Biological control of *Anopheles stephensi* and *An. gambiae* larvae with the entomopathogenic fungus *Metarhizium anisopliae*
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Preface

In this report I present the findings of my study on the effect of the entomopathogenic fungus *Metarhizium anisopliae* on the survival of larval stages of the malaria vectors *Anopheles gambiae* and *An. stephensi*. This study has been performed for my Master Biology Thesis at the Laboratory of Entomology. The thesis, which took 9 months, was a major part of my Master Biology. During this period, I have gained many new experiences and knowledge in performing scientific (entomological) research.

The present study was part of a larger study that is evaluating the potential of fungal entomopathogens as larval control agents against *Anopheles* malaria mosquitoes.

I have chosen this subject because of my personal interest in entomology. I came in contact with Bart Knols and he introduced me to the various studies that were taking place on mosquitoes. I made an appointment with one of his PhD students, Tullu Bukhari and decided to join her studies. What I liked most about the subject is the direct societal relevance of this research area. Malaria is still a huge problem in many tropical and sub-tropical countries and there is an urgent demand for practical and sustainable alternatives for the currently used chemical insecticides on bednets or through indoor residual spraying.
Summary

Introduction:
Several studies have already been conducted on the use of fungi as biocontrol agents of mosquito larvae. In most of these studies, the high pathogenicity of some fungal strains against larval stages shows the potential of fungal biological larval control (Daoust and Roberts 1982; Daoust et al., 1982; Sandhu et al., 1993; Alves et al., 2002; Clark et al., 1968; Pinnock, 1972; Scholte et al., 2004).

In this study, I investigated the potential of the fungal entomopathogen *Metarhizium anisopliae* against the larval stages of malaria mosquitoes as a means to reduce malaria transmission. The main research questions addressed were:

1. What is the effect of different spore concentrations when applied to different larval stages of both *An. gambiae* and *An. stephensi* larvae?
2. What is the effect of exposing the larvae to the fungus for different periods?
3. Can late stage larvae, that have been infected with spores prior to pupation, carry over the infection into adult mosquitoes?

Methods:
The fungus that was used in this study is *Metarhizium anisopliae* Metschnikoff. This fungus was applied on larvae of the Western Asian malaria vector *Anopheles stephensi* Liston and Africa’s principle malaria vector *Anopheles gambiae* s.s. Giles.

In the first experiment, the effects of exposing larvae to different fungal spore concentrations were compared. Early and late larval stages were infected with dry conidia in concentrations of 0.2, 2, or 20 mg spores/441 cm$^2$ (the surface of the water within the trays). In the second experiment, different exposure times were compared. In this experiment, larvae were exposed to fungal spores for 1, 2 or 3 days before being transferred to untreated water. In the last experiment, on the passage of fungus from the larval stage to the adult stage, pupae exposed to different concentrations of fungal spores were placed in a cage to develop into adults. After 5 days the adults were killed and checked for the presence of fungus. All experiments were carried out for both *An. gambiae* and *An. stephensi* and were replicated thrice.

For the data analysis, SPSS 15.0 was used. Cumulative percentages mortality were calculated. Survival curves using Kaplan-Meier were made. Furthermore, a Probit analysis was conducted in order to calculate LT$_{50}$ values.

Results:
In the first experiment, it was observed that for both mosquito species, there was a significant difference between cumulative percentage mortality of the different doses of spores applied.
With an increase in fungal dose, there was an increase in mortality. The highest dose (20 mg/441 cm² resulted in mortality reaching 100%. *An. gambiae* showed higher mortalities than *An. stephensi*. Overall, there was no significant difference between survival curves of the two larval (young, L₁/L₂; old L₃/L₄) stages for both species.

In experiment 2, the results showed that for *An. stephensi* there was no significant difference between the different exposure times. However, for *An. gambiae*, percentage mortality after 1 day of exposure was significantly lower than exposure times of 2 and 3 days.

In experiment 3, none of the adult mosquitoes that emerged from the pupae of treated larvae, showed fungal infection.

**Discussion/conclusion:**
A dose of 20 mg fungus/441 cm² causes the highest mortality in both *An. gambiae* and *An. stephensi*. For *An. gambiae*, one day of exposure is sufficient to cause mortality similar to 2 or 3 days of exposure. On the other hand, for *An. stephensi*, at least 2 days of exposure are required to cause comparable mortality. Infection cannot be transferred from the late larval stage into adult mosquitoes.

**General conclusion:**
In the present study, *M. anisopliae* was shown to be very effective in infecting larvae of *An. gambiae* and *An. stephensi*, killing up to 100% of the larvae within 7 days after exposure to a dose of 20 mg fungal spores at a surface of 441 cm² which is 454 mg spores translated to a surface area of one square meter. This is an encouraging finding which may form the basis for further research on the possible applications and effectiveness of this method in the field.
1. Introduction

1.1 Malaria
Malaria is one of the world’s most prevalent tropical infectious diseases. About 400-500 million people are infected with malaria every year, which results in 1-3 million deaths (Snow et al., 2005; Lopez et al., 2006; Coleman and Hemingway, 2007). In many countries in Africa, Asia and Mid- and South America, malaria is a huge human health and economic problem (Collins and Paskewitz, 1995; Sachs and Malany, 2002).

Malaria is a disease caused by infection with protozoan parasites of the genus *Plasmodium*. There are 5 recognized human parasites that cause malaria; *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* (White, 2008) among which *P. falciparum* is the most harmful and causes the highest mortality. Malaria parasites are transmitted by mosquitoes of the genus *Anopheles*. The parasite undergoes a complex developmental cycle within the mosquito. This cycle may take 8-14 days depending on the parasite species and ambient temperature (Collins and Paskewitz, 1995). Almost 20% of the 500 described species of *Anopheline* mosquitoes can be vectors of malaria, but most of them are of little importance. Major tropical mosquito hosts are: *An. gambiae*, *An. arabiensis*, *An. funestus*, and *An. stephensi* (Collins and Paskewitz, 1995; Coleman and Hemingway, 2007).

1.2 Malaria vector control
One of the earliest (beginning of 19th century) methods used for the control of *Anopheles* was the treatment of habitats with kerosene or the arsenic compound Paris Green (Floore, 2006). In the 1940s, the discovery of dichloro-diphenyl-trichloroethane (DDT) provided a large-scale approach to malaria control which is still in practice today (Sadasivaiah et al., 2007). It is regarded as a cost-effective and safe insecticide for indoor residual spraying (Coleman and Hemingway, 2007; Hay et al., 2004). Pyrethroids are also being used to control malaria vectors. They are mostly applied on bednets or on walls and ceilings (WHO, 2005; Coleman and Hemingway, 2007). DDT and pyrethroids are the only insecticides that are recommended by the World Health Organisation (WHO) for use in indoor residual spraying (IRS) and on insecticide impregnated bednets (ITN) (Coleman and Hemingway, 2007).

However, insecticide application is not without problems. Some of the problems are environmental pollution and uncertainty about the toxicity to humans and non-target organisms. However, the biggest problem is that the regular use of insecticides leads to vector resistance which threatens the ability to control disease vectors in the long term (Collins and Paskewitz, 1995).

In short, there is an urgent need for practical and sustainable alternatives for malaria vector control.
1.3 Anopheles larvae

1.3.1 Biology of Anopheles larvae

The genus *Anopheles* belongs to the family Culicidae in the order Diptera. The Culicidae are subdivided into 2 subfamilies: Culicinae, and Anophelinae. The latter is further subdivided into 3 genera among which *Anopheles* is one (Beaty and Marquardt, 1996).

*Anopheles* has a similar developmental cycle as most other mosquitoes. Within 2-3 days after a blood meal, the female produces between 50 and 200 eggs. Eggs are laid directly on the water. Larvae hatch within 2-3 days and they feed by filter feeding and collecting microorganisms and detritus from the water surface or from the bottom (Beaty and Marquardt, 1996).

*Anopheles* larvae develop through 4 instars. They can be recognized by their characteristic position in the water. In contrast to the larvae of other genera, their body lays parallel to the surface of the water when they are at rest (Snodgrass, 1959). The larvae have a sclerotised head capsule, a thorax and 9 abdominal appendages. Dorsally at the end of the abdomen, they have a spiraculum which ends in a tube, the siphon (Fig. 1A). In the *Anopheles* larva, the siphon is shorter than in other mosquito larvae. The end of the siphon with the spiracles is exposed to the air for respiration. The spiraculum at the abdomen is surrounded by 2 lateral lobes. These are the spiracular lobes which clamp tightly together when the larva submerges (Snodgrass, 1959; Beaty and Marquardt, 1996) (Fig. 1B,C).

The fourth instar larvae develop into an aquatic and mobile pupal stage which takes 2-4 days to complete metamorphosis into an adult. Both pupae and adults show sexual dimorphism. Males have mouthparts that are adapted to feed from liquid sugar sources and possess long feather-like antennae that are used to detect females for mating. Females have mouthparts adapted for blood feeding (Beaty and Marquardt, 1996).

