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Development of *Metarhizium humberi* in *Aedes aegypti* eggs

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ABSTRACT

The entomopathogenic fungus *Metarhizium humberi* affects *Aedes aegypti* adults, larvae and eggs, but its ovicidal activity is not yet well documented. Conidia of this fungus adhered to the chorion, initiated germination within 12 h, and germinating conidia were detected for up to 10 d after contact with the egg. Germ tubes either penetrated the chorion directly or formed appressoria at the end of a short hypha (<5 μm) or, subsequently, on longer, branched hyphae. Thin layers of what was most probably a fungal mucilaginous excretion were detected on the chorion adjacent to germ tubes, appressoria and hyphae. After 5 d eggs frequently appeared shriveled with ruptures in the chorion, and with the interior filled with hyphae that eventually produced mycelium and new conidia on the egg surfaces. Findings demonstrated that this fungus can infect *A. aegypti* eggs and subsequently recycle on their surface by producing large numbers of new conidia that should be infective for further generations of eggs, larvae and adults.

1. Introduction

Aedes aegypti (*Diptera, Culicidae*) oviposits on substrates close to the waterline of small- to medium-sized water volumes (Wong et al., 2011). At 25–30 °C and high ambient moisture embryogenesis is completed in the next 2–3 days (Farnesi et al., 2009; Vargas et al., 2014). After oviposition, the chorion of aedine eggs hardens within a few hours to an insoluble, densely packed, heavily melanized proteinaceous structure that also contains chitin-like components and protects the embryo and any enclosed, fully developed larva against desiccation. Without exposure to water, larvae inside eggs can survive for several months and generally eclose only after eggs are submerged in water (Fig. 1A; Luz et al., 2008; Denlinger and Armbruster, 2014). The eggshell consists of three distinct layers: (1) the outer exochorion, a fragile lamellar layer with protruding tubercles or a fibrillar network, (2) the middle endochorion, a homogeneous and dense layer (Christophers, 1960; Li and Li, 2006; Moreira et al., 2007), and (3) the inner serosal cuticle providing the remarkable resilience of the larva to desiccation (Fig. 1A; Rezende et al., 2008; Vargas et al., 2014). Aeropyles in the chorion allow the exchange of gases between the internal and the external environments (Mathew and Rai, 1975; Linley, 1989). Eggs of *A. aegypti* are easily distinguished from other aedine mosquitoes by morphological chorionic characteristics (Suman et al. 2011; Bova et al. 2016).

Aedine eggs–particularly those of *A. aegypti*–that account for unlimited vector dispersal and extended survival during drier seasons are underestimated as key targets for the biorational control of this important vector of viral pathogens to humans. Quiescent eggs are susceptible to injuries by natural antagonists such as predators and pathogens, particularly to entomopathogenic fungi. Ovicidal activity of *Metarhizium humberi* IP 46 (*Ascomycota, Hypocreales, Clavicipitaceae*) and other entomopathogenic fungi affecting this mosquito is well established (Luz et al., 2007; Santos et al., 2009; Albernaz et al., 2009; Leles et al., 2012; Sousa et al., 2013; Rocha et al., 2015; Flor-Weiler et al., 2017). Nevertheless, there is still no information about how these fungi act against *A. aegypti* eggs.

Entomopathogenic fungi, after the adhesion and germination of infective conidia to the cuticle, most commonly invade their insect hosts actively through the cuticle by the concerted actions of specific enzymes and mechanical pressure. Metabolites produced by the fungus on the cuticle or during penetration and subsequent development can affect the

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infected insect (Hajek and St. Leger, 1994; Wang and Wang, 2017). After host death, the fungus eventually emerges and sporulates on the cuticle. In previous studies, under conditions of high ambient moisture IP 46 produced mycelium and new conidia on *A. aegypti* eggs within about 5 and 10 days, respectively after their exposure to conidia, and only few larvae hatched from these eggs after submersion in water (Leles et al., 2012; Sousa et al., 2013). Because of the opacity of the black eggs, however, it was not clear at that time whether this fungus was developing only on the chorion and affected the embryo or larva inside the egg by the diffusion of secondary toxic metabolites as was reported for botanical insecticides applied to *A. aegypti* eggs (Benelli, 2015) or whether the fungus was actively able to penetrate the chorion, to develop inside the eggs and then to re-emerge to produce new conidia on their surface.

A better understanding on the mechanisms of ovicidal activity will contribute to the development of specific and efficient vector control techniques with mycoinsecticides that are also able to attack the eggs. Most importantly, this study confirms the ability of *M. humberi* IP 46 to cause successful infection of *A. aegypti* eggs.

2. Materials and methods

2.1. Origin and rearing of Aedes aegypti and preparation of eggs

The *A. aegypti* colony originated from larvae collected in an ovitrap in Goiânia, Brazil, in 2012, and mosquitoes were reared under laboratory conditions at IPTSP, UFG at 27 ± 5 °C, 75 ± 10% relative humidity (RH) and natural photophase as described by Lima et al. (2009) and Rocha et al. (2015). The technique was approved by the Ethics Commission for the Use of Animals (CEUA protocol 079/13, UFG).

