African Water Storage Pots for the Delivery of the Entomopathogenic Fungus *Metarhizium anisopliae* to the Malaria Vectors *Anopheles gambiae* s.s. and *Anopheles funestus*

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Abstract. We studied the use of African water storage pots for point source application of *Metarhizium anisopliae* against the malaria vectors *Anopheles gambiae* s.s. and *An. funestus*. Clay pots were shown to be attractive resting sites for male and female *An. gambiae* s.s. and were not repellent after impregnation with fungus. *M. anisopliae* was highly infective and virulent after spray application inside pots. At a dosage of $4 \times 10^{10}$ conidia/m², an average of 95 ± 1.2% of *An. gambiae* s.s. obtained a fungal infection. A lower dosage of $1 \times 10^{10}$ conidia/m² infected an average of 91.5 ± 0.6% of *An. gambiae* s.s. and 91.8 ± 1.2% of *An. funestus* mosquitoes. Fungal infection significantly reduced mosquito longevity, as shown by differences between survival curves and LT₁₀ values. These pots are suitable for application of entomopathogenic fungi against malaria vectors and their potential for sustainable field implementation is discussed.

INTRODUCTION

Human malaria, transmitted by female mosquitoes of the genus *Anopheles*, is a major public health challenge in developing countries. Malaria is Africa’s major cause of mortality in those younger than 5 years of age and constitutes 10% of the continent’s overall disease burden.¹ *Anopheles gambiae* s.s. Giles and *An. funestus* Giles are two of the most important malaria mosquito vectors in sub-Saharan Africa in terms of human morbidity and mortality, with high susceptibility to *Plasmodium* infection and high degrees of anthropophily.² *An. gambiae* s.s. has a continent-wide distribution, occupies temporary aquatic habitats, and is considered to be one of the most efficient species to develop and transmit malaria parasites.³,⁴ *An. funestus* is widely distributed throughout tropical Africa, occupies a wide range of ecological niches, and extends its activity even into the dry season when other malaria mosquito vectors are present in much reduced densities.⁵,⁶

Current vector control methods against adult mosquito populations are mainly based on insecticide application, particularly indoor residual spraying (IRS) and insecticide-treated bednets (ITNs). However, insecticide resistance poses a serious threat to sustainable insecticide-based vector control in many African countries.⁷ Both *An. gambiae* and *An. funestus* populations are showing increasing levels of resistance to one or more of the insecticide classes used in vector control.¹ There is a pressing need, therefore, to develop novel vector control methods that can complement or replace existing intervention tools.

*Metarhizium anisopliae* and *Beauveria bassiana* are hypomycetous insect-pathogenic fungi of which the conidia can infect insects by penetrating the cuticle, without the need for ingestion, and kill them within several days.⁸ *Metarhizium* spp. and *Beauveria* spp. are endemic worldwide and are not harmful to birds, fish, or mammals,⁹ and because of their opportunistic nature, lack of association with humans, and inability to survive at human body temperatures, are considered to cause no human health risks (S. De Hoog, personal communication). The US Environmental Protection Agency has declared no risk to humans when using products containing *M. anisopliae*, based on toxicity tests.¹⁰ Hypomycetous fungi can be cost-effectively mass-produced,¹¹ and several strains are already commercially available¹²,¹³ and in use for the control of agricultural and veterinary pests.¹⁴,¹⁵

Several studies have shown that conidia of *M. anisopliae* and *B. bassiana* are pathogenic to adult *Anopheles*, *Aedes*, and *Culex* mosquitoes, because fungal infection significantly reduces their longevity.¹⁶–¹⁸ This implies a high potential for use as biological control agents against vectors transmitting malaria, dengue, Chikungunya, and other mosquito-borne diseases. Besides a reduction in daily survival rates, fungal infection has also shown other effects that are likely to affect malaria transmission. First, infection of *Anopheles* with these fungi has an inhibitory effect on the development of malaria parasites in mosquitoes.¹⁹ Second, fungal infection reduces a mosquito’s feeding propensity and fecundity.²⁰ Another important potential benefit of biological vector control with entomopathogenic fungi is that fungi are slow-killing agents, which is considered to reduce the selection pressure on resistance formation by allowing adult mosquitoes partial reproductive success.²¹

