

Oviposition Deterrence and Immature Survival of Filth Flies (Diptera: Muscidae) When Exposed to Commercial Fungal Products

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Abstract

Filth flies are pests of livestock, and can transmit pathogens that cause disease to animals and their caretakers. Studies have shown successful infection of adult filth flies following exposure to different strains and formulations of entomopathogenic fungi. This study aimed to examine the effects of commercial formulations of *Beauveria bassiana* (Balsamo) (Moniliales: Moniliaceae) (i.e., BotaniGard ES, Mycotrol O, baEnce), and *Metarhizium brunneum* (Metsch.) (Ascomycota: Hypocreales) (i.e., Met52 EC), on filth fly oviposition and immature fly survival after exposure. House flies, *Musca domestica* L., laid significantly fewer eggs on Met52 EC-treated surfaces than on surfaces treated with all other products and the control. Similar numbers of eggs were laid on surfaces treated with all *B. bassiana* products, but egg production was half of the control. Stable flies, *Stomoxys calcitrans* (L.), laid the fewest eggs on Met52 EC- and Mycotrol O-treated surfaces. This species did not distinguish between the remaining products and the control. In a second experiment, house fly eggs were placed on treated cloths so that hatched larvae contacted the treatment prior to development. Met52 EC had the greatest effect on immature survival with a significant reduction in recovered pupae at the medium and high doses of fungi. Overall, Met52 EC, containing *M. brunneum*, had the greatest effect on house fly and stable fly oviposition deterrence and immature development of house flies. Management implications are discussed.

Key words: house fly, stable fly, *Beauveria bassiana*, *Metarhizium anisopliae*, *Metarhizium brunneum*, biological control

House flies [*Musca domestica* L.] and stable flies [*Stomoxys calcitrans* L.] (Diptera: Muscidae) are pests of medical and veterinary importance. These flies are not only a nuisance, but are capable of transmitting many pathogens mechanically (Greenberg 1971; Malik et al. 2007). Management of these filth fly pests has centered on insecticide use, but increasing resistance to many active ingredients has decreased efficacy of this control method (Pickens and Miller 1987; Cilek and Greene 1994; Kočišová et al. 2002; Marçon et al. 2003; Malik et al. 2007; Scott et al. 2013). Integrated pest management (IPM) programs incorporating biological control options commonly include the use of insect natural enemies, but can incorporate non-arthropod options as well.

Entomopathogenic fungi occur naturally in fly systems, and certain species are capable of causing mortality in both house and stable flies (Steinkraus et al. 1990; Skovgård and Steenberg 2002). *Beauveria bassiana* (Balsamo) (Moniliales: Moniliaceae), and *Metarhizium anisopliae* sensu lato (Metsch.) (Ascomycota: Hypocreales) have been investigated as potential biological control options for filth fly management.

Studies have shown successful infection of adult flies following exposure to different strains and formulations of fungi (Kuramoto and Shimazu 1992; Geden et al. 1995; Watson et al. 1995; Darwish et al. 2002; Lecuona et al. 2005; Mishra et al. 2011). However, most research has focused on adult mortality following exposure. Laboratory exposure is frequently unnatural, through dipping in solutions or dusting with conidial powders. Few studies have analyzed the behavioral or survivorship effects of these fungi on house and stable flies that have been exposed passively.

A substantial number of mycopesticide products have been developed for arthropod control, and the active ingredients in the majority of these products are *B. bassiana* and *M. anisopliae* sensu lato (de Faria and Wraight 2007). Infection with either of these two fungi takes ~4–6 days to kill an adult fly (Geden 2012). Therefore, females have opportunities after exposure to continue to lay eggs in suitable areas. Newly hatched offspring may be exposed to residues from applications of products, but nothing is known about the potential impact of such secondary exposures.

