Heat-stressed Metarhizium anisopliae: *viability (in vitro) and virulence (in vivo) assessments against the tick* Rhipicephalus sanguineus

Fabrício M. Alves, Cíntia C. Bernardo, Flávia R. S. Paixão, Lucas P. Barreto, Christian Luz, Richard A. Humber & Éverton K. K. Fernandes

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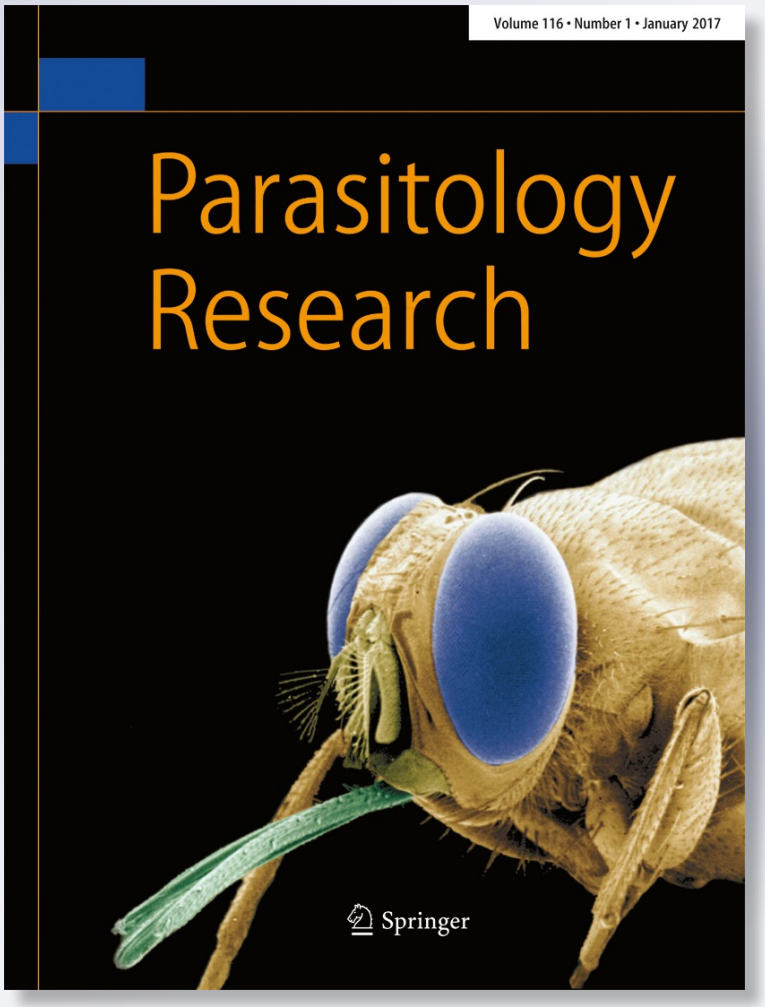

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ORIGINAL PAPER

Heat-stressed *Metarhizium anisopliae*: viability (in vitro) and virulence (in vivo) assessments against the tick *Rhipicephalus sanguineus*

Fabrício M. Alves[1] · Cíntia C. Bernardo[1] · Flávia R. S. Paixão[1] · Lucas P. Barreto[1] · Christian Luz[1] · Richard A. Humber[1,2] · Éverton K. K. Fernandes[1]

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Abstract The current study investigated the thermotolerance of *Metarhizium anisopliae* s.l. conidia from the commercial products Metarril® SP Organic and Metarril® WP. The efficacy of these *M. anisopliae* formulations against the tick *Rhipicephalus sanguineus* s.l. was studied in laboratory under optimum or heat-stress conditions. The products were prepared in water [Tween® 80, 0.01 % (*v*/*v*)] or pure mineral oil. Conidia from Metarril® SP Organic suspended in water presented markedly delayed germination after heating to constant 40 °C (for 2, 4, or 6 h) compared to conidia suspended in mineral oil. Metarril® SP Organic suspended in oil and exposed to daily cycles of heat-stress (40 °C for 4 h and 25 °C for 19 h for 5 consecutive days) presented relative germination of conidia ranging from 92.8 to 87.2 % from day 1 to day 5, respectively. Conversely, germination of conidia prepared in water ranged from 79.3 to 39.1 % from day 1 to day 5, respectively. Culturability of Metarril® WP decreased from 96 % when conidia were cultured for 30 min prior to heat exposure (40 °C for 4 h) to 9 % when conidia were cultured for 8 h. Tick percent control was distinctly higher when engorged females were treated with oil suspensions rather than water suspensions, even when treated ticks were exposed to heat-stress regimen. Oil-based applications protected fungal conidia against heat-stress. Although Metarril® is not registered for tick control, it may be useful for controlling *R. sanguineus*, especially if it is prepared in mineral oil.

Keywords Entomopathogenic fungi · Thermotolerance · Formulated fungi · Commercial bioproduct · Metarril · Tick control

Introduction

The brown dog tick, known in a broad taxonomic sense as *Rhipicephalus sanguineus* Latreille, 1806 (Acari, Ixodidae), is widely distributed in Brazil and in many other parts of the world. This tick has significant veterinary and medical importance; it preferentially parasitizes dogs but has also been found on other domestic animals and wild animals, as well as on humans (Dantas-Torres 2010; Dantas-Torres et al. 2006; Estrada-Pena and Jongejan 1999; Louly et al. 2006; Saxena and Maheshwari 1985). *R. sanguineus* is an important vector of several dog pathogens, including *Babesia canis* and *Ehrlichia canis* (Gothe et al. 1989; Smith et al. 1976). Additionally, it vectors important human pathogens, including *Rickettsia conorii*, the etiological agent of Boutonneuse fever in Europe (Merle et al. 1998), and *Rickettsia rickettsii*, a spotted fever agent in the USA and Mexico (Demma et al. 2005; Eremeeva et al. 2011). In Brazil, *R. sanguineus* is considered a potential vector to humans of both *R. rickettsii* (Rozental et al. 2002) and *Borrelia* sp. (Yoshinari et al. 1997).

