Entomologia
Experimentalis et Applicata

DOI: 10.1111/eea.12439

Attraction of female fungus gnats, *Lycoriella ingenua*, to mushroom-growing substrates and the green mold *Trichoderma aggressivum*

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Accepted: 10 February 2016

Key words: mushroom fly, Agaricales, Hypocreales, Diptera, Sciaridae, *Agaricus bisporus*, fungal mycelium, mushroom compost, mushroom mycelia, microbial metabolism, volatiles, mycoparasitic fungus

Abstract

To evaluate the attractiveness of several mushroom-growing substrates to the female mushroom fly *Lycoriella ingenua* (Dufour) (Diptera: Sciaridae), a pest of the cultivated white button mushroom, *Agaricus bisporus* (JE Lange) Emil J Imbach (Agaricales), we developed a two-choice, static-flow olfactometer. Behavioral assays using this olfactometer indicated that mushroom compost with *A. bisporus* mycelia growing in it was not more attractive than compost lacking growing mycelia. We also found that female flies were more attracted to compost lacking *A. bisporus* mycelia than to the actual commodity, the white button mushroom fruiting bodies. Flies were not, however, attracted to sterilized compost, suggesting the attraction is due to volatiles produced by microbial metabolism in the compost. We also found that female *L. ingenua* flies were attracted to the mycoparasitic green mold *Trichoderma aggressivum* Samuels & W Gams (Hypocreales). Flies preferred mushroom compost that had *T. aggressivum* growing in it over compost lacking *T. aggressivum*, providing an experimental outcome consistent with the anecdotal belief that *L. ingenua* flies are vectors of *T. aggressivum* spores that can infest mushroom-growing houses.

Introduction

Fresh market production of the white button mushroom, *Agaricus bisporus* (JE Lange) Emil J Imbach (Agaricales), in the USA reached 394 650 metric tons (877 million pounds) and grossed nearly US $1.4 billion between 2012 and 2013. Approximately 60% of the production occurred in Pennsylvania (USDA, 2014), where this crop has two major insect pests: the fungus gnats *Megaselia halterata* (Wood) (Diptera: Phoridae) and *Lycoriella ingenua* (Dufour) (Diptera: Sciaridae), formerly *Lycoriella mali* (Fitch) (Wetzel et al., 1982). Although little definitive experimental evidence exists, it is generally thought that *L. ingenua* damages mushrooms through (1) direct larval feeding on developing *A. bisporus* mycelia in the growing compost media (Cantelo, 1979; Kielbasa & Snetsinger, 1980; Grewal et al., 1993), (2) larval competition with developing *A. bisporus* mycelia for nutrients in the compost (Binns, 1980), thus reducing the water-holding capacity of the compost (Fletcher et al., 1989), (3) possible vectoring of several mycoparasitic *Trichoderma* spp. (Hussey, 1968); and (4) a negative impact of larval frass on mycelial growth (Hussey & Gurney, 1968). A significant reduction in crop yield is known to occur at a density of just 130 larvae per 30 g of spawned compost (Hussey, 1968), or 1 larva per 125 g of casing (White, 1986).

Females live on average 5 days (Wetzel et al., 1982) and deposit on average 200 eggs in their lifetime. Highly fecund females are able to produce eggs 24 h after emergence and mating (Cantelo, 1988), with a generation time of ca. 20 days (egg to adult) at normal *A. bisporus* growing temperatures (Lewandowski et al., 2004). Thus, for an economic problem to develop it only takes a few gravid female flies entering a growing house and laying eggs.