An understanding of commercial products containing entomopathogenic fungi on the behavior of adults and survivorship of offspring is needed to optimize the use of these products. One objective of this study was to test four commercial formulations of *B. bassiana* and *M. brunneum* on house fly and stable fly oviposition avoidance of products containing fungal conidia. The second objective was to test house fly immature survival after exposure to the products to better assess the effects of these strains and formulations.

Materials and Methods

House and Stable Flies.

House flies were from the USDA-ARS, Center for Medical, Agricultural and Veterinary Entomology (CMAVE) colony. This insecticide-susceptible colony ('Orlando Normal') was originally collected in the 1950's near Orlando, Florida. Flies were reared on a 13:1:6.5 ratio of wheat bran, Calf-Manna (Manna Pro Products LLC, Chesterfield, MO), and water. Adults were reared under laboratory conditions of $26 \pm 2^\circ\text{C}$, 45–55% RH, and a photoperiod of 8:16 (L:D) h.

Stable flies were from the USDA-ARS, CMAVE colony. This colony has been maintained for ~35 years at $26 \pm 2^\circ\text{C}$, $60 \pm 5\%$ RH, and a photoperiod of 12:12 (L:D) h. Adult stable flies were provided citrated bovine blood *ad libitum*. Larval diet was as described by Machtinger et al (2014).

Fungal Strains.

Four commercial fungal products and three strains were tested. *B. bassiana* strains tested were HF23, a house fly-derived strain in the formulation labeled balEnce (Terragena, Raleigh, NC) (1.12% active ingredient and 5.6×10^9 colony forming units per gram), and two formulations of the GHA strain, which was originally isolated from *Diabrotica* spp. (Coleoptera: Chrysomelidae). The GHA strain was tested in two formulations, BotaniGard ES and Mycotrol O (Laverlam International Corporation, Butte, MT) (11.3% active ingredient with 2×10^{13} colony forming units per gram and 10.9% active ingredient with 2×10^{10} colony forming units per gram, respectively). The *M. brunneum* strain tested was F52 isolated from *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) obtained in the formulation Met52 EC (Novozymes Biologicals, Inc., Franklinton, NC) with 11% active ingredient (5×10^{10} colony forming units per gram). Formulations were emulsifiable suspensions using petroleum distillates as the emulsifying agents. Mycotrol O was the exception, labeled as suitable for organic use and containing vegetable oils.

Viability of fungal cultures was confirmed by germination of propagules on agar. Subsamples of each commercial solution were spread on Sabouraud-dextrose agar with yeast (yeast concentration 0.5%) in a concentration gradient to ensure that accurate observation of the conidia was possible. After incubation at 22.5°C and 55% RH for 18–24 h, the percentage of conidia that germinated was calculated for each strain. All strains were confirmed to have germination rate of >90% except balEnce that had <20% germination.

Oviposition Experiment.

Seven- to ten-d-old house flies and stable flies were aspirated out of cages, and visually confirmed to be gravid (Machtinger et al. 2014). Adult flies were anaesthetized with CO_2 , and groups of 15 female house flies or 30 female stable flies were placed in five $17.5 \times 17.5 \times 17.5$ cm plastic cages (BugDorms, MegaView Science,

Taiwan) for 10 min prior to testing with a 10% sucrose solution provided *ad libitum*.

Seven-d-old medium used for house fly larval rearing was frozen at -20°C for a minimum of 1 wk to ensure all developing house flies had been killed. This substrate was thawed prior to use, and a 57 g ball of media was covered with an 8×8 cm black cotton cloth and secured with a rubber band to create an attractive oviposition site (oviposition ball). The oviposition ball was moistened with distilled water and warmed to a surface temperature of 33°C as added attraction for fly oviposition prior to adding fungal products. For stable flies, 3 ml of citrated bovine blood, along with water, also was added to the surface of the oviposition ball prior to warming.