2.2. Origin, growth and preparation of the fungus

Metarhizium humberi IP 46 was isolated in 2001 from soil collected in the central Brazilian State of Goiás (Rocha et al., 2013) and recently designated as the ex-type culture used to typify this new species in the *M. anisopliae* complex (Luz et al., 2019; access to Brazilian genetic heritage approved by SisGen, protocol A078C45). This strain is stored in the Collection of Entomopathogenic Fungi at IPTSP, UFG (Goiânia, Brazil), as IP 46; at Embrapa Genetic Resources and Biotechnology (Brasília, Brazil) as CG 620, and in the USDA-ARS Collection of Entomopathogenic Fungal Cultures (Ithaca, USA) as ARSEF 12874.

Before the tests, the strain was passed once through *A. aegypti* adults to standardize fungal virulence (Adames et al., 2011). Conidia were obtained from 15-day-old cultures grown on potato dextrose agar medium (PDA) in Petri dishes (100 × 20 mm) at 25 ± 1 °C and 12 h photophase (Luz et al., 2007). Conidia were scraped from the surface of the culture with a spatula, set on the bottom of a sterile Petri dish and dried in a chamber with silica gel at 4 ± 1 °C for 48 h. Afterwards, dried conidia were mixed and homogenized with a glass rod for 2 min, to

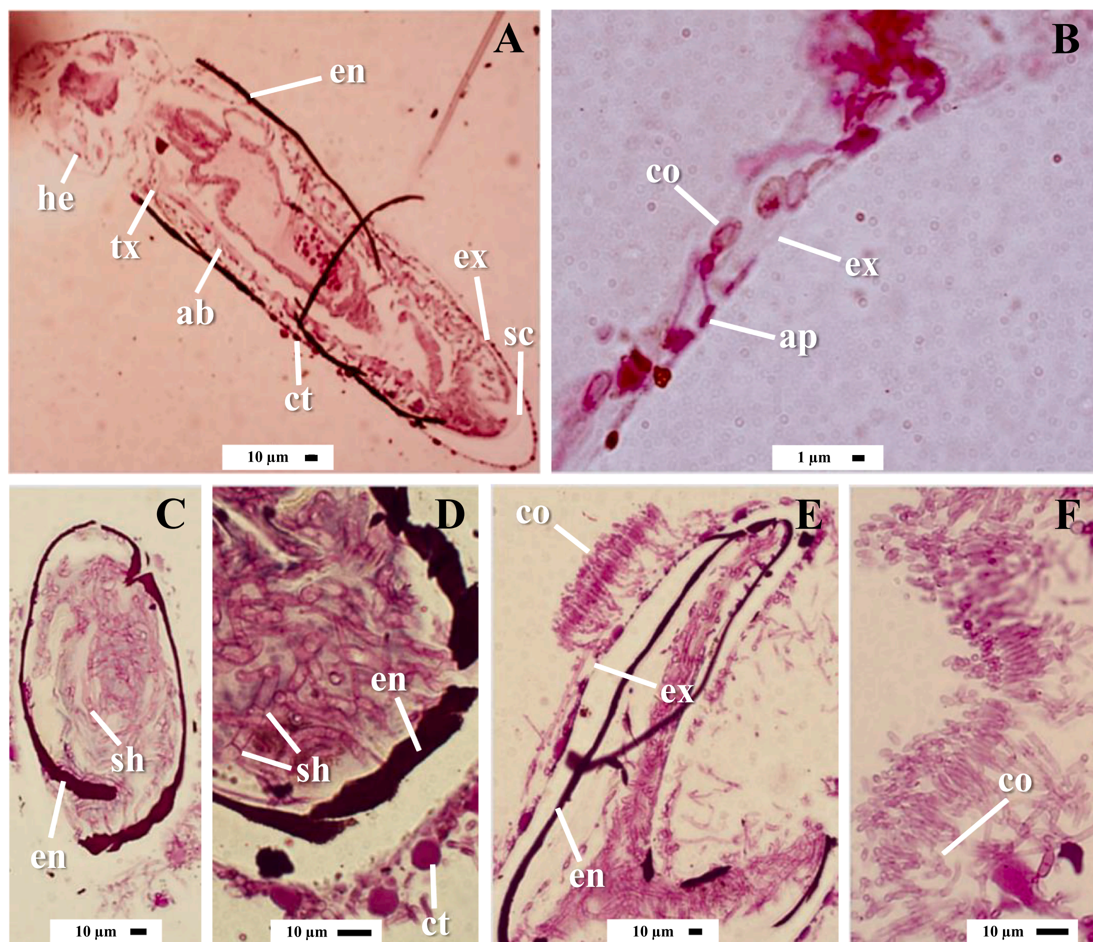

Fig. 1. Light micrographs of sections stained by PAS and counterstained with hematoxylin of *Aedes aegypti* eggs treated with water (control, A) or *Metarhizium humberi* IP 46 conidia (B–F) and incubated at > 98% relative humidity and 25 ± 1 °C for up to 5 d. (A) control eggs with eclosing larva at 48 h after oviposition showing free head (he), thorax (tx) and abdomen (ab) still inside the egg, serosal cuticle (sc), endochorion (en), exochorion (ex) and chorionic tubercles (ct); (B) germinated conidium (co) on exochorion with appressorium (ap) at 4 d; (C, D) extensive development of septate hyphae (sh) inside the egg at 5 d; (E, F) conidiogenesis on egg surface at 5 d.

separate the individual conidia that were then stored at 4 ± 1 °C until treatment.

