Exposure of Bed Bugs to *Metarhizium anisopliae* at Different Humidities

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ABSTRACT   Bed bugs *Cimex lectularius* L. were exposed to conidia (spores) of the entomopathogenic fungus *Metarhizium anisopliae* by feeding, aerosol spray, or contact with a treated surface. Feeding experiments demonstrated that bed bugs were innately susceptible to this fungus. However, only at 98% humidity were mortality rates high, regardless of whether bed bugs were sprayed with a fungal solution or contacted a treated surface. Mortality in treated bed bugs at ambient humidity did not increase when these bed bugs were kept in aggregation with other bed bugs that had recently blood fed to repletion. Based on these laboratory studies, we conclude that *M. anisopliae* is a poor pathogen for use in control of bed bugs, particularly at humidities that would likely be encountered under field conditions.

KEY WORDS   bed bug, *Cimex lectularius*, entomopathogenic fungi, *Metarhizium anisopliae*, humidity

The global resurgence in bed bug *Cimex lectularius* L. populations has demonstrated that controlling this hematophagous insect is challenging (Doggett et al. 2012). Bed bugs have evolved widespread resistance to pyrethroid insecticides (Romero et al. 2007, Zhu et al. 2010, Davies et al. 2012), and although other chemical classes of insecticides have been developed and registered for bed bug control, they have exhibited mixed results (Moore and Miller 2009, Romero et al. 2010, Haynes and Potter 2013, Koganemaru and Miller 2013, Wang et al. 2013). Nonchemical controls have also been proposed as part of integrated pest management strategies (e.g., Wang and Cooper 2011), and elimination of bed bugs by exposing infestations to heat (sustained lethal high temperatures) has proven effective (Pereira et al. 2009, Kells and Goblirsch 2011, Puckett et al. 2013).

Recently, the entomopathogenic fungus *Beauveria bassiana* has been shown to be pathogenic to bed bugs (Barbarin et al. 2012). Results demonstrated that the fungus was efficacious, and that due to the gregarious nature of bed bugs, infected individuals could transfer the fungus to uninfected bed bugs. Another entomopathogenic fungus *Metarhizium anisopliae* has been used to target a variety of insect species, including plant-feeding member of the Hemiptera (Shah and Pell 2003, Zimmermann 2007). This fungus has also been genetically modified to increase specificity and efficacy (St. Leger et al. 1996, St. Leger and Wang 2010).

In this laboratory study, we investigate the use of *M. anisopliae* as a potential pathogen against *C. lectularius* in the following ways: 1) by feeding conidia to bed bugs to bypass the cuticle and determine innate susceptibility to the fungus; and 2) by treating bed bugs with conidia (aerosol spray and contact) and determining mortality at three different humidities. Additionally, because the feeding status of natural infestations of bed bugs may vary (Reinhardt et al. 2010), we determined mortality of treated, unfed bed bugs that were comixed with bed bugs that had fed to repletion.

Materials and Methods

Insects.   A colony of *C. lectularius* was established from bed bugs originally obtained from Harold Harlan (Crownsville, MD). This pyrethroid-susceptible colony (Feldlaufer et al. 2014) was kept at ambient conditions (25 ± 5°C and 30 ± 5% relative humidity [RH]), and fed weekly on expired human red blood cells and plasma using an artificial (in vitro) feeding system (Feldlaufer et al. 2014). We assayed adult males, adult females, and nymphs (third–fifth instar) that had not been fed for 8 d before fungal treatments. For assays involving blood-fed bed bugs, nymphs and adults were fed to repletion <2 h before use. Bed bugs used in all experiments were kept as groups in 240-ml,

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wide mouth Mason jars (Jarden Home Brands, Daleville, IN), and provided with fan-folded, filter paper (40 by 140 mm; Whatman No. 1), which acted as a harborage.

