Explaining Mycoinsecticide Activity: Poor Performance of Spray and Bait Formulations of Beauveria bassiana and Metarhizium brunneum Against Mormon Cricket in Field Cage Studies*

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Abstract

The Mormon cricket (*Anabrus simplex* Haldeman) (Orthoptera: Tettigoniidae) is a historic pest on millions of acres in the western US. During outbreak years, annual losses attributed to it have amounted to millions of dollars. Nonchemical controls are needed for environmentally sensitive areas – an increasing concern. Myco-insecticides are available but show an unpredictable and unexplainable lack of effect on Mormon crickets. This research sought to understand performance of *Beauveria bassiana* and *Metarhizium brunneum* under field conditions, compared to studies showing excellent performance under laboratory conditions. Our objectives were: (1) to evaluate *B. bassiana* GHA and *M. brunneum* F52 for potential use against Mormon crickets; (2) to compare spray and bait formulations of each fungus against immature and adult Mormon crickets; and (3) to understand the effect of Mormon-cricket body temperatures on the speed of efficacy by the two fungi. No mortality of 3rd-instar Mormon crickets was detected in the field 14 d after treatment with fungal baits or sprays of either *B. bassiana* Strain GHA or *M. brunneum* Strain F52. Unexpected bait aversion may have adversely affected the success of the bait treatments. Lack of spray efficacy was also unexpected, given the laboratory infectivity of both fungi for the Mormon cricket. Possible body temperatures were estimated by thermal surrogates located on the ground and in the plant canopy within field cages. Surrogate temperatures below 18°C, as well as above 30°C, were common during the study. When cumulative amount of time at temperatures optimal for fungal growth (18 to 30°C) was calculated for the duration of the post-treatment observation period, there was an insufficient number of fungal growth hours to produce mortality within the 14-d post-treatment period. Our data suggest that at least 32 to 43 d (*M. brunneum*) or 58 to 75 d (*B. bassiana*) should have been required for insect mortality to occur under the conditions of the test. In a second test, targeting seventh-instar nymphs and adults, the in-field mortality was not followed, but treated individuals were incubated both indoors and in outdoor cages. Infections by *B. bassiana* and *M. brunneum* in the indoor-incubated insects caused 69 and 100% mortality (corrected for untreated controls) respectively, with 71 and 80% mycosis in cadavers. Mormon crickets in outdoor cages succumbed to unexpected high temperatures. Our data illustrate the importance of Mormon cricket body temperatures and their effects on fungal infection. Because physical location and thermoregulation may alter the environ inside the insect, at least a month may be required after treatment for infections by *B. bassiana* GHA and *M. brunneum* F52 to cause mortality. Future studies should take this longer observational time into account. There is some potential for faster fungal growth in Mormon crickets under natural conditions, as opposed to our experimental cages, particularly in the immature stages. Mormon crickets aggregate in sheltered locations under inclement weather and at night. This behavior may conserve and actually promote body temperatures more favorable to fungal development. The potential value of this aspect of Mormon cricket behavior will require additional study.

Key words

biocontrol, Orthoptera, *Metarhizium brunneum*, *Beauveria bassiana*, *Anabrus simplex*, Mormon cricket

Introduction

The Mormon cricket (*Anabrus simplex* Haldeman), a flightless shieldback katydid belonging to the family Tettigoniidae, is a historic pest in North America. In the United States it occurs in Washington, Oregon, California, Nevada, Idaho, Arizona, Utah, New Mexico, Colorado, Wyoming, Montana, Kansas, Nebraska, South Dakota, North Dakota and Minnesota. In Canada, it has been found in British Columbia, Alberta, Saskatchewan and Manitoba (Pfadt 2002). Annual agricultural losses due to Mormon crickets have amounted to millions of dollars. In 1938 the insect was estimated to have caused an average measurable loss of 15 per cent on almost 13 million acres of rangeland and to have damaged crops on 235,000 acres in the western United States (Wakeland & Parker 1952). More recently, from 2000 thru 2009, many millions of acres have been infested and a total of 1,088,524 acres were treated to control Mormon cricket by the USDA APHIS in the western United States (USDA APHIS PPQ Western Region, unpub.). The current control treatments used in USDA-APHIS sponsored control programs rely on carbaryl in a spray or bait formulation and diflubenzuron in a spray formulation (USDA 2002).

The need for nonchemical alternatives has become increasingly important in recent years. Environmentally sensitive situations commonly encountered within the western United States in potential control-program areas preclude traditional chemical insecticide treatments. These situations are increasing and in areas of grasshopper or Mormon cricket infestations, at best complicate, and more often prevent, much needed local or area-wide treatments of grasshoppers and/or Mormon crickets on rangeland. As a result, nonchemical options are highly desirable in the diverse Mormon cricket habitat in the western U.S. Two microbial agents, *Paranosema locustae* and *Beauveria bassiana*, are registered for use against Orthoptera on rangeland, but neither is economically efficacious against Mormon cricket.

In the early 1990's, extensive work on the development of the fungus *B. bassiana* Strain GHA by Mycotech Corporation and the USDA APHIS "Methods Development" group (Foster *et al.* 1991, 1992, 1993, 1996a, 1996-1999) led to registration of that fungus in the U.S., even though wide acceptance and use against grasshoppers was never achieved. Another insect pathogenic fungus, *Metarhizium brunneum* Strain F52, was registered in 2005 as a myco-insecticide for control of Coleoptera in horticulture and managed turf, and

soft-bodied ticks (USEPA 2003). Since its registration, F52 has demonstrated good efficacy against a range of insect orders, including Homoptera, Heteroptera, Lepidoptera, and Thysanoptera, (Novozymes Biologicals, pers. com.), and larval Diptera (Jaronski & Jackson 2008). Preliminary laboratory bioassays (Jaronski, unpub.) indicated the potential efficacy of these strains for Mormon crickets. A novel bait (Tast-E-Bait™, a combination of bakery, snack, cereal and confectionary waste) has been shown to be an excellent carrier for carbaryl against Mormon cricket (Foster *et al.* 2003, Foster *et al.* 2004). We hypothesize that such bait could enhance dose transfer of fungal conidia to Mormon crickets and thus increase efficacy over a conventional ultra-low volume spray of fungal conidia, as is the practice with *M. acridum* against locusts in Africa and Australia. If successful, baits would greatly decrease the cost of fungi used against this insect pest.

In this study we confirmed the infectivity of *B. bassiana* GHA (Bb GHA) and *M. brunneum* F52 (Mb F52) for nymphal Mormon cricket in laboratory bioassays and evaluated bait and spray formulations of the two fungi against Mormon crickets in cages under field conditions. Our objectives were threefold: 1) to evaluate *B. bassiana* GHA and *M. anisopliae* F52 for potential use against Mormon cricket; 2) to compare spray and bait formulations of each fungus against immature and adult Mormon cricket; and 3) to understand the effect of optimal and suboptimal Mormon cricket body temperatures on the speed of efficacy by the two fungi.