The control of ticks with chemical acaricides is widely practiced even though the indiscriminate and exclusive use of these products may lead to environmental contamination and tick resistance (Chandler et al. 2000; Fernandes and Bittencourt 2008; Samish et al. 2004); not surprisingly, the

✉ Éverton K. K. Fernandes
evertonkort@ufg.br

[1] Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Avenida Esperança s/n, Campus Samambaia, Goiânia, GO 74690-900, Brazil

[2] Emerging Pest and Pathogens Research Unit, US Department of Agriculture, Agriculture Research Service, Robert W. Holley Center for Agriculture and Health, Ithaca, NY 14853-2901, USA

resistance of *R. sanguineus* to chemicals has been reported (Borges et al. 2007; Miller et al. 2001). Microorganisms that occur in nature, such as entomopathogenic fungi, may be used as biological control agents of ticks; however, the use of entomopathogenic fungi has not been proposed as an exclusive method for tick control but in integrated pest management strategies to reduce acaricide resistance and environmental contamination resulting from the consistent use of chemicals (Bahiense et al. 2006; Fernandes et al. 2012; Samish et al. 2004; Sousa et al. 2011; Webster et al. 2015).

Natural abiotic factors, however, may limit the efficacy of entomopathogenic fungi in arthropod control programs (Braga et al. 2001b; Fernandes et al. 2010; Rangel et al. 2010a). Temperature is considered one of the most important environmental factors that may directly influence the effectiveness of fungi in biocontrol programs, e.g., high temperature exposures may delay or even prevent germination of conidia (Fernandes et al. 2010; Keyser et al. 2014; Rangel et al. 2005). To circumvent this limitation, many studies have been focused on improving the performance of fungi by formulating conidia with oil carriers. Oil-based formulations are claimed to protect conidia not only against extreme temperatures (Barreto et al. 2016; McClatchie et al. 1994) but also against low humidity (Bateman et al. 1993) and ultraviolet radiation (Alves et al. 1998; Moore et al. 1993); in addition, oil formulations favor wide dispersion and adhesion of conidia on the cuticle of arthropods (David-Henriet et al. 1998). The efficacy of oil-based conidial formulations was demonstrated against ticks (Angelo et al. 2010; Camargo et al. 2012), triatomine bugs (Luz and Batagin 2005; Luz et al. 2012), and other arthropods (Lobo et al. 2016; Lomer et al. 2001; Malsam et al. 2002).

The current study investigated the thermotolerance of the conidia of *M. anisopliae* s.l. from the commercial products Metarril® SP Organic and Metarril® WP [Koppert Biological Systems (formerly Itaforte Bioproducts), Piracicaba, São Paulo, Brazil] which claim to control ticks. Over the course of many years, Itaforte Bioproducts, which was acquired by Koppert Biological Systems in 2012, produced and sold several different formulations of Metarril®. The company recently reviewed these formulations, and Metarril® SP Organic is no longer available, whereas Metarril® WP is now accessible commercially in Brazil. Most tests reported in the current study used Metarril® SP Organic before the company stopped producing or marketing this formulation. One thermotolerance test, however, was conducted with the currently accessible Metarril® WP. The viability of dormant conidia suspended in water or mineral oil and of germinating conidia (germlings) was assessed after being exposed to single or multiple heat-stress tests (*in vitro* tests). In addition, the efficacy of these *M. anisopliae* formulations against *R. sanguineus* s.l. ticks was studied under heat-stress conditions (in vivo tests).

Material and methods

Commercial bioproducts, fungal strains, and preparation of conidial suspensions

Tests were performed with the commercial products: Metarril® SP Organic (which comprised a mixture of two strains of *Metarhizium anisopliae* s.l. ESALQ 1037 and E-9) and Metarril® WP (which is based on a single strain, *M. anisopliae* E-9, both as dried conidial formulations). The product was provided by the manufacturer and stored at −20 °C before being used.

Metarhizium robertsii ARSEF 2575 was isolated originally from *Curculio caryae* (Coleoptera, Curculionidae) collected in South Carolina, USA and was incorporated in the current study because its well-known thermotolerance (Fernandes et al. 2010; Rangel et al. 2005). ARSEF 2575, from the USDA-ARS Collection of Entomopathogenic Fungal Cultures (Ithaca, NY), was cultured on potato dextrose agar (Difco Laboratories, Sparks, MD, USA) supplemented with 1 g l^{-1} yeast extract (Technical; Difco Laboratories) (PDAY) in polystyrene Petri plates (90 × 15 mm, Cralplast, Cotia, SP, Brazil) in the dark at 27 ± 1 °C for 15 days.

Metarril® SP Organic was suspended in a warm (35 °C) 0.01 % (*v*/*v*) solution of Tween® 80 (polyoxyethylene sorbitan monooleate; Labsynth Prod. Lab. Ltda, Diadema, SP, Brazil) to avoid imbibitional damage of conidia, as recommended by Faria et al. (2009); the suspension cooled down for 15 min, at room temperature, before being used in experiments. In addition, the SP Organic formulation was suspended in non-emulsified mineral oil (Naturol®, Farmax Amaral Ltda, Divinópolis, MG, Brazil). The conidial suspensions were left for 5 min on the bench to allow the dissolved and lighter weight inert materials of the formulated product to be decanted; conidia were then quantified by hemacytometer, and the concentrations adjusted to 1.0×10^8 conidia ml^{-1}. The same amount (*w*/*v*) of conidia (product) used for preparation of water conidial suspensions was used to prepare oil conidial suspensions. Fresh conidia of ARSEF 2575 were harvested with a microbiological loop and suspended in water [Tween® 80, 0.01 % (*v*/*v*)] or mineral oil (Naturol®); the suspension was quantified, and the conidial concentration adjusted as described above. The viability of conidia was evaluated before each trial by inoculating 20 μl of conidial suspension onto the center of PDAY plates and incubating them at 25 ± 1 °C for 24 h. Germination was assessed at ×400 magnification. Conidia were considered germinated when the elongating germ tube was longer than the maximum conidial diameter.

