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Research article

Discovery, Development, and Evaluation of a Horn Fly-Isolated (Diptera: Muscidae) *Beauveria bassiana* (Hypocreales: Cordyciptaceae) Strain From Florida, USA

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Abstract

The horn fly, *Haematobia irritans* (L.) is an important cattle pest and traditionally has been managed using insecticides; however, many horn fly populations are insecticide-resistant in United States. Use of alternative control techniques has been limited because of the challenges of managing a fly pest on pastured cattle. After the discovery of a wild horn fly infected with *Beauveria bassiana* in Florida, the fungus was cultured and evaluated for efficacy against laboratory-reared horn flies. This fungal strain was selected for increased virulence by passage through laboratory-reared horn fly hosts to shorten interval from infection to fly death and subsequent conidia formation, properties important to future use of the fungus as a biological control agent against horn flies. After seven passages through horn fly hosts, fly mortality was not significantly accelerated as evaluated through LT_{50} values, but conidia were readily produced from these killed flies. Although further development is needed to improve fungal efficacy, this fungal strain holds promise as a biological control agent for inclusion in horn fly integrated pest management programs.

Key words: *Haematobia irritans*, biting fly, cattle, integrated pest management, biological control

The horn fly, *Haematobia irritans* (L.), is an important pest of pastured cattle in much of United States. Adult flies maintain a near continuous presence on animals and blood feed multiple times per day (Harris et al. 1974). The economic impact of horn flies was estimated to be over 876 million dollars in 1991 (Kunz et al. 1991) or $1.6 billion in 2016 dollars (Bureau of Labor Statistics 2016). Horn fly control has largely been reliant on insecticide applications, but resistance to many of the active ingredients is widespread (Sheppard and Joyce 1998, Guerrero 2000, Barros et al. 2001, Domingues et al. 2014). Cattle producers are left with few options, opening the door for innovative control tactics. The use of fungal pathogens is one underutilized option that could aid in management of this difficult pest. Considerable hurdles to implementation exist, including the location and isolation of horn fly-pathogenic fungal strains with high virulence and accompanying studies that document efficacy. There have been few published research articles on the use of entomopathogenic fungi as an alternative management tool for horn fly (Angel-Sahagún et al. 2005; Lohmeyer and Miller 2006; Mochi et al. 2010a,b; Galindo-Velasco et al. 2015).

Beauveria bassiana (Balsamo-Crivelli) Vuillemin (Hypocreales: Cordyciptaceae) is a fungal pathogen that has been reported from hundreds of arthropod species (Fargues and Remaudiere 1977). Steinkraus et al. (1990) were the first to report *B. bassiana* infection in a muscoid fly (house fly, *Musca domestica* L.). To our knowledge there is only one other report of *B. bassiana* infection naturally occurring in horn flies in the literature (Steenberg et al. 2001).

Several fungi have been isolated from field collections of other muscoid flies, but only balEnce™ (*B. bassiana*) has been formulated and registered as a commercially available product for house fly control (Andersen 2006). The use of fungal pathogens to manage muscoid fly populations has had inconsistent success. A continuing challenge with the use of fungal pathogens for filth fly management is that fly death following fungal exposure does not occur until 7 days after exposure. Although this may be nearer to when oviposition occurs with larger muscoid flies, it is well after horn fly oviposition begins (Krafsur and Ernst 1983). This lag in mortality, which allows for progeny production, greatly reduces the benefits of using entomopathogenic fungi against horn flies. Selection of fungal

strains with faster death rates could provide a means of overcoming this challenge.

The objectives of this study were to: 1) evaluate presence of patent infections of entomopathogenic fungi in horn flies collected from Florida cattle ranches; 2) compare fungal virulence against horn flies using a horn fly-isolated *B. bassiana* strain and two commercially available fungal strains under laboratory conditions; and 3) conduct a virulence study on a horn fly-isolated *B. bassiana* strain following an aggressive selection program designed to accelerate time-to-mortality.