Figure 1. *Anopheles maculipennis* larva. (A) tracheal system, dorsal. Sp=spiracle, al=anal lobes, dT=dorsal tracheal trunk. (B) End of abdomen of *Anopheles quadrimaculatus*, spiracular apparatus open. VIII-X=abdominal segments. (C) End of abdomen of Anopheles quadrimaculatus, spiracular apparatus closed (after from Snodgrass, 1959).
1.3.2 Habitat of Anopheles larvae

Larvae of *Anopheles* occur in a wide range of habitats. Compared to other mosquito genera, most *Anopheles* species are found in relatively unpolluted water. Common habitats of anophelines include margins of ponds, lakes, or slow flowing streams, temporary waters produced by rain, river flooding, small, flooded depressions in the soil and shallow ditches etc. (Fig. 2) (Mutuku et al., 2006; Takken and Lindsay, 2003; Beaty and Marquardt, 1996). Many species, for example *An. stephensi*, are also found in aquatic habitats created by humans, for example flooded agricultural fields, irrigation ditches, tyre tracks, containers, tanks, roof gutters, construction sites, room coolers, leaking water meter boxes, and valve chambers (Batra et al., 2006; Beaty and Marquardt, 1996).

Figure 2. Habitat types of *An. gambiae* that were sampled in a rural village in western Kenya. (A) Aggregation of cattle hoof prints. (B) Natural rain pool. (C) Drainage channel. (D) Pool in streambed (after Mutuku et al., 2006).
1.4 Infecting adults or larvae to control mosquitoes as disease vectors?

Malaria vector control targeting the larval stages of mosquitoes was applied successfully against many anopheline species until the 1940’s, when DDT and indoor residual spraying (IRS) were introduced. These new methods for adult mosquito control appeared to have a very powerful impact on mosquito populations which caused a shift of focus in malaria control programmes from larvae to adults (Walker and Lynch, 2007).

Controlling adult mosquitoes has some advantages. Adult females make themselves vulnerable to control interventions when they seek human hosts. Both the use of IRS and ITN’s can be very effective controlling methods by killing adult female mosquitoes when they come to take a blood meal. Furthermore, shortening the life of the adult stage has a major impact on its vectorial capacity. Only small reductions in adult daily survival rate can result in significant reductions of malaria transmission (McDonald, 1957; Killeen et al., 2002a,b).

The vectorial capacity (C) is a mathematic model of the transmission of malaria (McDonalds, 1957; Garrett-Jones, 1964) (Box 1). It is defined as the number of potentially infective contacts made by the mosquito population per case per day. This formula can be used to illustrate that small changes in daily survival rate (p) affects vectorial capacity (C).

A disadvantage of targeting adult mosquitoes is their mobility. Due to their wide distribution in and around human settlements, treating adult mosquitoes is always limited to a small proportion of the mosquito population unless the control intervention is applied at population level (e.g. when more than 80% of the population sleeps under bed nets) (Killeen et al., 2002a).

Concerns about insecticide resistance, environmental impacts and rising costs of IRS stimulated a renewed interest in larval control of malaria vectors (Walker and Lynch, 2007). Studies on the control of larvae as malaria vectors show the potential of this way of control. Controlling larvae instead of adults has the advantage that larvae are immobile, concentrated and easily accessible (CIA: Concentrated, Immobile, Accessible) in their aquatic stages, and therefore easier to attack. Another advantage of controlling larvae is that they cannot escape

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<td>[ C = \frac{ma^ap^n}{\log p} ]</td>
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<tr>
<td>C = Vectorial capacity, the number of infective bites received daily by a single host</td>
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<tr>
<td>m = Mosquito density per human</td>
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<td>a = Average number of humans bitten per day by any one mosquito</td>
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<td>p = Probability that a given mosquito survives over a given day</td>
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<td>n = Time taken for extrinsic parasite cycle in the mosquito</td>
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Box 1. Vectorial capacity (McDonalds, 1957; Garrett-Jones, 1964).
from their breeding sites until the adult stage. Unlike adult mosquitoes, they cannot avoid control measures (Killeen et al., 2002a,b). Larval control is promising in urban areas because of the high density of humans relative to the number of breeding sites (Walker and Lynch, 2007). *An. gambiae* for example, breeds in small and temporary puddles, for example hoof prints. These habitats are vulnerable to control because they are easy to locate and often found in close proximity to human habitation. Another example is the restricted larval habitats of the urban vectors *An. stephensi* and *An. claviger* which breed in cisterns, containers and wells. These habitats are ideal targets for treatment as well (Walker and Lynch, 2007).

However, before larval control can be implemented, the majority of the larval breeding sites must be identified. If the number of these sites is extremely large, or if many sites are inaccessible or difficult to locate, larval control may not be feasible (Walker and Lynch, 2007).

Larval control may be particularly promising in integrated programmes of mosquito control. In regions where adult mosquitoes are exophilic or when they bite before people are in bed; IRS and ITN’s are less effective under these circumstances (Killeen et al., 2000; 2002a,b, Walker and Lynch, 2007). In these areas, larval control may be of high value to complement existing measures. Moreover, it has been shown that once adult populations have been reduced through high ITN coverage, the larval population is also reduced. In this setting, breeding sites appear to be fewer and more aggregated. This suggests that larval control measures could be especially effective when there is a high level of ITN coverage (Walker and Lynch, 2007).

Predictions have been made of the combined impacts of ITN’s and larval control. Using the model by Killeen *et al.* (2000), it can be predicted that a 50% reduction in vector emergence from breeding sites could contribute to an overall 15-25 fold reduction in entomological inoculation rate (EIR), even in highly endemic areas.

### 1.5 Larval control

There are numerous methods for larval control. The most direct method is habitat modification e.g. damping and ditching. However, it is not practical to use this method since many larval habitats are economically and domestically important (Floore, 2006, Mutuku *et al*., 2006). Local villagers seem to be reluctant to participate in strategies to eliminate habitats if they are sources of domestic water supply (Mutuku *et al*., 2006). In such cases, modification is not an option (Floore, 2006; Walker and Lynch, 2007).

Other methods are the application of chemical larvicides and insect growth regulators (IGR’s). Larvicides that are currently available include microbial insecticides, organophosphate (OP) larvicides (temephos, malathion), methoprene (IGR), surface oils and films (Floore, 2006). Methoprene is a hormone that prevents the normal maturation process of an insect. It
is particularly effective against the larval stages. Mostly, larvae treated with IGR’s develop normally until they reach the pupal stage at which adults fail to emerge. Utilization of IGR’s poses low threat to mammals, birds, invertebrates and fish (Siddal, 1976; Henrick, 2007).

Furthermore, oils and films are effective larvicides. Arosurf MSF® and Agnique MMF® are Monomolecular film that are commercially available. The oil forms a thin layer on the surface and reduces the tension of the water surface. It prevents the larvae from breathing, causing suffocation and a rapid death (Batra et al., 2006; Floore, 2006).

Biological control is often being favoured over the use of chemical control because of its host-specificity and minimal non-target effects (Floore, 2006). Due to the biosafety and the ease of handling and application procedures, many biological control methods are appropriate for community-based malaria prevention programmes (Walker and Lynch, 2007).

A good example of biological control is the use of microbial insecticides like *Bacillus thuringiensis var. israelensis (Bti)*. This is an aerobic bacterium that can be found in a wide range of habitats. The bacteria produce toxins upon sporulation with which they are able to kill their host. *Bti* was discovered in 1976, isolated from *Culex pipiens* (Floore, 2006; Lacey, 2007). The bacterium produces a crystalline inclusion which release proteins called δ-endotoxins in the midgut after ingestion by insects. These proteins interact with the larval midgut epithelium causing disruption of membranes eventually leading to death of the insect (Gill et al., 1992). Due to its efficacy against mosquito larvae and black flies, and because it has no adverse effect on non-target organisms, *Bti* was soon commercialised. The first commercial products that were based on *Bti* were Vectobac®, Bacitmos® and Tekknar®. These were produced in a variety of formulations, varying from powders to liquids and tablets. *Bti* is effective against important malaria vectors like *An. albimanus, An. sinensis, An. stephensi, An. gambiae, An. maculatus, An. maculipennis* and *An. sundaicus* (Walker and Lynch, 2007). The advantages of microbial larvicides such as *Bti* is their environmental safety, the high efficacy and cost-effectiveness, along with the ease of handling and the capability of being produced locally (Fillinger et al., 2003). A limitation of *Bti* is the ability of mosquitoes to develop resistance. Another disadvantage is that only the early larval stages are affected. Moreover, the amount of toxins that are produced can vary depending on the temperature and seasonality (Lacey, 2007).

Another well known microbial insecticide is *Bacillus sphaericus (Bs)*. Like *Bti*, *Bs* is a gram-positive, spore forming and aerobic organism as well. It is found in a variety of soil and aquatic habitats. This bacterium has also been commercialised (Lacey, 2007). *Bs* is more specific than *Bti*, being mainly active against mosquitoes. An advantage of *Bs over Bti* is the better residual activity (Lacey, 2007). A disadvantage of *Bs* is the development of resistance (Singh and Prakash, 2008).
Larvivorous fish have also been proven successful for larval control in some places and has been used for nearly 100 years (Walker and Lynch, 2007). There are many fish species that can be used to control mosquito larvae. *Gambusia affinis* (mosquito fish) has been most widely used, but also *Poecilia reticulata* (guppy) and killifishes such as *Apocheilus* spp., *Aphanias dispar*, *Nothobranchius* spp., *Oryzias* spp., *Oreochromis* spp., (tilapias) and *Ctenopharyngodon idella* (grass carp) are species that can be used. The fish species can be reared and released into mosquito breeding sites. In habitats that do not have year-round water, they have to be released annually. However, the efficacy is highly variable and significant negative impacts on native fauna has been reported (Wickramasinghe and Costa, 1986; Floore, 2006).

Other biological control organisms include mosquito larvae that feed on other larvae (*Toxorhynchites* spp.), microcrustaceans (*Macrocyclops longisetus*), nematodes (*Romanomermis* spp.), the aquatic plant *Azolla* and viruses (cytoplasmic polyhedrosis virus) (Floore, 2006; Walker and Lynch, 2007).

