WAGENINGEN UNIVERSITY
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Evaluation of the entomopathogenic fungus *Beauveria bassiana* for the control of malaria mosquitoes

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Table of contents

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Summary

Entomopathogenic fungi are a promising new control tool for both the adult and larval stages of malaria mosquitoes. For adult control only batches with viability above 85% are used although it is unclear whether there is a difference in mosquito mortality between batches with viability above and below this cut-off value. Furthermore it is not known whether low spore viability can be compensated for with higher spore concentration. Spore persistence in relation to temperature and exposure time was also evaluated since spores are exposed to relatively high temperatures in the field. When treating larvae with *Beauveria bassiana* some mosquitoes were still able to emerge. It is unknown how fitness of these surviving females is influenced. In addition it was also tested whether larval exposed mosquitoes were still susceptible to re-exposure to *B. bassiana.*

Anopheles stephensi larvae and adults were treated with the fungus *B. bassiana* in all experiments. In the first experiment, bio-assays were performed in the laboratory by exposing females to two batches with different viability and spore concentration. In the second experiment spore viability was tested after exposing spores to different temperatures for two different exposure times. In the third experiment larvae were exposed to *B. bassiana* after which sex ratio, survival and reproductive success were monitored. Wing length and fungal infection were determined for all mosquitoes. In the fourth experiment larval exposed females were re-exposed to *B. bassiana* in a bio-assay after which survival was monitored.

No difference in survival of mosquitoes treated with the two batches of different viability was found. Therefore the experiment could not show whether lower viability can be compensated with higher spore concentration. Temperature, exposure time and the interaction had a negative effect on spore viability. Fitness of mosquitoes exposed to *B. bassiana* during the larval stage was affected. Sex ratio of larval exposed mosquitoes shifted to a larger number of females. Females which were infected had an intermediate to larger size compared to non-exposed females, had lower survival, laid an equal number of eggs and equal number of hatched larvae, compared to non-exposed females. Females which were not infected were larger in size, had equal survival and laid a larger number of eggs from which a higher number of larvae hatched. Larval exposed mosquitoes were re-exposed to *B. bassiana* and were able to live as long as non-exposed mosquitoes.

This study showed that even batches with relatively low viability are still efficient in killing mosquitoes. Spore viability will rapidly decline in the field when spores are exposed to temperature, although even spores with lower viability can still be virulent. Consequences for fitness of larval exposed mosquitoes depended on infection acquired during the larval stage. Larval exposed mosquitoes were still equal susceptible to *B. bassiana* compared to non-exposed mosquitoes.

Introduction

Malaria problem

In the tropics, malaria is one of the most important vector-borne diseases caused by protozoa of the genus *Plasmodium* (Collins and Paskewitz, 1995). The main five malaria parasites infecting humans are *P. falciparum*, *P. malariae*, *P. vivax*, *P. ovale* and *P. knowlesi* (Beier, 1998; Ta et al., 2010). Approximately 60 species of *Anopheles* mosquitoes have the potential of transmitting *Plasmodium* to human hosts, with *An. gambiae*, *An. funestus* and *An. arabiensis* causing almost 90% of the total malaria transmission in the world, mainly in sub-Saharan African countries (Cohuet et al., 2010; Collins and Paskewitz, 1995). According to the World Health Organization, almost 40% of the world population is at risk of being infected with malaria, which resulted in approximately 169 to 265 million cases of malaria and an estimated number of 781.000 deaths in 2009 (World Health Organisation, 2009, 2010)

Malaria is still a life-threatening disease which has not been eradicated from all parts of the world because there is yet no effective vaccine, antimalarial drugs are not available on a large scale and vector control has not been able to reduce malaria transmission worldwide (Michalakis and Renaud, 2009). The problem with malaria is the complex life cycle of *Plasmodium* which makes it difficult to develop an effective vaccine or antimalarial drug. In addition, large-scale resistance to drugs and insecticides and the diverse ecology of *Anopheles* mosquitoes, makes it hard to apply a universal vector control strategy (Beier, 1998; Michalakis and Renaud, 2009).

Malaria transmission

The lifecycle of mosquitoes consists of an egg, larval, pupal and adult stage. Only the adult stage is not aquatic. Together with the blood of an infected human, malaria parasites are ingested by a female during blood feeding. The sporogonic cycle, the sexual part of the life cycle of *Plasmodium*, completes inside the mosquito and takes approximately 10-14 days dependent on species and environmental conditions (Matuschewski, 2006). When parasite development is completed, sporozoites migrate to the salivary glands of the mosquito and are transmitted to a new person during blood feeding (Beier, 1998).

Current malaria control strategies

New control strategies can further contribute to the reduction of malaria morbidity and mortality, even though present control strategies have already resulted in the eradication of endemic malaria in some parts of the world (World Health Organisation, 2009). Control strategies are applied directly against the parasite or indirectly against the vector during the larval or adult stage, to prevent the further spread of malaria.

Two main control strategies directly applied against the parasite are vaccines and antimalarial drugs (Michalakis and Renaud, 2009). Development of a vaccine is still in progress. In the future, an effective vaccine can artificially increase the number of immune people and, as a result, morbidity and mortality due to malaria will decrease (Collins and Paskewitz, 1995). Even if an effective vaccine can be developed in the future, other factors like poverty could prevent the large-scale distribution of the vaccine resulting in only limited numbers of people becoming immune against malaria. Diagnosis and treatment of malaria is available although drugs are not available at a large-scale, especially in poor countries (World Health Organisation, 2009). Again poverty is a major problem together with the

wide-spread resistance against antimalarial drugs, resulting in failure of treatment and the need for other medicines or control strategies (Michalakis and Renaud, 2009).

Indirect control strategies are focused on decreasing the vector competence and the vector capacity of mosquitoes responsible for the transmission of *Plasmodium*. Vector competence is the susceptibility of mosquitoes to *Plasmodium* or the ability to replicate and transmit the parasite (Cohuet et al., 2010). Vector capacity depends on several factors including the density of suitable vectors, survival of vectors and human biting rate (Cohuet et al., 2010; Coosemans and Carnevale, 1995). Indirect control strategies can be aimed at the larval or adult stage.

Larval control reduces vector capacity by lowering the densities of malaria vectors. Environmental management can reduce malaria transmission by lowering the number of mosquito breeding sites which leads to a reduction in human-vector contact (Walker and Lynch, 2007). Another way of controlling larvae is by applying chemical or biological larvicides. Chemicals are applied in the water in order to kill mosquito larvae, although large scale resistance has already developed against larvicides (Walker and Lynch, 2007). Biological control of larvae involves the use of environmental friendly organisms such as the bacteria *Bacillus thuringiensis* (Majambere et al., 2007), predatory fish (Chandra et al., 2008) and the neem tree (Howard et al., 2009). These tools have been proven to be effective in the laboratory although application in the field is still complex.

Vector competence of *Anopheles* mosquitoes can also be reduced by genetic manipulation of adult mosquitoes, disabling the mosquitoes to replicate or transmit *Plasmodium* to new human hosts or by inducing sterility in males (Beier, 1998; Collins and Paskewitz, 1995). Several problems arise regarding to use of genetic manipulated mosquitoes, including political problems about the use of genetic manipulated organisms, low success rate in techniques to manipulate the genome, problems with manipulated mosquito populations in the wild and problems due to the high diversity in competent vectors of malaria and other related diseases (Michalakis and Renaud, 2009).

Insecticides are used on a large scale to reduce vector capacity by decreasing the survival and density of adult mosquitoes, resulting in a reduction of *Plasmodium* transmission. Insecticides are mainly used for indoor residual spraying or to impregnate bednets (Pluess et al., 2010). Indoor residual spraying is only effective when large areas are covered and when the vector shows resting behavior after blood feeding, during which it can be exposed to the insecticides (Coosemans and Carnevale, 1995). Insecticide-treated bednets have the advantage of both preventing mosquitoes to blood feed and to simultaneously kill mosquitoes which come into contact with the bed net, therefore reducing vector capacity by decreasing both human biting rate and mosquito survival (Michalakis and Renaud, 2009). Two major problems arise with the use of insecticides, which are public health problems and large-scale insecticide resistance (Hancock, 2009). Due to large-scale application of insecticides, resistance has occurred worldwide for several insecticides increasing the interest in other biological control strategies (Scholte et al., 2005).

Promising biological control strategies involve the use of entomopathogenic organisms such as bacteria, fungi and predatory fish (Collins and Paskewitz, 1995; Scholte et al., 2003). The main advantage of biological control strategies is the low probability of development of resistance against these organisms due to the complex mode of action (Bukhari et al., 2010; Hegedus and Khachatourians, 1995). Entomopathogenic fungi are already commercially produced and used in agriculture for biological pest control against insects such as beetles, flies and grasshoppers (Scholte et al., 2005; St. Leger and Wang, 2010) Studies on entomopathogenic fungi have shown promising

results in control of malaria mosquitoes either directed to the larval stage or adult stage (Blanford et al., 2005; Bukhari et al., 2010; Scholte et al., 2006; Scholte et al., 2005).

Entomopathogenic fungi

Once a fungus spore attaches to an insect, entomopathogenic fungi have the ability to develop specific infection structures and produce metabolites to overcome resistance and penetrate the different barriers (epicuticle, procuticle, epidermis and hemocoel) of the insect (Hajek and St Leger, 1994; St. Leger and Wang, 2010). Once the fungus has colonized the hemocoel a combination of fungal growth, production of toxins and exhaustion of nutrients become fatal for the insect (Hajek and St Leger, 1994). Infection of mosquito larvae mainly occurs by ingesting spores during filter-feeding, resulting in accumulation of spores in the gut which becomes fatal when endotoxins are produced (Hegedus and Khachatourians, 1995). Other ways of infection are through the spiracular opening or the anal region (Hegedus and Khachatourians, 1995).

According to the work of Scholte et al. (2004), an optimal fungus to be used for biological control of malaria mosquitoes should meet the following criteria. Both larvae and adult mosquitoes should be susceptible for the fungus resulting in high mortality of the mosquito population, the fungus should only need to be applied a few times per season, it should only kill mosquitoes and not affect other organisms especially humans, it should be able to persist in a large range of environmental conditions, mass-production should be possible at low costs and spores should be able to be stored for long periods of time (Scholte et al., 2004).

The most promising fungi for malaria control, currently subject of many studies, are *Metarhizium anisopliae* and *Beauveria bassiana*, satisfying most of the above-described criteria for an efficacious fungus. Studies with adult mosquitoes have shown a negative effect on vector capacity by reducing blood feeding propensity, fecundity (Scholte et al., 2006) and survival of *Anopheles* mosquitoes exposed to *M. anisopliae* and *B. bassiana* (Blanford et al., 2005; Scholte et al., 2005). In addition, entomopathogenic fungi can also support the effectiveness of insecticides by reducing insecticide resistance of mosquitoes (Farenhorst et al., 2009).

Although the effects of entomopathogenic fungi on *Anopheles* mosquitoes have been made clear by several studies, relatively little research regarding spore quality has been done. Several spore characteristics are involved in virulence of a fungus. One of those characteristics is the viability of fungal batches, which indicates the number of germinating spores. Only batches with a viability of 85% and higher were used in experiments, although the effect of batches which differ in viability on mosquito mortality is unclear (Farenhorst and Knols, 2010). Furthermore it is unclear whether low spore viability needs to be compensated with higher concentrations.

Another important factor regarding spore quality is spore persistence. Spores applied in the field are exposed to fluctuating environmental conditions such as temperature, relative humidity and solar radiation which all have a negative influence on spore viability (Walstad et al., 1970; Zimmermann, 2007). *B. bassiana* isolates from different geographic origins differ in their sensitivity to environmental conditions such as temperature (Fargues et al., 1997). Therefore it is unclear how spore viability of *B. bassiana* batches used in this thesis is influenced by increasing temperature and exposure time, to which spores are subjected in the field.

