HOUSE FLY (MUSCA DOMESTICA L.) MANAGEMENT IN POULTRY PRODUCTION USING FUNGAL BIOPESTICIDES

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by
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ABSTRACT

House flies, *Musca domestica* L., are economically and medically important insect pests in poultry production facilities. Standard, chemical pesticide-based control options for flies are becoming increasingly difficult due to insecticide resistance and regulatory constraints. Biopesticides based on naturally occurring fungal pathogens could provide an alternative tool for integrated pest management (IPM) in poultry production. Here we addressed several issues related to field application, persistence and performance of fungal biopesticides against flies. We demonstrated that residual spray treatments of fungal biopesticides, *Beauveria bassiana* and *Metarhizium anisopliae* could sustainably suppress fly populations through lethal and sub-lethal impacts on reproductive output. The study developed a cost-effective field delivery system whereby teneral adult flies are targeted via the application of oil-formulated spores to the lower portions of basement walls where flies momentarily rest to harden themselves following emergence. We also evaluated inexpensive, non-toxic contractor-grade plastic sheets as potential wall covering materials to support long-term spore viability. The infectivity of oil-formulated *B. bassiana* against flies on typical structural substrates of the poultry houses including plastic sheets declined rapidly within one or two weeks following repeated fly exposures. In further laboratory bioassays, conidia viability and enumeration tests demonstrated that flies reduced fungal persistence and infectivity through deactivation and physical removal of conidia, with higher fly densities and greater cumulative exposure hastening the decline. Nonetheless, flies’ detrimental effects were substantially reduced at realistic (lower) densities and overall fungal efficacy was only marginally affected since very low densities of viable
conidia were still able to cause rapid mortality, suggesting the potential for relatively long re-treatment intervals to achieve fly control. Fungal spray treatments remained viable for up to 13 weeks under laboratory conditions and periodic exposure of flies to the spray residue showed high levels mortality, with very little decline in mortality rate over time. Equivalent treatments placed in a commercial poultry house showed much more rapid decline. One trial at the end of summer showed conidia to remain viable up to seven weeks. However, repeats during the winter months revealed spore decay in one to two weeks, with fly mortality rates influenced accordingly. The exact reasons for the more rapid decay remain unclear but could be linked to high concentrations of ammonia in the basement areas, especially during winter when ventilation is minimal. While the rapid spore decay poses a challenge for operational use, our laboratory and field experimental results suggest the potential for adaptive treatment regimes with weekly spray intervals in conditions with very high fly populations and/or high ammonia levels, and potentially monthly spray intervals when fly populations and ammonia levels are reduced. Nevertheless, careful monitoring and adjustments of current housing and manure management to improve indoor air quality and create thermally stable environments would be necessary to increase the persistence and long term efficacy of biopesticide treatments. Overall, the study contributes to wider adoption of mycophage products as well as promotes commercial development of other potential fungal species/isolates and contributes to the reduction of pesticide consumption in the poultry industry.
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Chapter 1: Introduction/Literature review

1.1. Biology, ecology and behavior of house flies

House flies, *Musca domestica* L., are economically and medically important, non-biting, filth-loving synanthropic Dipterans (Family: Muscidae) with peridomestic habits that have a long history of co-existence with humans and their domestic animals. More than 700 species of Muscids exist in North America, and over 4,000 species in 100 genera occur worldwide. House flies are medium-sized, about 5-8 mm long, mostly dull gray in color and have four black stripes on the thorax (Skidmore 1985, Chapman 1998, Moon 2002). Their legs are covered with sensory hairs (setae), and the distal tarsomere of each leg bears a pair of claws and a sticky, sack-like, cushiony pad in between them called the pulvillus, which produces a sticky substance that enable flies to walk on smooth vertical surfaces (Hedges 1990, Sukontason 2006). Adults taste their foods through the gustatory hairs on legs; therefore, they have a tendency to crawl over foods while resting. They need to liquefy and/or pre-digest foods with regurgitated-salivary gland secretions (Graczyk 2001) and have developed modified sponging-type mouth parts designed to suck up liquid foods. Larvae (maggots) have mouth-hooks used to filter-feed on masses of bacteria (Broce and Elzinga 1984) and can utilize all kinds of human and animal foods and waste. Usually, females are larger than males and can easily be identified having a much wider space in-between compound eyes and absence of a black spot at the abdominal end (West 1951). The sex ratio in flies is roughly equal (West 1951). The female abdomen consists of nine segments, of which only the first five segments are visible and the last four are normally retracted inside and protruded during oviposition (Chapman 1998). The male abdomen, on the
other hand, consists of eight segments only. Adults can live up to two months under laboratory conditions (Rockstein 1957), but last only a few weeks under natural conditions (West 1951).