Methods

The fungi used in this study were *B. bassiana* GHA (Emerald BioAgriculture Corporation, Lansing MI, now owned by Laverlam International, Butte MT) and *M. brunneum* F52 (Earth BioSciences, New Haven CT, now owned by Novozymes Biologicals, Salem VA). Conidia of *B. bassiana*, as technical grade active ingredient, Lot 0304-1, were supplied by the company. Conidia of Mb F52 were produced as a dry conidial powder by USDA ARS, using a biphasic, liquid-solid substrate fermentation process (Bradley *et al.* 1992). Prior to formulation, conidial viabilities were determined by plating dilute aqueous suspensions of each technical powder onto potato dextrose agar, incubating at 27 to 28°C for 16 to 19 h, and then examining the conidia with 400X phase-contrast microscopy. The *M. brunneum* conidia required a preliminary step in which a small quantity of dry conidia was exposed to 100% relative humidity for 1 to 2 h before suspension and plating. A minimum of 400 conidia were examined for germination; a conidium was considered viable (germinated) if it had produced a visible germination peg during the specified incubation time. Viability of the Bb GHA technical powder was 95%, that of *M. brunneum*, 85%. Concentrations of conidia in our formulations were adjusted for conidial viability.

The bait consisted of Tast-E-Bait™ (Advanced Organics Inc., Maumee, OH) coated with fungal conidia, using a binder of methylated soybean oil. A 1-kg quantity of Tast-E-Bait was treated with a fine spray of Golden Pest Spray Oil® (Stoller Manufacturing Inc.) at the rate of 10% (v/w), and then mixed in a rotating v-cone blender for 15 min. The slightly sticky granules were spread out into a shallow layer and dusted with fungal conidia at a rate equivalent to 2.5×10^{12} viable conidia/kg carrier (2.8×10^6 conidia/bait particle), then mixed in the v-cone blender for 15 min. The baits were stored under cool temperatures and used within 2 wk.

Liquid formulations were prepared by adding sufficient conidia of Bb GHA or Mb F52 to achieve 1×10^{13} viable conidia/l of a paraffinic crop oil, Sun Spray Ultra Fine Oil® (Sun Oil Company). This concentrate was subsequently diluted in the field 1:4 (v:v) with canola oil for spraying. Bait and initial liquid formulations were made by the USDA ARS Northern Plains Agricultural Research Laboratory in Sidney, MT.

The field study area was located *ca* 29.8 km southeast of the Boise, Idaho airport, near the community of Mayfield. This location was selected because of a history of Mormon crickets on the property, proximity to current known populations of Mormon crickets and abundance of historically infested rangeland without livestock.

Preliminary Laboratory Bioassays.— Topical bioassays were conducted as a preliminary to the bait and spray trials to verify the efficacy of (Bb GHA) and (Mb F52) against Mormon crickets. The 'Green Muscle' strain of *M. acridum* (Mac GM) was included for comparative purposes. This latter fungus has been commercialized for the control of Orthoptera in Africa (Lomer *et al.* 2001). Conidia of Bb GHA and Mb F52 used for bioassays were obtained as described above; Green Muscle was provided as a dry conidial powder (5×10^{10} viable conidia g^{-1}) by CAB Biosciences, Silwood Park UK, and was imported and used under permit from APHIS PPQ following the permit conditions. The conidia were suspended in culinary canola oil with concentrations based on their viabilities and verified by hemocytometer counts of kerosene-diluted samples. Doses ranged from 1,500 to 42,500 (Mb F52), 7,570 to 354,000 (Bb GHA), and 3,150 to 52,300 (Mac GM) viable conidia per insect, with 5 doses in increments of either 50% (Mac GM, Mb F52) or 33% (Bb GHA). The doses were designed to bracket the median infectious doses (LD_{50}) for each fungus based on earlier bioassays.

Fifth instar Mormon crickets for the bioassay were collected from a population found east of Boise ID and shipped by overnight express to Sidney MT. Insects were maintained in the laboratory for 5 d to eliminate any injured or diseased individuals. Cold anesthetized Mormon crickets were dosed by application of a 1 µL droplet of conidial suspension to the first coxa of each insect, using a 0.25 cc glass syringe with blunted 21 G needle, mounted on an ISCO Model M microapplicator (Instrument Specialties Co.). Fifteen insects were used per dose, and the assays were replicated three times in their entirety. Treated and control insects were then incubated in individual containers at 26 to 28°C for 14 d, fed *ad libitum* with a mixture of bird seed and raw sunflower seed, and provided moisture by daily misting. Mortality was recorded daily, with all cadavers incubated at high humidity to identify mycoses.

Immature Mormon cricket studies.—Cages, 21.6 cm high by 17 cm diameter, fashioned from 0.032-cm mesh hardware cloth, were placed on untreated rangeland (Fig. 1a,b) to evaluate the potential of baits and sprays containing the separate fungal pathogens against Mormon cricket. Ten cages were established for each of 6 treatments (*Beauveria* spray, *Metarhizium* spray, *Beauveria* bait, *Metarhizium* bait, untreated control, oil-carrier control). Each of the treatments was replicated 4 times, for a total of 240 cages. The appropriate amount of bait was preweighed in the laboratory for each cage and placed in the cages prior to cricket introductions.

Sprays were applied using an airbrush (Paasche Type H with #75 regulator) modified with a customized hypodermic syringe needle for liquid injection, to produce droplets that simulate aerially applied sprays (Foster *et al.* 1996b, Reuter *et al.* 1996) to the area to be enclosed by each cage (Fig. 1). Desired spray deposition and coverage was documented by oil-sensitive spray cards placed in each sprayed area. Drift during spray application was prevented by using portable walls or a containment chamber (Fig. 2). All treatments were applied at the rate of 2.47×10^{13} conidia/ha (on 11.1 kg of bait/ha, or in 2.5 L of oil formulation/ha, diluted with canola

Fig. 1. a) (left) Close up of hardware cloth cage used to contain Mormon crickets exposed to sprayed vegetation or baits; b) (right) Cages set up on Idaho rangeland.

oil to deliver 12.4 L of spray/ha). The bait application theoretically delivered about 25 granules per cage; the theoretical delivery of the spray was 2.5×10^5 conidia/cm^2 to a planar surface. Baits were applied on April 29, 2005. Because of the time required to treat each of the cages with sprays, two replications of the spray portion of the study were applied on April 30 and the other two on May 1, 2005.

Immediately following treatment, each of the cages was stocked with a randomly selected single 3rd instar Mormon cricket previously collected from the vicinity. Stocking cages with a single cricket prevented cannibalism, a common occurrence when more than one cricket is confined in a cage. Cricket mortality within cages was monitored daily for 14 d. The study was conducted from April 29 through May 15, 2005. Crickets that died during the study were transferred to Petri dishes containing a cotton ball moistened with

distilled water to provide very high humidity to promote fungal outgrowth from the cadaver. The presence of fungal outgrowth characteristic of *B. bassiana* or *M. brunneum* was an indication of mycosis.

Mormon cricket thermal surrogates were used to estimate body temperatures that a cricket would experience on the ground or at canopy level, within or outside of a cage, on a 24-h basis. Surrogates consisted of 1.5-ml Eppendorff tubes filled with soy sauce and fitted with a copper-constantin thermocouple wire connected to a data logger (DualogR®, Cole Parmer). These thermal surrogates have been shown to reasonably represent the ability of Orthoptera to absorb solar radiant heat, as well as indicate typical body temperatures (Lactin & Johnson 1998). The thermocouples were calibrated against a National Institute of Standards and Technology liquid thermometer before being used. Temperatures of the surrogates were

Fig. 2. Airbrush system used to deliver fungal pathogen sprays and containment chamber to eliminate drift during airbrush applications.

recorded every 5 min for the duration of the field aspect of the study. The objective of the surrogate data was to estimate the amount of time daily during which Mormon cricket body temperatures were within thermal thresholds for good fungal vegetative growth. Our goal was to provide a range of estimates for the total time to death from infection: this was based on hours per day of temperatures that permitted fungal growth at specific insect habitat locations, in comparison with rates of growth and hours to onset of insect mortality under ideal laboratory conditions (28°C). In addition ambient air temperatures and relative humidity 1 m above ground were measured with a calibrated Hobo® H08 temperature and humidity logger (Onset Technologies Inc.).