Materials and Methods

Fungal Acquisition and Isolation From Wild Flies

Horn flies were collected from Florida, United States, cattle ranches and retained until death to screen for infection by entomopathogenic fungi. Adult flies were sweep-netted from the backs and bellies of mature cows and bulls at each collection site, placed into a cage and transferred to the Veterinary Entomology Laboratory at the University of Florida. Flies were held in 30 cm×30 cm×30 cm screened cages in a humidified incubator (27 °C and 80% RH) and provisioned with citrated bovine blood twice daily. Dead flies were removed daily. Adult flies were collected from ranches near the following seven Florida locations: four private commercial beef herds near Gainesville (∼250 flies), Kissimmee (∼300 flies), Labelle (∼5,000 flies), and Clewiston (∼500 flies); and three University of Florida properties, the Range Cattle Research and Education Center (Ona) (∼300 flies), Beef Teaching Unit (Gainesville) (∼300 flies), and Beef Research Unit (Starke) (∼400 flies).

In order to ensure that the field-collected flies carried the fungal infection internally, following death, these adult horn flies were surface sterilized by placement in 95% ethanol for 15 s, then transferred onto sterilized dry paper towels where the ethanol was allowed to evaporate. The fly cadavers were transferred to sterilized filter papers fitted into plastic Petri dishes and positioned such that they did not touch. Filter papers were dampened with distilled water and placed in humidity-saturated chambers to observe for flies that had fungal infections. If present, *Beauveria bassiana* infection would manifest after day 4 as an off-white hyphal growth covering part or all of the fly body. This hyphal growth formed conidia which were subsequently isolated using a sterile glass inoculating needle and plated onto Sabouraud Dextrose Agar with yeast extract (SDAY) plates as modified from Geden et al. (1995) and described later.

Fungal Strains, Viability, and Colony Maintenance

The EN1 (EN1-0, ARSEF 13303), GHA (ARSEF 6444) and HF23 (ARSEF 7940) strains were evaluated for horn fly infectivity and mortality. The EN1 strain was isolated from one field-collected horn fly captured near Micanopy, FL. This strain was confirmed as *B. bassiana* using procedures outlined in Castrillo et al. (2010) and the intergenic Bloc region sequence analysis of Rehner et al. (2006). The EN1 strain shared 99% similarity to other *B. bassiana* strains published by Rehner et al. (2011) with its sequence submitted to GenBank (accession number KY610216). The GHA strain was isolated from a BotaniGuard® 22 WP formulation (Laverlam International Corporation, Butte, MT). The HF23 strain, a commercially available strain used for house fly control, was received in pure culture from JABB of the Carolinas (Pine Level, NC). All fungal strains were maintained in culture as described later.

Fungal culture media, SDAY, was prepared from Sabouraud Dextrose Broth (Difco, Sparks, MD) prepared as per instructions with the addition of 30 g l^{-1} agar (Fisher Scientific, Fair Lawn, NJ) and 5 g l^{-1} technical yeast extract (Difco, Sparks, MD). Media was autoclaved at 121 °C for 20 min and, after slight cooling, 50 mg l^{-1} chlortetracycline was added and mixture poured onto plates. Plastic Petri plates (50 mm×10 mm) were used for isolation of strains, while larger plates (100 mm×15 mm) were used for routine culturing. Petri plates were inoculated with a sterile loop, using standard laboratory procedures. Inoculated plates were held at 25 (± 7) °C, a light: dark cycle of 12:12 h, and 50% relative humidity. Under these conditions, fungal conidia were produced beginning on day 7 with completion by day 30. Once conidiation was complete on SDAY plates, they were harvested using a bacteriological loop to scrape the surface of the media, to gather conidia. After harvest, this conidia-containing powder was used immediately in any bioassays. Fresh conidia powder was generated for use in all bioassays.

Germination rates of conidia were measured on SDAY plates at each use to ensure that conidia were viable. At least 100 conidia were counted per plate 12–24 h after inoculation under a compound microscope and assessed for germination. Germinated conidia appeared swollen and had germ tube hyphae emerging from individual conidia. Germination rates of at least 95% were observed in all assays.

