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Infection *of Aedes aegypti* (Diptera: Culicidae) larvae and Adults by the Entomopathogenic Fungus *Metarhizium anisopliae* (Metschn.) Sorokin

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Authors' contributions

This work was carried out in collaboration between all authors. Author LPV participated in bioassays, fungal culture and electron microscopy. Authors ARP and COP helped in the bioassays and maintenance of the mosquitoes. Author RAD helped with electron microscopy, preparation of the figures. Author RIS conceived the study, its design and coordination and wrote the draft the manuscript. All authors read and approved the final manuscript.

Short Communication

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ABSTRACT

Aims: The dengue vector *Aedes aegypti* is currently a target for control by entomopathogenic fungi. We have previously shown the susceptibility of both larvae and adult *A. aegypti* to fungal infection by *Metarhizium anisopliae* and strategies are being developed for control of both stages in the field.
Study Design: Few studies have been carried out on the early stages of the infection process of entomopathogenic fungi in mosquitoes, especially in the case of adult infection.
Place and Duration of Study: Department of Entomology and Plant Pathology and the Department of Cell Biology at the State University of North Fluminense. May 2010 – July 2011.
Methodology: Conidia were obtained for all experiments by culturing *M. anisopliae* in SDA.

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Conidial suspensions were standardized by serial dilution following quantification using a hemocytometer. Larvae were infected by addition of conidial suspensions to the water in which they were being maintained. Larvae were removed at specific time intervals for observation using a scanning electron microscope. Adult *A. aegypti* were infected by spraying with conidial suspensions at a concentration previously established. Following inoculation, mosquitoes were maintained in cages and cohorts were removed for observation using a scanning electron microscopy at specific time intervals.

Results: The initial stage of the infection process of larvae and adults (females) was described here. Conidia were found to attach to specific regions of the larvae, associated with thoracic hairs. Saprophytic fungal development was observed on the integument of larvae 36 hours after exposure to the fungus. For infection of adults, adhesion and germination of conidia were only observed on the thorax.

Conclusion: The pattern of fungal infection of mosquito larvae was different to that previously documented in the literature. The integument of the larvae was found to be a favorable environment for fungal development. This did not appear to be the case in colonization of the adult integument, which was a more hostile environment for the fungus. However, the microbial control of adult mosquitoes is thought to be more promising than that of larvae due to reduced half-life of the conidia in aqueous environments.

Keywords: Adhesion; germination; vector; Dengue; biological control.

1. INTRODUCTION

Dengue fever (DF), vectored principally by the mosquito *Aedes aegypti*, is a global concern. With predicted changes in world climate, this vector is likely to become an even more widespread problem than it is at the present moment, with two fifths of the world's population currently at risk [1]. Conventional vector control methods are failing to prevent DF epidemics and alternatives are urgently needed. One of the alternatives currently under investigation is the use of entomopathogenic fungi. Our recent studies have demonstrated the virulence of two species of entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana*, under laboratory and semi-field conditions to larvae and adult *A. aegypti* [2,3].

Many studies have been carried out to investigate the potential of entomopathogenic fungi for the control of adult malaria vector mosquitoes [4,5,6,7,8]. However the potential of entomopathogenic fungi for the control of dengue vector mosquitoes has received much less attention.

The infection process of entomopathogenic fungi can modify host physiology and behavior. Blood feeding propensity was reduced following infection of *Anopheles stephensi* [5] and *Anopheles gambiae* [9]. Fecundity was also negatively affected during fungal infections of *A. gambiae* [9] and *A. aegypti* [10]. Thus a reduction in “vectorial capacity” can occur even before the mosquitoes die, an extra benefit when using this type of approach.

The first stages of the infection process, adhesion and germination, have received little attention in mosquitoes, especially in the case of adult infection by entomopathogenic fungi. The current study described the time course of conidial adhesion and germination on the integument of both larvae and adult *A. aegypti* using scanning electron microscopy.

2. MATERIALS AND METHODS

2.1 Maintenance of Insect Colonies

A. aegypti (Rockefeller strain) colonies were reared in cages at 25ºC; 75% RH;16:8 L/D photoperiod and provided with a 10% sucrose solution. Insects were provided with blood meals by placing a mouse, immobilized in a wire mesh bag, in the adult mosquito cages. Following the blood meal, oviposition occurred in beakers half filled with water and lined with filter paper placed in adult cages. Egg eclosion was stimulated by total immersion of the filter paper in water to which mouse food had been previously added (24 h) to reduce oxygen levels.