Seventh instar to adult Mormon cricket studies.—Seventh instar and adult Mormon crickets collected from the vicinity were used in this second study. Cages were set up and sprays prepared and applied similarly to that described for the immature cricket study. No baits were evaluated against these older crickets. Due to time constraints, spray treatment replications were divided equally over 2 d: June 2nd and 3rd, 2005. Mortality within cages was monitored daily for 4 d, at which time all survivors were transferred within cooled styrofoam containers via express service to USDA ARS in Sidney, MT. Within the shipping container, all crickets within each treatment and replicate were combined in separately labeled, ventilated (46 cm long × 9 cm diameter) plastic-tube cages containing crumpled newspaper to increase the resting surface area and minimize cannibalism.

In Sidney, MT the Mormon crickets from each treatment and replicate were split into two groups. The first group, approximately 20 insects per fungus treatment, or 30 for each of the untreated and oil-carrier controls, was incubated indoors at nearly constant 26 to 28°C temperatures, optimal for fungal mycosis (Fargues *et al.* 1997, Kabaluk unpub.) and a 16:8 L:D photoperiod. A second group, 10 insects per treatment, chosen at random from those shipped, was held outdoors on vegetated ground, where the Mormon crickets could actively bask and thermoregulate. In each case individual insects were caged in plastic tubes, 20 cm long by 5 cm in diameter with screened caps at each end. All insects were provided a diet of sunflower seed, bird seed and Tetramin® tropical fish food (Spectrum Brands, Atlanta, GA), and cotton-stoppered vials of water. In addition, the interiors of the cages were sprayed with water mist each morning to simulate dew. Part of each cage was shaded to provide refuge during the hottest part of each day. Thermal surrogates were used in both field-cage studies and post-treatment laboratory incubations. A thermal surrogate connected to a data logger was placed in an empty cage, together with the outdoor-incubated insects. Observations of insects in both indoor and outdoor incubations were conducted for 14 d, June 8-22. Mortality was determined daily and all cadavers were removed, surface decontaminated by immersion in 0.5% NaOCl for 1 min, then placed at 100% humidity to allow any mycoses to become expressed.

Laboratory bioassays of bait granules.—We also conducted a laboratory bioassay of the granules. Individual Mormon cricket nymphs, starved for 24 h, were confined with 250 mg of treated or untreated bait for 48 h, then transferred to clean cages and provided seed/fish-food diet *ad libitum*. Fifteen or 16 crickets were used for each treatment, but the entire test was not replicated. Crickets were incubated for 14 d in the laboratory, at 26 to 28°C. Daily mortality was determined and all cadavers processed as described earlier to elicit fungal outgrowth.

Statistical analysis.—The bioassay data were analyzed by probit analysis (PriProbit, Sakuma 2000), using the day-14 mortalities to provide median lethal doses and associated statistics. Because the assays of each fungus were conducted separately not concurrently, and the log-dose probit mortality regressions were not all parallel, the fungi were compared by ANOVA of the replicate LD_{50}s and mean LD_{50} values, separated by Tukey's Honestly Significant Difference Test. Mortality data were adjusted for natural untreated mortality by the method of Abbott (1925). Field data were analyzed using an analysis of variance with the Tukey multiple comparison test to separate means when the ANOVA F was significant. Analyses were performed with SYSTAT for Windows® (SPSS Inc. 1977). Effects of fungal treatments on indoor-incubated Mormon crickets were assessed via Fisher's Exact Test and also in terms of Median Survival Time (Kaplan Meier method) and Cox's Proportional Hazards technique (Kleinbaum 1996). Latter analyses were performed using Statistix® 8 (Analytical Software Inc. 1985-2003). Mortality in the bioassay and laboratory incubation was manually analyzed with Fisher's Exact Test.

Results

Preliminary laboratory bioassays

Dose response regressions of the day-14 mortalities yielded mean LD_{50} values of 10,973 (± 1,739, S.D.) conidia/insect for Bb GHA, 4,110 (± 586, S.D.) conidia/insect for Mb F52, in comparison with an LD_{50} of 2,677 (± 1,092, S.D.) for Mac GM (Table 1). Differences among mean LD_{50}s for the three fungi were significantly different (ANOVA $F_{2,6}$ = 38.81, p = 0.0004). Both Mb F52 and Mac GM had significantly lower LD_{50}s than Bb GHA (Tukey's HSD Test, p = 0.05), but were not different from each other. Earlier mortalities were not used for the analyses because of excessive response heterogeneity in several of the individual bioassays. In general, F52 caused faster insect mortality than either GHA or Green Muscle (data not shown).

Immature Mormon cricket studies

Baits.—No significant mortality was detected 0 to 14 d after treatment (DAT) in the populations treated with bait formulations of *B. bassiana* or *M. brunneum,* when compared to the untreated population (ANOVA $F_{1,12}$=3.281, p > 0.05). No mortality occurred in untreated populations until 3 DAT. From then on, mortality in the untreated population increased to 27.5% at 14 DAT. Untreated mortality was slightly higher than expected at 12.5% from days 5 to 8, compared to other bait studies with Mormon crickets where untreated populations showed mortality of 5% at 7 DAT (Foster *et al.* 2003, 2004). However, at the end of field observations, 14 DAT, both fungal-treated and untreated populations showed an equivalent mean percentage mortality of 27.5%.

In addition, we observed that ants were very active in attacking Mormon cricket cadavers in the field cages. Even though we examined cages daily for cadavers, these were often covered with ants; in some cases only the larger exoskeletal bits left. There was no significant effect of mycosis on the frequency of subsequent ant predation (X^2 = 2.34, 3 df, p = 0.50).

Sprays.—No significant mortality was detected in the field 0-14 DAT in the populations sprayed with Bb GHA ($F_{1,12}$ = 3.28, p > 0.05), Mb F52 ($F_{1,12}$ = 0.86, p > 0.05), or oil only ($F_{1,12}$ = 2.03, p > 0.05), when compared to untreated populations. Mortalities from both Mb F52-treated and control populations were 27.5% and Bb

Table 1. Median lethal doses (LD_{50}) and associated probit analysis statistics from topical bioassays of *B. bassiana* GHA (Bb GHA), *M. brunneum* F52 (Mb F52) and *M. acridum* 'Green Muscle' (Mac GM) using 5th instar *A. simplex*. Analyses are based on day-14 mortalities.

Fungus	Test	LD50[1]	Lower 95%CL[1]	Upper 95%CL[1]	Slope	SE Slope	P (Pearson Chi²)
Bb GHA	1	12,900	4,100	22,900	2.88	0.94	0.07
	2	9,520	2,900	15,700	2.13	0.66	0.96
	3	10,500	3,100	18,400	2.97	0.92	0.63
	Mean	10,973 A[2]			2.66		
	S.D.	1,739			0.46		
	95% CL	1,968					
Mb F52	1	3,540	1,840	5,850	3.11	0.86	0.25
	2	4,080	2,080	7,080	2.83	0.79	0.70
	3	4,710	2,530	8,290	2.85	0.78	0.45
	Mean	4,110 B			2.93		
	S.D.	586			0.16		
	95% CL	663					
Mac GM	1	2,900	390	4,870	3.36	1.25	0.82
	2	3,640	840	6,070	3.06	1.06	0.56
	3	1,490	262	890	3.44	1.57	0.37
	Mean	2,677 B			3.29		
	S.D.	1,092			0.20		
	95% CL	1,236					

[1] conidia per insect
[2] Mean LD50's followed by different capital letters are significantly different (Tukey's HSD Test, p = 0.05).

