

An entomopathogenic fungus (*Metarhizium anisopliae*) for control of the adult African malaria vector *Anopheles gambiae*

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KEY WORDS

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Current indoor control for malaria 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. This manuscript describes a series of experiments to evaluate the potential impact of a new method, the use of an insect-pathogenic fungus (*Metarhizium anisopliae*) against adult *Anopheles gambiae* sensu stricto, both in the laboratory and in the field. Various experiments describe different aspects of this developing methodology, among which application techniques, sublethal effects, and the impact it may have on malaria transmission. Finally, it is discussed how this method can play a part in an integrated approach of mosquito vector control in the tropics.

Introduction

For a long time, the principal choice to reduce nuisance biting by mosquitoes or their transmission of parasitic or arboviral diseases has been the selective application of residual synthetic insecticides. The public health benefit delivered by this method, especially in tropical resource-poor settings, is huge, saving uncountable human lives. 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 grim reality of environmental impact and ever-developing resistance remains an issue of grave concern (Chandre et al. 1999, Hemingway & Ranson 2000,

Brooke et al. 2002). 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 protozoa (Chapman 1974, Legner 1995), nematodes (Kaya & Gaugler 1993), predatory fish (Legner 1995), and bacteria (Becker & Ascher 1998) has shown promise as a means to control mosquito populations, and some of these agents are successfully used in today's mosquito control programmes in various regions around the world (Becker et al. 2003). Unfortunately, in the one continent where malaria accounts for more than 1 million deaths each year, alternatives to insecticides are virtually non-existent. Recently, interest has increased in another group of biological

Fungus	mosquito species	affected stage	reference
Oomycota ¹			
<i>Leptolegnia</i> ² sp. (unidentified)	<i>An. gambiae</i>	larva	Nnakumusana 1986
<i>Pythium</i> sp.	<i>An. gambiae</i>	larva	Nnakumusana 1985
<i>Lagenidium giganteum</i>	<i>An. gambiae</i>	larva	Golkar et al. 1993
Chytridiomycota			
<i>Coelomomyces indicus</i>	<i>An. arabiensis</i>	larva	Service 1977
	<i>An. gambiae</i>	larva	Muspratt 1963
Zygomycota			
<i>Smittium</i> (unidentified)	<i>An. gambiae</i>	larva	Coluzzi 1966
Fungi Anamorfici			
<i>Aspergillus parasiticus</i>	<i>An. gambiae</i>	larva/adult	Nnakumusana 1985
<i>Metarhizium anisopliae</i>	<i>An. gambiae</i>	larva/adult	Roberts 1967, Scholte et al. 2003
<i>Beauveria bassiana</i>	<i>An. gambiae</i>	adult	Scholte et al. 2003

Table 1. Entomopathogenic fungi that have been reported to be pathogenic to African malaria vectors.

Table 1. Entomopathogene schimmels waar-
van gerapporteerd is dat ze pathogeen zijn
voor Afrikaanse malariavectoren.

¹ = Phylum, ² = Genus



1. *Metarhizium anisopliae*, sporulating from a cadaver of the African malaria mosquito *Anopheles gambiae* s.s. The green-whitish colour is caused by millions of conidia, produced by conidiogenous cells forming on hyphae growing out of the deceased mosquito. Photo: E-J Scholte (source: Knols & Thomas 2006).

1. *Metarhizium anisopliae*, sporulerend op een dood exemplaar van de Afrikaanse malaria-mug *Anopheles gambiae* s.s. De groen-witachtige kleur wordt veroorzaakt door miljoenen conidiën, die geproduceerd worden door conidiogene cellen, groeiend op de schimmelhyphen uit de dode mug.

control agents that opens up perspectives for vector control in Africa: fungi (Scholte et al. 2003a,b, 2004a,b, 2005, 2006, Blanford et al. 2005, Thomas et al. 2005, Knols & Thomas 2006).