Fungal Isolates. Wild-type *Metarhizium anisopliae* (ARSEF 1548) was obtained from the USDA Entomopathogenic Fungus Collection in Ithaca, NY. This strain was originally isolated from the rice black bug *Scotinophara coarctata* (F.) (Hemiptera: Pentatomidae). Fungal cultures were maintained in 100- by 15-mm sterile, plastic petri dishes (VWR International, Radnor, PA) on Difco potato dextrose agar (PDA; Becton-Dickinson, Sparks, MD) incubated at room temperature (Inglis et al. 2012). Virulence was maintained by passage through bed bug hosts every 2 mo (Butt and Goettel 2000). Cultures were incubated for 14 d and conidia harvested by scrapping colonies with a sterile spatula and were suspended in distilled water containing 0.01% Tween 80 (Sigma-Aldrich, St. Louis, MO). Spore suspensions were determined using a hemocytometer (Spencer, Buffalo, NY) and adjusted to desired concentrations by diluting in water containing 0.01% Tween 80.

Ingestion Assays. *M. anisopliae* conidia were added to a blood source and then fed to bed bugs to determine their innate susceptibility. For feeding experiments, 0.5 ml of a spore solution containing 5×10^7 conidia per milliliter was added to 49.5 ml of a blood–plasma mix to yield a final concentration of 5×10^5 conidia per milliliter, and fed to mixed stages of bed bugs ($n = 729$) using an in vitro feeding system (Feldlaufer et al. 2014). Concentrations of 1×10^5 conidia per milliliter, 1×10^4 conidia per milliliter, and 1×10^3 conidia per milliliter were also fed to bed bugs ($n = 354, 290,$ and 483, respectively) by first adding 0.5 ml of a spore solution containing 1×10^7 conidia per milliliter to 49.5 ml of a blood–plasma mix and then making appropriate dilutions. Control groups consisted of bed bugs ($n = 301$) fed either blood–plasma containing only 0.01% Tween 80, or bed bugs ($n = 282$) fed *M. anisopliae* spores that had been inactivated by autoclaving 15 min at 121°C. In all feeding experiments, any bed bugs that had not fed to repletion (by visual observation) were removed and not included in the analysis. Bed bugs were maintained at room temperature and humidity (25 ± 2°C and 30 ± 5% RH).

Humidity–Treatment Assays. We examined the effect of humidity on the mortality of bed bugs treated with *M. anisopliae* conidia by either spray application or by contact with a treated surface. Different humidity regimes were achieved by confining bed bugs in glass desiccators (150 mm i.d.; Fischer Scientific, Pittsburgh, PA) over saturated salt solutions (Wexler and Hasegawa 1954). Bed bugs were kept over a saturated solution of calcium chloride (32 ± 1% RH) to mimic ambient or room conditions; over a saturated solution of sodium chloride (74 ± 1% RH); and over distilled water (98 ± 1% RH). In all experiments, temperature (25 ± 2°C) and RH were verified by Traceable relative humidity–temperature meters (Fisher, Pittsburgh, PA).

Bed bugs were treated in one of the two ways: For spray applications, bed bugs were placed on filter paper in glass petri dishes and sprayed with *M. anisopliae* conidia suspended in sterile water containing 0.01% Tween 80. Using 30-ml amber bottles and a pump applicator (Specialty Bottles, Seattle, WA), we calculated that each group of bed bugs received ≈4.5×10^6 conidia. Treated bed bugs were transferred to petri dishes (60 by 15 mm) containing untreated (dry) filter paper (47 mm diameter; Whatman No. 1). Control bugs were sprayed with water containing 0.01% Tween 80 and maintained the same way as the treated bed bugs.

For contact assays, bed bugs were exposed to *M. anisopliae* by being placed on filter paper previously treated with conidia. In these experiments, conidial suspensions in sterile water containing 0.01% Tween 80 were applied to 47-mm (diameter) filter paper disks (Whatman No. 1) to yield a final concentration of 1×10^5 conidia per centimeter square. Filter papers were allowed to dry for 120 min before being placed in glass petri dishes (60 by 15 mm). Bed bugs were then placed on the dried, treated surface. Bed bugs placed on Tween 80-treated filter paper acted as controls. A total of 90–120 bed bugs (six trials of either 15 or 20 bed bugs per trial) were used for each humidity treatment, including controls.

In all experiments, mortality (see Feldlaufer et al. 2014) was assessed daily for 1 wk (168 h) posttreatment. Mycoses were confirmed by surface-sterilizing dead bed bugs with 70% ethanol–water followed by distilled water (Lacey and Solter 2012), and by subsequently maintaining dead bed bugs at 98% RH for an additional week. Dead individuals were then examined under a dissecting scope for the presence of fungus (Humber 2012).