Several integrated approaches to the control of *L. ingenua* primarily target the developing larval stages of the fly. They include compost drenches with the insecticides diazinon or imadocloprid (Shirvani-Farsani et al., 2013), treatments using the juvenile hormone analog methoprene, or the application of the chitin-synthase inhibitor diflubenzuron (Cantelo, 1983). Efforts have also been

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made to incorporate biocontrol agents such as predatory mites (Ali et al., 1999; Jess & Schweizer, 2009) and entomopathogenic nematodes (Shamshad et al., 2008) into the cropping regime to control the fly. The most common control measures still involve long-term use of compost drenches with chemical insecticides. This practice has had some negative consequences. For example, resistance to permethrin was reported for sciarid pests including *L. mali* (subsequently named *L. ingenua*) in commercial mushroom-growing houses after just 3 years of application (Brewer & Keil, 1989; Bartlett & Keil, 1997). Also, possible resistance to diazinon was reported for the mushroom pest *Lycoriella auripila* (Winnertz) (Diptera: Sciaridae) (White & Gribben, 1989).

Because of such resistance development, the high fecundity of female *L. ingenua*, and their almost impossible exclusion from growing rooms, novel tools are necessary to control this significant pest. It is anecdotally believed that females enter a growing house directly after 'phase II' composting, before the compost has *A. bisporus* added to it, i.e., is 'spawned' or 'filled' in a growing room (O'Connor & Keil, 2005; Shamshad, 2010). Although not experimentally proven, this belief exists because the first-generation flies emerge as adults 15–20 days after a room is filled. If this is the case, female flies may not be attracted to the volatile emissions of developing *A. bisporus* in the compost, but instead may be attracted to other volatiles released from microorganisms within the compost itself. We therefore developed a two-choice, static-flow olfactometer to conduct a series of bioassays that would allow us to find the natural substrates that are most attractive to female *L. ingenua*, so that we subsequently might begin to isolate and identify the volatile compounds that are responsible for this attraction. The bioassay apparatus proved to be discriminating in getting females to move toward some mushroom cultivation materials more than others at different stages of production and infestation with fungal mycelia or spores of different fungal species.

Materials and methods

Composting phases: preparation of mushroom-growing test substrates

Compost goes through various preparation phases before it can be used for mushroom production. These phases are outlined below as they are relevant to our experimental uses.

Phase I compost. The composting material was provided by Dr. John Pecchia (Mushroom Research Center, Pennsylvania State University, University Park, PA, USA). The compost consisted of a combination of switchgrass (*Panicum virgatum* L.) straw, wheat (*Triticum aestivum* L.) straw-bedded horse manure, and dried and pelletized poultry manure. The percentages of raw materials in the compost, on a dry-weight basis, were 72% wheat straw-bedded horse manure; 10% switchgrass straw; 6.5% dried distiller's grain; 6.5% dried, pelletized poultry manure; and 5% agricultural gypsum. This mixture underwent a 6-day aerated phase I composting outside made up of the previously mentioned materials plus additional wheat straw-bedded horse manure, additional dried and pelletized poultry manure, gypsum, and dried distillers grain. The primary goals of phase I composting are to break down the physical structure of the compost to increase its water-holding capacity, and to allow microbial activity to degrade complex proteins and polysaccharides (Pudelko, 2014).

Phase II compost. After the 6-day phase I composting regime, the composting material was brought inside a growing room and the temperature was raised to 52 °C for 9 days. The goal of phase II compositing is to allow for the degradation of ammonia by microorganisms within the compost, so that the nitrogen concentration is maintained at ca. 1.5–2% within the compost (de Siqueira et al., 2011). Nitrogen concentrations higher than 2% are detrimental to *A. bisporus* mycelial cultivation of the substrate (de Siqueira et al., 2011).

Phase III compost. After a 9-day phase II composting regime, the material is considered to be ready for 'spawning'. Mushroom grain spawn consists of pieces of sterilized millet grain that have been colonized by a specific clonal variety of *A. bisporus* mycelia. This spawn, plus a slow-release nitrogen supplement, is then added to the phase II compost at a 100:5:1 weight ratio of compost, spawn, and supplement. For our experiments, this phase III compost was mixed in a 10-l plastic autoclave bag (30.5 × 60 cm; VWR International, Atlanta, GA, USA) for 14 days at 21 °C and 70% r.h., after which the compost was thoroughly colonized by white *A. bisporus* mycelia (Fletcher et al., 1989).