At the beginning of tests, all of which were done with three independent repetitions, the viability (>95% germination) of conidia was confirmed by spreading 50 µL of suspended 10^6 conidia/mL onto SDAY medium (Sabouraud dextrose agar and 1% yeast extract). Germination of 100 conidia was scored from four separate areas each on this medium after an 18–24 h incubation at 25 ± 1 °C and 12 h photophase. Conidia were considered germinated when the length of the germ tube exceeded the diameter of the conidium.

2.3. Processing of eggs

Three- to 5-day-old eggs oviposited on filter paper by females were exposed to ultraviolet light (UV-C Lamp Germicidal Ultraviolet G30T8®) for 15 min to reduce the number of micro-organisms on their surface (Luz et al., 2007). Eggs were then carefully detached with a paint brush, and 30 eggs set on each of 10 double-sticky tapes (0.8 cm^2) for scanning electron microscope (SEM) examinations or 30 eggs on each of 10 filter papers (1 cm^2) for histopathological examinations by light microscopy (LM), all arranged on the bottom of Petri dishes.

A small amount of non-quantified dry conidia was spread evenly

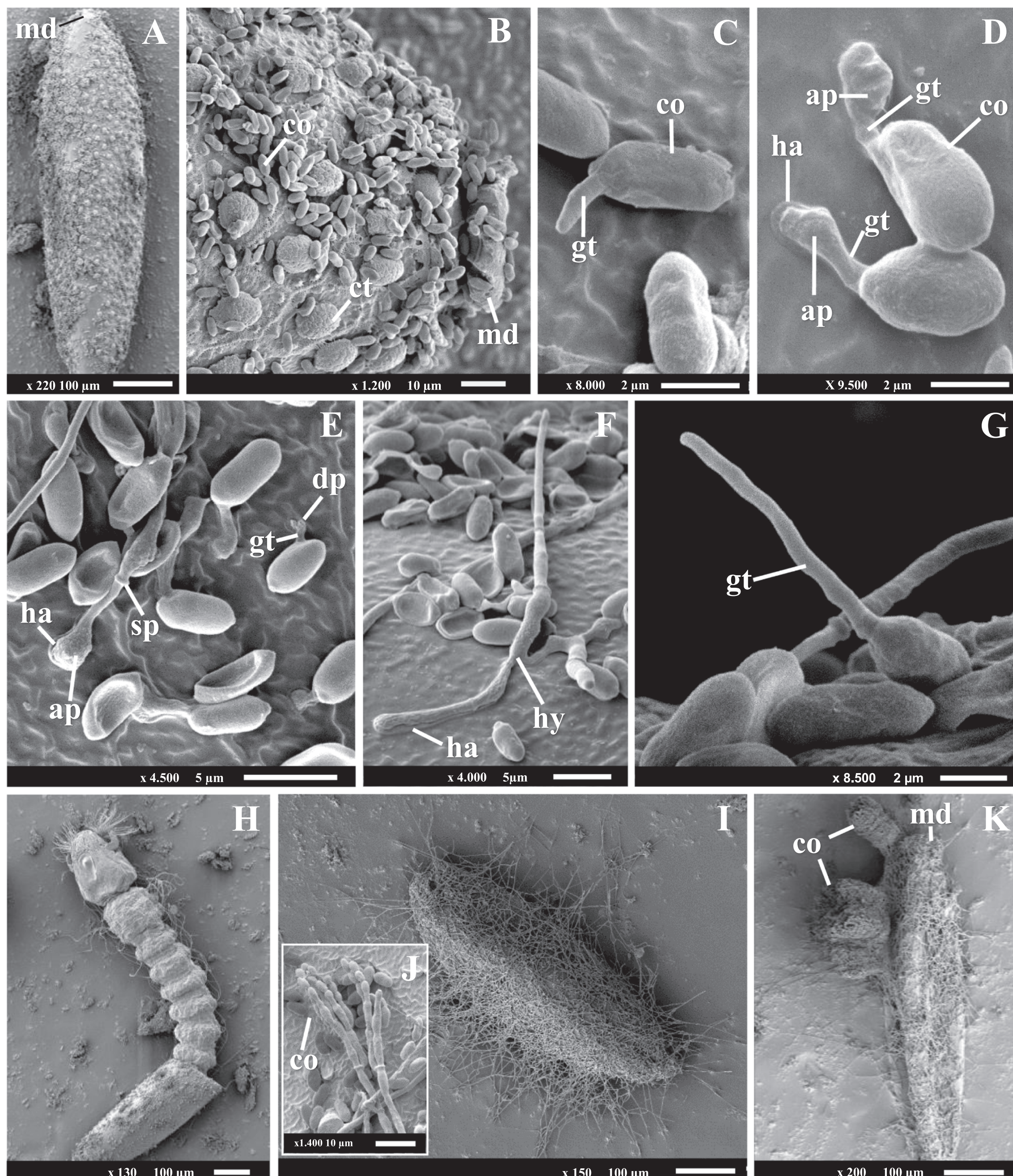

Fig. 2. Scanning electron micrographs of *Metarhizium humberi* IP 46 developing on *Aedes aegypti* eggs, after topical application of conidia and incubation at 25 ± 1 °C and > 98% relative humidity up to 10 d. (A–B) conidia (co) on chorion at 0 h; (A) conidia covering the entire egg; (B) micropylar disc (md) and chorionic tubercles (ct); (C) conidium with germ tube (gt) at 12 h; (D) germinating conidia with appressoria (ap) and amorphous halo (ha) at 24 h; (E) germ tube developing into septate hypha (sp) forming an appressorium or germ tube directly penetrating (dp) the chorion at 48 h; (F) ungerminated conidia and branching hypha (hy) with halo at 4 d. (G) aerial germ tube at 48 h; (H) prematurely eclosed larva at 3 d with no signs of fungal infective structures; (I) extensive hyphal growth on egg at 5 d; (J) detail: production of new conidia; (K) abundant new conidial production on shriveled egg (10 d).

with a paint brush onto the eggs using a Leica E24® stereomicroscope at 8–35 magnification. For the control, untreated eggs were tested. Petri dishes with eggs were then incubated at 25 ± 1 °C, RH > 98% and 12 h photophase. After 0 h, 12 h, 24 h, 36 h, 48 h, 3 d, 4 d, 5 d, 7 d and 10 d incubation eggs were fixed for SEM or LM examinations as explained below.

For the SEM studies, at each incubation time, 30 eggs were transferred into a 1.5 mL Eppendorf tube and fixed with 2% glutaraldehyde, 2% paraformaldehyde and 3% saccharose in 0.1 M sodium cacodylate buffer, pH 7.2, for at least 24 h at 4 ± 1 °C. Samples were then rinsed three times in 0.1 M sodium cacodylate buffer for 15 min each, dehydrated in a graded series of ethanol (30, 50, 70, 90, and twice 100%), 15 min each. Subsequently eggs were critical-point dried with CO_2 (Autosamdri 815®), mounted on a stub and gold-coated with a sputter coater (Denton Vacuum Desk V®). About 10–20% of the eggs were found with a damaged chorion as a result of previous extensive processing, and only intact samples were examined with a scanning electron microscope (Jeol JSM-6610®), at 20 kV acceleration voltage and photographed at LabMic, UFG.