Except for research on the effects of entomopathogenic fungi on adult mosquitoes, fungi can also be used for larval control. The study of Bukhari et al. (2010) showed that in the laboratory both *M.*

anisopliae and *B. bassiana* effectively decreased survival of *An. gambiae* and *An. stephensi* larvae, with only low percentages of emerging adults. A small fraction of the emerging mosquitoes still carried the infection although it is unclear what consequences this has on mosquito fitness. Furthermore it is not known whether adults which have survived exposure to spores during the larval stage are still vulnerable for exposure to *B. bassiana* during the adult stage.

Aim of the research and research questions

The main objective of this study is to expand the fundamental knowledge on entomopathogenic fungi used as a tool to control malaria mosquitoes. In this thesis, knowledge was gained on spore viability which is an important factor in spore virulence, spore persistence in relation to temperature and exposure time and consequences on mosquito fitness after exposure to *B. bassiana*. This knowledge is necessary to improve and optimize the use of entomopathogenic fungi as a relatively new biological control strategy in order to reduce malaria transmission and hopefully in the future, to control malaria worldwide.

Research questions:

1. What is the effect of spore viability and concentration on mosquito mortality?
2. What is the influence of temperature and exposure time on spore viability?
3. Which consequences does larval exposure to *B. bassiana* have on fitness of *An. stephensi*, regarding size, sex ratio, survival and reproductive success?
4. Are larval exposed females of *An. stephensi* equally susceptible to re-exposure to *B. bassiana* during the adult stage, compared to non-exposed females?

Because only batches with high viability have been used in previous studies (Farenhorst and Knols, 2010), it is hypothesized that *B. bassiana* batches with a relatively high spore viability (above 85%) are more efficient in killing mosquitoes compared to batches with a relatively low spore viability (below 85%). The absolute number of viable spores in a specific spore concentration is expected to be an important factor that determines the virulence of a concentration. Therefore, in order to keep the absolute number of viable spores in the concentration high, it is expected that low viability can be compensated by using a higher concentration

Both temperature and exposure time have a negative influence on spore viability (Walstad et al., 1970; Zimmermann, 2007). Therefore spores can only persist within a certain range of temperatures. Spore viability of spores used in field studies also declined over time (Scholte et al., 2005). Spore viability of batches used in these experiments is expected to decrease with increasing temperature and exposure time. Moreover, spores might be able to persist for a short amount of time at a relatively high temperature but when exposure time is increased, spores will lose their viability.

Mosquitoes which have survived exposure to *B. bassiana* during the larval stage are expected to have a lower fitness compared to non-exposed mosquitoes. Larvae have been into contact with *B. bassiana* and although the infection was not sufficient to kill the larvae, a small fraction of mosquitoes is still carrying the infection (Bukhari et al., 2010). There will be no difference in the sex ratio between non-exposed and larval exposed mosquitoes since there is expected to be no difference in fitness between males and females. Furthermore survival is expected to be lower compared to non-exposed mosquitoes. Reproductive success is also expected to be lower, which will be reflected by a lower number of eggs laid by larval exposed mosquitoes, as well as on average lower egg quality compared to eggs of non-exposed mosquitoes.

Mosquitoes which are re-exposed during the adult stage, after exposure during the larval stage, could have either lower or higher survival compared to mosquitoes which are only exposed during the adult stage. Survival will be lower when mosquitoes are still carrying a low dose of fungus from the larval stage. In that case mosquitoes might be more susceptible to re-exposure. On the other hand, mosquitoes might have become resistant to *B. bassiana* after exposure to a low spore dose during the

larval stage. Those mosquitoes will be less susceptible and will therefore live longer than mosquitoes which were only exposed during the adult stage.

Materials and methods

Mosquitoes

An. stephensi (Sind-Kasur strain, courtesy of Prof. R. Sauerwein, origin Pakistan) were reared in the laboratory under the same conditions as described in the article of Bukhari et al. (2010). All experiments were performed under identical climate controlled conditions with temperature: 27±1°C, relative humidity: 70 ± 5% and photoperiod: 12L:12D.

Eggs laid in a Petri dish filled with soaked cotton wool and a filter paper on top of it, were collected in plastic trays (25x25x8 cm) containing acclimatized water and 2 drops of Liquifry No.1. (Interpet Ltd., Dorking, Surrey, Uk). Water was acclimatized by keeping it for at least one night in a jerry can in the climate cell. One to two day old larvae were fed with one drop of Liquifry No. 1., three to five day old larvae were fed with 0.1-0.2 mg/larvae/day of Tetramin (Tetra, Melle, Germany) and six to nine day old larvae with 0.3mg/larvae/day. Pupae were collected daily from the trays and transferred to holding cages (30x30x30 cm). Adult mosquitoes were fed with ad libitum 6% glucose/water solution. Adult females were transferred to a blood feeding cage and were blood fed using the hemotek feeding system.

Fungus

Bioprocess Engineering, Wageningen University provided different batches with spores of *B. bassiana* (IMI-391510) in blue caps of 50 ml, kept and stored at 4°C.

Spore viability

Viability of *B. bassiana* batches was tested before the experiments were started. Thin plates of Sabouraud Dextrose Agar (SDA) were prepared according to the manual on the jar of agar powder and poured in Petri dishes (Ø = 9 cm). Of each batch 12.5 mg of spores were weighed, suspended in 5 ml of Shellsol oil (Shell, The Netherlands) and sonicated for 10 seconds. Under clean laboratory conditions and a burning flame, 50 µl of the spore suspensions was divided over two agar plates and spread with a clean glass rod. All Petri dishes were sealed with parafilm and incubated for 16 hours at 27°C. A cover glass was placed on the agar and a total number of approximately 300 spores was counted at 400x magnification using a light microscope. Spores were counted as germinated when the penetration tube was longer than 1.5x the spore itself. Viability was determined by calculating the percentage of germinated spores out of the total number of counted spores.

Spore concentration

In all experiments spore concentrations of spores suspended in Shellsol oil were verified using a Bürker-Türk haemocytometer (tiefe: 0.01 mm; 0.0025 qmm; 0.04 qmm). Figure 1 shows the total area of 1 mm^2 (A), which consists of 16 squares (B), in which all spores were counted in order to calculate the concentration of the spore suspension. The total number of counted spores was present in a volume of $1.0*10^{-5}$ ml, which has to be multiplied by the dilution factor to calculate the concentration of the spore suspension.

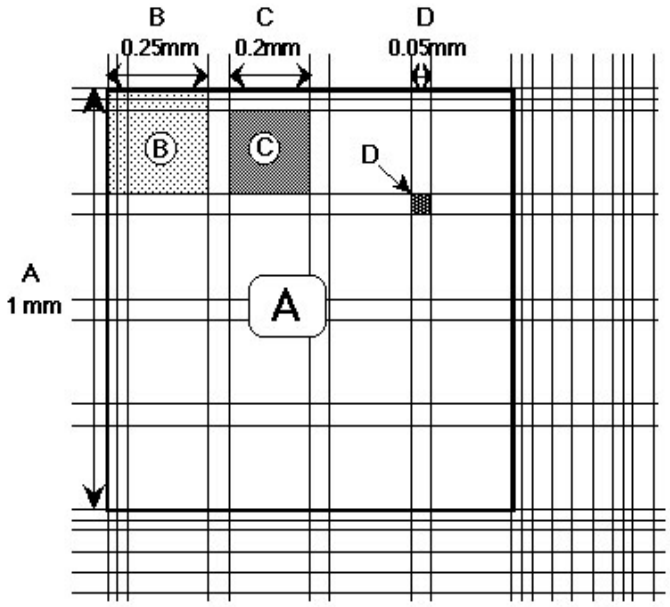

Figure 1: Schematic of Bürker-Türk haemocytometer. Spores in area A (1 mm^2) were counted in order to calculate the concentration of the spore suspension.

Spore viability and concentration

The effect of spore viability and spore concentration on mosquito mortality was tested by exposing *An. stephensi* females to two batches of *B. bassiana* with viability above and below 85%, at different concentrations. Viability of *B. bassiana* batch 2, 3, 4 and 22 was determined according to the method described in the 'spore viability' section, in order to select the two batches with a viability higher and lower than 85%. Details on these four batches can be found in appendix table 1. Batch 2 and 4 were selected since they meet the viability criterion and there was only a difference of two days in production time, respectively 07-04-2010 and 09-04-2010, which could have an influence on the quality of the spores. The only difference in the production process of the batches was the size of the Packed Bed Reactor (PBR) in which the spores were produced which was 0.2 L for batch 2 and 3.2 L for batch 4.

Viability of *B. bassiana* batch 4 has been determined at 89 ±1% and *B. bassiana* batch 2 at 59 ±1%. Figure 2 represents the total spore concentrations and viable spore concentrations used in each treatment. Treatment 2, 5 and 6 contained spores of batch 4 and treatment 3 and 4 spores of batch 2. Spore suspensions for treatments 1 to 6 were prepared with total spore concentrations of respectively 0.00; $5.00*10^{10}$; $5.00*10^{10}$; $7.50*10^{10}$; $3.33*10^{10}$ and $5.00*10^{10}$ sp/m^2. Treatment 6 consisted of a spore concentration of $3.33*10^{10}$ sp/m^2 together with a spore concentration of 1.66 sp/m^2 of spores which were damaged using the microwave at 1300 Watt for 3 minutes. Spores were checked on viability after the microwave treatment and no germination was observed. Details on calculations of spore concentrations and absolute numbers of spores per treatment can be found in table 2 in the appendix. Spore concentrations were checked using the Bürker-Türk haemocytometer as described in the 'spore concentration' section. If necessary, spore suspensions were diluted by adding an extra amount of Shellsol oil.

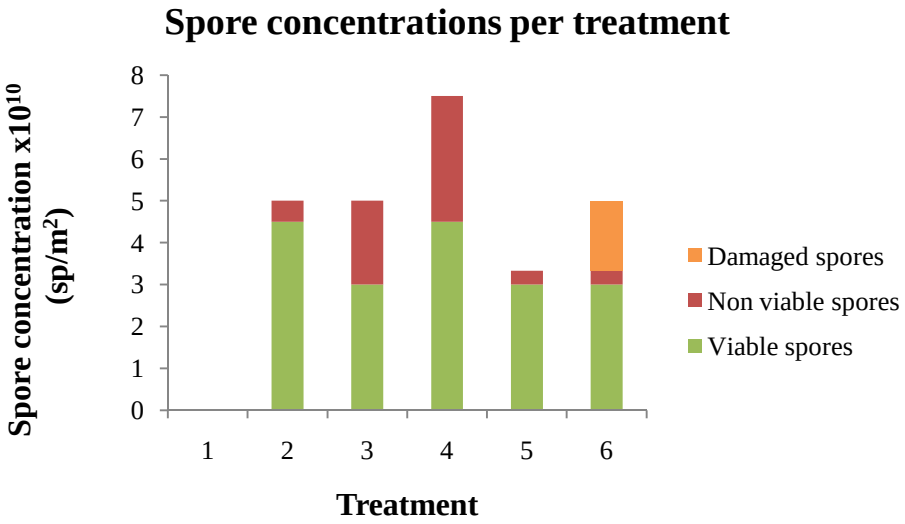

Figure 2: Total spore concentrations and viable spore concentrations used in the six treatments including the control (treatment 1) which consisted of Shellsol oil only. Treatment 2, 5 and 6: batch 4, treatment 3 and 4: batch 2.