After warming, each product was applied to the surface of the oviposition balls at 1×10^8 conidia per ml^{-1} and spread using sterilized paintbrushes. Products were diluted according to the conidial concentrations listed on the label in a solution of water (with Tween 80, 0.2%) and horticultural oil (SunSpray Ultra-Fine Spray Oil, HollyFrontier Refining and Marketing LLC, Plymouth Meeting, PA). This was chosen to provide the same viscosity as the emulsifying agents and inert oils found in the fungal products. As a control, horticultural oil, was added to sterile water and spread on the oviposition ball. The amount of oil (i.e., product plus horticultural oil) was equalized between all solutions (~1.8%). Every effort was made to have uniform coverage of the product over the material. This dose resulted in an estimated concentration of 1.25×10^7 conidia/ cm^2 on the treated oviposition ball. Oviposition balls were placed in 59 ml plastic cups (Instawares Holding Company, LLC., West Point, GA), ensuring the sides of the oviposition ball were touching the sides of the cup, preventing flies from laying eggs on the bottom of the cup or on the underside of the oviposition ball.

Cups with oviposition balls were added to the center of the plastic cages in a no-choice oviposition assay. House flies were permitted 1 h and stable flies 2 h to oviposit. After this time, the oviposition balls were removed. The medium was emptied out of the cloth, and eggs were washed off the cloth with distilled water into a 59 ml plastic cup and counted. For each fly species, six replicates of the oviposition experiment were conducted on different days using different cohorts of flies.

Immature Survival Experiment.

Each of the four commercial fungal products (described previously) were tested at a low dose (1×10^6 conidia per ml^{-1}), medium dose (1×10^8 conidia/ ml^{-1}), and high dose (1×10^9 conidia/ ml^{-1}), with horticultural oil (SunSpray Ultra-Fine Spray Oil, HollyFrontier Refining and Marketing LLC, Plymouth Meeting, PA) as a control at the respective dose in water (four treatments and one control total per dose per replicate). Products were diluted according to the conidial concentrations listed on the label in a solution of water (with Tween 80, 0.2%) and horticultural oil (SunSpray Ultra-Fine Spray Oil, HollyFrontier Refining and Marketing LLC, Plymouth Meeting, PA). The amount of oil (i.e., product plus horticultural oil) was equalized between all products and doses (~1.8%). One ml of each product and dose was applied to different 7×4 cm black cotton cloths.

Approximately 415 cm^3 of house fly medium (50% wheat bran, 30% alfalfa meal, 20% fine corn meal at a 5:6 medium:water ratio) was added to fifteen 473 ml plastic rearing containers (Instawares Holding Company, LLC., West Point, GA). Eggs were collected from colony house flies as described in Machtinger et al. (2014). Eggs were placed on treated cloths in groups of 100. Treated cloths with eggs were placed on the medium with the eggs facing up,

oriented away from the medium. Rearing containers were covered with muslin. House fly pupae were removed from the rearing medium by floatation after 7 d. Pupae were weighed individually after drying and subsequent adult fly emergence was recorded. Eight replicates of the immature survival study were conducted using eggs derived from the house fly colony on different dates.

Statistical Analysis.

Data on fly oviposition were subjected to a separate one-way analysis of variance (ANOVA) for each fly species. Means were separated post-hoc with Tukey's Honestly Significant Difference (HSD) test for comparison.

To compare the different products and their dose on immature fly survival, the effects of 'product' and 'dose' were examined together by full factorial, two-way ANOVA. Means were separated by Tukey's HSD test among comparisons. Unless otherwise specified, significance was determined at $\alpha=0.05$ (JMP v. 12.1.0, SAS Institute Inc., Cary, NC 2010).