Comparative Fungal Strain Experiments

Adult horn flies (Kerrville strain) obtained from a long-established colony of insecticide-susceptible horn flies maintained at New Mexico State University were used in all experiments. Horn fly adults were reared on citrated bovine blood and held at 27 °C and 12:12 L:D cycle. Flies used in comparative fungal strain experiments were between 1- and 3-d post-eclosion.

The EN1 and commercial *B. bassiana* strains were evaluated using two exposure methods; conidia-impregnated filter papers (filter paper) and conidia + inert carrier (cornstarch). In both experiments, assays were conducted in 100 mm×15 mm plastic Petri dishes with a 2.5-cm hole cut in the lid that was covered with a nylon mesh screen, which allowed for provisioning flies with blood.

For impregnated filter paper assays, conidial suspensions were generated by suspending conidial powder into a 0.1% Tween 80 (Fisher Scientific, Fair Lawn, NJ) and water solution to generate concentrations of 1.0×10^7, 1.0×10^6, and 1.0×10^5 conidia per milliliter. Tween 80 was added as a surfactant to aid in conidial suspension in a deionized water solution. Conidia suspensions were prepared by vortexing in a test tube until all visible particulates were homogenized. The conidia concentration of the initial suspension was determined using an improved Neubauer hemocytometer, and adjusted to desired concentrations using 0.1% Tween 80 and deionized water solution. The control treatments consisted of a 0.1% Tween solution with no conidia and an additional treatment where the adult flies were knocked down with CO_2 and held without exposure to solutions to establish baseline mortality.

A 1-ml aliquot of a conidia solution was applied to each filter paper (100 mm, P5, Fisher Scientific, Fair Lawn, NJ). Filter papers were allowed to dry for 1 h under a biological safety cabinet; after this time the treated filter papers were no longer visibly damp. For each *B. bassiana* strain, 10 adult horn flies, 1- to 2-d post-eclosion, were exposed in groups. Flies were immobilized by CO_2 and placed into a 100 mm×15 mm Petri plate, with a 2.5-cm hole in dish lid that was covered by 18×14 fiberglass mesh and contained a treated filter paper disc. During a contact time of 2 h no food was supplied to the flies. After the contact time had elapsed, flies were immobilized by CO_2 and the treated filter paper disc was replaced with an

untreated disc and blood was provided on cotton balls through the hole in the Petri dish lid.

In the second exposure method experiment, conidia were dispersed into cornstarch to create a dust formulation. To accomplish this, conidia were first weighed and suspended in 0.1% Tween 80 in deionized water, then the concentration of conidia in a known volume of solution was determined on an improved Neubauer hemocytomer. Thereafter, conidia-containing harvested material was diluted into the inert carrier, cornstarch (Great Value™, Walmart, Bentonville, AR), to achieve three desired concentrations. One-tenth of a gram of each concentration, 1.0×10^9, 1.0×10^8, or 1.0×10^7 conidia per gram, was applied to a filter paper disc (100 mm, P5, Fisher Scientific, Fair Lawn, NJ) that had been placed into a previously described modified Petri dish. Thus, final exposure concentrations in cornstarch were 1.0×10^8, 1.0×10^7, and 1.0×10^6 conidia per filter paper disc. Conidia:cornstarch mixtures were distributed as evenly as possible across the filter paper disc by lightly tapping the sides of the exposure chamber. Horn flies were immobilized by CO_2 and 10 adult flies were placed into the Petri plate chamber. After the contact time had elapsed, post-exposure handling was as previously described.

For these experiments, horn flies were blood fed daily and mortality was measured 1- to 2-h post-treatment and daily for 7 d. Horn flies appearing ataxic were considered dead. Each fungal strain assay was replicated 3 times for each fungal concentration and experiments were repeated 3 or 4 times, providing between 90 and 120 horn flies for strain and concentration combinations.

Fungal Virulence Selection and Evaluation

In an effort to improve virulence of the EN1 strain and generate sufficient inoculum, a series of seven selections that included alternate paired passage of the EN1 strain through the horn fly host and SDAY were conducted. *Beauveria bassiana* EN1 and subsequent selections were cultured on Sabouraud dextrose agar (65 g l^{-1}) (Fisher Scientific, Fair Lawn, NJ) with 5 g l^{-1} technical yeast extract added (Difco, Sparks, MD) in 100 mm×15 mm Petri dishes, at 25 °C. Conidiophores formed in 2–3 wk, at which time conidia and associated mycelia were harvested into two clean Petri dishes from enough cultures to yield 1 g of *Beauveria* conidia.