Of each spore suspension 1 ml was coated, using a K-hand coater (RK Print Coat Instruments Ltd., United Kingdom) (figure 3A), on gloss coated proofing paper (15x25 cm) according to the technique described by Farenhorst and Knols (2010). In total four papers were coated with 1 ml of the spore suspension for each treatment. The four control papers were coated with 1 ml of Shellsol oil only. All papers were dried one night in the fume hood. Dried coated papers were fixed with paperclips into PVC tubes (Ø = 8 cm and height = 15 cm) after which the tubes were closed with plastic foil and a rubber band at each side (figure 3B). At one side a small opening was made in the plastic foil and closed with a piece of cotton wool. Each treatment contains four replicates of each 30 *An. stephensi* females of 3-5 days old. Females were transferred to the PVC tube and were exposed for 3 hours. After exposure, females were transferred to holding buckets (Ø = 12.5 cm and height = 12 cm) closed with a nylon sock. Nutrition was provided by placing cotton wool, daily soaked in 6% glucose/water solution, on top of the nylon sock. Survival was monitored daily by counting the number of dead mosquitoes in each holding bucket.

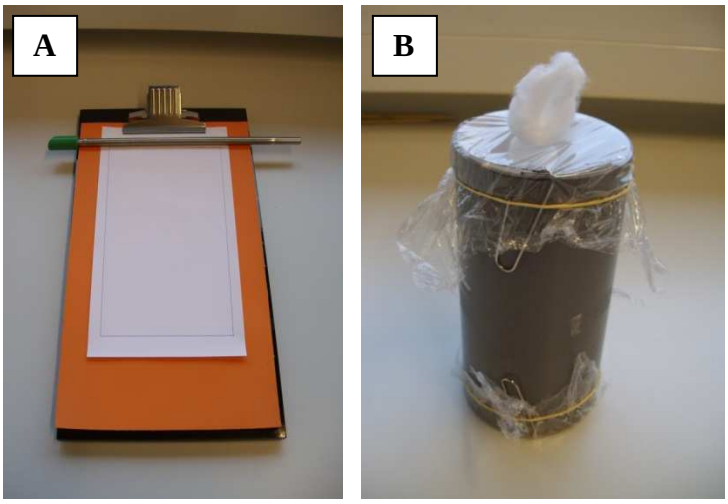

Figure 3: (A) K-hand coater and coating bed used to coat spore suspensions onto gloss coated paper. (B) PVC tube in which females are exposed to *B. bassiana* spores.

Infection with *B. bassiana* was determined for all mosquitoes by incubating dead mosquitoes within 0-2 days. Dead mosquitoes were placed on a filter paper inside a Petri dish (figure 4). In order to remove other undesirable bacteria and fungi, 70% ethanol was dripped on top of all dead mosquitoes. After the ethanol had evaporated, 1 ml of demiwater was added to each Petri dish and all Petri dishes were sealed with parafilm. After 3-5 days of incubation at 27 °C, infection could be determined using a binocular microscope.

Figure 4: Petri dish containing dead mosquitoes.
Fungus was growing out of the infected cadavers.

Temperature and exposure time

Spores of batch 4 with a viability of 89±1% when stored at 4°C, were exposed to 20, 30, 40, 50, 60 and 70°C for both 6 and 24 hours, in order to test the effect of temperature and exposure time on spore viability. For each of the 12 treatments 12,5 mg of spores were weighed and placed in a glass Petri dish (Ø = 9,5 cm). Spores were spread over the surface of the Petri dish in order to get an equal influence of temperature. The 20°C treatment was done by placing the Petri dish in the lab at room temperature of 20°C for 6 and 24 hours. The other temperature treatments were done by placing the Petri dish for 6 or 24 hours in a stove. After exposure, all Petri dishes were placed in the fridge before suspending the spores in 5 ml of Shellsol oil. Viability was determined by using the same method as described in the 'spore viability' section.

Mosquito fitness after larval exposure to *B. bassiana*

Pilot studies

Based on the results found by Bukhari et al. (2010), fungus species, fungal dose and larval density were determined. Pupation of L_{1-2} larvae in trays treated with *B. bassiana* was higher compared to trays treated with *M. anisopliae*, leading to a higher fraction of mosquitoes that emerged from trays treated with *B. bassiana* (Bukhari et al., 2010). Therefore larvae were exposed to *B. bassiana* in this thesis, since the focus was on fitness of females that survived exposure during the larval stage. The fungal dose used was established at 10 mg, since there appeared to be no significant differences in larval mortality between 5, 10 and 20 mg of *B. bassiana* added to trays containing L_{1-2} *An. stephensi* larvae (Bukhari et al., 2010). In this thesis each tray contained 250 larvae because there appeared to be no significant differences in mortality rates of larval densities of 50, 150 and 250 larvae (Bukhari et al., 2010). Furthermore it is more practical to have a relatively high larval density since a lower number of trays is needed to get enough females for the experiments.

In addition to these findings, three pilot studies have been done in order to select the most virulent *B. bassiana* batch, mosquito species and mode of spore application which will be used in subsequent experiments. First a comparison between five batches of *B. bassiana* was made in order to select the most virulent batch of *B. bassiana*. Viability was determined for all batches according to the method described in the 'spore viability' section. Six trays were filled with 1 liter of acclimatized water and to each tray 250 L_{1-2} larvae of *An. stephensi* were added. To one tray no spores were added since this was the control tray. To the other five trays spores of *B. bassiana* batch 1, 3, 4, 9 or 22 were added. Dead larvae were collected and counted each day. With these data a food schedule was set up that was adjusted to the number of (remaining) living larvae inside the trays (see appendix table 3). The number

of pupae collected from the trays was used to select the most virulent batch and to calculate the number of trays needed in order to get enough females which were necessary in the experiments. *B. bassiana* batch 4 was used in the next pilot experiments since spores in this batch have the highest viability.
Second, a comparison was made between susceptibility of *An. gambiae* (Suakoko strain, courtesy of Prof. M. Coluzzi) and *An. stephensi* to batch 4 of *B. bassiana*. Two plastic trays were filled with 1 liter of acclimatized water and to each tray 250 L_{1-2} larvae of one of the mosquito species were added. To both trays 10 mg of spores of batch 4 were added.
Third, a comparison was made between two modes of spore application, application of dry spores or spores suspended in Shellsol oil. Two plastic trays were filled with 1 liter of acclimatized water and to each tray 250 L_{1-2} larvae of *An. stephensi* were added. To one tray 10 mg of dry spores of batch 4 were added and to the other tray the same amount of spores of batch 4 suspended in 200 µl Shellsol oil, in order to see which mode of application is the most efficient in killing larvae.

Sex ratio
In total, 21 trays were filled with 1 liter of acclimatized water after which 250 one-day old larvae were placed in each tray. In eighteen trays, larvae were continuously exposed to spores of *B. bassiana* batch 1, the most virulent batch, by dusting 10 mg of dry spores on the water surface at the start of the experiment. No spores were added to the remaining three trays. Inside the experimental climate cell, non-exposed and fungus exposed trays needed to be kept separate in order to prevent spores from being blown from one tray to another by the ventilation system. Two different pipettes were used to collect pupae from non-exposed and fungus exposed trays, as well as two aspirators by which non-exposed and fungus exposed mosquitoes could be transferred. Larvae were fed according to the adjusted food schedule (table 3 in the appendix). Pupae were daily collected from each tray and placed in holding buckets or holding cages.
All mosquitoes emerging from non-exposed and fungus exposed trays were counted and the gender of each mosquito was determined in order to compare the sex ratio of non-exposed and larval exposed mosquitoes.

Survival
The effect of larval exposure on lifetime of surviving mosquitoes was tested by comparing lifetime of non-exposed and larval exposed mosquitoes. From the emerged mosquitoes fifty non-exposed and fifty larval exposed females were selected within 0-1 days and placed individually in paper cups ($Ø_{Top}$ = 7,5 cm, $Ø_{Bottom}$ = 5 cm and height = 8 cm) closed with netting and a rubber band, in order to monitor daily survival for each female. Cotton wool was soaked daily in 6% glucose/water solution and placed on top of the netting to provide nutrition.
Of all dead mosquitoes the right wing was removed with a forceps and pasted onto an object glass. The size of the wing was measured from the wing tip to the notch of the alula at 40x magnification using a light microscope with an eyepiece graticule. Fungal infection was determined for all dead mosquitoes according to the same method used in the 'spore viability and concentration' experiment.

Reproductive success
Pupae were placed in two holding cages, one for non-exposed mosquitoes and one for larval exposed mosquitoes. Mating was allowed for three to four days by keeping males and females together in the holding cages. After mating fifty non-exposed and fifty larval exposed females were selected and placed in paper cups closed with netting and a rubber band. Nutrition was provided by daily soaking cotton wool in 6% glucose/water solution and placing it on top of the netting of each paper cup. A blood meal was offered by placing the arms of a volunteer on top of six to eight paper cups for ten

minutes, allowing the females to blood feed. Females which did not blood feed were offered a second opportunity to take a blood meal for 10 minutes. Mosquitoes which did not blood feed after two opportunities, were excluded from the experiment. Two days after the blood meal a small oviposition cup ($Ø_{Top}$ = 4.5 cm, $Ø_{Bottom}$ = 3.5 cm and height = 3 cm) containing moist cotton wool and a filter paper was placed in each paper cup. The number of eggs were counted for each mosquito individually using a binocular microscope. Eggs were transferred to a small cup with water in which the eggs could hatch. After 2-4 days larvae were counted in order to determine the number of viable eggs. Wing length and fungal infection were both determined for all dead mosquitoes.

Mosquito survival after larval and adult exposure to *B. bassiana*

Again fifty non-exposed and fifty larval exposed females were selected and re-exposed to spores of *B. bassiana* batch 1, within 0-2 days after they emerged. Another fifty larval exposed females were also selected and exposed to Shellsol oil, within 0-2 days after emergence. A spore suspension of *B. bassiana* spores suspended in Shellsol oil with a concentration of $5.00*10^{10}$ spores/m^2 was prepared and coated onto gloss coated proofing paper using the same method as described in the ´spore viability and concentration' experiment. Papers were also coated with Shellsol oil in order to see the effect of exposure to Shellsol oil only. After exposure for 3 hours, all females were individually placed in paper cups in which survival could be monitored daily. Cotton wool soaked in a 6% glucose/water solution was again placed on top of the netting in order to provide for nutrition. Again, wing length and fungal infection were both determined for all dead mosquitoes.

Statistical analysis

Kaplan Meier analysis was used in the first experiment to pair wise compare survival curves of *An. stephensi* treated with different *B. bassiana* concentrations. A log rank test was performed in order to check for equality of survival distributions of the replicates. Only the replicates of treatment 2 were not equal and therefore replicate 2.1 was excluded from the analysis. All other replicates were pooled for each treatment.

Binary logistic regression was used in the second experiment in order to analyze the relation between temperature, exposure time and the interaction and spore viability. Binary logistic regression was used because germination of spores was noted by '0' if the spore did not germinate and '1' when it did germinate.

In the third experiment a Mann-Whitney U test was used to compare pupation in non-exposed and fungus exposed trays, since data were not normally distributed. Deviations in sex ratio were tested with a Binomial exact test with a test value of 0.5 in order to test whether the sex ratio deviated from 1:1. Kaplan Meier analysis was used again in order to compare survival curves of non-exposed and larval exposed *An. stephensi* females. Non normal data on wing length of these two groups were compared with a Mann-Whitney U test. In addition, the linear relation between survival and wing length was analyzed with Cox regression. Differences in blood feeding propensity of non-exposed and fungus exposed *An. stephensi* females and differences in the proportion of blood feeding females between donors were tested with Chi-square tests. Furthermore a Chi-square test was used to test for the difference in the proportion of females that laid eggs between non-exposed and fungus exposed females. Median number of eggs and larvae of non-exposed and fungus exposed females were compared with a Mann-Whitney test because these data were also not normally distributed. Differences in egg quality expressed by the proportion of hatched eggs was tested with a Chi-square test. Again differences in median wing length were compared using a Mann-Whitney U test. Using general linear models, the relation between the produced number of eggs and wing length was analyzed.

In the fourth experiment, Kaplan Meier survival analysis was used again to compare survival curves of larval & adult exposed *An. stephensi* females with females which were only exposed during the adult stage.
SPSS version 17 software was used for data analysis of all experiments.