Eventually, fungi are successful and promising agents in the biological control of mosquito larvae. About 450 fungus species are known to have insect pathogen properties (Ferron, 1978; Scholte *et al*., 2004). Many fungus species have been evaluated for their larvicidal potential. The water mold *Lagenidium giganteum* (oomycetes: lagenididles) for example, is commercially delivered as mycelium for biological control of mosquito larvae. It has the advantage of being able to recycle in stagnant water (Vyas *et al*., 2007). This organism has been shown to cause significant mortalities in mosquito (*Culex* and *Anopheles*) populations in several studies. However, *L. giganteum* has been found to be effective for only one week in reducing the number of larvae after application. This implicates the need for frequent application. Moreover, it infects and kills only a portion of larval mosquitoes. This might not be sufficient to decrease the risk of malaria infection (Kumar and Hwang, 2006). The use of specific entomopathogenic fungi against malaria mosquitoes will be discussed into more detail in the next paragraph.

### 1.6 Control of malaria vectors using fungi

#### 1.6.1 Entomopathogenic fungi

Within the phylum *Deuteromycetes*, a morphological group of fungi exists which is called *Hyphomycetes*. One of the characteristics of this group is the filamentous morphology and the production of conidia which are formed on conidiophores arising from the substrate. They have a very wide host range, including several mosquito species (Scholte *et al*., 2004). Recently, studies have shown that the hyphomycetes *Beauveria* and *Metharizium* are pathogenic to adult mosquitoes (Scholte *et al*., 2003; Scholte *et al*., 2005, 2006; Blanford *et al*., 2005).
1.6.2 Mode of infection

Infection takes place through the external integument (Fig. 3). Conidia attach to the cuticle (including the legs and tarsi) and subsequently germinate and penetrate the mosquito’s cuticle. Upon entering the haemocoel, the mycelium continues to grow and hyphal bodies are formed; the blastospores. The insect dies due to a combination of fungal toxins, obstruction of blood circulation, nutrient depletion and invasion of the organs. After death of the host and when conditions are permissive, hyphae become visible on the outside and conidia are produced. In case of infection with *Beauveria* and *Metarhizium*, the sporulating fungus gives the carcass a white and green appearance, respectively. The spores are dispersed by wind or water ready to infect the same, or another host species (Clark *et al.*, 1968; Ferron, 1978; Scholte *et al.*, 2004).

Due to the different morphology and habitat of *Anopheles* larvae compared to adults, infection is also different. It is thought that the larvae are infected with floating conidia when breaking the water surface with the perispiracular valves of the siphon while breathing (Clark *et al.*, 1968). The conidia germinate in the spiracula and the fungus will kill the host. It is, however, not completely understood how death occurs. It could be either by the production of toxins or by obstruction of the breathing mechanism (Clark *et al.*, 1968; Miranpuri and Khachatourians, 1991). However, death occurs before the fungus has reached the haemocoel and little hyphal formation has taken place by that time (Clark *et al.*, 1968; Daoust *et al.*, 1982).

1.6.3 Beauveria

*Beauveria* is a fungus with a global distribution and is an important natural pathogen of insects (Bidochka *et al.*, 1998). Many important insect pests are included in its host range and, as a result, much research has been conducted on the use of *Beauveria* for biological control of insects (Bidochka *et al.*, 1998; Rhener, 2005). The genus of *Beauveria* is known to infect more than 700 insect species, in both tropical and temperate areas around the world. However, natural occurrence on mosquitoes has been reported only four times (Rhener, 2005; Zimmermann, 2007a; Scholte *et al.*, 2004).

The fungus proved to be pathogenic to various mosquito species of the genera *Culex*, *Aedes*, *Ochlerotatus* and *Anopheles* (Clark *et al.*, 1968; Rhener, 2005).

A study of Blanford *et al.* (2005) showed that exposing *An. stephensi* mosquitoes to
surfaces treated with spores of *B. bassiana* resulted in >90% mortality.

In a study of Clark *et al.* (1968), it was shown that exposure to conidia of *Beauveria* even cause 100% mortality in adult mosquitoes compared to less than 50% in controls within five days after exposure (Clark *et al.*, 1968).

### 1.6.4 *Metarhizium*

For about 130 years, *M. anisopliae* has been used for biocontrol of insects (Zimmerman, 2007b). Like *Beauveria*, *Metarhizium* also has a global distribution and it belongs to the most common entomopathogenic fungi. *Metarhizium* is soil-borne and infects mainly soil-dwelling insects (Scholte *et al.*, 2004; Scholte *et al.*, 2006). *Metarhizium* consists of four varieties from which *M. anisopliae var anisopliae* is the best known. It has a large host range, including 5-7 orders with a total of more than 200 species, many of which are agricultural and forest insect pests such as spittlebugs, sugar cane borers, termites, scarab groups and grasshoppers (Bidochka and Small, 2005; Zimmermann, 2007b). Mosquitoes are not a natural host for *M. anisopliae*, but the fungus has proven to be pathogenic to several mosquito species, including anophelines (Scholte *et al.*, 2004; Kanzok and Jacobs-Lorena, 2006; Scholte *et al.*, 2007b).

Several studies have shown that *M. anisopliae* can be used as a biological control agent against adults of *Anopheles* mosquitoes. In a study by Scholte *et al.* (2003), it was shown that adults of *An. gambiae* are susceptible to *M. anisopliae*. After infection with an appropriate dose of conidia, the lifespan was significantly reduced compared to the control groups. Another study by Scholte *et al.* (2005) was a field study in which it was shown that the application of *M. anisopliae* conidia on black cotton cloths that were suspended from the ceiling inside local dwellings was able to infect and kill a significant proportion (ca. 23 %) of *An. gambiae* mosquitoes. In a study of Blanford *et al.* (2005), a mortality of 80% was achieved after infection of *An. stephensi* by *M. anisopliae*.

Scholte *et al.* (2006) also showed that fungal infection causes a significant reduction in bloodfeeding and lifetime fecundity. Both bloodfeeding and fecundity reduce the likelihood of pathogen transmission and thus are one of the most important factors for controlling the transmission of malaria (Knols and Thomas, 2006; Scholte *et al.*, 2006).

### 1.7 Using fungi as biological control agents against mosquito larvae

#### 1.7.1 *Beauveria* against mosquito larvae

Roubaud and Toumanoff (1930) were among the first scientist to experimentally infect mosquito larvae with a fungal pathogen. They infected larvae of *Cx. pipiens* and *An. maculipennis* with *B. bassiana* (Clark *et al.*, 1968). In their study, they concluded that *B. bassiana* would be of no value as a microbial control agent. The reason for this was that some individuals survived, even under conditions of infection. In the following decennia, only
a few further studies on *Beauveria* as a fungal pathogen for larval stages were conducted.

In a study by Clark *et al.*, (1968) *B. bassiana* was used in an experiment with *Cx. pipiens*, *Cx. tarsalis*, *An. sierrensis* and *Ae. nigromaculis*. Conidia were either submerged or applied as a dust to the surface of the water. The results showed that *B. bassiana* conidia that floated on the surface of the water killed the larvae more effectively than the same number of conidia being submerged in the water. The percentage mortality in the case of floating conidia was 88.3% whereas the mortality when conidia were submerged was 58.0%. The exact cause of death was not determined, but it was hypothesised that death was either caused by a fungal toxin or the mechanical blockage of the larval tracheal trunk that suffocated the larvae (Fig. 4). The conidia of *B. bassiana* showed to be effective in killing larvae of *Cx. pipiens*, *Cx. tarsalis* and *An. albimanus*. However, the authors concluded that *B. bassiana* when applied as conidial dust, is not a practical agent to control mosquito larvae. Doses required for significant mortality levels are high and the lack of residual effect would make the application economically prohibitive (Clark *et al.*, 1968).

Furthermore, Sandhu *et al.*, (1993) have been studying the effect of *B. bassiana* on *Cx. tritaeniorhynchus* and *Ae. aegypti*. In this study, it was shown that the fungus resulted in 82%-90% mortality.

Figure 4. (A) apex of the siphon of a fourth instar *Cx. pipiens* showing invasion of fungal hyphes after 48 hours of exposure to conidia of *B. bassiana*. Germ tubes have penetrated the perispiracular lobes of the siphon. (B) Melanized apex of siphon of fourth instar *C. pipiens* 72 hours after exposure to conidia of *B. bassiana*. Hyphae have grown into the siphon (after Clark *et al.*, 1968).
1.7.2 Metarhizium against mosquito larvae

Only a few studies have been done with *Metarhizium* as a fungal pathogen for mosquito larvae. Daoust and Roberts (1982) have been studying the virulence of different strains of *Metarhizium* to *Cx. pipiens*. They reported that isolates belonging to *M. anisopliae var. major* were generally avirulent. Strains of *M. anisopliae var. anisopliae* were highly virulent to *Cx. pipiens*, *Ae. aegypti* and *An. stephensi* larvae causing up to a 100% mortality.

Another study by Daoust et al. (1982) focussed on the effect of formulation on the virulence of *M. anisopliae* conidia against mosquito larvae. They reported that most granular and dust formulations were significantly less virulent than unformulated conidia after storage for 8-10 months at 4°C. In this study, it was also shown that all formulations as well as the unformulated dry conidia were significantly less virulent in the submerged application compared to the surface method. This supports the idea that the larvae are mainly affected via the siphon.

Sandhu et al. (1993) have been studying the effect of *M. anisopliae* on *Cx. tritaeniorhynchus* and *Ae. aegypti*. It was shown that the fungus caused 84-96% mortality.

