolated a number of times from *Oc. detritus* (Haliday), (Breaud *et al.* 1980; Hasan and Vago 1972) and proved to be virulent to *Cx. pipiens* in the laboratory, but later reports on the fungus are scarce.

Most of these infections however are either rare or the particular fungi are not considered highly pathogenic to mosquitoes.

DISCUSSION

The search for effective mosquito pathogens that can be used in mosquito control operations has been ongoing for several decades. Both laboratory and field studies of those fungi that appeared to have potential for operational use, have been evaluated. If promising, this was followed by development of methods for mass production and finally implementation in operational control programs, although few fungal species have reached this latter stage of development. From the studies on fungi reviewed in this chapter, three common characteristics emerge. First, pathogens are mainly effective against the larval stages of mosquitoes. Second, effective control requires repeated rather than a single application of the agent during the mosquito breeding season, and third, vector control programmes can only be cost-effective if the control agent can be produced *in vitro* (Federici 1995).

Concerning mosquito-pathogenic fungi, three genera are generally considered important; *Lagenidium*, *Coelomomyces*, and *Culicinomyces* (Roberts 1974; Lacey and Undeen 1986; Federici 1995). Each one of these (and others, discussed in this chapter) has one or more traits useful for mosquito control, but none of them possesses the full array of properties needed for general application and cost-effective control. *Coelomomyces* spp. are very effective in killing many mosquito species, although individual species have narrow host ranges, and have often been reported to cause epizootics, with the major obstacle of dependence on *in vivo* production, making mass-production difficult. *Lagenidium giganteum* can probably be regarded as the fungus with the best properties for larval mosquito control, though only for stagnant waters, like ricefields. The principle advantage of *Lagenidium* over the bacterium *Bacillus thuringiensis israelensis* (*Bti*) is that only a single application is required per season. Even less frequent application may be possible in some habitats, as there are indications that oospores can hibernate, initiating epizootics the following season (Federici 1995). Although low levels of infection are often observed after a *L. giganteum* population is established, recurrent epizootics have been reported (Glenn and Chapman 1978). It is the only fungus that has been produced commercially as a mosquito control agent (Laginex^{TM}). The fungal zoospores have been claimed to infect larvae of all species of mosquitoes but to be especially effective against *Culex* spp. (Khetan 2001). *Culicinomyces clavisporus* excited considerable interest initially, but this cooled down when the high dosages required for effective control and the low persistence of conidia in the environment became apparent (Service 1983; Lacey and Undeen 1986).

Many mosquito-pathogenic fungi are expected to be used for the control of medically important vector species (Service 1983; Lacey and Undeen 1986; Federici 1995). Unfortunately, none of the fungi described above are specifically adapted as a larvicidal agent against important vector species such as the African malaria vectors *An. gambiae s.l.*. The larval habitats of these mosquito species include a variety of transient, mainly sunlit, rain-water pools, like borrow-pits, drains, brick-pits, car-tracks, foot-and hoofprints around ponds and water-holes (Van Someren *et al.* 1956; Coene 1993; Lyimo 1993). Most of these sites are transient, and in some areas breeding is highly seasonal, following the rainfall pattern of that specific area. It is very unlikely that under normal field conditions, larvae of this mosquito species would contact any of the aquatic fungi discussed above, even though some infected females may contaminate a breeding site during oviposition. Moreover, *Lagenidium*, *Coelomomyces*, and *Culicinomyces* are all aimed at the larval stages of mosquitoes and not at the adult stage. In the field, mortalities of immature mosquitoes can commonly be 95% or more, yet the numbers of emerging adults may still be sufficient for maintaining substantial disease transmission. The important issue is how adult populations are affected (Service 1983). Although not often, and generally not considered to be of importance, some aquatic fungi have been found on adult mosquitoes, including species of *Coelomomyces*, *Culicinomyces* and *Smittium*. In these situations the infection took place on late-instar larvae or pupae, resulting in survival of the mosquito into the adult stage. They probably do not cause considerable adult mortality, but infected adults may disperse the fungus to other habitats (Lucarotti and Andreadis 1995; Shoulkamy *et al.* 1997). Pathogenic fungi that occur naturally on adult mosquitoes belong mainly to the Entomophthorales. Some species have caused epizootics in hibernating mosquitoes in relatively cool, humid, dark places in the northern hemisphere (Weiser 1988). Unfortunately, spores are short-lived. Moreover, the fungus' inability to grow *in vitro* makes it an unsuitable control agent. There are, however, some fungi that are normally not associated with mosquitoes, but have proven to be highly pathogenic to adult mosquitoes. The Hyphomycetes *Beauveria bassiana* (Clark *et al.* 1968) and *Metarhizium anisopliae* kill adult mosquitoes in the laboratory (Scholte *et al.*, 2003a) and the latter fungus also killed adult *An.gambiae s.l.* in the field in Tanzania (See Chapter 9) However, practical application methods need to be improved. We consider the potential for using Hyphomycetes such as *Metarhizium* or *Beauveria* for biocontrol of, especially African, vector mosquitoes is high: These fungi can be cost-effectively mass-produced (also locally) and many strains are already commercially available, circumventing the time-consuming and costly process of registration, including risk-assessment of 'new' fungal control agents. *Beauveria* (*B. bassiana* and *B. brongniartii*) are produced by more than 14, and *Metarhizium* (*M. anisopliae* and *M. anisoplaie* var. *acridum*) by more than 10 companies (including some in Africa), aimed at controlling various insect pests including termites, cockroaches, black vine weevil, white flies, aphids, corn borers, cockchafers, tsetse, locusts and other insects (Khetan 2001; Strasser *et al.*, 2000; Wraight *et al.* 2001). Moreover, the development of ULV-CDA formulations of conidia to control locusts in dry climates (Bateman 1992) opens up possibilities for the use of fungi to control mosquito vector species in dry areas in e.g. sub-Saharan Africa such as *An. gambiae* and *An. funestus* (malaria), *Ae. aegypti* (yellow fever, dengue), and *Cx. quinquefasciatus* (filariasis, viral encephalites).

Concerning the future of myco-insecticides, Burgess (1998) points out: "Improvements in shelf-life duration and formulations is the key to the future and should enable fungi to compete in efficacy with chemical insecticides on nearly equal terms, and should increase projected market sizes towards industrial viability". He then continues with "Budgets should cater for adequate research on strain viability, including reaction to formulation requirements. Research should target three areas that need improvement: application, storage, and production".

In summary, an ideal fungus for mosquito control should have the following characteristics. It a) kills both larval and adult stages, b) requires only one or a few applications per season, c) is actively dispersed by adult females to previously unoccupied breeding sites, d) shows residual activity and persistence in the mosquito population after introduction, e) selectively kills mosquitoes and no other organisms, f) is effective over a large range of salinities, temperatures, relative humidities and breeding sites with variable water quality, g) is easily and cost-effectively mass-produced and formulated, h) retains prolonged activity during storage (long shelf-life), and i) is not harmful to humans and other non-target organisms. None of the mosquito-pathogenic fungi presently known exhibit all these characteristics, but they all exhibit at least some. To determine which fungus could be used for the control of mosquito populations depends on many factors such as the biology of the target mosquito species, its distribution (isolated or uniform), the life-stage targeted, suitability of the ecosystem for survival and viability/virulence of the fungus (biotic and abiotic factors), application methodology and costs thereof, storage facilities, whether or not the fungus can be mass-produced, and whether or not the fungus is registered and available on the market. One question that needs to be addressed is the extent to which it is acceptable to risk infections in non-target organisms, and whether there may be other foreseeable side-effects.

In entomopathogenic fungi we find a huge area of only partially explored natural pathogens and undeveloped methodologies to apply for mosquito control. Entomogenous fungi, without the assistance of man, have been found affecting substantial proportions of many mosquito populations all over the world. This suggests that manipulation, such as increasing inoculum, could make fungi more efficient in mosquito control (Roberts 1970). Facing major problems associated with mosquito-borne human diseases (drug resistance, lack of vaccines) and mosquito control (especially insecticide resistance) it is of utmost importance to search for new/alternative agents and methods. In our view, entomopathogenic fungi, both new and existing ones with renewed/improved efficacies may contribute to an extension of the limited arsenal of effective vector control tools. These new agents and/or methods should preferentially be applied in integrated control strategies in order to gain maximum impact both on larval and adult mosquito populations.

CHAPTER 3

PATHOGENICITY OF FIVE EAST AFRICAN ENTOMOPATHOGENIC FUNGI AGAINST ADULT *ANOPHELES GAMBIAE S.S.* MOSQUITOES (DIPTERA, CULICIDAE)

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PATHOGENICITY OF FIVE EAST AFRICAN ENTOMOPATHOGENIC FUNGI AGAINST ADULT *ANOPHELES GAMBIAE S.S.* MOSQUITOES (DIPTERA, CULICIDAE)

ABSTRACT

Five different insect-pathogenic fungi, isolated in West Kenya, were screened for their pathogenicity to adult *Anopheles gambiae* Giles *sensu stricto* mosquitoes. The tested entomopathogens belong to the Hyphomycetes (Deuteromycota): *Beauveria bassiana* (Vuillemin), a *Fusarium* sp. (Link) and three different strains of *Metarhizium anisopliae* Metsch. (Sorokin). Infection percentages ranged from 46.5 ± 3.6 % (*M.anisopliae* soil sample) to 88.7 ± 3.3 % (*M. anisopliae* IC30). LT_{50} values ranged from 3.39 ± 0.37 (*M. anisopliae* IC30) to 5.87 ± 0.77 days (*M. anisopliae* soil sample) and were all significantly lower (p=0.003) than the control group (8.80 ± 0.65 days). Our results indicate that the IC30 strain of *M. anisopliae* is the most pathogenic of the fungi tested in this study, and therefore is considered the most appropriate fungus to be used for development of biological control strategies for adult African malaria vectors.

INTRODUCTION

Malaria is considered the most important mosquito-borne disease in the world, affecting millions of people, especially in sub-Saharan Africa, where 90% of the annual death toll from this disease, estimated at 0.5-3 million, occurs (WHO, 2000). A public health crisis due to resistance against affordable drugs (Trape *et al.*, 2002) and commonly used pyrethroid insecticides (Zaim & Guillet, 2002) is looming and calls for an expansion of the limited arsenal of environmentally safe vector control methods that can be used against *Anopheles gambiae* Giles *s.l.* (Diptera: Culicidae), Africa's most important malaria vector.

Considering that a female mosquito from the time of first infection will visit the house environment at least three times for a blood meal before transmission of malaria can occur, it has been argued that control should focus on adults in or near houses rather than on immature stages (MacDonald, 1957). The two techniques mostly applied for adult mosquito control are dependent on the use of residual insecticides either on bednets or on walls/roofs of houses, thus targeting the female mosquito either before or after she takes a blood meal. Though both methods have delivered major successes in curbing malaria morbidity and mortality (Lengeler, 1998), they do have fairly major limitations both technically (Zaim & Guillet, 2002), environmentally (WWF, 1998; WHO, 1999) and in terms of acceptance/cost (Karanja *et al.*, 1999).

It may thus be worthwhile to develop new methods that target adult females when visiting the house environment, preferably without the use of insecticides. Insect pathogens, like fungi, have been evaluated against disease vectors such as tsetse flies (Glossinidae; Maniania, 1998) and the search for effective species against mosquito vectors is ongoing. However, only a few fungi have been found on adult stages of mosquitoes in nature. Most of them belong to the order Entomophthorales (Zygomycota) (Roberts, 1974, Humber, 1997) such as *Entomophthora culicis* (Braun) (Kramer, 1983) and *Conidiobolus coronatus* (Cost.) Kev. (Low and Kennel, 1972), although these species have not been reported from Africa.

Moreover, these fungi are hard to culture *in vitro* and are therefore not considered practical for mosquito control. A few Deuteromycetes have been found on adult mosquitoes (Sur *et al.*, 1999), but it is generally assumed that fungal infections in the field other than Entomophthorales on adult mosquitoes are rare. No reports have been made of fungal infections in adult *An. gambiae* in the field. This does not imply, however, that adult mosquitoes, including *An. gambiae*, are not susceptible to certain fungi. In laboratory experiments, adults of *Culex tarsalis* Coquillett, *Cx. pipiens* L., *An. albimanus* Wiedemann, *Aedes aegypti* L., *Ae. sierrensis* Ludlow, and *Ae. nigromaculis* Ludlow, have been found susceptible to the Deuteromycete *Beauveria bassiana* (Clark *et al.*, 1968), *Ae. sierrensis* was found susceptible to *Tolypocladium cylindrosporum* (Soarés, 1982), and *Cx. quinquefasciatus* Say and *An. gambiae* to *M. anisopliae* (Scholte *et al.*, 2003b).

In this study we screened 5 different entomopathogenic fungi, isolated from East African soil samples and from insects, for their relative pathogenicities to adult stages of *An. gambiae s.s.*, in order to evaluate their potential as biological control agents of African malaria vectors.

MATERIALS AND METHODS

Mosquitoes

An. gambiae s.s. mosquitoes were obtained from a colony that originates from specimens collected in Njage village in 1996, Tanzania. In Kenya, the strain was maintained under ambient conditions at the Mbita Point Research and Training Centre of the International Centre of Insect Physiology and Ecology (ICIPE), with temperatures ranging from 18-30°C and relative humidity from 40-90%. Larvae were kept in plastic trays, filled with water from Lake Victoria, and fed Tetramin® fish food daily. Pupae were collected daily in small cups and allowed to emerge in 30 cm cubic cages covered with netting. Adult mosquitoes had access *ad libitum* to a 6% glucose solution. Female mosquitoes were allowed to feed on the arm of a human volunteer for 10 minutes every third day.

Isolation of fungi

A *M. anisopliae* strain was isolated from a white fly, *Trialeurodes vaporariorum* (Westwood), collected at Mbita Point, in November 2001. *B. bassiana* and a *Fusarium* sp. were isolated from dead *Busseola fusca* (Fuller) stemborers, collected from stems of harvested maize plants on Rusinga Island, West Kenya, in October 2001. Another *M. anisopliae* strain, IC30, was isolated in 1989 (courtesy N. K. Maniania), from Busseola fusca, at Kendu Bay, Western Kenya, and has been maintained under laboratory conditions since then. Mosquito carcasses were incubated individually on moist filter paper in a petri dish sealed with parafilm at room temperature, to allow fungi to grow and sporulate. Fungi, sporulating from the carcasses, were isolated and inoculated on Sabouraud Dextrose Agar (SDA) for the bioassays as described by Goettel & Inglis (1997).

A third strain of *M. anisopliae* originated from a soil sample, taken from the edge of a dry riverbed (Ghera river), approximately 5 km from Mbita Point, in October 2001. The fungi were isolated from the soil samples by insect baiting, using larvae of the greater wax moth (*Galleria mellonella* L.), as described by Goettel and Inglis (1997), and plated on SDA agars for the bioassays.

Bioassay procedure

Bioassays were conducted in the laboratory at Mbita under ambient conditions (average temperature 26.17 ± 0.11°C and RH 66.26 ± 0.63%). For each experiment 50 adult mosquitoes (age 2-5 days, sex ratio approximately 1:1) were gently placed in a transparent cylindrical plastic container (16 cm high, 7 cm diameter) from which the lid had been removed and replaced by an agar plate turned upside-down. This agar plate contained a sporulating culture of the fungus to be tested. The culture lid was replaced with the original lid after 3 days. Inside the container mosquitoes had constant access to a 6% glucose solution. Dead mosquitoes were removed daily and placed on moist filter paper in Petri dishes, sealed with parafilm, and examined for fungal growth after 3 days. Control groups contained a clean Petri dish lid throughout the experiment. Each bioassay of each fungal strain/species was repeated three times, each time using fresh cultures on SDA agar that had started sporulating 1 week earlier. The cultures were plated from the first clean culture of the isolate.

Statistical analysis

Mosquito survival data were plotted on a Gompertz model (Gompertz, 1825; Clements & Paterson, 1981), from which LT_{50} values were calculated (using software package Genstat 5.0). These LT_{50} values were analyzed using ANOVA, and pairwise compared in a LSD post-hoc test (using software package SPSS 11.0). This was done separately for all trials. The effect of the different entomopathogenic fungi on mosquito mortality was analyzed using Kaplan Meier survival analysis (SPSS 11.0).

RESULTS

All fungi were pathogenic to *An. gambiae s.s.*; survival analysis showed that all survival curves of fungus-contaminated mosquitoes were significantly different from the control group ($p<0.001$). Among all fungi, pathogenicities against *An. gambiae* differed significantly ($p<0.05$), except for *M. anisopliae* isolated from soil and *M. anisopliae* isolated from white fly ($p=0.913$) (Figure 1).

Mean infection percentages ranged from 46.5 ± 3.6 % (*M.anisopliae* soil sample) to 88.7 ± 3.3 % (*M. anisopliae* IC30) (Table 1). LT_{50} values were all significantly ($p=0.003$) different from the control group (Table 1). The fungus which was slowest to halve the number of surviving mosquitoes was the *M. anisopliae* isolated from the soil sample (LT_{50} 5.87 ± 0.77 days). This LT_{50} value was significantly lower ($p=0.003$) than the *M. anisopliae* isolate IC30 (LT_{50} 3.39 ± 0.37days), but not ($p=0.122$) from the *M. anisopliae* isolated from white fly (LT_{50} 4.71 ± 0.32days). *M. anisopliae* IC30 was the fungus with the lowest LT_{50}, with a difference of 5.5 days compared to the control group. Although the LT_{50} value of *B. bassiana* was only slightly, but not significantly ($p=0.886$), higher than that of *M. anisopliae* IC30 (3.49 ± 0.29 and 3.39 ± 0.37days respectively), survival analysis of the mosquito mortality curves showed that these were significantly different ($p<0.001$).

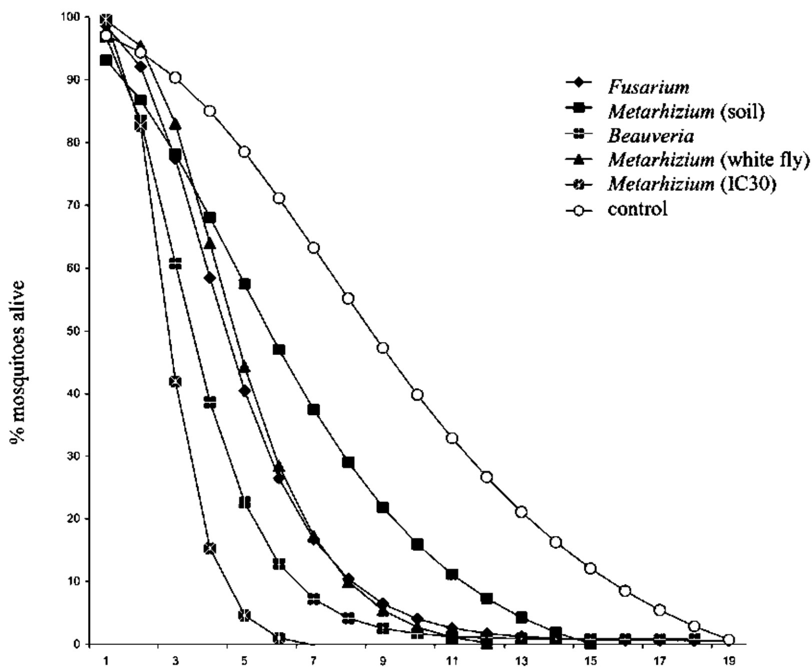

Figure 1. Survival curves (fitted on Gompertz survival function) of adult mosquitoes (*An. gambiae s.s.*) infected with various entomopathogenic fungi.

Table 1. Infectivity and pathogenicity of various fungal species to adult *Anopheles gambiae s.s.* mosquitoes

Fungus	% Infected ± SE[1]	LT_{50} ± SE (days)	Grouping[2] LT_{50}
control		8.80 ± 0.66	A
M. anisopliae (soil-sample)	46.5 ± 3.6	5.87 ± 0.77	B
M. anisopliae (white fly)	83.0 ± 3.4	4.71 ± 0.55	B C
Fusarium sp.	79.0 ± 4.3	4.48 ± 0.21	B C
B. bassiana	81.7 ± 0.9	3.49 ± 0.29	C
M. anisopliae (IC30)	88.7 ± 3.3	3.39 ± 0.37	C

[1]SE: Standard Error of the mean, [2]LT_{50} values without letters in common are significant at $p<0.05$

DISCUSSION

Several Hyphomycetes have been reported to infect the immature stages of mosquitoes (Roberts, 1974; Badran & Aly, 1995; Goettel *et al.*, 1984; Couch *et al.*, 1974; Sur *et al.*, 1999), but none have been reported from adult mosquitoes in the field. Clark *et al.* (1968) successfully infected adult *Ae. nigromaculis* with *B. bassiana*, Soarès (1982) infected adult *Ae. sierrensis* with *T. cylindrosporum* and recently Scholte *et al.* (2003b) infected *Cx. quinquefasciatus* and *An. gambiae s.s.* with *M. anisopliae*. The results of this study show that five entomopathogenic Hyphomycetes, found in a natural habitat of *An. gambiae* in Africa, significantly reduced the lifespan of the adult stage of this malaria vector. *M. anisopliae* (IC30) and *B. bassiana*, were relatively faster in killing than the other three fungi, although infection with neither of the two fungi resulted in a full 100% fungal sporulation on the mosquito cadavers as Clark *et al.* (1968) reported when they infected adult *Cx. pipiens*, *Cx. tarsalis* and *Ae. aegypti* mosquitoes with *B. bassiana*.

Varying pathogenicities among different strains of an entomopathogenic fungus, as in this study among the three strains of *M. anisopliae,* have been widely recognized and described earlier (St. Leger *et al.*, 1992; Vey *et al.*, 1982, Rath *et al.*, 1995), and are indicative of naturally occurring genetic variation (St. Leger *et al.*, 1992). Entomopathogenic Hyphomycetes (notably *M. anisopliae* and *B. bassiana*) are among the most commonly encountered insect pathogens (Goettel & Inglis, 1997), and are in use to control various arthropod pest species (Bartlett & Jeronski, 1988; Federici, 1995; Zimmermann, 1993, Khetan, 2001). Of the five Hyphomycetes tested in this study *M. anisopliae* (IC30), causing the highest infection percentage and most rapid death, should be considered a potential candidate in the development of biocontrol methods for anopheline mosquitoes. Moreover, this fungus, like all Hyphomycetes but unlike e.g. Entomophthoraleans, can be mass-produced easily and cheaply. *M. anisopliae* has demonstrated neither infectivity nor toxicity in mammals, although in laboratory experiments mice may develop allergic reactions when exposed to fungal antigens (Ward *et al.*, 1998). Another advantage for the use of this fungus as a control agent against adult anophelines in Africa, would be that an endemic organism is applied within its natural environment, therefore reducing the risk of disrupting fragile ecological equilibria.

We conclude that *M. anisopliae* may be developed as an environmentally harmless biological control agent in sub-Saharan Africa, particularly against adult anophelines, in order to reduce their life span. With the mosquito longevity being the single most important factor in the vectorial capacity equation (MacDonald, 1957), a reduction of which can greatly reduce transmission of malaria, prospects for developing this adult control strategy are promising. Questions regarding application methodology, with the aim of optimizing exposure of vector mosquitoes to sources of fungal spores/conidia, are currently being addressed.

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CHAPTER 4

INFECTION OF ADULT MALARIA (*ANOPHELES GAMBIAE S.S.*) AND FILARIASIS (*CULEX QUINQUEFASCIATUS*) VECTORS WITH THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE*

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INFECTION OF ADULT MALARIA (*ANOPHELES GAMBIAE S.S.*) AND FILARIASIS (*CULEX QUINQUEFASCIATUS*) VECTORS WITH THE ENTOMOPATHOGENIC FUNUGS *METARHIZIUM ANISOPLIAE*

ABSTRACT

Current intra-domiciliary vector control depends on the application of residual insecticides and/or repellents. Although biological control agents have been developed against aquatic mosquito stages, none are available for adults. Following successful use of an entomopathogenic fungus against tsetse flies (Diptera: Glossinidae) we investigated the potency of this fungus as a biological control agent for adult malaria and filariasis vector mosquitoes. In the laboratory, both sexes of *Anopheles gambiae* Giles *sensu stricto* and *Culex quinquefasciatus* Say were passively contaminated with dry conidia of *Metarhizium anisopliae* (Metsch.) Sorokin. Pathogenicity of this fungus for *An. gambiae* was further tested for two different exposure times and four different doses of oil-formulated conidia. Comparison of survival curves and LT_{50} values for treated and untreated specimens showed that for both species infected mosquitoes died significantly earlier ($p<0.0001$) than uninfected control groups. No differences in LT_{50} values were found for different exposure times (24/48 hrs or continuous exposure) of *An. gambiae* to dry conidia. Exposure of *An. gambiae* to oil-formulated conidia (doses ranging from $1.6x10^7$ to $1.6x10^{10}$ conidia/m^2) gave LT_{50} values of 9.69 ± 1.24 (lowest dose) and 5.89 ± 0.35 days (highest dose), with infection percentages ranging between 4.4-83.7%. Our study marks the first to use an entomopathogenic fungus against adult Afrotropical mosquitoes. Given its high pathogenicity for both adult *Anopheles* and *Culex* mosquitoes we envisage development of novel targeted indoor application methods to control endophagic host-seeking females, the rationale of which is discussed.

INTRODUCTION

Malaria and bancroftian filariasis rank amongst the world's most prevalent tropical infectious diseases. An estimated 500 million people are infected with malaria annually, resulting in 1.5-3 million deaths (WHO,2000). Filariasis is probably the fastest spreading insect-borne disease of man in the tropics, affecting about 146 million people (WHO, 1992). The use of residual insecticides for anopheline vector control, either through indoor house spraying (Curtis, 2002) or for bednet impregnation (Lengeler, 1998), has proven highly effective in various parts of Africa, but is not without obstacles. Emergence and spread of pesticide resistance in anophelines (WHO, 2000; Chandre *et al.*, 1999; Hargreaves *et al.*, 2000; Zaim and Guillet, 2002), environmental pollution (Ariee *et al.*, 2001), and unresolved issues pertaining to their toxicity to humans and non-target organisms (Curtis, 2002; WWF, 1999; Turusov *et al.*, 2002) hamper expanded use of these tools. Limited efficacy and rapid build-up of resistance to synthetic pyrethroids by culicine filariasis vectors (Chandre *et al.*, 1998), combined with the availability of effective anti-filarial drugs (Ramaiah *et al.*, 2002), has led to a gradual shift from vector control to mass-chemotherapy, though resurgence of transmission in the absence of vector control remains problematic (Burkot and Ichimori, 2002; Sunish *et al.*, 2002). A continued search for appropriate vector control strategies to augment this limited arsenal of tools is called for (Shiff, 2002), and includes biological control methods (Lacey and Undeen, 1986; Federici, 1995).

Many biological control agents have been evaluated against larval stages of mosquitoes, of which the most successful ones comprise bacteria such as *Bacillus thuringiensis israelensis* de Barjac and *B. sphaericus* Neide (Becker and Margalit, 1993; Fillinger *et al.*, 2003), mermithid nematodes such as *Romanomermis culicivorax* Ross and Smith (Zaim et al., 1988), microsporidia such as *Nosema algerae* Vavra (Undeen and Dame, 1987), and several entomopathogenic fungi (Federici, 1995). Among these fungi, the oomycete *Lagenidium giganteum* Couch has proven successful for vector control in rice fields (Hallmon *et al.*, 2000) and is currently produced commercially (Khetan, 2001). Other mosquito-pathogenic fungi that target mosquito larvae include the chytidriomycete *Coelomomyces* (Shoulkamy and Lucarotti, 1998), and the deuteromycetes *Culicinomyces* (Sweeney, 1981), *Beauveria* (Clark *et al.*, 1968), and *Metarhizium* (Roberts, 1970). Of the few fungi known to infect adult Diptera, the majority belongs to the group of Zygomycetes: Entomophthoraleans (Low and Kennel, 1972; Kramer, 1982; Eilenberg *et al.*, 2000). Unfortunately, problems associated with growing Entomophthoraleans *in vitro* have proven a major obstacle for these fungi to be used for biological control (Eilenberg, 2000).

Only a handful of studies evaluated biological control agents/methodologies to control adult stages of tropical disease vectors. Soarés (1982) infected adult *Aedes sierrensis* Ludlow with the deuteromycete *Tolypocladium cylindrosporum* Gams, resulting in 100% mortality after 10 days, whereas Clark *et al.* (1968) showed in a laboratory study that adult mosquitoes of *Culex tarsalis* Coquillett, *Cx. pipiens* L., *Ae. aegypti* L., *Ae. sierrensis* Ludlow, *Ae. nigromaculis* Ludlow, and *Anopheles albimanus* Wiedemann were susceptible to *Beauveria bassiana* (Bals.) Vuill.. Recently, Scholte *et al.* (2003) reported that adult *An. gambiae* is susceptible to *B. bassiana*, a *Fusarium* spp., and *Metarhizium anisopliae* (Metsch.) Sorokin.

M. anisopliae is a soil-borne fungus and it infects predominantly soil-dwelling insects (Veen, 1968; Zimmermann, 1993). It has a wide host-range, including arachnids and five orders of insects (Boucias and Pendland, 1998), comprising over 200 species. Although mosquitoes are not listed as natural hosts for *M. anisopliae* (Roberts, 1970; Veen, 1968) some strains have been shown to be virulent against mosquito larvae (Roberts, 1967; 1970; 1974; Ramoska, 1982; Daoust and Roberts, 1983; Ravallec *et al.*, 1989).

Spores (conidia) of *M. anisopliae* have been known for some time to be infectious to adult and emerging pupae of tsetse flies (Diptera: Glossinidae) (Kaaya, 1989; Kaaya and Munyinyi, 1995) and novel devices for exposing wild-caught specimens have been developed (Maniania, 1998). Following our initial observation that the same fungus is pathogenic to *An. gambiae* (Scholte *et al.*, 2003), we decided to study its effect on the adult bancroftian filariasis vector *Cx. quinquefasciatus*. Additional experiments with *An. gambiae* included the effect of different exposure times to conidia-treated substrates (24/48 hrs or continuous), and dose-response experiments using oil-formulations of conidia.

MATERIALS AND METHODS

Mosquitoes

Anopheles gambiae s.s. mosquitoes for bioassays 1 and 2 were obtained from a colony that originates from specimens collected in Njage village in 1996, 70 km from Ifakara town, in south-east Tanzania. All maintenance and rearing procedures have been described in detail elsewhere (Mathenge *et al.*, 2002; Knols *et al.*, 2002). *An. gambiae s.s.* mosquitoes for bioassay 3 originated from Suakoko, Liberia (courtesy Prof. M. Coluzzi). Rearing procedures for this strain were recently described by Mukabana *et al.* (2002). Climatic conditions were

18-30°C and 40-90% RH (bioassay 1), 28 ± 2°C and 70 ± 5% RH (bioassay 2) and 27 ± 1°C and 80 ± 5% RH (bioassay 3).

Bloodfed *Cx. quinquefasciatus* were collected daily from local houses in Mbita Point, western Kenya. For bioassays, only the F1 offspring from these wild mosquitoes was used. Larvae and adult mosquitoes were kept under similar conditions as the *An. gambiae s.s.* used in bioassay 1.

Fungus

Metarhizium anisopliae var. *anisopliae* (Metsch.) Sorokin, isolate ICIPE-30 (courtesy Dr. N. Maniania) was used for all three bioassays. The fungus was originally isolated from the stemborer *Busseola fusca* (Fuller) near Kendu Bay, western Kenya in 1989. Prior to use, fresh conidia were stored in the dark at 4°C.

Bioassay 1

For each of three replicates, 30-50 male and female *An. gambiae s.s.* or *Cx. quinquefasciatus* mosquitoes, aged 1-2 days at the start of the experiment, were placed in a 30 cm cubic iron-framed cage covered with white mosquito netting. These mosquitoes were offered a 6% glucose solution absorbed onto white laboratory filter paper placed in a glass vial (Figure 1).