For the LM studies, eggs were handled as mentioned above but fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2 for 24 h at 4 ± 1 °C (Arruda et al., 2003). Eggs were then washed twice in this buffer for 10 min, dehydrated in a graded series of ethanol solutions as mentioned for 10 min each, then embedded in historesin (Leica HistoResin Embedding Kit®) in subsequent concentrations of 2:1, 1:1 and 1:2 of 100% ethanol:historesin (v/v). Only intact eggs were finally submerged twice in historesin for 6 h each at ambient temperature and before polymerization in a plastic mold. Blocks were sectioned into 3-μm-thick samples with a Leica RM2245® microtome, stained with Periodic Acid-Schiff (PAS) and counterstained with hematoxylin (Pearse, 1960, modified), examined and digitally photodocumented mounted on an Olympus BX41®microscope.

3. Results

Within a maximum 15 min after topical application (0 h incubation) and subsequent fixation of the eggs, a high number of conidia appeared equally distributed on and fixed to the chorion (Fig. 2A); conidia were also detected on the micropylar disc (Fig. 2B). Conidia started to germinate at low proportions (10–25%) within the first 24 h and reached 18–35% after 48 h, and a 30–40% cumulative germination after 3 d (Fig. 2C−E); after 4 d there were still numerous ungerminated conidia on the chorion (Fig. 2F). Short germ tubes (1.5–5 μm; Fig. 2C,D) either penetrated the chorion directly (Fig. 2E) or formed a thickened appressorium at their apices (Fig. 1B; Fig. 2D,E) or even developed into septate hyphae (>5 μm; Fig. 2E,F) that also usually formed appressoria at their tips within 24 h after germination (Fig. 2E). Adjacent to the germ tubes, hyphae and particularly to the appressoria, some sort of a smooth-textured halo was clearly visible at the interface with the exochorion (Fig. 2D−F). Occasionally, conidia formed aerial germ tubes (Fig. 2G). After germination, conidial germ tubes, hyphae and appressoria on the chorion were frequently found to have a desiccated or deflated appearance (Fig. 2E,F).

The earliest spontaneous eclosion of the first larvae from fungus-treated eggs was observed after 3 d on the tape or filter paper (Fig. 2H), and neither hyphal fragments nor any evidence of possible hyphal penetrations were detected on the cuticles of these larvae. Spontaneous eclosion of larvae from fungus-treated eggs was more common in the following days and continued occasionally up to ca. 25–30% by the end of the tests. Spontaneous eclosion was never noticed in fungus-treated eggs from which external fungal recycling had started nor was observed in the control eggs.