Spawned compost. For all experiments, spawned compost is synonymous with phase III compost, i.e., it is the 14-day-old phase III compost as described above.

Unspawned compost. For all experiments, unspawned compost refers to 14-day-old phase II compost that was never spawned with mushroom grain spawn, but was still provided with the nitrogen supplement.

Sterilized compost. For all experiments, sterilized unspawned compost refers to the unspawned compost

that had been autoclaved at 250 °F for 20 min and then used in experiments immediately after it cooled to room temperature, i.e., ca. 5 h after autoclaving.

Agaricus bisporus mycelia

To determine the potential attractiveness of *A. bisporus* mycelia alone to *L. ingenua* females, mycelia from four grain-spawn particles were allowed to colonize a Petri plate of water agar (19 g of agar in 1 l of deionized water) for 12 days (21 °C, 70% r.h.).

Trichoderma aggressivum (green mold)

The green mold *Trichoderma aggressivum* Samuels & W Gams (Hypocreales) was obtained from the Department of Plant Pathology (Pennsylvania State University). To determine the potential attractiveness of *T. aggressivum* mycelia to *L. ingenua* females, *T. aggressivum* was grown on potato dextrose agar (PDA) and allowed to develop on this medium for 3 days in the dark (25 °C, 70% r.h.) in a growth chamber (Model 1-30 BL; Percival Scientific, Perry, IA, USA). For spore formation, *T. aggressivum* was allowed to develop for 3 days in constant light under the same temperature and humidity conditions. *Trichoderma aggressivum* was also grown on spawned compost by placing a 2-cm-diameter disk of 6-day-old *T. aggressivum* (grown on PDA) in a parafilm-covered PDA Petri plate and allowing the disk to colonize the spawned compost under constant light at 21 °C and 70% r.h. for 7 days.

Flies

The flies used in this study were from a 4-year-old laboratory colony maintained at the University Park Campus of Pennsylvania State University, Department of Entomology. Flies were positively identified by Dr. Seunggwan Shin (North Carolina State University, Department of Entomology, Raleigh, NC, USA). This colony was initiated in 2012 by using gravid adult female flies that had been aspirated from the beds of spawned *A. bisporus* compost in Burks County, PA, USA. The flies in the laboratory colony were reared on a mixture of phase II mushroom compost and nitrogen supplement (100:1) in an environmental growth chamber at 21 °C, 70% r.h., and L12:D12 photoperiod.

To initiate the *L. ingenua* colony, 355-ml (12-oz.) plastic cups (Solo, Riverview, MI, USA) were filled to the top with the above-described phase II compost–nitrogen supplement mixture. We found that for rearing *L. ingenua*, it was not necessary to spawn this compost with *A. bisporus* mycelia. We could maintain a robust and healthy colony without any *A. bisporus* at all (K.R. Cloonan, S.S. Andreadis & T.C. Baker, unpubl. data). Nine of these compost-filled cups were then placed into mesh cages (BioQuip, Rancho Dominguez, CA, USA; 30 × 30 × 30 cm), and ca. 50 male and 50 female *L. ingenua* flies were added to each cage. These cages were then left under colony conditions for 2 days to allow the females to oviposit in the compost mixture provided. After 2 days, the cages were covered with plastic autoclave bags to prevent the compost from drying out. The cages were left under colony conditions until future adult flies emerged ca. 21 days later. This process was repeated until we had a continuously emerging colony. For colony maintenance, cages with newly emerged flies had their cups of compost mixture moved to clean mesh cages. Nine cups of fresh compost mixture were added to the cage of newly emerged adults, and the cage was covered with an autoclave bag. The colonies were kept under the above-mentioned standard conditions until newly emerged adults again emerged ca. 21 days later, and then the process was repeated. Old colony cups (ca. 30 days old) were discarded.