Effect of single heat exposure on conidial germination

Conidial suspensions prepared with mineral oil (Naturol®) or water (Tween® 80, 0.01 %) were adjusted to 1×10^7

conidia ml^{-1}. Two milliliters of each suspension was transferred to four screw-cap glass tubes (16 × 125 mm). One tube was held at 27 ± 1 °C, while three other tubes were exposed to 40 ± 0.2 °C for 2, 4, or 6 h in a water bath. After being heated, 1 ml of the conidial suspension was transferred to screw cap 15-ml centrifuge plastic tubes (Gene, Ionlab Equip. Sup. Laborat. Hosp. Ltda., Curitiba, PR, Brazil) containing 9 ml of Tween® 80 0.05 % (*v*/*v*) and 100 μl of the surfactant Solub'oil® (General Chemicals and Service Ltd., Campo Mourão, PR, Brazil). Samples were vigorously agitated for 30 s and centrifuged for 5 min at 1972 g. The supernatant was discarded, and the process was repeated to ensure that the oil was washed out (Oliveira et al. 2015). The pellet was then re-suspended in 1 ml Tween® 80 0.05 % (*v*/*v*), vortexed, and 20 μl were inoculated into the center of polystyrene Petri dishes (35 × 10 mm) containing 8 ml PDAY plus benomyl 0.002 % (*w*/*v*) (50 % active ingredient; Benlate®, DuPont, São Paulo, SP, Brazil) (Braga et al. 2001b; Milner et al. 1991). The plates were incubated in the dark at 25 ± 1 °C for 8, 12, 16, 20, or 24 h, and a minimum of 300 conidia per plate was evaluated to determine the relative percent germination (viability) as described Braga et al. (2001b). Washing the conidia out of oil after heat exposure was necessary to assess the conidial germination on PDAY medium. Water suspensions also followed the "washing" procedure to standardize the method for both suspensions (oil and water). Three independent replicate trials were performed.

Effect of multiple heat exposures on conidial germination

Two milliliters of mineral oil (Naturol®) or water (Tween® 80, 0.01 %) suspensions of conidia (1×10^7 conidia ml^{-1}) were transferred to ten screw cap glass tubes (16 × 125 mm). Five of these tubes were held at constant 5 ± 1 °C (control), while the other five were daily exposed to the cycle of 40 ± 0.2 °C for 4 h and 5 ± 1 °C for 19 h, for consecutive 5 days; the tubes had a 30-min interval at 25 ± 1 °C immediately before and after heat exposures to circumvent the rapid change in temperature. Each day, after heat exposure, the oil suspension from one tube was processed to remove the oil, and 20 μl were inoculated into the center of Petri dishes containing PDAY plus benomyl, as described previously. At the same time, two suspensions (oil and water) from the control group were also processed. Plates inoculated with conidia were incubated at 25 ± 1 °C for 48 h, and germination was assessed as described in the "Effect of single heat exposure on conidial germination" section. Three independent replicate trials were performed.

Effect of heat on germlings (germinating conidia)

Aqueous conidial suspensions were prepared from *M. robertsii* ARSEF 2575 conidia produced on PDAY. The commercial product Metarril® WP was also prepared in water [Tween® 80 0.01 % (*v*/*v*)] as reported previously. Fungal suspensions were adjusted to 2.5×10^6 conidia/ml, and a 2-ml aliquot was transferred to 98 ml potato broth supplemented with yeast extract (1 l distilled water and 170 g sliced, unpeeled fresh potatoes autoclaved at 121 °C for 15 min and filtered through cheesecloth, supplemented with 1 g l^{-1} yeast extract, and re-autoclaved) in 250-ml flasks incubated at 130 rev min^{-1} and 25 ± 1 °C for 0 (control), 0.5, 1, 2, 3, 4, 6, or 8 h. After each incubation time, a 5-ml aliquot was removed and centrifuged for 5 min at 1972 g, and the pellet was re-suspended in 5 ml Tween® 80 0.01 % to remove the residue of medium from the conidia. Two milliliters of this suspension were then transferred to a screw cap test tube and exposed to 40 ± 0.2 °C for 4 h in water bath. After heated, 15 μl of suspension (approximately 380 propagules) were spread evenly with bent glass rods onto the surface of 23 ml PDAY supplemented with chloramphenicol (0.05 % *w*/*v*) in 95-mm Petri dishes. They were then incubated for 5 days, and the relative percent culturability determined by colony forming units (CFU) counts as described by Braga et al. (2001a). Three replicate plates were prepared for each heat exposure period, and three independent replicate trials were performed.

Virulence of heat-stressed conidia against *R. sanguineus* ticks

Engorged females of *R. sanguineus* were manually collected from naturally infested abandoned dogs that had been captured and held at the Center for Zoonosis Control, in Goiânia and Aparecida de Goiânia, Goiás, Brazil. The dogs were not exposed to any type of acaricides for at least 60 days. Living ticks were washed in distilled water and then surface-sterilized by immersion in 70 % ethanol for 30 s, 1 % sodium hypochlorite solution for 3 min, rinsed with sterile distilled water, and dried with sterile tissue paper. Ticks were weighed and distributed by weight as homogeneously as possible among the treatment and control groups.