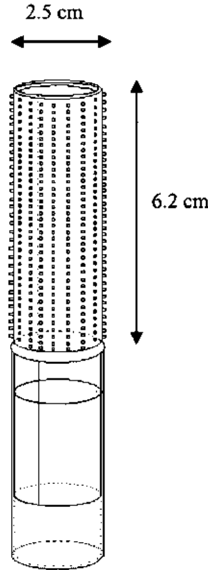

Figure 1. The 'suspensor', used in bioassays 1 and 2, a polyethylene perforated tube with short, protruding plastic hairs, dusted with dry conidia of the insect-pathogenic fungus *M. anisopliae* to infect adult mosquitoes.

The paper was enveloped by a locally available hair-roller ('suspensor'), basically a polyethylene perforated tube (height 6.5 cm, diameter 2.5 cm) with protruding, short plastic hairs that enlarged its surface area. This suspensor was dusted with 100 mg of dry conidia

using a small paintbrush and carefully placed over the filter paper. Mosquitoes landing on the suspensor to consume glucose would thus be exposed to conidia through tarsal contact or on the head and thorax region when feeding through the holes in the suspensor. The suspensor remained in the cage until the end of the bioassay (a minimum of 8 days), resulting in continuous exposure of mosquitoes to the conidia whenever they consumed glucose. The control group was exposed to a similar suspensor, but without conidia. An estimation of conidia density was made using a haemocyte counter (Fuchsrosenthal, 0.2 mm depth), showing that 100 mg of conidia approximated 6.0×10^8 conidia. Dead mosquitoes were removed from the cage, placed on moist filterpaper (distilled water) in a parafilm-sealed petri dish, and examined for fungal growth after a few days.

Bioassay 2

Experimental procedures were identical to those described above, except that the source of conidia was removed from the cages after either 24 or 48 hrs and replaced with an uncontaminated source of glucose.

Bioassay 3

Conidia were inoculated on oatmeal agar and placed in an incubator to grow for 2 weeks, after which fresh conidia were harvested using a 0.05% Triton-x solution and a glass rod. The solvent containing conidia was concentrated by removing the supernatant after centrifuging for 3 min at 5000 rpm. Dilutions were made using 0.05% Tween 20 to reach conidia concentrations of 1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia/ml. Sunflower oil was added to these solvents to reach 10% oil-formulations for all solvents. For the bioassay, 1 ml of the various oil formulations was pipetted evenly over a 8x6 cm piece of filter paper and left to dry at 70% RH for 48 hrs, resulting in spore densities of 1.6×10^7-1.6×10^{10} conidia/m^2. These papers were gently placed in cylindrical glass vials so that the paper coated the inside of the vial. Mosquitoes were tested individually by placing a 1-3 day old female *An. gambiae s.s.* in a vial, which was then sealed with cotton netting material. For each dose, 10-15 mosquitoes were tested each time, and all of the above procedures replicated three times. Mosquitoes had access to a 6% glucose solution by placing freshly soaked cotton wool pads on the netting daily. The filter paper was removed from the vial after three days, essentially delivering exposure of mosquitoes to conidia for 72 hrs. Dead mosquitoes were removed from the vials daily and placed in petri dishes containing moist filter paper. These were then sealed off and placed in an incubator at 27°C for three days, after which the cadavers were checked for sporulating *M. anisopliae* using a dissection microscope.

Data analysis

Survival curves were analysed by Kaplan-Meier pair-wise comparison (Everitt, 1994) and Cox regression analysis (using SPSS 11.0 and Genstat 5 software). For each trial, mosquito survival data were fitted to the Gompertz distribution model (Gompertz, 1825) (using Genstat 5) as described by Clements and Paterson (1981), from which LT_{50} values were calculated. LT_{50} values of treated versus control groups were compared using paired sample t-tests.

RESULTS

M. anisopliae were found to be pathogenic to adult male and female *An. gambiae s.s.*, both when mosquitoes were exposed to oil-formulated or dry conidia. Cox regression analysis showed that this pathogenicity was not dependant on exposure time. Mosquitoes exposed to conidia for 24 or 48 hrs or continuously, died significantly faster ($p<0.001$) than the untreated groups (Table 1). No significant differences in survival were found between groups exposed for either 24 or 48 hrs ($p=0.861$), but there appeared to be a trend that survival was lower under continuous exposure, compare to 24 or 48 hrs, although this difference was also not significant ($p=0.092$). Overall, males died faster than females ($p=0.020$). Following continuous exposure, 100% mortality of both male and female was observed by day 7, at which time an average of 41.7 and 82.3% of the respective sexes in the control treatment were still alive.

Table 1. LT_{50} ± SE values for adult *An. gambiae s.s.* and *Cx. quinquefasciatus* exposed to dry conidia of the entomopathogenic fungus *Metarhizium anisopliae* for various periods of time.

Species	Exposure time	Sex	LT_{50} ± SE[1] Control	LT_{50} ± SE[1] Treated	p-value[2]
An. gambiae s.s.	continuous	female	11.00 ± 0.58	5.08 ± 1.61	0.030
	continuous	male	7.65 ± 1.60	3.75 ± 0.29	0.042
	48 hrs	female	10.31 ± 1.30	3.80 ± 0.25	0.076
	48 hrs	male	11.66 ± 4.28	3.15 ± 0.37	0.125
	24 hrs	female	8.87 ± 1.32	3.39 ± 0.28	0.010
	24 hrs	male	11.68 ± 1.16	3.29 ± 0.59	0.048
Cx. quinquefasciatus	continuous	female	13.33 ± 2.91	3.88 ± 0.19	0.010
	continuous	male	18.00 ± 1.00	3.24 ± 0.23	0.010

[1] standard error; [2] paired t-test

In all bioassays where mosquitoes were exposed to dry conidia, fungal sporulation was observed in >95% of the cadavers. For conidia in adjuvant oil-formulations, percentage sporulation was positively correlated with conidial dose. Percentages ranged from 4.4 ± 4.4% for mosquitoes that had been exposed to the lowest, to 83.7 ± 8.3% for those that had been exposed to the highest dose (Table 2).

Table 2. Pairwise Kaplan-Meier survival curve and LT_{50} comparison for adult *Anopheles gambiae s.s.* exposed to four different doses of sunflower oil-formulated *Metarhizium anisopliae*.

Dose[1]	Survival curves[2]	LT_{50} ± SE[3]	LT_{50}-grouping[2]	% Infected
Control	a	9.86 ± 1.16	a	N/a
10^5	ab	9.37 ± 1.26	a	4.43 ± 4.4
10^6	bc	6.85 ± 0.44	b	32.63 ± 5.4
10^7	c	6.65 ± 0.43	b	59.74 ± 5.6
10^8	d	5.85 ± 0.26	b	83.70 ± 8.3

[1] conidia per ml of 10% oil formulation ; [2] treatments without letters in common are significantly different at $P<0.05$; [3] standard error. N/a: not applicable.

Mosquito survival data from all three bioassays closely fitted the Gompertz distribution model (variance accounted for ranged from 96.2-99.6%; Figure 2 and 3). Estimates of daily survival rates derived from the Gompertz model (Clements and Paterson, 1981) showed a dramatic reduction following exposure to conidia. In the dose-response experiment, daily survival rates were inversely related to the exposure dose. Table 3 estimates daily survival rates for *An. gambiae* at different ages for the doses tested.

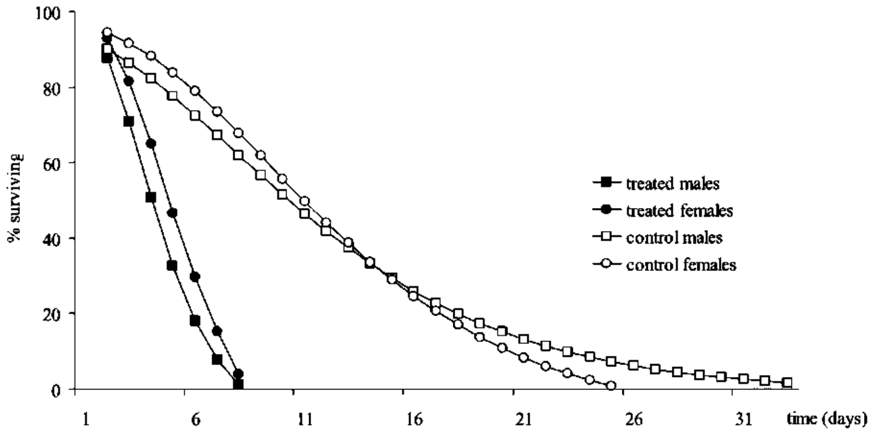

Figure 2. Mosquito survival curves of *An. gambiae s.s.* infected with *M. anisopliae* fitted to the Gompertz distribution model.

Pair-wise comparisons of Kaplan-Meier data and survival curves for the different doses of exposure (Table 2, Figure 3) showed that mosquito survival in the untreated group was not significantly different from that observed for mosquitoes exposed to the lowest dose (1.6 x 10^8 and 1.6 x 10^9 conidia/m^2) ($p=0.448$). Survival of mosquitoes exposed to the two intermediate doses (1.6 x 10^8 and 1.6 x 10^9 conidia/m^2) was significantly different from the lowest (1.6 x 10^7 conidia/m^2)($p=0.014$ and $p<0.001$), as well as the highest (1.6 x 10^{10} conidia/m^2) dose, ($p=0.001$ and $p=0.014$). LSD multiple comparison analysis of the LT_{50} values showed similar, but not equal cluster formation: the five groups appeared to be divided into two clusters (Table 2). One cluster contained the untreated group and the dose 1.6 x 10^7 conidia/m^2. Within this cluster the LT_{50} values were not significantly different from each other ($p=0.668$), but they were both significantly different from the three other doses (p-values ranging between 0.003 and 0.040). Within this other cluster the LT_{50}'s did not differ significantly from each other (p-values ranging between 0.261 and 0.880).

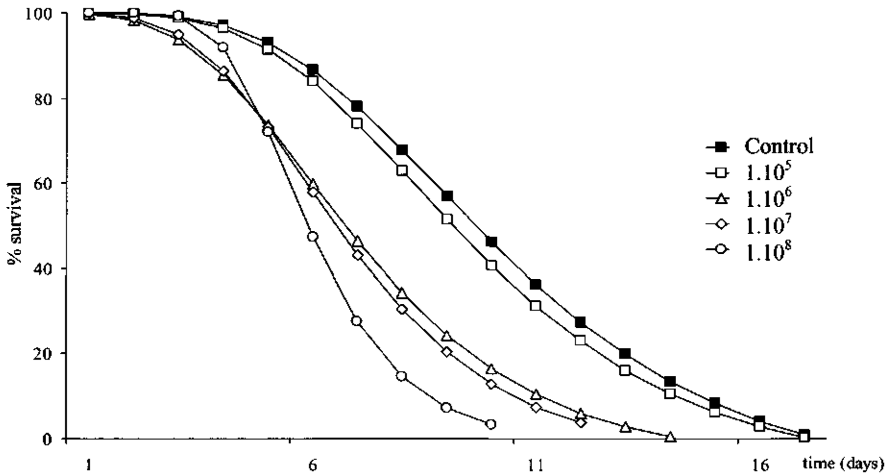

Figure 3. Gompertz survival curves for adult female *Anopheles gambiae s.s.* infected with various doses of conidia (formulated in 8% adjuvant oil) of the entomopathogenic fungus *Metarhizium anisopliae* (bioassay 3, for details, see text).

As for *An. gambiae*, male *Cx. quinquefasciatus* died faster than females ($p<0.001$). Under continuous exposure, 100% mortality was reached at day 6 for males and at day 7 for females, at which time 85.3% and 90.0% of the respective sexes in the control treatments were still alive. Highly significant reductions in both male and female survival were observed (Table 1).

Table 3. Estimated daily survival rates (± standard error), derived from the Gompertz model (see Clements and Paterson, 1981), for female *Anopheles gambiae s.s.*, following exposure to varying doses of oil-fomulated conidia of *Metarhizium anisopliae*.

Days after Exposure[1]	Dose (conidia/ml)				
	10^5	10^6	10^7	10^8	Control
3	0.98 (0.012)	0.93 (0.021)	0.92 (0.028)	0.93 (0.046)	0.97 (0.017)
7	0.83 (0.076)	0.73 (0.069)	0.69 (0.030)	0.51 (0.026)	0.87 (0.052)
10	0.80 (0.024)	0.77 (0.024)	0.37 (0.239)	0.22 (0.208)	0.81 (0.047)

[1] Mosquitoes were 1-2 days old at the start of the experiment (day 0).

DISCUSSION

Epidemiological studies on malaria and filariasis show that the adult survival rate is the most sensitive component of vectorial capacity (MacDonald, 1957; Garret-Jones, 1964; Miller *et al.*, 1973). Notable reductions in the life-span of female *An. gambiae* and *Cx. quinquefasciatus* will therefore have a considerable impact in reducing the transmission risk of malaria and lymphatic filariasis. For *An. gambiae*, daily survival rates (p) have been calculated for field populations, either from mark-release-recapture studies or physiological state ratios of wild-caught females. Takken *et al.* (1998), using mark-release-recapture methodology near Ifakara, southeast Tanzania, estimated p to be 0.78. Gillies (1961), using physiological state methodology in the coastal lowlands of Tanzania, found that female mortality rate was almost constant during the 1-parous to 6-parous age period, with p being 0.85, but that it is increased sharply thereafter. Killeen *et al.* (2000) arrived at a mean p value of 0.90, reflecting an approximate median of estimates for four holoendemic sites in Africa, which equals our estimates from the Gompertz model.

The results from the current study show that the daily survival rates of *M.anisopliae*-infected adult mosquitoes at any given moment in the mosquito's life span, is lower than uninfected mosquitoes, and that their life span is reduced, provided that the conidial dose is high enough.

If this is translated from a laboratory situation to the field it could result (under favourable circumstances) in reduced vectorial capacities of these mosquitoes. For *Cx. quinquefasciatus*, life-expectancies of 30.8 ± 3.4 and 44.1 ± 4.2 days were found for caged males and females in Tanzania (Kasule, 1986). Oda *et a*l. (2002) found mean longevities of 39.8 (males) and 64.4 days (females), when kept in the laboratory at 25°C. In our study we recorded lower survival of uninfected specimens, but when 100% of the treated female *Cx. quinquefasciatus* had died, 90.0 ± 6.0% of the untreated specimens were still alive, demonstrating that this species is highly susceptible to infection with *M. anisopliae*.

The correlltion of increasing proportion of sporulation with exposure to *An. gambiae* to increasing conidial doses suggests that the mosquitoes' immune system is only able to defend against the fungal infection at low doses, with diminishing effectiveness when exposed to increasing conidial doses. Mosquitoes may overcome a low infection level by melanization and/or encapsulation of hyphae and/or blastospores (Pathak, 1993). These defences would, however, have a cost on the mosquito's fitness (Schwarz and Koella, 2002; Hurd, 2003), which is a possible explanation for the positive correlation between conidial dose, mortality rate and proportion of sporulation found in bioassay 3. Unfortunately, in the present study we did not examine mosquitoes for melanization.

For successful conidial attachment, fungal penetration and, in the end, killing of a mosquito, a threshold number of conidia per unit surface area in required. In our dose-response experiments the lowest dose resulting in a significant effect on mosquito survival was 1.6×10^8 conidia/m^2, i.e. 160 conidia/mm^2. Apart from the two claws at the end of last tarsa, the legs/tarsae of mosquitoes are densily covered with hairlike structures (feathers), which makes it difficult for conidia to attach to the cuticle of the tarsa/leg. This was confirmed by observations under a light microscope at magnification 40x. Several mosquitoes were gently removed from the contamination cage, placed in a glass vial (they had been exposed for 24 hrs to 1×10^6 conidia/ml of adjuvant oil formulated conidia on impregnated filterpaper) and killed by adding a small droplet of chloroform in the vial. Many conidia were found attached to the 'feathers' of the last few tarsae, and several to the 'feathers'of the tibia,

but only few were actually attached to the cuticle. The ones that appeared to be attached to cuticle, were located near the end of the last tarsa, around the claws, and in between the tarsae, in the intersegmental areas of the tarsae, where very few 'feathers' are present. Apparently the effective conidial dose (i.e. conidia that actually attach to the mosquito's cuticle and invade the integument and haemocoel) is unknown, but presumably it is a rather small fraction of the conidial dosage that attaches to the mosquito. There appeared to be considerable differences in LT50 values between *An. gambiae* exposed to dry conidia or to conidia formulated in adjuvant oil. The dose of dry conidia that was used was estimated to be 6×10^8 conidia per suspensor. The suspensor has a surface area of approximately 48.7 cm^2, but has a 'hairy' surface area, which increases its surface area by an estimated 107 cm^2 to 155.7 cm^2, resulting in a conidial dose per surface area of approximately 5.6×10^{10} conidia/m^2. This is 3.5 times higher than the highest oil-formulated dose used in bioassay 3 (1.6×10^{10} conidia/m^2).

When comparing the effets of indoor application of residual insecticides, or bednets treated with synthetic pyrethroids, on mosquito mortality, *M. anisopliae* delivers lower overall mortality rates and speed of killing target insects. However, apart from its demonstrated pathogenicity to *An. gambiae* and *Cx. quinquefasciatus*, *M. anisopliae* exhibits several characteristics that make it an attractive agent for biocontrol of adult mosquitoes in sub-Saharan Africa. It can be mass-produced easily and cheaply (Khetan, 2001), has a considerable shelf-life if stored under proper conditions, and the fungus is not harmful to either birds, fish, or mammals (Zimmermann, 1993). Since the fungus is one of the most common entomopathogenic fungi, with a worldwide distribution, its use for biocontrol would be unlikely to result in the introduction of a non-endemic fungal species into the African ecosystem, although a thorough risk-assessment should be carried out for the particular fungal isolate.

CONCLUSIONS

The experiments clearly showed that both the malaria vector *An. gambiae s.s.*, and the filariasis vector *Cx quinquefasciatus* are susceptible to *M. anisopliae*. Their lifespan is greatly reduced if contaminated with an appropriate dose of conidia. As mosquito longevity is the single-most important factor in the vectorial capacity equation, prospects for developing this adult mosquito control strategy are promising and may in due course be developed into an adult mosquito control tool. Questions regarding application methodology, with the aim of optimizing exposure of vector mosquitoes to sources of fungal conidia, are currently being addressed.

ACKNOWLEDGEMENTS

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CHAPTER 5

INFECTION OF THE MALARIA MOSQUITO *ANOPHELES GAMBIAE* WITH THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE* REDUCES BLOODFEEDING AND FECUNDITY

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INFECTION OF THE MALARIA MOSQUITO *ANOPHELES GAMBIAE* WITH THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE* REDUCES BLOODFEEDING AND FECUNDITY

ABSTRACT

The entomopathogenic fungus *Metarhizium anisopliae* is being considered as a biocontrol agent against adult African malaria vectors. In addition to causing significant mortality, this pathogen is known to cause reductions in feeding and fecundity in a range of insects. In the present study we investigated whether infection with *M. anisopliae* affected blood feeding and fecundity of adult female malaria vectors *Anopheles gambiae* Giles *sensu stricto*. Mosquitoes were contaminated with either a low or a moderately high dose of oil-formulated conidia of *M. anisopliae*, and offered a single human blood meal 48, 72, or 96 hrs later to assess feeding propensity and individual blood meal size. In a second experiment, individual fungus-infected females were offered a blood meal every third day (to a total of 8 gonotrophic cycles), and allowed to oviposit after each cycle in order to quantify feeding propensity and fecundity. Infected females took smaller blood meals and displayed reduced feeding propensity. It was found that mosquitoes, inoculated with a moderately high dose of fungal conidia, exhibited reduced appetite related to increasing fungal growth. Of the fungus-infected females, the proportion of mosquitoes taking the second bloodmeal was reduced with 51%. This was further reduced to 35.3% for the 4th blood meal. The average total number of blood meals taken by uninfected females was 4.4, compared to 3.4 and 2.1 for low and high dose infections, respectively. Moreover, infected females produced fewer eggs per gonotrophic cycle. Our results show that the number of blood meals taken per lifetime are affected by infection with *M. anisopliae*. Epidemiological models show that both of these factors are among the most important factors affecting the likelihood of a mosquito transmitting malaria, which suggests that this fungus may have potential as biocontrol agent for vector-borne disease control.

INTRODUCTION

Insect-pathogenic fungi are able to infect and kill a large range of insects and some are used in biological control programs. The speed of killing is dose dependent, but even when using a high dose of conidia it may take several days from the moment of contamination to penetrate the cuticle, multiply, intoxicate and eventually kill the insect (Scholte *et al.*, 2004). Critics of the use of insect-pathogenic fungi for biocontrol often argue that the effect should be as immediate as possible, because in the pre-lethal (incubation) period these pests can still cause damage or transmit disease. In the case of *Anopheles* mosquitoes, this would be malaria parasite transmission. In epidemiological models of human malaria it is mosquito longevity that is the most important factor determining the probability of a vector to transmit *Plasmodium* parasites (McDonald, 1957). In these models it is assumed, that female mosquitoes take a blood meal every 2-3 days until they die (Killeen *et al.*, 2000). As a mosquito ages the number of blood meals which it has taken steadily increases, as does the probability of acquiring and subsequently transmitting the parasite. Reducing female longevity is thus considered the most effective way to reduce disease transmission. In fact, two of the most successful mosquito vector control methods are based on this principle: insecticide treated nets and indoor residual spraying (Sharp *et al.*, 2002; Lengeler, 1998). However, it is not the female mosquito mortality rate alone that determines effective control.

For instance, if because of pathogenic infection mosquito feeding behavior is changed in such a way that its vectorial capacity is reduced, then this may also contribute to reduction of transmission. In other words, if a mosquito survives certain periods of time without blood feeding before it dies, it remains, in epidemiological sense, inactive as a vector. The insect-pathogenic fungus *Metarhizium anisopliae* is a well-known insect-pathogenic fungus, causing mortality in a wide range of insects, including *An. gambiae*, for which it is currently studied as a potential biocontrol agent (Scholte *et al.*, 2003). In addition to causing significant mortality, several studies have shown that *M. anisopliae*, and several other entomopathogenic fungi, exhibit pre-lethal antifeedant properties. In fact, feeding rates are one of the first overt changes in infected hosts (Hajek and St. Leger, 1994). These anti-feedant properties are probably due to so-called destruxins (cyclic depsipeptides), secreted by the fungus (Pais *et al.*, 1981; Amiri *et al.*, 1997; 1999). This effect was shown for *M. anisopliae* infecting the thrips *Megalurothrips sjostedti* (Ekesi & Maniania, 2000), on the crucifer pests *Plutella xylostella* and *Phaedon coclearia*e (Amiri et al., 1997; 1999), but other studies did not report it (Rath & Worledge, 1995). Other examples of this phenomenon include the fungi *M. anisopliae* var. *acridum* on the grasshoppers/locusts *Zonocerus variegatus* (Thomas *et al.*, 1997), *Locustana pardalina* (Mueller, 2000), and *Schistocerca gregaria* (Moore *et al.*, 1992; Seyoum *et al.*, 1994), *Beauveria bassiana* (Balsamo) Vuillemin on the armyworm *Spodoptera exigua* Hübner (Hung and Boucias, 1992), and the microsporidium *Nosema locustae* Canning on the grasshopper *Melanoplus sanguinipes* (F.) (Johnson and Pavlikova, 1986).

In addition to causing death and reduced feeding in insects, some of these fungi may also reduce fecundity, which has been shown in the thrips *Megalurothrips sjostedti* upon infection with *M. anisopliae* (Ekesi and Maniania, 2000), and in the Colorado potato beetle when infected with *B. bassiana* (Fargues *et al.*, 1985). The only example for mosquitoes comes from a study by Nnakumusana (1985) who found reduced fecundity of three species of mosquitoes upon infection with *Aspergillus parasiticus*.

The aims of the present study were to investigate whether blood meal size, feeding propensity and fecundity of female *An. gambiae* mosquitoes are affected by infection with *M. anisopliae*. This was done in two separate experiments in which both the blood meal sizes, feeding propensity, the frequency of blood meal acquisition and number of eggs laid was determined, upon infection with varying doses of the fungus.

MATERIALS AND METHODS

Mosquitoes

An. gambiae s.s. mosquitoes were obtained from a colony that originates from specimens collected in Suakoko, Liberia (courtesy of Prof. M. Coluzzi). Rearing procedures were recently described by Mukabana *et al.* (2002). Insects were held at 27 ± 1°C and 80 ± 5% RH.

Fungus & inoculation

Metarhizium anisopliae var. *anisopliae* (Metsch.) Sorokin, isolate ICIPE-30 (courtesy of Dr. N.K. Maniania) was originally isolated in 1989 from a stemborer, *Busseola fusca* Fuller, near Kendu Bay, Western Kenya. Conidia were inoculated on oatmeal agar and placed in an incubator to grow for 2 weeks, after which fresh conidia were harvested using a 0.05% Triton-x solution and a glass rod. The solvent containing conidia was concentrated by removing the supernatant after centrifuging for 3 min at 5000 rpm. Dilutions were made using 0.05% Tween 20 to obtain conidial concentrations of 10^6 and 10^7 conidia ml^{-1} (henceforth called 'low' and 'high' dose). Vegetable (sunflower) oil was added to these solvents to obtain

10% oil-formulations. For the bioassays, 5 ml of the formulations was pipetted evenly over a 240 cm² piece of filter paper resulting in spore densities of $1.6x10^8$ and $1.6x10^9$ conidia m^{-2} for the low and high dose, respectively. The impregnated papers were left to dry at 70% rh for 48 hrs and were then placed on the inside of a plastic cylinder (height 11.3, diameter 3.4 cm). The tube was closed off with netting material. For contamination of mosquitoes with the fungus, 30-50 female *An.* gambiae, 6-7 d old, were placed inside this tube for 24 hrs, with access to a 6% glucose solution by placing freshly soaked cotton wool pads on the netting. Before contamination, the viability of conidia was assessed by placing a small piece of the paper with its impregnated side on a Petri dish containing Sabourad Dextrose Agar and incubated at 27° C in the dark for 16-20 hours, after which the germination percentage was determined using a light-microscope at magnification 400x.

Experiment 1: Effect of fungal infection on feeding propensity and egg production: 1 gonotrophic cycle.

Three groups of 20-30 (7-8 day-old) female mosquitoes that had just been exposed to the low dose of conidia for 24 hrs were placed in 3 identical cubic netting cages (30x30x30cm) and provided with water only for 24 hrs to ensure that the mosquitoes were starved. The conidial dose used in the current bioassay was considered sub-optimal for killing *An. gambiae s.s.*, resulting in 59.7 ± 5.6 % infection and an LT_{50} of 6.7 ± 0.43 days in a previous dose-response study (Scholte *et al.*, 2003). The control groups had not been exposed to the fungus, but were otherwise treated and held in the same way as the test mosquitoes. Prior to the bioassay, females were kept with 7-15 day old males to increase the probablity of insemination (Verhoek & Takken, 1994). To measure the effect of *M. anisopliae* infection on feeding propensity, blood meal size, and fecundity, the mosquitoes were offered one single blood meal either 48, 72, or 96 hours after the initial contact with the fungus. These groups will henceforth be referred to as '48', '72' and '96'. For blood feeding, a human volunteer placed his bare arms simultaneously in a test and a control cage for 15 minutes. Blood feeding took place during the last 15 minutes of the dark period in the insectary. The relative quantities of blood uptake were measured indirectly by determining the amount of hematin in post-diuresis excreta, by the method of Briegel (1980). To collect excreta, mosquitoes were placed individually into an Eppendorf tube immediately after blood feeding. An open Eppendorf tube, in which a second, smaller Eppendorf tube was inserted from which the tip had been cut off, contained the mosquitoes. A small cotton-wool plug moistened with 6% glucose solution was placed in the smaller tube. Blood fed mosquitoes remained in the tube for 72 hrs during which time the blood was digested, hematin excreted, and eggs developed. The females were then transferred individually to netting-sealed paper cups containing a 1cm layer of water, to allow oviposition. Any eggs were counted the next day.

To check whether possible differences in quantity of blood uptake and number of eggs produced were affected by mosquito size, one wing of every dead mosquito was removed, measured under a dissection microscope and separated in two classes (small [<3.1 mm] or large [≥3.1 mm]).

Experiment 2: Effect of fungal infection on feeding propensity and egg production: multiple gonotrophic cycles.

Seven to 8 day-old uninfected, starved female mosquitoes were offered a blood meal as described above. Half of the engorged mosquitoes were randomly removed and placed into the infection cylinder for 24 hrs, while the other half were placed in a similar cylinder without conidia. After 24 hrs the mosquitoes were placed individually in paper oviposition cups as described in experiment 1. Three days after this first blood meal the eggs in all the cups were counted and the mosquitoes gently transferred to new, clean oviposition cups, where they

were offered a second blood meal by placing the arm of a volunteer on top of the netting for 15 minutes. These procedures were repeated for a total of 8 gonotrophic cycles for both 'test' and 'control' mosquitoes. Mosquitoes that had died were placed on humid filter paper in a sterile Petridish, sealed off, and placed in an incubator at 27°C to allow fungal sporulation to verify infection. In this bioassay mosquitoes were tested using the same low and high conidial doses as described above.

Statistical analysis

Mosquito survival data from experiment 2 were fitted to the Gompertz distribution model (using Genstat 5 software) as described by Clements and Paterson (1981), which was used to determine differences in mosquito longevity between test and control groups, using Kaplan-Meier survival analysis (see Scholte *et al.*, 2003). Data on blood meal sizes and on mosquito size effects regarding fecundity (experiment 1) were analysed using univariate GLM analysis (SPSS 11.0). Feeding propensity in both bioassays and life-time fecundity (experiment 2) were analyzed using Chi-square tests.

RESULTS

For experiment 1 a total of 696 mosquitoes were offered a blood meal, in 5 trials. For experiment 2 we used 119 mosquitoes in three trials.

Mosquito survival

Mosquito survival was estimated from data collected in experiment 2. The curves describing raw data fitted Gompertz survival curves and mean survival times (Figure 1). Survival curves of mosquitoes that had been contaminated with either one of the two dosages of *M. anisopliae* were significantly different from those for the control groups, where mosquitoes lived longer as compared to treated mosquitoes ($F=10.3$, $p=0.001$, $df=2$) for low dose [experiment 1 and 2] and $F=43.1$, $p<0.001$ $df=2$ for high dose [experiment 2]. The average LT_{50} values were 8.44 days (low dose), 5.9 days (high dose), and 15.3 days (control).

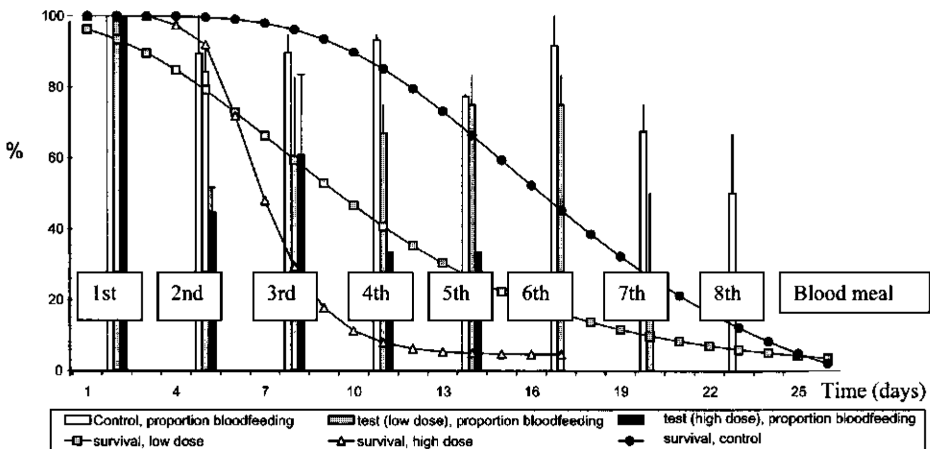

Figure 1. Survival curves from experiment 2 and proportions (± SEM) of *M. anisopliae*-infected female *An. gambiae s.s.* that took blood meals, as calculated from the mosquitoes alive at the time when each successive blood meal is due (experiment 2).