In a study of Alves et al. (2002), various isolates of *M. anisopliae* were tested against *C. quinquefasciatus*. It was found that one isolate was effective, killing 93% applied as a suspension and 75% applied as dry spores. This difference was, however, not significant. Applications of suspended spores was regarded as not practical because of the rapid loss of spores caused by sinking and loss of viability.
1.8 Present study

It is very important to keep searching and developing alternative agents and methods for vector control. Entomopathogenic fungi may contribute to an extension of the limited availability of effective vector control tools. To gain maximum impact on mosquito populations, new agents or methods should be applied in integrated control strategies (Scholte et al., 2004).

In this study, we investigated the potential of spores of a fungal entomopathogen to infect and kill larval stages of malaria mosquitoes. We used the fungus *M. anisopliae* (isolate ICIPE-30) and it was applied on larvae of the Asian malaria vector *An. stephensi* Liston and Africa’s principle malaria vector *An. gambiae* Giles.

The main research questions were:

1. What is the effect of different spore concentrations when applied to different larval stages of both *An. gambiae* and *An. stephensi* larvae?
   - Is there a difference between total percentage mortality of various doses?
   - Is there a difference in the LT$_{50}$ values for different doses?
   - Is there a difference in mortality between the species?
   - Is there a difference in mortality between the early (L$_1$/L$_2$) and late (L$_3$/L$_4$) larval stages?
   - Is there a difference in percentage fungal growth from the incubated dead bodies for different doses?
2. What is the effect of exposing the larvae to the fungus for different periods?
3. Can late stage larvae, that are infected with spores prior to pupation, carry over the infection into adult mosquitoes?
2. Methods

2.1 The fungus
Metarhizium anisopliae var. anisopliae, isolate ICIPE-30 was used in all experiments. The spores were stored in the fridge at 4C.

2.2 Killing of the fungus
Prior to the experiments, we tried to obtain non-virulent spores of M. anisopliae as a control for exposing the larvae to different concentrations of the living conidia, in order to examine whether death occurs due to the release of toxins or due to the physical blockage of the digestive tract and the breathing tube. 20 mg of dry spores was spread out in a glass petri dish and placed in an oven at 50°C with 100% humidity for an hour. After heating, the spores were plated on an agar plate and incubated at 28°C to check for germination. We found that after 4 days, the heated spores had germinated. The same procedure was repeated for 2 hours at 50°C, 60°C, 80°C and 100°C. It was found that even after heating for 2 hours at 100°C at 100% relative humidity, many spores survived and germinated (Fig. 5). Our findings are in conflict with results of other authors. Zimmerman (1982) for example found that after exposing dry conidia for 30 minutes to 50°C at 100% humidity, all spores were killed.

Figure 5. Plates containing fungal spores of M. anisopliae that were heated at different temperatures. (A) Spores were heated for 2 hours at 50°C. (B) Spores were heated for 2 hours at 60°C. (C) Spores were heated for 2 hours at 80°C. (D) Spores were heated for 2 hours at 100°C. (Picture: A. Middelman)
Subsequently, we tried autoclaving at 121°C for half an hour, but the spores became distorted in shape and colour which was not suitable for our experiments. Eventually, we used a solution of 10% formalin. The spores were suspended with the 10% formalin and incubated for 24 hours at 28°C. The suspension was poured into a glass petri dish and dried in an evaporation chamber. The dried spores were scraped off and crumbled to obtain the powder form again. Some of the spores were plated and after incubation, no germination was observed. So, formalin was found to be effective in killing spores of *M. anisopliae* (Fig. 6).

![Figure 6. Plate containing fungal spores of *M. anisopliae* that were killed in 10% formalin. There was no germination. (Picture: A. Middelman)](image)

### 2.3 Mosquito larvae

For the experiments, *An. gambiae* s.s. Giles and *An. stephensi* Liston were used. *An. gambiae* (Suakoko strain) was obtained from a colony originating from Liberia (courtesy Prof. M. Coluzzi). *An. stephensi* mosquitoes were obtained from MR4 (strain STE 2, MRA no. 128, Atlanta, USA).

Adult mosquitoes were offered a 6% glucose solution, absorbed into laboratory filter paper, placed in a glass vial. To obtain eggs, females were blood fed on the forearm of a volunteer. After blood feeding, a cup of water with a funnel shaped filter paper was introduced into the holding cage for oviposition. Eggs were collected 2-3 days after blood feeding. They were removed from the cups and placed in a tray filled with water with a filter paper attached to the walls of the tray to keep the eggs moist. After another 2 days the eggs hatched. The climatic conditions in which the larvae were reared were 27±1°C and 80±5% RH. After hatching, the
larvae were fed with yeast for the first two days followed by Tetramin® for the rest of the larval stage.

2.4 Experimental conditions
All experiments were carried out in trays that were 4 cm high with an upper edge of 24 cm by 24 cm, filled with 1 litre of tap water. The water was filled a day before the experiment to remove the chlorine residues which might negatively affect health of the larvae. The surface area exposed by 1 litre of water in the trays was 441 cm². Between each replica, the trays were cleaned with alcohol to remove all fungal spores.

2.5 Experiments
Prior to the experiments, a pilot experiment was performed to check if *M. anisopliae* spores were more effective if dusted on the water surface or applied in the form of a suspension. In this experiment, a total of 600 larvae were exposed to *M. anisopliae*. 50 L₁/L₂ larvae and 50 L₃/L₄ larvae of *An. gambiae* were exposed to the 0.2, 2 and 20 mg fungus applied in a suspension; another 50 L₁/L₂ larvae and 50 L₃/L₄ larvae were exposed to different doses of dry conidia. Suspensions of specific amounts (0.2, 2 and 20 mg) of spores were made with distilled water and few drops of surfactant (0.1% Tween 80) followed by mixing by a vortex. These suspensions were poured into the larval trays. There was a control for each larval stage and method of application. Using the suspension method, the spores were equally distributed in the water. However, after the first day, the spores moved to the bottom of the trays. Using the dry spores, we aimed for an equal distribution of the spores over the water surface. However in this method, after spreading the spores carefully over the surface, the majority of spores clustered together, due to their hydrophobic nature, so an equal distribution over the surface was not possible. Eventually we chose the second option (dry spore application) for the remainder of the experiments.

2.5.1. Experiment 1: The effect of different spore concentrations on different larval stages of *An. gambiae* and *An. stephensi* larvae
In this experiment, a total of 4800 larvae was used. To determine whether death after infection with the fungal spores occurs due to the release of fungal toxins within the body or due to mechanical obstruction of the breathing mechanism or digestive system, the effect of exposing the larvae to different doses of living fungus was compared with the effect of exposing the larvae to the same doses of dead fungus. On day 0, both larval stages of both *An. gambiae* and *An. stephensi* were exposed to varying spore concentrations: 0.2, 2 and 20 mg living and dead spores/441 cm². For both the treatments, species and larval stages there was a control without fungus. The fungus was applied by dusting the dry spores over the surface of the water. There were 50 larvae per tray and the experiment was replicated thrice.
Table 1 gives a schematic overview of the number of larvae that were exposed to fungal spores in one replica for one mosquito species.

Table 1. A schematic overview of the number of larvae that were treated in one replica, for one mosquito species.

<table>
<thead>
<tr>
<th></th>
<th>Living fungus</th>
<th>Dead fungus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2 mg</td>
<td>2 mg</td>
</tr>
<tr>
<td>L1L2</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>L3L4</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>total</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

During the subsequent days (up to day 7), the dead larvae were counted and removed from each tray. The corpses were washed in 70% ethanol and plated in a drop of distilled water on a object glass in a sealed petri dish. After incubation at 28°C for 5-7 days, they were checked for germination under both a light and stereo microscope. The number of larvae on which mycelial growth was visible, was recorded.

2.5.3 Experiment 2: The effect of exposing the larvae to the fungus for different time durations
The main purpose of this experiment was to examine whether there is a difference in mortality between different exposure times. We hypothesized that no significant difference in mortality would mean that one day exposure to the fungus would be enough for effective control. This information is important for field application were the persistence of fungus is a critical determinant of application frequency.

For this experiment, only L3/L4 larvae were used. A spore concentration of 20 mg spores/441cm² was used. The larvae were exposed to the fungus for 1, 2, or 3 days before being transferred to clean water. For each treatment there was a control. There were 50 larvae per tray and the experiment was carried out for both mosquito species. The experiment was replicated thrice.

2.5.3 Experiment 3: The ability of carrying over the infection from the late larval stage into adult mosquitoes
All pupae from the second replica of the first experiment were used. Every day, the pupated larvae were removed from the trays and placed in a cage. A separate cage was used for each fungal dose to which the larvae had been exposed and a control for the pupated larvae that had not been exposed to any fungus. A total of 14 and 20 An. gambiae pupae was used
which had been exposed as larvae to respectively 0.2 and 2 mg of fungus together with 21 control pupae that had not been exposed to fungus. A total of 7 and 18 An. stephensi pupae was used which had been exposed to these fungal doses together with 26 control pupae that had not been exposed to any fungus. In both cases there were no pupae for the 20 mg treatment because all the larvae had died. The adults that emerged from the pupae were kept alive until 5 days after the day that the last pupae from experiment 1 was included in present experiment. Then the mosquitoes were killed by removing the sugar water. After death they were incubated at 28°C on a wet filter paper placed in a petri dish and checked for mycosis.

2.6 Data analysis
For data analysis, SPSS 15.0 and Excel 2003 were used. For each replicate, species and larval stage, a dataset was made in excel. With this file, the data was explored, mortality percentages were calculated and graphs were made. Another, large dataset was made in SPSS to conduct the rest of the statistical analysis.