Engorged females were treated with 2.5 μl of fungal water or oil suspension applied directly onto their ventral cuticle with a semi-automatic micropipette (Nichiryo Nichipet EXII, Koshigaya-City, Japan). Then, a female was fixed in a dorsoventral position (dorsal side down) using a double-sided adhesive tape to an aluminum strip, 35 × 10 mm, cut from a beverage can, and placed in a glass vial (20 mm diameter × 45 mm high). The vial was closed with a cotton plug. The metallic support prevented the female from touching the glass walls. One vial had a probe connected to a HOBO® H8 data logger (Onset Computer Corporation, Bourne, MA, USA) to monitor the temperature and relative humidity (RH) inside the vial. Two control groups were treated with 2.5 μl oil or water (with no conidia), and two other control groups did not receive any treatment but were used to evaluate the effect of temperature on biological parameters of

R. sanguineus engorged females. Each treatment or control group was composed of ten replicates, i.e., ten vials with one female each. Three independent replicate trials were performed.

After treatment, five groups of females were incubated at constant 25 ± 1 °C, while five other groups were exposed to the cycle of 40 ± 0.2 °C for 4 h in water bath, and then incubated at 25 ± 1 °C and RH ≥80 % for 20 h, repeated for 4 consecutive days. From day 5 up to the end of oviposition, engorged females from both treated and control groups were held at 25 ± 1 °C and RH ≥80 %. The egg mass laid by each female was weighed daily and placed into individual test tubes. The eggs were incubated at 25 ± 1 °C and RH ≥80 % to allow larval hatch. The following parameters were evaluated: female initial weight, pre-oviposition period (number of days before laying eggs), oviposition period (number of days laying eggs), egg production (total weight of egg mass), egg incubation period (number of days before hatching), percentage of larval hatch, and residual weight of engorged female (engorged female weight 3 days after egg laying stopped). The egg production index (EPI), the nutritional index (NI) (Bennett 1974), the estimated reproduction (ER), and the "percent tick control" were calculated according to Drummond et al. (1971). The number of eggs per gram of eggs (20,000) used for calculation of ER was modified to 20,400 for *R. sanguineus* (Fujisaki et al. 1976).

Statistical analysis

Differences in heat tolerance (40 ± 0.2 °C for 2, 4, or 6 h) of Metarril® SP Organic conidial suspensions in oil or water for each incubation period (8, 12, 16, 20, 24, or 48 h) were assessed using analysis of variance (ANOVA). The difference in heat tolerance of Metarril® SP Organic conidia suspended in oil or water and exposed to five daily cycles of 40 ± 0.2 °C for 4 h and 25 ± 1 °C for 19 h was assessed using factorial ANOVA 2 × 6 design to measure if the combination of independent variables (conidial formulation and daily heat exposures) predicts the value of the dependent variable (relative germination) and the degree of their interaction. Differences in tolerances of germlings of Metarril® WP or ARSEF 2575 exposed to 40 ± 0.2 °C for eight exposure times (0, 0.5, 1, 2, 3, 4, 6, or 8 h) were assessed using factorial ANOVA 2 × 8 design. For all analyses, the Student-Newman-Keuls (SNK) test was used for pairwise comparisons among exposure-time means within each isolate. *P* values equal to or smaller than 0.05 were considered as significant.

Differences in engorged female initial weight, pre-oviposition period, oviposition period, and egg incubation period were assessed using factorial ANOVA 2 × 4 followed by the SNK test. Differences in egg production index and nutrient index were assessed by Kruskal-Wallis followed by the SNK test. *P* values equal to or smaller than 0.05 were considered as significant. All ANOVA models were fit using Statistica® version 10 (StatSoft Inc., USA). Kruskal-Wallis followed by the SNK test was carried out using BioEstat® version 5.3 (Mamirauá Institute, Belém, Pará, Brazil).

Results

Effect of single heat exposure on conidial germination

Conidia from Metarril® SP Organic suspended in water presented a marked germination delay after heat exposure (40 ± 0.2 °C for 2, 4, or 6 h) compared to conidia suspended in mineral oil (Fig. 1). Mean relative percent germination of 2 h heat-stressed conidia suspended in water was 9.5 % at 8 h incubation and reached 65 % at 12 h, whereas mean relative germination of conidia suspended in oil was 77.4 % at 8 h incubation and reached approximately 100 % at 12 h (8 h, $P = 0.005$, $F_{1,4} = 33.496$; 12 h, $P = 0.193$, $F_{1,4} = 2.911$). A significant delay in germination was observed at 6 h heat exposure, where mean relative germination of conidia suspended in water reached 2.2 % at 8 h and did not exceed 52 % at 24 h incubation, whereas conidia suspended in oil reached 32 % at 8 h incubation and 92.2 % at 24 h (8 h, $P = 0.0027$, $F_{1,4} = 58.359$; 24 h, $P = 0.0024$, $F_{1,4} = 64.914$). Mean relative percent germination of 2 h heated conidia suspended in water reached similar values to the conidia suspended in oil at 16 h incubation ($P = 0.578$, $F_{1,4} = 0.507$), while conidia suspended in oil or water exposed to heat for 4 h had analogous relative germination at 20 h incubation ($P = 0.296$, $F_{1,4} = 1.438$), and for 6 h of heat exposure, at 48 h incubation ($P = 0.348$, $F_{1,4} = 1.133$) (see Fig. 1).

Effect of multiple cycles of heat exposure on conidial germination

Metarril® SP Organic conidia suspended in oil and exposed to daily cycles of heat-stress (40 ± 0.2 °C for 4 h and 25 ± 1 °C for 19 h for 5 days) presented mean relative germination of conidia ranging from 92.8 % at day 1 to 87.2 % at day 5 (Fig. 2); conversely, mean relative germination of conidia suspended in water ranged from 79.3 % at day 1 to 39.1 % at day 5. No significance, however, was observed between conidial formulation and daily heat exposures ($P = 0.10$, $F_{4,20} = 2.17$), but significant variation was reported between water and oil conidial suspensions ($P = 0.0008$, $F_{1,20} = 15.24$).