Effect of fungal infection on feeding propensity: 1 gonotrophic cycle

Females infected with the fungus 48, 72 and 96 h before a first blood meal had a reduced feeding propensity (Figure 2): Out of 363 uninfected females (the 3 control groups '48', '72' and '96' pooled), 357 mosquitoes took a blood meal (98.3 ± 0.7 %). For the treated groups these percentages ranged from 84.4 ± 5.0% ('48') to 49.2 ± 9.0 ('72'). This anti-feedant effect was not immediate. It took 72 hrs for the fungus to cause a significant effect: p= 0.440 for the group of females that had been offered a bloodmeal 48 hrs after contamination, and p<0.001 and p=0.048 for the groups '72' and '96'. There were no significant differences between control groups.

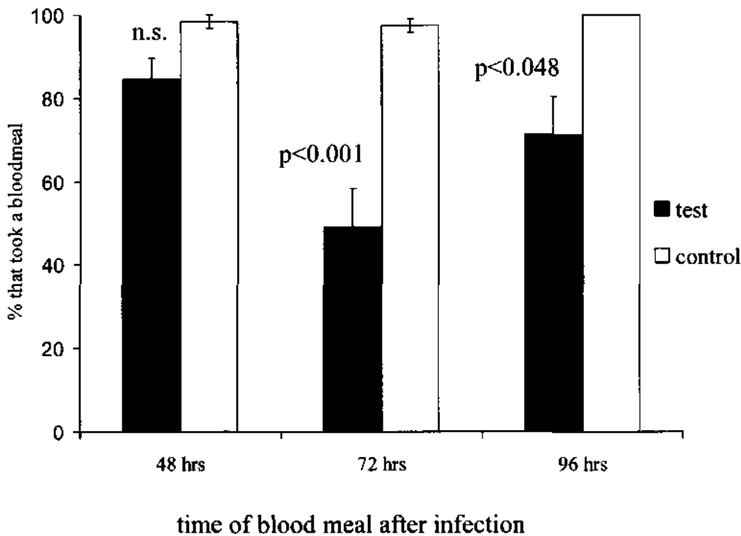

Figure 2. Feeding propensity (% ± SEM) of female mosquitoes that were offered a blood meal either 48, 72 or 96 hours after contamination with M. anisopliae (experiment 1).

Effect of fungal infection on feeding propensity: multiple gonotrophic cycles

Data from experiment 2 show that infection with *M. anisopliae* following a first blood meal resulted in a reduced feeding propensity in successive gonotrophic cycles (Figure 1). Since only those mosquitoes that had fed during the first blood meal were selected for subsequent blood meals, the proportion of blood fed females at the start of the experiment was set to 100%. If the proportions of females taking successive blood meals are calculated from the proportion of mosquitoes that were alive at the time of the blood meal (feeding propensity), we found that this proportion declines with age (Figure 1), suggesting that infected mosquitoes exhibit reduced appetite due to increasing effects of fungal growth. This was the case for the test groups, but also, although to a much lesser degree, for the control group. In the 'high dose' test group this decline started already with the second blood meal, when 87.5 ± 10.5% of the control group fed, against 44.8 ± 6.8% of the treated group. While 93.5 ± 1.5% of the control females alive at the time of the 4th blood meal ingested blood, only 67.1 ± 8.0% and 33.3 ± 0% did so for the low and high fungal doses, respectively.

Subsequent proportions of blood-ingesting mosquitoes from the control group, as calculated from all mosquitoes that were alive at the start of the bioassay, and hence of

epidemiological interest, ranged from 85.3% (3rd blood meal) to 8.8% at the 8th blood meal. For the low dose these proportions per blood meal were always lower, and in all except the 2nd blood meal, significant ($p<0.05$), with an overall significance level of $p<0.001$. These proportions were also significantly lower for the high dose compared to the control group (overall significance level of $p<0.001$), except for the 8th blood meal, where the effect was no longer significant ($p=0.119$) among the small number of females that were alive at this stage. During 8 feeding opportunities, the average total number of blood meals taken by uninfected females was 4.39, against 3.40 (low dose) and 2.07 (high dose) blood meals for the fungus-infected females.

Effect of fungal infection on blood uptake (quantity)

The excreta of all three groups of fungus-infected females contained less hematin then those of uninfected females ($F=3.124$, $p=0.026$, $df=3$), indicating that infected mosquitoes imbibed less blood during their first blood meal then uninfected ones (Figure 3). Multiple comparison tests showed that this overall effect was due only to the difference in hematin excretion between the test and control groups that had been offered a blood meal 96 hrs after first contact with the conidia ($p=0.018$). Infection with the fungus had no significant effect on blood meal size when offered either 48 ($p=0.075$) or 72 ($p=0.241$) hrs after infection. There was no significant difference in blood meal size between the three treated groups ($F=0.041$; $p=0.960$) although females in the control group of '96 hrs' consumed more blood than the other two control groups ($p=0.038$ between control '48' and control '96', and $p=0.064$ between control '72' and control '96'). From wing length measurements of blood fed females, two size classes could be distinguished (Table 1). A univariate analysis of variance showed that larger females generally appeared to consume larger quantities of blood, the overall effect approaching significance ($F=3.572$, $p=0.061$, $df=2$).

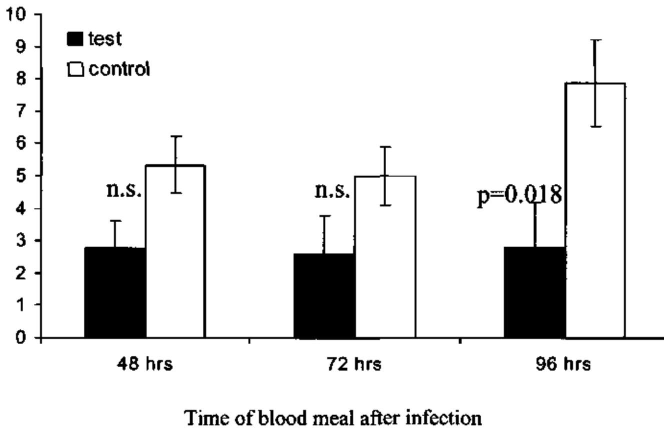

Figure 3. Mean quantity of hematin (µg ± SEM) found in the excreta of *M. anisopliae*-infected females that had taken a blood meal 3 days earlier. The terms '48', '72', and '96'hrs refer to the time between fungal inoculation and blood feeding (experiment 1).

Table 1. Relative quantities of blood ingestion of large and small *An. gambiae s.s.*, infected with the entomopathogenic fungus *M. anisopliae* (experiment 1).

		large (wing size ≥ 3.1mm)	small (wing size <3.1mm)
hematin (μg) ± S.E.	Control	7.78 ± 0.99	3.90 ± 0.82
	Test (pooled)	3.79 ± 1.34	2.88 ± 1.11

Effect of fungal infection on fecundity

Data from experiment 1 show that *M. anisopliae*-infected females laid significantly fewer eggs compared to healthy, uninfected ones (F=34.6, p<0.001, df=3) (Figure 4). Besides the effect of fungal infection also mosquito size affected the number of eggs, with small females laying significantly fewer eggs than larger females (F= 6.60; p=0.011, df=1). Mean numbers of eggs laid in the control groups ranged from 64.6 ± 4.7 and 42.2 ± 2.9 (pooled, large and small females, respectively). The number of eggs laid by females that had been infected 96 hrs previously decreased to 23.6 ± 7.9 and 17.7 ± 7.2 (large and small respectively). It should be noted, however, that by this time most infected mosquitoes had already succumbed to the fungal infection. There were no significant differences between the three control groups (F=1.78; p=0.179 for large females and F=0.93; p=0.398 for small mosquitoes), or amongst the treated groups (p>0.05). Multiple comparisons between test and control groups showed that in all three groups the differences were significant (p<0.001 for '48', p=0.020 for '72' and p<0.001 for '96').

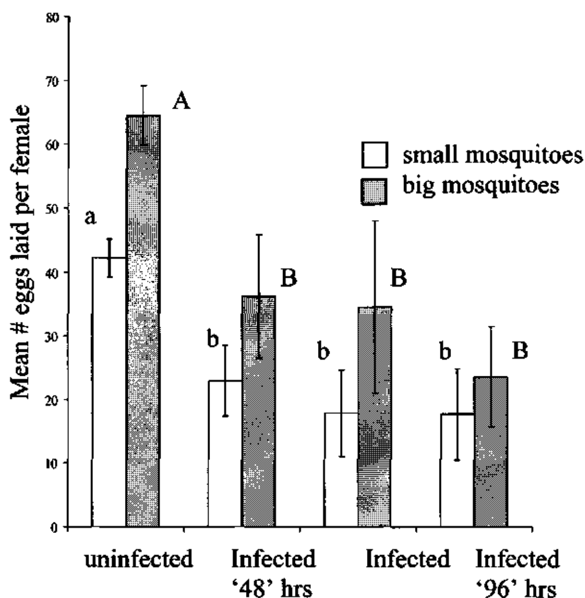

Figure 4. Mean number of eggs (± SEM) laid by female *An. gambiae s.s.* that had been offered a single blood meal either 48, 72, or 96 hours after contamination with *M. anisopliae* (experiment 1). Different letters beside the bars indicate significant differences between bars (small letters for small mosquitoes, capital letters for large mosquitoes).

Table 2. Mean number of eggs laid per blood meal, as calculated from those female mosquitoes alive at the time when oviposition is due in each gonotrophic cycle (experiment 2).

	Control	Low dosage	High dosage
Gonotrophic Cycle	Mean # eggs ± SEM (n*)	mean # eggs ± SEM (n*)	mean # eggs ± SEM (n*)
1	50.97 ± 4.17 (34)	50.13 ± 2.72 (56)	70.31 ± 4.79 (29)
2	65.09 ± 5.80 (32)	64.31 ± 5.56 (39)	36.65 ± 10.1 (23)
3	71.53 ± 7.27 (32)	58.29 ± 7.13 (28)	31.69 ± 9.89 (16)
4	80.96 ± 6.90 (27)	58.22 ± 8.93 (23)	24.80 ± 18.3 (5)
5	66.33 ± 9.87 (18)	44.91 ± 14.3 (11)	40.00 ± 13.3 (2)
6	59.23 ± 14.2 (13)	54.89 ± 15.6 (9)	0 (2)
7	51.67 ± 22.6 (3)	19.60 ± 6.53 (5)	0 (2)
8	0	0	0
average**	65.55 ± 2.96	54.81 ± 2.67	45.48 ± 4.70

* total number of mosquitoes alive at the time when oviposition is due in each gonotrophic cycle.
** significant overall effect of fungal infection on average number of eggs per bloodmeal (ANOVA): ($F=9.784$; $p<0.001$, df=2).

Similarly, the data from experiment 2 (Table 2) showed that *M. anisopliae*-infected females laid fewer eggs, both when calculated a) from the mean total number of eggs produced per mosquito during the total of 8 blood meals ($F=15.2$; $p<0.001$, df=2), irrespective of when the mosquito died (Figure 5), and b) when calculated from those mosquitoes that were alive at the time of oviposition ($F=9.78$; $p<0.001$, df=2) (Table 2). There were significant differences between the control and the two doses; for the lower dose and the control $p=0.007$, and for the higher dose with the control $p=0.001$.

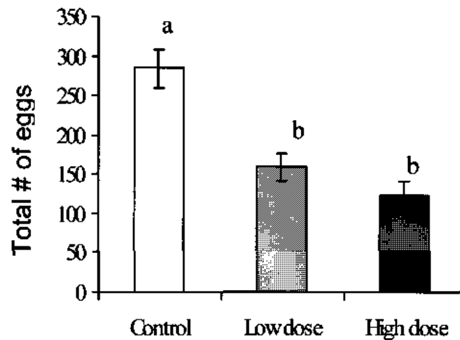

Figure 5. Average total number of eggs laid by *M. anisopliae*-infected female mosquitoes after a maximum of 8 bloodmeals, irrespective of time of death (experiment 2). Different letters indicate a significant ($p<0.05$) difference between bars.

DISCUSSION

Our findings show that infection of *An. gambiae* with *M. anisopliae* causes a significant reduction in blood feeding and lifetime fecundity. Studies with other insects indicated that reduction in feeding upon infection with this fungus may be attributed to degradation of tissues in combination with the production of secondary metabolites. These suggest that secondary metabolites produced by the fungus act on insect tissues including the midgut (Vey *et al.*, 1985; Samuals *et al.*, 1988; Vey and Quiot, 1989). Other studies suggest that 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; Zacharuk, 1971).

In microbial control of insect pests, secondary effects of the pathogen infection in addition to the mortality may play an important role in reducing the damage caused by the target insect (Thomas et al., 1997). In case this fungus will be used in malaria vector control programs, the secondary effect of reduced blood feeding upon infection may have an impact on *Plasmodium* transmission. Because the vectorial capacity of malaria is proportional to the square of the proportion of blood meals taken, this parameter is highly sensitive to any reduction in the total number of blood meals taken per mosquito (McDonald, 1957). The primary effect of the fungus, increased mortality, substantially reduces the probability of mosquito survival to any given feeding cycle, as is the probability of a fungus-infected mosquito reaching the infectious state. The secondary effects of the fungus, i.e. reduced number of blood meals prior to death and reduced feeding propensity as found in this study, further reduce the probability of a mosquito transmitting malaria. Experiment 2 showed that the average number of blood meals taken by uninfected females during their lifetime was 4.4, against 3.4 (low dose) and 2.1 (high dose) blood meals of *Metarhizium*-infected females, reducing the average total number of blood meals per fungus-infected female mosquito by 22.5% (low dose) and 52.8% (high dose), respectively, compared to uninfected females. It takes 10 days for ingested gametocytes to develop into infective sporozoites (Gilles & Warrel, 1993), with estimated values of 3.4-5.8 for the average number of feeding cycles required for parasite development (Killeen *et al.*, 2000). The results of our study, i.e. reduced survival and feeding propensity upon infection with *M. anisopliae*, indicate that if females become infected with the fungus early in their lives it becomes far less likely that they will transmit malaria. The combination of these primary and secondary effects of *M. anisopliae* on *An. gambiae* may have a considerable impact on malaria transmission if used as a biocontrol agent in the field.

Another secondary effect of *Metarhizium* infection in female *An. gambiae*, although of less epidemiological importance, is the observed reduction in fecundity. Several studies have shown that when larvae become infected with an entomopathogenic hyphomycete, reproductive fitness was reduced due to histological and cytological injuries to the ovaries (Sikura et al., 1972), and/or reduced resource availability for reproduction as a result of reduced fat bodies (Arthurs and Thomas, 2000). In the present study where adult insects were infected, we think the observed reduction in fecundity is more likely to be a direct effect of the reduced amount of blood ingested per blood meal. Egg production in insects is affected by the amount of protein in the diet (Engelmann, 1984; Klowden and Briegel, 1994). Moreover, reduction in feeding may indirectly result in reduction of egg laying, not only because fewer eggs are developed, but also because pre-lethal infection of *M. anisopliae* is possibly affecting the insects' flight ability. Arthurs and Thomas (2000) found that fat accumulation, needed to sustain flight, was reduced in pathogen-infected locusts. Seyoum *et al.* (1994) demonstrated flight impairment in desert locusts after 3-4 days of *Metarhizium* infection. In the case of

mosquitoes, sub-lethal *Metarhizium* infection might possibly reduce both the probability of a female engaging in host-seeking behavior and it might reduce the probability of a gravid female searching for, locate, and reaching a suitable oviposition site.

In addition to its primary insecticidal effect, the current study shows two secondary sub-lethal effects of *Metarhizium*-infection in adult female *An. gambiae* mosquitoes. It shows that subtle, yet potentially important effects of microbial agents may be overlooked if the only focus is short-term patterns of mortality. In the case of malaria epidemiology: a reduction in the number of mosquito bites per individual mosquito (even if the mosquito is remains alive) reduces the risk of malaria transmission.

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CHAPTER 6

AUTODISSEMINATION OF THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE* AMONGST ADULTS OF THE MALARIA VECTOR *ANOPHELES GAMBIAE S.S.*

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AUTODISSEMINATION OF THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE* AMONGST ADULTS OF THE MALARIA VECTOR *ANOPHELES GAMBIAE S.S.*

ABSTRACT

The entomopathogenic fungus *Metarhizium anisopliae* is being considered as a biocontrol agent for adult African malaria vectors. In the laboratory, we assessed whether horizontal transmission of the pathogen can take place during copulation, as this would enhance the impact of the fungus on target populations when compared with insecticides.
Virgin female *Anopheles gambiae sensu stricto* were exposed to conidia whilst resting on fungus-impregnated paper. These females were then placed together for 1 hr with uncontaminated males in proportions of either 1:1 or 1:10 shortly before the onset of mating activity. Males that had acquired fungal infection after mating indicate that passive transfer of the pathogen from infected females does occur, with mean male infection rates between 10.7 ± 3.2% and 33.3 ± 3.8%. The infections caused by horizontal transmission did not result in overall differences in survival between males from test and control groups, but in two of the three experiments the infected males lived significantly shorter than uninfected males ($P<0.05$). This study shows that autodissemination of fungal inoculum between *An. gambiae s.s.* mosquitoes during mating activity is possible under laboratory conditions. Field studies are required next, to assess the extent to which this phenomenon may augment the primary contamination pathway (i.e. direct contact with fungus-impregnated targets) of vector populations in the field.

INTRODUCTION

Control of the main African malaria vector *Anopheles gambiae* (Diptera: Culicidae) continues to rely heavily on application of residual insecticides, either for indoor residual house spraying (WHO/UNICEF, 2003) or bednet impregnation (Lengeler, 2001). These approaches have been highly effective in reducing malaria morbidity and mortality (Lengeler, 2001), but associated problems regarding environmental pollution (WWF, 1998; WHO, 1999), acceptability and cost (Karanja *et al.*, 1999; Conteh *et al.*, 2004) and the now widespread and continuing development of resistance (Zaim and Guillet, 2002, Corbel *et al.*, 2003, Hargreaves *et al.*, 2003; Weill *et al.*, 2003) underscore the need for alternative strategies, such as vector control with biological agents (WHO/UNICEF, 2003; Hougard *et al.*, 2002; Fillinger *et al.*, 2003).

Among the biological control agents against insect pests are the entomopathogenic fungi. Interest in using the Hyphomycete *Metarhizium anisopliae* against adult African malaria vectors has recently increased (Scholte *et al.*, 2004). The fungus has proven to be virulent for this vector, both in the laboratory (Scholte *et al.*, 2003) as well as in the field (See Chapter 9). The principal method of contamination of the target insect population with the fungus is through application of conidia on indoor resting targets. However, in order to achieve the highest possible impact on the target population, it is desirable that contamination pathways other than the primary mode of contamination are utilised, for instance horizontal transmission. Horizontal transmission of pathogens within the same host/target species is

called autodissemination, and this phenomenon has been suggested for biocontrol of several insect pests (Jackson *et al.*, 1992; Lacey *et al.*, 1994). Successful transmission of *M. anisopliae* by honeybees for infection of the pollen beetle *Meligethes aeneus* (Butt *et al.*, 1998), of *Beauveria bassiana* between adult flies of *Delia radicum* (Meadow *et al.*, 2000), and of *M. anisopliae* and *B. bassiana* between adult tsetse flies, *Glossina morsitans morsitans* (Kaaya and Okech, 1990) confirms the capability of insects to transmit fungi horizontally. Autodissemination of insecticidal biocontrol agents, such as insect-pathogenic fungi, provides an additional advantage over pesticides, as the impact on pest populations increases beyond direct contact. In several cases, autodissemination of entomopathogenic fungi within populations of insect pests, using attractant traps as the initial source of infection, has succeeded (Meadow *et al.*, 2000; Pell *et al.*, 1993; Furlong *et al.*, 1995; Klein and Lacey, 1999). The strategy envisaged for the use of *M. anisopliae* against adult *An. gambiae* is that host-seeking females, and occasionally also males that rest indoors, will receive primary infections while resting indoors on fungus-impregnated resting targets. Under optimal circumstances, prior to death, this infection may be transmitted to conspecifics upon contact (e.g. during mating). These mosquitoes are therefore not infected through direct contact with fungus-impregnated materials, but indirectly, upon physical contact with infected counterparts. It is estimated that approximately half of newly hatched, virgin females take a blood meal before mating (Gillies, 1954; Charlwood *et al.*, 2003). A female, with contaminated legs and mouthparts following the blood-feeding visit to a house containing fungus-impregnated resting targets, may contaminate male counterparts when she mates during the following dusk period, thereby spreading the fungus through the population.

The objective of this study was to investigate whether adult *An. gambiae* infected with *M. anisopliae* can transmit the fungus to uncontaminated mosquitoes of the opposite sex through physical contact during the mating process.

MATERIALS AND METHODS

Mosquitoes

The *Anopheles gambiae* Giles *sensu stricto* strain used originates from Suakoko, Liberia, (courtesy Prof. M. Coluzzi) and has been maintained in the Wageningen laboratories since 1989, under standardised conditions described by Mukabana *et al.* (2002). Experimental females were 4 days old and males were three days older. Virginity of the females was assured by collecting them within 24 hrs after emergence from cages and keeping them separate from the males prior to the experiments. In all experiments, mosquito mortality was checked daily, the mosquito cadavers placed on moist filter paper in Petri dishes that were sealed with parafilm. These were kept in an incubator at 27 ± 2 °C and checked for fungal sporulation after 3 days.

Fungus

Metarhizium anisopliae var. *anisopliae* (Metsch.) Sorokin, isolate ICIPE30 (courtesy Dr. N.K. Maniania) was originally isolated in 1989 from a stemborer, *Busseola fusca* Fuller, near Kendu Bay, Western Kenya. Conidia were inoculated on oatmeal agar (OA) and placed in an incubator to grow. Fungal virulence was maintained by passing it through *An. gambiae* mosquitoes every five cycles after growing on OA. Third instar larvae were infected by applying dry conidia on the water surface. After 48-72 hrs, moribund larvae were removed

and their thorax opened to remove tissue with blastospores. These were plated on OA and placed in a dark incubator at 27 °C. One week after the onset of sporulation of the colonies, conidia were harvested using a 0.05% Triton-X solution and a glass rod. The solvent with conidia was concentrated by removing the supernatant after centrifuging for 3 minutes at 5000 rpm. Dilutions were made using 0.05% Tween80 to obtain a conidial concentration of 10^8 conidia ml^{-1}. Vegetable (sunflower) oil was added to obtain an 8% adjuvant oil formulation. Five ml of this suspension was pipetted evenly over a 240 cm^2 piece of filter paper resulting in conidial densities of 1.6×10^{10} conidia m^{-2}. The impregnated paper was left to dry at 20 °C and 75 ± 5% RH for 48 hrs and was then placed on the inside of a plastic cylinder (height 11.3 cm, diameter 3.4 cm) in such manner that the paper neatly lined the upright wall of the tube. The top of the tube was covered with netting material. This setup was used only to infect female mosquitoes. Before any contamination, the viability of the impregnated conidia was checked by placing a 1 cm^2 piece of the impregnated paper on a Sabourad Dextrose Agar in an incubator at 27 °C in the dark for 16-20 hours. After incubation, the piece of paper was carefully removed and placed under a microscope (X 400) to determine the proportion of germination. For direct contamination of the female mosquitoes with *M. anisopliae*, around 30 individuals were placed in the cylinder for 24 hrs.

Bioassay 1

Thirty uninfected males were placed in a 30 cm cubic netting cage 3 hrs before the onset of mating. Half an hour before (artificial) dusk, which for *An. gambiae* is the time when mating activity occurs (Clements, 1999), thirty contaminated females were added to this cage by releasing them from the cylinder (see above) where they had spent the previous 24 hrs. By that time a large percentage of the males had the fibrillae on their antennae erect, which is considered a sign for upcoming mating activity (Charlwood and Jones, 1980). One hour after introduction of the females, all males were gently removed from the cage using a 2 cm diameter glass vial and placed in a clean cage where they had access to 6% glucose *ad libitum*. The experiment was replicated three times, on different days. Control groups were treated similarly, with the difference that the paper lining the contamination tube was uncontaminated with conidia. Mortality of males and females was checked daily to measure longevity. Dead mosquitoes (both sexes) were removed from the cages and placed on moist filter paper in a Petri dish, which was sealed and examined for fungal growth three days later. An additional similar experiment with only five females and five males was performed to determine whether conidia could be seen on the cuticle of the test males directly after the females were removed. Males were killed by a droplet of chloroform and placed under a microscope (X 400) and examined for attached conidia.

Bioassay 2

A single, uncontaminated 7-day old male was placed in a clean glass vial (diameter 2 cm, height 10 cm, sealed off with netting), to which one *M. anisopliae* contaminated female was added 30 minutes before the onset of mating activity. After one hour the couple was separated by gently removing the male, which was kept in a separate glass vial until it died. A wad of cotton wool moistened with 6% glucose was placed on top of the vial. Females remained in the vial and were provided with glucose in the same way. Mortality of both sexes was recorded daily. The control group consisted of an equal number of pairs that were

handled in the same way, with the difference that the females were not infected with the fungus. This was repeated with 35 male-female pairs on three different days.

Bioassay 3

Ten uncontaminated 7-day old males were placed in a 30 cm cubic netting cage. Half an hour before the onset of mating activity, a single infected female mosquito was added to this cage with the males. After 1 hr each male was gently removed using a clean 2 cm diameter glass vial, and kept alive individually as in bioassay 2. This was done 14 times for the test group, and 6 times for the control group.

Bioassay 4

To assess whether the males that had acquired fungal infection in the above bioassays from the contaminated females during the process of mating, or from resting on the substrate where fungus-contaminated females had rested previously (glass and netting), two experiments were carried out. In one experiment a total of 46 contaminated females (in two separate trials) were gently transferred to a 500 ml glass beaker, sealed with a glass Petri-dish. The females were killed after 1 hr by applying a droplet of chloroform in the beaker. They were then removed and placed in a Petri-dish on a moist filter paper which was sealed with parafilm, and checked 3 days later for fungal infection. Directly after removal of the contaminated females, a total of 47 uncontaminated males (in two separate trials) were placed in the beaker for 3 days, after which they were killed, removed and checked for fungal infection as described above. During the 3 days inside the beaker they had continuous access to a cotton wool wad moistened with a 6% glucose solution. The second experiment differed only in that a standard netting cage was used instead of a glass beaker, with 64 fungus-contaminated females and 67 uncontaminated males in two separate trials.

Data analysis

Mortality data were subjected to Kaplan-Meier pair-wise comparison survival analysis. Mosquito mortality data closely fitted the Gompertz distribution [28]. Comparisons of the average ages at the time of death were calculated and analyzed using GLM analysis. All analyses were carried out by using Genstat 7.0.

RESULTS

In all four bioassays, female mosquitoes that had been exposed to conidia in the cylinder setup died significantly faster than the control females ($F=104.4$; $p<0.001$), with an average of $96.4 \pm 2.0\%$ of the cadavers having sporulating *M. anisopliae*.

All three replicates of bioassay 1 indicated transfer of the fungus from contaminated females to uninfected males, with an average male infection rate of $26.1 \pm 5.3\%$ (Table 1). There was no difference in survival between the males of the control and test groups ($F=0.30$; $p=0.5844$), but when the group of test males was split into those that had been infected and those that had not, survival analysis showed a difference in survival approaching significance ($F=2.73$; $p=0.098$). Under a compound microscope, conidia were observed on 4 of the 5 males. Most were found on the lower parts (tibia, tarsi, uncinus (claws) and arolium) of the first and second pair of legs (Figure 1). A few conidia were found attached to the hairs of the

tip of the wings. No conidia were found on the head, thorax, abdomen or the hind legs. Per mosquito 0-25 conidia were found. Female mosquitoes that had spent 24 hrs on fungus-impregnated paper had conidia on legs, tips of their wings and mouthparts, but not on the thorax or abdomen.

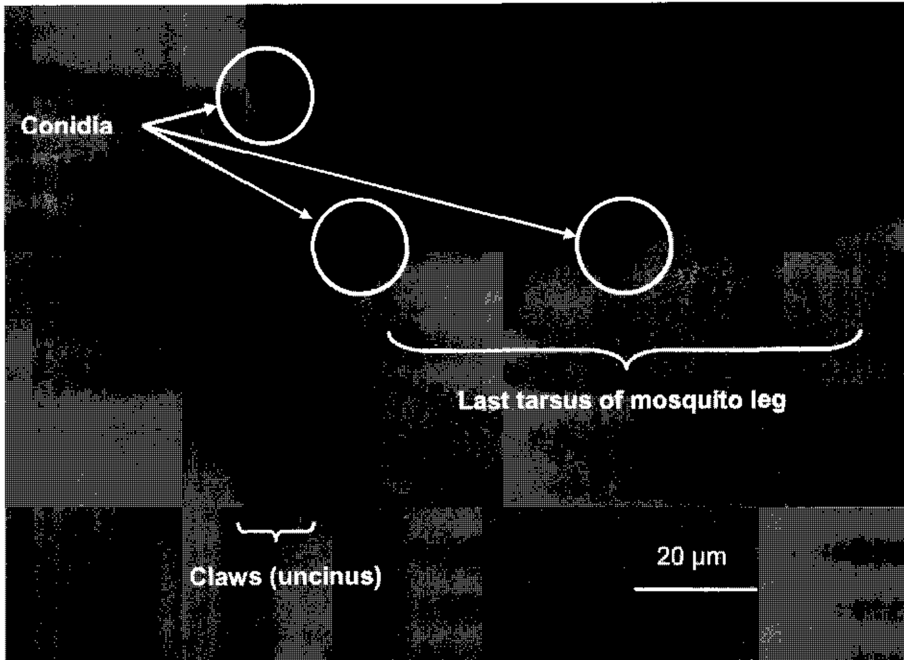

Figure 1. Conidia of the entomopathogenic fungus *Metarhizium anisopliae* on the lower part of the leg of a male *Anopheles gambiae s.s.*, after being horizontally transferred from an infected female during copulation.

In the second bioassay, with individual pairs in glass vials, an average of 34.2 ± 0.6% of the males acquired and died of the fungal infection. Survival of these males as a group was not significantly different from the control group ($F=1.27$; $p=0.259$). However, when the males from the test group were split into those that became infected and those that did not, a significant decrease in survival of the infected test males compared to the uninfected males was observed ($F=3.39$; $p=0.065$). Moreover, the average age of death of 11.3 ± 1.9 days of the fungus-infected males was lower than the 15.6 ± 1.6 days of the uninfected test males, although this was only approaching significance ($F=3.39$; $p=0.065$).

In the third bioassay, horizontal transmission occurred in 9 of the 14 replicates (64.3%). In those 9 trials an average of 16.7 ± 3.7% of the 10 males acquired an infection. Calculated over the 14 trials, the infection rate was 10.7 ± 3.2%. As in the other two bioassays, there was no significant difference in survival rates between the test and control groups ($F=2.19$; $p=0.139$). However, those males in the test group that were found with sporulating fungus died significantly faster than uninfected males ($F=13.02$; $p=0.001$) with

Table 1. Autodissemation of *M. anisopliae* from females to males *An. gambiae s.s.*. The proportions of male mosquitoes infected are shown with the differences in survival of fungus-infected males compared to uninfected males within the test groups.

Bioassay	Female: male ratio	Cage or Vial	Average % (± SEM**) of males infected	Survival analysis (Kaplan Meier)	Average age of males at time of death (days)	
					infected	uninfected
1	1 : 1	Cage	26.1 ± 5.3	F=2.73; p=0.098	10.3 ± 0.7	18.6 ± 0.8*
2	1 : 1	Vial	34.0 ± 0.6	F=3.39; p=0.065	11.3 ± 1.9	15.6 ± 1.6*
3	1 : 10	Cage/Vial	10.7 ± 3.2	F=13.02; p=0.001	13.1 ± 1.3	17.9 ± 0.6*

*: Significant difference ($p<0.05$) between infected and uninfected males using GLM analysis (Genstat 7.0).
**: SEM = standard error of mean

average age at the time of death of 8.4 ± 1.1 for infected and 17.9 ± 0.6 for uninfected males, respectively.