Larvae were maintained in plastic trays and fed on minced commercial mouse food until reaching the pupal stage. Pupae were separated into water filled beakers and transferred to cages before adult emergence. Recently hatched (2-3 days old) females were used for all experiments.

2.2 Fungal Isolate and Preparation of Suspensions

The isolate of *M. anisopliae* used here was obtained from the collection at ESALQ (ESALQ818) in Piracicaba (São Paulo), which had been previously demonstrated to have high virulence against adult *A. aegypti* (Paula et al., 2008). Fungi were cultured on Sabouraud Dextrose Agar (Dextrose 10 g; Peptone 2.5 g; Yeast Extract 2.5 g; Agar 20 g in 1L H_2O) at 27ºC for 15 days before being used in experiments. Fungal suspensions were initially prepared in Tween 80 (0.05% in sterile distilled water) and conidial concentration determined using a Neubauer hemocytometer. A final concentration of $1x10^8$ conidia mL^{-1} was prepared by serial dilution.

2.3 Exposure of Insects to Conidial Suspensions

Larvae: Larvae were infected by adding fungal suspensions to plastic cups (10 cm high x 6 cm diameter) containing 50 mL of “tap-water” with 10 larvae per cup. Each cup was inoculated with 1 mL of a fungal suspension with 1×10^8 conidia ml^{-1} (Tween 80 0.05% in sterile distilled water), giving a final concentration of 5×10^6 conidia ml^{-1}. The cups were maintained in an incubator at 25ºC; 70 ± 10 % RH; 12L; 12D. Control treatments were carried out by addition of Tween 80 (0.05% in sterile distilled water) without the fungi. Larvae were collected at 12, 24, 36 and 48 h following addition of conidia and prepared for SEM. Cohorts were maintained for evaluation of survival on a daily basis for 7 days (see survival curves in supplementary material Fig. 1) and insects were observed using SEM before high levels of mortality occurred.

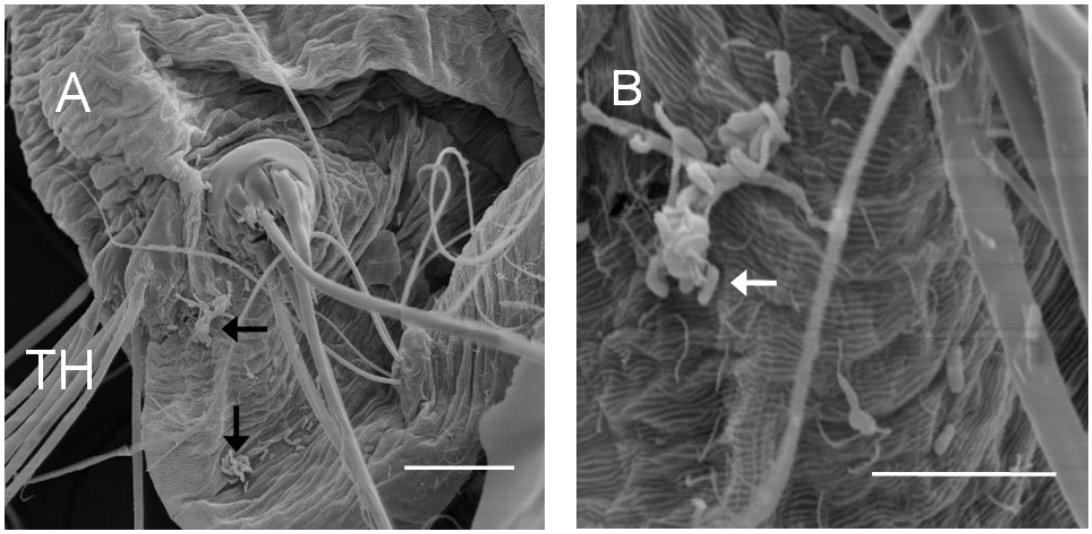
Adults: Two to three day old female mosquitoes were anesthetized with CO_2 and sprayed with fungal suspensions (1×10^8 conidia ml^{-1}) using a Potter spray tower (Burkhard Ltd. UK). Mosquitoes were then maintained in cages with access to sucrose (10%) and insects were collected for SEM preparation at 12, 48, 72 and 96 h post-inoculation. Cohorts were maintained for evaluation of survival on a daily basis for 7 days (see survival curves in supplementary material Fig. 2) and insects were observed using SEM before high levels of mortality occurred.