Results

There were significant differences among the number of eggs laid by house flies on surfaces treated with the tested entomopathogenic fungi products ($F=27.6$; $df=4,25$; $P<0.0001$) (Fig. 1A). The most eggs were laid on the control oviposition ball. There were no differences in house fly oviposition between the *B. bassiana* products (i.e., BotaniGard ES, Mycotrol O, and balEnce). Approximately half as many eggs were laid on the *M. brunneum* product (i.e., Met52 EC) than the *B. bassiana* products. The number of eggs laid on surfaces treated with the tested entomopathogenic fungi products also differed for stable flies ($F=16.8$; $df=2,25$; $P<0.0001$) (Fig. 1B), although unlike house flies there was a difference between *B. bassiana* products. Stable flies laid similar numbers of eggs on the oviposition ball treated with the control and two *B. bassiana* products, BotaniGard ES and balEnce. Fewer eggs were laid on the surfaces treated with Mycotrol O and the *M. brunneum* product, Met52 EC.

Product and dose applied to the treated cloth that the eggs were placed upon both had a significant effect on the number of house fly pupae recovered in the development experiment ($F=29.4$; $df=4,105$; $P<0.0001$ and $F=68.1$; $df=2,105$; $P<0.0001$, respectively). Additionally, there was a significant interaction between the effect of product and dose ($F=7.4$; $df=8,105$; $P<0.0001$). Weights of recovered pupae, and subsequent adult emergence, were not found to be significantly different with any treatment.

As expected, the pupal recoveries in the control treatments were not different regardless of dose (Table 1). There were no differences among any of the treatments at the low dose. Similarly, there was no difference in pupal recovery among the control and any *B. bassiana* product at the medium dose. Pupal recovery was significantly lower at the medium dose when eggs were placed on the surface treated with Met52 EC. At the highest dose tested, the number of pupae that were recovered from eggs placed on *B. bassiana* products were half that of the control. Pupal recovery was significantly lower with BotaniGard ES, Mycotrol O, and balEnce at this high dose than the lower doses. Significantly fewer pupae were recovered with the Met52 EC at the highest dose than all other treatments and doses, <16% of the control pupal recovery was recovered from Met52 EC at this high dose.

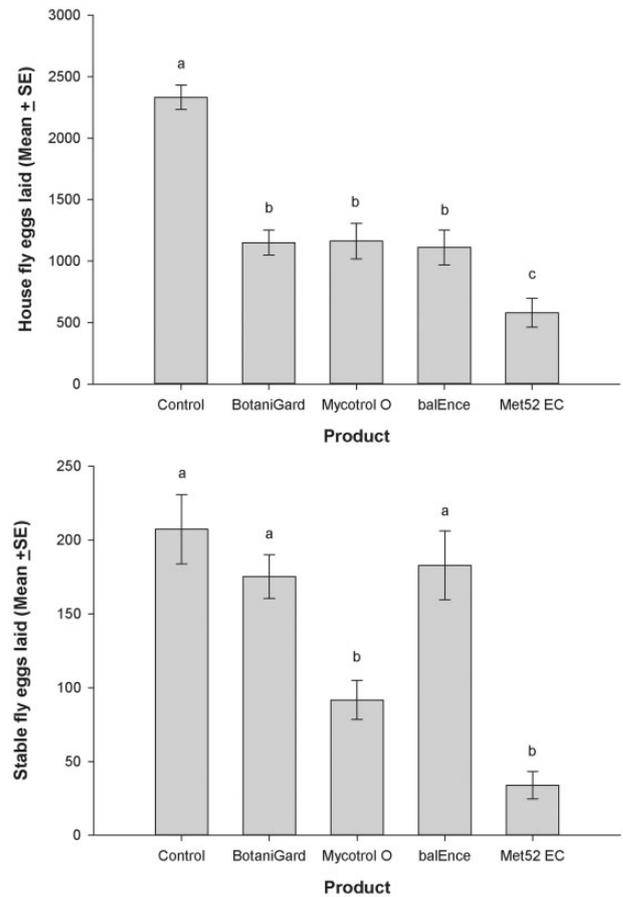


Fig. 1. House fly [*M. domestica* L.] and stable fly [*S. calcitrans* L.] oviposition on a surface treated with four commercial fungal products at 1×10^8 conidia per ml^{-1} dose diluted in horticultural oil and containing three fungal strains. BotaniGard ES and Mycotrol O included the *B. bassiana* strain GHA, and balEnce contained *B. bassiana* strain HF23. *M. brunneum* strain (F52) was tested as the product Met52 EC. All product formulations were emulsifiable suspensions using petroleum distillates, except Mycotrol O, which used vegetable oils. Horticultural oil was incorporated as a control. Means followed by the same letter are not significantly different (Tukey's HSD test, $\alpha=0.05$).