Effect of heat on germlings

Mean relative percent viability of Metarril® WP conidia decreased from 96 %, when conidia were cultured for 30 min in potato broth supplemented with yeast extract prior to heat exposure (40 ± 0.2 °C for 4 h), to 9 % when conidia

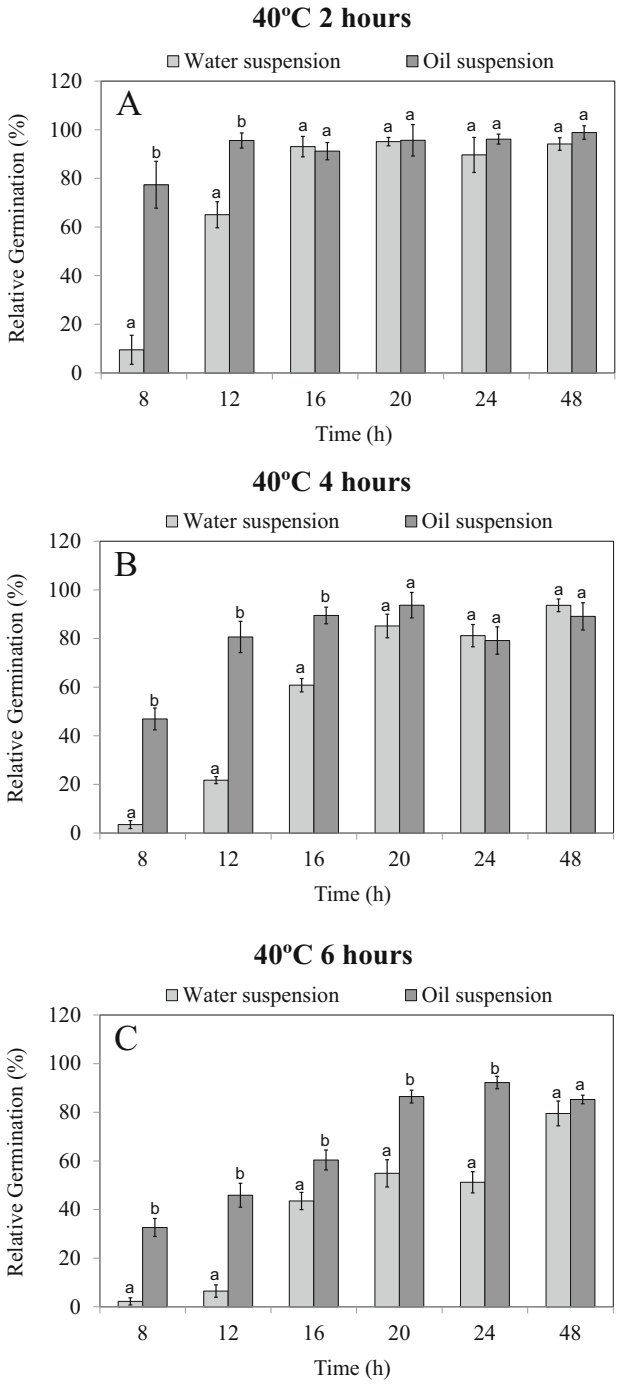

Fig. 1 Relative germination of *Metarhizium anisopliae* (Metarril® SP Organic) conidia suspended in mineral oil (Naturol®) or water (Tween® 80, 0.01 %), exposed to 40 ± 1 °C for 2 (**a**), 4 (**b**), or 6 h (**c**) and incubated for different periods of time (h) at 25 °C in the dark. Relative germination was calculated in relation to unheated controls. *Error bars* are standard errors of three trials. *Light* and *dark gray bars* with the *same letters* within the same incubation period do not differ significantly ($P \leq 0.05$)

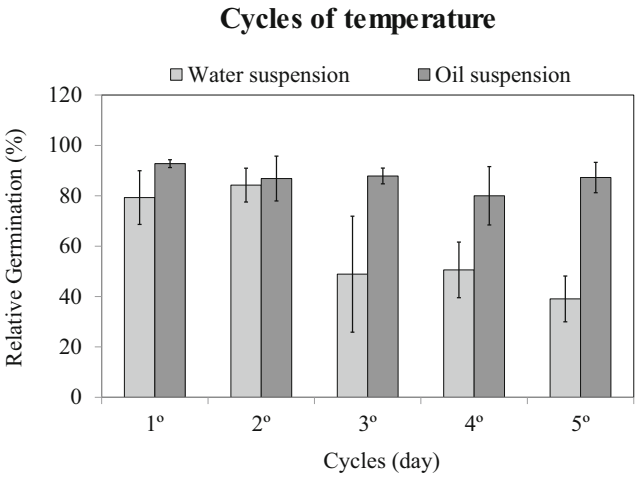

Fig. 2 Relative germination of *Metarhizium anisopliae* (Metarril® SP Organic) conidia suspended in mineral oil (Naturol®) or water (Tween® 80, 0.01 %) and exposed to daily cycles of 40 ± 1 °C for 4 h and 25 °C for 19 h for 5 days. The plates with fungi were incubated for 48 h at 25 °C in the dark before germination being assessed. Relative germination was calculated in relation to unheated controls. *Error bars* are standard errors of three trials. No significant interaction ($P = 0.24$; $F_{4,20} = 1.48$) between daily cycles and conidial suspensions was detected by factorial ANOVA. Variables analyzed separately showed no significant variation among the daily cycles ($P = 0.10$; $F_{4,20} = 2.17$) but significant variation between water and oil conidial suspensions ($P = 0.0008$; $F_{1,20} = 15.24$)

were cultured for 8 h ($P = 0.002$, $F_{6,14} = 10.13$) (Fig. 3). Similarly, mean relative viability of ARSEF 2575 germlings decreased from 95 %, when conidia were cultured for 30 min, to 4 % when conidia were cultured for 8 h prior to heat exposure ($P = 0.00002$, $F_{6,14} = 14.92$). The longer the growth time of conidia in liquid medium prior to heat exposure, the lower the mean relative viability of Metarril® WP and ARSEF 2575 conidia (see Fig. 3). No difference in thermotolerance of germlings was observed between Metarril® WP and ARSEF 2575 ($P = 0.67126$, $F_{6,28} = 0.67$).