Many entomopathogenic fungi have proven to be pathogenic to mosquitoes (Scholte et al. 2004b), and some of them have also been found in African malaria vectors (table 1). This list is likely to become much longer if fungal pathogens of the malaria vector *Anopheles gambiae* Giles sensu stricto are specifically being searched for in the field, and other fungi will be tested on this vector in the laboratory. While successful mosquito vector control in Africa is currently based on controlling adult mosquitoes, the vast majority of mosquitocidal fungi is aquatic and may only be used to control the aquatic stages of the insects. However, recent progress has been made in using anamorphic entomopathogenic fungi (Deuteromycetes) for controlling adult African mosquitoes (Scholte et al. 2003a,b, 2004a,b, 2005, Blanford et al. 2005). These fungi have a distinct advantage over the biological control agents mentioned above, in that they do not need to be ingested to infect and kill the insects – infection takes place through physical contact of the infective propagules (conidia) with the insect cuticle. As such, the contamination method is similar to conventional indoor residual spraying with insecticides. It is generally accepted that the use of these insect-pathogenic fungi, such as *Metarhizium anisopliae*, as a myco-insecticide is not harmful to the environment (Zimmermann 1993, Strasser et al. 2000), nor to humans. This was illustrated by the fact that in 2003, an *M. anisopliae* isolate (strain F52) was granted registration by the U.S. Environmental Protection Agency to be used against insect pests. The dossier stated that, after thorough (eco)toxicological testing, no harm is expected to be caused to humans or the environment (EPA 2003, Kanzok & Jacobs-Lorena 2006).

General life cycle of deuteromycetous entomopathogenic fungi

Deuteromycetous fungi are a group of fungi of which no sexual stage has been reported. Several insect pathogenic fungal genera such as *Beauveria*, *Fusarium* and *Metarhizium* belong to this group. The life cycle of entomopathogenic deuteromycetous fungi starts with conidia that attach to, germinate on, and penetrate the insect cuticle. Once in the haemocoel, the mycelium

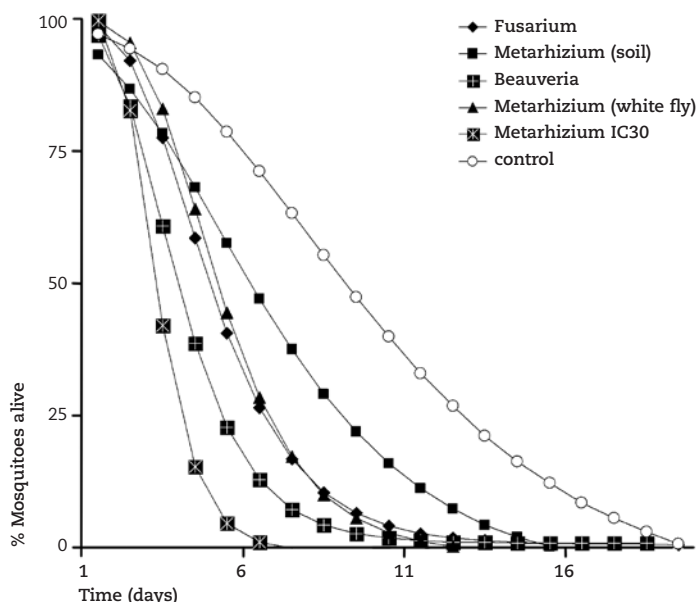
grows throughout the host, forming hyphal bodies called blastospores. Death of the insect is often due to a combination of the action of fungal toxins, physical obstruction of blood circulation, nutrient depletion and/or invasion of organs. After the host has died, hyphae usually emerge from the cadaver and, under suitable abiotic conditions, conidia are produced on the exterior of the host (figure 1). These are then dispersed by wind or water (Goettel & Inglis 1997).

First screening of candidate fungi

In order to obtain a suitable fungal candidate for controlling *An. gambiae* s.s., a search for mosquitocidal Deuteromycetes was conducted in Western Kenya (Scholte et al. 2003b). Four insect-pathogenic fungi were isolated. Three species were isolated from dead insects: *Beauveria bassiana* and *Fusarium* sp. from the stemborer *Busseola fusca* (Fuller), and an *M. anisopliae* strain from the white fly *Trialeurodes vaporariorum* (Westwood); one *M. anisopliae* isolate was found in a soil sample. A fifth fungus (*M. anisopliae* isolate ICIPE 30) was provided by the International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya (courtesy N.K. Maniania). Unformulated conidia of these fungi were screened for their virulence against adult *An. gambiae* s.s. mosquitoes. Survival analysis (Kaplan Meier) showed that survival of mosquitoes that were infected with either one of the five fungi was significantly lower than that of the control group, although virulence varied between fungi (figure 2) (Scholte et al. 2003b). Mean infection percentages are depicted in table 2. From this initial fungal screening experiment, the hyphomycete *M. anisopliae* isolate ICIPE 30 proved to be the most virulent for *An. gambiae*, and subsequent experiments were carried out using this isolate.