GHA-treated and oil-only treatments were 42.5 and 40% mortality, respectively, 14 DAT.

Temperature observations.—Daily temperature maxima recorded by the thermal surrogates regularly exceeded 30°C, with peaks as high as 44°C. Daily surrogate temperature minima were routinely as low as 10°C and occasionally as low as -4°C (Figs 3, 4). In comparison, daily minimum and maximum ambient air temperatures ranged between 0.7 to 10.2° C (mean 5.1° C), and 11.8 to 22.9 °C (mean 18.7° C), respectively, during the study period. More importantly, during the observation period, the number of daily hours of surrogate temperatures permissive for good fungal growth (18 to 30° C) ranged from 1.5 to 9.5 h, with a mean of 4.5 ± 2.10 (S.D.) h (inside cage, on ground); 0 to 10.8 h, mean 5.7 ± 3.25 (S.D.) h (inside cage, canopy); 2.3 to 6.3 h, mean 4.2 ± 1.20 (S.D.) h (outside cage, on ground); and 0.3 to 11.8 h, mean 6.7 ± 3.69 (S.D.) h (outside cage, canopy). There was no significant cage effect on either sensor location (on ground, t_{28}= 0.37, p (.05) = 0.71; canopy, t_{28} = -0.81, p (.05) = 0.43).

Subsequent laboratory bait bioassays.—Mortality of Mormon crickets confined with *Beauveria*-treated Tast-E-Bait for 48 h was only 18.8% after 14 d post-treatment incubation, while mortality from *Metarhizium*-treated bait was 6.7%. The corresponding control mortality was 13.3%. Tast-E-Bait granules formulated with 4× rate of *Beauveria* conidia caused only 12.5% mortality. None of these results were significantly different from each other (Fisher's Protected Test, X^2= 1.15, 3 df, p=0.76). Incidence of mycosis on cadavers was 100% (1/1), 67% (2/3), and 100% (2/2) for Mb F52, Bb GHA, and 4X Bb GHA respectively, indicating there was some infection.

Seventh instar to adult Mormon cricket studies

Indoor incubations.—Very high infection rates resulted from the field exposure to both fungi (Table 2). Mortalities from the *Metarhizium* and *Beauveria* treatments were 100 and 80% respectively at 14 d. In comparison, untreated and oil-carrier control treatments caused 30 and 15% mortality, respectively. Chi-square analysis of the untreated and oil-carrier controls indicated they were not significantly different from each other and the two treatments were pooled for further analysis. Both fungus-induced mortalities were significantly different from the pooled controls (X^2 = 48.72, 2 df, p < 0.001). Prevalence of mycosis among cadavers in fungus treatments was 71-80%. A low prevalence of mycoses (*Beauveria* only) was also present in both controls (Table 2). Median survival times (MST) of insects dosed with *Beauveria* and *Metarhizium* were 8 d, whereas the MST for the two control treatments could not be estimated because mortalities did not exceed 50% (Table 2). Use of Cox's Proportional Hazards technique with the daily mortality data revealed that Mb F52 was slightly more virulent than Bb GHA (Z = 2.12, p = 0.0344, Relative Risk of death = 2.11), while both fungi were significantly more virulent than either control (Table 3). Treatment with oil carrier did not affect subsequent Mormon cricket mortality in comparison with the untreated control. Cox's Proportional Hazards analysis showed no differences in survivorship between the two controls (Table 3).

Outdoor incubations.—Many of the crickets incubated outdoors died early during the study because of unexpected high temperatures, rendering these data useless.
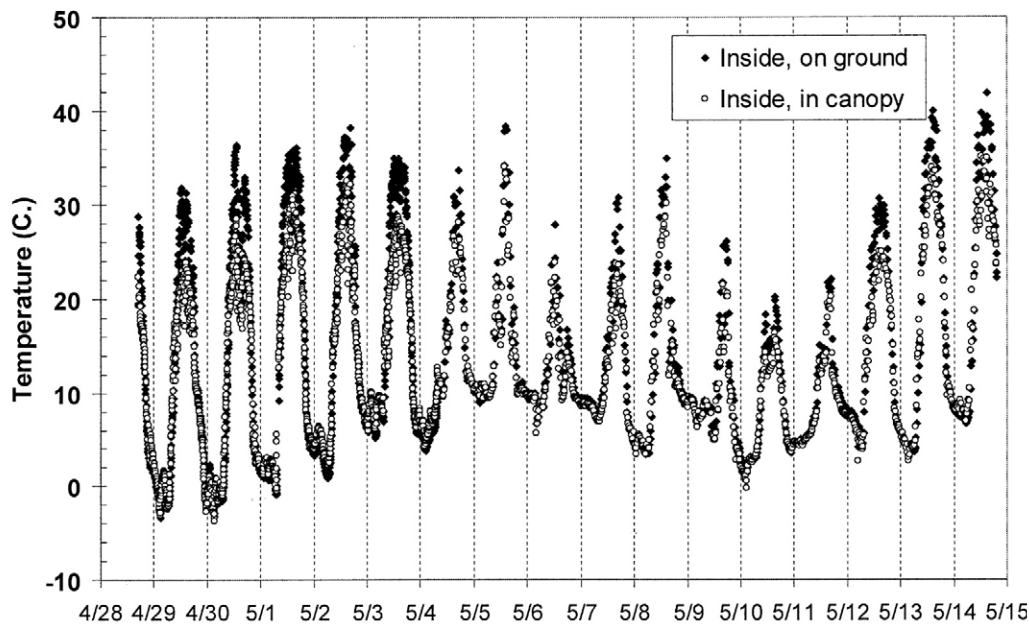
Fig. 3. Surrogate temperatures <u>inside</u> cages on the ground and at canopy level during the course of the immature Mormon cricket observation period. Symbols: ♦ "Inside, on ground" thermal surrogate positioned on the bare ground simulating basking insect; ○ "Inside, in canopy" thermal surrogate positioned on a bamboo skewer 10cm above the ground, simulating Mormon cricket roosting in plant canopy.

Discussion

In laboratory bioassays Bb GHA and Mb F52 were infectious and virulent for Mormon cricket nymphs, with F52 being as efficacious as the orthopteran-specific Green Muscle. The LD_{50} ratio of F52 to Green Muscle was 1.54, while the ratio of Bb GHA to Mac GM was 4.1. (Green Muscle, being nonindigenous to the United States, cannot currently be used outside of approved biocontainment.)

No mortality of immature Mormon crickets was detected in the field for 14 DAT with fungal baits or sprays. While we did not check conidial viability immediately after formulation of the bait, the binder has been successfully used as a carrier for sprayable formulations of both fungi in the past. The baits were stored under cool conditions and used within 2 wk of being prepared. The liquid formulations used a compatible paraffinic oil (Jaronski, unpub.), were prepared just before use and carefully stored until use. Thus we doubt that low conidial viability could have been the cause of our observed results.