Results

Spore viability and concentration

The effect of spore viability and concentration on mortality of *An. stephensi* females was tested at different concentrations of two batches of *B. bassiana*. In all treatments, survival distributions of the replicates were equal, except for treatment 2 (Log rank statistic = 8.789; df = 3; p = 0.032). In order to have equal replicates for treatment 2, replicate 1 was excluded from the analysis (Log rank statistic = 0.619; df = 2; p = 0.734). Figure 5 represents survival of mosquitoes in all replicates pooled over the six treatments. Significance is represented by capital letters next to the treatments. Survival of mosquitoes exposed to *B. bassiana* in all treatments is significantly lower than control mosquitoes ($p < 0.001$). With mosquitoes in treatment 1 surviving up to 33 days and mosquitoes in treatments 2 to 6 surviving up to 10 days. There was no significant difference in survival of mosquitoes exposed to batch 4 (treatment 2) and batch 2 (treatment 3) with equal spore concentrations of $5.00*10^{10}$ sp/m^2 (Log rank statistic = 0.215; p = 0.643). Therefore, it was not possible to determine whether low spore viability can be compensated by increasing the spore concentration, because this can only be determined when there is an initial difference in mosquito mortality between two batches of different quality (batch 2 and batch 4) but with equal starting concentrations.

Levels of infection were determined by use of a fungal sporulation test for all dead mosquitoes from all treatments. For treatment 1 to 6 mean infection percentages were respectively 3.7; 98.4; 97.4; 99.2; 94.0 and 96.1% out of the total number of dead mosquitoes per treatment.

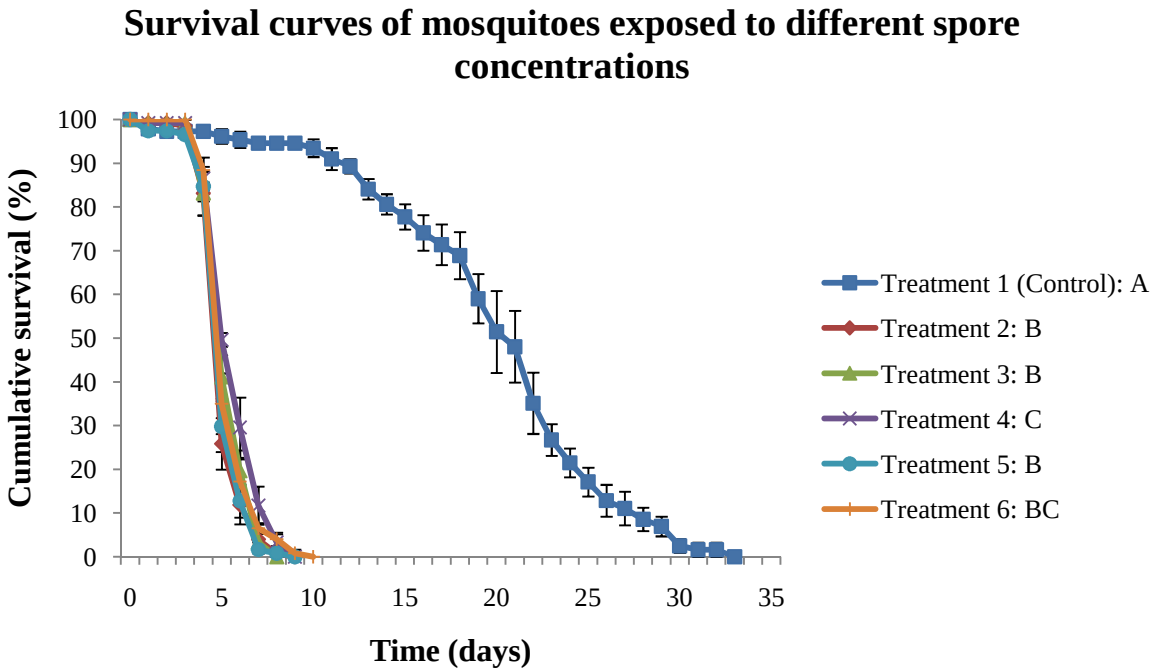

Figure 5: Cumulative survival of *An. stephensi* females exposed to two batches of *B. bassiana* with viability of 59 ±1% and 89 ±1% and different spore concentrations. Error bars represent the standard error of the mean.

Temperature and exposure time

Viability of *B. bassiana* batch 4 spores used in this experiment was determined at 89 ±1% when spores were stored at 4°C and incubated for 16 hours at 27°C. The effect of temperature and exposure time was tested in duplo at 6 different temperatures and 2 exposure times. Figure 6 shows that spore

viability decreased when the temperature was increasing and when exposure time was extended. Both temperature (Binary logistic regression; Wald = 1068.615; df = 1; $p < 0.001$) and exposure time (Binary logistic regression; Wald = 12.745; df = 1; $p < 0.001$) had a significant negative effect on spore viability. The two lines in the graph are not parallel to each other indicating that there was an interaction effect between temperature and exposure time. This means that there was a stronger negative effect of temperature for longer exposure time (24h) than for shorter exposure time (6h) . When testing for this interaction effect it was significant (Binary logistic regression; Wald = 63.229; df = 1; $p < 0.001$).

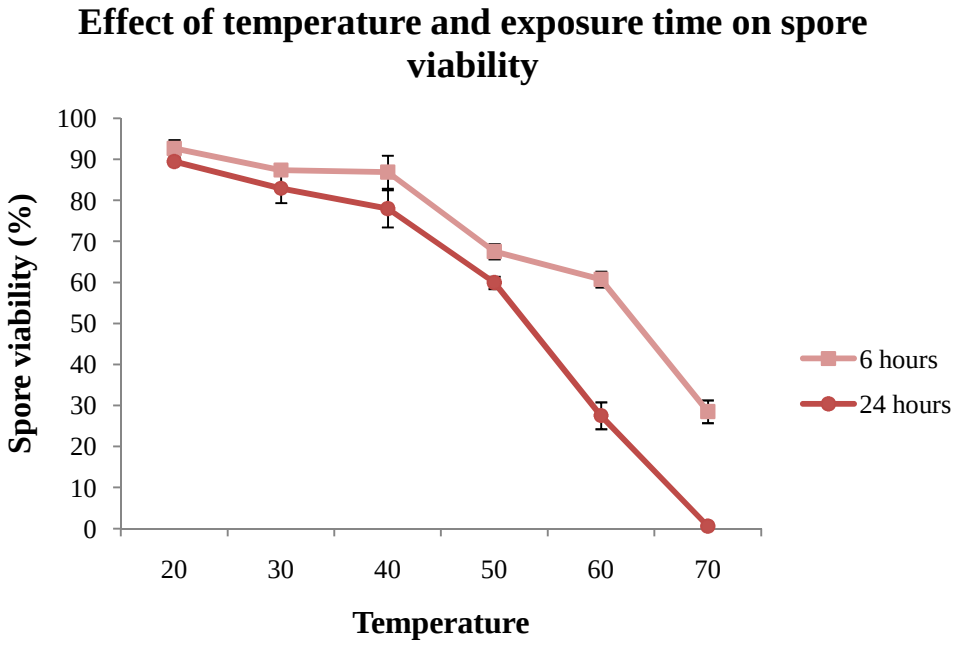

Figure 6: The effect of temperature and exposure time on *B. bassiana* spore viability. Spore viability was determined twice for each combination of temperature and exposure time. Error bars represent the standard error of the mean.

Mosquito fitness after larval exposure to *B. bassiana*

Pilot studies

In the first pilot experiment virulence of five *B. bassiana* batches was tested. By comparing the effect of *B. bassiana* batches 1, 3, 4, 9 and 22 on larval mortality, it appeared that batch 1 is the most virulent since the number of pupae is the lowest, although this was not the batch with the highest viability. Based on this result batch 1 was used in the mosquito fitness experiments.

In the second pilot experiment, *An. gambiae* was more susceptible to *B. bassiana* than *An. stephensi* since the number of *An. gambiae* pupae (6.8%) was lower than the number of *An. stephensi* pupae (23.2%). This result is consistent with the results found by Bukhari et al. (2010) that proportional pupation of fifty $L_{1\text{-}2}$ larvae of *An. gambiae* (0-1%) was lower than pupation of *An. stephensi* larvae (0-13%). Therefore *An. stephensi* was used in all the experiments since it was more likely that results found with *An. stephensi* can also apply for *An. gambiae* than vice versa. Furthermore for logistical reasons, it was hard to use *An. gambiae* because of the large number of larval exposed mosquitoes which were needed in the experiments. The number of plastic trays needed in the experiment would simply be too high when pupation is very low.

In the third pilot experiment the mode of spore application was tested. This pilot experiment showed that application of dry spores (23.2% pupation) was more efficient in killing larvae compared to

application of spores suspended in Shellsol oil (81.6% pupation). Dry spores were added to the trays in the following experiments. Bukhari et al. (2010) found that there were no significant differences between 5, 10 or 20 mg of dry spores applied to trays with 50 L_{1-2} *An. stephensi* larvae.

Sex ratio

One-day old larvae of *An. stephensi* were continuously exposed to *B. bassiana* during the larval stage. In trays exposed to *B. bassiana* high larval mortality significantly reduced the proportion of pupation per tray to 11.7% compared to 87.3% in non-exposed larvae (Mann-Whitney U; Z= -2.715; p = 0.007).

In total 519 mosquitoes emerged from the three trays which were not exposed and 475 mosquitoes from the eighteen trays which were exposed to *B. bassiana*. From non-exposed trays 243 males and 276 females emerged which was 46.8% and 53.2% respectively and from exposed trays 202 males and 273 females emerged which was 42.5% and 57.5% respectively (figure 7). For non-exposed mosquitoes there was no significant deviation from a 1:1 sex ratio (Binomial test; Test prop. = 0.5; p = 0.160), but for larval exposed mosquitoes there was a significant difference (Binomial test; Test prop. = 0.5; p = 0.001).

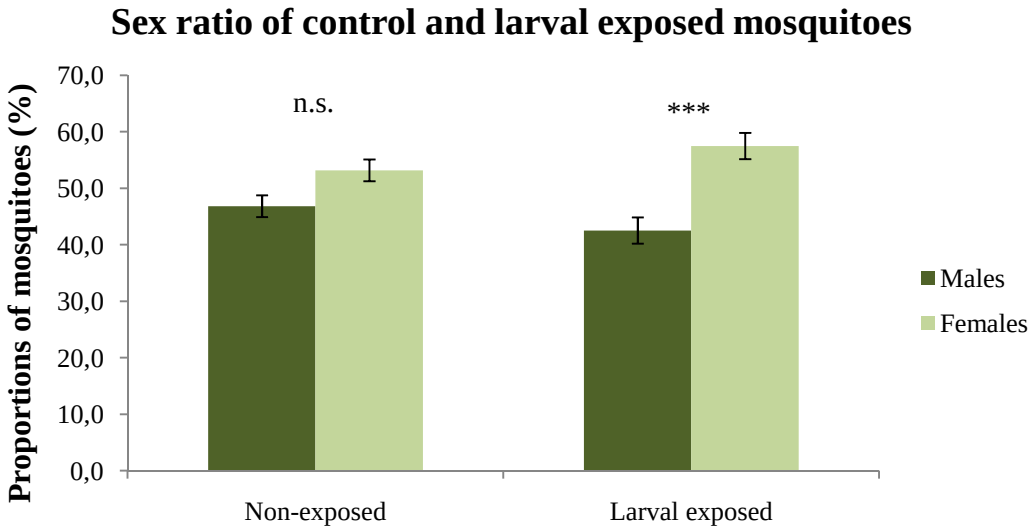

Figure 7: Percentage of males and females that emerged from non-exposed and trays which were exposed to *B. bassiana*. Asterisks represent significant differences in sex ratio tested with a Binomial test. N.s., no significance; ***, p < 0.001.