For all experiments, 2-day-old gravid females were used. To obtain gravid females of this age, cages of newly emerged adults had their emergence cups removed, and a cotton wick with a 10% table sugar solution was then provided at the bottom of the cage to allow adults to feed ad libitum. The cages were then kept under colony conditions for 2 days, thereby providing sufficient time to allow the adults to mate. Under such conditions, ca. 90% of females laid fertilized eggs (K.R. Cloonan, S.S. Andreadis & T.C. Baker, unpubl.). For all experiments, it was thus assumed that all females in the cage after 2 days were gravid.

Two-choice, static-flow olfactometer

We used a modified version of the olfactometer design developed by Tibbles et al. (2005); instead of testing the movement of multiple gravid females from a release arena onto test substrates, we tested the movement of individual females in each olfactometer. We used 20 or more identically machined olfactometers that consisted of several sections (Figure 1). Each olfactometer had a release arena in which an individual fly was placed to start the test. This arena consisted of a 5-cm-diameter plastic Petri plate (Corning Incorporated, Corning, NY, USA) with two 5-mm-diameter holes drilled into the bottom of the plate, spaced 4 cm apart. Each hole was connected to a pitfall trap on each side of the release arena via 1.5-cm-long, 6-mm-diameter plastic straws. The straws extended from the floor of the release arena into the pitfall trap (Figure 1). The base of each straw was 1 mm wider in diameter than the hole drilled in the release arena, such that each straw laid flush with the floor of the release arena when the arena was set on top of it. The pitfall traps consisted of 30-ml (1-oz.) plastic portion cups (Dart Solo, Harrisburg, PA, USA) fixed with a snap-top plastic lid (4 cm diameter, 3 cm high). A

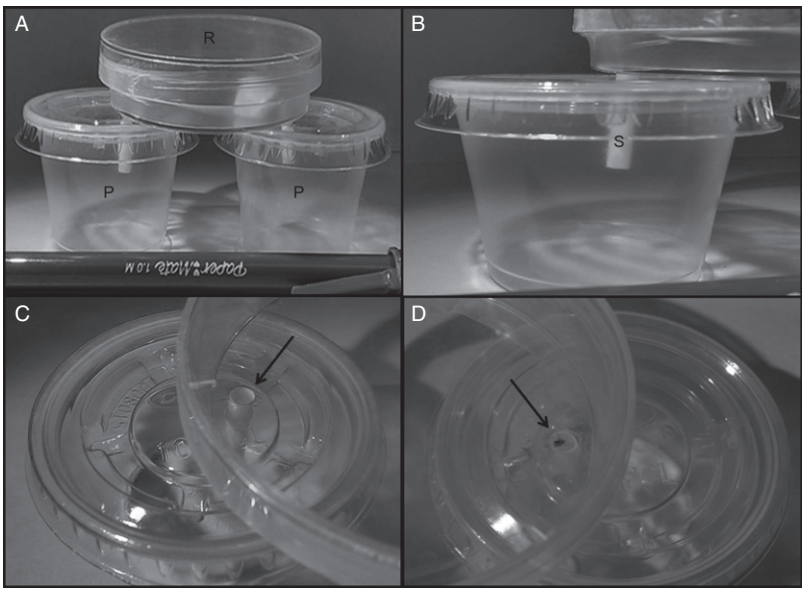

Figure 1 Two-choice, static-flow olfactometer. (A) The olfactometer consists of a 5-cm-diameter release arena (R) and two 4-cm-diameter, 3-cm-high pitfall traps (P). (B, C) A 5-mm-diameter, 1.5-cm-long plastic straw (S) extends from each of two holes (arrow in C) in the release arena floor to the interiors of the two pitfall traps. Note that the top of the straw lays flush with the release arena floor, this is critical in facilitating female *Lycoriella ingenua* entrance into the pitfall traps. (D) A single female fly (arrow) is shown here as she enters a straw leading to a pitfall trap.