2.4 Scanning Electron Microscopy

At least 15 individual larvae or adults were observed for each time point. For sample preparation, adults (females) and larvae placed in 1.5 ml tubes with Karnovsky fixative (4% of recently prepared formaldhyde, 2.5% of glutaraldehyde in 0.1 M of cacodylate buffer). Samples were then washed in 0.1 M cacodylate buffer solution and post-fixed in 2% osmium tetroxide solution in 0.1 M cacodylate buffer. The samples were then washed again in 0.1 M cacodylate buffer, and were dehydrated in graded series of acetone (30, 50, 70, 90 and 100%). Subsequently, samples were critical point dried using CO_2, mounted on stubs and coated with gold (20-30 nm). Samples were then observed using a Scanning Electron Microscope (Zeiss 962). Hundreds of images were captured and observations were based on these records.

3. RESULTS AND DISCUSSION

Broad spectrum fungal pathogens are capable of attacking terrestrial and aquatic insects, causing natural epizootics. Certain species of entomopathogenic fungi are naturally pathogens of mosquito larvae [11], although no products based on fungi are currently available for the control of vector species. Entomopathogenic fungi have an advantage over other insect pathogens as they can infect all developmental stages of their hosts such as eggs, larvae, pupae and adults. Although there has been many recent publications of the potential of entomopathogenic fungi for the control of disease vectors such as *Culex*, *Anopheles* and *Aedes* [12,8,2], few studies have been carried on the infection process. In the present study we observed *M. anisopliae* germination and colonization on the cuticle of larvae and adult *A. aegypti* (Figures 1 & 2).