An international consortium of seven research institutes was formed in 2005, with the main aim of reducing the burden of mosquito-borne diseases through the development and delivery of novel and sustainable approaches of adult mosquito control based on fungi.²² Although research on fundamental aspects regarding fungus production and fungus–mosquito interactions is underway, gaps remain between the scientific laboratory discoveries and a successful implementation of fungi in the field. Practical issues concerning the conidial viability, infectivity, formulation, and delivery of fungi need to be resolved before progress toward field research can be made.²³ The choice of delivery method will be an important determinant of the overall effectiveness of a fungus-based vector control method. An optimal delivery system requires
maximum exposure with minimal application of conidia and has to ensure an effective coverage through applying fungal conidia at sites that are both suitable for the fungus and attractive for mosquitoes. Several options exist for delivering fungal conidia to adult mosquitoes in the field, including indoor surface application, bednet application, and point source application. Point source application has certain benefits because it reduces human exposure to the fungus and overall costs by decreasing the amount of conidia needed. It also allows for application of fungi both indoors and outdoors, which could increase the effect on disease transmission through the ability to target both endophilic and exophilic mosquito species.

A recent study in western Kenya tested clay water storage pots as a mosquito sampling tool and showed that these pots are highly attractive resting sites for *Anopheles* mosquitoes in the field. Three clay pots, placed within 5 m of each sampled house, attracted relatively high numbers of both sexes of *An. gambiae s.s.* and *An. arabiensis* Patton, including females of all physiological stages (unfed, blood fed, gravid). Because they are attractive to resting mosquitoes, clay pots may be suitable objects for point source application of fungal conidia. Moreover, clay pots are widely available, low cost, portable objects with a dark, cool, and humid micro-climate that may prove an ideal environment for fungal conidia by reducing exposure to UV light and desiccation.

Research was conducted in the laboratory on clay pots as delivery systems for *M. anisopliae* against *An. gambiae s.s.* and *An. funestus*. Assessments were made to determine the attractiveness of a clay pot as a resting site for *An. gambiae s.s.* mosquitoes compared with other, similar yet smaller and lighter, potential resting sites, i.e., a PVC pipe and a small terra cotta pot, and the effect of fungal impregnation on the attractiveness of a clay pot. Two different dosages of oil-formulated *M. anisopliae* conidia were tested for infectivity and virulence after spray application inside clay pots against both sexes of *An. gambiae s.s.* and *An. funestus*.

MATERIALS AND METHODS

Mosquitoes. *Anopheles gambiae s.s.* mosquitoes originated from Suakoko, Liberia (courtesy of Prof. M. Coluzzi). Larvae were reared in plastic trays filled with tap water and were fed on Tetramin fish food (Tetra, Melle, Germany) once per day. Pupae were collected daily and transferred to holding cages of 30 × 30 × 30 cm. Eggs of *An. funestus*, originating from southern Mozambique, were obtained from a culture at the Vector Control Reference Unit of the National Institute for Communicable Diseases in Johannesburg (courtesy of Prof. M. Coetzee). They consisted of the FUMOZ strain, which shows high levels of pyrethroid resistance (0–1% mortality when exposed to 1% lambda-cyhalothrin for 1 hour), and were reared in plastic trays filled with tap water, supplemented with green algae. Larvae were fed on a mixture of finely crushed dog biscuits and brewers yeast, and pupae were collected daily and transferred to 30 × 30 × 30-cm holding cages. Both species were maintained at 27 ± 1°C and 80 ± 5% RH with a 12 L:12 D photoperiod. For experiments, 4- to 7-day-old mosquitoes were used, which were fed *ad libitum* on a 6% glucose solution.