The lack in detectable mortality from baits could be explained by crickets not readily feeding on the fungus-treated bait. An indication of bait avoidance was seen in the subsequent laboratory bioassays, where Mormon crickets showed no significant mortality at 14 d after fungal treatment, even when the dose of *B. bassiana* was increased four-fold, to an equivalent of 3.45×10^{13} ha^{-1} (the *B. bassiana* conidia on these granules had 95% viability at time of use). This result seems to conflict with data of excellent mortality from exposure to Tast-E-Bait containing carbaryl in several other studies (Foster *et al.* 2003, 2004) and the susceptibility of the insect to Bb GHA and Mb F52 in the topical bioassays described in our present study. If given equal value and taken together, the earlier studies would seem to suggest that Mormon crickets may have been attracted to the carbaryl or the additive used to incorporate the chemical toxicant into the bait, rather than the bait itself. In the current study this apparent nonfeeding (avoidance) behavior alone may explain the lack of mortality with the fungi. However, only additional testing will fully provide that answer.
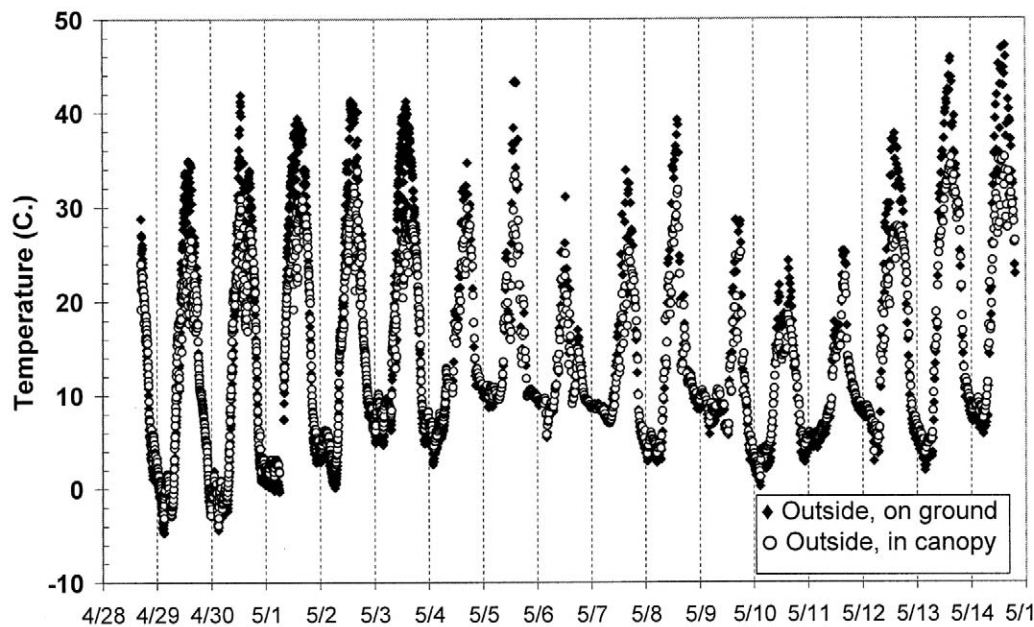

Fig. 4. Surrogate temperatures <u>outside</u> cages on the ground and at canopy level during the course of the immature Mormon cricket observation period. Symbols: ♦ "Outside, on ground" thermal surrogate positioned on the bare ground simulating basking insect; ○ "Outside, in canopy" thermal surrogate positioned on a bamboo skewer 10 cm above the ground, simulating Mormon cricket roosting in plant canopy.

Table 2. Mortality, prevalence of mycosis, and Median Survival Time (MST) of Mormon cricket adults collected from field cages 4 d after exposure, shipped overnight to USDA ARS, Sidney, MT and incubated indoors for 14 d at 27 to 28° C.

Treatment	Number of insects	Per cent mortality	Corrected per cent mortality[1]	Prevalence of mycosis among dead	MST (days) ± 95% Confidence Limits
Untreated	30	30%	-	14%	-
Oil Control	30	15%	-	25%	-
Beauveria	20	80%	74.2%	71%	8 (8-11)
Metarhizium	20	100%	100%	80%	8 (7-9)

[1] Untreated and oil-carrier control data pooled before correction for natural mortality (Abbott 1925).

The low mortality among immature Mormon crickets treated with fungus sprays in the first test remains to be explained. Topical bioassays using 5th instar Mormon crickets yielded mean LD_{50}s of 10,973, and 4,110 conidia per insect for Bb GHA and Mb F52, respectively, at 14 d (Table 1). In the adult Mormon cricket field study, the corresponding laboratory mortalities from mycosis were 74-100% at 14 DAT at the same application rate, 2.47×10^{12} conidia ha^{-1}. Therefore, it is clear these insects are physiologically susceptible to both fungi. The observed high infection and mortality of the Mormon crickets brought into the laboratory after 2 d of exposure to sprayed vegetation indicates good infection rates can be achieved merely by contact with sprayed vegetation and without direct impingement.

Data from the thermal surrogates may explain the apparent ineffectiveness of both fungal treatments. The temperatures measured in the surrogates inside and outside cages, on the ground and in the plant canopy, demonstrate the possible length of time each day during which fungal growth could occur within the insects, depending upon their positions. Although the surrogates are fixed while insects are mobile, the surrogates represent the daily duration of body temperatures that could be experienced at the two fixed positions. The goal was to provide a range of estimates for the total time to death from infection, based on hours per day with temperatures permissive for fungal growth at the specific insect locations, in comparison with rates of growth and onset of insect mortality under ideal laboratory conditions (constant 28° C., no thermal regulation).

Normally, the insects would move to maintain preferred body temperatures, but not when tethered with a thermocouple wire. We believe the "grab and stab" procedures of several grasshopper studies (*e.g.*, Kemp 1986) do not accurately reflect body temperatures. These procedures were impractical in our case given the destructive nature of such sampling methods and a limited supply of insects. Our compromise was to measure body temperatures using the surrogates in the two extreme positions where we observed crickets in our cages – on the ground or in the canopy. The more important parameter is length of time at temperatures that permit fungal growth, rather than the actual temperatures themselves.

Optimal growth rates (>50% of maximum) of both fungi occur at 18 to 30°C with greatly reduced growth (<50%) at the intervals of 31 to 32°C and 9 to 17°C. No fungal growth occurs above 32°C or below 9°C (Fargues *et al.* 1997, Jaronski unpub.). We converted the observed surrogate temperatures from absolute values, recorded at 5-min intervals, to the number of hours each day during which the defined fungal growth temperature ranges were observed (Figs 5-8). Breakdown of each surrogate dataset illustrates the actual number of hours each day that could produce optimal fungal growth. For example, on April 29 the in-cage, on-ground surrogate (Fig. 5) recorded 1 h during which temperatures were above 30°C, 7 h during which temperatures were 18 to 30°C, and 16 h when temperatures were below 18°C. Thus, on April 29 there were only 7 h during which fungal growth would be ≥ 50% of the fastest (*in vitro*) growth rate, and 17 h when fungal growth would be <50% the fastest rate.