None of the 114 males in bioassay 4 were found infected with the fungus.

DISCUSSION

It is generally believed that fungal dissemination within a host population occurs due to activities and movements of the host. The fungus can exploit host behaviour such as trophallaxis, tactile communication, grooming (in social insects) (Watanabe, 1987; Schmid-Hempel, 1998), and mating (Andreadis, 1987) to spread through a host population. Taking into account the physiological state of the females and behaviour at the time of the bioassays, we assume that the observed autodissemination of *M. anisopliae* from female to male *An. gambiae s.s.* was the result of mating. This is strongly supported by the findings from experiment 4 where none of the males, that had stayed on the surface area where fungus-contaminated females had rested previously, acquired an infection. The average age at death of fungus-infected mosquitoes was quite high when compared to mosquito survival reported by Scholte *et al.* (2003). We think this is probably due to the relatively low level (a maximum of 25 conidia) of inoculum transferred. From those mosquitoes that were checked under the microscope, 4 out of 5 males contained conidia. It is thus likely that many males become contaminated, but that only a relatively small proportion of these males will actually succumb to the infection: in many cases the number of conidia was low, resulting in marginal infections that were successfully countered by the immune responses of the males.

In order to achieve the highest possible impact of the fungus on the mosquito population, it is desirable that other pathways besides the primary mode of (direct) contamination are utilised. The results of this study show that under laboratory conditions horizontal transmission can occur, which suggests that it may occur in the field. When these experiments were carried out, we presumed that it would be mainly females which would be infected directly from the indoor resting targets in the field. From a recent field experiment (See Chapter 9), however, it was found that a large proportion (44.9%) of the *An. gambiae s.l.* found indoors were males. This suggests that not only females can deliver fungal inoculum to uninfected males, but that also infected males may infect uninfected females. Further research is needed to determine to what extent this secondary pathway of fungal contamination may contribute to spreading the fungus within mosquito field populations.

CONCLUSION

Our study has shown that horizontal transfer of fungal inoculum between mosquitoes is possible during copulation and may contribute to spreading of the fungus within target mosquito populations in the field. However, since conditions under which horizontal transmission is likely to occur are quite specific, field experimentation is required to measure the real impact that autodissemination may have. At present we conclude that the relatively low infection levels recorded in this study suggest that the impact of biological control with *M. anisopliae* against African anophelines will predominantly depend on direct contamination of adult mosquitoes from conidia-impregnated resting targets such as walls, ceilings and sheets.

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CHAPTER 7

A STUDY ON AVOIDANCE AND REPELLENCY OF THE AFRICAN MALARIA VECTOR *ANOPHELES GAMBIAE* UPON EXPOSURE TO THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE*

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A STUDY ON AVOIDANCE AND REPELLENCY OF THE AFRICAN MALARIA VECTOR *ANOPHELES GAMBIAE* UPON EXPOSURE TO THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE*

ABSTRACT

Metarhizium anisopliae is being considered as a biocontrol agent against the adult stage of the malaria mosquito *Anopheles gambiae*. In the current study we investigated behavioral effects of female *An. gambiae* in the presence of the fungus. In three different bioassays we tested whether the behaviour of female *Anopheles gambiae s.s.* mosquitoes was affected by conidia of the entomopathogenic fungus *Metarhizium anisopliae*. From the first bioassay where mosquitoes were allowed to escape from a cylinder containing dry conidia, and the second bioassay, where mosquitoes could choose between two cages (one containing dry conidia, the other not) to feed from a glucose-solution, it became clear that dry conidia have a significant repelling effect ($p<0.05$). When conidia were applied in a suspension of 8% adjuvant vegetable-oil formulation and impregnated on paper, it appeared that these formulated conidia had no repelling effect ($p=0.205$). In addition to the already known positive effects of formulating conidia in oil (preventing conidia from becoming airborne and protection from drying out), we here show another positive effect, namely absence of repellency, suggesting that for field application the conidia should be oil-formulated.

INTRODUCTION

Entomopathogenic fungi can be effective in biocontrol of harmful insects (Hajek and St. Leger, 1994), and recently they have been suggested for the control of African malaria vectors (Scholte *et al.*, 2003; 2004). Especially the hyphomycete *Metarhizium anisopliae* isolate ICIPE30 was found virulent against *Anopheles gambiae* under laboratory conditions, and preliminary field-data indicate that this fungus may work in the field against wild anophelines, encouraging further study (Chapter 9). *M. anisopliae* is a non-specific entomopathogenic fungus infecting a large group of different insect species (Veen, 1968, Zimmermann, 1993), although it has never been found on mosquitoes. This does not necessarily mean that mosquitoes are not susceptible. In fact, numerous mosquito species are found susceptible to infections (Roberts, 1970; Ramoska, 1982; Scholte *et al.*, 2003; 2004). It is more likely that mosquitoes do not often contact the fungus because they do not spend time in those habitats where almost all infective conidia are present: in upper layers of soil.

Those insects that live sympatrically with the fungal conidia in, or close to, soil are likely to co-evolve, selecting strategies either to become resistant or to reduce the probability of infection by behavioral traits. An example of the first strategy is that several species of collembolans are not susceptible to infection of different entomopathogenic Hyphomycetes (Dromph and Vestergaard, 2002). An example of the second strategy is that several insect species have the ability to detect conidia or mycelia from a distance, and avoid them: termites (Milner and Staples, 1996), ants (Kermarrec and Decharme, 1982), groundnut beetles (Ekesi *et al.*, 2001) and larvae of scarab beetles (Villani *et al.*, 1994) are all repelled by *M. anisopliae*. If *M. anisopliae* is to be used as a biological control agent against African anophelines, it is important to study whether this fungus may trigger any behavioral effects in the mosquito, such as avoidance of conidia; a behavioral trait which would hamper its use in the field. The aim of the current study was, therefore, to test whether conidia of this fungus have a repelling effect on adult females of *Anopheles gambiae s.s.*.

MATERIALS AND METHODS

The study was carried out at the ICIPE (International Centre of Insect Physiology and Ecology) Mbita Point Training and Research Centre, in western Kenya. All experiments were carried out from 07.00-9.30 hrs in a field-laboratory consisting of timber framework, cement floor, iron roof, and walls consisting of cane mats. The entire laboratory was sealed with mosquito netting on the inside to prevent mosquitoes from escaping. There were no windows and artificial light sources, resulting in a poorly lit environment: dark enough for female *An. gambiae* to take a blood meal, but light enough for mosquito behavior to be observable.

Fungus

Metarhizium anisopliae var. *anisopliae* (Metsch.) Sorokin, isolate ICIPE-30 (courtesy Dr. N.K. Maniania) was used in all bioassays. The fungus was originally isolated in 1989 from a stemborer, *Busseola fusca* Fuller, near Kendu Bay, western Kenya. The fungus was cultured on oatmeal agar plates at 27°. Conidia were harvested from recently sporulating cultures and stored in the dark at 4°C. The viability of both dry and oil-formulated conidia was checked at the start of each bioassay (Lacey and Brooks, 1997) by inoculating conidia on SDA agar and counting the proportion of germinating conidia after 20-24 hrs. In all cases this proportion was higher than 95%.

Mosquitoes

Anopheles gambiae Giles *sensu stricto* (henceforth termed *An. gambiae*) mosquitoes were obtained from a colony that originates from specimens collected in Njage village, 70 km from Ifakara town, in south-eastern Tanzania in 1996. All maintenance and rearing procedures have been described elsewhere in detail (Knols *et al.*, 2002). In this study only female mosquitoes, aged 4-8 days, were used.

Experimental procedures

Bioassay 1

Ten female *An. gambiae* mosquitoes were gently placed in a plastic cylinder (20 x 12 cm diameter). The cylinder was covered with black fabric to create a dark environment inside. It contained one circular opening (diameter 15 mm) through which daylight entered the cylinder. Mosquitoes could escape from the cylinder through this opening (Figure 1). One flat side of the cylinder could be unscrewed and removed, but was kept closed during bioassays. Inside the cylinder an open Petri-dish (diameter 6 cm) was placed in which either no conidia (control), or 0.1, 0.5, 1.0 or 5.0 gram freshly harvested dry conidia of *Metarhizium anisopliae* (strain IC30) were placed. The cylinder was placed on its side during the experiment (Figure 1).

Figure 1. Cylinder used to test repellent effect of dry conidia of the entomopathogenic fungus *Metarhizium anisopliae* to female mosquito *Anopheles gambiae s.s.* (bioassay1). (a) plastic cylinder containing adult mosquitoes; (b) Petri-dish containing dry conidia; (c) dark cloth covering the cylinder; (d) exit hole from which mosquitoes can escape (e). NB Exit hole is connected to a 30 x 30 x 30cm netting cage (not shown).

Just before the start of an experiment the cylinder was unscrewed, the Petri-dish containing conidia placed inside, and the cylinder closed again. After the mosquitoes were placed inside the cylinder the exit was kept closed for 1 minute. On a few occasions a mosquito escaped when placing them in the cylinder resulting in only 9 mosquitoes per cylinder. After opening the mosquitoes could escape to an adjacent 30 x 30 x 30 cm netting cage, away from the conidia. The exit was kept open for 3 minutes in each trial. In each trial two cylinders were used simultaneously: one control in which no conidia were placed, and the test cylinder containing the disc with conidia. This method had been successful in previous experiments where the repelling effect of other substances were tested on *An. gambiae* and *Culex quinquefasciatus* Say (Scholte *et al.*, unpublished data). The method is based on the presumption that these mosquitoes prefer dark surroundings (i.e. inside the cylinder) and will only move to lighter surroundings (i.e. escape from the cylinder) when they are disturbed either physically (e.g. by direct physical danger) or chemically (by e.g. repelling substances). Mosquitoes that are induced to escape the repellent substance move to the opening and exit.

Bioassay 2

A transparent Perspex box was divided into two identical compartments, each compartment contained a carbohydrate (6% glucose solution) source and had one circular exit at the front (Figure 2). In one compartment an open Petri-dish was placed containing either 0.1, 0.5, 1.0 or 5.0 gram of dry conidia of *M. anisopliae*. The Petri-dish in the other compartment did not contain conidia. A transparent cylindrical container (18cm length, 12 cm diam.) was placed facing the two openings of the compartments. In each test a single mosquito was released in this container and allowed to fly into either one of the compartments. To increase the probability that the mosquitoes would fly towards one of the two cages they had been deprived of sugar water 3 hrs before the bioassay. Only those mosquitoes that had entered one of the two compartments within 3 minutes after release were scored. Percent repellency (PR) values were calculated as PR= $[(N_C - N_T)/(N_C + N_T)] \times 100$ (Ekesi *et al.*, 2001). Control and test compartments were cleaned and interchanged regularly. Control bioassays were carried out where both compartments were without conidia. For each conidial quantity >1000 mosquitoes were tested.

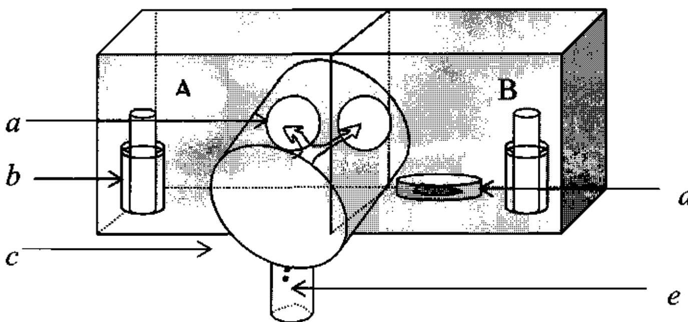

Figure 2. Two compartments (A and B) used to test repellency of dry conidia of *M. anisopliae* to female *An. gambiae s.s.* (bioassay 2). (a) entrance of compartment A; (b) 6% glucose solution; (c) cylinder from which mosquitoes choose (white arrow) compartment; (d) Petri-dish containing dry conidia; (e) release cup.

In addition, 25 trials were carried out where a sock, worn by a human for 48 hrs as an attractive bait for a human blood meal (Pates *et al.*, 2001), was placed inside both cages. This was done to test whether a possible repelling effect of dry conidia was strong enough to prevent female mosquitoes in search of a blood meal from entering the cage containing 1.0 grams of dry conidia.

Bioassay 3

As in bioassay 2, two cages were used, one of which contained a 625 cm^2 piece of paper impregnated with conidia suspended in a 8% vegetable oil suspension (dissolved using a 0.05% Tween20 surfactant), resulting in a conidial density of 1.0×10^9 $conidiam^{-2}$. The other cage contained a similar piece of paper impregnated with the 8% oil-suspension but without conidia. Each cage contained 6% glucose solution as in bioassay 2. The two cages were placed 50 cm apart, and were connected by a transparent plastic cylinder of 8 cm diameter. In the middle of the cylinder a single mosquito was released. A data point was recorded when a mosquito entered one of the two cages within 3 minutes of release. Six hundred mosquitoes were tested, plus 200 for the controls (no conidia in either cage). The cages switched position after 25 trials.

Data analysis

Proportions of mosquitoes escaping from the cylinder (bioassay 1) were analyzed using GLM (SPSS 11.0). Proportions of mosquitoes choosing either one of the two cages in bioassays 2 and 3 were analyzed by Chi-square tests (SPSS 11.0).

RESULTS

Bioassay 1

From a total of 826 mosquitoes in the control group, 81 (10.9 ± 0.08 %) had moved out of the cylinder (Table 1). In all of the test groups more mosquitoes escaped from the cylinder as compared to the control group. This difference was significant ($p<0.001$, $F=9.146$, $dF=4$), indicating that mosquitoes tended to escape exposure to dry conidia. Least Significant Difference (LSD) post-hoc analysis showed that there were no significant differences between the three lowest conidial quantities, but that the proportion of mosquitoes escaping from the cylinder containing the highest conidial quantity was significantly higher than from the other quantities ($p<0.05$).

Table 1. Number of mosquitoes that escaped from a cylinder where mosquitoes were exposed to different quantities of dry conidia of *M. anisopliae* (bioassay 1).

Conidial quantity (g)	Mosquitoes remaining in cylinder	Mosquitoes escaping from cylinder					# of replic. (N)	% (± SEM) that escaped cylinder	
		0	0.1	0.5	1	5			
0	745 control	81 control					83	10.9 ± 0.08 control	a*
0.1	194		163				36	45.7 ± 0.28	b
0.5	122			79			20	39.3 ± 0.18	b
1.0	98				59		16	37.6 ± 0.25	b
5.0	21					87	11	80.6 ± 0.19	c

* = Proportions followed by the same letter do not differ significantly by LSD post-hoc analysis with GLM ($P>0.05$).

Bioassay 2

Mosquitoes entered the compartment without conidia more often than the compartment containing conidia (Table 2). This was significant for all 4 conidial quantities ($p<0.05$). In the control assays mosquitoes did not prefer one compartment above the other. PR values ranged between 7.0 and 15.4% against 3.2% of the control group. This indicates that dry conidia of *M. anisopliae* have a moderate repelling effect on female *An. gambiae s.s.*. When the worn socks were placed inside the cages, 11 mosquitoes (44.0 ± 4.9%) flew into the cage without conidia, and 13 (52.0 ± 4.9%) into the cage containing 1.0 gram of dry conidia. One mosquito did not enter either one of the compartments. This difference was not significant (χ^2=0.391; df=2; p=0.532).

Table 2. Proportions of *An. gambiae* that, in a choice-bioassay, entered a cage containing dry conidia of *M. anisopliae* as opposed to a cage without conidia. These proportions are used as a measure of avoidance (PR) of the fungus by the mosquitoes (bioassay 2). Proportions were analyzed in an unpaired t-tests.

Quantity of conidia (g)	% ± SEM	PR	χ^2	p
Control*	48.4 ± 0.01a	3.2	1.02	0.312
0.1	45.7 ± 0.01b	8.6	7.37	0.007
0.5	42.4 ± 0.01c	15.2	23.4	<0.001
1.0	42.3 ± 0.01c	15.4	23.9	<0.001
5.0	46.5 ± 0.01b	7.0	5.07	0.024

* control bioassays where both cages did not contain conidia.

Bioassay 3

The oil-formulated conidia impregnated on paper used in bioassay 3 had no repelling effect on the mosquitoes. From the 510 mosquitoes that had entered one of the compartments, 269 had entered the compartment containing the paper without oil-formulated conidia and 241 the other one (PR=5.49%). This difference was not significant (χ^2=1.537; df=1; p=0.215). Control assays (neither cage containing conidia) demonstrated that there was no preference for either of the compartments: from the 177 mosquitoes that entered, 93 (52.5 ± 1.9%) had entered one and 84 (47.5 ± 1.9%) the other (χ^2=0.458; df=1; p=0.499).

DISCUSSION

The study demonstrated that, under the two experimental set-ups of the first two bioassays, dry conidia have a moderate repelling effect on female *An. gambiae*, but conidia suspended in 8% adjuvant vegetable oil, impregnated on paper, have not. There is a limited amount of data available on repellency effects of entomopathogenic fungi on insect pests. In Australia Milner & Staples (1996) found a repelling effect of *M. anisopliae* against termites. This trait of the fungus is now used to repel termites by mixing soil with conidia to prevent damage to susceptible timber. In the case of using *M. anisopliae* as a biological control agent for African malaria mosquitoes the goal is to achieve the highest possible infection level in the targeted mosquito populations in order to reduce mosquito densities. Therefore, a repellent effect of the fungus is not desirable unless the effect is strong enough to repel mosquitoes from houses to prevent them from biting humans. Bioassay 2, however, showed that that was not the case: when a sock, worn by a human, was placed in both cages, mosquitoes were no longer repelled by the fungus. Apparently the mosquitoes' feeding drive was stronger than the repelling effect of the dry conidia. Bioassay 3 showed that when paper, impregnated with an

8% adjuvant vegetable-oil conidial formulation, was used, *An. gambiae* females were not repelled. Also data from the field-experiment (Chapter 9), where a large proportion of the mosquitoes caught indoors was found on a fungus-impregnated sheet, suggest that there is no repelling effect of oil-formulated conidia, on wild anophelines in Tanzania. So if there is any repelling effect of *M. anisopliae* on *An. gambiae,* it weighs little in a trade-off against host seeking: mosquitoes are not disturbed by the presence of (oil-formulated) conidia. Possibly the oil film prevent conidia from free dispersion in the air and thus reduce the probability of a flying mosquito encountering conidia. When dry conidia are used, minimal air-movement will blow some conidia into the air, causing 'conidial dust'. Although we kept the movement of air inside the compartments as low as possible, it was difficult to prevent some of the conidia being blown into the air. These air-borne conidia may be detected by the flying mosquito and avoided if possible. Another possibility, although less likely, is that certain odors, originating from dry conidia, are detected by female *An. gambiae.* This, however, remains speculation since to our knowledge, there are no reports on odors originating from dry conidia.

Despite the moderate repellent property of dry conidia against female *An. gambiae,* the results of this study support the idea of using *M. anisopliae* against these mosquitoes, especially when oil-formulated conidia are used. With the knowledge that a) the strain of fungus used is highly virulent against this mosquito species, b) the indoor use of the fungus minimizes non-target infections, c) it is considered safe towards humans and the environment and d) there was no repellent effect of oil-protected conidia on the target insect, we think that the fungus is a suitable candidate as a biocontrol agent against malaria vectors in Africa.

CONCLUSION

The experiments showed that dry conidia of the fungus *M. anisopliae* have a moderate repellent effect on female *An. gambiae.* This effect was not observed when human odor emanating from socks was located close to the conidia, suggesting that the observed repellency against conidia has little impact on a mosquito's host-seeking behaviour. Moreover, when conidia were suspended in an 8% adjuvant oil-formulation and impregnated on paper, no repellent effect was observed, indicating that an oil-formulation should be used to prevent any repellent effects if the fungus is to be used as a biocontrol agent against Afro-tropical malaria vectors.

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CHAPTER 8

A STUDY ON THE VIRULENCE OF *METARHIZIUM ANISOPLIAE* CONIDIA ON *ANOPHELES GAMBIAE S.S.* OVER TIME

A STUDY ON THE VIRULENCE OF *METARHIZIUM ANISOPLIAE* CONIDIA ON *ANOPHELES GAMBIAE S.S.* OVER TIME

ABSTRACT

The virulence of conidia of the entomopathogen *Metarhizium anisopliae* isolate IC30 against adult female *Anopheles gambiae s.s.*, impregnated on two different materials (filter paper and mosquito netting), was investigated over a period of 3 months. Also, viability of conidia stored at three different temperatures, formulated either as 1) dry powder with silica gel, 2) in an emulsifiable adjuvant oil formulation of 8% vegetable oil, and 3) in a 0.05% Tween 80 suspension was monitored over a period of 6 months. Conidia, impregnated on paper and netting and kept at 27 ± 2°C and 65 ± 5% RH gradually lost viability over 3 months, with virulence against female mosquitoes remaining significantly different from unimpregnated control materials up to 1 month. Dry conidia stored with silicagel retained viability for the entire 6 months. Conidia stored in 0.05% Tween 80 exhibited only slightly reduced viability after 3 months at 27° and after 6 months at 4°C. Conidia kept in 8% oil remained viable up to at least 1 month, but clumped together after that, which made viability checks impossible. The study shows that this isolate of *M. anisopliae*, applied on material as a source for indirect contamination of adult mosquitoes, remains virulent for one month. It is discussed that this may be improved by using oil-based conidial formulations instead of only 8% oil.

INTRODUCTION

As alternatives to chemicals for mosquito vector control, interest in alternative strategies such as biological control has increased over recent decades (Service, 1983; Hougard *et al.*, 2002; Fillinger *et al.*, 2003; WHO/UNICEF, 2003). Among these, entomopathogens are currently being considered as bioagents against adult stages of malaria vectors in tropical Africa (Scholte *et al.*, 2003; 2004). Recent field experiments in rural Tanzania showed that wild *Anopheles gambiae s.l.* can become infected with the fungus and die as a result of it (Chapter 9). Since many African mosquito vectors are found indoors, potential indoor contact points to deliver conidia to the mosquitoes include impregnated cotton sheets (Chapter 9), curtains, bed nets, walls and ceilings.

For effective biological control, conidia of the entomopathogenic fungus must retain high viability and virulence (McClatchie *et al.*, 1994). Nothing is known about longevity of *M. anisopliae* conidia applied indoors in Africa as proposed by Scholte *et al.* (2003), but much basic information regarding shelf life, formulation and abiotic factors on the effectiveness of *Metarhizium* conidia was obtained from the LUBILOSA program, where *M. anisopliae* var. *acridum* was studied as replacement for chemical pesticides against desert locusts and grasshoppers. Important findings were that formulating the lipophilic conidia in oil had advantages over conventional water-based suspensions (Prior *et al.*, 1988; Bateman *et al.*, 1993). Especially in arid areas, such as many *An. gambiae* habitats in Sub-Saharan Africa, the oil protects conidia from desiccation (Bateman *et al.*, 1993; Burges, 1998). During storage, survival of conidia proved to be best at extreme humidities approaching 100 and 0%, where temperature is an important factor (Bateman *et al.*,1993; McClatchie *et al.*, 1994; Burges, 1998). Conidia can be stored protected from UV light, either unformulated as dry powder or in oil where pre-drying can improve survival in oil (Hedgecock *et al.*, 1995; Morley-Davies et al., 1995; Alves *et al.*, 2002).

Since the isolate IC30 of *M. anisopliae* was highly virulent against the most important African malaria vector, *An. gambiae*, and the fungus is considered for future use against adult mosquitoes in Africa, we monitored the virulence of this isolate on female *An. gambiae s.s.* for four times over a period of 3 months, stored at 27°C. Conidia were suspended in an emulsifiable adjuvant oil formulation of 8% vegetable oil and impregnated on filter paper and netting. In a second experiment the conidial viability of this fungal isolate, stored either as 1) dry powder with silica gel, 2) in a 8% oil-formulation' and 3) in a 0.05% Tween80-based suspension at 3 different temperatures were monitored 5 times over a period of 6 months.

MATERIALS AND METHODS

Experiment 1: fungal virulence against An. gambiae s.s. over time

The pathogenicity of *M. anisopliae* isolate IC30 on female *Anopheles gambiae s.s.* was monitored over a period of 3 months. Conidia were impregnated either on filter paper (the methodology used in Scholte *et al.* (2003)), as well as on cotton mosquito netting. The impregnated paper and netting was wrapped in aluminum foil, and stored at 27 ± 2°C at 65 ± 5%RH. Pathogenicity tests were carried out at intervals of one day, one week, one month or three months after conidia had dried on the impregnated surface.

To obtain conidial suspensions for impregnating the filter paper and the netting, we harvested conidia of a colony that had been sporulating for two weeks, using a 0.05% Triton-X solution and a glass rod. For each trial 2 petri-dishes containing sporulating conidia were used to obtain a stock suspension. In total 3 trials were performed, using three different stock suspensions. The solvent containing conidia was concentrated by removing the supernatant after centrifuging for 3 min at 5000 rpm. Dilutions were made using 0.05% Tween80 to obtain conidial stock concentrations of 10^8 conidia ml^{-1}. Vegetable (sunflower) oil was added to these solvents to obtain 8% oil-formulations. For the bioassay where filter paper was impregnated, 5 ml of the formulations was distributed evenly over a 240 cm^2 piece of paper resulting in spore densities of 2.1 x 10^{10} conidia m^{-2}. The impregnated materials were left to dry slowly for 48 hrs at high humidity (>70%), a method recommended by Hong *et al.* (2000) to increase conidial longevity. To increase the surface area of the netting material, the netting was folded to obtain a 'double layer'. Unlike the impregnation on filter paper where 5 ml of the oil-formulation was absorbed by the paper, only 3.5 ± 0.2 ml was absorbed by the netting material, resulting in a conidial density of 1.5 x 10^{10} conidia m^{-2}. The impregnated papers and netting were left to dry in an incubator at 70% RH for 48 hrs at room temperature and were then placed on the inside of a plastic tube (height 11.3, diameter 3.4 cm). The tube was closed off with clean netting material. For contamination of mosquitoes with the fungus, female *An. gambiae*, 6-7 d old, were placed inside this tube for 24 hrs, with access to a 6% glucose solution by placing freshly soaked cotton wool pads on the netting. After inoculation the mosquitoes were transferred gently to 30 x 30 x 30 cm mosquito cages. A 6% glucose solution was available *ad libitum*. Mortality was checked daily, cadavers removed and placed on moist filterpaper in small Petri-dishes that were sealed with parafilm and checked for *M. anisopliae* sporulation after 3 days.

Experiment 2: residual viability

First, conidial viability of the impregnated paper and netting as used for pathogenicity tests in experiment 1, was measured over a period of 6 months. Impregnated paper and netting was wrapped in aluminum foil and stored in an incubator at 27 ± 2°C and 65 ± 5%RH. Conidial viability was tested 1 day, 1 week, 1 month, 3 months and 6 months after the 48 hrs drying of the impregnated substrates by pressing pieces of 1cm^2 (conidial side down) lightly on fresh SDA agar plates. After 18-20 hrs at 27°C the paper or netting was carefully removed

from the agar, after which the proportion of conidia germinating was determined under a microscope at 400x magnification. This method of pressing netting or filter paper containing conidia on agar is not optimal since there are always some ungerminated conidia sticking to the paper or netting. To check whether conidia had remained on the paper or the netting, the materials were checked under the microscope. Conidia that had remained on the substrates were included in the calculations. Propagules were considered viable when the germ tube was at least twice the size of the conidium (Goettel and Inglis, 1997). A minimum of 200 conidia were checked per plate.

Second, conidia in suspensions were tested for viability. Conidial suspension from the stock was diluted to a concentration of 1.10^6 conidia m^{-2} and kept in two different suspensions in closed 80ml glass jars (Schott®) 1) in an 8% vegetable oil formulation, and 2) in a 0.05% Tween80 water solution (henceforth referred to as kept in 'water'). To assess the effects of temperature on residual viability, all samples were stored at temperatures of 27°C, 21°C and 4°C. After stirring on a vortex mixer, 100 µl of the conidial suspension was pipetted on a fresh SDA plate and distributed evenly over the middle of the plate. Conidial viability was determined as described above.

Third, viability of dry conidia was determined on the same time schedule as for the conidia in suspension. About 100 mg of fresh conidia were scraped off from a 2-week sporulating plate. These were placed in a 35mm diameter Petri-dish, together with 5 granules of indicating silica gel, sealed with parafilm and stored in the same incubators as the conidial suspension desribed above, at the three different temperatures. Prior to inoculation on medium, the sample was suspended in 10 ml 0.05% Tween 80, shaken well, and 100 µl was pipetted onto fresh SDA agar to determine conidial viability as described above. Moisture content of the conidia was not determined.

Mosquitoes

An. gambiae s.s. mosquitoes were obtained from a colony that originates from specimens collected in Suakoko, Liberia (courtesy of Prof. M. Coluzzi) and had been cultured in the Laboratory of Entomology in Wageningen since 1988. Rearing procedures were recently described by Mukabana *et al.* (2002). Insects were held at 27 ± 1°C and 80 ± 5% RH.

Fungus and inoculation

Metarhizium anisopliae var. *anisopliae* (Metsch.) Sorokin, isolate ICIPE-30 (courtesy of Dr. N.K. Maniania) was originally isolated in 1989 from a stemborer, *Busseola fusca* Fuller, near Kendu Bay, Western Kenya. Conidia were inoculated on oatmeal agar and placed in an incubator at 21± 2°C to grow for 2 weeks, after which fresh conidia were harvested to inoculate fresh OA medium. Every 5 'generations' the fungus were passaged through the target insect, *An. gambiae s.s.*, to enhance the virulence of the fungus.

Statistical analysis

Mosquito longevity data were analyzed using Kaplan Meier survival analysis (Everitt, 1994). Mosquito survival data were fitted to the Gompertz distribution model (Gompertz, 1825), using Genstat 7th Ed., as described by Clements and Paterson (1981). Germination data were analyzed using General Linear Models for binomial data, using Genstat 7th Edition.

RESULTS

Experiment 1: persistence of fungal virulence against An. gambiae s.s.

Conidia impregnated on filter paper and on netting remained pathogenic for 1 month, significantly reducing the mosquito life-span ($p<0.05$) as compared to uninfected mosquitoes.