After 5 d most eggs had lost their ovoid shape and showed signs of desiccation and cracking of the chorion (Fig. 1C,D). Numerous were filled with septate hyphae, and no larvae were visible inside these eggs (Fig. 1C,D). Abundant mycelium grew on the egg surface, and conidiogenesis had started with distinct single chains of new conidia (Fig. 1E,F; Fig. 2I,J). Within the next 5 days eggs eventually shriveled, and columnar blocks of long laterally condensed conidial chains that are typical of the genus *Metarhizium* developed on the egg surface, concentrated at sites where the chorion was physically damaged or in areas with thinner chorionic layers such as the opercular suture of the micropylar disk (Fig. 2K).

4. Discussion

Findings support an active and specific infection of *A. aegypti* eggs by the entomopathogen *M. humberi*. Ovicidal activity of IP 46 has been reported previously (Luz et al., 2007; Leles et al., 2012; Albernaz et al., 2009; Sousa et al., 2013) and is due to the invasion of the egg by the fungus through the chorion. Conidia visibly adhered to and germinated on the egg surface. Whether the fungus produced appressoria or not hyphae eventually penetrated the chorion and proliferated in the interior. IP 46 hyphae developed in a more limited, directed and nonrandom manner on the egg and colonized the egg's interior with hyphae. Nonspecific and specific mechanisms for conidial adhesion by *Metarhizium* spp. or other entomopathogenic fungi on the chorion, similar to those on the cuticle (Wang and St. Leger, 2007; Zhang et al., 2011; Wang and Wang, 2017), are probable but had not yet been demonstrated until now. Amorphous halos along developing fungal structures in contact with the chorion observed here have often been reported on the host cuticle during the first steps of infection, particularly beneath and extending slightly away from appressoria (Zacharuk, 1970; Butt et al., 1995; Moino et al., 2002; Arruda et al., 2005). A high metabolic activity and the excretion of mucilaginous cuticle-degrading enzymes in germ tube tips and appressoria, where the fungus initiates penetration of the cuticle (Butt et al., 1995; St. Leger et al., 1986, 1991; Zacharuk, 1970; Goettel et al., 1989), apparently lead to the formation of such presumably mucoid halos that increase adhesion of fungal structures to both the cuticle and chorion and that obviously also support hyphal invasions of the chorion. Germlings developing on the chorion in the present study were clearly capable to penetrate the different layers and to reach the inner egg area with the larva. The desiccated appearance of the greatest part of germinated conidia, hyphae and appressoria on the chorion was probably due to the forward movement of the fungal cytoplasmic content from conidia and germ tubes into the penetration structures. Processes of self-inhibition among the numerous conidia deployed on the chorion are known for conidia of other entomopathogenic fungi applied in high numbers on insect cuticles or culture media (Luz et al., 1999; Boyle and Christopher Cutler, 2012); such self-inhibition here may have contributed to the low and delayed rates of conidial germination that were continuously observed on the chorion up to 4 d.

The spontaneous premature eclosion of larvae from treated eggs − without any contact with water − observed in the present study has been reported for this and other fungi that were tested previously against *A. aegypti* eggs. Fungal development on the egg surface may have possibly interfered with the exchange of gases between the interior egg and the surrounding environment, and subsequently affected still-living larvae or induced eclosion. This spontaneous eclosion has been reported before and also may be a possible escape reaction of the larva to the imminent fungal infection by hyphae or by specific metabolites diffusing through the egg chorion (Leles et al., 2012; Sousa et al., 2013; Flor-Weiler et al., 2017). Our findings suggest that fully developed larvae (usually 3–5 days old) in the eggs may be stimulated to eclose after exposure to conidia developing on the cuticle or by invading hyphae. It seems that some larvae in the eggs were able to escape by spontaneous eclosion while other larvae became infected and eventually succumbed to infection or died before infection due to unspecified fungal activities.

The characteristic shrinkage and mummification of fungus-treated eggs after advanced periods of incubation that may have resulted in the death of the larva inside the egg is also common in insects that succumbed to fungal infection (Marcandier and Khachatourians, 1987;

Luz and Fargues, 1998). Initial desiccation of the infected moribund or dead insect or egg reduces competitive growth especially by bacterial antagonists and, thereby, favors the development of the fungus in and on the insect or egg after host death. The final recycling of the fungus on the insect or egg cadavers, however, needs high moisture (Luz and Fargues, 1998; Luz et al., 2007, 2008; Santos et al., 2009). The fungus was obviously able to disturb the mechanisms protecting eggs from desiccation. Eggshells were plainly disrupted or the suture of the micropylar disk opened by fungal action or by later dehydration, and the fungus finally sporulated abundantly on the egg surface using nutrient sources from the embryonated or unembryonated interior contents of the egg.