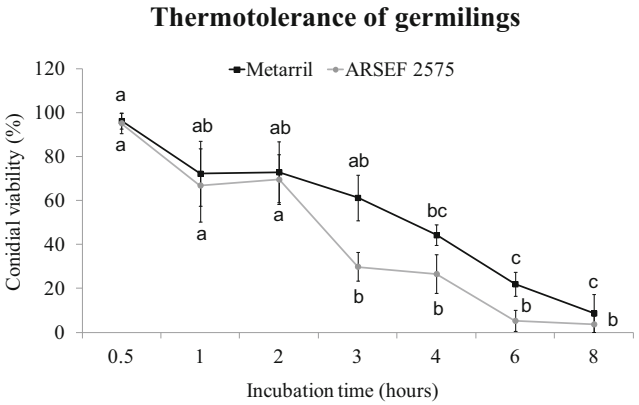

Fig. 3 Mean relative viability of *Metarhizium* sp. *(Metarril*® WP or ARSEF 2575) conidia cultivated in potato broth supplemented with 1 g l^{-1} yeast extract for 0, 0.5 (30 min), 1, 2, 3, 4, 6, or 8 h; suspended in Tween® 80 (0.01 %); and exposed to 40 ± 0.2 °C for 4 h. An aliquot of suspension was spread onto the surface of PDAY medium supplemented with chloramphenicol (0.05 %, *w*/*v*) and the plates incubated at 25 ± 1 °C in the dark. The number of colony forming units (CFUs) was assessed 5 days after sample inoculation, and the mean relative percent viability was calculated in relation to unheated controls. *Error bars* are standard errors of three trials. Means followed by the *same letters* did not differ significantly among the incubation times of the bioproduct ($P = 0.00020$; $F_{6,14} = 10.13$) or ARSEF 2575 ($P = 0.00002$; $F_{6,14} = 14.92$)

Virulence of heat-stressed conidia against *R. sanguineus* tick

The initial weights of engorged females did not differ among treatment and control groups, thus confirming the homogeneity of groups (Table 1). Engorged females treated with conidial oil suspensions demonstrated shorter oviposition period ($P < 0.0001$, $F_{3,20} = 45.373$), lower EPI ($P < 0.0001$, $F_{3,20} = 16.451$), and lower nutrient index (NI) ($P = 0.0003$, $F_{3,20} = 9.800$) when compared with engorged ticks treated with conidial water suspensions or to the control groups. "Percent control" (Drummond et al. 1971) was significantly higher when ticks were treated with oil suspensions than with water suspensions (see Table 1). Ticks treated with conidial water suspensions and held in constant optimum temperature or exposed to the heat-stress cycles had percent control ranging from 33.4 to 29.6 %, respectively, and when treated with conidial oil suspension tick percent control, these ranged from 63.6 to 54.0 %, respectively. Fungal treatments associated to the two temperature regimen did not alter most of the biological parameters investigated, except for the pre-oviposition period. Ticks that were not treated with fungi or with adjuvants (oil or Tween® 80, 0.01 %) presented similar biological parameters at both temperature regimens (Table 2).

Discussion

Few bioproducts claim to control ticks anywhere in the world, and only one product line, Metarril®, is registered in Brazil (Faria and Wraight 2007). This product is registered for controlling the sugarcane spittlebug, *Mahanarva fimbriolata* (Hemiptera, Cercopidae), but a few studies have shown its potential for tick control (Camargo et al. 2012; Camargo et al. 2014; Lopes et al. 2007). Metarril® SP Organic was used in most tests in the current study, and even though this specific product has been withdrawn by its current owners, this study indicated that oil-based applications of both Metarril® formulations tested here were more effective than water-based applications for tick biocontrol. These oil-based applications reduced the number of ticks generated from treated individuals (increased percent tick control according to Drummond et al. 1971), and protected the fungal conidia against heat-stress. Constant changes in the composition, formulation, and even the availability of specific biologically based products for pest control can be expected as a natural outcome of dynamic needs to improve commercial product lines.

The use of entomopathogenic fungi in biological control programs depends not only on the selection of strains with high virulence against the target host but also on their tolerance to environmental abiotic factors such as high temperatures, low relative humidity, and solar ultraviolet radiation (Braga et al. 2001b; Fernandes et al. 2012; Rangel et al. 2005; Xie et al. 2012). In fact, these conditions may limit the efficacy of fungal agents by delaying or preventing germination of conidia (Braga et al. 2001b; Braga et al. 2002; Fernandes et al. 2008; Keyser et al. 2014; Rangel et al. 2005). As expected, the delayed germination of *M. anisopliae* conidia from Metarril® SP Organic exposed to heat (40 ± 1 °C) was also reported in the current study. However, when conidia were suspended in oil, germination was notably faster than conidia suspended in water (Tween® 80, 0.01 %), i.e., relative germination of conidia suspended in oil reached the maximum germination in a shorter period of incubation (see Fig. 1). Evidence of the thermal protection conferred to fungal conidia by oil was reported by McClatchie et al. (1994) and more recently by Barreto et al. (2016).

