Graphical Abstract

**Effect of heat stress and oil formulation on conidial germination
of *Metarhizium anisopliae* s.s. on tick cuticle and artificial
medium**

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Abstract

The effect of heat stress (45 °C) or non-heat stress (27 °C) on germination of *Metarhizium anisopliae* sensu stricto (s.s.) IP 119 conidia were formulated (suspended) in pure mineral oil or in water (Tween 80, 0.01%), and then applied onto the cuticle of *Rhipicephalus sanguineus* sensu lato (s.l.) engorged females or onto culture medium (PDAY). In addition, bioassays were performed to investigate the effect of conidia heated while formulated in oil, then applied to blood-engorged adult *R. sanguineus* females. Conidia suspended in water then exposed to 45 °C, in comparison to conidia formulated in mineral oil and exposed to the same temperature, germinated less and more slowly when incubated on either PDAY medium or tick cuticle. Also, conidial germination on tick cuticle was delayed in comparison to germination on artificial culture medium; for example, germination was 13% on tick cuticle 72 h after inoculation, but 61.5% on PDAY medium. Unheated (27 °C) conidia suspended in either water or oil and applied to tick cuticle developed appressoria 36 h after treatment; whereas only heat-stressed conidia formulated in oil, developed appressoria on tick cuticle. In comparison to conidia heated in mineral oil, there was a strong negative effect of heat on germination of conidia heated in water before being applied to arthropod cuticle. Nevertheless, bioassays [based primarily on egg production (quantity) and egg hatchability] exhibited high percentages of tick control regardless of the type of conidial suspension; i.e., water- or oil-formulated conidia, and whether or not conidia were previously exposed to heat. In comparison to aqueous conidial preparations, however, conidia formulated in oil reduced egg hatchability irrespective of heat or no-heat exposure. In conclusion, mineral-oil formulation protected conidia against heat-induced delay of both germination and appressorium production when applied to the cuticle of *R. sanguineus*.

Keywords: *Rhipicephalus sanguineus*, thermotolerance, formulation, entomopathogenic fungi, *Metarhizium anisopliae* s.s., Ixodidae.

1. Introduction

The brown dog tick, *Rhipicephalus sanguineus* Latreille, 1806 (Acari, Ixodidae), is widely distributed throughout the world, and it is considered one of the main ectoparasites of domestic dogs. Besides its preference for dogs, *R. sanguineus* occasionally parasitizes other domestic and wild animals, including humans (Estrada-Peña and Jongejan, 1999; Dantas-Torres et al., 2006; Dantas-Torres, 2008, 2010; Louly et al., 2006).

Rhipicephalus sanguineus vectors many dog pathogens such as *Babesia canis* and *Ehrlichia canis* (Dantas-Torres, 2008), and high infestations, even without vectoring pathogens, may cause skin irritation, anemia and lethargy. In spite of its low anthropophily (Palmas et al., 2001), it is known to trigger inflammatory or allergic reactions to the bites of larvae and nymphs. Transmission of pathogens to humans by *R. sanguineus* is well documented; for example: it is an important vector of the bacterium *Rickettsia conorii*, the etiologic agent of Mediterranean spotted fever or Boutonneuse fever in Europe (Merle et al., 1998; Matsumoto et al., 2005); and *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever in the United States (Demma et al., 2005) and Mexico (Eremeeva et al., 2011). In Brazil, *R. sanguineus* is considered a potential vector to humans of *R. rickettsii* (Rozental et al., 2002), agent of the Brazilian spotted fever (Rocky Mountain spotted fever in North America), and *Borrelia* sp., agent of Lyme-like disease (Yoshinari et al., 1997). Laboratory tests confirmed that *R. sanguineus* is a competent vector of *R. rickettsii* (Piranda et al., 2011), and molecular studies and isolation in cell culture demonstrated natural

infection of this tick with *R. rickettsii* in endemic areas of Brazilian spotted fever in Brazil (Cunha et al., 2009; Ogrzewalska et al., 2012).

The control of *R. sanguineus* is mainly based on the use of chemical acaricides applied directly on infested dogs and/or in the dogs' environments (Labruna and Pereira, 2001; Dantas-Torres and Figueiredo, 2006). However, over the years, many tick populations developed tolerance to most chemicals used. Misuse, e.g., mistakes during preparation of the product, inappropriately low application rates, and overly repeated applications of chemical acaricides, are situations that contributed to the selection of chemical-acaricide-resistant tick populations (Labarthe, 1994). Resistance of *R. sanguineus* against chemical products has been demonstrated worldwide (Burrige et al., 2004; Miller et al., 2001; Estrada-Pena, 2005), as well as in Brazil (Borges et al., 2007).

Tick pathogens, in particular entomopathogenic fungi, have been investigated as promising biocontrol alternatives to the current widespread excessive and almost exclusive use of chemicals for tick control. The objectives of these studies are to reduce both acaricides resistance and environmental contamination. Fungi have been recognized repeatedly as the most promising microorganisms for microbial control of ticks, e.g., *Rhipicephalus microplus* (Bittencourt et al., 1999ab; Arruda et al., 2005; Fernandes and Bittencourt, 2008; Leemon and Jonsson, 2008), *R. sanguineus* (Samish et al., 2001; Prette et al., 2005; Garcia et al., 2004; Garcia et al., 2005), *Amblyomma cajennense* s.l. (Souza et al., 1999; Reis et al., 2001; D'Alessandro et al., 2012), *Amblyomma cooperi* (Reis et al., 2003) and *Amblyomma variegatum* (Kaaya et al., 1996; Maranga et al., 2005).