To improve virulence, approximately 350 adult Kerrville strain horn flies of two post-emergence age groups, 3–4 and 5–6 d, were exposed to sequentially selected generations of the EN1 *B. bassiana* strain. Flies, anesthetized with CO_2 were placed in a Petri dish with the selected fungus, and the plate was shaken to distribute the fungus. A 1-min time period followed during which flies recovered and actively walked, flew, and groomed while exposed to conidia and mycelia. Flies were again anesthetized to facilitate transfer to 1.9-l holding cage with a screened lid and were provisioned daily with a cotton ball soaked in citrated bovine blood.

As flies died during a 7-d post-exposure period, cadavers were collected daily, surface sterilized in 95% ethanol, and placed individually in a single well of a 96-well plate. Plates were placed into a plastic box (18.5 cm×13.3 cm×9.5 cm) which contained paper towels soaked in distilled water to maintain humidity in the chamber. Emergence of mycelia from the cadavers and subsequent development of conidiophores was recorded daily starting 3-d post-death until 7-d post-death, and then once more after an additional 4–7 d.

Horn flies from the shortest interval between fungal exposure to both post-treatment death and subsequent production of conidiophores on their cadavers formed the parental lineage for the subsequent generation. Beginning with the EN1 strain, this process was repeated across seven exposure selections. Four samples were taken from each cadaver using a sterile glass inoculating needle, sterilized between each use, and the fungus was reared on an SDAY agar plate to re-isolate the cadaver-isolated fungus without contamination. Cultures obtained from these four isolates that contained only desired fungal growth were then plated on a second passage of SDAY to generate material for horn fly inoculations. Mixtures of between 3 and 17 of these isolates served as the source material for the next *B. bassiana* EN1 sub-strain used for fly inoculation. Selections ended after the EN1-7 strain selection. As strains were generated, through this process, conidia were preserved on silica chips under standard practices (Nakasone et al. 2004).

Fungal strains EN1-0, EN1-1, EN1-3, EN1-5, EN1-7, representing the original isolate, and virulence selections from the first, third, fifth, and seventh horn fly passages, respectively, were recovered from silica chips and fresh cultures grown on SDAY media (EN1-1 ARSEF 13304; EN1-3 13305; EN1-5 13306; EN1-7 13307). For each of the three study repetitions, conidia from each selection strain and the original isolate were propagated as described previously. Treatments consisted of conidia placed into attapulgite clay (Attagel 25; BASF, Research Triangle Park, NC), a material planned for future field experiments and is the base material for use in cattle dust bag insecticide dispensers, at a concentration of 1×10^8 conidia per gram, as well as a clay only control.

In groups of 50, 3-d post-eclosion horn flies were anesthetized with CO_2 and exposed to one of the five fungal strain isolates in 1 g of clay:conidia formulation or a clay-only control. Flies and formulation were placed in a Petri dish and gently shaken for 60 s. These groups of horn flies were transferred to a recovery chamber and held for 2 h, where they were allowed to groom the clay:conidia mixture. Thereafter, flies were separated into two groups of 25 flies, with one group placed in a plastic holding chamber (118 ml) with a screened lid that contained 12 g of fresh cattle manure, while the other was placed into a similar holding chamber without manure. Each holding chamber was provided citrated bovine blood daily via a cotton ball placed on top of the chamber. Flies were monitored daily for 10 d to assess mortality and those that died were recorded, removed, surface sterilized in 95% ethanol, put into a 96-well plate, and monitored for generation of conidia, as described previously. Within each repetition, four replications were completed ($n = 100$ flies). The three repetitions resulted in 300 horn flies per treatment being exposed to each fungal strain formulated in clay either with or without manure.