Fungi. *Metarhizium anisopliae* var. *anisopliae* (Metsch.) Sorokin, isolate ICIPE-30 (courtesy of Dr. N. Maniania) was used. The fungus was produced through solid state fermentation with glucose-impregnated hemp (courtesy of F. van Breukelen), of which conidia were dried and stored in the dark at 4°C. For application, dry conidia were suspended in a highly refined mineral oil (Shell Ondina Oil 917, Shell, Capelle aan de IJssel, The Netherlands). The suspension was mixed by vortexing and briefly sonicating at a low frequency (Branson sonifier B12; G. Heinemann, Schwäbisch Gmünd, Germany). Conidial concentrations of each stock were determined using a Bürker-Türk hemocytometer counter (W. Schreck, Hofheim/TS), after which the conidial viability was assessed by plating a small drop of the conidial formulation on a petri dish containing Sabouraud dextrose agar and subsequently incubating the plate at 27°C in the dark for 22–26 hours. The proportion of germinated conidia was determined using a light microscope at a magnification of ×400. Stocks showing 85% or higher sporulation were used for experiments.

**Clay pot bioassays.** Handmade Ghanaian clay pots (Afrikaad, Barendrecht, The Netherlands) ∼38 cm in diameter and 35 cm in height were used. The size of the pot opening was on average 14 cm, and the inside surface was ∼0.45 m². Conidial formulations were applied inside the pots 1 day before experiments using a SATA minijet 4 HVLP spray gun (vd Belt, Almere, The Netherlands) at a constant pressure of 1.5 bars. A specially designed apparatus rotated the pot while spraying at a speed of ∼16 rotations/min, while also automatically moving the spray gun vertically up and down during spraying (Figure 1). The pot opening was covered during spraying with thin plastic with a small hole in the center for the spray gun nozzle. Test pots were first sprayed with 30 mL of oil, left to dry for 2 hours, and subsequently treated with an additional 15 mL of either the 1.2 × 10⁹ or 3 × 10⁹ conidia/mL formulation, reaching an end concentration of 4 × 10¹⁰ or 1 × 10¹⁰ conidia/m², respectively. Control pots were sprayed with 30 mL, and subsequently 15 mL, of Ondina oil without conidia.

In each pot, sealed off with plastic, 50 male and 50 female mosquitoes were released with a mouth aspirator through a small hole drilled in the bottom of each pot. Mosquitoes were exposed for 17 hours, having access to a 6% glucose solution.
through freshly soaked cotton wool pads on the rim of the pot opening, against the plastic. Subsequently, mosquitoes were transferred to a holding cage by replacing the plastic with a holding cage sleeve secured over the pot opening, lifting the pot and cage vertically, and blowing softly into the pot. Most mosquitoes took flight into the holding cage, and the remaining individuals were retrieved through aspiration. Mosquitoes that died because of handling were discarded ~2 hours after transfer to the holding cage and were not included in data analyses.

Mosquito survival was determined by recording mosquito mortality in each holding cage. Dead mosquitoes were removed daily from the cages, dipped in 70% ethanol, placed in sealed petri dishes containing moist filter paper, and incubated at 27°C for 3 days. Mosquito cadavers were checked for sporulating M. anisopliae, i.e., emerging hyphae, using a dissection microscope to verify fungal infection.16

For An. gambiae s.s., a total of nine test replicates with 4 × 10^10 conidia/m² and three control replicates were conducted on 3 consecutive days, besides three test and control replicates in total on 2 consecutive days with a concentration of 1 × 10^10 conidia/m². For An. funestus, three test replicates with 1 × 10^10 conidia/m² and two control replicates were conducted on a single day.