Abiotic factors, especially high temperatures, may be important obstacles to the effectiveness of entomopathogenic fungi applied for arthropod-pest control. High temperatures may reduce the virulence and/or the survival of these microorganisms; and,

consequently, limit their effectiveness (Fernandes et al., 2010; Rangel et al., 2010; Ment et al., 2011). The optimum temperature range for *Metarhizium anisopliae* s.l. is between 24 and 30 °C (Walstad et al., 1970; Dimbi et al., 2004). Rangel et al. (2010) reported that several *Metarhizium* isolates germinated well at constant 35 °C, but mycelial growth was very slow at 36 °C in comparison to growth at 28 °C; also, the exposure of different isolates to 37 °C for 96 h (Santos, 1978) or 45 °C for 8 h (Fernandes et al., 2010) totally inhibited conidial germination. Nevertheless, *Metarhizium* species and isolates may vary greatly in thermotolerance. For example, heat-treated conidia may undergo significantly delayed germination, depending mostly on the fungal species, isolate and the heat-exposure time (Rangel et al., 2005; Fernandes et al., 2010; Keyser et al., 2014). Of particular interest in this regard is the markedly elevated thermotolerance of all *M. acridum* isolates tested to date (Fernandes et al. 2010).

The temperature, as expected, may vary considerably from one locality to another; e.g., in dog kennels and enclosures, high temperatures may be reached at least for a few hours a day due to direct or indirect sunlight. High temperatures may reduce the viability of conidia (bioproduct) applied locally for tick control, and also may seriously influence the biology of ticks (Bellato and Daemon, 1997a; Bellato and Daemon, 1997b), e.g., reducing the molting periods of larvae and nymphs, longevity of nymphs and adults, period of pre-oviposition and oviposition of engorged females, and length of egg incubation periods, e.i., eggs incubated at high temperature (32 °C) hatched quicker than those held at low (18 °C) or optimum (27 °C) temperature (Bellato and Daemon, 1997a).

Formulation is a much studied approach to protecting fungal propagules from environmental adversities and, thereby, increase their efficiency as biocontrol agents of arthropods. The combination of oil and conidia, for example, assists in protecting the

fungus against rapid dehydration in low-humidity environments (Bateman et al., 1993; Bateman, 1994); high temperatures (McClatchie et al., 1994); and ultraviolet radiation (Moore et al., 1993; Alves et al., 1998; Bateman and Alves, 2000). In comparison with conidia conventionally prepared in water, oil-based formulations favor the spreading and adhesion of conidia on the hydrophobic cuticle of host arthropods (David-Henriet et al., 1998). In addition, oil extends the viability of conidia held at ambient conditions or under refrigeration (Prior et al., 1988). The positive effects of formulating entomopathogenic fungi in oil have been reported as, **(a)** increased fungus-induced mortality against cocoa weevil (Prior et al., 1988), triatomine bugs (Luz and Batagin, 2005), grasshoppers (Lomer et al., 2001) and whiteflies (Malsam et al., 2002); **(b)** increased ovicidal activity against *Aedes aegypti* mosquito (Albernaz et al., 2009) and *Rhipicephalus annulatus* tick (Samish et al., 2014), and **(c)** increased virulence against different life stages of the tick *R. microplus* (Angelo et al., 2010; Camargo et al., 2012).

Unfortunately, the information currently available on conidial thermotolerance was generated using *in vitro* studies, with viability being assessed by germination/cultivation of heat-treated conidia on artificial culture media, rather than observing them on the cuticle of ticks or other arthropods. The research reported here, therefore, assessed the effect of heat stress on germination **(a)** when conidia of *M. anisopliae* sensu stricto IP 119 were suspended in pure mineral oil or water (Tween 80, 0.01%), then heated or not heated, and finally applied onto the cuticle of *R. sanguineus*. In addition, as a comparison with previous heat-tolerance studies, **(b)** we investigated the performance of conidia heat-treated in water and then incubated on artificial medium (PDAY). We also assessed **(c)** the effect of heat treatment of *M. anisopliae* conidia on their appressorium formation on *R. sanguineus* engorged female cuticle; and we **(d)** compared responses (primarily egg production and egg

hatchability) of ticks following exposure to heat-treated (45 °C) and non heat-treated (27 °C) conidia.

2. Material and methods

2.1. Fungal isolate, cultivation, preparation of conidial suspensions, and assessment of conidial viability

The fungal isolate used in this study was *M. anisopliae* s.s. IP 119, which was obtained from Brazilian Savanna (Cerrado) soil collected from Goiás State, Center-West Brazil and identified by Rocha et al. (2012). IP 119 has been deposited in the Fungal Research Collection of the Laboratory of Invertebrate Pathology, at the Institute of Tropical Pathology and Public Health of the Federal University of Goiás; and, at Embrapa Collection of Entomopathogenic Fungi, Brasília, Brazil, under the code name CG 764. This isolate was selected from a group of ten Brazilian *Metarhizium* isolates, due to its exceptional virulence against *R. microplus* engorged females when conidia were formulated in oil, and moderate virulence when suspended in Tween 80[®] 0.01% (Muniz and Fernandes, unpublished data).

IP 119 was cultured on 23 mL potato dextrose agar medium (Difco Laboratories, Sparks, MD, USA) supplemented with 1 g L⁻¹ yeast extract (Technical, Difco) (PDAY) in glass Petri plates (90 × 15 mm) in the dark at 27 ± 1 °C for 15 days. Conidia were dislodged with a microbiological loop, harvested, weighed, and suspended in Tween 80[®] (Labsynth Prod. Lab. Ltda, Diadema, SP, Brazil) solution (0.01% v/v) or pure mineral oil (Impex, Labimpex Ind. e Com. de Prods. p/ Lab. Ltda., Diadema, SP). Aqueous conidial suspensions were quantified by hemocytometer counts, and concentration adjusted to 1 × 10⁸ conidia mL⁻¹. Due to difficulties in making hemocytometer counts of conidia suspended

in mineral oil, these suspensions were prepared using the same weight of conidia used for preparation of aqueous conidial suspensions.