Data Analysis

For EN1 strain and commercial strain evaluations (filter paper and cornstarch studies), all replicates were aggregated and analyzed in a multiple factor ANOVA (SAS version 9.2, SAS Institute, Cary, NC) (SAS Institute 2004) to test for strain, dose and strain×dose interactions. Additionally, orthogonal contrasts were applied to determine differences between the EN1 and two commercial strains, with a second evaluation between the two commercial strains.

EN1 post-selection fungal strain virulence was evaluated for clay:conidia-exposed flies by generating lethal time for 50% mortality (LT_{50}) values through statistical analysis in JMP® Pro 9.0.2 (SAS Institute, Cary, NC) (JMP 2007). Analysis was conducted separately for each strain and manure combination. A linear regression that included the inverse estimate function was used to generate an LT_{50} value and associated 95% confidence intervals.

Results

Over 7,000 horn flies were collected from Florida cattle ranches and only one individual horn fly yielded an isolate of *B. bassiana*, EN1. This fly was collected from a beef cattle ranch near Gainesville, FL on 24 November 2010 and was 1 of 250 horn flies examined for infection from that collection. No other fungal pathogens were observed among these 7,000 flies.

In the filter paper bioassay, control mortality was 17% on post-treatment day 4 and 24% at day 7. Aggregated and analyzed by post-treatment day, horn fly percent mortality (Mean ± SEM) following *B. bassiana* exposure to the liquid suspension formulation dispersed onto filter papers was very low, even in the high dose 10^7 treatments where 15.3 ± 2.7, 31.2 ± 4.5 and 29.8 ± 5.2 uncorrected mortality was achieved with the EN1, GHA and HF23 strains, respectively. Even with these low mortality responses, the overall model strain variable was significantly different between EN1, HF23, and GHA; while concentration and the interaction between concentration and strain were not significant (Table 1). The *F* values presented document that at post-treatment days 4 through 7, GHA and HF23 preformed similarly, with no differences found between them, whereas mortality in the EN1 was significantly lower.

Control mortality in the cornstarch-conidial trials was at 21% on post-treatment day 4, rising to 29% by post-treatment day 7. Data uncorrected for control mortality demonstrated peak mortality in the EN1 strain of 58% on post-treatment day 7, with the GHA (30%) and HF23 (42%) strains also peaking at this post-exposure time point in the 1×10^9 conidia per gram concentration. No significant differences were found between concentrations, strains or their interaction (data not shown).

The LT_{50} values generated from clay:conidia post-selection bioassays are presented in Table 2. Overlapping 95% confidence intervals were present across all strain isolates and treatments, thus no change in virulence occurred across the selection process. The EN1-5 and the EN1-5 + M were numerically larger representing a longer time to mortality, however, as the confidence limits overlapped with several other strains, no significant difference can be inferred. Of horn flies that died during the 10-d post-exposure period, >90% expressed *B. bassiana* conidia, with the exception of the EN1-5 strain treatments.

Table 1. F values for mean percent mortality of adult *Haematobia irritans* exposed to three strains of *Beauveria bassiana* conidia impregnated on filter paper in a 0.1% Tween 80 and water solution and evaluated for mortality over 7 d

Model effect	Post-treatment day			
	4	5	6	7
Concentration	0.10	0.11	0.52	0.19
Strain	13.54*	15.77*	13.61*	15.07*
Concentration × strain	0.35	0.24	0.28	0.40
Orthogonal contrasts				
EN1 vs. GHA + HF23	25.90*	31.03*	26.96*	30.02*
GHA vs. HF23	1.48	0.74	0.41	0.03

Concentrations included conidial levels of 1×10^5, 10^6 and 10^7 in each exposure chamber. EN1 (ARSEF 13303) is a field-collected horn fly *B. bassiana* strain, GHA (ARSEF 6444) and the HF23 (ARSEF 7940) are commercially available strains. Uncorrected mortality of 15.3, 31.2 and 29.8% was achieved with the EN1, GHA and HF23 strains, respectively, at post-treatment day 7.

*$P < 0.01$.