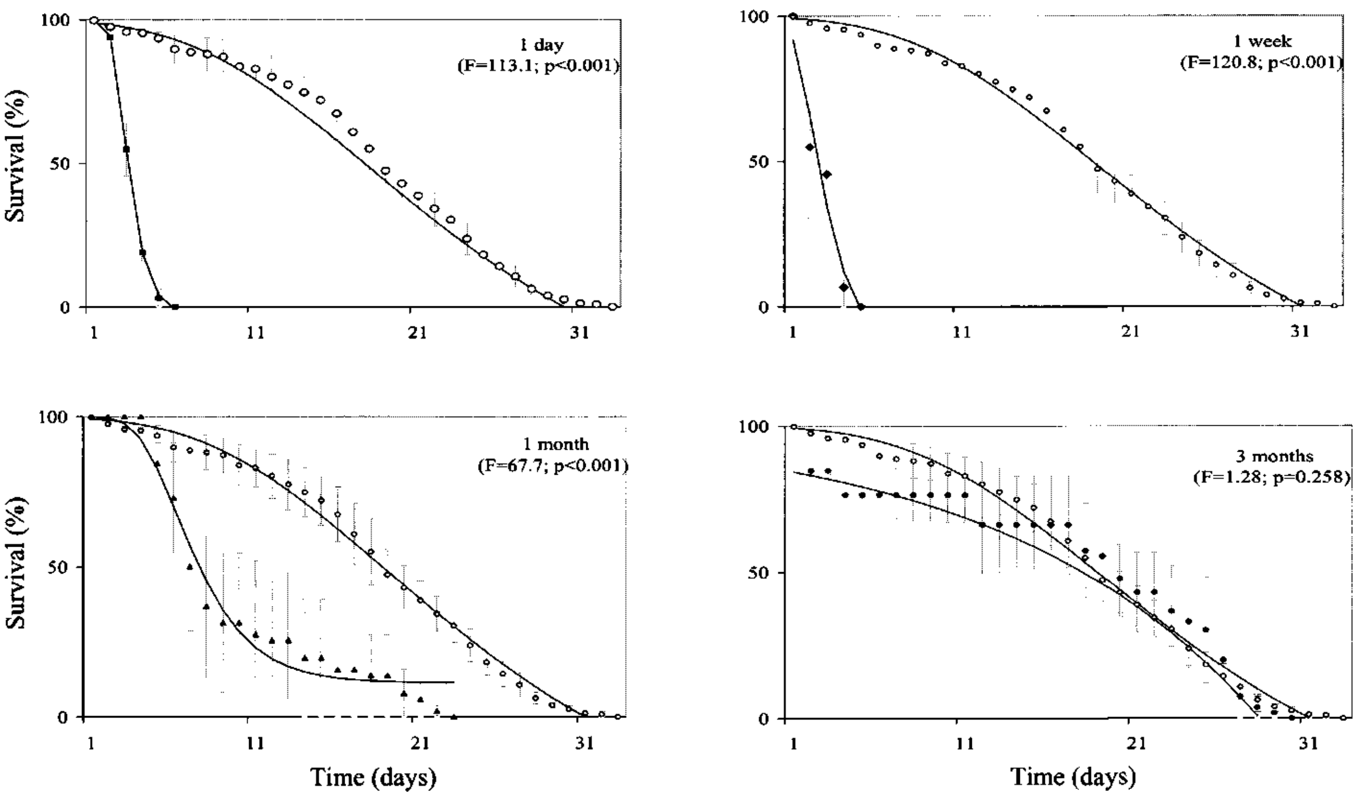

Figure 1. Virulence of 8% adjuvant-oil formulated *M. anisopliae* conidia, impregnated on filterpaper and tested either 1 day, 1 week, 1 month, or 3 months after impregnation on *An. gambiae s.s.* Black symbols indicate treated mosquitoes, open symbols indicate are uninfected mosquitoes (control). Error bars represent SEM's of mosquito survival data. In brackets are displayed the test-statistic and the p-value of Kaplan Meier survival analysis.

Infection percentages significantly decreased with time after impregnation (F=10.612; p=0.04 and F=6.935; p=0.013 for impregnated paper and netting respectively) (Table 1).

Table 1. Mean proportions of *An. gambiae s.s.* females infected with *M. anisopliae*. Mosquitoes were tested either 1 day, 1 week, 1 month or 3 months after the fungus-impregnated materials (paper or netting) had dried after impregnation.

Time of bioassay: after impregnation	Infection % ± s.e.m. of mosquitoes exposed to conidia while resting on	
	paper	netting
1 day	93.9 ± 0.6 a*	84.8 ± 5.4 A
1 week	97.7 ± 1.5 a	44.5 ± 12.9 A
1 month	67.8 ± 25.4 a	67.2 ± 23.6 AB
3 months	6.9 ± 3.7 b	1.1 ± 1.1 B

* for each impregnation material, rows without letters in common are significantly different at p<0.05 (ANOVA, LSD post-hoc test).

Pathogenicity was lost 3 months after impregnation both on paper (F=0.128; p=0.258) and netting (F=0.02; p=0.876) (Figures 1 & 2). Impregnated papers that were tested 1 day and 1 week after drying were not significantly different from each other in terms of pathogenicity towards *An. gambiae* (F=1.52; p=0.217), but both (pooled) were significantly different from the ones tested either 1 month or 3 months after impregnation (F=171.8; p<0.001 and F=231.9; p<0.001 respectively). Impregnated netting material that was tested 1 day after drying resulted in the largest reduction in mosquito survival compared to those tested later (F=226.4; p<0.001). The netting tested 1 week and 1 month after impregnation was significantly different in virulence against mosquitoes from the netting tested 1 day (F=144.3; p<0.001) and 3 months (F138.8; p<0.001) after impregnation, although they were not different from each other (F=2.81; p=0.094).

Experiment 2: residual viability

Viability rates of conidia impregnated on paper and netting gradually declined from 100% germination one day after impregnation, to 0% after 6 months (Table 2). All of these differences in time were significant (p<0.05) with an overall difference of p<0.001. All of the observed differences in viability between conidia impregnated on paper and netting were significant with an overall difference of p<0.001.

Table 2. Conidial viability of *M. anisopliae* stored for 1 day, 1 week, 1 month, 3 months or 6 months. Conidia had been impregnated either on filterpaper or on cotton mosquito netting and kept at 27 ± 2°C and 65 ± 5% RH.

		1 day	1 week	1 month	3 months	6 months
paper	trial 1	100	96	63	40	0
	trial 2	100	85	70	21	0
	trial 3	100	89	40	18	0
	average ±SEM	100 ± 0	90.0 ± 3.2	57.0 ± 9.1	26.3 ± 6.9	0
netting	trial 1	100	80	30	10	0
	trial 2	85	82	62	9	0
	trial 3	96	84	53	12	0
	average (±SEM)	93.7 ± 4.5	82.0 ± 1.2	48.3 ± 9.5	10.3 ± 0.9	0

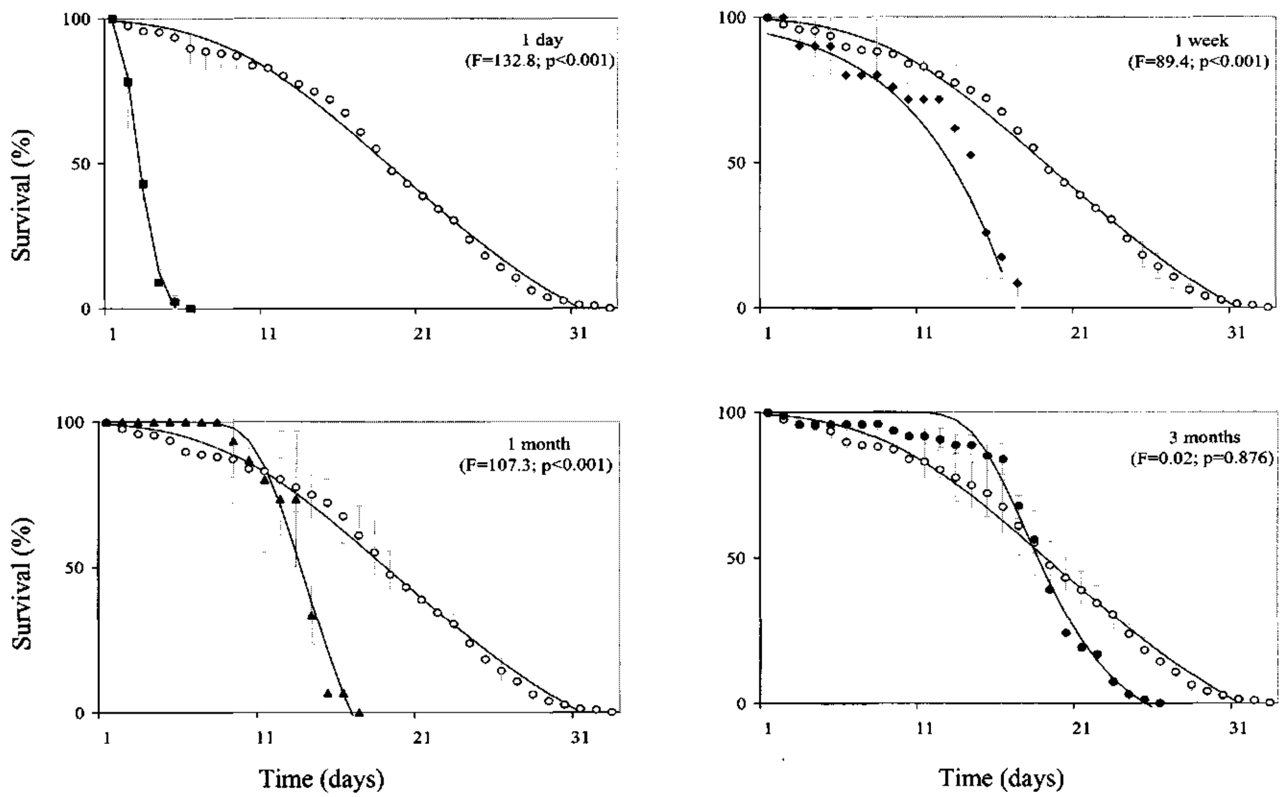

Figure 2. Virulence of 8% adjuvant oil-formulated *M. anisopliae* conidia, impregnated on netting and tested either 1 day, 1 week, 1 month, or 3 months after impregnation on female *An. gambiae s.s.* Black symbols represent treated mosquitoes, open symbols represent uninfected mosquitoes (control). Error bars represent SEM's of mosquito survival data. In brackets are the test-statistic and the p-value of Kaplan Meier survival analysis.

Viability rates of conidia kept as dry powder or in suspension are listed in Table 3. There were significant effects of time ($p<0.001$) and treatment ($p=0.046$), although the interaction of these two factors was only approaching significance ($p=0.052$). Conidia kept in the 8% vegetable oil formulation did not lose viability the first month, and there were no significant differences in conidial viability between the three different storage temperatures ($p=0.547$). For conidia that had been stored for longer than 3 months, it had became impossible to assess conidial viability since the oil had changed from a thin liquid film into a 'leathery' kind of sheet where all conidia were clumped together.

Also for the conidia kept in 0.05% Tween80 there were no significant differences in viability between the three different temperatures up to 1 month. After that there were a few significant differences found within the same temperature regime. For example the viability of conidia that were stored at 27°C up to 1 month was significantly different ($p=0.025$) from those stored for 3 months ($p=0.025$) and for 6 months ($p=0.019$). The difference in viability between conidia stored at 4°C for 6 months compared to the other (pooled) storage-periods was significant with $p=0.008$. Between the three temperatures, differences only became significant after 3 months of storage, where the ones kept at 21°C remained the most viable with 98.7 ± 0.7% germinating against 82.0 ± 8.5% ($p=0.019$) and 73 ± 17.1% ($p=0.005$) of those kept at 27°C and 4°C respectively.

There were no overall significant differences in viabilities of conidia kept in 0.05% Tween80, in 8% oil (measured up to one month), or as dry powder. There were no significant differences in viability of dry conidia between the different storage periods, or between the three different temperatures.

Table 3. Conidial viability of *M. anisopliae* stored for 1 day, 1 week, 1 month, 3 months or 6 months at 27°, 21° or 4°C. Conidia were kept either in a 0.05% Tween 80 (water) suspension or a 8% vegetable oil formulation (oil).

			1 day	1 week	1 month	3 months	6 months
oil	27°C	trial 1	100	96	96	--	--
		trial 2	100	98	98	--	--
		trial 3	100	98	98	--	--
	average ± SEM		100.0 ± 0	97.3 ± 0.7	97.3 ± 0.7	--	--
oil	21°C	trial 1	98	98	98	--	--
		trial 2	100	100	100	--	--
		trial 3	100	97	98	--	--
	average ± SEM		99.3 ± 0.7	98.3 ± 0.9	98.7 ± 0.7	--	--
oil	4°C	trial 1	100	98	98	--	--
		trial 2	100	96	96	--	--
		trial 3	100	100	98	--	--
	average ± SEM		100.0 ± 0	98.0 ± 0.9	97.3 ± 0.7	--	--

Table 3 (continued)

			1 day	1 week	1 month	3 months	6 months
water	27°C	trial 1	100	100	100	96	95
		trial 2	100	98	98	68	66
		trial 3	100	99	99	85	85
	average ± SEM		100.0 ± 0	99.0 ± 0.3	99.0 ± 0.3	83.0 ± 6.7	82.0 ± 8.5
water	21°C	trial 1	100	98	100	100	100
		trial 2	100	100	98	96	85
		trial 3	99	99	99	100	100
	average ± SEM		99.7 ± 0.3	99.0 ± 0.3	99.0 ± 0.3	99.3 ± 0.7	98.7 ± 0.7
water	4°C	trial 1	100	100	100	96	96
		trial 2	100	98	98	98	40
		trial 3	100	100	98	98	85
	average ± SEM		100.0 ± 0	99.3 ± 0.7	98.7 ± 0.7	97.3 ± 0.7	73.7 ± 17.1
dry	27°C	trial 1	100	98	99	96	96
		trial 2	100	100	98	98	94
		trial 3	100	99	100	92	91
	average ± SEM		100.0 ± 0	99.0 ± 0.3	99.0 ± 0.3	95.3 ± 1.8	93.7 ± 1.5
dry	21°C	trial 1	100	98	100	100	100
		trial 2	100	100	98	98	98
		trial 3	99	99	99	100	98
	average ± SEM		99.7 ± 0.3	99.0 ± 0.3	99.0 ± 0.3	99.3 ± 0.7	98.7 ± 0.7
dry	4°C	trial 1	100	98	98	98	97
		trial 2	100	100	100	100	100
		trial 3	100	100	98	98	99
	average ± SEM		100.0 ± 0	99.3 ± 0.7	98.7 ± 0.7	98.7 ± 0.7	98.7 ± 0.7

DISCUSSION

The results indicate that the isolate of *M. anisopliae* which was studied, when impregnated with 8% vegetable oil on filterpaper or netting material and kept continuously at a temperature of 27 ± 2°C and 65 ± 5% RH, remains pathogenic for 1 month, significantly reducing survival of female *An. gambiae s.s.*. Experiment 2 revealed that after one month 57.0 ± 9.1% (paper) and 48.3 ± 9.5% (netting) of the conidia were still viable, but that after 3 months these proportions were already reduced to 26.3 ± 6.9% and 10.3 ± 0.9%. Results from a recent field study (Chapter 9), where *M. anisopliae* conidia were suspended in the same emulsifiable adjuvant oil formulation and impregnated on cotton sheets inside houses in rural Africa showed that viability was reduced to 63% three weeks after impregnation. This is consistant with the results from the current study, where conidia, one month after

impregnation on paper and on netting had viability proportions of 57 ± 9.1% and 48.3 ± 9.5% respectively. The viability data of these two studies suggest that conidia could be impregnated on bednets, sheets, and possibly other materials such as walls and ceilings, but that re-impregnations should be carried out monthly.

Since the viability of conidia kept in suspension at any of the 3 temperatures had not significantly declined after 1 month, the factor most likely to have caused this loss in viability was the relatively low humidity. Humidity is critical for all stages of the life cycle of a fungus, including survival of conidia. To protect conidia against desiccation in areas with low humidity, formulation in non-evaporative diluents such as oils is required to prolong viability (Bateman *et al.*, 1993). Although the emulsifiable adjuvant oil fungal formulation we used (8% vegetable oil, emulsified with 0.05% Tween80) is based primarily on water, we suspect that the oil-film surrounding conidia protects them to a certain extent from dessiccation. Climatological data collected indoors in rural Tanzania (Chapter 9) show that relative humidities ranged from 27.7% in the afternoons to 78.1% at night. These low RH's in the field, at temperatures rising over 33°C for 4 hours every afternoon represent a hostile climate for impregnated conidia with considerable risk of dessiccation. Based on data described by Alves *et al.* (2002) and Burges (1998) we believe the level of protection of oil against desiccation and thus conidial longevity can be enhanced by immersion of conidia in pure oil instead of only 8% before applying them to the contact material.

Views on how long the longevity of a mycoinsecticide is required may differ, but it is generally believed that there should be minimal loss of viability for at least three months of storage at 30°C (Burges, 1998). If at least twelve months of storage are required, cooled storage would be necessary (Moore *et al.*, 1996). Several studies showed that oil-formulated *Metarhizium* conidia retained viability for many weeks (Bateman *et al.*, 1993; Morley-Davies *et al.*, 1995; Alves *et al.*, 2002), up to 30 months (Moore *et al.*, 1995). In our study, conidia formulated in 0.05%Tween80 retained >73% viability after 6 months of storage at 27°C. Alves *et al.* (2002) mention that emulsifiable adjuvant oil fungal formulations, such as the 8% vegetable oil formulation we used in this study, can be used to formulate and store conidia for medium-term, and probably for long-term storage under cooled conditions, similar to oil-based formulations. The results from the current study show that conidia in 8% oil retained viability for at least one month, although clumping of conidia was observed. This clumping became progressively worse over time and was probably due to the surfactant not being optimal in emulsifying. Since other studies that used oil-formulations have found long storage periods without much loss in viability (Moore *et al.*, 1995) it may be worthwhile to monitor the conidial viability of this particular *M. anisopliae* strain that are suspended in pure oil instead of 8% oil. We found that when dry conidia were stored with silica gel they retained >93% viability over the 6 months period at all three temperatures. This is in accordance with other studies, where a RH of 0% resulted in high conidial longevity (Daoust *et al.*, 1983; McClatchie *et al.*, 1994; Hong *et al.*, 2001).

Due to the absence of any suitable outdoor anopheline mosquito trap, the most effective methodology for use of this fungus against adult stage mosquitoes in Africa would be to apply the fungus indoors. Although this is prone to many practical obstacles there is also an important advantage regarding formulation when compared to outdoor use of this fungus against agricultural pest insects: It is very dark inside African houses and therefore there is no concern of UV light damaging conidia, thus eliminating the need to include sunscreens in the formulation.

In practical terms the results from this study suggest that conidia of this isolate, once harvested, can be stored easily in dry-air and that conidia should be formulated only shortly before impregnation to retain viability as high as possible. As temperatures inside buildings in Africa may exceed 35°C, it may be helpful to pre-dry conidia as described by McClatchie *et*

al. (1994) and Alves *et al.* (2002) to enhance temperature tolerance. Formulation can be pure oil, as suggested by studies from the LUBILOSA project (a research programme on the biological control of locusts and grasshoppers using *Metarhizium flavoviride*), or in emulsifiable adjuvant oil. These methods should be studied in more detail for the proposed indoor use of *M. anisopliae* against mosquitoes.

CONCLUSIONS

The results of the current study on fungal virulence against *An. gambiae s.s.* and conidial viability showed that the conidia of *M. anisopliae* isolate IC30, when impregnated on filterpaper and netting, remain virulent for 1 month at 27°C and 65 ± 5% RH and retain viability over a period of at least 6 months if stored as a dry powder with silica gel or in 0.05%Tween80. It is proposed to study the impact that oil-formulations may have in improving shelf life and conidial virulence on impregnated materials.

ACKNOWLEDGEMENTS

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CHAPTER 9

REDUCING LONGEVITY OF ADULT MALARIA (*ANOPHELES GAMBIAE S.L.*) AND FILARIASIS (*CULEX QUINQUEFASCIATUS*) VECTORS USING INDOOR RESTING TARGETS TREATED WITH THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE*

To be submitted as: Scholte E-J, Ng'abi KR, Kihonda J, Knols BGJ, Takken W, Paaijmans K, Abdulla S, and Killeen, GF: Reducing longevity of adult malaria (*Anopheles gambiae s.l.*) and filariasis (*Culex quinquefasciatus*) vectors using indoorresting targets treated with the entomopathogenic fungus *Metarhizium anisopliae*.

REDUCING LONGEVITY OF ADULT MALARIA (*ANOPHELES GAMBIAE S.L.*) AND FILARIASIS (*CULEX QUINQUEFASCIATUS*) VECTORS USING INDOOR RESTING TARGETS TREATED WITH THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE*

ABSTRACT

Current indoor control for malaria and filariasis vectors in Africa depends on the use of insecticides and/or repellents. Although several biological control agents can reduce larval mosquito populations, none are appropriate for targeting the adult stage. Following successful laboratory evaluations of the impact of the entomopathogenic fungus *Metarhizium anisopliae* on adult *Anopheles gambiae* and *Culex quinquefasciatus*, we field-tested two alternative domestic applications of *M. anisopliae* in houses in a region holoendemic for malaria and lymphatic filariasis in southeastern Tanzania. The fungus was applied on black cotton sheets, attached to ceilings as indoor mosquito resting targets (novel format), and as residual application on walls (conventional format). Over half of all indoor resting mosquitoes were caught on the targets. Of mosquitoes resting on treated targets, 33.6% (n=181) of *An. gambiae sensu lato* and 10.0% (n=6) of *Cx. quinquefasciatus* became infected with the fungus and died significantly sooner than uninfected mosquitoes ($p<0.001$; $F=178.9$ for *An. gambiae s.l.* and $p<0.001$; $F=16.30$ for *Cx. quinquefasciatus* by Kaplan-Meyer analysis). More than 94% of the collected *An. gambiae* were *An. gambiae sensu stricto*. Laboratory-reared *An. gambiae s.s.* exposed to conidia on indoor walls showed similar infection percentages and longevities. This study was the first to show that the entomopathogenic fungus *M. anisopliae* can infect and kill adult endophilic Afrotropical malaria vectors under natural conditions. These results also suggest that black cloth indoor resting targets may have considerable potential for delivery of biocontrol agents and other contact insecticides.

INTRODUCTION

Malaria and bancroftian filariasis rank amongst the world's most prevalent tropical infectious diseases. At least 0.7 – 2.7 million people die from malaria each year, of which 75% are African children (Breman, 2001, WHO/UNICEF, 2003). Filariasis is distributed in much of the tropics, affecting about 146 million people (WHO, 1992). These numbers are increasing due to environmental changes as a result of human activity (deforestation, agriculture and urbanization), breakdown in health care due to socio-economic problems or wars, and continued increase of resistance to drugs (Snow *et al*, 2001; Hougard *et al.*, 2002; Korenromp *et al.*, 2003). Until now, the most effective and important vector control interventions for malaria prevention have relied upon the application of insecticides in houses: insecticide treated nets are the top priority intervention in Africa today (Lengeler, 1998; UNICEF/WHO, 2003) and indoor-residual spraying finds more limited application but is highly effective (Kouznetsov, 1977; Goodman et al., 2001; Charlwood *et al.*, 2001; Booman *et al.*, 2003). The use of these compounds for mosquito vector control is undeniably of enormous value for public health, saving thousands of lives each year. Unfortunately, the compounds available for indoor spraying (including DDT) in vector management programmes create risks due to their toxic properties for humans and other mammals (WWF,

1999). Moreover, the ever-developing resistance against these compounds remains an issue of grave concern (Chandre *et al.*, 1999; Hemingway and Ranson 2000; Brooke *et al.*, 2002). It is therefore not surprising that interest in alternative non-chemical strategies such as biological control has increased over recent decades (Service, 1983; Hougard *et al.*, 2002; Fillinger *et al.*, 2003; WHO, 2003; Scholte *et al.*, 2004).

In addition to the available biological vector control agents, such as predatory fish (Legner, 1995) and bacteria (Becker and Ascher, 1998), that are aimed at the larval stage of mosquitoes, we have studied the potential of a fungal biocontrol agent aimed at the adult stage of African mosquito vectors which might be used indoors as an environmentally safe residual myco-insecticide. Laboratory studies showed that the entomopathogenic fungus *Metarhizium anisopliae* can effectively infect and kill adult-stage *Anopheles gambiae* Giles *sensu stricto* and *Culex quinquefasciatus* Say, and significantly reduce their survival (Scholte *et al.*, 2003a,b). This fungus is soil-borne and predominantly affects soil-dwelling insects, although it is a generalistic insect pathogenic fungus, capable of infecting a large array of insect species (Veen, 1968). The fungus is of interest to vector control for several reasons. First, it is very common worldwide, thus avoiding the introduction of a non-endemic organism as in classical biological control (Howarth, 1991). There are also no known toxicological or pathological symptoms in birds, mice, rats, or guinea pigs after exposure to conidia of the fungus (Zimmermann, 1993). Strasser *et al.* (2000) conclude from a risk-assessment study that the fungus poses no obvious risk to humans or the environment. However, in aquatic environments, the fungus can cause significant mortality in embryos of shrimps, frogs and fish exposed to high dosages of conidia (Genthner *et al.* 1994, 1998). Although infecting a large range of insects, its indoor use would be unlikely to impact on non-target organisms. Moreover, the fungus can be easily and economically produced *in vitro*, with minimal infrastructure and equipment. Here we report the results of a small-scale field study in which a conidial suspension was compared in two ways: In the first we impregnated mosquito resting targets: pieces of black cotton fabric, attached indoors to the lower part of ceilings in households in rural Tanzania. In the second, we applied the fungus directly to the wall, in a manner analogous to indoor residual spraying. In both cases, we tested whether wild mosquitoes, after resting on the fungus-impregnated cloth, became infected and died as a result of the infection.

MATERIALS AND METHODS

Ethics

Ethical clearance for the study was obtained from the Institutional Review Board of the Ifakara Health Research and Development Centre (IHRDC), the Medical Research Coordination Committee of the National Institute for Medical Research (NIMR) and the Tropical Pesticide Research Institute (TPRI) of Tanzania. Prior to any practical work the Lupiro community, its village leaders, and Government representatives of Kilombero and Ulanga Districts were sensitized to the intended study. A community meeting was organized by the village chairman where the project was discussed with the research team and permission was granted to begin enrolling study participants. Houses were enrolled only after obtaining informed consent by the household head and the individual household members.

Study site

The studies were carried out in Lupiro village (Ulanga District), 40 km south of Ifakara, in the southern Kilombero valley, a flat plain some 20 km wide in south-eastern Tanzania at latitude 8.38°S and longitude 36.67°E as described previously (Charlwood *et al.*, 2000). This village of about 11,400 inhabitants (year 2004) lies on a low plateau of about 10m above the surrounding area at an elevation of 324 m above sea level. The village lies amidst a relatively intact area of dry wooded grassland 10 km west of the Selous Game Reserve. Habitations in the village are relatively sparse with large numbers of mature trees and along the edge of the village there is farming of mainly maize and cassava. These are generally mud-walled thatched huts with small windows and two or three rooms. Most houses remain habitable for a relatively short period (2-6 years). During our study (October-December 2003) almost all households used bednets (Armstrong Schellenberg *et al.*, 2001), although less than 10% of these are impregnated with sufficient levels of insecticide (Erlanger *et al.*, 2004). Most people sleep on string beds with woven straw mats, although some use mattresses. The majority of villagers are farmers, the main crops being rice, which is cultivated in a permanent swamp (near the Ndolo River), as well as maize and cassava. There are relatively few domestic animals and cattle in the area, partly due to endemic trypanosomiasis associated with the local wildlife, which is abundant especially in the dry season, when they move out of the nearby Selous Game Reserve in search of food and water. About 500 m east of the village flows the Ndolo River, which, downstream, joins the Kilombero River. In 2002 the natural wetland between the river and the village was cleared for rice-cultivation, creating abundant anopheline breeding sites. Many people spend their time in the rice fields, in close proximity to mosquito breeding sites. In the evening most people return to the village, although some remain for the night to chase away birds and large herbivores that feed on the rice crop. They spend the night on simple elevated platforms about 1.5m above ground level that are built in the rice-fields.

The climate is dry with two rainy seasons; the long rains from April to June and the short rains normally in October and November, although in 2003 rains started about a month later, in December. The average indoor temperatures, measured by two Tinytag dataloggers (Gemini®) placed in two of our study houses ranged between minima of 24.4°C in the early morning until sunrise and maxima of 38.0°C around 13.00 hrs. The average indoor relative humidity in these houses was 52.6 ± 0.13% with minima of 27.7 during the hot part of the day and maxima of 78.1% at night.

During the first few weeks of mosquito resting catches (pre-intervention period) we observed that the majority of mosquitoes were found hanging from oblique and vertical planes, and often on materials of a dark coloured rough material such as black curtains or clothes. These observations, and historical reports of the attraction of Afrotropical *Anopheles* to black fabric (Anonymous, 1975), prompted us to use sheets of black cotton fabric to act as resting targets to achieve a high proportion of indoor resting mosquitoes contaminated with conidia of the fungus (Figure 1). In five of the households
M. anisopliae-impregnated sheets were fitted (henceforth called 'test' houses) and the other five households received control cloths (without fungus). One room was selected for fitting of a cloth target in each house. Of the 10 selected houses, 8 households used untreated bednets, all of which were maintained poorly, having numerous holes. Two households did not use a bednet. Although bednets themselves are potential objects on which to apply conidia, safety reasons, time constraints, availability of conidia, and preference of the inhabitants made us decide to impregnate cloth sheets.

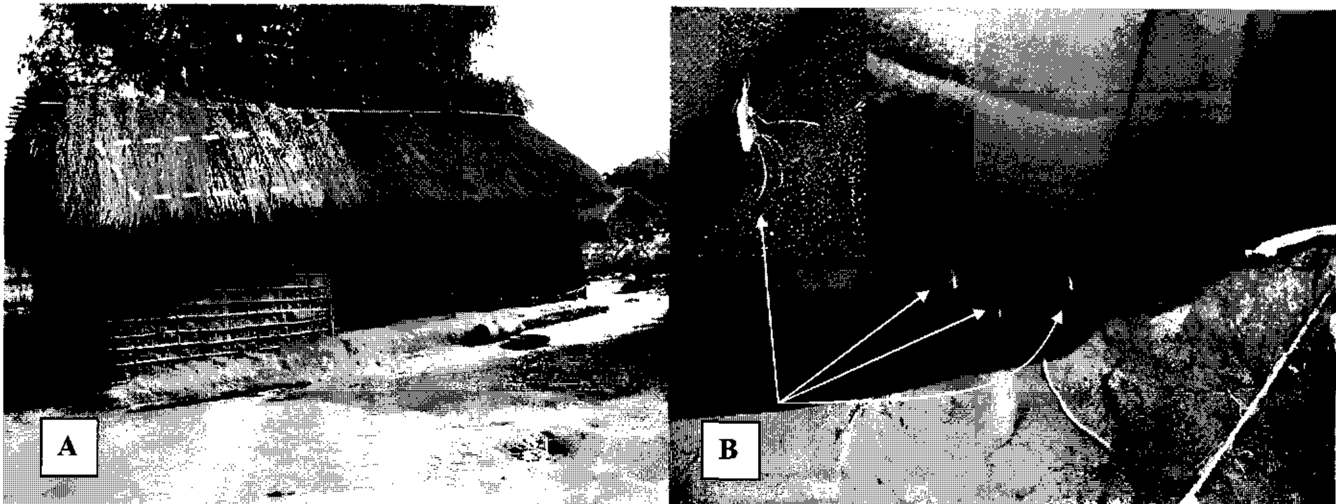

Figure 1. A: A house in Lupiro, Tanzania, where a black cotton sheet, impregnated with conidia of the entomopathogenic fungus *Metarhizium anisopliae*, was placed indoors on the ceiling (in the picture the sheet is drawn superimposed to show its size relative to the house) to act as a mosquito resting target.
B: Blood fed *Anopheles gambiae s.l.* resting on one of the cloth sheet.

Fungus

Metarhizium anisopliae var. *anisopliae* (Metsch.) Sorokin, isolate ICIPE-30 (courtesy Dr. Maniania, International Centre of Insect Physiology and Ecology) was originally isolated in 1989 from a stemborer, *Busseola fusca* Fuller, near Kendu Bay, Western Kenya. In the Laboratory of Entomology at Wageningen University, conidia were inoculated on oatmeal agar (OA) and placed in an incubator to grow for two weeks, after which fresh conidia were harvested using a 0.05% Triton-X solution and a glass rod. These conidia were used to infect a laboratory strain of *An. gambiae s.s.* The conidia grown from these fungus-killed mosquito cadavers were again inoculated on OA. This procedure was repeated three times to enhance virulence. The solvent containing conidia from the third repetition was transported to Tanzania, where it was grown again on OA in the laboratory of the IHRDC in Ifakara. Conidia were harvested using a glass rod and 0.05% Triton-X. After counting, dilutions were made using 0.05% Tween-20. For both experiments we used a stock solution of 8.0×10^7 conidia ml^{-1}.

