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Fitness consequences of larval exposure to *Beauveria bassiana* on adults of the malaria vector *Anopheles stephensi*

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ABSTRACT

Entomopathogenic fungi have shown to be effective in biological control of both larval and adult stages of malaria mosquitoes. However, a small fraction of mosquitoes is still able to emerge after treatment with fungus during the larval stage. It remains unclear whether fitness of these adults is affected by the treatment during the larval stage and whether they are still susceptible for another treatment during the adult stage. Therefore, we tested the effects of larval exposure to the entomopathogenic fungus *Beauveria bassiana* on fitness of surviving *Anopheles stephensi* females. Furthermore, we tested whether larval exposed females were still susceptible to re-exposure to the fungus during the adult stage. Sex ratio, survival and reproductive success were compared between non-exposed and larval exposed *A. stephensi*. Comparisons were also made between survival of non-exposed and larval exposed females that were re-exposed to *B. bassiana* during the adult stage. Larval treatment did not affect sex ratio of emerging mosquitoes. Larval exposed females that were infected died significantly faster and laid equal numbers of eggs from which equal numbers of larvae hatched, compared to non-exposed females. Larval exposed females that were uninfected had equal survival, but laid a significantly larger number of eggs from which a significantly higher number of larvae hatched, compared to non-exposed females. Larval exposed females which were re-exposed to *B. bassiana* during the adult stage had equal survival as females exposed only during the adult stage. Our results suggest that individual consequences for fitness of larval exposed females depended on whether a fungal infection was acquired during the larval stage. Larval exposed females remained susceptible to re-exposure with *B. bassiana* during the adult stage, indicating that larval and adult control of malaria mosquitoes with EF are compatible.

1. Introduction

Malaria remains a life-threatening disease because the complex life cycle of the *Plasmodium* parasite makes it extremely difficult to develop an effective vaccine or antimalarial drug (Girard et al., 2007; Michalakis and Renaud, 2009). In addition, large-scale resistance to insecticides and the diverse ecology of *Anopheles* mosquitoes, makes it hard to apply a universal vector control strategy (Beier, 1998; Michalakis and Renaud, 2009).

Promising strategies for biological control of the adult and larval stages of mosquito vectors involve the use of entomopathogenic fungi (EF) (Collins and Paskewitz, 1995; Scholte et al., 2003). The main advantage of this strategy is the low probability of development of resistance due to the complex mode of action of the fungi (Thomas and Read, 2007). Once a fungal spore attaches to a mosquito host, the pathogen has the ability to develop specific structures to penetrate the cuticle and to colonize the hemocoel (Hajek and St Leger, 1994; St. Leger and Wang, 2010). Then, the combination of fungal growth, toxins and nutrient exhaustion becomes fatal for the mosquito (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 (Clark et al., 1968; Hegedus and Khachatourians, 1995). Other paths of infection in the aquatic larvae are through the spiracular opening or the anal region (Clark et al., 1968; Hegedus and Khachatourians, 1995).

At present, studies on biological control of malaria mosquitoes with EF mainly focus on *Metarhizium anisopliae* and *Beauveria bassiana*. EF have a negative effect on vector capacity by reducing blood feeding propensity, fecundity and survival of adult *Anopheles* mosquitoes as well as suppression of *Plasmodium* development (Blanford et al., 2005; Mnyone et al., 2009; Mnyone et al., 2010;

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Scholte et al., 2006, 2005). In addition, EF can also support the effectiveness of insecticides by countering resistance of mosquitoes against insecticides (Farenhorst et al., 2009; Howard et al., 2010, 2011).

The study of Clark et al. (1968) was one of the first to show the effects of exposure to *B. bassiana* on larval and adult stages of different mosquito species (Clark et al., 1968). Thereafter, several other studies also found that under both laboratory and field conditions, *M. anisopliae* and *B. bassiana* can be used to decrease survival of mosquito larvae from a wide variety of species, with very low percentages of emerging adults (Bukhari et al., 2010, 2011; Daoust et al., 1982). Bukhari et al. (2010) showed that a small fraction of the emerging mosquitoes carried the fungal infection. It remains unclear what consequences such an infection has on mosquito fitness. Furthermore, it is not known whether mosquitoes that have survived exposure to spores during the larval stage are still vulnerable for exposure to *B. bassiana* during the adult stage. For example, only the fittest mosquitoes that are no longer or less susceptible to treatment with EF may survive. On the other hand, those mosquitoes that survive larval exposure may already be weakened by the fungal infection that they take with them from the larval stage. These may therefore be more susceptible to another dose of EF. The present study focuses on the consequences of larval exposure to fungus for females only, as insights in these factors may play an important role in evaluating the efficacy of EF for malaria vector control. The main objectives of this study were (a) to assess the consequences of larval exposure to *B. bassiana* on the fitness of surviving *A. stephensi* (sex ratio, survival and reproductive success of females) and (b) to assess the susceptibility of larval exposed *A. stephensi* females to re-exposure to *B. bassiana* during the adult stage.