Table 2. Horn fly, *Haematobia irritans*, lethal time (days) to 50% mortality (LT_{50}) and percentage of flies that died and subsequently expressed conidia following exposure to the EN1 *Beauveria bassiana* strain

Treatment	LT_{50} (±95% CI)	Cadavers expressing conidia percent (±SEM)
EN1-0	5.49 (5.29–5.63)	93.0 (1.29)
EN1-O+M	5.68 (3.32–8.04)	91.3 (2.65)
EN1-1	5.95 (2.82–9.08)	95.3 (1.62)
EN1-1+M	6.02 (3.32–8.73)	93.7 (1.46)
EN1-3	6.05 (3.58–8.53)	95.1 (1.58)
EN1-3+M	6.01 (3.58–8.46)	96.0 (1.51)
EN1-5	10.88 (5.61–16.47)	72.8 (4.60)
EN1-5+M	8.06 (4.13–12.07)	81.7 (2.55)
EN1-7	7.34 (4.33–10.38)	89.4 (1.77)
EN1-7+M	6.27 (3.64–8.92)	93.0 (1.36)

Evaluation of virulence occurred following progressive selection for the first mortality and conidia event at each of seven horn fly passages.

EN1-0 = original isolate, EN1-1 = first passage, EN1-3 = third passage isolate, EN1-5 = fifth passage, EN1-7 = seventh passage (ARSEF 13303-13307, respectively). The inclusion of +M indicates the addition of 12 g of fresh cattle manure to post-exposure holding chambers. Horn flies evaluated daily for mortality over 10 d and carrier-only controls (attapulgite clay) mortality did not exceed 28% on day 10.

Discussion

Our *B. bassiana* isolate (EN1) represents only the second report of fungi isolated from field-collected horn flies; the first report is from Steenberg et al. (2001). The behavior of adult horn flies, which spend their entire lives on pastured cattle hosts and the resultant immature development in undisturbed cattle dung pats, has hindered the exploitation of fungal pathogens for this species (Foil and Hogsette 1994). Given adult behavior and ecology, wherein horn flies cannot be easily collected from a centralized location as has been done with other muscid species (Steinkraus et al. 1990, Geden et al. 1995), little research has been done to isolate fungal pathogens from this species.

The life expectancy of adult horn flies reported in literature is somewhat variable. Krafsur and Ernst (1983) reported that adult flies only lived for 6.6 d on an average; however, life spans of 28 d to 8 wk have been reported (summarized in Butler and Okine 1999). Because the horn fly is reported to begin oviposition 3.5-d post-eclosion (Krafsur and Ernst 1983), an entomopathogenic fungus must act quickly to be effective. Thus, our objective was to accelerate the mortality rate of *B. bassiana* due to their rapid reproductive capacity to determine if significant mortality could be achieved prior to the onset of oviposition.

Of the few studies evaluating entomopathogenic fungi in horn flies, a majority of the focus has been upon the immature stages; eggs, larvae, and pupae; with little attention given to infecting adult flies. Angel-Sahagún et al. (2005) were the first to show artificial infection of adult horn flies with fungi, and did so with several isolates of *Metarhizium anisopliae* (Metschnikoff) Sorokin, *B. bassiana*, and *Isaria fumosorosea* (Wize) (Brown and Smith) (formerly *Paecilomyces fumosoroseus)*. Their study used manure inoculations to infect eggs and larvae with conidia, as well as continuous exposure assays for pupae and adults. In their study, at doses of 1×10^8

several *Metarhizium* and *Isaria* strains provided >90% mortality. However, at similar doses with *B. bassiana*, mortality ranged from 40 to 74%, although post-exposure holding time was not provided. In our study, day 10 post-exposure mortality in the clay:conidia experiment ranged from 85 to 99.7% in all treatments outside of the EN1-5 strain (data not shown). Taken together, these studies demonstrate the need for strain-specific data generation, as mortality is contingent on specific strains, rather than fungal genera.