The longer bioproducts persist in the field (e.g., on dog kennel or enclosures for *R. sanguineus* control or on cattle for *Rhipicephalus microplus*), the higher their efficacy against the target host because it increases the chances of the arthropod being infected. After field applications, however, conidia may be exposed to variable periods of heat on a daily basis that may compromise their viability. The current study indicated that conidia suspended in oil tolerated heat exposure for longer periods compared to conidia suspended in water (see Fig. 2); on the other hand, if the conidia reach the arthropod (e.g., a tick), they will germinate, but the fungal penetration through the cuticle will not be accomplished before the germlings are exposed to heat, since this penetration process usually takes more than 24 h (Arruda et al. 2005; Bittencourt et al. 1999). The current study demonstrated that germlings are more sensitive to heat than dormant conidia (see Fig. 3), as indicated previously (Rangel et al. 2010b), thereby revealing the vulnerability of this initial stage in the fungal infection process; it showed that the beginning of the fungal infection is a crucial phase needing protection from high temperatures exposures. Germlings of *M. anisopliae* were also more sensitive to UV-B radiation than dormant conidia as reported by Braga et al. (2001a). In fact, as reviewed by Valdes-Santiago and Ruiz-Herrera (2013), in the early steps of germination, fungal spores are expected to be more sensitive to abiotic environmental stresses.

Environmental abiotic factors also drive the biology of ticks (Bellato and Daemon 1997; Dantas-Torres 2010; Socolovschi et al. 2009), e.g., engorged females of *R. sanguineus* held at constant low temperature (17 °C) laid fewer eggs than females incubated at optimal temperature, and lower estimated reproduction (ER) was also reported for females held at low temperature, whereas shorter egg incubation periods were reported at high temperature (32 °C) (Bellato and Daemon 1997). Similar responses were reported for *R. microplus* incubated on comparable temperature regimens (Gloria et al. 1993). Conversely, no significant changes in biological parameters of engorged females were reported in the current study (see Table 2),

Table 1 Biological parameters of *Rhipicephalus sanguineus* engorged females treated with water (Tween 80, 0.01 %) or oil (Naturol®) suspension of *Metarhizium anisopliae* (Metarril Organic®), both at 1.0×10^8 conidial ml^{-1} and held at constant optimal temperature (25 °C) or at heat-stress condition (40 °C for 4 h) for 4 days

Treatment[a]	Engorged female mean initial weight (g)	Pre-oviposition period (days)	Oviposition period (days)	Egg incubation period (days)	Larval hatch (%)	EPI 1[b]	EPI 2[b]	NI	Control (%)[c]
Oil suspension 40/25 °C	0.1040 ± 0.006Aa ($n = 30$)	7.65 ± 0.36Aa ($n = 20$)	6.90 ± 0.32Aa ($n = 20$)	26.1 ± 0.62Aa ($n = 20$)	52.5Aa ($n = 20$)	36.12Aa ($n = 20$)	23.30Aa ($n = 30$)	32.57Aa ($n = 30$)	54.09
Oil suspension 25 °C	0.1064 ± 0.006Aa ($n = 30$)	6.29 ± 0.32Ba ($n = 17$)	5.80 ± 0.48Aa ($n = 17$)	25.4 ± 0.38Aa ($n = 17$)	50.8Aa ($n = 17$)	27.84Aa ($n = 17$)	15.26Aa ($n = 30$)	23.03Aa ($n = 30$)	63.69
Water suspension 40/25 °C	0.1023 ± 0.006Aa ($n = 30$)	6.38 ± 0.22Aa ($n = 26$)	9.10 ± 0.58Ab ($n = 26$)	26.0 ± 0.35Aa ($n = 26$)	65.2Aa ($n = 26$)	41.60Ab ($n = 26$)	34.90Ab ($n = 30$)	46.35Ab ($n = 30$)	29.62
Water suspension 25 °C	0.0996 ± 0.006Aa ($n = 30$)	5.92 ± 0.30Ba ($n = 25$)	9.60 ± 0.59Ab ($n = 25$)	26.3 ± 0.48Aa ($n = 25$)	58.1Aa ($n = 25$)	44.68Ab ($n = 25$)	37.48Ab ($n = 30$)	54.48Ab ($n = 30$)	33.47
Oil control 40/25 °C	0.1091 ± 0.007Aa ($n = 30$)	8.86 ± 0.45Ab ($n = 24$)	13.1 ± 0.47Ac ($n = 24$)	26.3 ± 0.48Aa ($n = 24$)	70.4Aa ($n = 24$)	51.42Ac ($n = 24$)	38.15Abc ($n = 30$)	64.35Ab ($n = 30$)	–
Oil control 25 °C	0.1034 ± 0.006Aa ($n = 30$)	7.79 ± 0.48Bb ($n = 24$)	13.4 ± 0.57Ac ($n = 24$)	24.9 ± 0.26Aa ($n = 24$)	69.0Aa ($n = 24$)	55.47Ac ($n = 24$)	43.00Abc ($n = 30$)	65.87Ab ($n = 30$)	–
Water control 40/25 °C	0.1069 ± 0.006Aa ($n = 30$)	6.07 ± 0.14Aa ($n = 28$)	12.7 ± 0.52Ac ($n = 28$)	26.9 ± 0.60Aa ($n = 28$)	73.4Ab ($n = 28$)	53.10Ac ($n = 28$)	46.75Ac ($n = 30$)	53.51Ab ($n = 30$)	–
Water control 25 °C	0.1010 ± 0.006Aa ($n = 30$)	5.72 ± 0.16Ba ($n = 25$)	12.0 ± 0.60Ac ($n = 25$)	26.6 ± 0.65Aa ($n = 25$)	84.0Ab ($n = 25$)	52.17Ac ($n = 25$)	45.00Ac ($n = 30$)	60.11Ab ($n = 30$)	–

Optimal and heat-stress conditions were conducted under relative humidity superior to 80 %. Means followed by the same uppercase letters in the same column do not differ statistically ($P \leq 0.05$) for comparisons between the two temperature regimens (cycles 40/25 °C and constant 25 °C). Means followed by the same lowercase letters in the same column do not differ statistically ($P \leq 0.05$) for comparisons between treatments with fungal suspensions and controls (oil suspension, water suspension, oil control, and water control). Bioassay was conducted three times, and each bioassay had ten replicates ($n = 30$). $n < 30$ means that females died before data were collected