2.6.1 Probit analysis
Data from bioassay experiments such as the proportions of insects killed by different concentrations of an insecticide are one of the many biological responses that often form a sigmoid curve. These sigmoid relationships can be linearised using Probit transformation (Throne et al., 1995). The slope and the intercept of the regression line of the Probit-transformed data are calculated and the goodness-of-fit indicates by the chi-square. For many systems, Probit gives closely fitting results. In this study, LT$_{50}$ values were calculated using Probit analysis in SPSS (Finney, 1971).

The Probit model is defined as:

$$\Pr(y=1|x) = \Phi(xb)$$

$\Phi$ is the standard cumulative normal probability distribution and $xb$ is called the Probit score or index (Finney, 1971).

2.6.2 Kaplan-Meier
Survival curves were made using Kaplan-Meier survival analysis. Kaplan-Meier was used for analysing survival data. An important advantage of the Kaplan-Meier curve is that the method takes censored data into account. The censored data are losses from the sample before the final outcome is observed (Chan, 2004).

Significant differences between different curves were calculated using the pairwise comparison function in the Kaplan-Meier analysis in SPSS 15.0.
3. Results

3.1 The effect of different spore concentrations on different larval stages of *An. gambiae* and *An. stephensi* larvae

3.1.1 Mortality after 7 days

The purpose of this experiment was to investigate the effect of different fungal doses on larval mortality. Percentage mortality after 7 days of exposure was calculated for the different treatments, for the different larval stages and for both species. When comparing these percentages, a clear dose effect was observed. In general, for both *An. gambiae* and *An. stephensi* and for both larval stages, an increase in amount of fungus caused an increase in mortality. The results of the percentage mortality after 7 days are depicted in Figure 7. After 7 days of exposure to 20 mg fungal spores per 411 cm², 100% mortality was obtained in the *L₁/L₂* larvae of *An. gambiae*. The mortality after 7 days of exposure to the same dose of fungus of *L₃/L₄* *An. gambiae* larvae was 95.3% and the mortality of *L₁/L₂* and *L₃/L₄* *An. stephensi* larvae after exposure to the same dose was 94.6% and 91.3%, respectively. In contrast to the highest dose, the percentages mortality for the doses of 0.2 mg/441 cm² ranged from 35.3%-57.3% and the mortality of the 2 mg dose was intermediate. All mortality data are shown in Table 2.

3.1.2 Survival

To obtain a more detailed overview of the mortality of the larvae over the 7 days of the experiment, survival curves were made. Survival curves of both larval stages and both species showed the same dose effect over the 7 days of exposure. In general, the higher the dose of applied fungus, the higher the mortality rate (Fig. 8 and 9).
Figure 8. Percentage cumulative survival (±SE) of young (A) and old (B) *An. gambiae* larvae after exposure to different doses of fungus at day 0.
Figure 9. Percentage cumulative survival (±SE) of young (A) and old (B) An. stephensi larvae after exposure to the different doses of fungus at day 0.
LT\textsubscript{50} values were calculated (Table 2). In line with previous results, for both species and both larval stages, LT\textsubscript{50} values decrease with an increasing fungal dose.

**Table 2.** Mean percentages mortality (±SE) after 7 days of exposure to the fungus and median lethal time (days) to death (LT\textsubscript{50}) of the early and late larval stages of *An. gambiae* and *An. stephensi*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Larval stage</th>
<th>Fungal concentration</th>
<th>Treatment living fungus</th>
<th>Treatment dead fungus</th>
<th>LT\textsubscript{50} living fungus</th>
<th>LT\textsubscript{50} dead fungus</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>An. gambiae</em></td>
<td>L\textsubscript{1}/L\textsubscript{2}</td>
<td>control</td>
<td>39.60 ± 9.33</td>
<td>34.00 ± 19.63</td>
<td>9.558</td>
<td>8.119</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2</td>
<td>57.33 ± 16.34</td>
<td>48.00 ± 27.71</td>
<td>5.789</td>
<td>6.480</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>83.33 ± 9.33</td>
<td>49.33 ± 28.48</td>
<td>3.646</td>
<td>6.112</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>100.0 ± 0</td>
<td>40.67 ± 23.48</td>
<td>1.619</td>
<td>7.634</td>
</tr>
<tr>
<td></td>
<td>L\textsubscript{3}/L\textsubscript{4}</td>
<td>control</td>
<td>34.44 ± 5.70</td>
<td>32.67 ± 18.86</td>
<td>11.959</td>
<td>8.970</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2</td>
<td>54.67 ± 15.59</td>
<td>48.67 ± 28.10</td>
<td>6.602</td>
<td>6.525</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>67.33 ± 14.89</td>
<td>30.67 ± 17.71</td>
<td>5.083</td>
<td>9.745</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>95.33 ± 1.33</td>
<td>36.37 ± 21.17</td>
<td>2.008</td>
<td>8.232</td>
</tr>
<tr>
<td><em>An. stephensi</em></td>
<td>L\textsubscript{1}/L\textsubscript{2}</td>
<td>control</td>
<td>23.81 ± 22.34</td>
<td>19.33 ± 11.16</td>
<td>15.599</td>
<td>9.159</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2</td>
<td>58.67 ± 27.72</td>
<td>30.00 ± 17.32</td>
<td>4.399</td>
<td>8.736</td>
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<tr>
<td></td>
<td></td>
<td>2</td>
<td>73.33 ± 13.78</td>
<td>18.67 ± 10.78</td>
<td>4.485</td>
<td>9.549</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>94.67 ± 3.33</td>
<td>19.33 ± 11.16</td>
<td>2.841</td>
<td>12.697</td>
</tr>
<tr>
<td></td>
<td>L\textsubscript{3}/L\textsubscript{4}</td>
<td>control</td>
<td>20.92 ± 11.57</td>
<td>16.00 ± 9.24</td>
<td>21.838</td>
<td>15.639</td>
</tr>
<tr>
<td></td>
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<td>0.2</td>
<td>35.33 ± 25.33</td>
<td>20.00 ± 11.54</td>
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<tr>
<td></td>
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<td>2</td>
<td>44.00 ± 11.02</td>
<td>26.00 ± 15.01</td>
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<td>91.33 ± 6.77</td>
<td>17.33 ± 10.01</td>
<td>2.982</td>
<td>13.391</td>
</tr>
</tbody>
</table>

(The adults that emerged from the pupae were considered alive resulting in an increase in LT\textsubscript{50} values.)

Differences were calculated between the survival curves for the different doses. There was a significant difference between all doses for both species, except for the difference between 0.2 and 2 mg fungus applied to both larval stages of *An. stephensi* (Kaplan-Meier pairwise comparison; p=0.069 and p=0.149) (for all p-values, see Appendix 2).

Furthermore, survival curves of both species were compared. There was a significant difference between the curves of *An. gambiae* and *An. stephensi*. For both larval stages, *An. gambiae* showed a higher overall mortality (pairwise comparison, Kaplan-Meier; for all fungal concentrations p<0.001). The differences in survival curves between the different species are shown in Figure 10.
Figure 10. Survival of young (A) and old (B) larvae of An. gambiae and An. stephensi after exposure to the different doses of fungus at day 0.
When comparing the effect of the different doses of fungus on the different larval stages, it was found that for both species and for all concentrations, in general there were no significant differences between the different larval stages. Exceptions were the 2 mg dose for *An. gambiae* and the 20 mg dose for *An. stephensi*, in which there was a higher mortality in the first and second instar larvae compared to the third and fourth instar larvae (for all p-values, see Appendix 3).

In Figure 11, the differences in mortality between the effects of living and dead spores in the different doses are compared. In general, there was a significant difference between the living and the dead fungus for each particular dose (pairwise comparison, Kaplan-Meier; p<0.001) except for the 0.2 dose in both larval stages of *An. gambiae* (p=0.832 and p=0.360) (for all p-values, see Appendix 4).

Furthermore, there appears to be a general equal mortality for the different doses of the dead fungus. Most of the curves of the different doses of dead fungus are not significant different, except for 0.2 and 20 mg for the L_1/L_2 stages of *An. gambiae*, the 0.2 and 2 mg for the L_3/L_4 stage of *An. gambiae* and 0.2 and 2 mg for the L_1/L_2 stage of *An. stephensi* (for p-values, see Appendix 5).

Moreover, there was no significant difference between the 3 doses of dead fungus and the controls with no fungus (for all doses: p<0.001).
3.1.3 Fungal growth from the dead larvae

There was substantial variation in the percentages of plated and incubated dead larvae that showed fungal growth (Fig. 12 and Appendix 6) without an apparent correlation between fungal dose and sporulation.

![Figure 12](image)

Figure 12. Percentages of larvae that showed fungal growth after plating and incubation. (A) *An. gambiae*, (B) *An. stephensi*.
3.2 The effect of different exposure times on mortality

The main purpose of this experiment was to examine whether there is a difference in mortality between different exposure times. L₃/L₄ larvae were exposed to the fungus for 1 (treatment 1), 2 (treatment 2) or 3 days (treatment 3) before being transferred to clean water. Mortality after 6 days was calculated for each treatment. For treatment 1, the mortality after 6 days was 69.5% for An. gambiae and 22.6% for An. stephensi. For treatment 2, the mortality was respectively 49.2% and 64.8% and for treatment 3 these percentages were 49.3% and 45.7%. The results are shown in Figure 13 and Table 3.