Aggregation Assays. Because bed bug aggregations can generate humidified boundary layers (Benoit et al. 2007), we conducted an experiment to determine if bed bugs fed to repletion would produce an aggregation microclimate (i.e., raise the humidity) that increased mortality in fungal-treated, unfed bed bugs kept at a low RH. Ten unfed bed bugs were sprayed with conidia of *M. anisopliae*, as described in the humidity-treatment assays and kept in filter-paper-lined glass petri dishes with five untreated bed bugs that had recently (<2 h) fed to repletion. Nine trials were run: 90 unfed bed bugs (sprayed with conidia) and 45 untreated blood-fed bed bugs were used. Controls consisted of two trials (15 bed bugs per trial) of unfed bed bugs sprayed with conidia. Treated and control groups were kept over a saturated solution of calcium chloride (25 ± 2°C and 32 ± 1% RH) to mimic ambient humidity.

Data Analyses. We used a generalized linear model logistic regression (R Core Team 2013) to test for mean differences in treatment combinations. In this model, humidity level, conidia application methods, and their interaction were independent variables; the binomial dependent variable was the number of dead for each trial. An over-dispersion parameter was included in the model to accommodate the large dif-
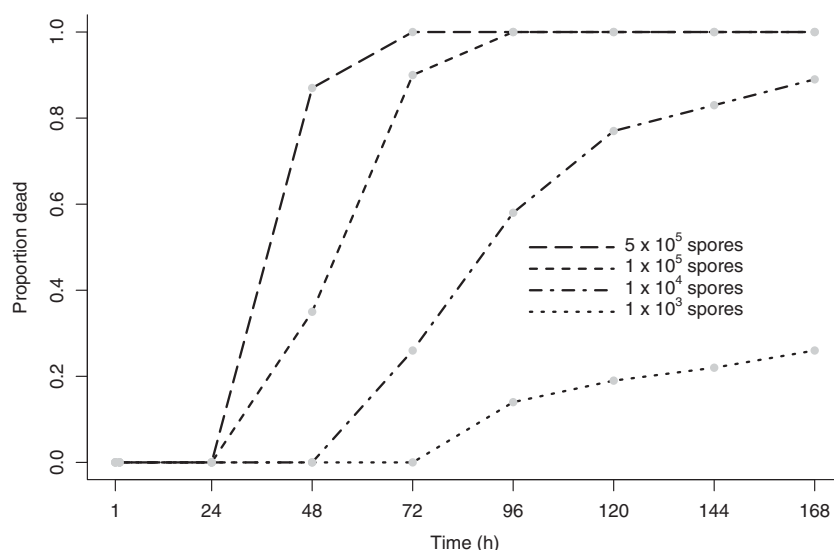
Fig. 1. Mortality in bed bugs fed spores of *M. anisopliae*. Conidia of *M. anisopliae* were added to blood products to achieve the final concentrations indicated, and fed to bed bugs (see Materials and Methods). Mortality (expressed as proportion dead) was recorded from 1–168 h (1 wk) postfeeding. As bed bugs ingest 2–8 microliter per insect (see Usinger 1966), the number of spores theoretically ingested at the different concentrations were 1,000–4,000 spores (at 5×10^5 spores; $n = 729$ bed bugs); 200–800 spores (at 1×10^5 spores; $n = 354$ bed bugs); 20–80 spores (at 1×10^4 spores; $n = 290$ bed bugs); and 2–8 spores (at 1×10^3 spores; $n = 483$ bed bugs). Control bed bugs ($n = 301$) fed only 0.01% Tween 80 exhibited <1% mortality after 168 h; bed bugs ($n = 282$) fed inactivated *M. anisopliae* spores (autoclaved 15 min at 121°C) had 2.8% mortality at 168 h.

ferences among the replicates of the contact filter treatment method, allowing for trial-to-trial variability. Tests for mean differences were performed using the R multcomp package (Hothorn et al. 2008). In the analysis, for each percent relative humidity the controls for aerosol spray and contact were combined; there was negligible mortality in any control group where bed bugs contacted Tween 80 by spray or by contact. All results were back-transformed to the original scale (either "proportion dead" or "% mortality") for clarity.