EPI egg production index (Bennett 1974), *NI* nutrient index (Bennett 1974)

[a] 25 °C refers to the groups of engorged females held in constant temperature; 40/25 °C refers to the groups exposed daily to cycles of 40 °C for 4 h and 25 °C for 19 h over 4 days after treatment, and from the fifth day until the last day of oviposition, the groups were held in constant 25 °C

[b] EPI 1 was calculated considering only the engorged females that laid eggs, and EPI 2 was calculated considering all the engorged females treated (the females that did not lay eggs had zero for weight of eggs in EPI calculations)

[c] Percent tick control was calculated according to Drummond et al. (1971), with modification of the constant to 20.400 which refers to the number of larvae per gram of eggs of *R. sanguineus* (Fujisaki et al. 1976)

Table 2 Biological parameters of *Rhipicephalus sanguineus* engorged females held at constant optimal temperature (25 °C) or at heat-stress condition (40 °C for 4 h) for 4 days

Temperature[a]	Engorged female mean initial weight (g)	Pre-oviposition period (days)	Oviposition period (days)	Egg incubation period (days)	Larval hatch (%)	EPI 1[b]	EPI 2[b]	NI[b]	ER[b]
Cycles 40/25 °C	0.1006 ± 0.006a ($n = 30$)	6.40 ± 0.17a ($n = 25$)	13.5 ± 0.44a ($n = 25$)	26.2 ± 0.43a ($n = 25$)	68.2a ($n = 25$)	54.7a ($n = 25$)	44.2a ($n = 30$)	60.6a ($n = 30$)	617,832.6a ($n = 30$)
Constant 25 °C	0.1034 ± 0.006a ($n = 30$)	5.38 ± 0.22a ($n = 26$)	13.8 ± 0.42a ($n = 26$)	26.0 ± 0.45a ($n = 26$)	85.0a ($n = 26$)	59.2a ($n = 26$)	49.6a ($n = 30$)	69.1a ($n = 30$)	857,956.0a ($n = 30$)

Optimal and heat-stress conditions were conducted under relative humidity superior to 80 %. Means followed by the same letter in the same column do not differ significantly ($P \leq 0.05$). Bioassay was conducted three times, and each bioassay had ten replicates ($n = 30$)

EPI egg production index (Bennett 1974), *NI* nutrient index (Bennett 1974), *ER* estimated reproduction

[a] 25 °C refers to the groups of engorged females held in constant temperature; 40/25 °C refers to the groups exposed daily to cycles of 40 °C for 4 h and 25 °C for 20 h over 4 days after treatment, and from the fifth day until the last day of oviposition, the groups were held in constant 25 °C

[b] EPI 1 values were calculated considering only engorged females that laid eggs, and EPI 2 values were calculated considering all the engorged females treated (the females that did not lay eggs had zero for weight of eggs in EPI calculations). ER was calculated according to Drummond et al. (1971), with modification of the constant to 20.400 which refers to the number of larvae per gram of eggs of *R. sanguineus* (Fujisaki et al. 1976)

possibly because females were exposed to high temperatures (40 °C) for a few hours in daily cycles for four days (prior to oviposition) compared to the constant high temperatures (32 °C) reported previously (Bellato and Daemon 1997; Gloria et al. 1993). These evidences suggest that engorged females may search for places with the best possible conditions (especially temperature and humidity) for laying their eggs (Brovini et al. 2003; Chagas et al. 2001); immature stages may also search for similar conditions for molting.

Many studies have demonstrated the potential of entomopathogenic fungi to control *R. sanguineus* and other ixodidae (Angelo et al. 2010; Fernandes et al. 2011; Gindin et al. 2002; Reis et al. 2008; Rot et al. 2013). Studies have also reported that conidia formulated in oil were more efficient against ticks than conidia prepared in water; these laboratory studies, in general, treated ticks by immersion in conidial suspension (Angelo et al. 2010; Camargo et al. 2012; Fernandes and Bittencourt 2008; Kaaya and Hassan 2000; Leemon and Jonsson 2008; Polar et al. 2005). In the current study, however, each engorged female of *R. sanguineus* was treated with an application of a single drop of conidial suspension onto their cuticle. The topical application of conidial suspensions on tick cuticles in laboratory tests seems to mimic the effect of the application of bioproducts on tick-infested settings. In fact, the commercialized *M. anisopliae*, Metarril® SP Organic, was able to control *R. sanguineus* in laboratory tests, especially when the product was suspended in mineral oil, even when treated ticks were exposed to heat-stress regimen; a reduced oviposition period, nutrient index, and larval hatch were detected (see Table 1). In a previous study, Metarril® SP Organic formulated in 10 % mineral oil was also efficient against *R. microplus* in pen trials; calves artificially infested by these ticks were sprayed with the formulated product, and the efficacy of tick control reached ca. 70 % (Camargo et al. 2014). The efficacy of oil-formulated fungi may be attributed to improved adhesion of conidia on the tick cuticle and to the protection of these spores against desiccation (Bateman et al. 1993; David-Henriet et al. 1998; Leemon and Jonsson 2008).

In conclusion, the current study demonstrated the efficacy of a commercial *M. anisopliae* to control *R. sanguineus* and pointed out serious concerns about conidial formulation and the detrimental effects of heat on tick control. Although Metarril® is not registered for tick control, the results suggested that it may be useful for controlling *R. sanguineus*. In fact, there is no product registered for tick control in Brazil. Therefore, the commercialization of entomopathogenic fungi for tick control is expected to advance in formulation of selected strains and to improve the strategies for product application to establish efficient control programs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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