Mosquitoes

For experiment 2 we used *An. gambiae s.s.* colonised in the mosquito insectary at the IHRDC, Ifakara. This colony was established from mosquitoes collected at Njage village, south-eastern Tanzania in 1996 and maintained using standard rearing procedures at 28 ± 2 °C and 60-70% RH.

Mosquito species identification

Wild-caught mosquitoes were initially identified to species morpohologically (Gillies and De Mellion, 1968), following which DNA was extracted from each *An. gambiae s.l.* specimen according to the protocol of Collins *et al.* (1987). DNA pellets were suspended in 100µl ddH_2O. Two µl of this extract was used to determine the sibling species, using the species diagnostic rDNA-PCR protocol described by Scott *et al.* (1993).

Experimental procedures

Experiment 1

After the granting of official permission by the authorities, over 30 local houses were visited for suitability for inclusion in the study (experiment 1). From this group a selection was made for houses in which mosquitoes were abundant, insecticide was not used either on walls or on mosquito nets, that were constructed similarly with mud walls and thatched roofs, and were located in the eastern part of the village near the rice fields. From these, a total of 10 households were eventually included in the study after written consent was obtained .

Indoor resting mosquitoes were collected manually using torches and mouth aspirators six days a week for a period of 6 weeks (3 weeks pre- and intervention) at the end of the dry period. During the entire period of mosquito resting catches no precipitation occurred. These resting catches were carried out for 15 minutes per house between 07.00 and 10.30 hrs by two of the investigators (EJS, KN). In cases where the selected houses consisted of multiple rooms, mosquito catches were restricted to only one room. These mosquitoes were placed gently in polystyrene cups covered with netting material. On each cup the date of collection and the house number were written, and a wad of cotton wool, soaked in 6% glucose solution was placed on top. These cups were placed in a cardboard box to be protected from the sun while walking from one house to the next. Each day, directly after finishing the resting catches in all the 10 houses, a layer of 1 cm of ground water from a nearby bore-hole and

pump was added to the cups, providing humidity and the possibility for gravid females to oviposit. All cups were placed in an improvised on-site shelving system where the boxes were protected from direct sunlight and predatory ants. A moist towel was placed on top of each box to maintain a sufficiently high humidity in the boxes. The cotton wool containing the glucose solution was moistened daily with fresh solution and replaced with a fresh wad when needed. Mosquito mortality was checked daily. Dead mosquitoes were removed from the cups. From dead *An. gambiae s.l.*, the heads were removed and placed in Eppendorf vials containing silica gel for species identification (Scott 1993). The remaining part of the mosquito cadavers were then placed individually in glass tubes containing a piece of wet clean filterpaper, and sealed off to check for fungal growth four days later (Boucias and Pendland, 1998).

Black cotton sheets of 3 m^2 were impregnated with the *M. anisopliae* stock solution described above. In total 700 ml of this stock solution was used to impregnate five sheets, resulting in a conidial density of 3.7×10^9 conidia/m^2. Prior to impregnation, vegetable oil was added, resulting in an 8% oil-formulation. Impregnation was done by shaking the flask containing the conidial oil-formulated suspension vigorously and then sprinkling it carefully on the cloths, kneading it manually using latex gloves before the suspension was absorbed by the sheet. This was performed in Ifakara in an enclosed room at room temperature with high humidity (>90%) where the sheets were left to dry slowly for 48 hrs. The five control sheets were treated equally with the difference that the oil formulation did not contain conidia. After drying, all sheets were transported to the houses in Lupiro village and suspended from the lower part of the roofs indoors (Figure 1). Of the five households in which treated sheets were placed, one did not use a bednet. In the remaining 5 houses (four with and one without a bednet) the unimpregnated control sheets were fitted. At the start of the study two dataloggers were placed in two different houses between the thatched roof and the cloths to monitor temperature and humidity. Daily indoor mosquito catching continued until 3 weeks after the intervention. To determine conidial viability during the testing period we carried out two different checks. First, we kept 3 small glass test tubes, each containing 1 ml of the original oil-formulated conidial suspension used for impregnation. We inoculated 0.1 ml of these suspensions (with 3 replications) on SDA-agar plates to count the proportion of conidia that had germinated 18-20 hrs later using a light microscope at 400x magnification. Secondly, conidial viability on the cloths was assessed by cutting off small pieces (1cm^2) of 3 different sheets. These three pieces were placed in a small plastic container and transported the same day to Ifakara and placed on SDA-agar. 18-20 hrs later the pieces of cloth were carefully removed from the agar, after which the percentage germination was determined as described above. Both checks were carried out at the IHRDC laboratory in Ifakara on the day the sheets were placed inside the houses and three times weekly after that.

Experiment 2

Laboratory-reared *An. gambiae s.s.* pupae were taken from the IHRDC mosquito rearing facility and transported to Lupiro village where they eclosed in a standard cubic netting cage. At the age of 2-4 days both males and females were placed gently into two identical flat rectangular netting cages (2.5 x 40 x 50 cm) and placed for 24 hrs into one of the houses we selected for experiment 1 (Figure 2). Inside the house both cages were placed tightly against the mud wall. One cage was placed against a section of the wall that, two days earlier, had been impregnated, using a paint brush. A total of 9.3 ml of the stock solution was applied, resulting in the same conidial density per surface area as in experiment 1 (3.7×10^9

con. m^{-2}). The cages used were 'flat' in order to increase the probability that mosquitoes would rest on the wall and thus be infected with the fungus. After 24 hrs the mosquitoes were removed from the cages and placed into two separate polystyrene cups. They were then treated similarly to the mosquitoes kept in experiment 1, to measure longevity and determine the proportion of *M. anisopliae*-infected mosquitoes. In total 116 mosquitoes were tested in two separate trials. The first trial of experiment 2 was carried out on the last day of experiment 1 in which cloths and conidial suspensions were checked for conidial viability (the end of week 3). Since the same conidial suspension was used for both experiments, we used the oil-formulated conidial suspension of experiment 1 to assess conidial viability in experiment 2.

Figure 2. Rectangular cages containing laboratory reared *An. gambiae s.s.* placed indoors in a village house. The cage on the left was placed on a section of the wall that was impregnated with the entomopathogenic fungus *M. anisopliae*. Mosquitoes were kept here for 24 hrs after which longevities and infection proportions were determined (experiment 2).

Data analysis

Mosquito longevity data were plotted on Gompertz survival functions (Genstat 7.0) from which LT_{50}-values were calculated. Mosquito survival was analyzed using Kaplan Meyer survival analysis (SPSS 11.0). Differences in longevity between mosquitoes caught in the 6 different weeks were analysed using Cox Regression survival analysis. Differences in the numbers of *M. anisopliae*-infected mosquitoes each week that were caught during the post-intervention period, and the distributions of mosquitoes caught either on cloth or elsewhere in the room were analysed using Chi-Square Tests (SPSS 10.0). Daily survival rates were calculated from Gompertz survival functions.

RESULTS

Mosquito species identification

From a total of 190 random samples, 113 could be successfully identified. Of those, *An. gambiae s.s.* represented 94.7 ± 0.5% of all *An. gambiae s.l.* collected. The remaining 5.3 ± 0.5% was *An. arabiensis*. This proportion appeared to be constant over time.

Experiment 1

From all 10 study houses a total of 2939 mosquitoes were caught, 1052 during the pre-intervention and 1887 during the intervention period. Of these 88.9% were *An. gambiae s.l.* and 10.7% *Cx. quinquefasciatus* (Table 1). Overall, 53.6% of the mosquitoes were caught on the targets, and 46.4% elsewhere in the rooms.

Table 1. Species distribution of mosquitoes caught indoors by resting catches.

	Total	male	female
An. gambiae s.l. *	2612 (88.9%)	1174 (44.9%)	1438 (55.1%)
An. funestus	2 (0.1%)	0	2
Culex spp	314 (10.7%)	153 (48.7%)	161 (51.3%)
unidentified Culicids	11 (0.4%)	-	-
Total	2939 (100%)		

* : of these 94.7% were *An. gambiae sensu stricto* and 5.3% were *An. gambiae arabiensis*

A total of 980 *An. gambiae s.l.* and 109 *Cx. quinquefasciatus* were collected during the intervention period in the 5 treatment houses. Of these, 181 *An. gambiae s.l.*, 6 *Cx. quinquefasciatus* and one unidentified Culicid were found infected with *M. anisopliae*. The total number of mosquitoes caught on the 5 treated sheets were 539 *An. gambiae s.l.* and 60 *Cx. quinquefasciatus*. When the fungus-infected mosquitoes were calculated as a proportion (± SEM) of the total number of mosquitoes caught on the treated sheets during the intervention period we found this was 33.6 ± 0.01% for *An. gambiae s.l.* and 10.0 ± 0.01% for *Cx. quinquefasciatus* with a mean proportion of 31.2 ± 0.01% of all mosquito species included. When calculated as a proportion of the total number of mosquitoes caught anywhere in the treated rooms during the intervention period we found that 18.5 ± 0.01% of *An. gambiae s.l.* and 2.2 ± 0.01% of *Cx. quinquefasciatus* were infected with the fungus, with a mean of 17.1 ± 0.01% of all mosquito species included. Of the 188 *M. anisopliae*-infected mosquitoes, 124 (66%) were caught on the cloths, and the remaining 64 (34%) elsewhere in the rooms. Not a single fungal infection was found on mosquito cadavers during the pre-intervention period. Of the 188 infected mosquitoes most were caught during the first two weeks after the start of the intervention; 80, 79 and 29 in the first, second and third week, respectively. The overall differences between these numbers was significant ($F=27.14$, $p<0.001$).

Mosquito survival

An. gambiae s.l.: There was no significant difference in longevity between mosquitoes that had been caught before versus after the intervention ($F=2.903$, $p=0.088$), and no significant difference between mosquitoes caught from the control houses versus un-infected mosquitoes from the test houses during the intervention period ($F=0.91$, $p=0.3411$). Also, there was no overall significant difference in survival between mosquitoes caught during intervention in the treated versus the control houses ($F=2.26$, $p=0.1328$). However, from the mosquitoes caught during the intervention period from treated houses, infected mosquitoes lived for significantly shorter mean times than non-infected ones (Figure 3) (overall effect of male/female $F=178.9$, $p<0.001$). LT_{50}-values ranged between 3.70 and 3.49 days (*M. anisopliae*-infected males and females respectively) against 5.88 and 9.30 days (uninfected, males and females). The maximum number of days that *An. gambiae* remained alive, counted from the day of capture, was 47 for males and 39 for females.

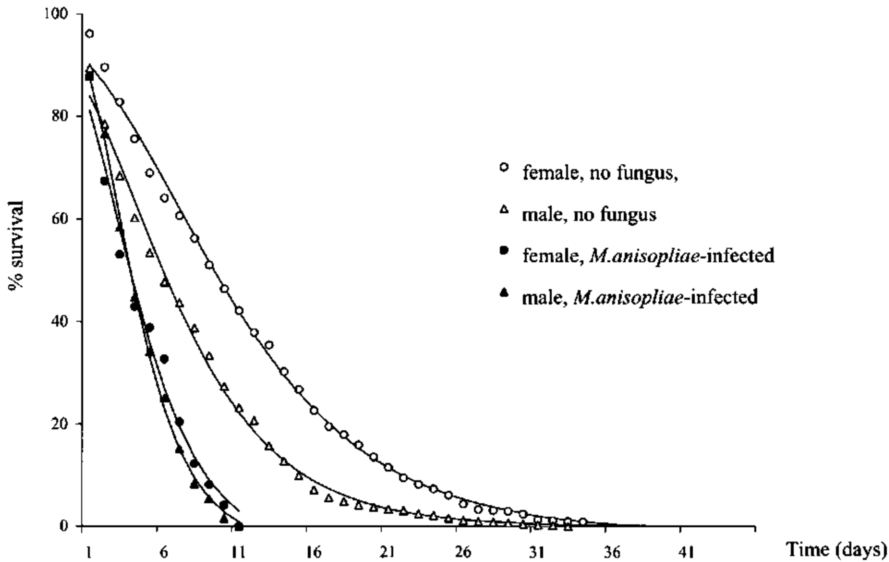

Figure 3. Survival curves (fitted on Gompertz survival-distribution model) of *M. anisopliae*-infected and uninfected wild *An. gambiae s.l.* mosquitoes (males and females).

Cx. quinquefasciatus: There was no significant difference in longevities between mosquitoes that were caught before or during the intervention (F=2.10, p=0.1470). However, of the mosquitoes caught in the treated houses (during the intervention) the 6 *M. anisopliae*-infected mosquitoes lived for significantly shorter times than uninfected ones (Figure 4) (F=16.30, p<0.001). The LT_{50} value for the *M. anisopliae*-infected mosquitoes (since only 6 specimens were infected we decided to combine male and female data), calculated from the Gompertz distribution model was 12.0 days, against 20.6 and 24.1 days (males and females respectively) for mosquitoes that had not been infected with the fungus.

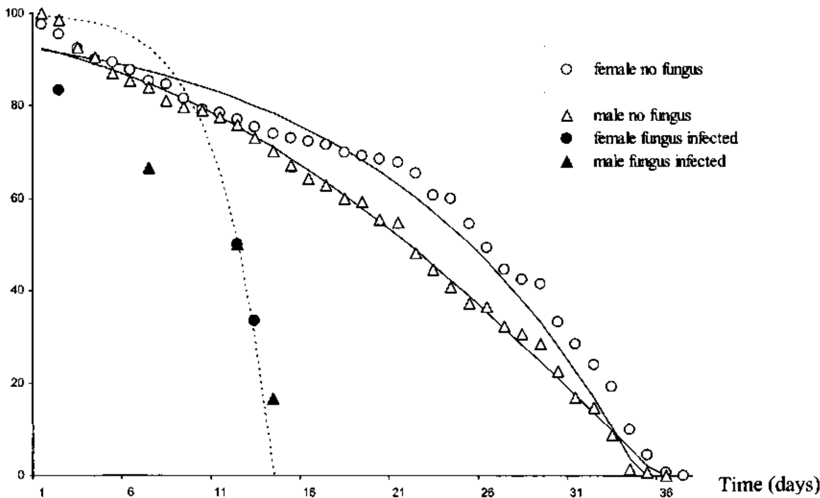

Figure 4. Survival curves (fitted on Gompertz survival distribution model) of *Metarhizium anisopliae*-infected and uninfected wild *Culex quinquefasciatus* mosquitoes.

Conidial viability

Conidia in the suspension hardly lost any viability during the period of experiment 1: from 96.3 ± 0.88% to 93.7 ± 0.88% germinating (Table 2). However, there was a loss of conidial viability from conidia impregnated on the sheets from 95.0 ± 1.0% to 63.0 ± 6.7% germinating over the three weeks of the intervention. Table 3 shows the temperatures and relative humidity of the two houses with the data-loggers.

Experiment 2

From 58 mosquitoes kept in the test cage, 22 cadavers (8 males and 14 females) showed sporulation of *M. anisopliae* (37.9 ± 2.0%). There were no differences in survival between the two groups (F=0.06, p=0.811). There was also no significant difference in survival between caged mosquitoes on the treated wall and mosquitoes on the control wall (F=0.48, p=0.487), but within the group that was kept on the treated part on the wall the *M. anisopliae*-infected mosquitoes (both sexes pooled) had significantly shorter life spans than

uninfected ones (F=7.16, p=0.008). LT_{50}-values of fungus-infected mosquitoes were 7.6 and 7.1 days compared with 7.4 and 11.6 days for the uninfected (males and females respectively).

Table 2. Conidial viability expressed as the proportion of germinating conidia of both the conidia from the cloth and the original oil-formulated suspension used for impregnation in experiment 1.

conidia impregnated	at day1	after 1 week	after 2 weeks	after 3 weeks
cloth nr 1	93	85	77	75
cloth nr 2	96	92	79	62
cloth nr 3	96	71	56	52
mean ± S.E.M.	95.0 ± 1.00	82.7 ± 6.17	70.7 ± 7.35	63.0 ± 6.66
Conidial suspension	at day 1	after 1 week	after 2 weeks	after 3 weeks*
Trial 1	95	95	96	94
Trial 2	98	94	89	92
Trial 3	96	96	96	95
mean ± S.E.M.	96.3 ± 0.88	95.0 ± 0.58	93.7 ± 2.33	93.7 ± 0.88

* The test tubes containing the suspension used to assess the conidial viability of 'after 3 weeks' derive from the same suspension used for bioassay 2.

Table 3. Temperatures and relative humidities of two local houses, measured by data-loggers placed indoors hanging at a height of about 2.0m. during the entire duration of the experiment.

	Temperature (°C)			Relative humidity (%)		
	mean	min.	max.	mean	min.	max.
House nr 1	30.36	24.74	37.97	52.43	27.65	76.47
House nr 2	30.32	24.40	37.49	52.69	28.40	78.10
Mean ± S.E.M.	30.34 ± 0.02°C			52.56 ± 0.13%		

DISCUSSION

The results of this study confirm that *M. anisopliae* conidia, formulated in 8% vegetable oil and impregnated on cloth and placed in village houses can successfully contaminate, infect and kill wild *An. gambiae* and *Cx. quinquefasciatus*.

The proportion of *An. gambiae s.l.* that was killed by the fungus after contact with the impregnated sheet in this study (33.6 ± 0.01% in experiment 1 and 37.9 ± 2.0% in experiment 2) was lower than in an earlier laboratory study in which almost 60% was found infected when exposed to a dosage 2.3 times lower than the one used in the current study (Scholte *et al.*, 2003). In that laboratory study an infection percentage of 32.6 ± 5.4% (similar to the one found in the current study) was found when the mosquitoes were exposed to 1.6×10^8 conidia m^{-2}, a dosage 23.3 times lower than the one used in our field study. It is possible that the application method used for the fungal suspension to impregnate the cloth resulted in uneven distribution of conidia. From observations of conidial distribution on the samples cut weekly

from the cloth to check for conidial viability, we noticed that conidia were sometimes clustered and unevenly distributed, although on those 1 cm^2 samples none were found where no conidia were present. This resulted in areas with higher or lower conidial concentrations, increasing the probability that a mosquito made contact with an area with a relatively low number of conidia. We are uncertain how to interpret the fact that only 10% of the *Cx. quinquefasciatus* that were caught from the fungus-impregnated cloths were found infected, against 33.6% for *An. gambiae s.l.*. Possibly the immunocompetence of *Cx. quinquefasciatus* was higher in eliminating the fungal infection than in *An. gambiae*, although previous data (Scholte *et al.*, 2003), where both mosquito species were infected with dry conidia of the same fungus under laboratory conditions, do not support this speculation.

Also in experiment 2 we found that the proportion of infected mosquitoes was lower than expected. In this case the design of the mosquito cage may have influenced the outcome. The thin but wide shape of the cage resulted in a theoretical probability of 44.9% for a mosquito to rest on the impregnated side, assuming that the mosquitoes remained on the same side of the cage throughout the 24 hours of the exposure. Since the movements of mosquitoes in the cage were not monitored, it remains uncertain what proportion actually rested on that particular side of the cage in contact with the impregnated surface. In spite of these cage effects, it nevertheless was clear that a large proportion of the mosquitoes became contaminated with the fungus in this bioassay.

When the survival data of female *An. gambiae s.l.* from experiment 1 are compared with those obtained from laboratory experiments where female *An. gambiae s.s.* were infected with oil-formulated conidia (Scholte *et al.*, 2003), we see that the LT_{50} values for the control group in the field and the laboratory were similar: 9.3 (field) against 9.9 ± 1.2 days (laboratory). However, when using the same oil-formulation, the females caught in the field succumbed faster to the fungal infection than those in the laboratory. LT_{50}-values of the two highest fungal dosages in the laboratory (1.6 x 10^9 and 1.6 x 10^{10} con/m^2) were 6.7 ± 0.4 and 5.9 ± 0.3 days, against 3.5 days (3.7 x 10^9 con/m^2) in the field study. These values are more similar to the laboratory results obtained from infected female *An. gambiae s.s.* with dry conidia, where the LT_{50} was 3.4 ± 0.3 (conidial exposure of 24 hrs).

Mosquito survival is often expressed as the daily probability of survival (p), (e.g. see Charlwood *et al.* (1995)). Mosquito survival is often assumed to die off exponentially and a constant daily survival rate for a mosquito is obtained from linear regression. In our case, and as reported previously for *An. gambiae* in the Kilombero valley during the dry season (Charlwood *et al.*, 2000), the survival data fitted better to a Gompertz distribution than to a simple exponential decay model. For example, in the case of wild female *An. gambiae s.l.* of experiment 1 the mean deviance of the residual is 1.26 when fitted to a Gompertz model, compared with 21.11 when fit on the exponential. Since the Gompertz distribution is not linear, it is only possible to calculate survival rates day per day, resulting in a daily survival rate-curve (Figure 5).

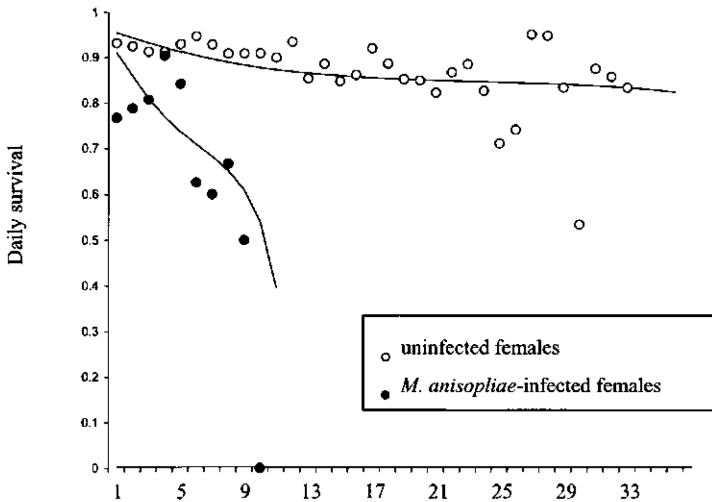

Figure 5. Daily survival rates of wild caught female *Anopheles gambiae s.l.* with or without infection of the entomopathogenic fungus *M. anisopliae.*

The daily probability of survival of *M. anisopliae*-infected females declines fast, whereas that of uninfected females remains at an average of 0.85 ± 0.01 for up to 42 days. This value is similar to that reported by Gillies (1961) (p=0.84 based on mark-release- recapture studies from East Africa). It should, however, be noted that wild mosquitoes were kept alive under semi-controlled conditions which may have influenced survival: once the mosquitoes were caught and kept in the cups, their energy intake (glucose only, no blood) and energy consumption (reduced flight activity) became different from their 'free' siblings in the village. Also, the absence of risks involved with blood feeding and the micro-climatological conditions in which the mosquitoes were kept in the cups will have influenced survival compared to wild mosquitoes. Notwithstanding these differences, the effect of *M. anisopliae* in reducing daily survival rates of *An. gambiae s.l.* is clear.

This leads us to suppose that application of this method on a larger scale (Killeen *et al.*, 2003) is likely to have a reducing effect on the survival of wild anopheline mosquitoes, and possibly on *Plasmodium* transmission. To assess the actual impact of the method discussed in this study it is necessary to perform a large-scale field trial, applying conidia on proportionally large surface areas in all houses in an area the size of at least an entire village (Killeen *et al.*, 2003; Gimnig *et al.*, 2003; Hawley *et al.*, 2003). For applying the fungus there are several options. As in the current study, the use of larger impregnated cloths is an option. This method has the advantage that the houses themselves remain clean of an oil-based layer on the walls/roof, the cloths can be easily removed and washed before new impregnation, but has the disadvantage of leaving uncovered large surface areas where mosquitoes may be resting, and remain uncontaminated. Another option is bednet impregnation, having the advantage of using human odour as an attractant, thereby increasing the probability of

mosquitoes becoming infected while trying to enter the bednet. However, to minimize safety risks, impregnating bednets should be avoided. Another possibility is by indoor spraying on walls and/or roofs (for spraying techniques, see Bateman and Alves, 2000), having the advantage of covering a large proportion of the indoor surface area but having the disadvantage that its oil-layer on walls/and or roofs may be unacceptable to the inhabitants. With 33.6% of *An. gambiae s.l.* being infected and killed with this new method and biocontrol agent, the results of this study are encouraging. There is however much room for improvement, both in application methodology, conidial viability and increase in infection percentage.

A promising intervention identified in this study is the mosquito resting target itself. The use of fabrics impregnated with an insecticidal agent and placed indoors is not new. Other studies have shown reductions of mosquito numbers and biting rates when using carbosulfan-impregnated polyester curtains (Fanello *et al.*, 2003), and permethrin-impregnated curtains made of sisal, polyester, nylon, or cotton (Lines *et al.*, 1987; Mutinga *et al*, 1993; Oloo *et al.*, 1996; Modiano *et al.*, 1998). However, in none of these studies was a biological control agent applied, nor were mosquitoes found more on the fabric than elsewhere in the room. On the contrary: in many cases the curtains, even unimpregnated ones, repelled mosquitoes. Sampling by aspiration is undoubtedly less sensitive on substrates such as mud walls, thatched roofs, furniture and other household contents as compared to sampling from cloth. Nevertheless, the attractiveness of black cloth for African malaria vectors has been reported previously (Anonymous, 1975) and it is striking that over half the mosquitoes caught resting in houses were aspirated from the targets (Figure 1), even though these represent a much smaller fraction (~ 10%) of the resting habitat in the houses they were placed in. We therefore suggest that such simple, cheap and widely available materials that attract resting malaria vectors could be used to selectively target residual applications of biocontrol agents and, if desired, mainstream synthetic insecticides alike. If indeed mosquitoes could be drawn to selectively treated surfaces such as these cloth targets, it might be possible to overcome and even reverse the problem of mosquito avoidance which leads to reduced effective coverage of the targeted mosquito population and reduced impact (Garret-Jones, 1964; Killeen *et al.*, 2002). Furthermore, treatment of a specific target rather than walls may be preferred by householders (Booman *et al.*, 2003) and increase acceptance of residual spraying programmes.

CONCLUSIONS

The experiments clearly showed that wild adult *An. gambiae s.l.* and, to a lesser degree, the filariasis vector *Cx. quinquefasciatus* mosquitoes can be contaminated with conidia of *M. anisopliae* from fungus-impregnated materials placed indoors in houses in rural Africa, and die as a result of their infection. Their life spans are considerably reduced with a dosage of 3.7×10^9 con.m^{-2}. For *An. gambiae* the results indicate that the daily survival rates of *M. anisopliae*-infected females were reduced rapidly, which suggests that fungal infection may have an impact on population density if only enough wild females could be infected. Effects on the entire vector population remain to be studied in a more extensive field study covering a larger area for a longer period, using repeated fungal applications. A coincidental, but interesting finding, was the black cloth mosquito resting target itself: By attracting mosquitoes to selectively treated surfaces, it may be possible to improve the impact, cost

effectiveness and acceptability of domestically applied residual (biological) insecticide interventions.

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CHAPTER 10

GENERAL DISCUSSION AND CONCLUSIONS

A part of this chapter will be submitted as: Scholte EJ, Ng'abi KR, Kihonda J, Takken W, Paaijmans K, Abdulla S, Killeen GF, Knols BGJ. An entomopathogenic fungus against adult African malaria mosquitoes.

GENERAL DISCUSSION

1. FINDINGS

Aim of study

There are at least two biological control agents that have been proven to effectively reduce larval populations of *Anopheles gambiae*: *Bacillus thuringiensis israelensis* and *B. sphaericus* (Becker, 1998; Fillinger *et al.*, 2003). Currently, studies are ongoing to improve and implement these agents in mosquito vector control programmes in Africa (U. Fillinger & G. Killeen, personal communication). In the present study we focused on the use of microbials for the control of adult stage malaria and filariasis mosquitoes, which is a new field of biological control of these important vectors. For this purpose, Hyphomycetous entomopathogenic fungi were selected, as these possess a characteristic that gives them an important advantage over insect-pathogenic bacteria or viruses by infecting and killing an insect without the need for ingestion (Samson *et al.*, 1988). Physical contact alone is enough for infection, resulting eventually in the death of the insect, a characteristic shared with insecticidal residual chemicals. Knowledge of both entomopathogenic fungi and of mosquito bionomics, and of techniques using residual chemicals is extensive. In this thesis efforts to combine these three fields are described, in order to develop a method in which a mosquito-virulent, but environmentally safe, biocontrol agent can be applied in parallel with already existing tools to reduce adult stage malaria and filariasis vector populations in Africa.

Choice of fungus

Three different species of entomopathogenic fungi were tested for their effects on *Anopheles gambiae*. All three are naturally present in Western Kenya, an area with endemic malaria and filariasis. For one of these fungi, *Metarhizium anisopliae*, three different isolates were tested. Although the doses used in that study were not standardized, *M. anisopliae* isolate ICIPE30, with its high degree of infectiousness and effect on mosquito survival, proved to be a candidate with high potential. In order to find the most virulent and promising fungus it would be preferable to test a larger range of fungal species and isolates than we did.

In Chapter 2 (Scholte *et al.*, 2004), a number of characteristics of 'the ideal fungus for mosquito control' are listed. Although this list was not specified to any particular mosquito species, it is here used to consider the advantages and disadvantages of *M. anisopliae*, targeted mainly against *An. gambiae* in sub-Saharan Africa. In the first place both larvae as well as adults should be susceptible to the fungus. *M. anisopliae* has been shown to be pathogenic to both (Roberts, 1970; Daoust *et al.*, 1982; Scholte *et al.*, 2003a,b), although I focused on the adults. Secondly, a control programme using an ideal fungus should require only one or a few applications per season. Based on data from Chapters 8 and 9 on conidial longevity and virulence over time it is suggested that in the field, the fungus should be applied monthly.

Related to this is the question about timing of fungal application. Most anopheline populations in sub-Saharan Africa increase and decrease following wet and dry seasons. When breeding sites start to dry up, populations decrease drastically. Most mosquitoes do not survive longer than two months, but a small proportion of females enters a physiological stage called senescence and survives by hiding in refugia (Charlwood *et al.*, 2000), feeding only sporadically on humans. At the end of the dry season only a fraction of the population will have survived, which is the time when any type of vector control strategy that targets adult mosquitoes will have the highest possible impact on mosquito populations, when compared with other times of the year. The end of the dry season would therefore be a suitable time to start a vector control campaign where the method described in Chapter 9 is included. This

does not apply to areas with continuous high levels of anopheline populations, such as near river valleys, irrigation areas, river deltas and lakesides.

Also, the ideal fungus should be dispersed actively by adult females to previously unoccupied breeding sites in order to spread the fungus. This does occur with *Coelomomyces*-infected female mosquitoes (See Chapter 2), but not for those infected with *M. anisopliae*. Mosquitoes that die as a result of infection with the fungus may sporulate, thereby dispersing the fungus in the environment. There are no indications that *M. anisopliae*-infected mosquitoes will move to a certain area to die (as e.g. with flies infected with *Entomophthora* spp. that move up to branches or grass just before they die (Eilenberg, 2000; 2002)). This suggests that *M. anisopliae*-infected mosquitoes are likely to die anywhere. Sporulation of the fungus from an insect cadaver occurs only when the relative humidity is close to 100% (Arthurs and Thomas, 2001). In the climate of sub-Saharan Africa that means that sporulation is only likely to occur after it has rained, dispersing conidia close to the ground as the fungus does under natural conditions. Since mosquitoes rarely land on the ground, it seems unlikely that the fungus will spread through the mosquito population that way. The results of Chapter 6 show that horizontal transmission does occur under laboratory conditions, and suggest that it may play a moderate role in dispersing the fungus within the mosquito population in the field, thereby contributing to the primary mode of infecting wild mosquitoes with the fungus as described in chapter 9.