Based upon the bioassays with nymphal Mormon crickets, and adults (Jaronski, unpub.), Bb GHA, at its LD_{50}, will produce mor-

Fig. 5. Daily accumulated hours of temperatures optimal and suboptimal for fungal growth, based on thermal surrogates inside cages and on the ground.
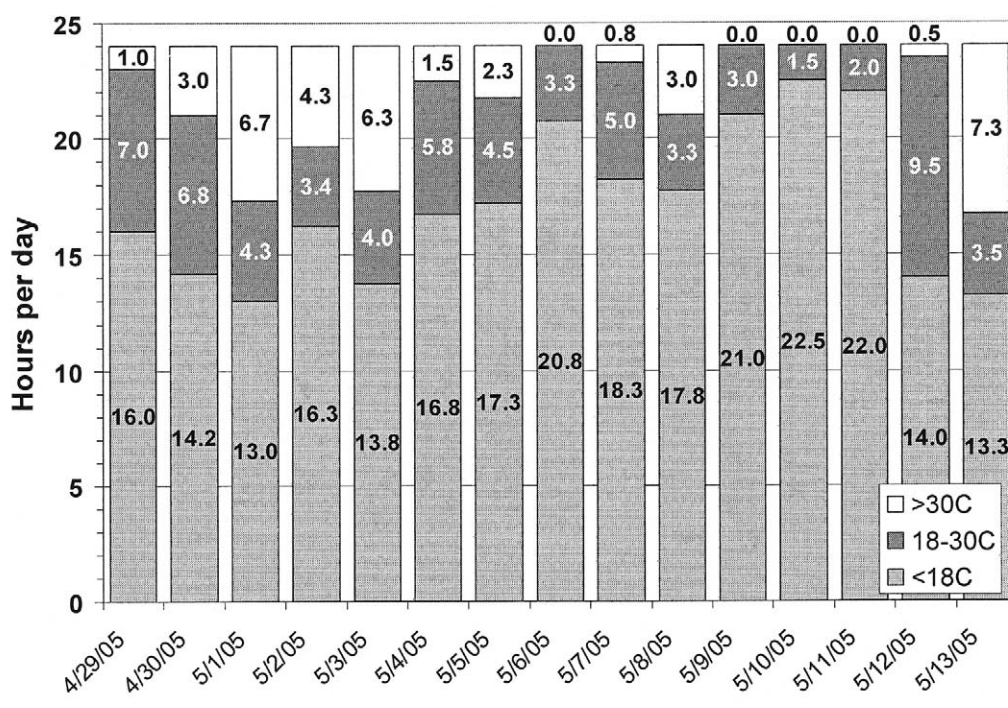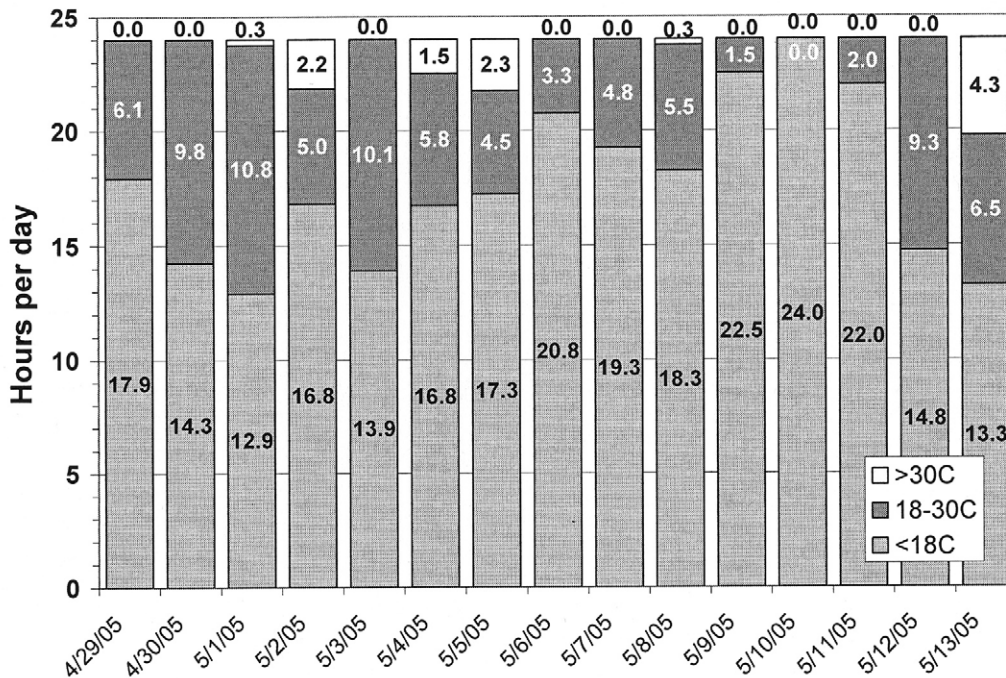
Fig. 6. Daily accumulated hours of temperatures optimal and sub-optimal for fungal growth, based on thermal surrogates inside cages and at canopy level.

tality in 10 to 14 d (240 to 336 h) at the constant, optimal 26 to 28°C and Mb F52 will grow to produce mortality in crickets in 6 to 8 d (144 to 192 h) at its LD_{50}. With the lab-incubated Mormon crickets from the second spray test, median survival time was 196 h for both fungi. When the number of hours for optimal growth are accumulated for the duration of the post-treatment observation period (immature cricket spray study), an insufficient number of hours of growth-permissive temperature occurred during the 15 d of the study to produce mortality (Table 4). Accumulated hours of potential fungal growth for the entire 14-day observation period, based on the thermal surrogates inside a cage, were only 67 h (on ground) and 85 h (in canopy). Corresponding data for the surrogates outside the cage were 63 h (on ground) and 100 h (in canopy).

Treatments with Mb F52 at the temperatures we observed during our study should have resulted in mortality only after 26 to 34 d (based on surrogates inside cages and in plant canopy) or 32 to 43 d (based on the surrogates inside cages, on the ground). Treatments with *B. bassiana* at the temperatures we observed, would have required 42 to 60 d or 58 to 75 d to kill its hosts inside cages at vegetation canopy and on the ground level, respectively. Therefore, temperatures experienced by the Mormon crickets, as represented by the thermal surrogates, indicate that a sufficient number of accumulated hours at temperatures appropriate for fungal development and host mortality did not occur during the study and the necessary post-treatment period should have been at least a month. We terminated the field portion of the immature Mormon cricket studies at 14 d because our expectations based on the bioassays were not met and because of logistical problems in continuing the studies for a longer period.