Holometabolous house flies undergo four distinct life stages, i.e. egg, larva or maggot, pupa and adult. They are prolific breeders and can utilize almost all kinds of food and waste of animal and plant origin. Mating usually begins one to three days post-emergence from puparium; males are attracted by a female sex-pheromone called Z-9-tricosene (Rogoff et al. 1973). Volatile compounds emitted from decaying organic matter (Cosse and Baker 1996) and semio-chemicals emitted from egg-associated symbiotic bacteria (Lam et al. 2007) stimulate oviposition in females. The multiplying symbiotic bacteria on the surface of eggs provide ovipositional cues for other gravid flies as well as providing an enriched larval-growing substrate (Lam et al. 2007). Gravid females lay small, creamy white, elongated eggs on moist rotting organic matter and substrates with 60-75% moisture content are optimum for oviposition and maggot development (Miller et al. 1974). Each female can produce up to six to eight batches of 75-200 eggs during her lifetime (Fletcher et al. 1990). Eggs usually hatch within 24 hours, and maggots undergo three instars before turning into puparia. Third instars, mature maggots crawl out to drier areas particularly on the edge or top of breeding substrates and contract into puparia after voiding gut contents (Frankel and Bhaskaran 1973). After emergence from puparia, adults remain teneral for a few hours. In high-rise, layer barns, ready-to-pupate maggots move out of the manure pile and pupate at the edge near basement walls and after emergence; they walk to rest on walls or nearby vertical surfaces where they spend a considerable time to harden themselves before moving elsewhere.

The house fly development cycle, population density and daily activities including flight in a particular locality depend on resource, temperature and other biotic and abiotic factors. If
food is not limiting, flies will complete their life cycle in about ten days at 29.5\(^\circ\)C, 21 days at 21\(^\circ\)C and 45 days at 15.5\(^\circ\)C (Stafford 2008). The optimum temperature for fly development is around 26\(^\circ\)C with the lower and upper thermal limits of 12 and 45\(^\circ\)C, respectively (Davidson 1944, Keiding 1976). Eggs can hatch within nine hours after oviposition and take about seven to ten days to complete egg to adult stage under ideal conditions (Schoof 1964, Keiding 1976). However, cooler weather, dry media and scarce food may increase development time until two weeks or more. Flies produce multiple generations per year and the generations overlap; all stages are present at the same time (Keiding 1976, Stafford 2008). Even if the development depends on temperature, multiple generations per year are possible in tropical and temperate regions due to their peridomestic habits (Merchant et al. 1987). Flies are diurnal and mostly active during the day at temperatures of 26-32\(^\circ\)C and become inactive during the night and at temperatures below 12.5\(^\circ\)C (Stafford 2008). Fly numbers in a particular locality vary with availability of foods and breeding resources, sunshine hours, temperature and humidity regime. For instance, high fly activities are seen at around 20-25\(^\circ\)C, and they are undetectable below 10\(^\circ\)C or above 45\(^\circ\)C (Stafford 2008). They can fly a considerable distance in a random pattern and flight capability is usually temperature-dependent (Sacca 1963). Different studies have reported different distances that flies can travel, ranging from 3.22 km up to 32.19 km (Schoof and Silverly 1954, Sacca 1963). The flights are mostly aimed at searching for food and oviposition sites. Flies travel relatively longer in rural areas than urban areas due to widely scattered human settlements (Hindle and Merriman 1914, Murvosh and Thaggard 1966). Thus, it is common in the countryside that flies escape from animal rearing facilities and invade nearby residential areas (Winpsinger et al. 2005).
1.2. House flies as pests and disease-vectors