Moreover, survival curves were made which are shown in Figure 14. For An. gambiae and An. stephensi, there were no significant differences between the treatments (for p-values, see Appendix 7). For An. stephensi on the other hand, mortality in treatment 1 was significantly lower compared to treatments 2 and 3 (p<0.001) (Appendix 7). Furthermore, there was a significant difference between the controls and the treatments (for An. gambiae for all treatments p<0.001; for An. stephensi treatment 1: p=0.026 and treatments 2 and 3: p<0.001). Eventually the species were compared and there appeared to be a significant difference between the species, mainly for treatment 1 (treatment 1: p<0.001; treatment 2: p=0.049 and treatment 3: p=0.575). In treatment 1, mortality in An. gambiae was higher, whereas in treatments 2 and 3, mortality in An. stephensi was higher.
Table 3. Mean percentage mortality at days 6 after 1, 2 and 3 days of exposure to the 20 mg of fungus.

<table>
<thead>
<tr>
<th>Species</th>
<th>Mean percentage mortality</th>
<th>LT-50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>treatment</td>
<td>treatment</td>
</tr>
<tr>
<td>An. gambiae</td>
<td>1</td>
<td>69.5 ± 2.73</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>49.2 ± 12.42</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>49.3 ± 12.18</td>
</tr>
<tr>
<td>An. stephensi</td>
<td>1</td>
<td>22.6 ± 8.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>64.8 ± 11.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>45.7 ± 4.3</td>
</tr>
</tbody>
</table>
Figure 14. Survival of *An. gambiae* and *An. stephensi* larvae after exposure to 20 mg of fungus for different time periods. (A) survival curves for *An. gambiae*, (B) survival curves for *An. stephensi*. 
3.3 Carrying over the infection from the late larval stage into adult mosquitoes.
For this experiment, all pupae from the second replicate of the first experiment were used. 14 and 20 An. gambiae pupae were used that had been exposed as larvae to respectively 0.2 and 2 mg of fungus together with 21 control pupae that had not been exposed to any fungus. A total of 7 and 18 An. stephensi pupae was used that had been exposed to these fungal doses together with 26 control pupae that had not been exposed to any fungus. After death of the mosquitoes that had emerged from the pupae, they were plated incubated at 28 C. None of the mosquitoes showed any fungal growth.
4. Discussion

In this discussion, first the results of present study will be interpreted and compared with results of other studies. Then, drawbacks of present study will be mentioned. Eventually, there will be a section about implementing this method of larval control of in the field.

4.1 Killing of the spores

4.1.1 Heat resistance

Prior to the experiments, we tried to obtain non-virulent spores of *M. anisopliae* as a control for exposing the larvae to different concentrations of the living spores. It was found that after heating for 2 hours at 100°C at 100% humidity, many spores survived and germinated (Fig. 5). Our findings are in conflict with results of some other authors. Zimmermann for example found that the thermal death point for 30 minutes exposure was between 50°C and 55°C at 100% RH. At 76% RH, the thermal death point increased to 70°C-75°C and at 33% RH to 80°C-85°C (Fig. 15).

Figure 15. The effect of high temperature and different moisture conditions on the germination of conidia of *M. anisopliae*. After incubation for 24 hours, spores were plated on agar medium (after Zimmerman, 2004).
Differences between findings of different laboratories may be due to different methodologies. The volume of the spores exposed to heat and the isolate of the fungus have been found to result in highly variable outcomes (Rangel et al., 2004). We used an amount of about 100 mg of spores whereas Zimmermann (1982) reported to use a small amount, although the exact volume was not mentioned. The possible difference in volume of spores might have resulted in different outcomes. Moreover, Zimmermann (1982) heated the spores in a tube in a bath of warm water whereas we used an oven.

It is well known that heat resistance of fungal spores is closely related to moisture conditions (Zimmermann, 2004; Rangel et al., 2005). When heat is accompanied by high humidity, protein denaturation and membrane disorganisation are the major causes of death (Rangel et al., 2004). To reach 100% humidity, we placed the fungal spores in a petri dish placed in a closed container filled with water. We assumed the humidity to be 100%, however, the exact moisture content within the container was not measured.

4.1.2 Formalin
Formalin is it known to be capable of destroying all forms of microbial life even when greatly diluted (Fahmy et al.,1983). Formalin is well known for its desinfective capacities in general. Very few studies however have been performed on the exposure time and dilution of aqueous formalin on the viability of fungi. In a study of Fahmy et al. (1983), it was found that even after exposure to 10% formalin for 5 minutes, the formalin destroyed all fungal organisms in infected animal tissue.

In present study, the 10% formalin method proved to be an easy and excellent way in effectively killing fungal spores as well. The method was found to be simple to apply and it can be an excellent way of obtaining non-virulent fungal spores in many studies on the effects of entomopathogenic fungi on several insect species.

4.2 Mode of infection

4.2.1 Primary infection site
It has been found that the two main sites of infection of B. bassiana are the head and the anal region. However, fungal growth has been found to be far more abundant in the perispiracular lobes than in the mouth regions (Miranpuri and Khachoatourians, 1991).

In present study, observations are in line with these findings. While observing the infected larvae under the microscope, the siphon seemed to be the main infection site (Fig. 16 B and C; and Fig. A and B in Appendix 1), although the head seemed to be a source of infection in many cases as well. The primary infection site was determined by the developmental stage of the fungal growth at different sites of the larval corpses. It was assumed that the regions where fungal growth was most developed would be the primary infection sites (Miranpuri and Khachoatourians, 1991).
4.2.2 Cause of death

For *B. bassiana*, it has been found that applying dry spores on the surface of the water kills larvae more effectively than the same number of conidia submerged in the water (Clark *et al*., 1968; Daoust *et al*., 1982). It is known that after infection, conidia remain fixed at the perispiracular lobes and are prevented from being washed off by the folding of the perispiracular lobes as the larvae submerge (Clark *et al*., 1968). Within 2 to 3 days after application, germ tubes penetrate the perispiracular lobes (Fig. 3). Hyphae grow within the siphon and by the 4th day, the siphon appears to be filled with mycelium (Clark *et al*., 1968). The exact cause of death has not yet been proven. It has, however, been shown that relatively little mechanical damage occurs prior to death (Clark *et al*., 1968). From these findings, it can be concluded that either death is caused by a mechanical blockage of the tracheal trunks which caused suffocation of the larvae, or death was caused by the production of toxins within the infected larvae.

In our study, the purpose of comparing mortality after exposure to both living and dead spores of *M. anisopliae* was to examine whether death occurs due to the release of toxins or due to the physical effects of the spores in the digestive tract or in the breathing mechanism. Our results show that, overall, there was a significant difference between the living and the dead fungus for each particular fungal dose. Mortality increased with an increase in the amount of living fungus applied to the larvae. Mortalities were virtually similar when comparing the different doses of dead fungus. Moreover, there was no significant difference
between the effects of applying the different doses of dead fungus and the controls with no fungus at all. These results show that applying dead fungal spores to the larvae has no effect on survival. This result provides evidence that death is not caused by suffocation or blockage of the digestive system due to direct mechanical effects of the spores. From our findings, we can, however, not conclude that death occurs due to the release of toxins. It could be that mechanical effects do play a role. Hyphae from spores germinating in the siphon might still cause blockage of the breathing mechanism. A study on the pathogenic processes of entomopathogenic fungi within mosquito larvae could help to generate a more detailed understanding of the mechanical and/or toxical aspects of invasion or tissue penetration in the host.

4.3 The effect of different spore concentrations on different larval stages of \textit{An. gambiae} and \textit{An. stephensi} larvae

4.3.1 Dose effect

Only two other studies investigated mortality after application of different fungal spore doses (Daoust \textit{et al}., 1982; Daoust and Roberts, 1982). It has been found that application of \textit{M. anisopliae} on \textit{L}_3 larvae of \textit{Cx. pipiens} in doses of 10 mg, 5 mg and 2.5 mg fungal spores per 150 cm\(^2\) resulted in a mortality of 92%, 59% and 20% (Daoust \textit{et al}., 1982). In another study, it was found that application of 1 mg, 0.5 mg and 0.25 mg fungus per 16 cm\(^2\) (calculated to 9 mg, 4.5 mg and 2.25 mg at a surface of 441 cm\(^2\)) on the same species resulted in a mortality of 100%, 96% and 33% (Daoust and Roberts, 1982). In our study, application of 20 mg, 2 mg and 0.2 mg per 411 cm\(^2\) on \textit{L}_3/\textit{L}_4 larvae resulted a mortality of 95%, 67% and 55% in \textit{An. gambiae} and 91%, 44% and 35% in \textit{An. stephensi}.

However, findings of different studies are difficult to compare because of great differences between replicates, different species and due to different sizes of larval containers.

One study has been performed in which \textit{M. anisopliae} was applied on \textit{An. stephensi} (Daoust and Roberts, 1982). In this study, 1 mg fungus was applied on a surface of 16 cm\(^2\) (which translates to 9 mg per 441 cm\(^2\)). This dose resulted in a mortality of 100% which is even more than the 91% mortality obtained after application of 20 mg/441 cm\(^2\) in present study. This suggests that one could use 10 mg rather than 20 mg and still obtain the same high mortality.

An interesting follow up to this study would be investigating the effect of intermediate doses like and 15, 10 or 5 mg of spores to investigate if application of lower doses can result in the same high mortality that was obtained using 20 mg.

In our study, there was a significant difference between all different doses except for the difference between 0.2 and 2 mg fungus applied on both larval stages of \textit{An. stephensi}. This
would mean that for *An. stephensi*, applying 0.2 mg/441cm² will result in the same mortality compared to a dose of 2 mg/441cm². In case of aiming for a relatively low dose for larval control, for example when combining the application of *M. anisopliae* with some other way of vector control (which will be discussed in a later section), this could be beneficial. However, more replicates are needed to confirm this observation.