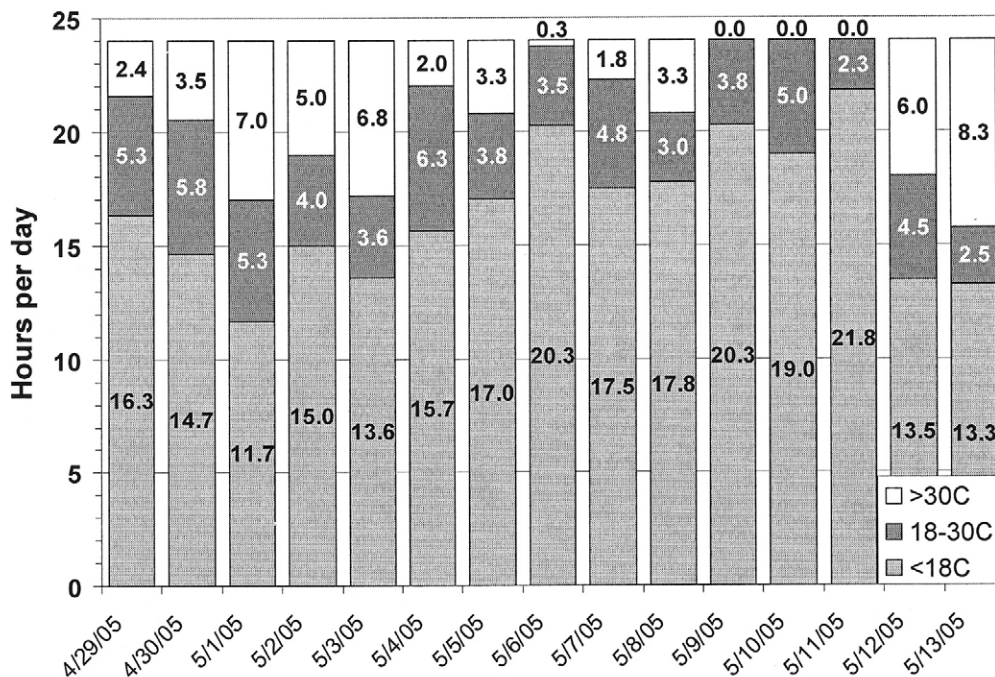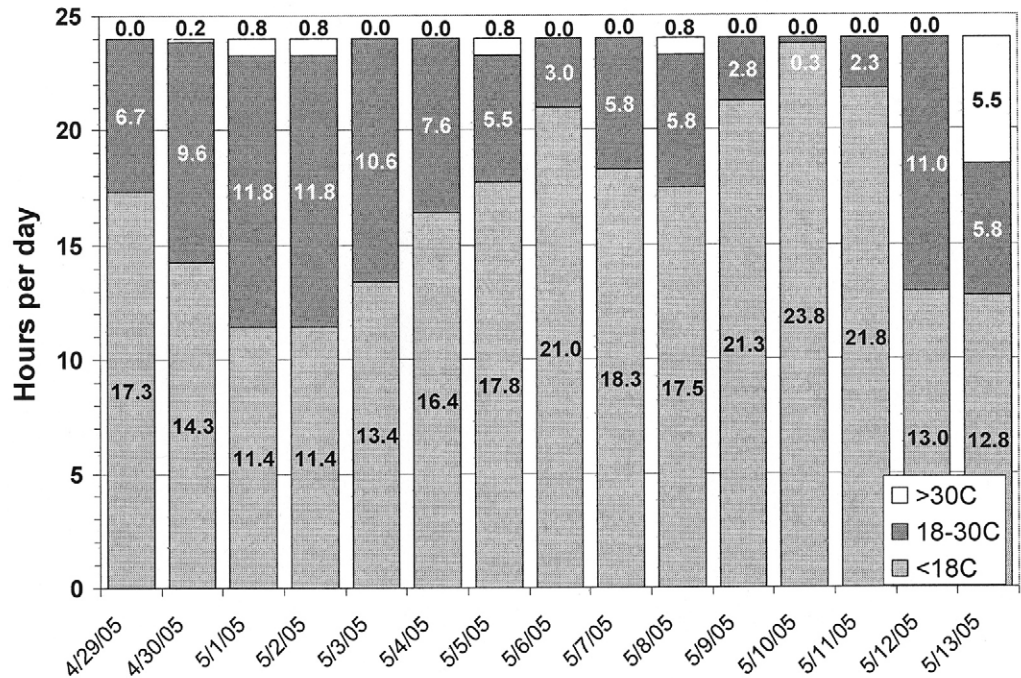The study of adult Mormon crickets was inconclusive due to

Fig. 7. Daily accumulated hours of temperatures optimal and suboptimal for fungal growth, based on thermal surrogates outside cages and on the ground.

Fig. 8. Daily accumulated hours of temperatures optimal and suboptimal for fungal growth, based on thermal surrogate outside cages and at canopy level.

premature death caused by excessive temperatures that occurred in the Sidney portion of the experiment. Infection and mortality (corrected for control mortality) of adult Mormon crickets collected after field sprays and incubated under ideal temperatures for the fungi, were in excess of 70%. The unexpected presence of *B. bassiana* in the untreated controls was unclear (Table 2).

The severe ant predation of Mormon cricket cadavers we observed has important implications about the potential for entomopathogenic fungi to spread after the initial application. Thomas *et al.* (1996) proposed that *M. acridum* could persist in grasshopper cadavers and fragments of cadavers during the Sahelian dry season to provide significant inoculum for natural infections in the following year. Their conclusions were based on the persistence of cadavers with sporulating fungus deliberately placed in grassland habitat in one year and persistence assessed by caging grasshoppers on the plots at the start of the next rainy season. In our study 39 to 41% of the fungus-killed Mormon crickets were attacked by foraging ants within one day of death. Our observations imply that few cadavers would persist long enough for the fungus to sporulate, even assuming favorable environmental conditions, and the potential for persistence until the next year would be minimal.

Early-season use of these US-registered, commercial fungi was anticipated to yield better efficacy than late-season use, because we knew that warmer air temperatures, more intense solar heat, and elevated Mormon cricket body temperatures (Turnbow 1998) would become a serious problem mid-to-late season for fungal infection (*e.g.*, Arthurs & Thomas 2001). Based on our observations, early season use against young Mormon crickets may also not be appropriate because cool temperatures would cause prolonged onset of mortality, which is unacceptable to the user community.

The rate used in this study (2.47×10^{13} conidia ha⁻¹) was the midlevel label rate for the commercial Bb GHA; there was no comparable rate for Mb F52. Based just on the mortalities of the lab-incubated Mormon crickets, thus ignoring thermal aspects, this rate may have satisfactory efficacy. The rate however, is not economic for most rangeland in the U.S. Use of either commercial fungus in environmentally sensitive areas, where traditional insecticides are not permitted, may justify higher cost to protect the integrity of adjacent control investments. *M. acridum*, used economically in Africa and Australia, cannot currently be employed in the U.S. because of its nonindigenous status and associated regulatory constraints.

Our data illustrate the importance of insect physical location

Table 3. Cox's Proportional Hazards analysis based on the survivorship of adult Mormon crickets collected postapplication and incubated indoors (see Table 2).

Variables	Coefficient	Std Error	Z	P	Rel. Risk
Untreated *vs* Oil	0.05343	0.19526	0.27	0.7843	1.05
Untreated *vs* Beauveria	1.76189	0.46437	3.79	0.0001	5.82
Untreated *vs* Metarhizium	1.52518	0.29794	5.12	0.0000	4.6
Beauveria vs Metarhizium	0.74506	0.35219	2.12	0.0344	2.11

Table 4. Expected number of days required for Mormon cricket mortality from *M. brunneum* F52 and *B. bassiana* GHA, based on the accumulated hours of temperature optimal for fungal development that were recorded during the 15 d of the study by thermal surrogates.

	Days Needed for Onset of Mortality			
	Inside Cage		Outside Cage	
Fungus	In Canopy	On Ground	In Canopy	On Ground
Metarhizium brunneum F52	26-34	32-43	21-29	35-45
Beauveria bassiana GHA	42-60	58-75	36-50	57-80

and suboptimal temperatures for fungal development in preventing practical efficacy. Because physical location may alter the thermal environ inside the insect, at least a month at this time of year may be required after treatment for infections by either fungus, to cause mortality in the field. Future studies should take this longer observational time into account and/or include evaluation of other strains with more favorable temperature requirements. There is some potential for faster fungal growth in Mormon crickets under natural conditions, as opposed to our experimental cages, particularly in the immature stages. Mormon crickets aggregate in sheltered locations under inclement weather and at night (Cowan 1929). This behavior may conserve and actually promote body temperatures more favorable to fungal development. Aggregation that may trap body heat under cool temperatures has been observed in the Brown locust, *Locusta pardalina* (Blanford & Thomas 2000) and the Australian plague locust, *Chortoicetes terminifera* (Blanford 2001). The potential value of this aspect of Mormon cricket behavior will require additional study.

Acknowledgements

References

Abbott W.S. 1925. A method of computing the effectiveness of an insecticide. Journal of Economic Entomology 18: 265-267.