Formulated fungal strains developed and sold for biological control of other insect species were evaluated by Lohmeyer and Miller (2006) and included *M. anisopliae* (strain ESCI) and *B. bassiana* (strain GHA). These authors used a 2-h contact exposure and reported a lethal time for 50% mortality, LT_{50}, of 2.70, 4.98, and 9.42 d for *B. bassiana*, *M. anisopliae*, and untreated controls, respectively. In our study, peak mortality with the GHA strain was 30%, 7 d after exposure, quite different from the estimated 2.70-d LT_{50} as reported in Lohmeyer and Miller (2006). Certainly, exposure concentration and application technique impacts fungal lethality, and the Lohmeyer and Miller (2006) studies were conducted at ~340-fold higher concentrations than our high dose, but their exposure concentration was considerably higher than realistic levels for field applications 5×10^9 per gram. Galindo-Velasco et al. (2015) evaluated multiple strains of *M. anisopliae* and *I. fumosorosea*, previously evaluated in Angel-Sahagún et al. (2005) and formulated in a liquid suspension and applied to the backs of individually caged cattle. In this study, >90% horn fly mortality was achieved at 13-d post-application. However, horn fly mortality for most applications did not exceed 50% until after the 4-d post-treatment evaluation. It is unfortunate that mortality readings were not conducted between the 4- and 8-d post-treatment period when one would expect fungal mortality events to manifest. Furthermore, mortality in the control animals was 50–85% at day 13, suggesting that adult horn fly life expectancy was approaching its terminus, perhaps increasing the fungal mortality effect. Given that horn fly oviposition occurs as early as 4-d post-emergence, the expression of high mortality 9 d thereafter suggests that this treatment may not impact horn fly populations in a meaningful manner.

Some *B. bassiana* isolates have been shown to be highly virulent to the hosts from which they were isolated, but this is not always the case (Feng et al. 1994). Initially, the EN1 strain was not passed through a horn fly host so that we could study the inherent virulence of the strain. In our evaluations, the commercial strains appeared to cause greater mortality than the EN1 horn fly-isolated strain. This is not unusual, as several isolates from divergent hosts have shown variability in their virulence without host passages to other insects (Feng and Johnson 1990, Watson et al. 1990, Khachatourians 1992). Following these results where significant differences between the three *B. bassiana* strains were not observed, we selected the EN1 strain to produce a faster time to fly death effect by passing the fungus through successive horn fly hosts and culturing the isolates that resulted in the first mortality with associated sporulation. Failure of this selection process to improve efficacy may have been the result of the initial isolate being collected from a single horn fly adult, thus lacking genetic variability. Additionally, it is unknown what role environmental bacteria in manure may play in increasing mortality in filth flies. Our inclusion of a cattle manure variable was an attempt to introduce bacteria to a subset of fungus-exposed horn flies; however, this exposure did not improve mortality.

Hall (1980) reviewed the early literature on increasing the virulence of insect fungal pathogens and noted great variability in success through selection in only agar, only insects or rotations of both. Samsinakova and Kalalova (1983) utilized single spore *B. bassiana* isolation as a means to identify increased virulence, and after identifying six isolates, determined that these isolates were stable in their increased virulence. There appears to be little consensus on technique as well as expected outcome. We utilized an aggressive selection method wherein the first fly to die and subsequently produce conidia was selected to serve as the foundation for the next generation. Although our selection process ended at the F7 selection, our study documented that selection of the EN1 strain through horn fly hosts did not significantly decrease time to mortality of this *B. bassiana* strain. It is possible that similar selections with other *B. bassiana* strains could result in accelerated death rates. Given the behavioral and ecological aspects of the horn fly, which limits treatment options, a combination of high mortality and hyphal expression will be necessary if *B. bassiana* is to be used in a biological control program targeting this important pest. Although we were unable to accelerate mortality, nearly all flies that died in *B. bassiana*-exposed treatments produced conidia (Table 2).

The current work is the first evaluation of a horn fly-obtained *Beauveria* isolate examined against horn flies. The search for highly pathogenic fungal isolates against the horn fly should continue such that an improved control program can be developed. Although the EN1 isolate or subsequently selected variants did not result in increased mortality in our study, the generation of improved formulations should be evaluated for their potential role to accelerate speed of kill. Integrated pest management seeks to continually develop and utilize novel methods for pest control; and horn flies essentially have been overlooked in development of biological control agents over the past 30 yr. Due to their biology, ecology, pasture-dwelling behavior, and ability to develop insecticide resistant populations, alternative methods should continue to be investigated and developed to provide for improved management of horn fly populations.

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