4.3.2 Differences between the early and late larval stage

Very little research has been done in comparing effects of fungal entomopathogens on different larval stages of mosquito species. In our study, a small difference between survival of the early and late larval stages was observed. In general, the L₁/L₂ larvae showed higher mortality compared to the L₃/L₄ larvae, however only partially significant. These results correspond with findings of Sandhu *et al.* (1993). In their study, mortality due to both *B. bassiana* and *M. anisopliae* was compared for the four different larval stages of *Ae. aegypti* and *Cx. tritaeniorhynchus*. It was found that for both fungal pathogens and for both mosquito species, there was a difference in susceptibility of the different larval stages. First instar larvae appeared to be most susceptible while fourth instar larvae were least susceptible.

4.3.3 Differences between the different species

For all fungal concentrations, *An. gambiae* showed a higher mortality for both larval stages compared to *An. stephensi* (*p*<0.001). An explanation for this finding could be genetic differences between the species or the difference in time they have been reared in the Laboratory. The *An. gambiae* strain has been reared in the Laboratory for 21 years whereas the *An. stephensi* strain for only 1 year. It is likely that the *An. gambiae* larvae are weaker due to inbreeding.

4.4 The effect of different exposure times on mortality

For *An. gambiae*, there were no significant differences between exposing the larvae 1, 2 or 3 days to the fungus before transferring them to clean water. For *An. stephensi* however, mortality after 1 day of exposure to the fungal spores was significantly lower compared to 2 and 3 days of exposure. This would lead to the conclusion that for *An. gambiae*, one day of exposure seems to be enough to cause mortality equal to 2 or 3 days of exposure. On the other hand, for *An. stephensi*, at least 2 days of exposure are required to cause comparable mortality.

In general, there was a significant difference between the species. In treatment 1, mortality in *An. gambiae* was higher, whereas in treatment 2 and 3, mortality in *An. stephensi* was higher. These results are not in line with what could have been expected after experiment 1 in which *An. gambiae* showed an overall higher mortality compared to *An. stephensi*. Present difference between the species might be a random effect. More replicates are needed to obtain more consistent results.
4.5 Carrying over the infection from fourth instar larvae into adult mosquitoes

In this experiment it was shown that no adult mosquitoes that emerged from the pupae of the larvae that had been treated with different concentrations of the fungus, showed fungal infection. This leads to the conclusion that late stage larvae that are exposed to fungal spores of this strain of \textit{M. anisopliae} prior to pupation cannot carry over the infection into adult mosquitoes. However, we cannot state that the larvae that survived during 7 days after application of the fungus had actually been infected. It could be that in some way, they survived by simply not having been in direct contact to the fungus. This could be the case, since the spores were largely clustered together on the water surface.

Very little can be found in the literature about the possibility of carrying over infection from the larval stage into adult mosquitoes. It has, however, once been reported that larvae surviving the fourth day of exposure often pupated successfully and emerged as apparently healthy adults. Infected pupae have been observed, however, these pupae never survived (Clark \textit{et al.}, 1968). Infected pupae all died before emerging into adults (Sandhu \textit{et al.}, 1993).

4.6 Larval stages

In our experiments, larvae from all 4 larval stages were used. First and second instar larvae were combined in one group and third and fourth instar larvae within another. However, during the 7 days of the experiment, larvae progressively moulted into the next stage(s). So, at the end of the experiments, L\textsubscript{1}/L\textsubscript{2} larvae had become L\textsubscript{2}/L\textsubscript{3} (or even L\textsubscript{4}) larvae and L\textsubscript{3}/L\textsubscript{4} larvae had turned into L\textsubscript{4} larvae and pupae/adults. The pupae were removed from the experiment. However, in calculating the percentages mortality, all larvae participating at the start of the experiment were included. The L\textsubscript{4} larvae that had pupated and excluded from the experiment, have been regarded as being alive at the end of the experiment. This decision has been made on basis of the third experiment, in which it was shown that fourth instar larvae exposed to the fungus prior to pupation that pupated during the experiment, did not show any fungal infection as adults. So, for the pupae that were removed from the experiment, we assumed them to be alive at the end of day 7.

4.7 Fungal growth from the incubated dead larvae

Fungal infection was determined by the presence / absence of germinated spores and the growth of mycelium. In the majority of the dead larvae, fungal growth was visible (Fig A-F in the appendix). The differences in percentage of larvae that showed fungal growth that are observed between the species, the larval stages and the fungal doses seem to be random. An explanation could be the way of handling the dead larvae. After picking the larvae out of the water, they were dipped into 70\% ethanol to rinse off / kill spores attached to the outside
of the body. However, many corpses were very soft and weak. Therefore, it could be that many of the corpses had degenerated to such an extend that the ethanol sterilised the whole body instead of solely spores attached to the outside.

4.8 Drawbacks of this study

4.8.1 Variation between the replicates
There was relatively large variation in the data between different replicates. This can be explained by variation in larval health. Differences in health of the larvae might be due to fluctuations of environmental conditions. Although we strived for constant conditions for the rearing, there might have been some variation in temperature, diet, larval densities and quality of the water.

4.8.2 Number of replicates
Data of the present study show a clear effect of different doses to different larval stages of both mosquito species. However, to obtain more reliable data, it would be nice to include more replicates. More replicates would also moderate the large variations observed in some of the experiments.

4.9 Feasibility in the field
The present study showed high pathogenicity of the fungus *M. anisopliae* against different larval stages of *An. gambiae* and *An. stephensi*. However, several issues have to be examined before fungal spores can be applied in the field for larval control.

It is important to investigate if the proportion of the larval population that can be targeted when applying dry fungus on pools and ditches in the field is high enough to be an effective way of controlling. This effectiveness depends on, for example, fungal dose, persistence and delivery methods or combining the application of dry spores on water surfaces with other ways of malaria vector control.

4.9.1 Larval density
In present study, in all experiments, a larval density of 50 larvae/441cm² was used. Little is known, however, about larval densities in nature (Gimnig et al., 2002). In a study of Gimnig et al. (2002) in Western Kenya, it was found that there was an average of 0.13 *An. gambiae* larvae per square cm of habitat. The experimental conditions in our larval trays was 0.11 larvae per square cm water, comparable to the natural densities that were found in the study of Gimnig et al. (2002).

There are several reasons for the importance of gaining more insight in the impact of different larval densities on mortality as a response to fungal infection. A breeding site with high larval concentrations could simply demand for a higher dose of fungal spores for
effective larval control compared to the same surface area of a site with lower larval concentrations. Moreover, it has been shown that larval survivorship decreases with an increase in density (Schneider and Takken, 2000). Therefore, it might be possible that larvae that are grown in lower densities have a higher survival rate and might therefore be less vulnerable to fungal infection.

Furthermore, an increase in larval densities results in an extension of their development time (Gimnig et al, 2002) which in turn increases the probability of becoming infected due to exposure to fungal spores during the larval stage.

So, before translating experiments from the lab into the field, the impact of different larval densities on mortality as a response to fungal infection has to be investigated in more detail.

4.9.2 Persistence
An important factor in fungal efficacy is spore persistence in infectivity over time. For many fungal strains, conidia lose their infecting potency after a few months. High persistence will result in lower efforts and costs of re-treatment. The lifespan of spores has been shown to be relatively stable in suspensions, but rapidly decreases after application on surfaces as spores. In this application they are susceptible to high temperature, low humidity and UV-B radiation (Scholte et al., 2004; Zimmermann, 1982; Zimmermann, 2007b). This would include that the application of spores on the water surfaces should be repeated frequently which would be time consuming, expensive and it might be impractical due to the many and sometimes inaccessible breeding places.

A possible strategy to counteract the harmful effect of exposure to sunlight is to incorporate ultraviolet protectants in spore formulations (Fargues et al., 1996; Fernandes et al., 2008). Another strategy is selecting fungal isolates with increased resistance to irradiation through the selection of the most UV resistant isolates. Through these approaches, in combination with optimising storage conditions, it might be possible to significantly prolong the persistence and efficacy of these fungi (Fernandes et al., 2007; Daoust et al., 1983).

4.9.3 Combining larval control with fungal entomopathogens with other controlling strategies
Larvae of An. gambiae can be found in rice fields, temporary pools, drinking water vessels and even in small holes like hoof prints and tyre tracks filled with water (Walker and Lynch, 2007; Mutuku, 2006; Takken and Lindsay, 2003). If numbers of breeding sites are extremely large, for example during the rainy season, it might be not feasible to apply spores of M. anisopliae on all those water surfaces. Management of the more limited number of dry season habitats might be more effective alternative (Fillinger et al., 2004).

Furthermore, as explained in the introduction, larval control measures could be more effective in combination with existing methods for adult control like the use of ITN’s and IRS (Killeen et al., 2000; Walker and Lynch, 2007).
4.10 Safety of *M. anisopliae* for application in the field

To develop a fungus-based control measure for a large-scale field implementation that is socially acceptable, sufficient attention is required of the effects on non target organisms. *M. anisopliae* is a non-specific entomopathogen and thus pathogenic to a wide range of insects (Strasser *et al*., 2000). The fungus is recognised as less hazardous to non-target organisms than most chemical pesticides (Roberts and Leger, 2004). However, some beneficial insects may be at risk. Generally, *An. gambiae* for example, will breed in temporary sites with marginal diversity of aquatic life. Nevertheless, it is important that before implementation in the field, sufficient knowledge is obtained on the effects on non target organisms like insects, plants, other soil and water organisms, mammals, and human health (Strasser *et al*., 2000; Roberts and Leger, 2004; Zimmermann, 2007b).