The viability of conidia was assessed by dropping (without spreading) 20 μ L of aqueous conidial suspension on PDAY medium supplemented with 0.002% (w/v) of benomyl (50% active ingredient; Benlate[®], São Paulo, SP, Brazil) and 0.05% (w/v) chloramphenicol (Officinal, Goiânia, GO, Brazil) in Petri dishes (55 $\times$ 15 mm). The plates were then incubated for 24 h at 27 ± 1 °C and RH $\geq$ 80%. A drop of lactophenol cotton blue and a coverslip were placed over the inoculum, and conidial germination observed under a phase contrast microscope with a magnitude of 400 $\times$. A minimum of 300 conidia was evaluated and the percent germination calculated according to Braga et al. (2001) and Milner et al. (1991).

2.2. Collection of *R. sanguineus* s.l. engorged females

Engorged females of *R. sanguineus* s.l. were collected directly from naturally tick-infested abandoned dogs that were rescued and held at the Center for Zoonosis Control in Goiânia, GO, Brazil. Ticks were carefully examined, and injured or apparently unhealthy ones were discarded. Ticks were surface sterilized by immersion in a 1% (v/v) sodium hypochlorite solution for 3 min, rinsed with sterile distilled water and dried with sterile tissue paper. The engorged females were held in glass Petri plates (90 $\times$ 15 mm) and immediately used in bioassays.

2.3. Tick fixation and dissection for fluorescence microscopy (FM) or scanning electron microscopy (SEM)

Each fungus-treated *R. sanguineus* engorged female was fixed with 2% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde, 3% (w/v) sucrose in 0.1M sodium cacodylate buffer solution, pH 7.2; first by inoculation of 50 μ L of fixative using insulin syringes (13 $\times$ 0.45 mm; Descarpack, São Paulo, SP, Brazil), followed by immersion of the specimen into 2 mL of fixative, in a 15 mL centrifuge tube (Gene, Curitiba, PR, Brazil), for 10 days at 4 °C. After fixation, rectangles (ca. 6.0 $\times$ 5.0 mm) of dorsal cuticle were dissected, removed with tweezers, and rinsed three times (15 minutes each) in sodium cacodylate buffer solution (0.1 M, pH 7.2).

For FM, the cuticles were placed individually in each well of a 12-well cell-culture plate with 1.5 mL 2% (v/v) Calcofluor White M2R (Sigma-Aldrich Ltda., São Paulo, SP, Brazil). The cuticles were held overnight in the dark at room temperature. Immediately before microscopy, each cuticle was placed on a glass slide, covered with a coverslip, and analyzed and photographed with a Nikon Fluorescence Microscope (Eclipse 80i).

For SEM, the cuticles were dehydrated in a graded series of ethanol solutions (30, 50, 70, 80, 90 and 100%), held for 15 minutes in each solution and passed twice in 100% ethanol. Subsequently, the samples were placed separately in 2.5 micro-centrifuge tubes, containing approximately 500 μ L of hexamethyldisilazane (Electron Microscopy Sciences, Hatfield, PA, USA), and maintained immersed for 6 min. Then, the solution was removed and the tubes kept open for evaporation. After drying, the samples proceeded to metallization, being mounted on a stub, and coated with carbon in a sputter-applicator (Denton Vacuum, Desk V). The cuticles were then analyzed and photographed under a scanning electron microscope (Jeol JSM 6610), at an acceleration voltage of 5 kV.

214 *2.4. Experiment 1: Germination on PDAY of water-suspended versus oil-formulated conidia*
215 *exposed to heat*

216 Conidial suspensions prepared with oil or water (0.01% Tween 80[®]) were adjusted
217 to 1×10^7 conidia mL⁻¹. Two milliliters of stock suspension were transferred to four screw-
218 cap glass tubes (15 × 150 mm; Pyrex, Corning, São Paulo, SP, Brazil). One tube of each
219 fungal preparation was held at 27 ± 1 °C (control, unheated) while other three tubes of each
220 were exposed to 45 ± 0.5 °C for 2, 4 or 6 h in a water bath, with the bottom 3 cm immersed.
221 Temperature was monitored with a HOBO H8[®] data logger (Onset Computer Corporation,
222 Bourne, MA, USA) throughout the heat exposure. After heat exposure, 1 mL of conidial
223 suspension was transferred to screw-cap 15-mL centrifuge plastic tubes (Gene, Ionlab
224 Equip. Sup. Laborat. Hosp. Ltda., Curitiba, PR, Brazil) containing 9 mL of 0.05% (v/v)
225 Tween 80[®] and 100 µL of surfactant (General Chemicals, Campo Mourão, PR, Brazil).
226 Samples were vigorously agitated for 30 sec and centrifuged at 3157 RCF for 5 min. The
227 supernatant was discarded and the process repeated to ensure the oil was washed out
228 (Oliveira et al., 2015). The pellet was then suspended in 1 mL Tween 80 0.05% (v/v),
229 vortexed, and 20 µL were inoculated into the center of Petri plates (55 × 15 mm) containing
230 8 ml PDAY amended with 0.002% (w/v) benomyl (Braga et al., 2001; Milner et al., 1991).
231 The plates were incubated in the dark at 27 ± 1 °C and RH ≥ 80% for 0, 12, 18, 24, 36 or 48
232 h, and a minimum of 300 conidia per plate were evaluated by light microscope at 400 ×
233 magnification; the relative percent germination was calculated as described by Braga et
234 al. (2001). Washing the conidia out of oil after heat exposure was appropriate to assess the
235 conidial germination on PDAY medium. Aqueous suspensions also followed the ‘washing’
236 procedure to standardize the method for both suspensions (oil and water). Three

independent trials were conducted on different occasions, with new batches of conidia for each assay.

