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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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 *An. gambiae* s.s. mosquitoes was affected by conidia of the entomopathogenic fungus *M. anisopliae*. From two bioassays where mosquitoes (1) were allowed to escape from a cylinder containing dry conidia, and (2) 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 oil-treated conidia had no repelling effect ($p=0.205$). In addition to the already known positive effects of suspending 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 in field applications the conidia should be oil-formulated.

Keywords: Metarhizium anisopliae, malaria mosquito, avoidance, repellency, behaviour

Entomopathogenic fungi can be effective in biocontrol of harmful insects (Hajek & St. Leger 1994), and recently they have been suggested for the control of African malaria vectors (Schulte *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 (Scholte 2004). *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 & 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 & Staples 1996), ants (Kermarrec & 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 *An. gambiae*.

MATERIALS AND METHODS

The study was carried out at the Mbita Point Training and Research Centre of the International Centre of Insect Physiology and Ecology (ICIPE), in western Kenya. All experiments were carried out from 07.00-09.30 h 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, ICIPE) 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°C. 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 1997) by inoculating conidia on SDA agar and counting the proportion of germinating conidia after 20-24 h. 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 × 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. 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 g freshly harvested dry conidia of *M. anisopliae* (strain IC30) were placed. The cylinder was placed in a horizontal position during the experiment.

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 released in 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 × 30 × 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 was 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. In one compartment an open Petri dish was placed containing either 0.1, 0.5, 1.0 or 5.0 g of dry conidia of *M. anisopliae*. The Petri dish in the other compartment did not contain conidia. A transparent cylindrical container (18 cm length, 12 cm diameter) 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 h 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.

In addition, 25 trials were carried out where a sock, worn by a human for 48 h 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 g of dry conidia.

Bioassay 3

As in bioassay 2, two cages were used, one of which contained a 625 cm^2 piece of filter paper impregnated with conidia suspended in a 8% vegetable oil suspension (dissolved using a 0.05% Tween20 surfactant), resulting in a conidial density of 10^9 conidia/m^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 ($p<0.05$).

Bioassay 2

Mosquitoes entered the compartment without conidia more often than the compartment containing conidia (Table 2). This was significant for all 4 conidial

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	Number of replicates	Percentage escapees (± SEM)*
0 (control)	745	81	83	10.9 ± 0.08a
0.1	194	163	36	45.7 ± 0.28b
0.5	122	79	20	39.3 ± 0.18b
1.0	98	59	16	37.6 ± 0.25b
5.0	21	87	11	80.6 ± 0.19c

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

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	Chi^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

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*. 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 ($Chi^2=0.391$, df=2, p=0.532).

Bioassay 3

The oil-formulated conidia impregnated on paper 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 ($Chi^2=1.537$, 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 ($Chi^2=0.458$, df=1, p=0.499).

DISCUSSION

The study demonstrated that, under the two experimental set-ups of bioassay 1 and 2, dry conidia caused a moderate repelling effect on female *An. gambiae*, but conidia suspended in 8% adjuvant vegetable oil, impregnated on paper, did 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 (Scholte 2004), 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.

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.

Acknowledgements We thank Godfrey Sune for help in carrying out the experiments, and Romanus Odhiambo for assisting in constructing the laboratory. Hassan Ali Akelo, Jackton Arija and Peter Ong'ele are acknowledged for rearing *An. gambiae* mosquitoes, and Dr. Ulrike Fillinger for assistance during the experiments.

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