6-mm-diameter hole was drilled into the lid, and the plastic straw was snuggly inserted into this hole so that it extended 7.5 mm into the plastic-cup pitfall trap. We observed that once females entered a pitfall trap, none of them were able to move back in the opposite direction into the release arena. Thus, the measurement of the attractiveness of the material in one pitfall trap compared to another was measured by each female's irrevocable choice for that trial.

A layer of parafilm was used to seal the release arena and thereby prevent flies from escaping and also to maintain sufficient humidity within the arena such that none of the flies would die during the 24 h of each replicate. Our previous experiments found that without this parafilm covering, ca. 10% of flies would die during the 24-h period. An individual 2-day-old gravid female was then aspirated into the arena through one of the holes drilled in the bottom, and the arena was gently placed on top of the two pitfall traps so that it rested on top of the straws that were fixed into the lids of the pitfall traps. These traps were then placed in one of two growth chambers under colony conditions (21 °C, 70% r.h., L12:D12 photoperiod) for 24 h. After 24 h, the location of each female fly in the individual olfactometers was recorded (i.e., whether in either of the pitfall traps or in the release chamber). At the end of the experiments, all pitfall traps and plastic straws were discarded, and the release arenas were washed with hot water and detergent, soaked in a 70% ethanol solution, and allowed to air dry for 24 h before reuse.

Olfactometer experiments

Six sets of choice tests were performed over several weeks in the following combinations: (1) spawned vs. unspawned compost, (2) unspawned compost vs. *A. bisporus* white button mushrooms (fruiting bodies; i.e., the white button mushroom that is sold in stores) (ca. 3 cm diameter), (3) unspawned non-sterilized compost vs. unspawned sterilized compost, (4) *A. bisporus* growing on water agar vs. blank water agar, (5) *T. aggressivum* growing on PDA vs. blank PDA, and (6) *T. aggressivum* growing in spawned compost vs. spawned compost alone.

For each set of choice tests, 20 two-day-old females were tested each night in individual static-flow olfactometers. The 20 olfactometers were divided into two identical groups of 10 that were placed in separate growth chambers. The positions of olfactometers within both growth chambers varied randomly. This procedure was carried out over three consecutive nights, resulting in 60 replicates for each experiment, e.g., three groups of 20 females on three consecutive nights were allowed to choose between two substrates whose attractiveness was being compared.

Statistical analysis

All analyses were conducted using PRISM v.5.0 (GraphPad Software, San Diego, CA, USA). Differences among percentages of female flies found in individual pitfall traps for each choice-test experiment were assessed via conventional goodness-of-fit χ^2 analysis, testing frequencies observed vs. expected ($\alpha = 0.05$). Non-responders remaining in the release chamber were excluded from analysis.

Results

In the olfactometer choice tests, the presence of *A. bisporus* mycelia in compost did not increase attraction of gravid female *L. ingenua* (spawned vs. unspawned compost:

$\chi^2 = 0.019$, d.f. = 1, P = 0.88; Figure 2A). Mature, unspawned compost is more attractive to *L. ingenua* females than sterilized unspawned compost ($\chi^2 = 8.64$, d.f. = 1, P = 0.003; Figure 2B). *Lycoriella ingenua* females were more attracted to unspawned compost (lacking mycelia or other life stages of *A. bisporus*) than to an *A. bisporus* fruiting body ($\chi^2 = 4.92$, d.f. = 1, P = 0.02; Figure 2C). Hence, volatiles released from actively metabolizing microorganisms within the compost may explain female attraction to compost, with or without *A. bisporus*.