Another characteristic of the ideal fungus for mosquito control should be that the fungus has long residual persistence in the mosquito population after introduction. Unlike classical biological control, where introduction and permanent establishment of an non-endemic organism for long-term pest management is intended, we propose an inundative biological control method: the release of large amounts of a mass-produced biological control agent to reduce a pest (mosquito) population without necessarily achieving continuing impact or establishment. Once applied, conidia remain on the impregnated resting targets. Numbers of effective (viable) conidia decrease in time until the number of viable conidia drops to levels that are unable to have an effect on mosquito survival. The results of Chapters 8 and 9 indicate that, when *M. anisopliae* is applied on resting targets, the fungus significantly reduces *An. gambiae* survival for approximately one month. This is four times as long as the effect of some larval control agents (e.g. *B.t.i.*), but not as long as the effect of chemical residual insecticides such as permethrin that remain effective for approximately 6 months (Marchant *et al.*, 2001).

Furthermore, the ideal fungus for mosquito control should be easily and cost-effectively produced and formulated. In general, Hyphomycetes such as *M. anisopliae*, are easy to mass-produce. This contrasts for example with the more host-selective entomopathogenic fungi of the Entomophthorales, which may cause spectacular epizootics but are difficult to mass-produce (Eilenberg, 2000). As mentioned in Chapter 2, several isolates of *M. anisopliae* are currently being commercially produced, and used against a range of insect pests (Khetan, 2001; Wraight *et al.*, 2001).

Epidemiology - the importance of mosquito survival rates and number of blood meals

Fundamental to the design of malaria control programmes is a basic understanding of relationship between malaria transmission by mosquito vector populations and malaria prevalence, incidence, morbidity, and mortality. There are two main entomological parameters that describe the intensity of malaria transmission, namely vectorial capacity and the Entomological Inoculation Rate (EIR) (Molineaux *et al.*, 1988; Beier *et al.*, 1999). Vectorial capacity is deduced from Macdonald's basic reproduction rate (Macdonald, 1957), and is the capacity of a vector population to transmit malaria in terms of the potential number

of inoculations originating per day from an infective person. The entomological inoculation rate (EIR) is the rate at which sporozoite positive bites are received.

Both functions express the importance of mosquito survival and the number of blood meals taken, reductions of which have high impact on reductions in the intensity of malaria transmission (Charlwood *et al.*, 1997; Killeen *et al.*, 2000b; Charlwood, 1997; Drakeley *et al.*, 2003). The observed reductions in daily survival rates of wild *An. gambiae s.l.* (Figure 5 in Chapter 9) and numbers of blood meals taken by *M. anisopliae*-infected females (Figure 1) will thus have a high impact on vectorial capacity and EIR, as this will reduce the intensity of *Plasmodium* transmission, provided that enough mosquitoes become infected with the fungus.

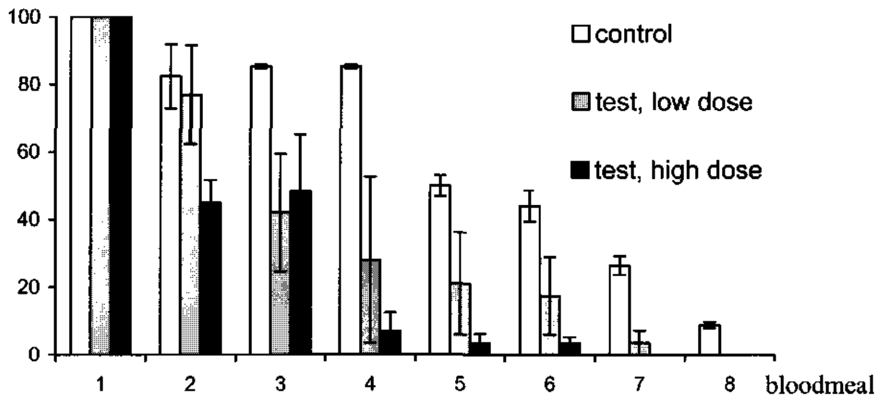

Figure 1. Proportion of *M. anisopliae*-infected female *An.gambiae s.s.* mosquitoes that took bloodmeals, as calculated from mosquitoes alive at the start of the experiment.

The differences in longevities found between uninfected females in Chapters 4, 8 and 9 are probably due to the fact that in Chapter 4 mosquitoes were kept individually in small, confined spaces, whereas those in Chapter 8 were kept in larger cages. In all three studies, however, the fungus-infected females died significantly faster than the uninfected females, resulting in drastic reductions of daily survival rates. Average daily survival rates measured 7 days after infection, at a dose of 1.6 x 10^{10} conidia/m^2, were reduced to 0.51 compared to 0.87 of the control females in the laboratory (Chapter 4). In the field (Chapter 9), this rate was 0.60 for the infected against 0.92 for the uninfected females at a dose of 3.7 x 10^9 conidia/m^2. The field study further showed that the daily survival rate of the infected females was reduced to zero by day 10, whereas that of the control females continued at rates of around 0.84 for over a month (Figure 5 in Chapter 9).

Furthermore, a reduction in the number of blood meals is caused by the feeding inhibition effect of the fungal infection (Chapter 5). Female mosquitoes that are infected with the fungus take fewer blood meals compared to healthy, uninfected females. When females became infected with the fungus after the first blood meal, less than half of the females, infected on a surface with 1.6 x 10^{10} conidia m^{-2}, that were alive at the start of the experiment took a second blood meal, compared to 82.4% of the uninfected ones. At the time of the 4th blood meal, only 6.9 ± 5.5% took a blood meal, whereas 85.3 ± 0.6% of the uninfected females did so. This effect shifts the incubation period to a lower level.

Effective vector control measures decrease the prevalence of malaria infections because there is a log linear relationship between EIR and malaria prevalence (Beier *et al.*, 1999; Lusingu *et al.*, 2004). In different parts of Africa, malaria transmission intensity is highly variable with annual EIR ranging from less than 1 to over 1000 infective bites per person per year, where even almost undetectably low levels of EIR can be associated with sufficiently high prevalence rates of *P. falciparum* malaria to seriously impact on public health (Beier *et al.*, 1999). Substantial reductions in transmission intensity are necessary to reduce the prevalence of malaria in human populations. In some holoendemic areas, reductions in EIR by 95% or more are required before any decrease in the prevalence of *P. falciparum* infection can be expected (Molineaux, 1997; Lengeler *et al.*, 1998; Modiano *et al.*, 1998). In some parts of Africa it may even not be possible to achieve reductions in the prevalence of *P. falciparum* unless control measures reduce EIR to well below 1 infective bite per person per year (Beier *et al.*, 1999; Killeen *et al.*, 2000b). To predict what proportion of a vector population should be reduced to achieve such a low EIR varies in different areas, since it depends largely on factors such as vector competence, the number of new vectors per site per year, and the proportion of infectious humans (Killeen *et al.*, 2000a). A simulated campaign with modestly effective vaccine coverage, bednet use, and larval control indicated that such campaigns would reduce EIR by 30-50 fold, where the method aimed at reducing adult female life span (indoor spraying) and prevent blood meals (bed nets) had the highest impact (Killeen *et al.*, 2000b; 2004). For these reasons it is crucial to assess the extent to which this fungus may reduce anopheline populations, in combination with other vector control strategies:

Epidemiology - estimating the impact of M. anisopliae on malaria transmission using an EIR model

EIR can be estimated as the product of the human reservoir infectiousness (κ), the life-time transmission potential of individual mosquitoes (L) and the rate at which they emerge from larval breeding sites (E) relative to human population size (E/N_h) (Killeen *et al.*, 2000a):

$$EIR = \kappa LE/N_h \qquad 1$$

In Killeen *et al.* (2000a) the EIRs are calculated for four different malaria-endemic sites. One of these sites is Namawala, a village in the same valley (Kilombero) as the village (Lupiro) in which the field trial (Chapter 9) was carried out. The potential effects of fungus-treated targets, at village level, was modelled as described above except that the predicted survival to a given feeding cycle (P_i) was calculated as a function of the survival probability per feeding cycle (P_f), the number of feeding cycles completed (i) and the additional hazards that are accrued from the presence of the fungus (H_i):

$$P_i = (P_f)^i (1-H_i) \qquad 2$$

The potential hazard to mosquitoes resulting from the presence of fungus-treated targets is then calculated as a function of the effective coverage (C) of the resting population they achieve and the time delay, in terms of the minimum number of feeding cycles required between infection of exposed mosquitoes and their subsequent death (D):

$$H_i = H_{i-1} + C\,(1-H_{i-1}) \text{ if } i>D, \text{ otherwise } H_i=0 \qquad 3$$

D was estimated by dividing the mean survival time observed in field infected specimens (=4.58 days, see Figure 5 of Chapter 9) by the expected feeding cycle length (2.7 days, as

determined by Charlwood *et al.*, 1997 for the same mosquito species complex in the nearby village of Namawala). Since mosquitoes are only infected immediately before or right after their first blood meal, D is calculated as (4.58 / 2.7) + 1 = 2.7. Effective coverage (C) of the method is calculated as the proportion of fungus-infected females (132) of the total number of females collected in the test houses during the intervention period (580), resulting in an estimate of C=0.2276.

In our field study (Chapter 9) the daily survival rates (P) were not determined from parous rates (as is normally done to estimate P), and the sporozoite rates, length of a gonotrophic cycle (f), and number of human bites per year (H_{Bt}) were not determined. Although the two villages Namawala and Lupiro differ in many respects, they do share many characteristics that influence malaria epidemiological parameters, the most important being that the two villages are at relatively close distance (about 65 km) from each other, are both in the Kilombero valley, areas with comparable ecological and social structure, and both are home to the same mosquito vector species: *An. gambiae s.l.*. Therefore, the values of several parameters from Namawala, such as P_f=0.62 (probability of survival for one feeding cycle), Q=0.95 (human bites per bite), κ=0.018 (infective bites per human bite), f=2.7 (interval between blood meals), and δ=3 (number of previous blood meals that occurred too recently for ingested parasites to have become infectious) were used for the model of the 'fungus-uninfected females' in Lupiro.

Figure 2 shows the transmission models of both the fungus-infected, as well as the uninfected females. Figure 3 shows that when a fungus intervention is applied at village level for a whole year, the EIR would decline from 262 infective bites per person per year to 64. The proportion of mosquitoes with infectious sporozoites in the overall population (S) would decrease from 0.011 to 0.0036.

Relatively simple modifications regarding the application of the fungus may increase its efficacy of transmission. For instance, increased size of the impregnated sheets will increase the proportion of mosquitoes with fungal infection. Field experiments should quantify this relationship, but if the effective coverage due to this method modification increases from 0.23 to (a hypothetical) 0.45, this will result in an EIR of 14.5 infective bites per person per year. In addition, improved formulations and fungal application methods will enhance the efficacy of the fungus. Again, field experiments should be carried out to quantify this relationship, but if these improvements could add 10% to the effective coverage, this would result in an EIR of 7 and a reduction of H_{Bt} (bites per person per year) of more than 50%.

A potential limitation of the model derives from the method of mosquito sampling. The proportion of mosquitoes collected from the cloth (these having a higher probability of being contaminated with the fungus) as compared to the mosquitoes collected from the rest of the house is probably biased since it was more likely to overlook a mosquito resting elsewhere in the room. The sampling method also misses mosquitoes that took a blood meal but rested outdoors afterwards. On the other hand, the effect of reduced blood feeding described in Chapter 5, which increases the hazard, is not incorporated. To assess the actual impact of the fungus, the field trial needs to be followed up with experimental hut trials and a village scale intervention trial.

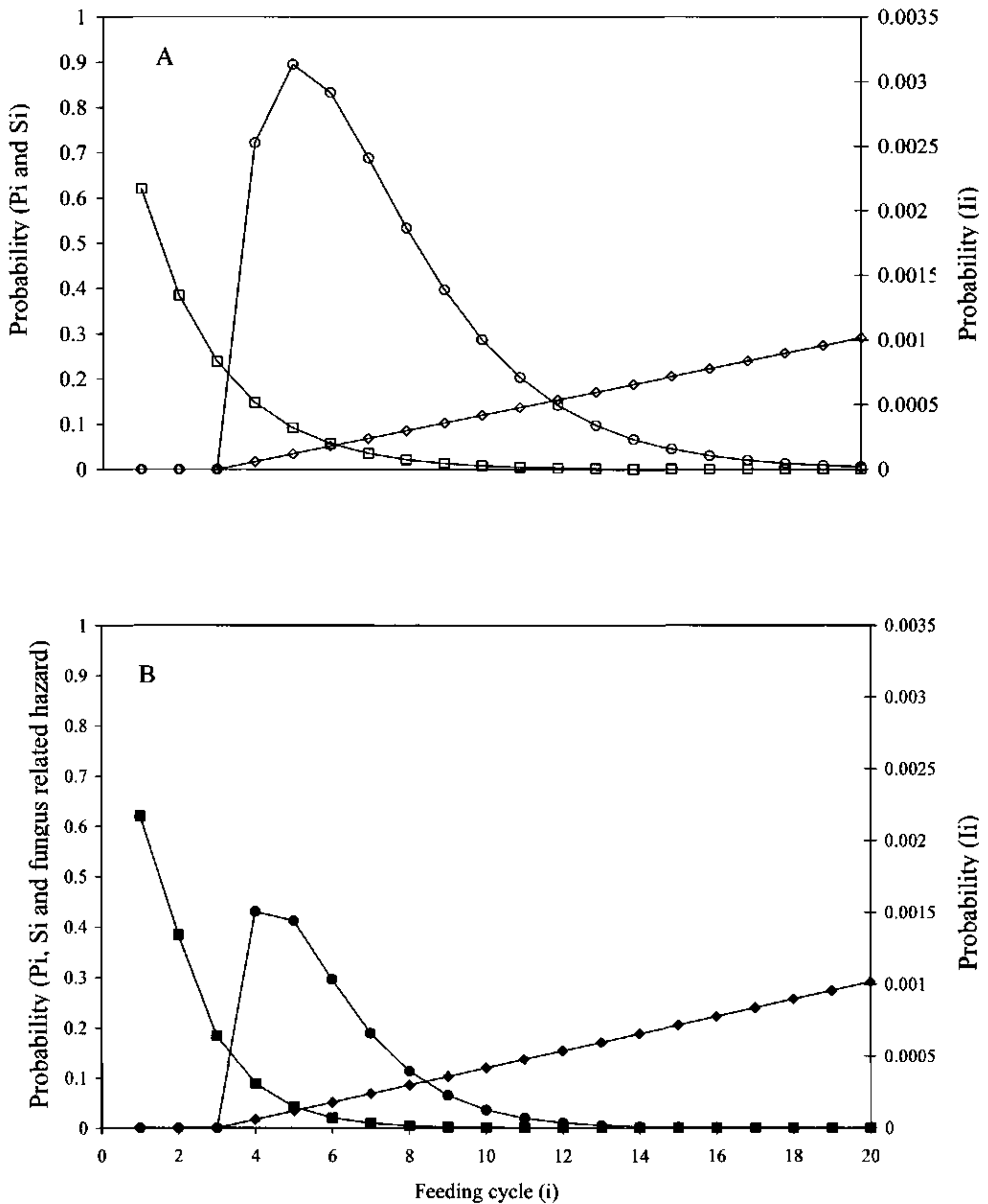

Figure 2. Predicted proportion of emerging *An. gambiae* that are alive (Pi), infectious for malaria (Si), or both (Ii) over the course of their lifetime A) without intervention and B) with intervention of *M. anisopliae* at village level at an effective coverage of 22.8% and a lag-time of 2 gonotrophic cycles.

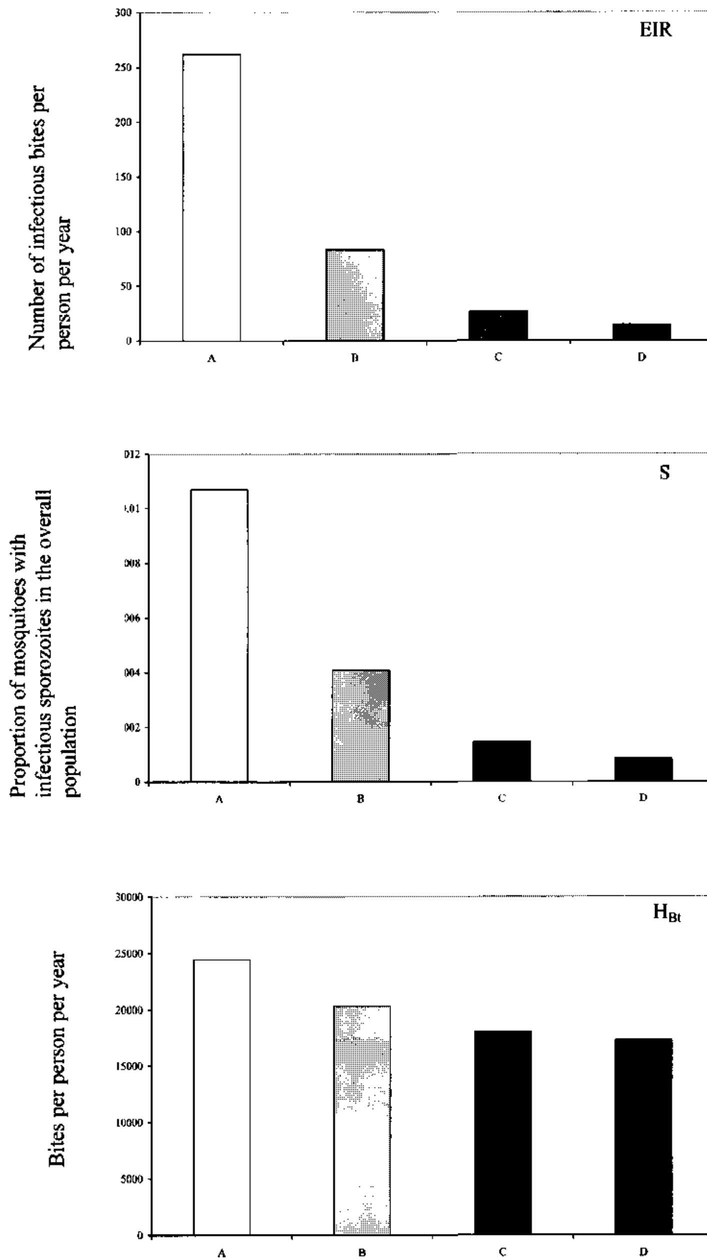

Figure 3. Epidemiological parameters of EIR, S, and H_{Bt} at four fungal coverage levels: A: without intervention, B: with fungus coverage of 22.8% (data of field experiment), C: values if resting targets are increased in surface area (hypothetical effective coverage of 45%), D: values with larger resting targets plus improved fungal formulation and application method (hypothetical effective coverage of 55%).

2. CONSIDERATIONS

Ecological risk assessment

An important aspect concerning the use of entomopathogenic fungi for biological control is environmental risk assessment (Howarth, 1991; Hokkanen and Hajek, 2003). In general it is accepted that the use of *M. anisopliae* as a mycoinsecticide is not harmful to the environment (Zimmermann, 1993; Strasser *et al.*, 2000). In Brazil it is produced under the name Metaquino®, where it is extensively sprayed on sugar cane fields to control spittle bugs (Moscardi, 1989) and in 1993 the fungus was registered by the U.S. Environmental Protection Agency, patented by Bio-Path® for control of nuisance flies and cockroaches. In 2003 *M. anisopliae*, strain F52, was granted registration by the same agency, mentioning in the biopesticide fact-sheet that no harm is expected to humans or the environment (EPA, 2003). The factors that affect the degree of risk to non-target organisms have long been known (Howarth, 1991), but it was only recently that these factors were quantified (Van Lenteren *et al.* 2003). These authors proposed a general framework of an environment-based risk assessment methodology for biological control agents that integrates information on five criteria: a) the potential of an agent to establish itself, b) its ability to disperse, c) its host range, and d) its direct and e) indirect effects on non-target organisms. Although the stage of the proposed biocontrol system described in this thesis is still premature in terms of actual application in integrated vector management (IVM), I tried to estimate the likelihoods (on a scale from 1 [very unlikely] to 5 [very likely]) and magnitude (on a scale from 1 [minimal] to 5 [massive]) levels for the parameters as proposed by Van Lenteren *et al.* (2003):

I estimate the likelihood of establishment of the fungus in a non-target habitat as likely (4), with moderate (3) magnitude, and the likelihood of dispersal potential of the fungus as likely (4) but of minor magnitude (2). The host range restriction I estimate as none (5) but with minor magnitude (2), the likelihood of direct effects on non-target populations as unlikely (2) at a minimal (1) magnitude, and indirect effects on non-target species influenced by *M. anisopliae* as unlikely (2) at minor magnitude (2). The overall risk index, calculated as the sum of the multiplied likelihoods and magnitudes is thus estimated as 4x3=12 for 'establishment', plus 4x2=8 for 'dispersal', plus 5x2=10 for 'host range', plus 2x1=2 for 'direct effects', plus 2x2=4 for 'indirect effects, which makes a total of 36 points. Hokkanen *et al.* (2003) write that risk indices lower than 35 points will generally result in a proposal of no objection against release of the agent, a risk index higher than 70 points will generally result in advice not to release the agent, and intermediate risk indices between 35 and 70 points will result in a request for additional information before a conclusion concerning release will be made. In the case of *M. anisopliae* against African anophelines, without empirically determined estimates of these parameters, the risk assessment values estimated here remain open to debate. The overall risk index we estimated here is lower than the two examples of *M. anisopliae* given in Table 5 of Van Lenteren *et al.* (2003), where *M. anisopliae* was used in inundative control in Sahelian Africa (based on Lomer *et al.*, 2001) and in Finland (Husberg and Hokkanen, 2000) with risk-index estimates of 45 and 43. The difference between the proposed usage of the fungus described in this thesis and the two studies mentioned above is that we propose to use the fungus indoors, which reduces the likelihoods and magnitudes of the factors 'dispersal' and 'direct effects'.

The ideal fungus should be selective for mosquitoes and not kill other organisms. *M. anisopliae* is a generalistic insect-pathogenic fungus, infecting over 200 insect species (Boucias and Pendland, 1998). Therefore, if this fungus is to be applied, caution is required. With the method proposed in this thesis (i.e. conidia-impregnated sheets in the intra-domiciliary domain) we tried to minimize as much as possible the risks of infecting non-target organisms. During our work inside houses (Chapter 9), we encountered very few insects other

than mosquitoes. These are possibly repelled by wood smoke, derived from cooking either indoors or outdoors under the window (Bockarie *et al.*, 1994). Other insects found indoors are ants, termites, cockroaches, flies, and bedbugs. Ants and flies occurring inside are considered as unwanted by the residents of the houses, but generally not as pests and are thus to be considered as non-target organisms.

In aquatic environments, the fungus can cause mortality of embryos of shrimps, frogs and fish when these are exposed to high doses of conidia (Genthner *et al.* 1994, 1998), but since its intended use is indoors it is unlikely that large quantities of conidia will end up in aquatic environments. The results of some studies with *M. anisopliae* on non-target organisms in Africa (with other isolates) suggested that these isolates were unlikely to cause epizootics in field hives of Africanized honey bees and most likely will not cause bee mortality following field applications for pest control (Alves *et al.*, 1996), nor for several other African Hymenoptera (Stolz, 1999).

Biosafety

Also important to assess is the acceptability of a microbial agent in terms of its effect on humans and other vertebrates. *M. anisopliae* is not naturally associated with humans or other vertebrates, has demonstrated neither infectivity nor toxicity in mammals, and is regarded as non-pathogenic to humans (Zimmermann, 1993; Ward *et al.*, 1998; 2000a). Zimmermann (1993) summarized eight safety studies in which conidia of *M. anisopliae* were administered to mammals by skin- and eye application, acute oral, acute dermal application, and by injection, inhalation, feeding, and drinking. In none of those studies were any adverse effects reported. However, it has been demonstrated in mice that crude extracts of the fungus contain one or more allergens (Ward *et al.*, 2000b; Selgrade, 2000). Even though the risk of exposure and thus of allergy is not so much to oil-formulated conidia (which stick to the impregnated material) but predominantly to dry, air-borne conidia, it is good practice to reduce the risk of exposure to humans as much as possible. It was for this reason that in the field experiment (Chapter 9) we preferred to impregnate cotton sheets, which were then placed on ceilings, instead of bednets. In summary, after extensive testing on mammals, *M. anisopliae* is generally regarded as a safe organism, not harmful to man or other mammals. The fact that the fungus is registered by the U.S. Environmental Protection Agency and has been granted permission to be used indoors in the U.S.A. against cockroaches and nuisance-flies can be seen as strong indications for this. It should be noted however, that registration normally applies to a single defined isolate, for which mammalian toxicity data is necessary (often, however, published data for closely related isolates can be accepted at the discretion of the registration authorities) (Lomer *et al.*, 2001). Therefore, before *M. anisopliae* would be used on a large scale for the control of adult mosquitoes in Africa, the necessary steps regarding official registration of the intended fungal isolate should be followed.

Synthetic insecticides versus M. anisopliae: where do we stand

In the years following WWII, the use of synthetic insecticides intensified throughout Africa for malaria and filariasis vector control, saving large numbers of human lives (Utzinger *et al.*, 2002). In general, the types of insecticides shifted over the decades; in the 1950's organochlorines (among which DDT, HCH, and dieldrin) were predominantly used, followed in the 1960's by organophosphorus compounds (among which malathion, fenthion, chlorpyrifos, methoprene and temephos), carbamates such as thiodicarb, and in the 1970's by synthetic pyrethroids such as permethrin. Initially, almost all of these were used for indoor residual spraying, followed by impregnation of bednets, curtains and other fabrics. Until 1990 DDT was the most widely used insecticide for malaria vector control in the world (Walker, 2000). In the recently adopted Stockholm Convention on Persistent Organic Pollutants (POPs)

an exception was made for the continued use of DDT for malaria control, in recognition that in the absence of better and more effective methods of malaria control, indoor spraying should not be abandoned before effective alternative tools are becoming available (WHO, 1999; Christen, 1999; Curtis, 2002a,b). Today, indoor residual spraying finds more limited application but remains effective (Goodman *et al.*, 2001; Charlwood *et al.*, 2001; Booman *et al.*, 2003) and insecticide treated nets are one of the recommended methods of malaria vector control in Africa (Lengeler, 1998; UNICEF, 2004).

Advantages of synthetic insecticides are that some of them, such as DDT and pyrethroids, are relatively cheap (Walker, 2000; Conteh *et al.*, 2004), they are easy to apply, have relatively long residual activity, upto a minimum of 6 months (Lengeler, 1998; Ehiri *et al.*, 2004; Maharaj *et al.*, 2004), act fast (upon contact it will kill the insect in much less than 24 hrs (Koffi *et al.*, 1998)), are easily produced, are widely available, and, in the absence of resistance, will kill 100% of the insects. This last point however, touches on the main disadvantages of the use of insecticides: the continuing development of resistance (Koffi *et al.*, 1998; Chandre *et al.*, 1999; Hargreaves *et al.*, 2000; Hemingway and Ranson 2000; Hemingway *et al.*, 2002; Brooke *et al.*, 2002; Corbel *et al.*, 2003; Weill *et al.*, 2003; McAbee *et al.*, 2004). Also, many of these compounds have serious negative impacts on the environment (WWF, 1999; Vale and Grant, 2002; Binelli and Provini, 2003; Nakamaru *et al.*, 2003) and on human health (WHO, 1998; Weistrand and Noren, 1998; Hardell and Ericsson, 1999; Settimi *et al.*, 2003; Siddiqui *et al.*, 2003; Calvert *et al.*, 2004; Ehiri *et al.*, 2004; Kunisue *et al.*, 2004).

Although the use of a living organism in biocontrol is less likely to result in the development of resistance, it is not excluded from happening. Petersen (1978) and Woodard & Fukuda (1977) reported development of resistance of respectively *Cx. quinquefasciatus* against *R. culicivorax* and *An. quadrimaculatus* against *Diximermis peterseni* and there are recent reports of developing resistance of mosquitoes to bacterial products of *B. sphaericus* (Wirth *et al.*, 2000; Yuan *et al.*, 2000; Mulla *et al.*, 2003) and even, although at low levels and not by anophelines, to *Bti* products (Saleh *et al.*, 2003).

One might argue that a disadvantage of using *M. anisopliae* for mosquito vector control is the relatively long incubation-period (or 'lag-time') as compared to synthetic insecticides: a *Plasmodium*-infectious mosquito that becomes infected with *M. anisopliae* may still consume one or a two blood meals (although the probability that it will is significantly reduced (Chapter 5)), and thus for several days continues to pose a risk for malaria transmission. However, the EIR model described above, where the impact of using a *M. anisopliae* vector control method was estimated, suggested an estimated 18-fold reduction in EIR from 262 to 64 infectious bites per person per year, even at the relatively low effective coverage of 22.8%. Even though these are extrapolated data from a relatively small-scale experiment, they show that a programme based on this method using *M. anisopliae* against adult anophelines, may have a considerable impact on malaria transmission.

The relatively short residual viability of the fungal conidia at present would require re-treatment at monthly intervals (Chapter 8). This may be a disadvantage compared to bednet use, where re-treatment with an insecticide is at 6 months intervals or even more (Kroeger *et al.*, 2004). However, the fungus can be produced locally with little difficulty, and this would rank fungal mosquito control high on the list of sustainable tools for malaria control. As no large-scale experiments have yet been carried out it is difficult to estimate the cost of using this fungus and it is therefore too early to compare economics of this with other vector control strategies.

Integrated control

To decrease the malaria burden, the availability of health care services for diagnosis and treatment is crucial, but it can do little to reduce the risk of malaria infection in areas of high transmission because it deals only with malaria infection after it occurrs and has a low impact on the infectiousness of the human reservoir (Killeen *et al.*, 2000b). Large-scale prophylaxis has been shown to be a powerful instrument in some successful control programmes (Kitron and Spielman, 1989; Alonso *et al.*, 1998; Romi *et al.*, 2002) but may also have dangerous consequences such as the emergence of drug resistance (Krogstad, 1996; Molineaux *et al.*, 1999). Even intensive control of infections by active case detection, prompt drug treatment, and follow up cannot eliminate endemic malaria from most parts of sub-Saharan Africa unless rapid re-infection can be prevented by effective vector control (Carter *et al.*, 2000; Killeen *et al.*, 2000; 2002).

There are only a few examples where one control method alone was sufficient to eradicate *An. gambiae* from large areas: from Brazil in the 1930s and early 1940s (Soper and Wilson, 1943) and from Egypt in the 1940s (Shousha, 1948). In both cases the mosquito immigrated from elsewhere, and was eradicated using larval control with Paris green. To this day, these eradications were permanent. Also, DDT has been credited with a high degree of suppression of, and even complete eradication of some vector species (Curtis and Lines, 2000; Curtis and Mnzava, 2000). However, the Garki project in Nigeria revealed that although indoor spraying was indeed highly effective in reducing the ability of the vector population to transmit malaria, this was not enough to destabilize transmission in the savannah zone of northern Nigeria (Molineaux and Gramiccia, 1980). If the ambition is to truly control malaria in Africa, it will be necessary to combine several tools (Carter *et al.*, 2000; Killeen *et al.*, 2000; Shiff, 2002).

Killeen *et al.* (2004) showed that EIR can be drastically reduced with methods that do not require chemicals but are based on integration of a few moderately efficient vector control methods such as environmental management-based larval control and zooprophylaxis (placing cattle in or close to a human dwelling which reduces the probability that a mosquito vector will feed on humans). In a similar way we envisage an integrated vector control programme (IVM) without any use of chemicals, where the application of *M. aniospliae* against the adult stage mosquito vectors (this thesis) is used in addition to biological larval control using *B.t.i.* and/or *B. sphaericus* (Fillinger *et al.*, 2003), the use of untreated bednets, and push-pull with zooprophylaxis (Seyoum *et al.*, 2002) and repellent plants (Seyoum *et al.*, 2003). Larval control is likely to ensure a reduction in the number of emerging adults, whereas the cattle in or near houses will prevent some of the vectors from feeding on humans. The repellent odours from live, potted, or burned plants around the house will reduce the numbers of entering mosquitoes even further. Those that remain unaffected by the distracting odours will encounter a bednet which, even unimpregnated, will reduce the number of mosquito bites (Guyatt and Snow, 2002; Takken, 2002). Some of those that entered will be exposed to conidia of *M. anisopliae* when they land on impregnated resting targets, which will reduce their daily survival. None of these techniques, as stand-alone tools, are perfect, but the combination of these vector control methods may be very powerful and greatly enhance reductions in EIR. If such a strategy is backed up by substantial financial, political and local support for long-term and large-scale application, the use of *M. anisopliae* for adult control of mosquito vectors in Africa can be a valuable and viable aspect of the IVM strategy. Such a strategy may result in a more sustainable strategy of vector-borne disease control than the current one based on chemicals, especially when integrated with a policy that enables immediate access to healthcare for diagnosis and treatment. The use of biological control agents that have proven effective in killing mosquito vectors such as those described in this thesis, opens new prospects for developing alternatives for disease control.