Arthurs S.P., Thomas M.B. 2001. Effect of dose, pre-mortem host incubation temperature and thermal behaviour on host mortality and mycosis and sporulation of *Metarhizium anisopliae* var. *acridum* in *Schistocerca gregaria*. Biocontrol Science and Technology 11: 411-420.

Blanford S. 2001. "Report on collaborative field research on Australian plague locust during field trials with *M. anisopliae* var *acridum* in New South Wales, 19th October- 26th November 2000." Report for Australian Plague Locust Commission and Commonwealth Scientific Research Organization, 34 pp.

Blanford S., Thomas M.B. (2000). Thermoregulation by two acridid species: effects of habitat and season on thermal behaviour and the potential impact on biocontrol with pathogens. Environmental Entomology 29: 1060-1069.

Bradley C.A., Black W.E., Kearns R., Wood P. 1992. Role of production technology in mycoinsecticide development, pp. 160-173. In: Leatham G.E. (Ed.), Frontiers in Industrial Microbiology. Chapman & Hall, New York.

Cowan F.T. 1929. Life history, habits and control of the Mormon cricket. USDA Technical Bulletin 161: 1-28.

Fargues J., Goettel M.S., Smits N., Ouedraogo A., Rougier M. 1997. Effect of temperature on vegetative growth of *Beauveria bassiana* isolates from different origins. Mycologia 89: 383-392.

Foster R.N., Reuter K.C., Black L., Britton J. 1996a. Evaluation of the fungus *Beauveria bassiana* with selected insecticide stressors for control of unconfined rangeland grasshoppers, 1995. Arthropod Management Tests. 21: 280.

Foster R.N., Reuter K. C., Black L.R., McNeal D., McChesney R. 2004. Comparison of wheat bran, apple pumice and food waste (combined bakery, snack, cereal, and confectionary) baits containing carbaryl for controlling Mormon crickets: studies of longevity and primary and secondary mortality. United States Department of Agriculture-Animal and Plant Health Inspection Service-Plant Protection and Quarantine-Center for Plant Health Science and Technology-Decision Support and Pest Management Systems Laboratory, Phoenix, AZ. 12 pp.

Foster R.N., Reuter K.C., Black L., Kahler E., Fuller B., Flora E.A. 1996b. Laboratory evaluation of dose and manner of pickup of Spinosad on the migratory grasshopper, 1995. Arthropod Management Tests. 21: 416.

Foster R.N., Reuter K.C., Bradley C., Britton J., Black L., Drake S., Leisner L., Tompkins M., Fuller B., Hildreth M., Kahler E., Radsick B. 1992. Development of the fungus *Beauveria bassiana* as a bio-insecticide for grasshoppers on rangeland, pp 207-215. In: Cooperative Grasshopper Integrated Pest Management Project, 1992 Annual Report. United States Department of Agriculture-Animal and Plant Health Inspection Service, Boise, Idaho.

Foster R.N., Reuter K.C., Bradley C.A., Wood P.P. 1991. Preliminary investigations on the effect of *Beauveria bassiana* on several species of rangeland grasshoppers, pp. 203-210. In: Cooperative Grasshopper Integrated Pest Management Project 1991 Annual Report. USDA-APHIS, Boise Idaho.

Foster R.N., Reuter K.C., Britton J., Bradley C. 1996-1999. Lab studies and field trials with the fungus *Beauveria bassiana* against grasshoppers. In: Cunningham G.L., Sampson M.W. (Tech. Coord.). Grasshopper Integrated Pest Management User Handbook. Technical Bulletin 1809. Washington DC: United States Department of Agriculture, Animal and Plant Health Inspection Service: VII.5-1—VII.5-8.

Foster R.N., Reuter K.C., McNeal D. and McChesney R. 2003. Comparison of flakey wheat bran and apple pumice baits for controlling Mormon cricket: evaluating initial and secondary mortality with baits containing 2% and 5% carbaryl. United States Department of Agriculture-Animal and Plant Health Inspection Service-Plant Protection and Quarantine-Center for Plant Health Science and Technology-Decision Support and Pest Management Systems Laboratory, Phoenix, AZ. 10 pp.

Foster R.N., Reuter K.C., Tompkins M.T., Bradley C., Britton J., Underwood N., Black L.R., Vigil E., Daye G., Radsick B., Fuller B., Brinkman M., Kahler E. 1993. Development of the fungus *Beauveria bassiana* as a bio-insecticide for grasshoppers on rangeland, pp 233-238. In: Cooperative Grasshopper Integrated Pest Management Project 1993 Annual Report. United States Department of Agriculture-Animal and Plant Health Inspection Service, Boise, Idaho.

Jaronski S.T., Jackson M.A. 2008. Efficacy of *Metarhizium anisopliae* microsclerotial granules. Biocont. Sci. Technol. 18: 849-863.

Kemp W.P. 1986. Thermoregulation in three rangeland grasshopper species. Canadian Entomologist 118: 335-343.

Kleinbaum D.G. 1996. Survival Analysis, A Self-Learning Text. Springer Verlag, New York NY.

Lactin D.J., Johnson D.L. 1998. Environmental, physical, and behavioral determinants of body temperature in grasshopper nymphs (Orthoptera: Acrididae). Canadian Entomologist 130: 551-577.

Lomer C.J., Bateman R.P., Johnson D.L., Langewald J., Thomas M.B. 2001. Biological control of locusts and grasshoppers. Annual Review of Entomology 46: 667-702.

Reuter K.C., Foster R.N., Black L., Britton J. 1996. Laboratory evaluation of *Beauveria bassiana* with an added chemical stressor, 1995. Arthropod Management Tests: 21: 415.

Pfadt R.E. 2002. Field Guide to Common Western Grasshoppers, Third edition. Wyoming Agricultural Experiment Station Bulletin 912, February 2002. University of Wyoming 288 pp.

Sakuma M. 2000. Probit analysis of preference data. Applied entomology and zoology 33: 339-347. PriProbit ver. 1.63 (C) available from James E. Throne USDA-ARS-GMPRC, Manhattan KS at http://www.ars.usda.gov/sp2userfiles/place/54300530/probit/PriProbit.zip

SPSS Inc. 1997. SYSTAT for Windows: Statistics, SPSS Inc., Chicago, Illinois.

Statistix 8.0. 1985-2003. Analytical Software Inc. Tallahassee, Florida.

Thomas M.B., Gbongboui C., and Lomer C. J. 1996. Between-season survival of the grasshopper pathogen *Metarhizium flavoviride* in the Sahel. Biocontrol Science and Technology 6: 569-574.

Turnbow J. H. 1998. Temperature-sensitive *Beauveria bassiana* mycosis in the Mormon cricket, *Anabrus simplex*. M.S. Thesis. Montana State University, Bozeman, MT.

United States Department of Agriculture. 2002. Rangeland grasshopper and Mormon Cricket Suppression Program Final Environmental Impact Statement-2002. United States Department of Agriculture, Marketing and Regulatory Programs, Animal and Plant Health Inspection Service.

United States Environmental Protection Agency. 2003 *Metarhizium anisopliae* strain F52 (029056) biopesticide fact sheet. http://www.epa.gov/pesticides/biopesticides/ingredients/factsheets/factsheet_029056.htm cited 27 Oct 2009.

Wakeland C., Parker J.R. 1952. The Mormon cricket, pp. 605-608. In: Insects, The Yearbook of Agriculture, 1952 ed. Alfred Stefferud (Ed.). United States Department of Agriculture. Washington, DC.