Survival

Fungal infection was determined for all mosquitoes and used to determine the effect of exposure to *B. bassiana* on survival. Fungal infection in non-exposed mosquitoes, larval exposed, larval & adult exposed and adult exposed mosquitoes was 0, 14, 94 and 98%, respectively (Figure 8). Out of the 11.7% of mosquitoes which survived exposure to *B. bassiana* during the larval stage, fifty females were selected from which 86% was not infected and 14% was still carrying the infection in the adult stage. Out of the starting number of 250 larvae per tray, 5.79% of all larvae developed in non infected females and only 0.94% developed in females infected with *B. bassiana*. Out of trays which were not exposed to *B. bassiana* 46.44% emerged as non infected females.

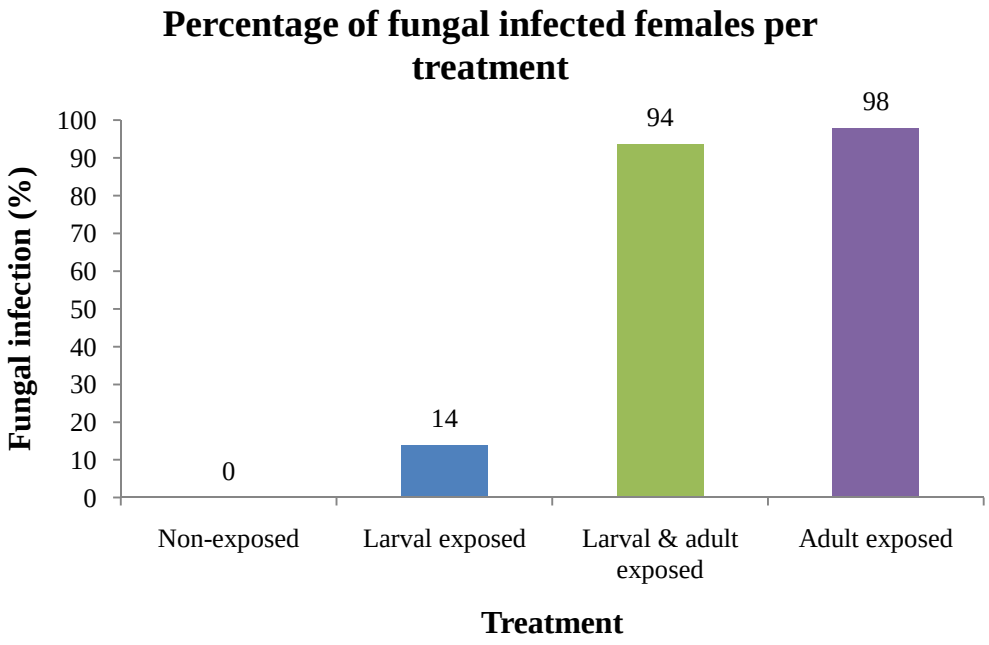

Figure 8: Percentage of *An. stephensi* females in which fungal sporulation was determined after 5 days of incubation at 27°C.

Females which were exposed to the fungus during the larval stage could be split in two groups. In one group fungal infection was not detected with the sporulation test and in the other group infection was detected. With Kaplan Meier pairwise comparison survival of these two groups was compared with mosquitoes which were not exposed to *B. bassiana* during the larval stage (Figure 9). There was no significant difference in survival between mosquitoes which were not exposed to *B. bassiana* during the larval stage and mosquitoes which were exposed but were not infected (Log rank statistic = 0.262; p = 0.609). Survival of larval exposed mosquitoes which were infected was significantly lower compared to both non-exposed (Log rank statistic = 45.770; $p < 0.001$) and larval exposed mosquitoes which were not infected (Log rank statistic = 32.933; $p < 0.001$).

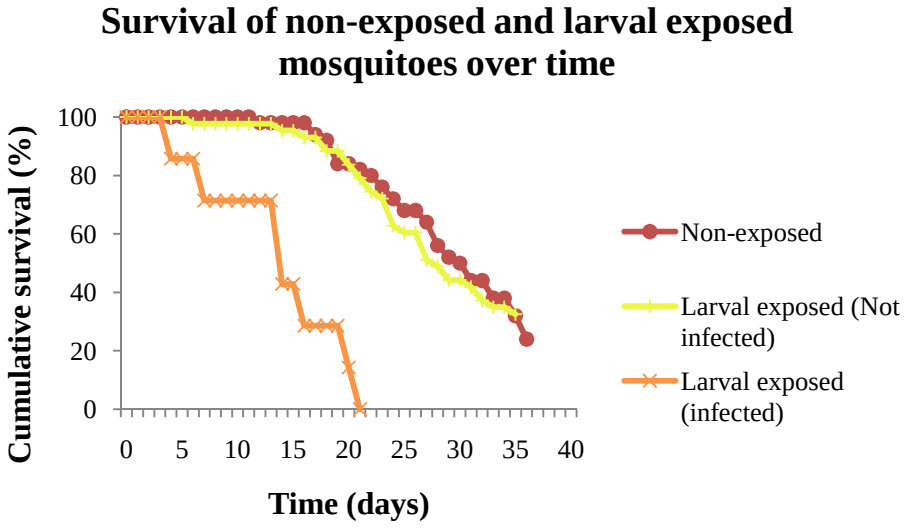

Figure 9: Cumulative survival of non-exposed and *An. stephensi* females exposed to 10 mg of *B. bassiana* during the larval stage. Females which were still alive after 35 days were included in the curves.

Exposure to *B. bassiana* during the larval stage had an influence on size of surviving mosquitoes which is represented by wing length in figure 10. Median wing length of both non infected and infected mosquitoes which were exposed to *B. bassiana* during the larval stage was significantly larger compared to non-exposed mosquitoes (Not infected: Mann-Whitney U; Z = -7.649; p < 0.001; infected: Mann-Whitney U; Z = -3.803; p < 0.001). Within both larval exposed groups of non infected and infected mosquitoes there was no significant difference in wing length (Mann-Whitney U; Z = -1.054; p = 0.308).

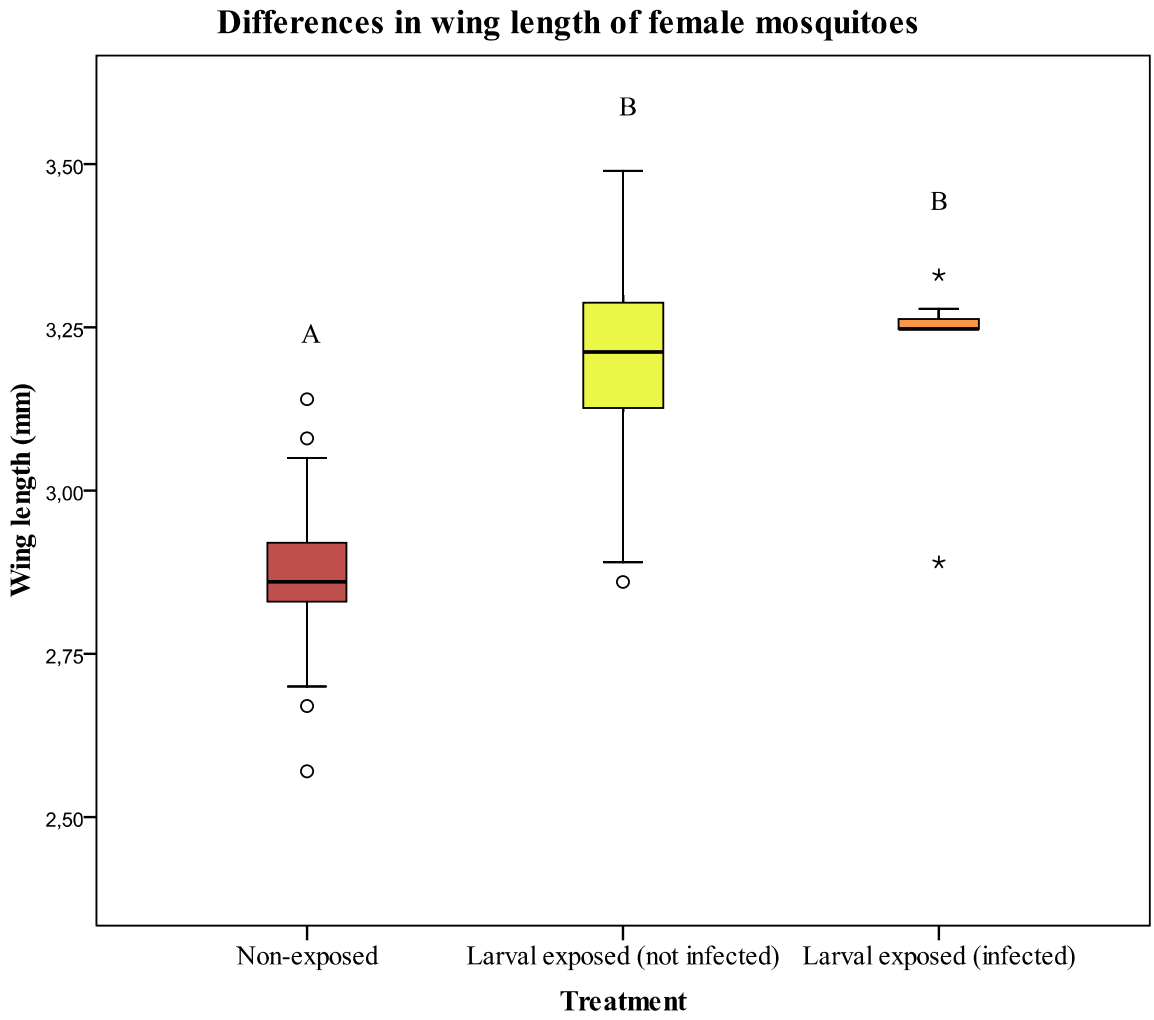

Figure 10: Boxplots of wing length of non-exposed and larval exposed *An. stephensi* females which were either not infected or infected with *B. basssiana*, from which survival was monitored. Boxes represent the lower quartile (25%), median and upper quartile (75%) and whiskers the minimum and maximum wing length. Outliers are indicated by open circels and extreme values by asterices. Different capital letters represent significance between treatments.

Analysis with Cox regression showed that there was no significant relation between wing length and survival of non-exposed mosquitoes (Cox regression; Wald = 2.850; df = 1; p = 0.091), while there was a significant relation for larval exposed mosquitoes, although this was borderline significance (Cox regression; Wald = 3.855; df = 1; p = 0.050). After removal of one outlying mosquito (which had a small wing length and short lifetime) from the analysis, the difference was no longer significant (Cox regression; Wald = 2.737; df = 1; p = 0.098). This relation was also tested separately for non infected and infected mosquitoes which showed that non infected mosquitoes did have a significant relation (Cox regression; Wald = 4.954; df = 1; p = 0.026), but infected mosquitoes did not have a

significant relation (Cox regression; Wald = 0.928; df = 1; p = 0.335). When further investigating the correlation of wing length with survival of non infected mosquitoes, the correlation appeared to be weak although it was significant (Spearman; correlation coefficient = 0.434; p = 0.004).

Reproductive success

Out of 50 non-exposed and 50 larval exposed mosquitoes which were offered a blood meal, only one non-exposed and three larval exposed mosquitoes did not take up any blood. Furthermore, 44 non-exposed mosquitoes took a blood meal the first time and five mosquitoes took up blood the second time. For larval exposed mosquitoes, 42 mosquitoes took blood the first time and five the second time a blood meal was offered. Blood feeding propensity was not influenced by exposure to *B. bassiana* during the larval stage (Chi square = 1.047; df = 2; p = 0.593).

Donor also did not have an effect on blood feeding propensity since there were no significant differences in the total number of mosquitoes which took a blood meal from each of the donors (Chi square = 1.313; df = 2; p = 0.519) and there was no relation between donor and the first or second time a mosquito took a blood meal (Chi square = 2.178; df = 2; p = 0.337).