Development of standard infection methodology

For contamination of adult mosquitoes with fungal conidia of a known concentration, a standard protocol was developed similar to screening of residual pesticides on mosquitoes: a filter paper was impregnated on one side with a known concentration of conidia, formulated with an 8% adjuvant vegetable oil suspended in 0.05% Tween-80 and dried slowly during 48 hrs at room temperature. The paper was rolled up and inserted into a



2. Survival curves (fitted on Gompertz survival distribution) of adult mosquitoes (*Anopheles gambiae* s.s.) infected with various entomopathogenic fungi (source: Scholte et al. 2003).

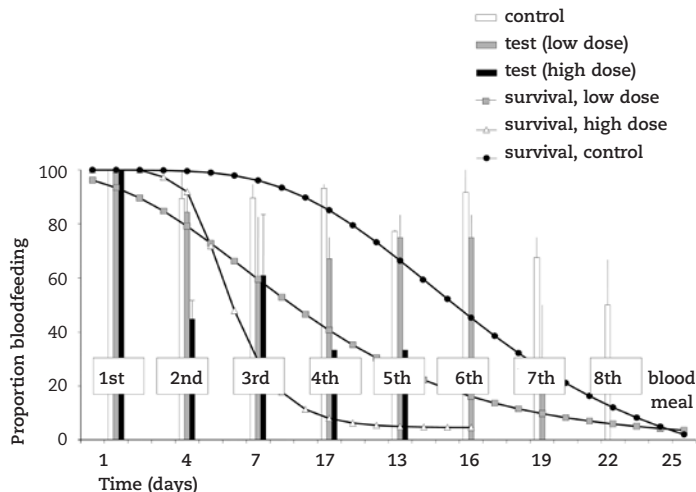
2. Overleving (geplot op de 'Gompertz-overlevingsverdeling') van volwassen *Anopheles gambiae* s.s., geïnfecteerd met verschillende soorten entomopathogene schimmels.

Table 2. Infectivity and pathogenicity of various fungal species to adult *Anopheles gambiae* s.s. mosquitoes (source: Scholte et al. 2003).

Table 2. Mate van besmettelijkheid en ziekteverwekkend vermogen van enkele schimmelsoorten tegen volwassen stadia van *Anopheles gambiae* s.s. muggen.

Fungus	% Infected ± SE ¹	LT ₅₀ ± SE (days)	Grouping ² LT ₅₀
control		8.80 ± 0.66	A
<i>M. anisopliae</i> (soil-sample)	46.5 ± 3.6	5.87 ± 0.77	B
<i>Fusarium</i> sp.	79.0 ± 4.3	4.48 ± 0.21	B C
<i>B. bassiana</i>	81.7 ± 0.9	3.49 ± 0.29	C
<i>M. anisopliae</i> (white fly)	83.0 ± 3.4	4.71 ± 0.55	B C
<i>M. anisopliae</i> (ICIPE 30)	88.7 ± 3.3	3.39 ± 0.37	C

¹SE: Standard error of the mean, ²LT₅₀ values without letters in common are significant at p<0.05



3. Survival curves (fitted on Gompertz distribution), from the second experiment on blood-feeding propensity, and proportions (± SE) 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 was due (source: Scholte et al. 2006).

3. Overleving (geplot op de 'Gompertz-overlevingsverdeling'), uit het tweede experiment dat de neiging tot bloedvoeden onderzocht, en de relatieve aantallen (± SE) van *M. anisopliae*-geïnfecteerde vrouwelijke *An. gambiae* s.s. die een bloedmaaltijd namen, berekend op basis van de aantallen muggen die een volgende bloedmaaltijd namen.

glass cylinder in such a way that the side containing conidia was the surface on which mosquitoes would rest once inserted in the glass cylinder. Subsequent laboratory experiments on female *An. gambiae* s.s. showed that a dose of 1.6×10^{10} conidia/m² resulted in infection percentages between 83.7 and 97.7, and LT₅₀-values (i.e., the time it takes for 50% of a group to die) ranging from 3.2 to 5.9 days, while uninfected females lived up to 31 days with LT₅₀s between 9.9 and 18.5 days (Scholte et al. 2003a, 2004a). This dosage was considered suitable for field application, because the expected infection proportions in targeted mosquitoes were high, and the quantities of required conidia were of an acceptable magnitude.