Results

Ingestion Assays. After ingesting spores of *M. anisopliae*, bed bugs died in a dose- and time-dependent manner, proving them innately susceptible to this entomopathogenic fungus (Fig. 1). Concentrations of 5×10^5 and 1×10^5 spores per milliliter produced 100% mortality in 72 h and 96 h, respectively. Bed bugs fed lower concentrations of conidia exhibited lower mortalities; a concentration of 1×10^4 spores per milliliter produced a maximum mortality of 89% after 1 wk (168 h), while a concentration of 1×10^3 spores per milliliter achieved a mortality of only 26% after 1 wk. Mortality in bed bugs fed blood containing 0.01% Tween 80 (3 dead of 301; <1%), or inactivated *M. anisopliae* spores (8 dead of 282; <3%) was negligible.

Humidity–Treatment Assays. Bed bug mortality at 1 wk postexposure to *M. anisopliae* conidia varied, depending largely on the humidity at which the bed bugs were kept (Fig. 2). By both spray and contact, mortality was greatest in bed bugs kept at 98% RH.

Mortality in these groups was 71.1% (64/90) by spray and 97.8% (88/90) by contact, and these differed from each other and from all other treatments. Mortalities at the two lower humidities never exceeded 25% (25/100; aerosol spray at 74% RH), and all but one were not significantly different from the Tween 80-treated controls. Mycoses were confirmed (noticeable mycelia growth emanating from appendage joints, etc.) in all treated bed bugs that died. Although a few control bed bugs died, no evidence of fungal infection was found in control groups.

Aggregation Assays. Because of the high water content of recently fed bed bugs, we mixed unfed bed bugs sprayed with *M. anisopliae* conidia with untreated bed bugs recently blood-fed to repletion in an effort to determine if mortality in these treated bugs could be increased over what was observed in the previous experiment at low humidity (32 ± 1% RH). Mortality in these fungal-treated, unfed bed bugs that were comixed with untreated, blood-fed bed bugs was 11.1% (5.3%, 21.7%; lower and upper 95% confidence intervals, respectively). This was not significantly different than the 13.3% mortality (4.1%, 35.4%; lower and upper 95% confidence intervals, respectively) in the fungal-sprayed, unfed bed bugs kept separate from fed bugs, and similar to the 17.8% mortality we observed in fungal-sprayed bed bugs kept at 32 ± 1% RH in the previous humidity–treatment assays (Fig. 2).

Discussion

M. anisopliae exhibits a broad invertebrate host range, making this entomopathogenic fungus an at-
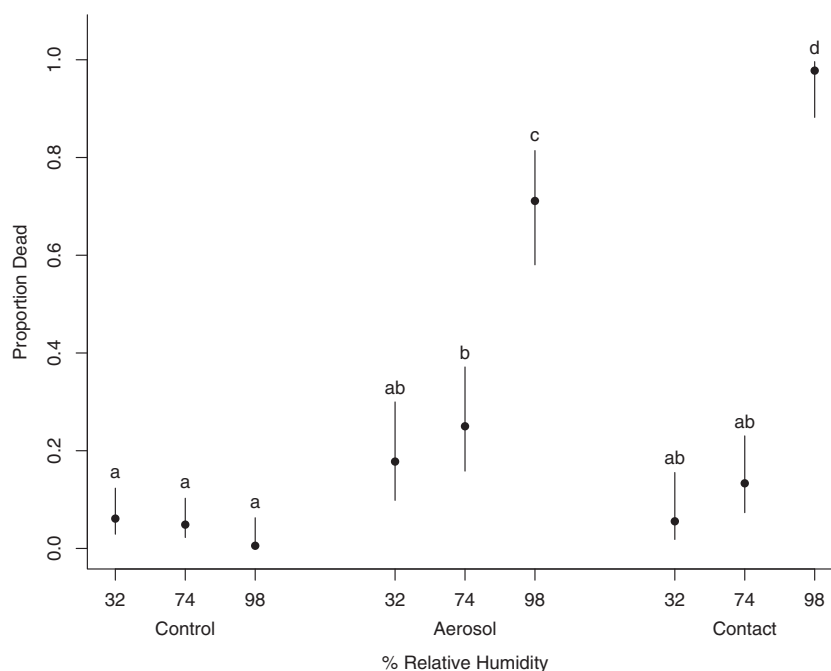
Fig. 2. Mortality in bed bugs treated with spores of *M. anisopliae* and kept at different humidities. Bed bugs were treated with *M. anisopliae* conidia by either aerosol spray ("Aerosol") or by placing the bed bugs on a conidia-treated surface ("Contact"), and kept at three different relative humidities. Mortality (expressed as proportion dead) was assessed 1 wk (168 h) posttreatment. Control groups consisted of bed bugs that were either sprayed with 0.01% Tween or placed on filter paper previously treated with 0.01% Tween 80. Ninety to 120 bed bugs were used for each humidity treatment, including controls. Vertical bars indicate 95% confidence intervals. Means that differ significantly have different letters.