2.5. Experiment 2: Germination on tick cuticle and PDAY of water-suspended conidia exposed to heat. Germination evaluated by fluorescence microscopy

Aqueous conidial suspensions (2 mL) were transferred to a 15 × 150 mm glass screw-crap tubes (Pyrex, Corning, São Paulo, SP, Brazil) and immediately exposed to heat (45 ± 0.5 °C) in a water bath for 4 h, with the bottom 3 cm immersed. Control (unheated) conidial suspensions were held at 27 ± 1 °C for 4 h. Temperatures were monitored with HOBO H8® data loggers (Onset Computer Corporation, Bourne, MA, USA).

Fourteen *R. sanguineus* engorged females were weighed and distributed in two groups of seven and attached to two Petri plates (90 × 15 mm) in dorsal side up position using double-sided adhesive tape on their ventral surface. Each of the seven females was treated topically with 2 µL of heated conidial suspension, while each of the seven females from the control group was treated with 2 µL of an unheated conidial suspension. The plates were then incubated in a humid chamber in the dark at 27 ± 1°C and a RH higher than 90%. One female from each group was removed after 0, 12, 18, 24, 36, 48 or 72 h; each tick was immediately fixed and processed for fluorescence microscopy as described in details in section 2.3. Conidial germination on tick cuticle was assessed by evaluation of a minimum of 300 conidia per cuticle, and percent germination calculated as indicated above.

In addition, conidial suspensions used for treating ticks were diluted to ca. 1.0×10^5 conidia mL⁻¹ and a 20 µL aliquot of each was individually placed onto 4 ml PDAY medium amended with 0.002% benomyl and 0.05% chloramphenicol in Petri plates (55 × 15 mm). Plates were incubated in the dark at 27 ± 1 °C for the same incubation time periods as

described for the *in vivo* test (0, 12, 18, 24, 36, 48 or 72 h). Germination was assessed and relative percent germination determined by relating germination of heated to unheated conidia as previously described (Braga et al., 2001). Four independent repetitions were conducted in different days using fresh batches of conidia.

2.6. Experiment 3: Germination on tick cuticle of water-suspended versus oil-formulated conidia exposed to heat. Germination evaluated by scanning electron microscopy

M. anisopliae IP 119 conidia in aqueous suspension (0.01% Tween 80[®]) or in oil formulation were heat treated as described previously. Twelve *R. sanguineus* engorged females were attached to the plates and conidial suspensions applied to the dorsum of each. The containers were held as before, and specimens removed after 36, 48 or 72 h. One female tick was removed from each group within each incubation time, fixed, dissected, and the cuticle processed for scanning electron microscopy (SEM), as described in detail in section 2.3. Each sample was photographed and investigated for conidial germination and appressorium formation. Germination on tick cuticle was assessed by evaluation of a minimum of 300 conidia per cuticle, and percent germination determined. The whole experiment was repeated twice at different times, using new batches of conidia for each test.

2.7. Experiment 4: Effect of heat and formulation on virulence of M. anisopliae to R. sanguineus

Aliquots of 2 mL of 1×10^8 conidia mL⁻¹ collected from water or oil suspensions were individually transferred to glass test tubes with screw caps and heated for 4 h, as described previously. Engorged females of *R. sanguineus* weighing between 0.0867 and

0.1905 g were separated in seven groups (six treatment, and a control group) that had similar ranges of weights in each group, with 15 females in each. The bioassay protocol called for each female being weighed again, and then attached to the bottom of glass Petri plates (130 × 20 mm) with double-sided tape. Precise weights are needed because part of the bioassay results is expressed as ratio of egg-mass weight produced by individual ticks to the tick weight.

The groups were treated as follows: 1) controls, whose females were treated individually with 2 µL of water [Tween 80 0.01% (v/v)] or pure mineral oil (without addition of conidia); 2) females treated individually with 2 µL of aqueous or oil suspension, at 10^8 conidia mL⁻¹, with no previous exposure to heat; 3) females treated with 2 µL of the aqueous or oil suspension at 10^8 conidia mL⁻¹, previously exposed to heat (45 ± 0.5 °C for 4 h), as described earlier. The plates were then incubated in a humid chamber at 27 ± 1 °C and RH superior to 90%.

The mass of eggs laid by each female was collected daily, weighed, and immediately transferred to their respective test tube (15 × 150 mm); each tube was closed with a cotton plug. The tubes were incubated in a humid chamber at 27 ± 1 °C and RH > 90% for subsequent evaluation of larval hatch. The eggs were observed daily and percentage of larval hatching for each tube was visually estimated through microscopic examination and expressed as varying from 0% to 100% with 5% intervals in relation to the total mass of eggs (Fernandes et al., 2006). At the end of the oviposition, the females were weighed, assembled according to the treatment groups, and placed in Petri plates. All treatment-group tubes were incubated at 27 ± 1 °C and RH > 90% for evaluation of fungal growth on the tick cuticle.

The following biological parameters were investigated: female initial weight; pre-oviposition period, oviposition period, egg production, egg incubation period, percentage of hatch, hatching period, and residual weight of engorged female after oviposition. Most of these data were used for calculating the egg production index (Bennett, 1974), the nutritional index (Bennett, 1974), and the estimated reproduction (Drummond et al., 1971); but the number of eggs per gram of eggs (20,000) for calculating the estimated reproduction was modified to 20,400 for *R. sanguineus* (Fujisaki et al., 1976). In addition, the “percent tick control” was calculated by comparing the estimated reproduction of treated with untreated (control) groups (as recommended by Drummond et al., 1971). Two independent repetitions were performed on different days, using fresh batches of conidia in each bioassay.