More females responded to *A. bisporus* mycelia growing on a water agar medium than to water agar medium alone, but this trend was not significant ($\chi^2 = 3.1$, d.f. = 1, P = 0.07; Figure 3A). Gravid female *L. ingenua* were attracted to *T. aggressivum* growing both on spawned compost compared to spawned compost alone ($\chi^2 = 4.74$, d.f. = 1, P = 0.03; Figure 3B) and on PDA compared to PDA alone ($\chi^2 = 27.9$, d.f. = 1, P<0.0001; Figure 3C). Hence, something emitted by this fungus, even without compost, appears attractive.

Discussion

This is the first evidence, to our knowledge, of attraction of sciarid flies to *T. aggressivum* actually growing in mushroom compost. Future experiments should build on this relationship to examine the spore- or mycelium-carrying vectorial capacity of *L. ingenua* adults that might visit this compost and be exposed to *T. aggressivum* life stages. Also, the vectorial capacity of *L. ingenua* larvae should be examined once this mold has become established in mushroom-growing beds.

The results of the first three choice-test experiments suggest that flies entering a mushroom house might not be attracted to developing *A. bisporus* mycelia or fruiting bodies, but rather might be attracted to the compost itself.

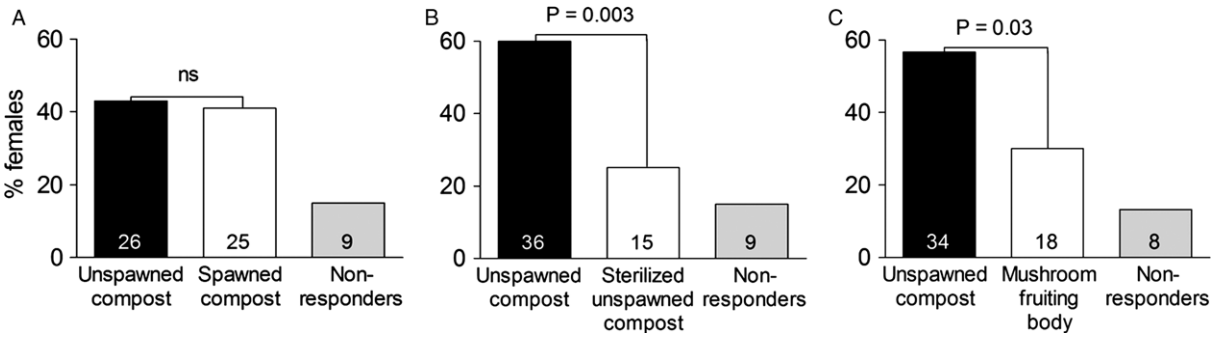

Figure 2 Percentage of female *Lycoriella ingenua* gravid females attracted to various mushroom compost–related substrates in two-choice, static-flow olfactometer assays: (A) spawned vs. unspawned compost, (B) unspawned vs. sterilized unspawned compost, and (C) unspawned compost vs. fruiting bodies of *Agaricus bisporus*. Numbers in the bars indicate the number of females choosing either chamber or staying in the release arena (not responding).