In semi-protected indoor settings of animal production units, the generation of manure and waste nourishes and multiplies filth flies by providing favorable dwelling and breeding sites, and the fly population frequently reaches pest and vector status imposing a significant economic cost in affected rearing facilities (Axtell 1986b, 1999, Axtell and Arends 1990). In dairy facilities, for instance, excessive numbers of flies cause annoyance and reduce feed consumption and milk production (Morgan and Bailie 1980, Ahmad et al. 2007, Scott et al. 2009). In high-rise layer production barns, constant irritation by flies stress birds and can reduce feed consumption, egg production and compromise weight gain (Axtell 1986b, 1999, Miller et al. 1993a). At high populations, flies in hen house could migrate from the manure-containing basement to the second story, where birds are housed and contaminate eggs with body excretions, which reduces the eggs’ attractiveness to customers and compromises market value (Axtell 1999, Malik et al. 2007). Flies can cause financial loss through vectoring different diseases and increasing the costs of disease management. They annoy farm workers, which leads to poor animal husbandry and increases costs of production (Dhillon et al. 2004). Similarly, excessive regurgitation spots can cause corrosion and degradation of metal equipment, structural materials and reduced illumination from light sources (Axtell and Arends 1990, Axtell 1999).

Flies are capable of travelling several kilometers from development sites (Winpisinger et al. 2005). During heavy outbreaks, they can escape from animal rearing facilities and invade nearby residential areas leading to poor community relations and damage the community standing of even well established farms, sometimes leading to the boycott of farm products and lawsuits and farm closure in extreme cases (Axtell 1999, Renn et al. 1999, Chakrabarti et al. 2010). The invading flies may become a threat to public health and increase the risk of disease.
epidemics in the communities (Hanec 1956, Pickens et al. 1967, Lysyk and Axtell 1985). Flies are also responsible for spreading *Salmonellosis* in and around layer farms (Olsen and Hammack 2000), and the *Salmonella*-contaminated eggs increase the risks of food-borne illnesses to consumers (FDA 2009). Although it is very difficult to estimate direct production losses caused by flies, except for their role as disease-vectors, they are responsible for damage and control costs to the US poultry industry in excess of a billion dollars annually (Geden et al. 2001).

House flies are important mechanical and propagative vectors for more than 65 human and animal diseases (Malik et al. 2007). They can transmit disease-causing organisms through mouthparts, body and leg hairs, pulvilli, and bodily excretions. Flies can pick up pathogenic microbes during walking or feeding on waste, garbage and rotting materials and spread them by harboring on their external body surfaces including minute hairs and tarsal pulvilli (Barro et al. 2006, Sukontason et al. 2006, Getachew et al. 2007), and salivary glands or the digestive tract (Sasaki et al. 2000). High electrostatic charges of body hairs and high viscosity of feces enhance adhering capacity of pathogens to body surfaces (Graczyk et al. 2001). Further, flies liquefy solid foods before ingestion and may leave regurgitation spots on the surfaces which they feed contaminating the substrates (Graczyk et al. 2001). Ingested viruses or bacteria can retain their virulence in the gut and contaminate the substrates when excreted in feces (Greenberg 1973, Getachew et al. 2007). Bacteria such as *E. coli* O157: H7 can persist at least for four days in fly crop after ingestion and propagate inside the pseudotrachae (Sasaki et al. 2000). In addition, prestomal teeth inside mouthparts enhance their capacity to transmit bacteria (Broce and Elzinga 1984). Flies defecate excessively, usually two to ten times per day during their active period (Sasaki et al. 2000), and their feces contain a myriad of pathogenic microbes such as