2.8 - Statistical analyses

Data were submitted to parametric analysis of variance (ANOVA) followed by the Student-Newman-Keuls test (SNK) to determine: 1) Differences, in each incubation time, between mean relative germination or percent conidial germination on PDAY medium from conidia prepared in water or oil exposed or not exposed (control) to heat for 2, 4 or 6 h (section 2.2); 2) Differences between percent conidial germination on the cuticle of *R. sanguineus* and on artificial medium (PDAY), when conidia were suspended in water and exposed or not exposed (control) to heat for 4 h (section 2.3); 3) Differences in percent conidial germination on the cuticle of *R. sanguineus*, when conidia were suspended in water or mineral oil and exposed or not (control) to heat for 4 h (section 2.4); 4) Differences in initial weight of engorged females or oviposition period (section 2.4). Non-parametric data (i.e., egg production index, nutrient index and percentage of hatch,

described in section 2.4) were analyzed by the Kruskal-Wallis test followed by the SNK test. *P* values less than 0.05 were considered as significant. ANOVA followed by the SNK were fit using Statistica[®] version 7.1 (StatSoft, Inc., USA), while Kruskal-Wallis followed by the SNK test was carried out using BioEstat[®] version 5.3 (Mamirauá Institute, Belém, Pará, Brazil).

3. Results

3.1. Germination on PDAY of water-suspended versus oil-formulated conidia exposed to heat

M. anisopliae IP 119 was considerably more tolerant to heat (45 °C) when suspended in mineral oil than when suspended in water, at all exposure times investigated (Fig. 1). The heat exposure was sufficient to cause a significant delay in germination of conidia suspended in water (Tab. 1). In general, a significant difference was observed in the mean relative germination of conidia of the two suspensions at all incubation times of heat exposure (2, 4 or 6 h). For example, germination of conidia formulated in oil and exposed to heat for 2 h was 66% in the first 12 h of incubation, while germination of conidia suspended in water and heated for 2 h was 7.5% ($P = 0.0113$; $F_{1,4} = 19.75$). Also, germination of conidia formulated in oil and exposed to heat for 4 h was 64.4% in the first 12 h, whereas germination of conidia suspended in water and heated for 4 h was 0% at the same period of incubation ($P < 0.001$; $F_{1,4} = 1263.03$). After 24 h of incubation, very few conidia suspended in water had germinated (2.2%), while the conidia suspended in oil had approximately 95% germination ($P = 0.0005$; $F_{1,4} = 108.01$). Conidia suspended in water and exposed to heat for 6 h also had low germination at 24 h of incubation (2%), while conidia formulated in oil presented germination of 83% in the same incubation time ($P =$

0.0065; $F_{1,4} = 26.98$). Only conidia that were formulated in oil and exposed to heat (2, 4 or 6 h) reached approximately 100% germination at 48 h incubation; conversely, conidia suspended in water reached 84,9% when exposed to heat for 2 h and incubated for 48 h, and 23,4% when heated for 6 h and incubated for the same period (see Fig. 1).

3.2. Germination on tick cuticle and PDAY of water-suspended conidia exposed to heat. Germination evaluated by fluorescence microscopy

The germination of *M. anisopliae* conidia on PDAY differed from the germination of conidia on tick cuticle, regardless if they were heated or not prior to application (Fig. 2). The percent germination of unheated conidia (controls) applied to tick cuticle was significantly less than that of conidia on artificial culture medium at all incubation periods tested (12, 18, 24, 36 or 48 h), with exception of the incubation period of 72 h ($P = 0.003$; $F_{3,12} = 9.39$) when germination on both substrates reached approximately 80 to 100% (see Fig. 2). However, when conidia suspended in water were heat-stressed (45 ± 0.5 °C for 4 h), the percent germination of conidia on culture medium did not differ significantly from germination of conidia on tick cuticle at most of the incubation periods tested (12, 18, 24, 36 or 48 h), but at 72 h incubation a considerably higher percent germination was observed for conidia inoculated on PDAY ($61.5 \pm 15.3\%$) compared with conidia on tick cuticle ($13 \pm 6.6\%$). Appressoria developed on many germ tubes growing on tick cuticle at 36 h incubation, but only when conidia in water were not previously exposed to heat. No appressoria were seen on cuticles treated with conidia exposed to heat stress (45 °C for 4 h), even when conidia were incubated for 48 (Fig. 3B) or 72 h (Fig. 3D).

379 *3.3. Germination on tick cuticle of water-suspended versus oil-formulated conidia exposed*
 380 *to heat. Germination evaluated by scanning electron microscopy*

381 Conidial germination on tick cuticle of conidia suspended in mineral oil and heated
 382 (45 °C) for 4 h was greater at all incubation times in comparison to conidia suspended in
 383 water (Fig. 4). For example, at 36 h incubation, the percent germination of conidia
 384 suspended in oil was 16.3%, while the percent germination of conidia suspended in water
 385 was 2.2% ($P = 0.0011$; $F_{3,4} = 54.78$). At 48 h incubation, no significant difference was
 386 noted between the germination of conidia heated in oil, and the germination of conidia not
 387 heated in water. In the control groups (ticks treated with conidia not exposed to heat), the
 388 percent germination of conidia in oil suspension was greater at 48 and 72 h incubation (61.7
 389 and 71.1 %, respectively) than conidia in water suspension (32.7 and 32.6%, respectively).
 390 When conidia were suspended in mineral oil, a large amount of conidia germinated and
 391 produced highly developed germ tubes (Fig. 3E and 3F). At 36 h incubation, appressoria
 392 were seen on germ tubes from conidia not heated in water or oil prior to application to tick
 393 cuticle; while with heated conidia, only germ tubes from conidia heated in oil produced
 394 appressoria (Fig. 3B, 3D and 3F).