Out of 49 non-exposed females, 38 larval exposed, but uninfected females and nine larval exposed females that were infected, which all took up blood, respectively 81,63%, 78,95% and 77,78% of females laid eggs. There was no significant difference in the proportion of mosquitoes in each treatment which have laid eggs (Chi square = 0.134; df = 2; p = 0.935).

Figure 11 shows at which day after the blood meal females from both treatments laid eggs. Both non-exposed and larval exposed females started to lay eggs 3 days after they had a blood meal. For non-exposed mosquitoes, the last female laid eggs at day 13 and for larval exposed mosquitoes the last female laid eggs at day 21. By counting the number of larvae, it appeared that eggs laid after day 11 did no longer hatch.

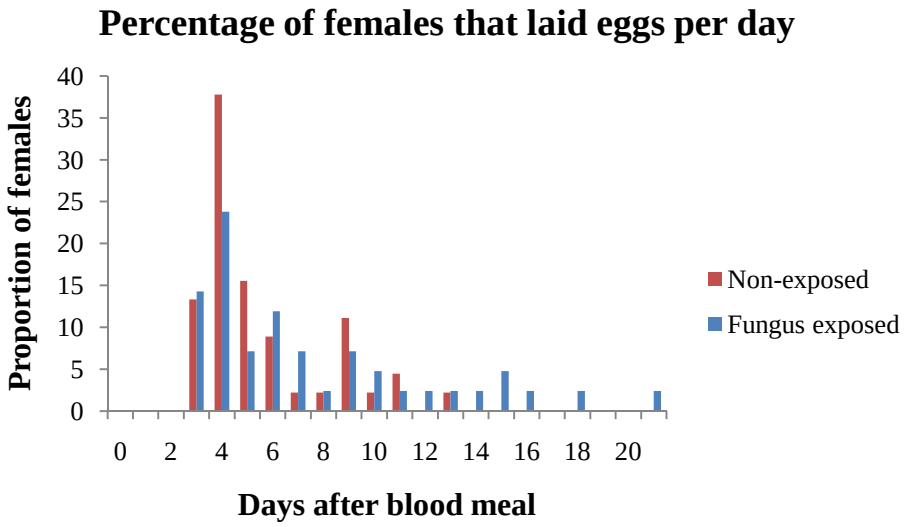

Figure 11: Percentages of non-exposed and larval exposed *An. stephensi* females which have laid eggs per day. All females have taken a blood meal at day 0.

When comparing the median number of eggs laid per female (figure 12), only those females which laid eggs were included in the analysis. The median number of eggs laid by a larval exposed female was significantly higher than a female which was not exposed to *B. bassiana* (Mann-Whitney U; Z = -

4.706; p < 0.001). However, there was no significant difference in the number of larvae of non-exposed and larval exposed females (Mann-Whitney U; Z = -1.082; p = 0.279).

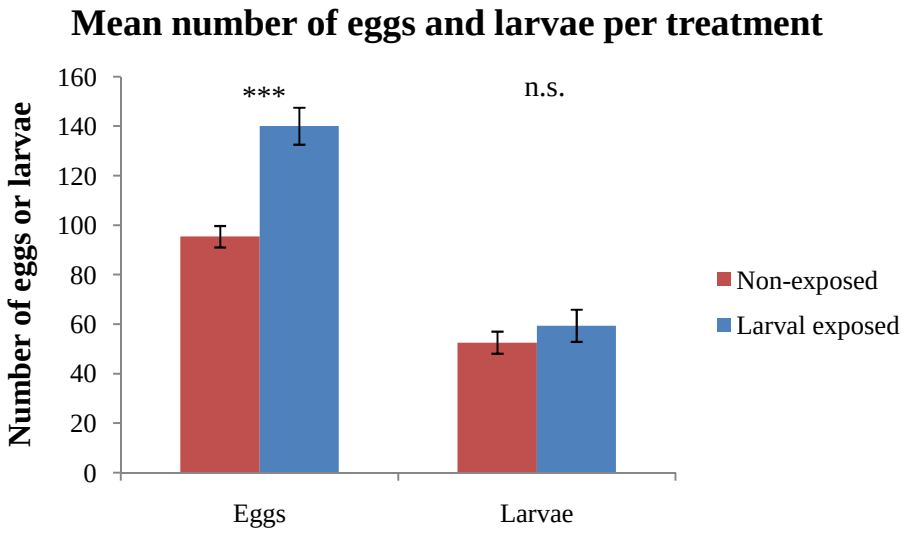

Figure 12: Mean number of eggs and larvae per treatment. All data of *An. stephensi* females which laid eggs were included. Asterisks represent significance tested with a Mann-Whitney U test. N.s., no significance; ***, p < 0.001.

This indicated that the quality of eggs of larval exposed mosquitoes might be lower than eggs of non-exposed mosquitoes, because there was a significantly lower percentage of hatched eggs. This result could be biased by the fact that all eggs laid after 11 days did not hatch anymore. Therefore the data on larvae from mosquitoes that laid eggs after day 11 were excluded from the next analysis in order to see whether these larvae were the explanation for the difference in egg quality (figure 13). By excluding those data, there was a significant difference in the median number of larvae between both treatments (Mann-Whitney U; Z = -2.712; p = 0.007). Average quality of eggs laid by larval exposed females was lower compared to non-exposed females since there was a significantly lower fraction of eggs that hatched, 42.38% compared to 55.10% respectively (Chi square = 40.235; df = 1; p < 0.001).

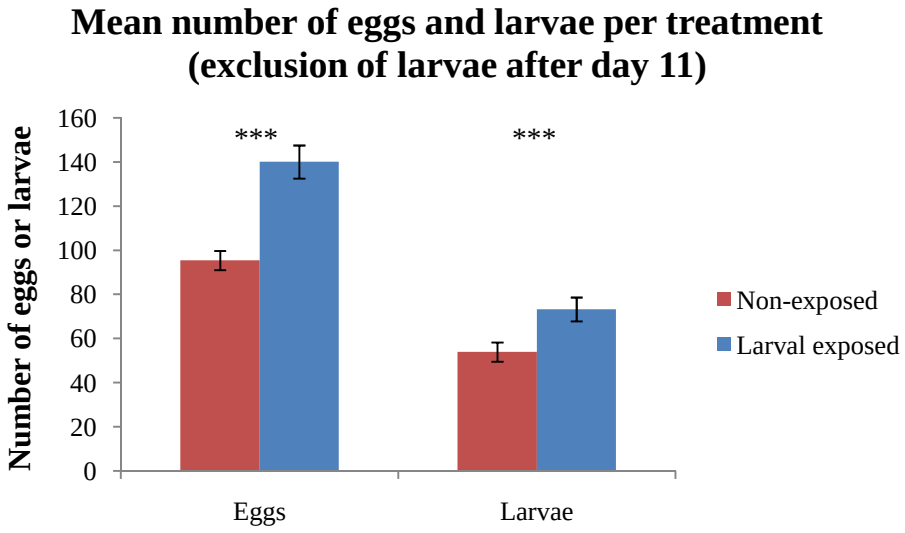

Figure 13: Mean number of eggs and larvae per treatment. Data of eggs laid by *An. stephensi* females 11 days after the blood meal, which did not hatch, were excluded. Asterisks represent significance tested with a Mann-Whitney U test. ***, p < 0.001.

When the larval exposed group was divided in 2 groups of 'non infected' and 'infected' females (figure 14), there was a significant difference in median number of eggs between non-exposed and larval exposed mosquitoes which were not infected (Mann-Whitney U; Z = -4.973; p < 0.001) and a significant difference in median number of larvae between non-exposed and larval exposed mosquitoes which were not infected (Mann-Whitney U; Z = -2.853; p = 0.004).

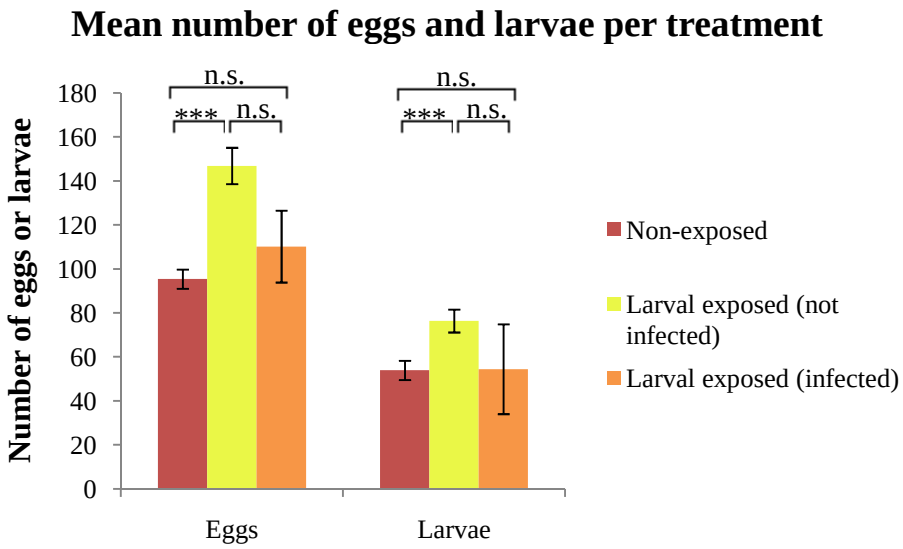

Figure 14: Mean number of eggs and larvae per treatment. Larval exposed *An. stephensi* are split in two groups of 'not infected' and 'infected' females. Asterisks represent significant differences between treatments tested with a Mann-Whitney U test. N.s., no significance; ***, p < 0.001.

Again there was an influence of exposure to *B. bassiana* during the larval stage on mosquito size, expressed by wing length (figure 15). In this experiment there was only a significant difference between non-exposed and larval exposed females which were not infected with *B. bassiana* (Mann-Whitney U; Z = -6.059; p < 0.001). The pattern in median wing size of each group was equal to the pattern of mean number of eggs of the three groups. In both there was only a significant difference between non-exposed and larval exposed females which were not infected.

When testing for the relation between mosquito size (wing length) and number of eggs, only mosquitoes which had laid eggs were included in the analysis. General linear models showed no significant relation between mosquito size (wing length) and number of eggs in the total model nor in any of the three separate groups (Total model; GLM; F = 0.521; p = 0.473; Non-exposed; GLM; F = 0.170; p = 0.683; Larval exposed (not infected); GLM; F = 0.642; p = 0.430; Larval exposed (infected); GLM; F = 0.024; p = 0.884).