**Attractiveness tests.** The attractiveness of a dry clay pot and a wet clay pot (i.e., a pot containing ~1.5 L of water) was compared with a small terracotta pot (22 cm high, 8 cm diameter, 6 cm opening diameter) and a dark-gray PVC pipe (50 cm long, 15 cm diameter), which were considered as other, perhaps more convenient point source objects for field application, because of their smaller size and reduced weight. These four objects were placed on the floor in the center of a large cage of 3 × 3 × 2.5 m inside climate-controlled rooms (temperature, 26–29°C; humidity, 70–90% RH). Subsequently, 80–90 male and female An. gambiae s.s. mosquitoes were released for 3 hours, after which the number of resting mosquitoes in each object was determined. Three replicate tests were performed in which the position of the four tested objects was randomized.

The effect of the conidial formulation on the attractiveness of clay pots was tested in three replicate tests in which a clay pot impregnated, as described above, with 4 × 10^10 conidia/m², and a clean clay pot were placed 1 m apart in the center of the cage, in which 80 male and 80 female mosquitoes were released for 4 hours. Resting proportions were determined from the mosquito numbers found resting inside each pot.

**Data analysis.** In the test on attractiveness, mosquito numbers found resting in the four objects were compared using a χ² test on the assumption that resting mosquitoes would be evenly distributed over all test objects. Mosquito survival data were fitted to the Gompertz distribution model16 using GenStat 9.2 software. Differences in the computed survival curves of treated and control mosquitoes were analyzed using Kaplan-Meier pairwise comparisons16 with SPSS 15.0 software. Each test replicate was compared with the corresponding control replicates separately.

### RESULTS

**Clay pot bioassays.** A total of 858 An. gambiae s.s. mosquitoes were exposed to clay pots with a conidial dose of 4 × 10^10 conidia/m², of which 95.0 ± 1.2% (SE) acquired a fungal infection. The lower conidial dose, of 1 × 10^10 conidia/m², was able to infect 91.5 ± 0.6% of the 267 exposed An. gambiae s.s. mosquitoes. For An. funestus, a total of 233 mosquitoes were exposed to 1 × 10^10 conidia/m², of which 91.8 ± 1.2% showed fungal infection after death.

Mosquito survival curves showed a close fit to the Gompertz distribution model, with the percentage of variance accounted for exceeding 96% for all converted survival curves. For both fungal dosages, the survival curves of male and female An. gambiae s.s. and An. funestus mosquitoes showed a significant reduction in longevity compared with the control mosquitoes (Figure 2). Kaplan-Meier pairwise comparison of the survival curves showed that in all the tested groups longevity of mosquitoes exposed to the fungus was significantly reduced compared with their control counterparts (P < 0.0001). There were no significant differences between male and female mosquito longevity within the control and treatment replicates of An. gambiae s.s. and An. funestus (P > 0.05).

LT_{50} values of the control and test treatments (Table 1) were calculated using the parameters of the computed survival functions. For all tested mosquito species and fungal dosages, pairwise comparisons of the LT_{50} values showed highly significant differences between the control and infected mosquitoes (P < 0.0001). Within the control and treated groups of An. gambiae s.s. and An. funestus, there were no significant differences between male and female LT_{50} values (P > 0.05).

**Attractiveness.** Of the 219 male An. gambiae s.s. mosquitoes that were released in the experimental cage and retrieved, on average, 47.4 ± 7.2% was found resting inside one of the four tested objects. For females (N = 245), this was 81.8 ± 2.0%. The remaining mosquitoes were found resting on the cage netting. Resting male and female mosquitoes showed a high preference for the dry and the wet clay pots compared with the small terracotta pot and the PVC pipe (Figure 3), shown by highly significant differences for both the number of male (P < 0.0001) and female (P < 0.0001) mosquitoes resting in them. The dry and the wet clay pot were shown to be equally attractive, because there was no significant difference between male (P = 0.88) and female (P = 0.42) presence in either of these treatments.