tractive biological control candidate (Faria and Wraight 2007). Use of *M. anisopliae* has been investigated for controlling a number of arthropods of medical and veterinary importance including mosquitoes (Blanford et al. 2005; Scholte et al. 2005, 2006; Mnyone et al. 2009), biting flies (Kaaya and Munyinyi 1995, Ansari et al. 2011), and ticks (Kaaya et al. 1996, Frazzon et al. 2000, Kaaya and Hassan 2000, Benjamin et al. 2002, Kirkland et al. 2004). Use of entomopathogenic fungi to control urban pests such as termites (Rath 2000, Wang and Powell 2004) and cockroaches (Quesada-Moraga et al. 2004, Hernandez-Ramirez et al. 2008) has also been reported, though laboratory studies indicate termites may possess defensive mechanisms to fungal infection (Chouvenc et al. 2009, Chouvenc and Su 2010). The use of the entomopathogenic fungus *B. bassiana* for bed bug control has been demonstrated in the laboratory (Barbarin et al. 2012). Because we do not know the humidity in the *B. bassiana* experiments, direct comparisons with our study cannot be made, though growth and infection of both fungi can be limited by relative humidity (Maximiano et al. 2006).

In general, outdoor field effectiveness of *M. anisopliae* can be limited by unsuitable physical conditions such as heat, humidity, and sunlight (Jaronski 2010). Thus, although laboratory bioassays demonstrate that entomopathogenic fungi have the potential to significantly reduce pest populations, results of field trials

have been inconsistent. For instance, Benjamin and colleagues (2002) found *M. anisopliae* killed 96% of adult *Ixodes scapularis* (deer or black-legged) ticks in the laboratory, but only 53% of ticks treated in the field. These authors further caution that the 53% mortality in these ticks may be artificially high, as the treated ticks were subsequently maintained in the laboratory at relatively high humidity. The need for high humidities for entomopathogenic fungi to be effective killing agents has been previously demonstrated in two species of beetles (Walstad et al. 1970, Doberski 1981). Our laboratory results with bed bugs also demonstrate that high humidity is necessary for mortality.

Because natural infestations of bed bugs usually contain bed bugs that are of mixed feeding status (unfed to fully fed), we attempted to mimic field infestations by combining fungal-treated, unfed bed bugs with bed bugs that had recently fed to repletion. Our rationale was that the high water content of blood-fed individuals might raise the humidity of the aggregation microenvironment sufficiently to result in increased mortality in the fungal-treated bed bugs. This was not the case, as mortality in these treated bed bugs was not significantly different from treated bed bugs kept with other unfed individuals. A caveat to this conclusion is that we cannot say whether true aggregations formed or whether any avoidance was exhibited by either fungal-infected or noninfected bed

bugs. For whatever reason, the microenvironment in the individual dishes did not promote fungal growth in treated bed bugs. A detailed review of insect behavior as it applied to fungal pathogens is given by Baverstock et al. (2010).

We conclude that mortality in bed bugs treated with the fungal isolate (ARSEF 1548) of *M. anisopliae* used in this study is humidity dependent. Although this fungal pathogen can kill bed bugs, the humidity level required to exceed 70% mortality is deemed impractical under natural, indoor conditions, and that low humidity would impede practical use of this strain of *M. anisopliae* in a control program aimed at these indoor, urban pests. Additional studies are needed to identify other strains of *M. anisopliae* that are less impacted by low humidity. Alternatively, the use of *M. anisopliae* in settings that possess higher relative humidities, such as poultry houses, might prove feasible (see Oliveira et al. 2014).

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