Discussion

The number of eggs laid on a surface treated with products containing *B. bassiana* and *M. brunneum* was influenced by the product tested. In addition, the product and dose used had an effect on subsequent house fly immature survival. These results suggest that the tested products effected house and stable fly fitness and survivorship, and this should be considered when developing an IPM program that includes products containing entomopathogenic fungi.

Unfortunately, we were unable to separate the effect of the fungi from the other product ingredients due to formulations without fungi not being available from the suppliers, and the effect of irradiation and heat having unknown consequences to the active ingredients and formulations. However, it is plausible that female flies were reacting to microbial volatile organic compounds (MVOC's) from the fungi and avoiding contact with the oviposition ball or distinguishing the presence of fungal conidia harmful to larval survival. Conversely, the inert product ingredients may be repellent to female flies assessing suitable oviposition sites.

House fly oviposition on all products was significantly lower than the control and so females could be deterred by the presence of inactive ingredients. Stable fly oviposition on Met52 EC and

Table 1. Pupal recovery from house fly eggs placed on the surface of media treated with three doses of four commercial fungal products.

Product ^c	Pupal recovery by dose (%) \pm SE ^{a,b}		
	1×10^6 (Conidia ml ⁻¹)	1×10^8 (Conidia ml ⁻¹)	1×10^9 (Conidia ml ⁻¹)
Horticultural oil (control)	82.5 \pm 4.3a	79.1 \pm 2.4a	87.0 \pm 1.1a
BotaniGard ES	74.8 \pm 4.0a	76.5 \pm 4.5a	44.9 \pm 5.5c
Mycotrol O	86.4 \pm 3.4a	73.9 \pm 5.1a	45.9 \pm 4.1bc
balEnce	77.1 \pm 4.9a	75.1 \pm 3.5a	42.3 \pm 7.1c
Met52 EC	67.4 \pm 5.2ab	51.6 \pm 4.9bc	13.5 \pm 3.2d

^aMeans followed by the same letter are not significantly different (Tukey's HSD test, $\alpha = 0.05$).

^b100 eggs were tested in each replicate. Eight replicates of each treatment were performed ($N = 800$ total eggs per treatment).

^cBotaniGard ES and Mycotrol O included the *B. bassiana* strain GHA, and balEnce contained *B. bassiana* strain HF23. *M. brunneum* strain (F52) was tested as the product Met52 EC. All product formulations were emulsifiable suspensions using petroleum distillates except Mycotrol O, which used vegetable oils. Dilutions were prepared using horticultural oil, which was also incorporated as a control.

Mycotrol O were significantly less than the control, but females of this species appeared to be more tolerant of the other *B. bassiana* products and similar numbers of eggs were laid among BotaniGard ES, balEnce, and the control. As Mycotrol O and BotaniGard ES contain the same fungal strain yet differ in the type of oil they contain, vegetable and petroleum, respectively, it is possible that oil plays a larger part in this difference in oviposition deterrence.