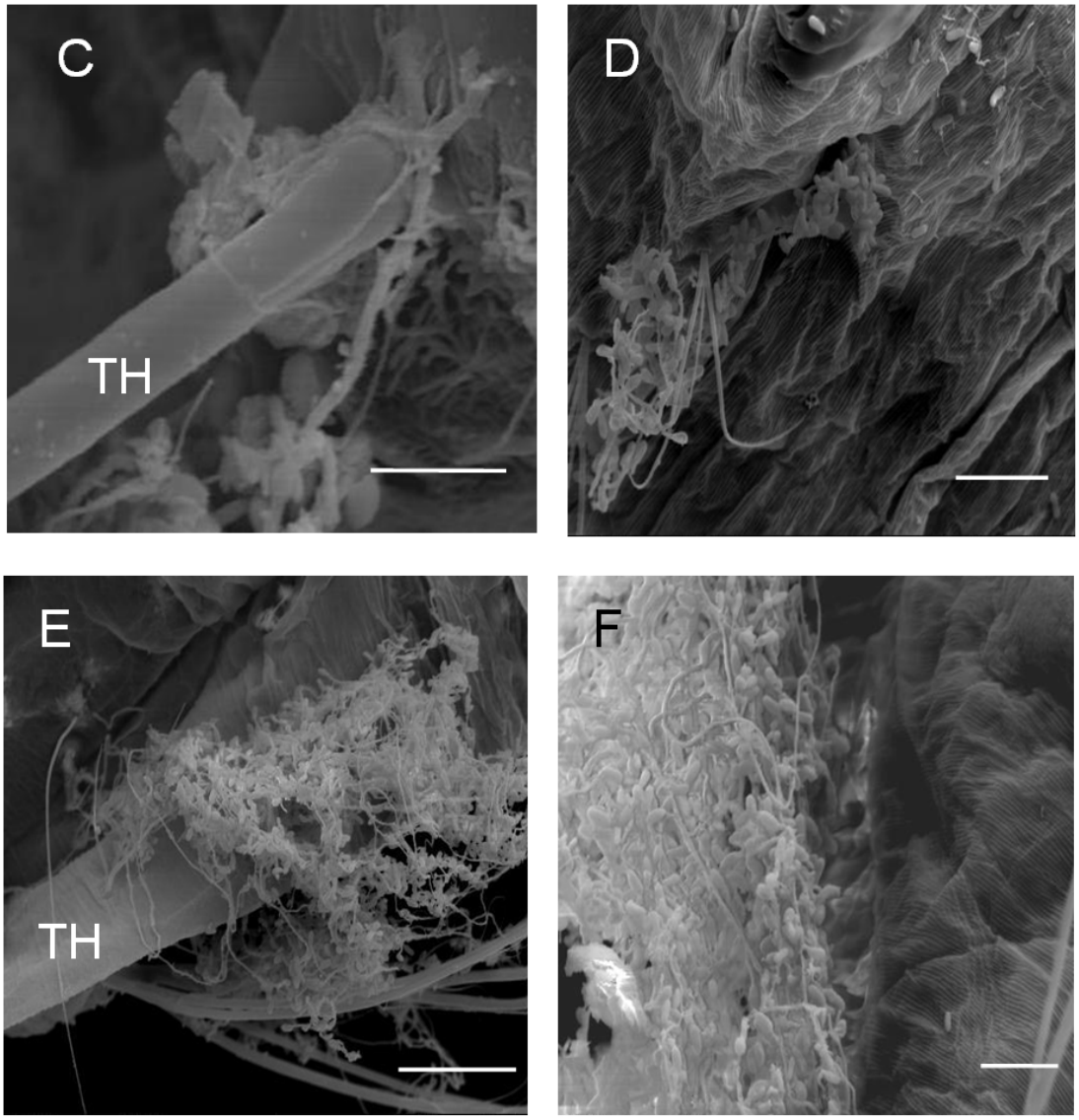


Figure 1. Larvae of *Aedes aegypti* exposed to *M. anisopliae* conidia and observed by scanning electron microscopy at different times following infection. A and B: Presence of conidia 12h after exposure to fungal suspension (bar = 50 and 20µm respectively) The arrows point to fungal bodies. TH= Thoracic hairs. C and D: Larvae 24h after exposure (bar = 10 and 20µm respectively). E: larvae 36h after exposure (bar = 50µm). F: External colonization of the integument at 48h (scale bar = 20µm)