In a separate comparison, the attractiveness of a fungus-impregnated and a clean clay pot was compared. Both male and female mosquitoes were found resting inside the two objects after 3 hours (Figure 4), although significantly more females than males were attracted to both the impregnated pot (P = 0.003) and the control pot (P = 0.004). The attractiveness of a clay pot as a mosquito resting site was not affected by impregnation with oil-formulated fungal conidia, because there were no significant differences for both male (P = 0.71) and female (P = 0.77) mosquito numbers in either pot.
DISCUSSION

The results of this study showed the potential usefulness of clay water storage pots as point source application objects for entomopathogenic fungi against adult anophelines. Clay was shown to be suitable material for spray application of oil-formulated conidia. Fungal conidia remained both infective and virulent after formulation in mineral oil and spray application inside clay pots. The significant differences in survival curves and LT₅₀ values between control and fungus-exposed mosquitoes showed a significant reduction in mosquito longevity of both male and female *An. gambiae s.s.* and *An. funestus* mosquitoes. The obtained survival curves and LT₅₀ values were consistent with the results of previous studies with the same fungal strain and *An. gambiae s.s.* The clay pot bioassays were highly consistent in infecting mosquitoes with a pathogenic fungus, reaching high infection percentages with low variation between results. Observations indicated that concentrations < 10¹⁰ conidia/m² were insufficient to reach such consistency in infections. It may, however,

<table>
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<tr>
<th>Species</th>
<th>Dosage</th>
<th>Sex</th>
<th>Mean LT₅₀ ± SE</th>
<th>P*</th>
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<td></td>
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<td>Control</td>
<td>Fungus</td>
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<tr>
<td><em>A. gambiae s.s.</em></td>
<td>4 × 10¹⁰ conidia/m²</td>
<td>Male</td>
<td>19.26 ± 4.49</td>
<td>3.54 ± 0.15</td>
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<td></td>
<td>Female</td>
<td>16.01 ± 2.82</td>
<td>3.84 ± 0.17</td>
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<tr>
<td><em>A. gambiae s.s.</em></td>
<td>1 × 10¹⁰ conidia/m²</td>
<td>Male</td>
<td>14.97 ± 0.22</td>
<td>4.03 ± 0.11</td>
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<tr>
<td></td>
<td></td>
<td>Female</td>
<td>13.37 ± 0.34</td>
<td>4.07 ± 0.08</td>
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<tr>
<td><em>A. funestus</em></td>
<td>1 × 10¹⁰ conidia/m²</td>
<td>Male</td>
<td>19.54 ± 2.56</td>
<td>3.91 ± 0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>16.01 ± 2.82</td>
<td>3.91 ± 0.07</td>
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* ANOVA, pairwise comparison.
be worthwhile to test if shorter exposure times are sufficient for infecting high proportions of mosquitoes. We observed similar infection results with 6-hour exposure compared with 17 hours (data not shown). Nevertheless, we believe it is reasonable to assume that most mosquitoes that use the pots as a resting site will remain inside from at least dawn till dusk (J. D. Charlwood, personal communication). We would like to point out that, for field implementation, clay pots should not be sealed off with plastic, because this was only necessary for bioassay purposes. Furthermore, the rotating spray application apparatus, although very effective in applying spores in the laboratory, might not be the most efficient method for large-scale field application. In that respect, we propose that manual spray application, through hand-held spray guns, could be used to reach equally effective spore dosages inside the clay pots.

Significantly more mosquitoes were found resting in clay pots compared with a small terracotta pot and a PVC pipe. Fungus impregnation did not affect the attractiveness of clay pots, which is consistent with results from experiments where M. anisopliae conidia showed no repellent effect on An. gambiae mosquitoes. Although dry clay pots have already been shown to be attractive resting sites for both sexes of Anopheles mosquitoes under dryer environmental conditions, because it is generally found that mosquitoes prefer to rest in cool and humid places. In our experiments, there were no differences in attractiveness between the wet and the dry clay pot, although this could be due to the high humidity in the experimental room. In the field, under more arid conditions, we expect wet pots to be more favorable for both mosquitoes and conidial longevity.