396 *3.4. Effect of heat and formulation on virulence of M. anisopliae to R. sanguineus*

397 The virulence tests of *M. anisopliae* against *R. sanguineus* had homogeneous groups
 398 of engorged females, as evidenced by no significant differences in the initial weights of
 399 engorged females among the groups ($P = 0.998$; $F_{5,6} = 0.04$) (Tab. 2). Engorged females
 400 treated with conidia suspended in water or formulated in oil, exposed or not to heat before
 401 tick inoculation, laid eggs for shorter periods in comparison to the no-conidia control
 402 groups ($P = 0.0045$; $F_{4,5} = 16.37$). The percent egg hatchability also differed among the

groups ($P < 0.0001$; $H = 27.61$), with the highest percentages found in the controls and the lowest percentages in the groups treated with fungal suspensions. The egg production index from females treated with either fungal preparation (water suspension or oil formulation) was lower than those untreated (control) females ($P < 0.0001$; $H = 74.02$). The nutrient index also differed among treatments ($P < 0.0001$; $H = 55.49$); the groups treated with oil or water conidial preparations, previously exposed or not exposed to heat, had significantly lower nutrient index in relation to the control groups. Groups of females treated with *M. anisopliae* exhibited high percent “tick control”, regardless of the type of conidial suspension (water or oil) or the heat exposure of conidia prior to tick treatment (see Tab. 2).

4. Discussion

It should be pointed out that in this study heat was applied to conidia only while they were in suspension (mineral oil or aqueous suspension), after which their germination was assessed on PDAY medium or tick cuticle at 27 °C. Our findings demonstrated that conidia of *M. anisopliae* IP 119 formulated in mineral oil germinated faster than conidia suspended in water, when both conidial formulations were exposed to heat and, then, inoculated on the cuticle of *R. sanguineus* or onto artificial medium (PDAY). IP 119 conidia, therefore, were more thermotolerant when formulated in mineral oil than in water. Delayed germination of heat-stressed conidia may be related to the time required for the fungus to recover from the damage caused by the heat before starting the germination process (Nicholson et al., 2000); this recovery time varies among isolates (Rangel et al., 2005; Rangel et al., 2010; Fernandes et al., 2010; Keyser et al., 2014). Many studies on thermotolerance of *Metarhizium* spp. used conidia suspended in water, and germination was assessed only on artificial culture media (Zimmermann, 1982; Rangel et al., 2005;

Fernandes et al., 2010; Rangel et al., 2010); the current study, however, also reports impacts on germination of conidia suspended in water or oil and exposed to heat stress previous to inoculation onto tick cuticle.

M. anisopliae conidia in water germinated faster when inoculated onto artificial substrate than onto tick cuticle, whether the conidial suspensions were previously exposed to heat (test) or not (control). There was a delay in germination of heat-stressed conidia suspended in water of about 24 h on both substrates (cuticle or culture medium), but conidial percent germination was much higher on culture medium than tick cuticle when observed up to 72 h. Culture medium provides nutrients and other conditions favorable for fungal development and growth. Conversely, an arthropod cuticle is a physical barrier and affords difficult-to-digest nutrients for entomopathogenic fungi (Tiago and Furlaneto, 2003). The very thick cuticle of engorged females, a feature which occurs due to hormonal stimuli during the engorgement process (Furlong et al., 2007), in addition to the presence of natural inhibitors and fungistatic compounds in epicuticle of hard ticks (Ixodidae), may compromise the success of fungal infection (Kirkland et al., 2004). According to Kirkland et al. (2004), the ability of fungi to overcome these natural barriers may determine the probability of successful infection and the virulence of certain strains.

The hydrophobic nature of arthropod epicuticle is, in general, considered important for adhesion of fungal spores to it. However, many studies have explored other important factors in the complex interaction between antimicrobial molecules present or secreted in the cuticle and the adhesion/germination of spores. For example, the presence of cuticular lipids in the cuticle of insects and other arthropods may support or inhibit adhesion and spore germination (Kirkland et al., 2004; Ortiz-Urquiza and Keyhani, 2013). Some molecules inhibit conidial germination of *Beauveria bassiana* (Lecuona et al., 1997;

Kirkland et al., 2004) and *M. anisopliae* s.l. (Sosa-Gomez et al., 1997). In hard ticks, particularly, cuticular lipids extracted from *Amblyomma americanum* significantly inhibited *B. bassiana* germination (Kirkland et al., 2004). Accordingly, the delay in conidial germination noted in our current study (even when conidia were not previously exposed to heat) may be due to the presence of natural barriers on the cuticle of *R. sanguineus*, or possibly to the limited availability of nutrients from cuticle in comparison to artificial culture medium.

The differences in germination rates of conidia suspended in water in experiments 2 and 3 (Fig. 2 and 4) probably are due to the use of different techniques for assessing the conidial germination on tick cuticle, viz. fluorescence microscopy or scanning electron microscopy. Fluorescence microscopy was not possible for conducting experiment 3 because Calcofluor White did not stain conidia covered with oil. Also, differences in conidial germination may occur in ticks from different populations, since ticks from different populations may have different susceptibilities to fungal infection (Fernandes et al., 2011; Perinotto et al., 2012). In the current study, experiments 2 and 3 were conducted on different occasions with ticks from different locations.

M. anisopliae IP 119 developed appressoria on the cuticular surface of *R. sanguineus* under optimal conditions of temperature and humidity. In fact, the time required for appressorium formation may vary among fungal isolates, as well as with the target host and abiotic factors. Bittencourt et al. (1999a) demonstrated for the first time the penetration of *M. anisopliae* s.l. through the tick cuticle of *R. microplus*, and detected appressorium at 72 h post-treatment. Other studies of the infection process of *M. anisopliae* sensu lato toward *R. sanguineus* reported appressorium formation at 18 h post-treatment (Garcia et al., 2004); and 24 h post-infection with *R. microplus* (Arruda et al., 2005). In the current study,

appressoria were not observed on cuticle of ticks treated with heated conidia suspended in water at any incubation time assessed (including 72 h). On the other hand, with conidia heated in oil, appressoria were first detected on cuticle at 36 h post-treatment, as well as in the control groups (without heat exposure). Possibly, heated conidia suspended in water formed appressoria after longer incubation times due to their delayed germination. At 72 h incubation, in general, germ tubes were shorter than germ tubes of unheated conidia. Nevertheless, bioassays have demonstrated infection in ticks treated with heated conidia formulated in water.