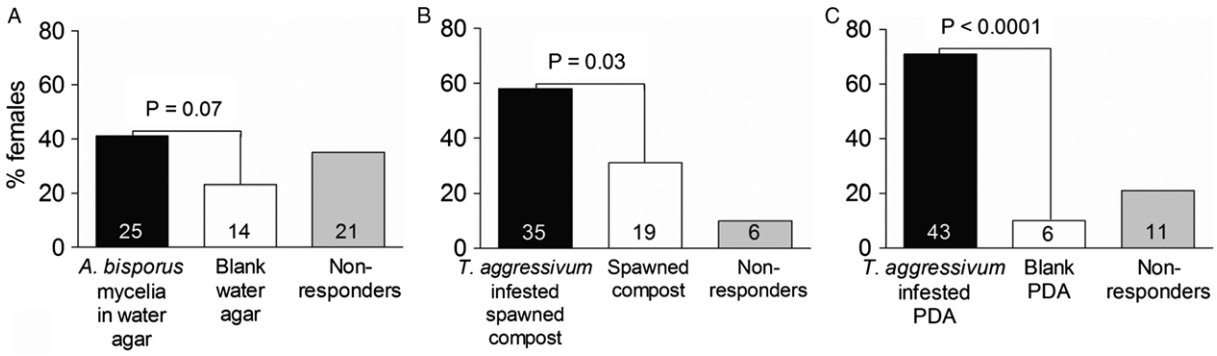

Figure 3 Percentage of female *Lycoriella ingenua* gravid females attracted to *Agaricus bisporus* or *Trichoderma aggressivum* mycelia in two-choice, static-flow olfactometer assays: (A) *A. bisporus* growing on water agar vs. water agar alone, (B) *T. aggressivum* mycelium growing in spawned compost vs. spawned compost alone, and (C) *T. aggressivum* mycelia growing in potato dextrose agar (PDA) vs. potato dextrose agar alone. Numbers in the bars indicate the number of females choosing either chamber or staying in the release arena (not responding).

Thus, in our study with *L. ingenua* (Figures 2A–C) and in two-choice assays with *Lycoriella castanescens* (Lengersdorf) (Tibbles et al., 2005), flies of both species were found to be similarly attracted to mushroom compost with and without *A. bisporus*. However, other studies have shown that compost that is densely colonized by *A. bisporus* mycelia is actually repellent to ovipositing *L. ingenua* females (Kielbasa & Snetsinger, 1981). A possible ecological explanation may be that a negative relationship exists between larval survival and the amount of *A. bisporus* mycelia growing in compost, as suggested by Tibbles et al. (2005).

The weak difference in attraction to *A. bisporus* mycelia growing on water agar and water agar alone may have been due to a relative paucity of volatiles from either chamber, as suggested by the unusually high number of non-responders in this experiment. Sciarid flies have been shown to achieve ca. 50% survival on just *A. bisporus* mycelia and fruiting bodies (O'Connor & Keil, 2005); thus, being attracted to these fungi is not totally detrimental to survival (Figure 3A). The goal of the last two-choice test experiments (Figures 3B, C) was related to the anecdotal evidence that *L. ingenua* is a vector of *T. aggressivum* and transports this noxious green pest mold into mushroom-growing houses, leading to infestations. If *T. agressivum* spores or mycelia themselves were attractive to *L. ingenua* females or males, then the flies might acquire green mold life stages and carry them into mushroom houses and infect the mushroom crop.

Previous studies showed that *L. castanescens* development is significantly reduced on compost heavily colonized by *A. bisporus* mycelia (Tibbles et al., 2005), suggesting that if fly populations are excluded from a mushroom-growing house in the beginning of the cropping cycle, the chances of a large infestation may decrease as mycelia develop. These results, plus the currently demonstrated attraction of females to mature phase II compost over sterilized compost, may guide mushroom growers in their efforts to exclude *L. ingenua* flies. Growers may use this information to control the incoming colonizing female flies in the early crop stages before they oviposit on the fresh phase II spawned compost that has just been added.

A study with another sciarid species, *Bradysia impatiens* (Johannsen), indicated that survival was significantly reduced when flies were forced to develop in sterilized compared to non-sterilized peat moss (Olson et al., 2002), suggesting that there is a fitness disadvantage to flies that lay their eggs in sterilized compost. Our future work will be aimed at isolating and identifying the odorant compounds of unspawned compost that are attractive to gravid *L. ingenua*. Our static-flow olfactometer design, with its ability to determine the attraction-related behavior of individual flies, may prove to be an effective tool for screening synthetic compounds for their potential attractiveness to *L. ingenua* as well as to other mushroom fly species, such as the phorid pest *M. halterata*.

Acknowledgments

This project was supported by USDA/NIFA/SCRI grant 2012-51181-19912 (project director David M Beyer). We thank David Beyer, John Pecchia, and Nina Jenkins for their contributions to this work, and Dr. Julie Todd for editing a penultimate version of this manuscript.

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