For future implementation, the attractiveness of clay pots could be further enhanced through application of additional mosquito attractants inside the pots. Mosquitoes have been shown to be attracted to several host odors, including carbon dioxide (CO₂) and a suite of other kairomones. The possibility of adding attractants or compounds that induce landing responses by An. gambiae (e.g., 2-oxo-pentanoic acid) inside clay pots could potentially enhance the number of mosquitoes resting in the pots and increase the number that can be targeted with the fungus.

Because point source application requires only small areas to be impregnated, the use of clay pots could be an improvement on the cotton cloths that were tested as an application method in Tanzania or indoor spraying of large surfaces by reducing the amount of fungi required and thereby the overall costs. However, more knowledge on how many pots per household will reach the same effective coverage as these other potential delivery methods will have to be obtained from field studies. Nevertheless, pots have the advantage of including possibilities for outdoor application of fungi. Placing impregnated pots both inside and outside houses could increase the infection coverage and therefore the effectiveness of this delivery method. Furthermore, clay pots have been shown to be attractive resting sites for both sexes of anophelines, implying that high numbers of male mosquitoes can be targeted. This may result in a higher infection coverage of females through horizontal transmission of conidia during mating, which was shown under laboratory conditions. Moreover, we believe that, because of the dark inside environment with lower temperatures and higher humidity, conidial persistence will be higher in clay pots under field conditions compared with indoor spraying or application of conidia on cotton cloth material, especially when a layer of water can be maintained inside the pots. This needs to be confirmed by measurements in the field.

This study also showed significant impact of M. anisopliae on the survival of pyrethroid-resistant An. funestus mosquitoes, indicating high potential of fungal biocontrol agents...
against insecticide-resistant mosquitoes. These findings support the general idea that through complementing or replacing existing intervention tools, fungal vector control measures may overcome problems in areas with increasing levels of insecticide resistance and thereby may increase the overall impact of vector control measures against mosquito-borne diseases.

Because clay material is suitable for fungal application, and clay pots are highly attractive to *Anopheles* mosquitoes, substantial infection percentages and therefore a significant impact on malaria transmission could potentially be reached with this delivery system. Ultimately, field experiments are needed to determine the actual effectiveness of this novel vector control method. In the near future, field studies in Tanzania and Ghana will test the effectiveness of entomopathogenic fungi against malaria vectors under natural environmental conditions and will incorporate experiments with clay pots as delivery systems. Evidently, however, it can be stated that the greatest disease control benefit will be reached when such novel vector control measures based on entomopathogenic fungi are applied in integrated vector management strategies. In that sense, clay pots show great potential, because they have many features that will allow for the incorporation with existing control measures. For instance, outdoor use of fungus-impregnated pots can be easily combined with the use of ITNs or IRS. Considering that these insecticidal methods exert an excito-repellent effect, the provision of outdoor lethal resting sites may substantially improve the overall impact on mosquito populations.

In conclusion, we believe that biological control with entomopathogenic fungi through the use of innovative delivery systems, such as clay pots, shows great promise for complementing existing malaria vector control methods and increasing the impact of these interventions on malaria transmission.

Received December 17, 2007. Accepted for publication February 22, 2008.

Acknowledgments: The authors thank Dr. John Gimnig for early discussions on this line of research; Leo Koopman, André Gidding, and Frans van Aaggelen for help in rearing the experimental mosquitoes; Frank van Breukelen for providing *Metarhizium* anisopliae and Frans van Aggelen for help in rearing the experimental mosquitoes.

Financial support: This research was supported by the Adessium Foundation (Rotterdam, The Netherlands). MC is supported by the South African Research Chairs Initiative of the Department of Science and Technology and the National Research Foundation. BGJ is supported by the Dutch Scientific Organization through VIDI Grant 864.03.04.

Disclaimer: Any opinion, findings, and conclusions or recommendations expressed in this material are those of the authors, and the NRF and DST do not accept any liability with regard thereto.

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