Most studies on microbial control of ticks available report the performance of fungal formulations in bioassays held in optimal conditions of temperature and RH (Kaaya and Hassan, 2000; Maranga et al., 2005; Lopes et al., 2007; Fernandes and Bittencourt, 2008; Angelo et al., 2010; Camargo et al., 2012). The current study, however, reports the performance against the tick *R. sanguineus* of *M. anisopliae* formulated in oil or water, and exposed or not exposed to heat stress. Our results report a high virulence (negative impact on egg production and hatchability) of IP 119 against *R. sanguineus* and emphasize that exposures to heat of conidial suspensions did stress the conidia, but did not totally inactivate them. Despite the delayed germination caused by prior exposure of conidia to heat, conidia formulated in water were able to cause infection and kill ticks. A single heat exposure (45 °C for 4 h) was emphasized in the current study; in the environment, repeated daily heat exposures may limit the period required for the fungus to recover from the stress. Fungal conidia which survive a given environmental stress factor (either heat, UV radiation, nutrient deprivation, dehydration or others) may have reduced vigor to infect the target pest (for example, retarded growth). As a result, reduced potential of the fungus as a biological control agent is expected in environments where a specific stress factor is

frequent (Keyser et al., 2014). Selection of appropriate fungi for field use, therefore, must consider the tolerance of fungal strains to such stress factors (Fargues et al., 1997; Ouedraogo et al., 1997; Braga et al., 2001; Rangel et al., 2005; Fernandes et al., 2010; Keyser et al., 2014).

The application of *M. anisopliae* IP 119 conidia in mineral oil onto *R. sanguineus* engorged females killed many ticks before they started laying eggs. Likewise, formulating conidia of *M. anisopliae*, *B. bassiana* (Camargo et al., 2012) or *Lecanicillium lecanii* (Angelo et al., 2010) in mineral oil increased the efficacy of these fungi in controlling various life stages of *R. microplus*. Oil-based formulation ingredients can be toxic to arthropods, such as the mortality of *R. microplus* engorged females treated with coconut oil (Polar et al., 2005). In the present study, however, the biology of ticks treated with mineral oil or water (control groups, without addition of conidia), did not differ significantly, demonstrating that the mineral oil tested was not toxic to *R. sanguineus* engorged females. In addition, the groups treated with oil-formulated conidia had shorter oviposition periods, lower egg production indexes, lower nutritional indexes and lower percentage of larval hatches in comparison to the groups treated with oil only (test) or water only (controls). These results indicate that the high percentage of ‘tick control’ in the groups treated with oil conidial suspension was due to fungus exposure.

Fungal formulations, especially oil-based formulations, have been studied for improving the performance of fungal entomopathogens by (a) favoring the spreading and adhesion of conidia onto the hydrophobic cuticle of host arthropods (David-Henriet et al., 1998), and (b) protecting these propagules from detrimental environmental factors, such as heat (McClatchie et al., 1994), desiccation (Bateman et al., 1993; Bateman, 1994), and ultraviolet radiation (Moore et al., 1993; Alves et al., 1998; Bateman and Alves, 2000;

Hedimbi et al., 2008). Our data revealed that oil formulation protected conidia of *M. anisopliae* from heat by favoring faster germination and enhancing the efficacy of the fungus to control *R. sanguineus* in comparison to conidia suspended in water. Accordingly, these traits (faster germination and protection from detrimental environmental factors) are important to providing reasons why the association of conidia of entomopathogenic fungi with oils improves their performance against arthropods.

Most currently extant studies of thermotolerance of conidia were conducted *in vitro*, and predicted that the damage caused by heat exposures could affect the effectiveness of fungal-based biopesticides in arthropod-pest-control programs (Zimmermann, 1982; Rangel et al., 2005; Fernandes et al., 2010; Rangel et al., 2010; Keyser et al., 2014). Our study demonstrated that the damage caused by heat on fungal biocontrol agents are possibly underestimated, since the delayed germination of conidia exposed to heat stress was more pronounced on the tick cuticle than that demonstrated in *in vitro* assays where germination was assessed on artificial-culture medium.

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Captions for figures

Figure 1. Relative germination of *Metarhizium anisopliae* (IP 119) conidia suspended in mineral oil (Impex[®]) or water (Tween 80[®], 0.01%), exposed to heat stress (45 ± 0.5 °C) for 2, 4 or 6 h and incubated on PDAY medium for 0, 12, 18, 24, 36 or 48 h at 27 ± 1 °C and a RH higher than 80%. Relative germination was calculated in relation to unheated controls. Error bars are standard errors of three trials.

Figure 2. Percent germination of *Metarhizium anisopliae* IP 119 conidia in aqueous suspension exposed to 45 ± 0.5 °C (heat) or to 27 ± 1.0 °C (control) for 4 h, applied immediately to the cuticle of *Rhipicephalus sanguineus* engorged females or onto artificial culture medium (PDAY), and incubated at 27 ± 1.0 °C for 12, 18, 24, 36, 48, and 72 h. Line

markers followed by the same letter in the same incubation time do not differ significantly ($P < 0.05$). Error bars are standard errors of four trials.

Figure 3. Photomicrographs by fluorescence microscopy (A and B) and scanning electron micrographs (C, D, E and F) of *M. anisopliae* (IP 119) conidia on cuticle of *Rhipicephalus sanguineus*. A) and C) Unheated (27 °C, control) conidia suspended in water (Tween 80, 0.01%) and incubated for 48 h and 72 h after tick treatment, respectively; B) and D) Heated (45 °C for 4h) conidia suspended in water and incubated for 48 h and 72 h after tick treatment, respectively; E) Unheated (27 °C, control) conidia suspended in mineral oil and incubated for 72 h after tick treatment; F) Heated (45 °C for 4h) conidia suspended in mineral oil and incubated for 72 h after tick treatment.