4.10.1 Persistence

The persistence of a biocontrol agent can be seen from two different perspectives; one from the perspective of minimising side effects to ecosystems and environment and the other from the biocontrol user. The first prefers a low persistence of the fungus with respect to possible undesirable environmental impacts while the second is mainly interested in a long persistence for better and prolonged product efficacy.

Results of studies have shown that spores of *M. anisopliae* may persist in the soil for several months and even years depending on the strain and soil conditions (Rangel *et al*., 2005; 1985; Milner *et al*., 2003). It was also found that the number of spores per gram of soil decreases rapidly with soil depth after watering. At 10 cm, the spore number is reduced by 10-100 fold. Results have shown that contamination of groundwater by *M. anisopliae* is very unlikely. However, no information is available on the fate and persistence of unformulated spores of *M. anisopliae* drifting onto natural water surfaces (Zimmermann, 2007b).

4.10.2 Effects to non-target organisms

So far, no phytopathogenic effects of *M. anisopliae* have been found (Zimmermann, 2007b). Research on the pathogenicity on organisms in the soil have shown that several mite species are susceptible to the fungus (WeiBing and MingGuang, 2004; Brooks and Wall, 2001).

Furthermore, it has been found that a few collembolan species (*Folsomia candida, Folsomia fimetaria, Hypogastrura assimilis* and *Proisotoma minuta*) are not affected by *M. anisopliae* (Broza *et al*., 2001, Dromph and Vestergaard, 2002). No infection was observed after infection of two tenebrion beetles (*Pimelia senegalenis* and *Trachyderma hispida*), however species of hymenopteran parasitoids (*Bracon hebetor* and *Apoanagyrus lopzi*) were infected by the fungus (Danfa, 1999).

Relatively few studies have been performed on the toxicity of *M. anisopliae* to aquatic organisms. The fungus is found to be harmful for embryos of an estuarine decapode (*Palaemonetes pugio*), living along the eastern and southern coasts of the USA (Genthner *et al*., 1998). Harmful effects to some cladoceran species have also been reported (Milner *et
Moreover, several toxic effects of the applications of spores of *M. anisopliae* to water were observed in embryos and larvae of the silverside fish (Genthner et al., 1998; Genthner and Middaugh, 1995). It was also found that the fungus was toxic to juvenile mosquito fish and to grass shrimp species (Genthner et al., 1998). However the fungus had no adverse effects on juvenile rainbow fish (Milner et al., 2002).

Some studies have been performed on the toxicity of spores after ingestion by amphibian species (*Xenopus laevis*) in which it no mortality was observed; the viscera were free of fungal elements (Genthner et al., 1998).

Many studies on toxicity or pathogenicity to mammals and human health have been carried out with *M. anisopliae*. Until 1990, *Metarhizium anisopliae* has never been reported as infecting mammals or humans. During the last years, some cases have been described. However, none was associated with the use of *Metarhizium anisopliae* as a biocontrol agent (Zimmermann, 2007b).

Compared to *B. bassiana*, there are fewer records on the allergenicity of the fungus. In inhalation studies with rats, pigs and mice, no allergic reactions were observed (Roberts and Leger, 2004). However, further studies with allergenic extract inoculation obtained from *M. anisopliae* into mice demonstrated that this extract contained components that induce immunological, inflammatory and histopathological responses and thus has an allergenic potential (Ward et al., 1998, 2000). Due to the wide-scale use of *M. anisopliae* for biocontrol of sugar cane pests in Brazil, several persons showing asthmatic symptoms due to *Metarhizium* were recorded (Barbieri et al., 2005). Furthermore, 2 cases of human corneal infection have been reported (DeGarcia et al., 1997, Jani et al., 2001). Moreover, in a factory in which employees were massively exposed to conidia, some employees reported an allergic response after continuous exposure to conidia of *Metarhizium*. However, no cases of infection by the fungus was reported by any of these workers (Roberts and Leger, 2004).

It can be concluded that *M. anisopliae* has some allergenic potential to humans, but it is regarded as safe for using as a biological control with minimal risks (Zimmermann, 2007b).

Knowledge on side effects of the fungus to organisms in the pools and ditches on which *Metarhizium* is to be applied is however scarce. More elaborate research on side effects on many organisms is needed before large-scale implementation in the field can be realised.
Conclusions

In the present laboratory study, *M. anisopliae* was shown to be highly effective in infecting larvae of *An. gambiae* and *An. stephensi*, killing up to 100% of the larvae within a week after exposure to a dose of 20 mg of dry spores on a water surface of 441cm².

In the first experiment, it was demonstrated that for both mosquito species, there was a significant difference between cumulative percentage mortality of the different doses of applied fungus. Dose and mortality were positively correlated; increase in fungal dose gave an increase in mortality. In general, there were no significant differences between the survival curves of the two larval stages for both species.

In experiment 2, different exposure times did not result in different percentage mortality for *An. stephensi*. However, for *An. gambiae*, percentage mortality after an exposure time of 1 day was significant lower compared to percentages mortality after an exposure time of 2 and 3 days. So for *An. stephensi*, one day of exposure seems to be enough to cause a mortality equal to 2 or 3 days of exposure. For *An. stephensi* however, at least 2 days of exposure are required to cause comparable mortality.

In experiment 3, it was shown that none of the adult mosquitoes that emerged from the late larval stage that had been exposed to different concentrations of the fungus, showed fungal infection. This suggests that late stage larvae that are exposed to fungal spores prior to pupation cannot carry over the infection into adult mosquitoes

Larval control with entomopathogenic fungi might provide an excellent opportunity for biologically controlling malaria vectors in the future. Controlling *Anopheles* larvae as a vector using fungi is promising in urban areas, but also in rural communities, were larval control might function as a supplement to IRS and ITN’s, particularly in the dry season when vector larvae are concentrated.

It is very important to keep searching and developing alternative agents and methods for vector control. This is indispensable for finding the most optimal way of infecting larvae and thereby contributing to an effective, biological malaria vector control. The application of entomopathogenic fungi on the larval stage of mosquitoes may contribute to an extension of the limited available control agencies like *Bti* and *Bs*. To gain maximum impact on mosquito populations, this new method of larval control should be applied in integrated control strategies.

Present study shows the potential of *M. anisopliae* in killing larval stages of *An. gambiae* and *An. stephensi*. This is an outstanding finding which might be a base for further research on the possibilities and effectiveness of using this method in the field.
I have had a nice time at the Department of Entomology. One of the things I liked the most was the atmosphere in which one can easily feel at home. Another thing I have definitely appreciated was the great guidance and supervision. I would like to thank Bart Knols for his enthusiastic, warm and personal supervision which raised the quality of my study to a higher level. Furthermore I want to thank Tullu Bukhari for her help and guidance. She was always available to discuss both practical and theoretical matters and supported me with good advices and comments. Thanks is also due to Jeroen Spitzen, Niels Verhulst, Bruce Schulitz, Karel van Roey, Tullu Bukhari and Bart Knols for blood feeding the mosquitoes. I would like to thank Sander Koenraadt for his help with the statistical analyses. And in the end I would like to thank everyone at Entomology for their interest, support and help during my thesis work.
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Appendix 1

Images of larvae infected with *M. anisopliae*
### Appendix 2

P-values computed with Kaplan-Meier pairwise comparison. The values indicate the significance differences in the different doses for the 2 species and 2 larval stages.

<table>
<thead>
<tr>
<th></th>
<th>An. gambiae</th>
<th>An. stephensi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>0.2</td>
</tr>
<tr>
<td>$L_1/L_2$</td>
<td>0.2</td>
<td>0.004*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.000*</td>
</tr>
<tr>
<td>$L_3/L_4$</td>
<td>0.2</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

*significant at $p<0.05$

### Appendix 3

P-values computed with Kaplan-Meier pairwise comparison. The values indicate the significant differences between survival curves of the different larval stages.

<table>
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<td>2</td>
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<tr>
<td>20</td>
<td>0.436</td>
<td>0.037*</td>
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*significant at $p<0.05$
Appendix 4

P-values computed with Kaplan-Meier pairwise comparison. The values indicate the significance differences between the curves of the living and the dead spores of each particular dose.

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<td>0.832</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.000*</td>
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<tr>
<td></td>
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<td>0.000*</td>
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<tr>
<td>L₃/L₄</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>20</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

*significant at p<0.05

Appendix 5

P-values computed with Kaplan-Meier pairwise comparison. The values indicate the significant differences between the curves of the dead spores for the different doses.

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*significant at p<0.05
Appendix 6

Percentage of larvae that showed fungal growth after plating and incubation

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<td>2</td>
<td>20</td>
<td>0.2</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>L1/L2</td>
<td>46.82</td>
<td>83.82</td>
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<td>37.99</td>
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<tr>
<td>L3/L4</td>
<td>47.4</td>
<td>75.85</td>
<td>83.7</td>
<td>47.82</td>
<td>25.84</td>
<td>70.41</td>
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</table>

Appendix 7

Differences between different exposure times (Kaplan-Meier, pairwise comparison)

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<th>An. stephensi</th>
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<th></th>
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<td>1</td>
<td>2</td>
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<tr>
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<td>-</td>
<td>0.000*</td>
<td>-</td>
<td></td>
<td></td>
</tr>
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<td>0.000*</td>
<td>0.576</td>
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<td></td>
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</tbody>
</table>

*significant at p<0.05