Few studies have tested the impact of oils on insect behavior, but those that have mostly recorded a repellent effect (Severin and Severin 1914; Shultz et al. 1983; Luz and Batagin 2005; Hidayat et al. 2013). Oviposition deterrence has been found to occur associated with petroleum (Shultz et al. 1983), and vegetable oils (Severin and Severin 1914; Hidayat et al. 2013). Severin and Severin (1914) compared petroleum and vegetable oils as trap baits and found that while kerosene (petroleum oil) was highly attractive to male fruit flies, *Ceratitis capitata* Wied., a vegetable oil bait did not improve trap catch. The use of vegetable oil-based formulations for application of *B. bassiana* was studied in detail with *Triatoma infestans* (Klug). A 5–10% oil concentration was found to promote *B. bassiana* infection through increased germination and infection even at unfavorable relative humidity. Luz and Batagin (2005) found all 11 vegetable oils to be repellent at concentrations above 10% in water to *T. infestans*, with variation between the oils. At all concentrations *T. infestans* preference for resting on leaves decreased from corn to castor to olive to sunflower oil (Luz and Batagin 2005). Although concentrations above 10% oil were repellent, nymphs were indifferent to an oil concentration of 10% and attracted to a 1% oil concentration (Luz and Batagin 2005). However, a 1% vegetable oil concentration was found to deter oviposition in fruit flies, *Bactrocera tyroni* (Froggat), resulting in less fruit punctures and less eggs laid on oil treated fruit (Hidayat et al. 2013). In our study the concentration of total oil was maintained at 1.8%, throughout testing of the different products and the doses, so unlikely to be highly repellent but may have resulted in oviposition deterrence in the house fly and increased deterrence in the stable fly associated with vegetable oil.

In the current study, fewer house fly and stable fly eggs were laid on the *M. brunneum* product and fewer house fly puparia were recovered from treatments with this product at the medium and high doses. However, although *B. bassiana* products seemed to reduce oviposition, they had no significant effect on the mortality of developing immature house flies until the high dose. As both Met52 EC and several of the other products are formulated in petroleum oil it is likely that the high level of oviposition deterrence and larval mortality was caused by differences in the fungi itself.

Termites and Japanese beetles are known to detect and avoid soil containing entomopathogenic fungi (Villani et al. 1994; Staples and

Milner 2002). Although such an ability is clearly adaptive for soil-inhabiting arthropods where the risks of infection are high due to prolific bacteria, generalist predators such as ladybird beetles and anthorcidids also can detect the presence of prey items infected with fungal pathogens, and the parasitoid *Aphidius ervi* (Haliday) (Hymenoptera: Aphidius) can detect the presence of aphids infected with the fungal pathogen *Erynia neophidis* (Remaudière and Henneber) (Entomophthorales: Entomophthoraceae) (Pope et al. 2002; Meyling and Pell 2006; Ormond et al. 2011). These examples represent adaptations to common and substantial hazards, but the pathogens tested here are rare in the flies' environment. Natural prevalence of *B. bassiana* and *M. anisopliae* sensu lato in field populations of house flies and stable flies is well below 1% (Steinkraus et al. 1990; Geden et al. 1995; Skovgård and Steenberg 2002). However, these flies are known to assess the quality of potential development sites before committing to oviposition.

MVOCs have been shown to have a direct influence on muscoid fly oviposition. House flies detect the odor profiles of harmful fungi on otherwise suitable animal feces and avoid oviposition (Lam et al. 2007). Romero et al. (2006) demonstrated that some bacterial isolates stimulate stable fly oviposition and subsequently supported larval development, whereas bacteria that do not support development are not attractive to stable flies for oviposition. *B. bassiana* and *M. anisopliae* sensu lato produce volatile secondary metabolites that can be detected by other insects, and direct contact subsequently avoided (Meyling and Pell 2006; Yanagawa et al. 2009, 2011; Hussain et al. 2010; Mburu et al. 2012). In several studies *B. bassiana* and *M. anisopliae* conidia were found to produce volatiles that were repellent to termites. Repellency to a conidial VOC extract treated surface and defensive and protective behavior in individuals towards treated conspecifics were observed in multiple studies by different authors (Yanagawa et al. 2009, 2011; Hussain et al. 2010, Mburu et al. 2012). The VOC's produced by conidia of various fungal species including *B. bassiana* have been identified as 1-octen-3-ol and 3-octanone (Chitarra et al. 2004; Yanagawa et al. 2011).