Figure 4. Percent germination of *M. anisopliae* IP 119 conidia suspended in water or oil, and exposed to heat (45 ± 0.5 °C) or not (27 °C) for 4 h, previous to application on cuticle of *R. sanguineus*. Ticks were incubated at 27 ± 1 °C and RH > 90%. Line markers followed by the same letter in the same incubation time do not differ significantly ($P < 0.05$). Error bars are standard errors of two trials.

Table 1

Table 1. Mean percent germination of *Metarhizium anisopliae* (IP 119) conidia suspended in mineral oil or water (Tween 80[®], 0.01%), exposed to heat stress (45 ± 0.5 °C) for 2, 4 or 6 h, and then incubated on PDAY medium in Petri dishes for 12, 18, 24, 36 or 48 h at 27 ± 1 °C and RH higher than 80%. Experiments were conducted three times, each on a different day.

Treatments	Heat exposure	Germination (%)				
		12 h	18 h	24 h	36 h	48 h
Aqueous Suspension	0 h	42.4 ± 21.7 abc	89.1 ± 3.6 ab	95.9 ± 0.8 a	97.2 ± 1.5 a	99.8 ± 0.2 a
	2 h	2.9 ± 2.9 ce	9.0 ± 5.11 d	18.2 ± 6.8 b	67.4 ± 9.8 b	84.7 ± 2.5 b
	4 h	0 de	0 de	2.1 ± 2.1 c	26.3 ± 7.9 c	39.9 ± 12.1 c
	6 h	0 ef	0 e	1.9 ± 1.9 c	5.8 ± 1.0 d	23.3 ± 4.2 c
Oil formulation	0 h	40.5 ± 16.8 b	87,5 ± 6,3 b	96.1 ± 1.4 a	97.5 ± 1.2 a	99.0 ± 1.0 a
	2 h	28.7 ± 13.7 abdf	72,3 ± 4,6 ac	95.1 ± 1.64 a	96.2 ± 1.9 a	98.2 ± 1.8 a
	4 h	31.9 ± 6.5 abd	59,0 ± 5,6 c	90.2 ± 2.9 a	92.5 ± 3.4 a	96.6 ± 2.1 a
	6 h	2.6 ± 2.6 abcdef	48,4 ± 5,5 c	76.2 ± 13.6 a	92.9 ± 2.8 a	94.5 ± 1.7 ab
F _{7,16}		5.28	56.05	49.83	36.89	34.44
P		0.0028	< 0.0001	< 0.0001	< 0.0001	< 0.0001

(*) Means and standard errors followed by the same letter in the same column do not differ significantly (*P* > 0.05).

Table 2

Table 2. Biological parameters of *Rhipicephalus sanguineus* engorged females treated topically with *Metarhizium anisopliae* s.s. (IP 119) suspended in water (0.01% Tween 80) or pure mineral oil (Impex[®]) at a concentration of 1×10^8 conidia ml⁻¹. The conidial suspensions were either held at 27 ± 1 °C (unheated) or 45 ± 0.5 °C (heated) for 4 h before application to tick cuticle. Controls were water or oil without conidia. After treatment, females were incubated at constant 27 ± 1 °C.

Treatment ¹	Engorged females mean initial weight (g)	Oviposition period (days)	EPI ²	NI ³	Egg hatchability (%)	Tick control (%) ⁴
Control (water without conidia).	0.1217 ± 0.004 a (n= 30)	12.4 ± 0.43 a (n= 26)	35.84 ± 3.71 a (n= 30)	60.14 ± 6.55 a (n= 30)	77.3 ± 3.95 a (n= 26)	-
Unheated water suspension.	0.1233 ± 0.004 a (n= 30)	4.0 ± 0.39 b (n= 11)	7.23 ± 2.36 bc (n= 30)	20.85 ± 5.77 bc (n= 30)	59.09 ± 6.13 ab (n= 11)	81.8
Heated water suspension.	0.1271 ± 0.004 a (n= 30)	5.5 ± 0.55 b (n= 14)	12.96 ± 3.30 c (n= 30)	31.49 ± 7.11 c (n= 30)	34.28 ± 5.04 bc (n= 14)	84.2
Control (oil without conidia).	0.1237 ± 0.004 a (n= 30)	10.7 ± 0,58 a (n= 27)	38.22 ± 3.41 a (n= 30)	62.69 ± 4.64 a (n= 30)	68.07 ± 5.56 a (n= 27)	-
Unheated oil suspension.	0.1240 ± 0.005 a (n= 30)	4.0 ± 0.31 * (n= 03)	2.15 ± 1.22 b (n= 30)	7.69 ± 4.39 b (n= 30)	10.0 * (n= 03)	98.9
Heated oil suspension.	0.1285 ± 0.004 a (n= 30)	4.0 ± 0.50 b (n= 06)	3.85 ± 1.99 bc (n= 30)	14.29 ± 6.38 bc (n= 30)	13.33 ± 3.59 c (n= 06)	94.9

Means in the same column followed by the same letter do not differ statistically ($P \leq 0.05$). When $n < 30$, some females died before laying eggs. (1) Unheated refers to conidia not heat exposed and heated refers to conidia exposed to 45 ± 0.5 °C for 4 h. (2) EPI = Egg Production Index (Bennet, 1974); (3) NI = Nutrient Index (Bennet, 1974). (4) Percent tick control was calculated according to the method proposed by Drummond et al. (1971), with modification of the constant to 20,400 for *R. sanguineus* (Fujisaki et al., 1976). (*) Refers to parameters that could not be used in this statistical analysis due to the absence of variance, because in one of two repetitions the females of this group did not lay eggs.

Figure 1

Figure 2

Figure 3

Figure 4