M. humberi–a fungus with high potential for biological control of *A. aegypti*–developed on the chorion at high humidity and successfully penetrated the egg membranes to infect and to kill the larva, and finally grew out onto the egg surface to produce new, presumably infective conidia able to disperse and to perpetuate infections of healthy hosts. This pattern of activity on mosquito eggs appears to be completely consistent with that of the stages of transcuticular infection and further development on fungus-killed arthropod hosts commonly reported for this and other genera of hypocrealean entomopathogenic fungi (Goettel et al., 1989; Butt et al., 1995; Moino et al., 2002; Arruda et al., 2005). Other entomopathogenic fungi with known ovicidal activity (Luz et al., 2007; Rocha et al., 2015; Flor-Weiler et al., 2017) might have a similar course of development of infection of mosquito eggs or on the eggs of other arthropods (Garcia et al. 2005; Rodrigues et al. 2015). The concerted action of *M. humberi* IP 46 against all of the developmental stages and adults of *A. aegypti* makes this fungal strain a particularly interesting agent for biological control of this important vector.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Adames, M., Fernández, M., Peña, G., Hernández, V.M., 2011. Effects of passages through a suitable host of the fungus, *Metarhizium anisopliae*, on the virulence of acaricide-susceptible and resistant strains of the tick, *Rhipicephalus microplus*. J. Insect Sci. 11 (21), 1–13. https://doi.org/10.1673/031.011.0121.

Albernaz, D.A.S., Tai, M.H.H., Luz, C., 2009. Enhanced ovicidal activity of an oil formulation of the fungus *Metarhizium anisopliae* on the mosquito *Aedes aegypti*. Med. Vet. Entomol. 23, 141–147. https://doi.org/10.1111/j.1365-2915.2008.00792.x.

Arruda, W., Oliveira, G.M.C., Silva, I.G., 2003. Toxicidade do extrato etanólico de *Magonia pubescens* sobre larvas de *Aedes aegypti*. Rev. Soc. Bras. Med. Trop. 36 (1), 17–25. https://doi.org/10.1590/S0037-86822003000100004.

Arruda, W., Lübeck, I., Schrank, A., Vainstein, M.H., 2005. Morphological alterations of *Metarhizium anisopliae* during penetration of *Boophilus microplus* ticks. Exp. Appl. Acarol. 37 (3-4), 231–244. https://doi.org/10.1007/s10493-005-3818-6.

Benelli, G., 2015. Plant-borne ovicides in the fight against mosquito vectors of medical and veterinary importance: a systematic review. Parasitol. Res. 114 (9), 3201–3212. https://doi.org/10.1007/s00436-015-4656-z.

Bova, J., Paulson, S., Paulson, G., 2016. Morphological differentiation of the eggs of North American container-inhabiting *Aedes* mosquitoes. J. Am. Mosquito Contr. 32, 244–246. https://doi.org/10.2987/15-6535.

Boyle, D., Christopher Cutler, G., 2012. Effects of insect activity, soil, and cuticular factors on virulence of *Beauveria bassiana* toward *Blissus leucopterus hirtus*. J. Pest. Sci. 85 (4), 505–512. https://doi.org/10.1007/s10340-012-0453-y.

Butt, T.M., Ibrahim, L., Clark, S.J., Beckett, A., 1995. The germination behaviour of *Metarhizium anisopliae* on the surface of aphid and flea beetle cuticles. Mycol. Res. 99 (8), 945–950. https://doi.org/10.1016/S0953-7562(09)80754-5.

Christophers, S.R., 1960. *Aedes aegypti* (L.) The Yellow Fever Mosquito its Life History, Bionomics and Structure. Cambridge University Press, London.

Denlinger, D.L., Armbruster, P.A., 2014. Mosquito diapause. Ann. Rev. Entomol. 59 (1), 73–93. https://doi.org/10.1146/annurev-ento-011613-162023.

Farnesi, L.C., Martins, A.J., Valle, D., Rezende, G.L., 2009. Embryonic development of *Aedes aegypti* (Diptera: Culicidae): influence of different constant temperatures. Mem. Inst. Oswaldo Cruz 104 (1), 124–126. https://doi.org/10.1590/S0074-02762009000100020.

Flor-Weiler, L.B., Rooney, A.P., Behle, R.W., Muturi, E.J., 2017. Characterization of *Tolypocladium cylindrosporum* (Hypocreales: Ophiocordycipitaceae) and its impact against *Aedes aegypti* and *Aedes albopictus* eggs at low temperature. J. Am. Mosqu. C. Assoc. 33 (3), 184–192. https://doi.org/10.2987/16-6596R.1.

Garcia, M.V., Monteiro, A.C., Szabo, M.J.P., Prette, N., Bechara, G.H., 2005. Mechanism of infection and colonization of *Rhipicephalus sanguineus* eggs by *Metarhizium anisopliae* as revealed by scanning electron microscopy and histopathology. Braz. J. Microbiol. 36, 368–372. https://doi.org/10.1590/S1517-83822005000400012.

Goettel, M.S., St. Leger, R.J., Rizzo, N.W., Staples, R.C., Roberts, D.W., 1989. Ultrastructural localization of a cuticle-degrading protease produced by the entomopathogenic fungus *Metarhizium anisopliae* during penetration of host (*Manduca sexta*) cuticle. J. Gen. Microbiol. 135, 2233–2239. https://doi.org/10.1099/00221287-135-8-2233.