3. SUGGESTIONS FOR FURTHER RESEARCH

The findings of the studies described in this thesis are encouraging and may form the basis for further research on this topic, such as:

1) The search for more virulent fungi. Although *M. anisopliae* isolate IC30 was found highly virulent against the targeted mosquitoes, it is possible that other isolates of this species, or another Hyphomycetous species altogether will prove to be even more virulent. Studies on the efficacy of various isolates at different temperatures and humidities may result in finding a more efficient strain or species under the relevant field conditions.

2) Studies on formulation and application. Improving conidial formulation and application methods for impregnating materials are likely to increase fungal efficacy. For instance, complete immersion of conidia in oil instead of using an emulsifiable adjuvant oil formulation may increase both conidial attachment to the insect cuticle, as well as increasing conidial longevity.

3) Studies aimed to improve effective coverage. To increase the likelihood that endophilic mosquitoes will acquire fungal contamination, modifications of the impregnated sheets should be studied, like type of textile, increased size, and the application of mosquito-attracting substances such as oxo-carboxylic acids (Healey *et al.*, 2002). After the laboratory experiments, semi-field experiments are needed to assess to what extent horizontal transmission may occur in the field and how much it would contribute to spreading the fungus within mosquito populations. Also the observed reduction in blood feeding of infected females in the laboratory should be investigated in the field.

4) Studies on risk-assessment. Before large-scale applications are carried out, the effects of the fungus on non-target organisms should be carried out such as honeybees and intra-domiciliary insects such as ants and flies. There are already several reports where *M. anisopliae* has been studied to control domestic insect pests such as cockroaches (Pachamuthu *et al.*, 1999), termites (Milner *et al.*, 1998a,b; Rath, 2000; Maniania *et al.*, 2002), and bedbugs (Luz *et al.*, 1998). The impact of *M. anisopliae,* targeted against vector mosquitoes may have additional impacts on these other intra-domestic pest insects.

5) Studies on the effect of the fungus on the vector-stage of *Plasmodium*. Possibly the toxins, released by the growing fungus have negative impacts on the developing parasites. Such effects may have implications for the infectiousness of *An. gambiae* (objective of the research group of A. Read, Edinburgh).

4. CONCLUSIONS

Virulence to the targeted mosquitoes, the absence of mosquito resistance to the agent, and its safety towards humans, mammals and the environment are characteristics of *M. anisopliae* that satisfy key requirements for alternative vector control tools. The experiments described in this thesis indicate the potential that *M. anisopliae* has for controlling mosquito vectors in Africa. The fungus proved highly virulent for *An. gambiae*, significantly reducing its life span. During the incubation period fewer blood meals are taken, and the fungus may be actively dispersed through the mosquito population, resulting in increased efficacy and coverage. When applied on a large-scale, the observed life-span reduction at an effective coverage of 22.8%, implemented in an Entomological Inoculation Rate (EIR) model, predicted a major reduction in malaria transmission intensity. The data suggest that if this novel biological control method was applied as part of large-scale integrated vector management programmes, malaria transmission levels may decrease to such low levels that malaria prevalence would be affected.

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* = not read

SUMMARY

***Insect-pathogenic fungi for mosquito control* (Chapters 1-3)**

Malaria and lymphatic filariasis impose serious human health burdens in the tropics. Up to 500 million cases of malaria are reported annually, resulting in an estimated 1.5-2.7 million deaths, of which 90% occur in sub-Saharan Africa. Malaria is caused by protozoa of the genus *Plasmodium* and is transmitted through bites of mosquitoes belonging to the genus *Anopheles*. Lymphatic filariasis is caused by helminths, the most widespread species being *Wuchereria bancrofti*, and is transmitted through bites of mainly *Culex quinquefasciatus* and certain *Anopheles* species. Worldwide, approximately 146 million people are infected with the disease.

Mosquito vector control is an important way to fight these diseases. In Africa, vector control is almost exclusively based on chemical insecticides, used predominantly to impregnate bed nets and for indoor residual spraying. Growing concerns about their negative impact on human health, on the environment, and about insecticide resistance are the reasons for increasing interests in vector control methods that are not based on chemicals, such as biological control.

Several biological control agents are known to be effective against mosquitoes such as predatory fish (e.g. *Gambusia affinis* and *Poecilia reticulata*), nematodes (e.g. *Romanomermis culicivorax*), microsporidia (e.g. *Nosema algerae*), bacteria (e.g. *Bacillus thuringiensis israelensis* and *B. sphaericus*), and insect- pathogenic fungi (e.g. *Lagenidium, Coelomomyces* and *Culicinomyces*). All of these, however, target the larval stages of mosquitoes. To date, there is no biological control agent for use against the adult stage of mosquitoes. However, reduction in survival of adult mosquitoes is considered to have a much higher impact on transmission than a reduction in the number of mosquito larvae. The objective of this PhD thesis was therefore to search for a biological control agent for adult mosquitoes, and to develop a method to use such an agent in integrated vector management (IVM) in Africa. The primary targets for this research were the major malaria vector *Anopheles gambiae s.l.*, and, to a lesser degree, the lymphatic filariasis vector *Culex quinquefasciatus*.

In Chapter 2 the most important insect-pathogenic fungi for (mostly) larval mosquito control are reviewed. Of these, the Hyphomycetes possess a characteristic that gives them a major advantage over other biocontrol agents to be used for killing adult mosquitoes: Unlike with bacteria, nematodes or microsporidia, the infectious propagules of these fungi do not need to be ingested. Instead, contact with the cuticle is enough for the infective propagules (conidia) to infect the mosquito. A conidium penetrates the insect cuticle by secreting cuticle-degrading enzymes. Once inside, the fungus grows rapidly and secretes toxins, which kill the mosquito. Depending on temperature, fungal dosage, and susceptibility of the mosquito to the fungus, the process from inoculation to host death may take between approximately three and ten (or even more) days. After host death, and under favourable conditions of high humidity, the fungus will grow out of the cadaver and produce conidia asexually (sporulation).

The strategy envisaged to infect and kill wild mosquitoes in sub-Saharan Africa is based both on the characteristic of Hyphomycetous fungi to infect insects through contact by penetrating the cuticle, and on the behavioural characteristic of *An. gambiae* mosquitoes to blood feed predominantly inside houses during the night, and remain indoors for at least several hours afterwards to rest and digest the blood meal. If conidia are applied indoors on so-called 'mosquito resting targets' (see Chapter 9), mosquitoes are expected to acquire an infection of the fungus by resting on those targets.

In Chapter 3, five different Hyphomycetous insect pathogenic fungi were tested on adult *An. gambiae*, including *Beauveria bassiana*, a *Fusarium* sp. and three isolates of *Metarhizium anisopliae*. Four of these fungi were isolated from insects in western Kenya, an area of endemic malaria. Isolate ICIPE30 of *M. anisopliae* proved to be highly virulent for the tested mosquito species, and it was decided to continue further studies with this isolate.

***The effect of the insect-pathogenic fungus Metarhizium anisopliae on African mosquito vectors* (Chapters 4-7)**

As described in Chapter 4, *M. anisopliae* was tested both on *An. gambiae* as well as on *Cx. quinquefasciatus*, and a standard contamination technique to infect adult mosquitoes was developed. Using this technique, the effect of the fungus on *An. gambiae* was quantified in more detail by a dose-response bioassay. This experiment showed that at a dose of 1.6×10^{10} conidia m^{-2}, >83% were infected (i.e. mosquito cadavers with sporulating fungus), with a mean LT_{50} value of 5.6 ± 0.4 days. Later experiments (Chapters 6 and 8) showed that the fungus could be even more effective at that same dose, with infection levels up to 96.4%, and all mosquitoes dead by day 6, whereas uninfected female *An. gambiae* lived much longer with LT_{50} values >18 days.

Apart from the principal effect of the fungus, causing mosquito death by direct contact with conidia, infection with *M. anisopliae* also caused at least two secondary effects (Chapter 5). One of those secondary effects is a reduction in feeding propensity. In one of the experiments of Chapter 5, individual female *An. gambiae s.s.* were offered a total of 8 blood meals. It was found that mosquitoes, inoculated with a moderately high dose of fungal conidia (1.6×10^9 conidia m^{-2}), exhibited reduced appetite upon increasing effects of fungal growth. Of the fungus-infected females, the proportion of mosquitoes taking a second blood meal was reduced with 51%. This was further reduced to 35.3% for the 4th blood meal. The other observed secondary effect was that infected females took smaller blood meals, resulting in fewer eggs per gonotrophic cycle.

In order to achieve the highest possible impact on mosquito populations, it is desirable that other contamination pathways besides the primary mode of contamination are utilized to spread the fungus through the population, such as horizontal transmission. The results of experiments described in Chapter 6 showed that, under laboratory conditions, conidia can be transferred from an inoculated female to a 'clean' male during the process of mating, with mean male infection rates between $10.7 \pm 3.2\%$ and $33.3 \pm 3.8\%$.

Since the mosquito inoculation method described above is based on mosquitoes that rest on conidia-impregnated sheets, it is desirable that mosquitoes are not repelled by conidia. To test this, behavioural effects of female *An. gambiae* in close vicinity of the fungus were investigated (Chapter 7). The results showed that dry conidia have a significant repellent effect ($p<0.05$). However, when conidia were applied in a suspension of 8% adjuvant vegetable-oil formulation and impregnated on paper, this effect ceased ($p=0.205$). The results suggest that if the fungus is to be applied as a biological control agent against Afrotropical mosquitoes, conidia should be impregnated on e.g. cotton sheets in an oil-based formulation to avoid repellency effects.

Practical approach to mosquito vector control in Africa using *M. anisopliae* (Chapters 8-10).

From a practical and economic point of view, the interval between applications of the control agent should ideally be as long as possible, without the agent losing too much efficacy. In the case of commonly used chemical residual insecticides such as permethrin this is about 6 months. Laboratory experiments (Chapter 8) showed that *M. anisopliae* conidia impregnated on paper and on netting material remained virulent to *An. gambiae* up to one

month after impregnation. Experiments on conidial shelf life under different conditions showed that conidia kept in 8% vegetable oil remained viable up to at least 1 month. Conidia stored in 0.05% Tween 80 exhibited only slightly reduced viability after 3 months at 27° and after 6 months at 4°C. Dry conidia stored with silica gel retained viability for at least 6 months. The results suggest that, if applied in the field, re-impregnation should be carried out monthly, but dry conidia can be stored for at least 6 months under conditions of very low relative humidity.

Chapter 9 of this thesis describes a field study of domestic application of *M. anisopliae* in houses in south east Tanzania, a region holoendemic for malaria and lymphatic filariasis. The fungus was applied on black cotton sheets, attached to ceilings as indoor mosquito resting targets. Indoor resting catches of mosquitoes were carried out daily and collected mosquitoes were kept alive in small containers as long as possible to determine survival. Almost 90% of all collected mosquitoes were *An. gambiae s.l.* (of which 94.7% were *An. gambiae s.s.* and 5.3% *An. arabiensis*). In total, 181 wild *An. gambiae s.l.* and 6 wild *Cx. quinquefasciatus* were infected with the fungus. Infected mosquitoes died significantly sooner than uninfected mosquitoes, with an average daily survival rate of 0.722 for infected female *An. gambiae*, against 0.869 for uninfected females. Calculated from the total number of *An. gambiae s.l.* and *Cx. quinquefasciatus* that were caught from the fungus-impregnated resting targets, respectively 33.6 and 10.0% had acquired fungal infection. Of the total number of 580 female *An. gambiae* collected from the houses containing fungus-impregnated sheets, 132 were infected, which is an effective coverage of 22.8%. If this same coverage level is assumed at village level, and, together with the reduced daily survival rate, is introduced into a malaria transmission model, the total number of infectious bites per person per year (Entomological Inoculation Rate; EIR) drops from 262 to 14 (Chapter 10).

Although the field experiment was on a relatively small scale and of short duration, the predictions of the malaria transmission model strongly indicate that application of *M. anisopliae*, aimed at the adult stage of African mosquito vectors can have a high impact on transmission intensity. It is argued that large-scale application of this method, implemented as part of an integrated vector management (IVM) strategy including larval control using biological control agents, the use of repellent plants and of unimpregnated bednets, malaria can effectively be controlled without the use of chemical insecticides. This thesis may form a first step towards such a strategy. Further research is necessary, especially in 1) searching for a fungal isolate that has even higher virulence against the targeted mosquito species, 2) testing of non-target effects and safety of the most effective fungal strain for registration, 3) searching for the most optimal formulation and application method to increase infection percentages.

SAMENVATTING

Insectpathogene schimmels voor muggenbestrijding.
Malaria en filariasis zijn gevaarlijke humane ziektes in de tropen. Jaarlijks worden bijna 500 miljoen malaria infecties gerapporteerd, waarvan een geschatte 0.7-2.7 miljoen tot een dodelijke afloop leiden, 90 % hiervan in Afrika. De ziekte wordt veroorzaakt door Protozoën van het geslacht *Plasmodium* en worden overgebracht door beten van muggen behorende tot het geslacht *Anopheles*. Filariasis is een ziekte die veroorzaakt wordt helminthen (wormen) waarvan *Wuchereria bancrofti* de meest voorkomende soort is. Ook deze ziekte wordt overgebracht door muggen, voornamelijk van het geslacht *Culex*, maar ook door enkele *Anopheles* soorten. Wereldwijd zijn ongeveer 146 miljoen mensen geïnfecteerd met deze ziekte.

Muggenbestrijding is een belangrijk onderdeel van de strijd tegen deze ziektes. In Afrika is muggenbestrijding vrijwel geheel gebaseerd op chemische insecticiden, gebruikt bij het impregneren van klamboe's en het binnenshuis spuiten op muren en plafonds. Groeiende bezorgdheid wat betreft hun negatieve invloed op menselijke gezondheid, het milieu, en de zich almaar ontwikkelende resistentie van muggen tegen insecticiden zijn de reden van hernieuwde interesse in muggenbestrijdings methoden die niet zijn gebaseerd op chemicaliën, zoals bijvoorbeeld biologische bestrijding.

Er zijn een aantal biologische bestrijdings organismen die effectief zijn tegen muggen, zoals roofvisjes (bijvoorbeeld *Gambusia affinis* en *Poecilia reticulata*), nematoden (bv. *Romanomermis culicivorax*), microsporidiën (bv. *Nosema algerae*), bacteriën (bv. *Bacillus thuringiensis israelensis* en *B. sphaericus*), en insectpathogene schimmels (bv. *Lagenidium*, *Coelomomyces* en *Culicinomyces*). Deze zijn echter allemaal gericht op het larvale stadium van muggen. Tot op heden is er geen biologisch bestrijdings organisme dat is gericht op het volwassen stadium van muggen. Dit is jammer, omdat volgens malaria transmissie modellen een afname in de overleving van volwassen muggen een veel grotere invloed heeft op transmissie dan een afname in het aantal muggenlarven. Het doel van dit promotie onderzoek was daarom om een biologisch bestrijdings organisme te zoeken dat volwassen muggen kan doden, en om een methode te ontwikkelen waarbij dit een organisme kan worden ingezet in geïntegreerde bestrijding van muggen in Afrika. Vooral de malaria muggen behorende tot het soortencomplex *Anopheles gambiae s.l.*, en, in minder mate, de filariasis mug *Culex quinquefasciatus* vormden de doelgroepen.

In hoofdstuk 2 wordt een uitgebreid overzicht gegeven van de insectpathogene schimmels die met muggen bestrijding van belang zijn. Binnen insectpathogene schimmels is er een groep (Hyphomyceten) die een eigenschap bezit die hen een enorm voordeel geeft ten opzichte van andere biologische bestrijdings organismen: in tegenstelling tot bacteriën, nematoden en microsporidiën, behoeven de conidiën (sporen) van deze schimmels niet opgezogen te worden door de mug om haar te doden. Fysiek contact van het uitwendige skelet (cuticula) met de conidiën is voldoende om een infectie te bewerkstelligen die het insect zal doden. Een conidium dringt de mug binnen door cuticula-afbrekende enzymen af te scheiden. Eenmaal in de mug groeit de schimmel, waarbij het een scala aan toxines afscheidt die de mug uiteindelijk doden. Afhankelijk van de temperatuur, de oorspronkelijke dosis conidiën en de gevoeligheid van de mug voor de schimmel duurt het proces van besmetting tot dood zo ongeveer 3 tot 10 (en soms nog meer) dagen. Nadat de gastheer dood is, mits onder gunstige omstandigheden van heel hoge luchtvochtigheid, zal de schimmel weer 'naar buiten' groeien om, asexueel, conidiën te vormen.

De strategie die we voor ogen hebben om in het wild voorkomende malariamuggen in Afrika ten zuiden van de Sahara te infecteren en te doden is gebaseerd op zowel de eigenschap van de schimmel om insecten via contact met het uitwendig skelet te infecteren,

als de gedragseigenschap van de meeste malariamuggen om 's nachts, binnenshuis, mensen te steken, waarna ze tenminste een aantal uren binnenshuis blijven rusten. Als conidiën binnenshuis worden aangebracht op zogenaamde 'rust plekken voor muggen' is de verwachting dat muggen worden besmet met de schimmel als ze op deze rustplekken met conidiën in aanraking komen.

In hoofdstuk 3 zijn vijf verschillende Hyphomycete insect-pathogene schimmels getest op *Anopheles gambiae*, waaronder *Beauveria bassiana*, een *Fusarium* soort en drie stammen van *Metarhizium anisopliae*. Vier van deze schimmels waren geïsoleerd van insecten in west Kenia, een malaria-endemisch gebied. Stam ICIPE30 van *M. anisopliae* bleek hoog virulent te zijn voor de geteste muggen soort, en er werd besloten om deze stam te gebruiken voor verdere experimenten.

Het effect van de insectpathogene schimmel *Metarhizium anisopliae* op Afrikaanse ziekte-overbrengede muggen (Hoofdstukken 4-7).

In hoofdstuk 4 zijn experimenten beschreven waarin *M. anisopliae* werd getest op zowel *An. gambiae* als *Cx. quinquefasciatus*. In datzelfde hoofdstuk is een standaard techniek beschreven die ik ontwikkeld heb om muggen te besmetten met deze schimmel. Met behulp van deze techniek werd het effect van de schimmel op *An. gambiae* verder gekwantificeerd door het uitvoeren van een dosis-effect experiment.

Dit experiment liet zien dat met een dosis van 1,6 x 10^{10} conidiën/m^2 meer dan 83% van de muggen werd geïnfecteerd (d.w.z. muggen kadavers die sporuleerden), met een gemiddelde LT_{50} waarde van 5.6 ± 0.4 days. Latere experimenten (Hoofdstukken 6 en 8) lieten zien dat de schimmel zelfs nog efficiënter kon zijn bij dezelfde dosis, met infectieniveaus tot 96.4% en alle geïnfecteerde muggen dood op dag 6, terwijl ongeïnfecteerde *An. gambiae* vrouwtjes muggen veel langer leefden met LT_{50} waarden van meer dan 18 dagen.

Behalve de belangrijkste functie van de schimmel, namelijk het doden van muggen door direct contact met conidiën, resulteerde de schimmelinfectie ook in tenminste twee secondaire effecten (Hoofdstuk 5). Eén van die effecten is verminderde eetlust. In één van de experimenten die in Hoofdstuk 5 zijn beschreven werden individuele muggen een totaal aantal van 8 bloedmaaltijden aangeboden. Wanneer muggen waren geïnfecteerd met een redelijk hoge dosis conidiën (1,6 x 10^9 conidiën/m^2) bleken ze, naarmate de schimmelinfectie vorderde, steeds minder eetlust te hebben. De proportie van schimmelgeïnfecteerde muggen die een tweede bloedmaaltijd namen was afgenomen met 51%. Dit nam verder af tot 35.3% voor de vierde bloedmaaltijd. Het andere secundaire effect was dat geïnfecteerde muggen minder bloed per bloedmaaltijd opnamen, wat resulteerde in een verminderd aantal eieren per gonotrofische cyclus.

Om een zo groot mogelijk effect te hebben op muggen populaties, is het nuttig dat er, behalve directe contaminatie, ook andere manieren worden benut om de schimmel binnen muggen populaties te verspreiden, zoals horizontale transmissie (waarbij conidiën van mug tot mug worden doorgegeven). De resultaten van Hoofdstuk 6 laten zien dat, onder laboratorium omstandigheden, conidiën kunnen worden overgebracht van een besmette vrouwtjes mug naar een ongeïnfecteerde mannetjes mug tijdens het paren, met infectiepercentages van de mannetjes tussen de 10.7 ± 3.2% and 33.3 ± 3.8%.

. Omdat de primaire besmettingsmethode, hierboven beschreven, gebaseerd is op muggen die op schimmelgeïmpregneerde doeken zitten, is het wenselijk dat muggen niet worden afgestoten door conidiën. Om dit te testen, werden gedragsexperimenten uitgevoerd met muggen die zich dicht bij conidiën bevonden (Hoofdstuk 7). Uit de resultaten blijkt dat droge conidiën een significant afstotend effect hebben ($p<0.05$). Echter, wanneer conidiën worden gesuspendeerd in 8% (plantaardige) olie en vervolgens geïmpregneerd worden op papier, dan verdwijnt dit effect ($p=0.205$). Deze resultaten geven aan dat wanneer deze

schimmel in Afrika bij muggenbestrijdings programma's zou worden ingezet, conidiën bij het impregneren van doeken in olie gesuspendeerd moeten worden, om afstoting te voorkomen.

Praktische benaderingen betreffende muggenbestrijding met de schimmel *M. anisopliae* (Hoofdstukken 8-10).

Vanuit zowel praktisch als economisch oogpunt gezien zou het interval tussen twee applicaties van een bestrijdingsmiddel zo lang mogelijk moeten duren, zonder dat het te veel aan werkzaamheid verliest. In het geval van de veelvuldig gebruikte chemische insecticiden zoals permethrine is dat ongeveer 6 maanden. Uit laboratorium experimenten (Hoofdstuk 8) blijkt dat *M. anisopliae*, wanneer het geïmpregneerd is op papier of op muggengaas, een maand na impregnatie nog steeds virulent is voor *An. gambiae* muggen. Bij experimenten naar de houdbaarheid van conidiën bleek dat conidiën, gesuspendeerd in 8% plantaardige olie, na tenminste een maand nog kiemkrachtig waren. Wanneer ze werden bewaard in 0.05% Tween 80, was de levensvatbaarheid na 3 maanden bij 27°C en na 6 maanden bij 4°C nog maar weinig afgenomen. Wanneer droge conidiën werden bewaard bij zeer lage vochtigheid, konden ze zonder problemen tenminste 6 maanden overleven. Deze resultaten suggereren dat, als de schimmel zou worden ingezet, her-impregnatie maandelijks zouden moeten plaatsvinden. Droge conidiën kunnen onder veldomstandigheden voor tenminste 6 maanden worden opgeslagen, mits de luchtvochtigheid laag is.

Hoofdstuk 9 van dit proefschrift beschrijft een veldexperiment waarin *M. anisopliae* binnenshuis werd aangebracht in huizen in een dorpje het zuidoosten van Tanzania (een gebied dat holo-endemisch is voor malaria en filariasis). De schimmel werd aangebracht op zwarte katoenen doeken en bevestigd aan de 'plafonds'. De doeken dienden om muggen op te laten rusten, waardoor de muggen zichzelf besmetten met de conidiën. Dagelijks werden alle waargenomen muggen gevangen, zowel van de doeken als uit de rest van de kamers. Deze werden in bekertjes gedaan en vervolgens zo lang mogelijk in leven gehouden om hun overleving te meten. Bijna 90% van alle gevangen muggen was An. gambiae s.l., waarvan 94.7% *An. gambiae s.s.* en 5.3% *An. arabiensis*. In totaal waren er 181 *An. gambiae* en 6 *Cx. quinquefasciatus* muggen die geïnfecteerd waren met de schimmel. Geïnfecteerde muggen gingen sneller dood dan ongeïnfecteerde muggen, met de kans om de dag levend door te komen (daily survival rate) 72.2% (geïnfecteerde), tegen 86.9% van de ongeïnfecteerde vrouwtjes. Gerekend van het totale aantal *An. gambiae s.l.* en *Cx. quinquefasciatus* muggen die van de schimmelgeïmpregneerde doeken waren gevangen hadden respectievelijk 33,6% en 10% een schimmelinfectie opgelopen. Van het totale aantal van 580 *An. gambiae s.l.* vrouwtjes muggen die waren gevangen in de huizen met de schimmelgeïmpregneerde doeken, waren 132 geïnfecteerd (22.8%). Als ditzelfde percentage wordt aangehouden op dorpsniveau en het, samen met de waargenomen verkleinde kans voor een geïnfecteerde vrouwtjesmug om een dag te overleven, word ingevoerd in een transmissie model voor malaria, dan neemt het aantal infectieuze muggenbeten per persoon per jaar af van een geschatte 262 naar slechts 14 (Hoofdstuk 10).

Alhoewel het veldexperiment slechts op vrij kleine schaal was, en van relatief korte duur, doen de voorspellingen van het malaria transmissie model ten sterkste vermoeden dat het gebruik van *M. anisopliae* ter bestrijding van volwassen malaria muggen in Afrika een groot effect zal hebben op transmissie intensiteit. Als deze methode op grote schaal zou worden toegepast als onderdeel van een geïntegreerde muggenbestrijdings campagne met onder andere larvale bestrijding met behulp van biologische bestrijdingsmiddelen, het gebruik van muggenafstotende planten en het gebruik van ongeïmpregneerde klamboe's, is de verwachting dat malaria effectief zou kunnen worden bestreden zonder gebruik te maken van chemische insecticiden. Dit proefschrift zou een eerste stap kunnen zijn voor een dergelijke campagne. Verder onderzoek is nodig, met name om 1) de schimmelstam te vinden die het

meest virulent is voor de beoogde muggensoorten, om 2) 'non-target effecten en veiligheidsaspecten te bestuderen, om 3) te zoeken naar de meest optimale formulering en toepassingsmethode, om zodoende het infectiepercentage te kunnen verhogen.

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CURRICULUM VITAE

Op 9 Februari 1974 werd ik, Ernst-Jan Scholte, geboren in Ermelo (Gelderland). Via MAVO en HAVO haalde ik in 1994 m'n diploma VWO aan het Christelijk College Groevenbeek te Ermelo. In datzelfde jaar begon ik met de studie biologie aan de toenmalige Landbouwuniversiteit Wageningen. In 1996 haalde ik m'n propedeuse, waarna ik koos voor de specializatie 'organisme'. Ik ging me meer en meer interesseren voor medische entomologie, en ben in 1998 begonnen met een eerste afstudeervak bij Dr. Willem Takken en Marieta Braks op de vakgroep Entomologie, waar ik heb gekeken naar de rol van menselijk zweet en huidbacteriën bij het gastheerzoekgedrag van de malaria mug *Anopheles gambiae*. Een stage heb ik in het najaar van 1998 uitgevoerd bij Dr. Bart Knols aan het ICIPE in Nairobi (Kenia), waar ik heb gekeken naar de rol die grondbacteriën spelen bij het ovipositie-gedrag van malaria muggen. Daaropvolgend heb ik een tweede afstudeervak gedaan bij het veldstation van ICIPE in Mbita (west Kenia), waar ik heb gewerkt aan de effecten van infectie met de insect-pathogene schimmel *Metarhizium anisopliae* op *An. gambiae* en op *Culex quinquefasciatus*. De resultaten van die studie zouden later de basis vormen van het huidige promotie-onderzoek. In het voorjaar van 1999 ben ik voor m'n derde afstudeervak naar het laboratorium van Prof. Paul Ward aan de Universiteit van Zürich (Zwitserland) gegaan, om een moleculair-ecologische studie te doen naar PGM genotypes van gele mestvliegen *Scatophaga stercoraria*. Direct na het behalen van m'n bul in September 1999 heb ik met een VSB beurs voor een jaar in Bologna (Italië) gewerkt in het laboratorium van Dr. Romeo Bellini van het CAA (Crevalcore) aan de steriele mannetjes techniek (SIT) bij de tijgermug *Aedes albopictus*. Bij terugkomst in Nederland in September 2000 ben ik aan het promotie-onderzoek begonnen onder begeleiding van Prof. Van Lenteren, Dr. Takken en Dr. Knols, en gefinancierd door WOTRO. In dit AIO-schap heb ik gekeken of de entomopathogene schimmel *M. anisopliae* ingezet zou kunnen worden als biologisch bestrijdings organisme tegen malaria en filariasis muggen. Na een half jaar 'inlezen' heb ik een jaar lang experimenten uitgevoerd bij het ICIPE veldstation in Mbita (Kenia). De daaropvolgende anderhalf jaar heb ik in op de Laboratorium voor Entomologie verdiepende experimenten uitgevoerd om meer informatie te krijgen over de interactie tussen de schimmel en de muggen. Om te bestuderen of de voorgestelde toepassing daadwerkelijk werkt heb ik 3 maanden bij het IHRDC in Ifakara (Tanzania) een veldstudie uitgevoerd. De resultaten van het promotie-onderzoek staan beschreven in dit proefschrift.

CURRICULUM VITAE

On the 9th of February 1974 I, Ernst-Jan Scholte, was born in Ermelo, the Netherlands. After having finished the Dutch equivalent of High school in 1994, I went to study biology at the Wageningen University, the Netherlands (at that time called Agricultural University of Wageningen). I soon started to specialize in entomology, particularly medical entomology. My MSc consisted of three separate projects plus an internship. The first project I started in 1998 at the Laboratory of Entomology, supervised by Dr. Willem Takken and Marieta Braks, where I studied the effect of human sweat and skin-bacteria on the host-seeking behavior of the malaria mosquito *Anopheles gambiae*. In September 1998 I went for my internship to the ICIPE in Nairobi (Kenya), where I studied the role that soil-bacteria may play on oviposition-behavior of *An. gambiae*, supervised by Dr. Bart Knols. This was followed by the second part of my MSc, which was carried out at the fieldstation of the ICIPE at Mbita, western Kenya. There I studied the effect of the insect-pathogenic fungus *Metarhizium anisopliae* on adult stage malaria (*An. gambiae*) and filariasis (*Culex quinquefasciatus*) mosquitoes. The results of that study would later form the basis of the current PhD project. In the spring of 1999 I went for the third part of my MSc to the laboratory of Prof. Ward at the University of Zürich, Switzerland, where I worked on a molecular ecological (field and lab) study on different PGM genotypes in the yellow dung fly (*Scathophaga stercoraria*). After obtaining my MSc in September 1999, I won a VSB-scholarship and went to Bologna, Italy, where I worked for one year at the Centre of Agriculture and Environment, Crevalcore, on the Sterile Male Technique for the tiger mosquito, *Aedes albopictus*. In October 2000 I started my PhD-study at the Laboratory of Entomology, Wageningen University, with the objective to study whether the insect pathogenic fungus *M. anisopliae* could be used against the adult stage of African malaria vectors. In the spring of 2001 I went for one year to the Mbita fieldstation of ICIPE, western Kenya. After having returned to the Netherlands I did some more extensive laboratory studies on the interaction of the fungus and the mosquitoes. In October 2003 I went for 3 months to the IHRDC in Ifakara, Tanzania, to carry out field experiments to determine whether we could infect and kill wild mosquitoes with the fungus. The results of this PhD-study are presented in this dissertation.

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