2. Material and methods

2.1. Mosquitoes

A. stephensi (Sind–Kasur strain, courtesy Prof. R. Sauerwein, origin Pakistan) were reared in the laboratory of Entomology at Wageningen University and Research centre, under the same conditions as described by Bukhari et al. (2010). Experiments were performed at 27 ± 1 °C, a relative humidity of 70 ± 5% and a photoperiod of 12:12 (light:dark). Eggs laid in a Petri dish filled with soaked cotton wool and filter paper on top, were transferred to plastic trays (25 × 25 × 8 cm) containing acclimatized water and 2 drops of Liquifry No. 1. (Interpet Ltd., Dorking, Surrey, UK). One to two day old larvae were fed with one drop of Liquifry No. 1, 3–5 day old larvae were fed with 0.1–0.2 mg/larva/day of Tetramin (Tetra, Melle, Germany) and 6–9 day old larvae with 0.3 mg/larva/day. Pupae were collected daily from trays and transferred to holding cages (30 × 30 × 30 cm). Adult mosquitoes were fed 6% glucose solution *ad lib*. Adult females were transferred to a blood feeding cage and fed with human blood (Sanquin, Nijmegen, the Netherlands) using a Hemotek membrane feeding system.

2.2. Entomopathogenic fungi

Spores of *B. bassiana*, isolate IMI-391510, were provided by the Department of Bioprocess Engineering of Wageningen University. These were stored dry at 4 °C.

2.3. Mosquito fitness after larval treatment

To determine the impact of fungal infection on the adult stage, while the infection itself was obtained during the larval stage, we carried out the following experiment. In total, 21 trays were filled with one liter of acclimatized water after which 250 one-day old *A. stephensi* larvae were placed in each tray. To obtain approximately equal and sufficient numbers of surviving adult females per treatment, 18 trays with larvae needed to be exposed to *B. bassiana*, whereas three trays were sufficient for the non-exposed larvae (control). These numbers were sufficient to do all the experiments at once. In the exposed trays, larvae were continuously exposed to spores of *B. bassiana* by dusting 10 mg of dry spores on the water surface at the start of the experiment (Bukhari et al., 2010). No spores were added to the remaining three non-exposed trays. The amount of food added to the trays was corrected for the estimated number of larvae that died or pupated, based on a pilot study (unpublished data) during which the number of living larvae over time was monitored in non-exposed and fungus exposed trays. Pupae were collected daily from each tray and placed in holding buckets or holding cages.

2.3.1. Sex ratio

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.

2.3.2. Survival

The effect of larval exposure to the fungus on longevity of surviving females was tested by comparing the lifetime of non-exposed and larval exposed females as follows: from the emerged mosquitoes, fifty non-exposed and fifty larval exposed females were randomly selected 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. Survival was monitored daily for each female. Cotton wool was soaked daily in 6% glucose 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, for estimating adult size. Wing length was measured (in mm) from the wing tip to the notch of the alula at 40× magnification using a light microscope equipped with an eyepiece micrometer. Fungal infection with *B. bassiana* was determined for all dead mosquitoes by placing them in Petri dishes and removing undesirable bacteria and fungi on the outside of the corpses with 70% ethanol. After the ethanol had evaporated, 1 ml of demineralized water was added and Petri dishes were sealed with parafilm. After 5 days of incubation at 27 °C, infection could be determined by observing the typical hyphal growth of *B. bassiana*.

2.3.3. Reproductive success

Pupae were placed in two holding cages, one for non-exposed mosquitoes and another for larval exposed mosquitoes. Mating was allowed for three to four days by keeping males and females together. After mating, fifty non-exposed and fifty larval exposed females were selected and placed individually in paper cups closed with netting and a rubber band. Nutrition was provided by daily soaking cotton wool in 6% glucose solution and placing it on top of the netting of each paper cup. A blood meal was offered by placing the arms of one of three human volunteers on top of six to eight paper cups simultaneously for ten minutes. Females which did not blood feed during this first round were offered a second opportunity to take a blood meal for another 10 min. Mosquitoes that did not blood feed after these 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 laid on this filter paper was counted for each mosquito individually using a stereo microscope. Eggs were transferred to a small cup with water in which the eggs were allowed to hatch. After 2–4 days larvae were counted in order to