Hajek, A.E., St. Leger, R.J., 1994. Interactions between fungal pathogens and insect hosts. Ann. Rev. Entomol. 39, 293–322. https://doi.org/10.1146/annurev.en.39.010194.001453.

Leles, R.N., D'Alessandro, W.B., Luz, C., 2012. Effects of *Metarhizium anisopliae* mixed with soil against the eggs of *Aedes aegypti*. Parasitol. Res. 110, 1579–1582. https://doi.org/10.1007/s00436-011-2666-z.

Li, J.S., Li, J., 2006. Major chorion proteins and their crosslinking during chorion hardening in *Aedes aegypti* mosquitoes. Insect Biochem. Molec. 36 (12), 954–964. https://doi.org/10.1016/j.ibmb.2006.09.006.

Lima, W.P., Chiaravalloti Neto, F., Macoris, M.L.G., Zuccari, D.A.P.C., Dibo, M.R., 2009. Estabelecimento de metodologia para alimentação de *Aedes aegypti* (Diptera-Culicidae) em camundongos swiss e avaliação da toxicidade e do efeito residual do óleo essencial de *Tagetes minuta* L. (Asteraceae) em populações de *Aedes aegypti*. Rev. Soc. Bras. Med. Trop. 42 (6), 638–641. https://doi.org/10.1590/S0037-86822009000600005.

Linley, J.R., 1989. Comparative fine structure of egg of *Aedes albopictus*, *Ae. aegypti* and *Ae. bahamensis* (Diptera: Culicidae). J Med Entomol 26, 510–521. https://doi.org/10.1093/jmedent/26.6.510.

Luz, C., Fargues, J., 1998. Factors affecting conidial production of *Beauveria bassiana* from fungus-killed cadavers of *Rhodnius prolixus*. J. Invertebr. Pathol. 72 (2), 97–103. https://doi.org/10.1006/jipa.1998.4774.

Luz, C., Silva, I.G., Cordeiro, C.M.T., Tigano, M.S., 1999. Sporulation of *Beauveria bassiana* on cadavers of *Triatoma infestans* after infection at different temperatures and doses of inoculum. J. Invertebr. Pathol. 73 (2), 223–225. https://doi.org/10.1006/jipa.1998.4827.

Luz, C., Tai, M.H.H., Santos, A.H., Rocha, L.F.N., Albernaz, D.A.S., Silva, H.H.G., 2007. Ovicidal activity of entomopathogenic Hyphomycetes on *Aedes aegypti* (Diptera: Culicidae) under laboratory conditions. J. Med. Entomol. 44, 799–804. https://doi.org/10.1603/0022-2585(2007)44[799:oaoeho]2.0.co;2.

Luz, C., Tai, M.H.H., Santos, A.H., Silva, H.H.G., 2008. Impact of moisture on survival of *Aedes aegypti* eggs and ovicidal activity of *Metarhizium anisopliae* under laboratory conditions. Mem. Inst. Oswaldo Cruz 103 (2), 214–215. https://doi.org/10.1590/S0074-02762008000200016.

Luz, C., Rocha, L.F.N., Montalva, C., Souza, D.A., Botelho, A.B.R.Z., Lopes, R.B., Faria, M., Delalibera, I.J., 2019. *Metarhizium humberi* sp. nov. (Hypocreales: Clavicipitaceae), a new member of the PARB clade in the *Metarhizium anisopliae* complex from Latin America. J. Invertebr. Pathol. 166, 1–9. http://doi.org/10.1016/j.jip.2019.107216.

Marcandier, S., Khachatourians, G.G., 1987. Susceptibility of the migratory grasshopper, *Melanoplus sanguinipes* (Fab.) (Orthoptera: Acrididae), to *Beauveria bassiana* (Bals.) Vuillemin (Hyphomycete): Influence of relative humidity. Can. Entomol. 119 (10), 901–907. https://doi.org/10.4039/Ent119901-10.

Mathew, G., Rai, K.S., 1975. Structure and formation of egg membranes in *Aedes aegypti* (L.) (Diptera: Culicidae). Int. J. Insect Morphol. 4 (5), 369–380. https://doi.org/10.1016/0020-7322(75)90037-9.

Moino Jr., A., Alves, S.B., Lopes, R.B., Neves, P.M.O.J., Pereira, R.M., Vieira, A.S., 2002. External development of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in the subterranean termite *Heterotermes tenuis*. Sci. Agr. 59, 267–273. https://doi.org/10.1590/S0103-90162002000200010.

Moreira, M.F., Santos, A.S., Marotta, H.R., Mansur, J.F., Ramos, I.B., Machado, E.A., Souza, G.H.M.F., Eberlin, M.N., Kaiser, C.R., Kramer, K.J., Muthukrishnan, S., Vasconcellos, M.H., 2007. A chitin-like component in *Aedes aegypti* eggshells, eggs and ovaries. Insect Biochem. Molec. 37, 1249–1261. https://doi.org/10.1016/j.ibmb.2007.07.017.

Pearse, A., 1960. Histochemistry, Theoretical and Applied. second ed. J. and A. Churchill, London.