Fungal infection effects on blood-feeding and fecundity of mosquitoes

In addition to causing significant mortality, *M. anisopliae* is known to cause reductions in feeding and fecundity in a range of insects. Since a change in blood-feeding propensity of malaria vectors could have important implications regarding malaria transmission, we investigated whether blood feeding of *An. gambiae* s.s. was also affected by infection with *M. anisopliae*

Gonotrophic cycle	Treatment		
	Control	Low fungal dosage	High fungal dosage
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	6.33 ± 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

*significant overall effect of fungal infection on average number of eggs per bloodmeal (ANOVA): F=9.784; p<0.001, df=2.

Table 3. Mean number of eggs (± SE) laid per blood meal, as calculated from the female mosquitoes alive at the time when oviposition was due in each gonotrophic cycle (n) (source: Scholte et al. 2006).

Table 3. Gemiddelde aantal eitjes (± SE) dat gelegd werd per bloedmaaltijd, berekend op grond van het aantal vrouwtjesmuggen dat levend was op het moment waarop ze, per gonotrofische cyclus, verwacht werden eitjes te leggen (n).



4. (a): Position of black cloth (dotted line) impregnated with conidia of the entomopathogenic fungus *Metarhizium anisopliae* inside a Tanzanian house. (b): Blood-fed *An. gambiae* females on a *M. anisopliae*-infected cloth (source: Scholte et al. 2005).

4. (a): De positie van de met de entomopathogene schimmel geïmpregneerde zwarte doek (gestreepte lijn) in een Tanzaniaans huis. (b): Volgezogen *An. gambiae* vrouwtjes op een *M. anisopliae*-geïmpregneerd doek.

(Scholte et al. 2006). The mosquitoes were contaminated with either a low (1.6×10^8 conidia/m²) or a moderately high (1.6×10^9 conidia/m²) dose of oil-formulated *M. anisopliae* conidia, and offered a single human blood meal 48, 72, or 96 hrs later to assess feeding propensity and individual blood meal size. Females infected with the low dose of conidia ingested less blood than uninfected females [$3.79 \pm 1.34 \mu\text{g}$ vs. $7.78 \pm 0.99 \mu\text{g}$ hematin/blood meal for large females (wing size ≥ 3.1 mm) and $2.88 \pm 1.11 \mu\text{g}$ vs. $3.90 \pm 0.82 \mu\text{g}$ hematin for small females (wing size < 3.1 mm)]. This experiment also showed that females took significantly fewer blood meals than uninfected females 72 hrs after they had been contaminated.

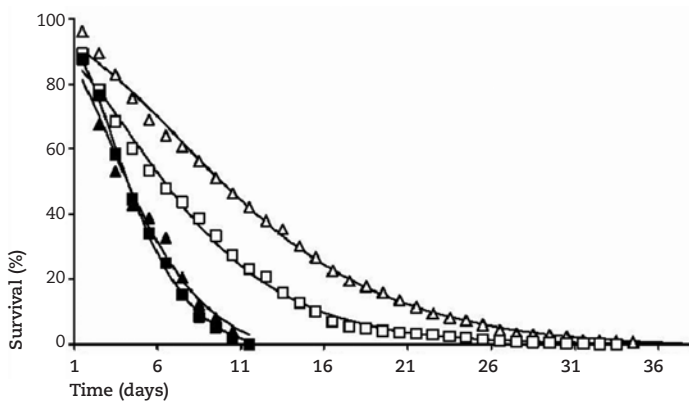
In a second experiment, individually kept fungus-infected females were offered a blood meal every third day (to a total of eight gonotrophic cycles), and allowed to oviposit after each cycle in order to quantify feeding propensity and fecundity. After each feeding opportunity it was scored whether the

female had fed or not. It was found that mosquitoes, inoculated with the moderately high dose of conidia, exhibited reduced appetite (figure 3). 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 fourth blood meal. During eight feeding opportunities, the average 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. Moreover, infected females produced fewer eggs per gonotrophic cycle (table 3).

Application in the field – can entomopathogenic fungi reduce malaria transmission?