Overall, the most effective tested commercial mycopesticide in reducing fly oviposition and inhibiting house fly survival was Met52 EC containing *M. brunneum*. Other studies have documented *M. anisopliae* sensu lato to be more pathogenic than *B. bassiana* to house flies (Barson et al. 1994; Mishra et al. 2011). It is possible that flies were able to detect the presence of fungal conidia through MVOCs and avoid laying eggs on the Met52 EC-treated oviposition site in order to protect their offspring from the increased pathogenicity of this fungus.

Future research will focus on separating the effects of the formulation and fungi on oviposition through obtaining blank formulation

from the suppliers of the products. If this proves to not be possible, experiments to attempt to remove the fungi from the product through centrifugation and other methods will be completed. Although oviposition may be reduced by application of products containing entomopathogenic fungi in the treatment areas, flies may oviposit in untreated areas. If VOC's from formulations or fungi, such as 1-octen-3-ol and 3-octanone, could be identified as fly oviposition deterrents, they could assist in a push-pull strategy for fly control in small areas.

One product, balEence, had a low germination rate of <20%, which was not taken into consideration when calculating concentrations. This product was as repellent to ovipositing house flies and as pathogenic to house fly larvae at the highest dose as the other *B. bassiana* products, Mycotrol O and BotaniGard ES, which had >90% germination. Despite low viability and low pathogenicity observed in laboratory studies (unpublished data), field applications of balEence have been relatively successful (Kaufman et al. 2005). It is possible that the more septic environment in the media caused increased levels of mortality associated with this product. Future studies could explore the impact of septic versus aseptic environments in promoting mortality through fungal treatment.

The immature survival experiment was not completed with stable flies due to the difficulty rearing this species in small containers with the tested medium, but the effect of these products on stable fly survival should be examined to explore if there is a similar connection between oviposition avoidance on the Met52 EC and Mycotrol O and reduced immature survival.

Similar to previous studies, the high conidial doses provided the most effective dose for reducing house fly survival for all products (Watson et al. 1995; Mwamburi et al. 2010). At lower doses, the applied fungi may have been outcompeted by other microbes in the development medium, and unable to proliferate and kill the immature flies. Following the label of the product that had the strongest effect on oviposition and larval survival, Met52 EC, surface treatment is ~\$2.00/30 m², but this cost does not incorporate the amount of product necessary to reach deeper into the substrate where immature flies develop. Although effective, this application rate likely is not economically feasible for large areas, and the duration of effectiveness is not known. Greater mortality may have been achieved at the lower doses if the products had been mixed in the development medium instead of applied as surface treatments, or if high doses are applied as spot treatments to areas of high fly activity. Comparison of application methods could provide useful information on maximizing the effectiveness of commercial fungal products.

B. bassiana and *M. anisopliae* sensu lato require several days after exposure to kill adult flies, but secondary effects could reduce population growth by inhibiting oviposition and reducing immature survival success (Geden 2012). Overall, Met52 EC containing *M. brunneum* was the most effective product in reducing fly oviposition and house fly survival, but field trials are necessary to determine optimal locations and delivery methods for applications of these products. Field trials on the use of *B. bassiana* for fly control have produced encouraging but mixed results. Watson et al. (1996) found greatly increased prevalence of *B. bassiana* in house flies after applications of conidia to calf bedding. Applications in poultry houses resulted in either control similar to that achieved with pyrethrins or in total elimination of the fly population (Kaufman et al. 2005; Cova and Scorza-Dagert 2006). Applying the tested strain of *M. brunneum* may increase fly control in certain situations as part of an IPM program, but care should be given to the selection of treatment areas because of the wide host range of the fungi and potential for exposure to non-target organisms (Geden 1995).

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