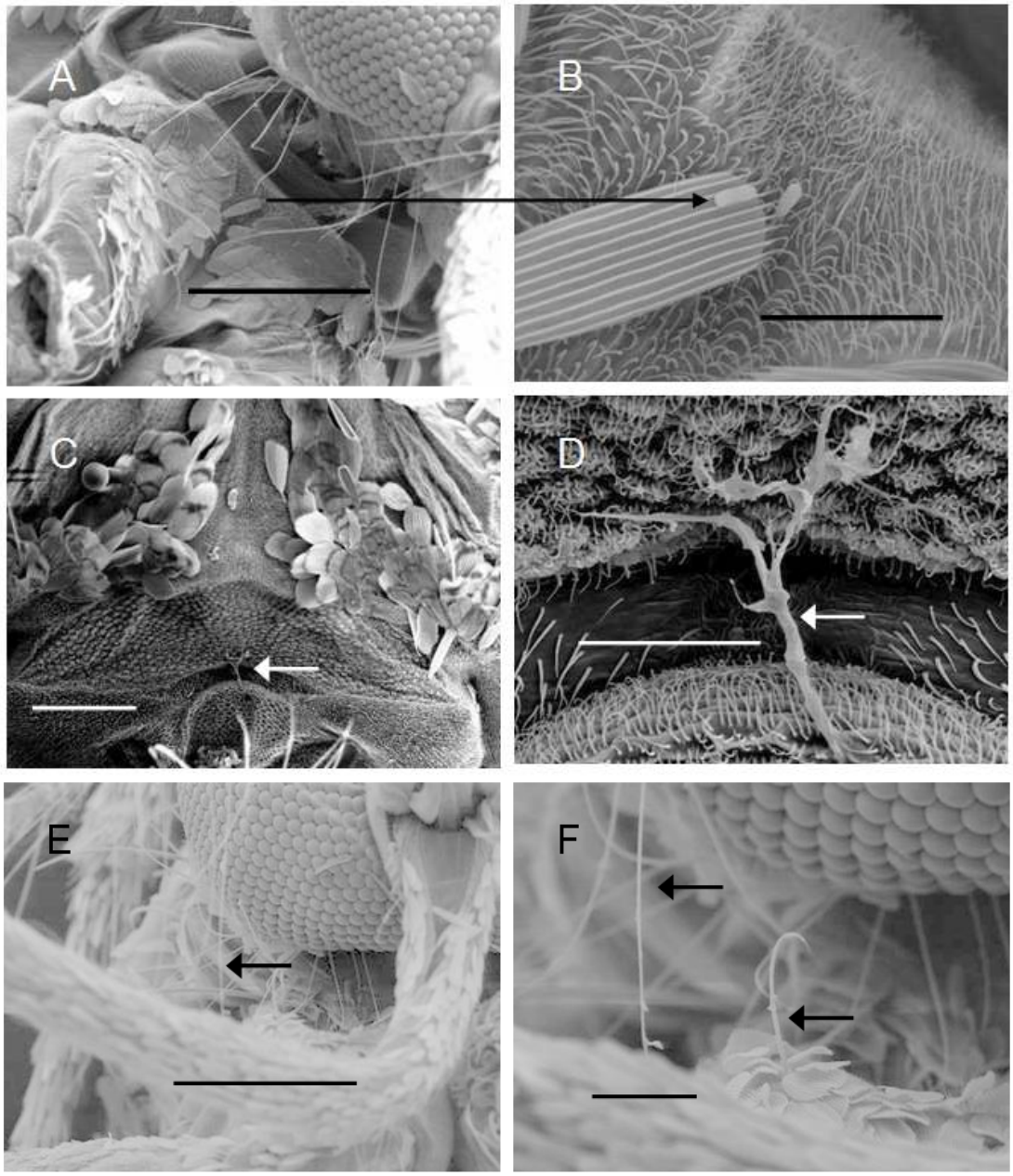


Figure 2. *A. aegypti* adults (females) inoculated with *M. anisopliae* conidia and observed by scanning electron microscopy. A and B: Presence of ungerminated conidia 12 h after inoculation of the pathogen (scale bars 200 and 20 µm respectively). C and D: germination with penetration of the germ tube after 48 h on the ventral thorax (scale bars 100 and 20 µm respectively). E and F: hyphal re-emergence from the moribund insect at 96 h post-infection showing the head region (scale bars 200 and 50 µm respectively). Arrows point to fungal bodies

Larvae were infected by adding conidial suspensions to the water in which the insects were maintained. Previous studies have shown the virulence of fungal isolates to *A. aegypti* larvae [3] and that exposure to fungi, when not considered lethal to larvae, can result in reduced frequency of adult emergence. Although *M. ansiopliae* can kill 50% of the larvae in 2-3 days, fungi are not currently being commercialized for used in larval control due to low half-life (approximately 10 days).

Lacey et al. [13] observed blockage of the siphon of *Culex quinquefasciatus* larvae caused by *M. anisopliae* mycelial growth and mortality was therefore thought to have been caused by asphyxiation. These authors also documented conidial germination in the midgut of the larvae, with mortality between 6 and 24 hours of infection. Here we observed that the fungus appeared to prefer sites on the thorax, associated with thoracic hairs (Figs. 1C & E). This association could have been due to the concentration of thoracic hair characteristic of this species of mosquito facilitate the adhesion of conidia. Other regions had very low numbers of fungal bodies (data not shown). Saprophytic growth was seen on the cuticle of larvae before host mortality (Figs. 1E & F). No fungal development was observed near or in the siphon (results not shown). Goettel [14] described the infection process of the hyphomycete *Tolypocladium cylindrosporum* colonizing *A. aegypti* larvae and showed that the head was the principal infection site.

Initially following direct spray application of conidia to adult *A. aegypti*, it was possible to visualize the adherence of conidia on the surface of the adults after 12 hours (Fig. 2A & B). This interaction represents a complex event and is the first step in the infection cycle, followed by preparation for the penetration phase.

Conidial germination and penetration on adult *A. aegypti* occurred between 24 and 48 hours (Fig. 2C & D) on the thorax of the mosquito. During this period it is likely that appressoria formation occurred, with dilatation of hyphae at the end of the germ tubes. It is interesting to note the apparent development of mycelium, reemerging from the insect body even before host death (Fig. 2F) at 36 h post-infection. This type of growth has been previously observed in Entomophthoraceae [15]. Rhizoids and pseudocystidia of *Erynia conica* were observed to emerge from infected adult black-flies (Simulium) before host death [15].

4. CONCLUSION

The pattern of fungal infection of mosquito larvae was different to that previously documented in the literature. The integument of the larvae was found to be a favorable environment for fungal development. This did not appear to be the case in colonization of the adult integument, which was a more hostile environment for the fungus. However, the microbial control of adult mosquitoes is thought to be more promising than that of larvae due to the reduction in half-life of the conidia in aqueous environments.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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