Fundamental to the design of malaria control programmes is a basic understanding of the relationship between malaria transmission by mosquito vector populations and malaria prevalence, incidence, morbidity and mortality. One parameter that describes the intensity of malaria transmission is the Entomological Inoculation Rate (EIR), which is the rate at which sporozoite-positive bites are received. This function expresses the importance of mosquito survival and the number of blood meals taken, reductions of which have high impact on the intensity of malaria transmission (Charlwood 1997, Charlwood et al. 1997, Killeen et al. 2000, Drakeley et al. 2003). In a small-scale field study in Tanzania, we placed *M. anisopliae*-impregnated cloths indoors of local houses (figure 4) (Scholte et al. 2005). Of all *An. gambiae* sensu lato collected in these houses, 23% were found to be infected with the fungus. The infected mosquitoes had significantly shorter life spans compared to those of non-infected ones (figure 5). LT₅₀-values were 3.70 and 3.49 days for infected males and females, respectively, and 5.88 and 9.30 days for uninfected males and females. The reductions in daily survival rates of wild *An. gambiae* s.l. and numbers of blood meals taken by *M. anisopliae*-infected females may thus have a high impact on the EIR, provided that enough mosquitoes become infected. Extensive field studies on village scale have recently begun to assess the impact of this fungus on EIR and morbidity.

Important aspects concern the system of delivering the fungus to the mosquito population, and effective coverage of the



5. Survival of uninfected (open symbols: Δ = females, \square = males) and *M. anisopliae*-infected (closed symbols: \blacktriangle = females, \blacksquare = males) wild *An. gambiae* s.l. mosquitoes collected from rural Tanzanian houses (source: Scholte et al. 2005).

5. Overleving van ongeïnfekteerde (open symbolen: Δ = vrouwtjes, \square = mannetjes) en *M. anisopliae*-geïnfekteerde (gesloten symbolen: \blacktriangle = vrouwtjes, \blacksquare = mannetjes) wilde *An. gambiae* s.l. muggen, verzameld van huisjes in een Tanzaniaans dorp.

targeted insect population with the fungus. In the field study in Tanzania, conidia were suspended in an 8% adjuvant oil formulation and applied on 3-m² black cotton sheets. These sheets were placed inside houses on the ceiling and contaminated indoor-resting mosquitoes with the fungus. Increased size of these 'resting targets' and application in larger parts of human habitations may maximise coverage.

Application strategies

Killeen *et al.* (2004) showed that the 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. In a similar way we envisage an integrated vector-control programme without any use of chemicals, where *M. anisopliae* is used against adult mosquito vectors in addition to biological larval control with *Bacillus thuringiensis* subsp. *israelensis* and/or *B. sphaericus* (Fillinger *et al.* 2003), and application of

untreated bed nets, push-pull methods with zooprophylaxis (Seyoum *et al.* 2002) and repellent plants (Seyoum *et al.* 2003). Larval control will reduce the number of emerging adults, whereas cattle in or near houses may prevent some of the vectors from feeding on humans. Repellent odours from live, potted, or burned plants around houses will diminish the numbers of mosquitoes entering the houses. Mosquitoes which are able to enter dwellings will be prevented to bite by bed nets (Guyatt & Snow 2002, Takken 2002). And if some of these mosquitoes that entered houses will be infected by *M. anisopliae* when they land on the 'resting targets', this will reduce their survival rate. None of these techniques, as stand-alone tools, is perfect, but the combination of these vector control methods may be very powerful and greatly enhance reductions of the EIR. Such a strategy may result in a more sustainable vector-borne disease control than the current ones based on potentially harmful chemicals. However, in order to be successful, substantial financial, political and local support for long-term and large-scale application is necessary.

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Samenvatting

Een insectpathogene schimmel (*Metarhizium anisopliae*) ter bestrijding van volwassen Afrikaanse malariamuggen (*Anopheles gambia*)

Bestrijding van malariamuggen in Afrika gebeurt vrijwel geheel met chemische insecticiden. Hoewel er diverse biologische bestrijdingsorganismen bestaan die larvale populaties kunnen decimeren, zijn er geen organismen die kunnen worden ingezet voor de bestrijding van het volwassen stadium. Dit artikel beschrijft een serie laboratorium- en veldexperimenten waarin een nieuwe biologische bestrijdingsmethode wordt geëvalueerd. Deze methode gebruikt een insect-pathogene schimmel (*Metarhizium anisopliae*) die wordt ingezet tegen adulten van de Afrikaanse malariamug *Anopheles gambiae* sensu stricto. Diverse aspecten van deze nieuwe methode worden besproken, waaronder de beoogde toepassings-techniek, sublethale effecten van de schimmel op een geïnfecteerde mug, de impact die gebruik van deze methode kan hebben op malariaoverdracht, en hoe deze nieuwe methode deel kan uitmaken van een geïntegreerde benadering van muggenbestrijding in de tropen.



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