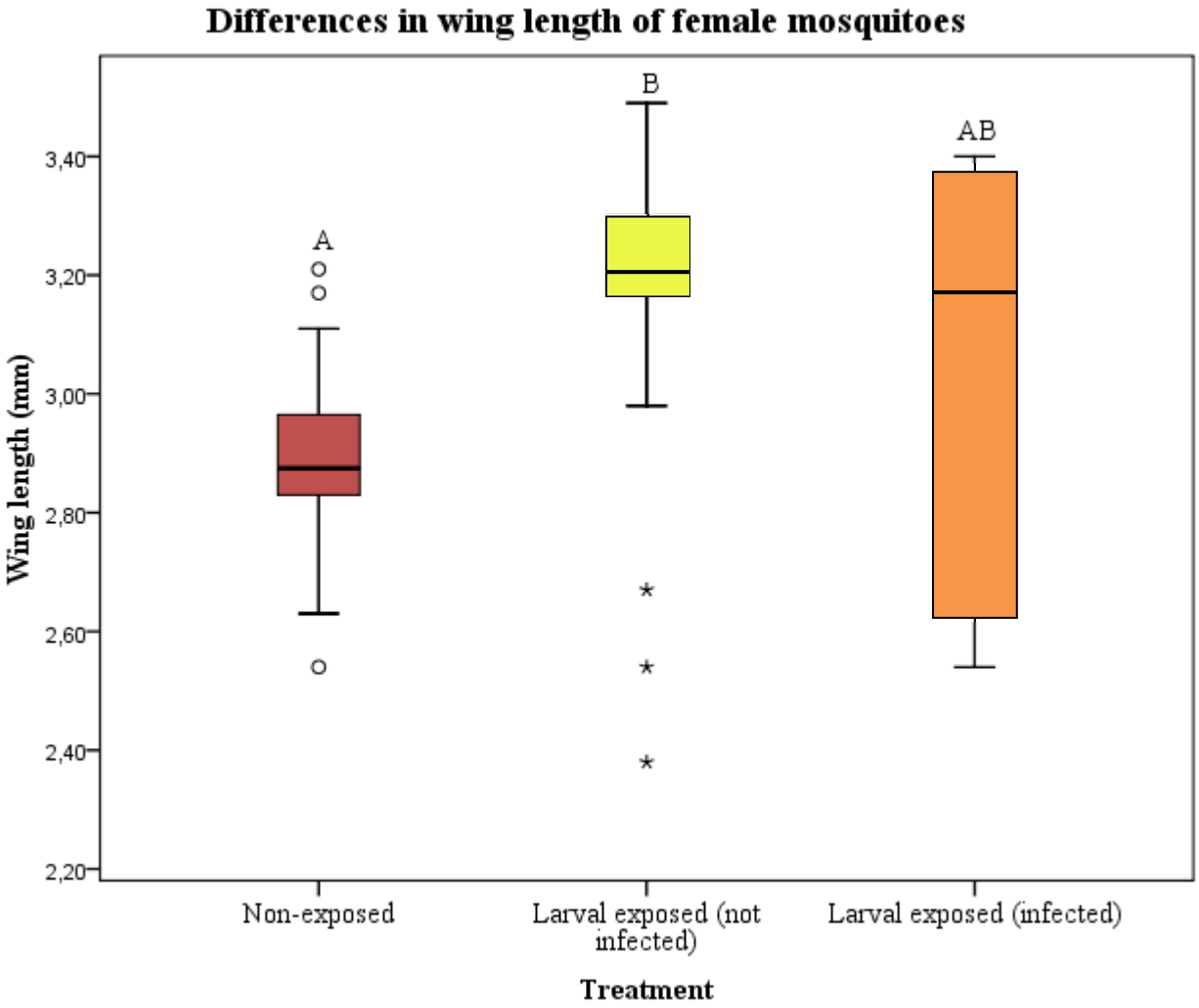

Figure 15: Median wing length of non-exposed and larval exposed *An. stephensi* females which were either not infected or infected with *B. basssiana*, from which reproductive success was monitored. Boxes represent the lower quartile (25%), median and upper quartile (75%) and whiskers the minimum and maximum wing length. Outliers are indicated by open circels and extreme values by asterices. Different capital letters represent significance between treatments.

Mosquito survival after larval and adult exposure to *B. bassiana*

In order to test whether exposure to Shellsol oil had a negative effect on survival of mosquitoes, larval exposed mosquitoes were exposed for 3 hours to gloss coated papers coated with Shellsol oil only. Compared to mosquitoes which were only exposed during the larval stage, there was no significant difference in survival (Log rank statistic = 0.562; p = 0.453).

Mosquitoes which survived larval exposure could be more susceptible or could have developed resistance against *B. bassiana* when they were re-exposed during the adult stage. Survival of mosquitoes which were only exposed during the adult stage was therefore compared with mosquitoes which were exposed during both the larval and adult stage (figure 16). There was no significant difference in survival of mosquitoes which were only exposed during the adult stage and mosquitoes which were exposed during both the larval and adult stage (Log rank statistic = 1.666; p = 0.197).

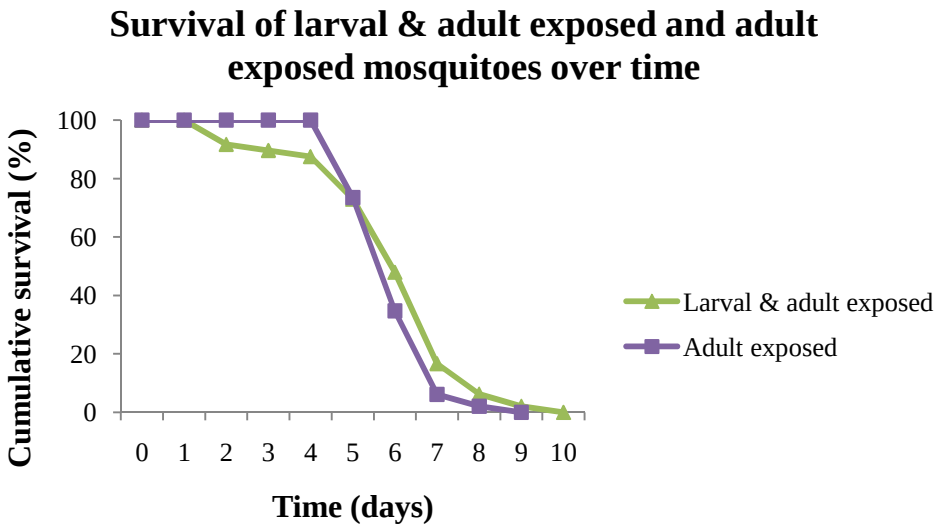

Figure 16: Cumulative survival of *An. stephensi* females which were exposed during both the larval and adult stage to *B. bassiana* and females that were only exposed during the adult stage.

Discussion

This thesis focused on spore quality of the entomopathogenic fungus *B. bassiana* by evaluating the effect of spore viability and concentration on mortality of *An. stephensi* females and the effect of temperature on spore viability. Furthermore the focus was on *B. bassiana* used for larval control of *An. stephensi*, by providing new insights in fitness of mosquitoes which had been exposed during the larval stage to *B. bassiana*. Susceptibility of *An. stephensi* was also tested by re-exposing females which had already been exposed to *B. bassiana* during the larval stage.

In this study there was no difference in mosquito mortality between two batches of *B. bassiana* with viability of 89 ±1% and 59 ±1%. In previous experiments only batches with a viability of 85% and higher were used because it was expected that virulence of batches with viability below this cut off value was lower compared to batches with relatively high viability (Farenhorst and Knols, 2010). In this experiment both batches limited survival of all females until a maximum of 10 days, which can strongly reduce malaria transmission since development of *Plasmodium* spp. takes approximately 10-14 days dependent on the species and environmental factors (Beier, 1998; Matuschewski, 2006). The study of Scholte et al. (2006) showed that blood feeding propensity of *An. gambiae* females infected with *Metarhizium anisopliae* was reduced by the fungus, which lowers the chance of malaria transmission even more. Findings of Blanford et al. (2005) support that the same applies for *An. stephensi* females infected with *B. bassiana.* The combination of survival reduced to a maximum of 10 days, together with reduced blood feeding propensity of females infected with *B. bassiana* could lead to a much lower malaria transmission compared to non infected females.

The effect of increased spore concentration in order to compensate for relatively low spore viability could not be displayed by this experiment. This effect can only be evaluated when there is a difference in mosquito survival when mosquitoes are treated with the same concentration of the two batches, which was not the case in this experiment. In order to test whether spore viability can be compensated by higher spore concentrations, two batches with a larger difference in viability should be used in future research.

A small proportion of mosquitoes in the control (treatment 1) was infected with *B. bassiana* although these mosquitoes should not be infected. The same aspirator was used to transfer mosquitoes from all treatments between holding buckets and PVC tubes. This could have led to infection of the small percentage of the control mosquitoes. In order to prevent infection of control mosquitoes, special measures regarding spore application and use of pipettes and aspirators need to be taken when working with fungi. After implementing these measures, they were successful since no control mosquitoes in the following experiments were infected.

Temperature and exposure time were shown to have a major impact on spore viability. In contrary to continuous exposure to a certain temperature in this experiment, spores are exposed daily to fluctuating temperatures in the field. Peak temperatures in traditional grass-thatched and corrugated iron-roofed houses in Western Kenya, have been measured of 30 and 37°C respectively (Okech et al., 2004). Viability will decline when spores are exposed to these temperatures for long periods of time, which has already been shown after 24 hours of continuous exposure. Furthermore, under field conditions spores are not only exposed to high temperatures but also to changing humidity and UV radiation. Both factors have a negative influence on spore viability and therefore spore viabilities found in this study are optimistic compared to spore viability expected to be found in the field (Zimmermann, 2007).

Interestingly, although spore viability declines under field conditions, it appears that even spores with a relatively low viability are still virulent, as has been shown by the first experiment. In that case, spores can be used for longer periods of time in the field without losing virulence. However, virulence of spores which were exposed to high temperatures was not tested in a bio-assay and therefore further research on the influence of environmental conditions on spore virulence is necessary.

In the third pilot experiment high pupation was found in trays to which *B. bassiana* spores suspended in Shellsol oil were added, which is in contrast to findings of Bukhari et al. (2011) (Bukhari et al., 2011). A spore suspension was prepared by suspending 20 mg of *B. bassiana* in 400 µl Shellsol oil, from which 200 µl was added to the tray. If the suspension was not mixed properly, the 200 µl could have contained only a low spore dose and therefore pupation could have been relatively high.

Bukhari et al. (2010) found pupation of 50 L_{1-2} *An. stephensi* larvae treated with different amounts of *B. bassiana* (2.5 – 20 mg), ranging between 0 – 13%. In this research pupation of 250 L_1 *An. stephensi* larvae exposed to 10 mg of *B. bassiana* ranged between 3.2 and 23.6%. It is not completely clear what has caused this difference, apart from the fact that variation in pupation between the trays is relatively high, which could be an explanation for the difference. Another explanation is the difference in larval density, 50 compared to 250 larvae per tray respectively, resulting in a five times lower relative amount of spores per larvae in this thesis. This lower mortality at higher larval density has also been shown with *Aedes aegypti* exposed to *Leptolegnia chapmanii* (Pelizza et al., 2007).

Sex ratio of larvae exposed to fungus showed a shift towards a higher number of females. If female larvae are stronger than males it would be likely that more females were able to survive exposure to *B. bassiana*. On the other hand, a similar trend towards a higher number of females was visible in the sex ratio of non-exposed mosquitoes. Latter sex ratio was determined for a lower number of trays (three instead of 18) and there might have been a significant difference when a larger number of trays was used.

Exposing *An. stephensi* larvae to *B. bassiana* greatly reduced the number of emerging adult mosquitoes. From the 250 larvae per tray at the start of the experiment, 5.79% developed in non infected females which could live for more than 35 days and 0.94% developed in infected females which could live up to 21 days, compared to 46.44% of non-exposed females which could live for more than 35 days. When comparing the potential growth of two mosquito populations starting with 1000 non-exposed and 1000 fungus exposed larvae, then the non-exposed population is able to grow up to 25 times its initial size after one generation, compared to population growth of only five times in the fungus exposed population. In the laboratory, mosquito populations which were not treated with fungus during the larval stage were therefore able to increase in size five times faster than larval exposed populations. If population growth can be inhibited in the field this could result in smaller mosquito populations and therefore reduced malaria transmission. However, 5.79% of females being exposed to *B. bassiana* during the larval stage were still able to live as long as ‘normal’ mosquitoes. This could have implications for malaria control since transmission is not completely stopped.

The sporulation test was not 100% accurate since fungal infection could only be demonstrated when the fungus was growing out of the dead mosquito. The fungus was not detected in mosquitoes in which the amount of fungus was not sufficient to cause or detect sporulation. PCR used to detect fungal infection would be more accurate but is also more labor intensive and much more expensive (Bell et al., 2009). For mosquitoes which were exposed to *B. bassiana* during the adult stage, infection rates were very high since mosquitoes were forced to come into contact with the fungus in

the bio-assay. It is unclear whether mosquitoes which were exposed during both the larval and adult stage, carried the infection from the larval stage since the same, genetic identical, *B. bassiana* batch was used for both larval and adult exposure. This distinction can only be made by using two genetically different strains of *B. bassiana* or two different fungus species for exposure during the larval and adult stage, after which presence of fungus can be demonstrated by use of PCR. This can be useful in order to see differences in survival of mosquitoes which were infected during both times they were exposed to the fungus or only once during the adult stage.