Rezende, Gustavo L, Martins, Ademir J, Gentile, Carla, Farnesi, Luana C, Pelajo-Machado, Marcelo, Peixoto, Alexandre A, Valle, Denise, 2008. Embryonic desiccation resistance in *Aedes aegypti*: presumptive role of the chitinized serosal cuticle. BMC Dev. Biol. 8 (1), 82. https://doi.org/10.1186/1471-213X-8-82.

Rocha, L.F.N., Inglis, P.W., Humber, R.A., Kipnis, A., Luz, C., 2013. Occurrence of *Metarhizium* spp. in Central Brazilian soils. J. Basic Microbiol. 53 (3), 251–259. https://doi.org/10.1002/jobm.v53.310.1002/jobm.201100482.

Rocha, L.F.N., Sousa, N.A., Rodrigues, J., Catão, A.M.L., Marques, C.S., Fernandes, É.K. K., Luz, C., 2015. Efficacy of *Tolypocladium cylindrosporum* against *Aedes aegypti* eggs, larvae and adults. J. Appl. Microbiol. 119 (5), 1412–1419. https://doi.org/10.1111/jam.12945.

Rodrigues, J., Lobo, L.S., Fernandes, É.K.K., Luz, C., 2015. Effect of formulated *Metarhizium anisopliae* on eggs and eclosing nymphs of *Triatoma infestans*. J. Appl. Entomol. 139 (1-2), 146–153. https://doi.org/10.1111/jen.12183.

Santos, A.H., Tai, M.H.H., Rocha, Luiz F.N., Silva, H.H.G., Luz, C., 2009. Dependence of *Metarhizium anisopliae* on high humidity for ovicidal activity on *Aedes aegypti*. Biol. Contr. 50 (1), 37–42. https://doi.org/10.1016/j.biocontrol.2009.01.018.

Sousa, N.A., Lobo, L.S., Rodrigues, J., Luz, C., 2013. New insights on the effectiveness of *Metarhizium anisopliae* formulation and application against *Aedes aegypti* eggs. Lett. Appl. Microbiol. 57 (3), 193–199. https://doi.org/10.1111/lam.12097.

St. Leger, R.J., Charnley, A.K., Cooper, R.M., 1986. Cuticle-degrading enzymes of entomopathogenic fungi: Mechanisms of interaction between pathogen enzymes and insect cuticle. J. Invertebr. Pathol. 47 (3), 295–302. https://doi.org/10.1016/0022-2011(86)90099-6.

St. Leger, R.J., Goettel, M., Roberts, D.W., Staples, R.C., 1991. Prepenetration events during infection of host cuticle by *Metarhizium anisopliae*. J. Invertebr. Pathol. 58 (2), 168–179. https://doi.org/10.1016/0022-2011(91)90061-T.

Suman, D.S., Shrivastava, A.R., Pant, S.C., Parashar, B.D., 2011. Differentiation of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) with egg surface morphology and morphometrics using scanning electron microscopy. Arthropod Struct. Dev. 40 (5), 479–483. https://doi.org/10.1016/j.asd.2011.04.003.

Vargas, H.C.M., Farnesi, L.C., Martins, A.J., Valle, D., Rezende, G.L., 2014. Serosal cuticle formation and distinct degrees of desiccation resistance in embryos of the mosquito vectors *Aedes aegypti*, *Anopheles aquasalis* and *Culex quinquefasciatus*. J. Insect Physiol. 62, 54–60. https://doi.org/10.1016/j.jinsphys.2014.02.001.

Wang, C., St. Leger, R.J., 2007. The MAD1 adhesin of *Metarhizium anisopliae* links adhesion with blastospore production and virulence to insects, and the MAD2 adhesin enables attachment to plants. Eukaryot. Cell 6, 808–816. https://doi.org/10.1128/EC.00409-06.

Wang, C., Wang, S., 2017. Insect pathogenic fungi: Genomics, molecular interactions, and genetic improvements. Ann. Rev. Entomol. 62 (1), 73–90. https://doi.org/10.1146/annurev-ento-031616-035509.

Wong, J., Stoddard, S.T., Astete, H., Morrison, A.C., Scott, T.W., 2011. Oviposition site selection by the Dengue vector *Aedes aegypti* and its implications for Dengue control. PLoS Negl. Trop. Dis. 5, e1015. https://doi.org/10.1371/journal.pntd.0001015.

Zacharuk, R.Y., 1970. Fine structure of the fungus *Metarhizium anisopliae* infecting three species of larval Elateridae (Coleoptera) II. Conidial germ tubes and appressorial. J. Invertebr. Pathol. 15, 81–91. https://doi.org/10.1016/0022-2011(70)90101-1.

Zhang, S., Xia, Y.X., Kim, B., Keyhani, N.O., 2011. Two hydrophobins are involved in fungal spore coat rodlet layer assembly and each play distinct roles in surface interactions, development and pathogenesis in the entomopathogenic fungus, *Beauveria bassiana*. Mol. Microbiol. 80, 811–826. https://doi.org/10.1111/j.1365-2958.2011.07613.x.