When wing length of non-exposed and larval exposed mosquitoes was compared, it can be concluded that non-exposed females are smaller than larval exposed females. When exposing larvae to *B. bassiana* it could be that only the largest larvae are able to persist exposure to the fungus, resulting in selection of only the relatively large larvae which were able to survive. Such selection has not occurred in trays in which no spores were applied, so relatively small mosquitoes also emerged. However, larval density in fungus treated trays was eventually lower compared to non-exposed trays, resulting in lower competition between larvae for food and space and therefore on average larger mosquitoes, as already has been demonstrated with *Anopheles dirus* (Kitthawee et al., 1990).
Wing length does not appear to have an influence on mosquito survival because non-exposed and larval exposed females which were not infected were both able to live for more than 35 days, despite their difference in wing length. Larval exposed females which were infected had a shorter lifetime which was probably due to the fungus infection since there was no influence of wing length on survival. Only within the group of larval exposed mosquitoes which are not infected there was a significant relation between wing length and mosquito lifetime. In addition, the correlation between wing length and survival was tested which also was significant. Despite this statistical significance, the correlation coefficient was very weak and there were no differences in lifetime visible between the groups, suggesting that there was no biological relation between wing length and survival which is in line with findings of Walker et al. (1987) and Manoukis et al. (2006) (Manoukis et al., 2006; Walker et al., 1987). However, this is in contrast to previous studies which did show the positive relation between mosquito size and survival (Hawley, 1985; Kitthawee et al., 1990).

Eight days after the last non-exposed mosquito laid eggs there was still one larval exposed mosquito which laid eggs. This could be due to the fact that egg development in larval exposed mosquitoes took longer or it could also be a coincidence. More replicates are necessary in order to see whether exposure to fungus can delay egg development. The design of the experiment probably also influenced the day at which eggs were laid. Oviposition cups placed in paper cups of blood fed mosquitoes were small and water evaporated fast, so each day water had to be added to the cups. Egg laying could have been delayed when oviposition cups were not sufficiently moist. However, this does not explain the differences between the last day at which non-exposed and fungus exposed females laid eggs, since the experimental design was equal for both.

Reproductive success expressed by the number of eggs laid per female was highest for larval exposed mosquitoes. Quality of eggs of larval exposed mosquitoes was on the other hand lower compared to non-exposed mosquitoes since a lower proportion of eggs had hatched, even when data from the eggs which did not hatch 11 days after the blood meal were excluded from the analysis.
The difference in reproductive success between non-exposed and larval exposed mosquitoes was caused by the higher number of eggs laid by larval exposed mosquitoes which were not infected with *B. bassiana*. The same pattern was visible when looking at wing length. Wing length was only significantly higher for larval exposed females which were not infected with *B. bassiana* than wing length of non-exposed females. This indicates that wing length and thus mosquito size had an

influence on the number of eggs which are laid by a female. Larger females were able to take up a larger quantity of blood and could therefore be able to produce more eggs (Briegel, 1990). When testing for the linear correlation between wing length and the number of eggs laid by a female there appeared to be no significant relation, although there appears to be an association between wing length and the number of eggs.

Exposure to Shellsol during the adult stage did not reduce survival since larval exposed mosquitoes exposed to Shellsol were able to live as long as mosquitoes which were exposed to *B. bassiana* during the larval stage and to clean papers (without fungus) during the adult stage. In addition, transferring mosquitoes from holding buckets to PVC tubes and again back to the holding bucket did also not seem to have a negative influence on survival.

Mosquitoes which survived exposure to *B. bassiana* were still susceptible when being re-exposed during the adult stage. In other words, mosquitoes which were exposed during the larval stage did not become resistance against *B. bassiana*. In addition there were also no negative effects caused by fungus exposure during the larval stage. These results show that larval and adult control with *B. bassiana* can complement each other and can both be implemented in integrated vector management without having a negative influence on each other.

Entomopathogenic fungi are a promising new tool for control of malaria mosquitoes in the future. In these laboratory experiments only the malaria vector *An. stephensi* was used but results could also apply for other *Anopheles* species such as *An. gambiae*, since the number of emerging *An. gambiae* mosquitoes being exposed to *B. bassiana* during the larval stage is even lower (Bukhari et al., 2010). However, in the field differences in ecology between *An. stephensi* and *An. gambiae* have large consequences for application of fungus in larval habitats. *An. stephensi* mainly breeds in containers, tanks, ponds and pools in urban areas while *An. gambiae* breeds in more diverse habitats ranging from small water bodies such as small pools, rain pools and tire tracks to large water bodies such as rice fields and flood plains (Dash et al., 2007; Keiser et al., 2004; Kumar and Thavaselvam, 1992; Majambere et al., 2008; Sharma, 1996; Walker and Lynch, 2007). Especially during the wet season the number of breeding sites of *An. gambiae* is high because of the high number of small water bodies, making larval control operationally difficult (Collins and Paskewitz, 1995; Fillinger et al., 2003). Large water bodies on the other hand, make larval control with entomopathogenic fungi also complicated since the area which needs to be treated might be too large for fungus application, as has already been shown with application of *Bacillus thuringiensis* (Majambere et al., 2010). *An. stephensi* is therefore more suitable for larval control with entomopathogenic fungi because the number of breeding sites is less influenced by environmental conditions and water bodies are defined and better accessible.

The advantage of larval control is that larvae cannot move from one site to another and cannot avoid control interventions compared to adult mosquitoes which are able to move freely (Killeen et al., 2002). Another advantage is the low toxicity of fungi compared to chemical larvicides, which decreases harmful effects on the environment, humans and non-target species (Walker and Lynch, 2007). Risk of resistance development is low because of the slow-acting and complex mode of action of fungi (Walker and Lynch, 2007; Zimmermann, 2007). Except for complications with application of fungi, another negative aspect regarding the use of entomopathogenic fungi is low spore persistence in the field, since exposure to environmental factors reduces spore viability (Zimmermann, 2007). As a result spores need to be reapplied after a certain period of time, which is labor intensive and results in

higher costs compared to control tools which could be applied for longer periods of time (Walker and Lynch, 2007).

In the future entomopathogenic fungi should be implemented in malaria control programs. Use of *B. bassiana* in the laboratory has been shown to be promising in the control of malaria mosquitoes. In the future research needs to be focused on field trails in which entomopathogenic fungi are applied in a large variety of larval habitats. This research is necessary in order to optimize spore formulations and to select mosquito species for which larval control with fungi can contribute to lower malaria transmission.
I believe that in the future entomopathogenic fungi, in combination with other malaria control tools, can result in the control of malaria worldwide!

Conclusions

This study showed that the difference in spore viability of 89 ±1% and 59 ±1% of two *B. bassiana* batches is not reflected in lifetime of *An. stephensi* females. Batches with a viability lower than the cut off value of 85% were previously not used in experiments, although this study showed that lower quality batches have equal virulence.

Temperature and exposure time, as well as the interaction between both has a negative influence on spore viability. Persistence of spores in the field is therefore low, especially when humidity and solar radiation are also considered. However decline in spore viability up to 60% has been proven to have no effect on spore virulence.

The sex ratio of larval exposed mosquitoes shifted to a higher number of females. Furthermore females which have been exposed to *B. bassiana* during the larval stage only suffered from reduced survival when the infection was carried in the adult stage. In fact, the majority of females surviving larval exposure were actually not infected and were still able to live as long as non-exposed females. Larval exposed females appeared to be larger than non-exposed females which could be explained by survival of only the largest larvae in trays exposed to *B. bassiana*, or because of the lower larval densities in fungus exposed trays. Reproductive success was higher for larval exposed females which were not infected because a larger number of eggs was produced which resulted in a larger number of offspring, although overall egg quality was relatively lower compared to non-exposed females. Larval exposed females that were infected laid an equal number of eggs compared to non-exposed females. Only size of larval exposed females which were not infected appeared to be larger than non-exposed females. Therefore differences in reproductive success could be explained by mosquito size, since larger females were able to take up a larger volume of blood, resulting in production of a larger number of eggs.

Larval exposed females were still equally susceptible to re-exposure to *B. bassiana* compared to non-exposed females. Exposure to *B. bassiana* did not induce development of resistance nor did it result in negative effects on survival compared to females which have not been exposed before.

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Appendix

Table 1: Details on *Beauveria bassiana* batches 2, 3, 4 and 22.

Batch	Species	Production date	Volume PBR	Viability (21-12-2010)
2	*Beauveria bassiana*	07-04-2010	0.2 L	59.0%
3	*Beauveria bassiana*	07-07-2010	3.2 L	89.3%
4	*Beauveria bassiana*	09-04-2010	3.2 L	89.2%
22	*Beauveria bassiana*	30-09-2010	10 L	25.2%

Table 2: Spore viability; calculations of spore concentrations per treatment.

Treatment	Batch	Percentage of viable spores	Concentration (sp/m^2) of all spores	Concentration (sp/m^2) of viable spores	Total number of spores	Number of viable spores
1 (Control)	-	0%	0 sp/m^2	0 sp/m^2	0	0
2	4	90%	$5.00*10^{10}$ sp/m^2	$4.50*10^{10}$ sp/m^2	$1.875*10^9$	$1.688*10^9$
3	2	60%	$5.00*10^{10}$ sp/m^2	$3.00*10^{10}$ sp/m^2	$1.875*10^9$	$1.125*10^9$
4	2	60%	$7.50*10^{10}$ sp/m^2	$4.50*10^{10}$ sp/m^2	$2.813*10^9$	$1.688*10^9$
5	4	90%	$3.33*10^{10}$ sp/m^2	$3.00*10^{10}$ sp/m^2	$1.249*10^9$	$1.124*10^9$
6	4	90%	$5.00*10^{10}$ sp/m^2	$3.00*10^{10}$ sp/m^2	$1.875*10^9$	$1.124*10^9$

Table 3: Pilot study: food schedule

Number of pupated and dead larvae were counted during the pilot experiment in order to calculate the number of living larvae per day. The amount of food in fungus exposed trays is higher because non-exposed larvae pupated earlier and therefore the remaining number of larvae in the trays was lower.

Day	Non-exposed tray	Fungus exposed tray
1	1 drop of Liquifry	1 drop of Liquifry
2	1 drop of Liquifry	1 drop of Liquifry
3	25 mg	25 mg
4	25 mg	25 mg
5	25 mg	25 mg
6	75 mg	75 mg
7	71.5 mg	75 mg
8	52.5 mg	64 mg
9	41.5 mg	52.5 mg

Data

All data obtained during this thesis can be found in three separate excel files.

Experiment 1

File name: Data spore viability and concentration

- Tab: Treatments; overview of spore concentrations of all treatments used in this experiment.
- Tab: Data; data set on survival of mosquitoes exposed to the six treatments.
- Tab: Fungal infection; data on fungal infected females per replicate and treatment.
- Tab: Legend; explanation of used symbols and numbers in the other tabs.

Experiment 2

File name: Data temperature and exposure time

- Tab: Data viability; data on viability of spores exposed to different temperatures and for two different exposure times.
- Tab: Data SPSS; binomial representation of data from the 'data viability' tab, as it is used in SPSS.
- Tab: Legend; explanation of used symbols and numbers in the other tabs.

Experiment 3 & 4

File name: Data mosquito fitness after exposure to *B. bassiana*

- Tab: Food schedule; overview of the daily amount of food added to the trays in this experiment.
- Tab: Spores in trays; quantification of the amount of spores present in the trays during the last days of the experiment.
- Tab: Pupae; data on daily pupation of larvae in trays.
- Tab: Sex ratio; number of males and females emerging from the trays.
- Tab: Survival; combined data on survival of females used in experiment 3 and 4.
- Tab: Reproductive success; data on donor, blood meal and daily number of eggs and larvae of females used in experiment 3.
- Tab: Legend; explanation of used symbols and numbers in the other tabs.