Flies pose a serious health hazard to people and animals by spreading various diseases in and around animal rearing facilities and nearby residential areas. In dairy barns, flies feed on milk leaking from the udders of diseased animals and spread diseases such as Bovine mastitis to healthy ones (Sanders 1940, Hillerton and Bramley 1985). They could transmit pig parasites (Forster et al. 2009); human intestinal parasites (Oyerinde 1976, Graczyk et al. 1999, Forster et al. 2007), *Helicobacter pylori* (Gruebel et al. 1997); mycobacteria (Fischer et al. 2001); *Escherichia coli* (Iwasa et al. 1999, Moriya et al. 1999, Sasaki et al. 2000) and *Corynebacterium pseudotuberculosis* (Braverman et al. 1999, Zurek et al. 2001). Flies are also an important mechanical or biological vector for Shigellosis (Cohen et al. 1991, Levine and Levine 1991); Salmonellosis (Olsen and Hammack 2000, Forster et al. 2007, Fetene and Worku 2009); Rotaviruses (Tan et al. 1997); rikettsia, fungi and worms (Oyerinde 1976, Dipeolu 1982, Umeche and Mandah 1989) in and around animal confinements. In poultry, flies are reported to transmit Necrotic enteritis (Dhillon et al. 2004), New castle disease virus and Turkey corona virus (Calibeo-Hayes et al. 2003), Campylobacteriosis (Hald et al. 2004), Reticuloendotheoliosis (Davidson and Braverman 2005), H5N1 Avian influenza virus (Sawabe et al. 2006, Wanarata et al. 2011), chicken tapeworms (Abrams 1976, Mullen and Durden 2002), Coccidiosis (Greenberg 1973, Milushev 1978), and several other contagious diseases (Malik et al. 2007).

Flies harbor and spread antibiotic-resistant bacteria both on livestock farms and in hospital environments (Macovei and Zurek 2006, Graham et al. 2007). More than 80% of poultry production units in the United States use antibiotics in feed to protect birds against bacterial diseases (Silbergeld et al. 2008), and the indiscriminate use of antibiotics can create selection
pressure on bacteria resulting in emergence of resistant strains. Flies invading nearby residential areas from poultry farms are responsible for spreading diseases in the community as flies acquire the resistant bacteria such as *Staphylococci, Enterococci* from antibiotic-fed poultry feces (Graham et al. 2007). They are also involved in transmission of multiple antibiotic-resistant bacteria in hospital environments (Fotedar et al. 1982, 1992, Rady et al. 1992). Flies near residential and urban fast-food restaurants commonly carried genetically diverse populations of *Enterococci* with antibiotic resistance and raised a serious concern about public health (Graczyk et al. 2001).

### 1.3. Poultry production system

Modern commercial poultry production systems are highly integrated with intensive production techniques that change the environments in which birds are reared, and the ecology of flies is closely tied with this artificial environment (Axtell 1999). Different production systems have differential requirements for temperature, housing type, flock management and production practices. Hence, the complexity of fly problem differs between systems (Parkhurst and Mountney 1988, Axtell and Arends 1990). For example, fly problems are severe in high-rise caged-layer production systems but not in manure belt caged-layer and littered-floor, non-caged systems (Axtell 1986b). In littered-floor, non-caged systems, manure is less concentrated due to use of absorbent materials and is frequently disturbed by birds, which reduces manure suitability for oviposition and larval development. Several species of filth flies are common in poultry production facilities; however, the most problems caused are associated with common house fly, *Musca domestica* L. and partly with little house fly, *Fannia canicularis*. Although there are many variations in structure, design and type of housing, below are the most common poultry production systems in US.
1.3.1. Cage system of layer production

This is a popular egg production system where egg-laying hens are housed in wire mesh cages in one or two-storied houses and fresh manure either accumulates at ground floor or falls onto a conveyer belt beneath the cages. About 70% of current caged-layer houses are high-rise and only 30% are manure-belt, although most new houses are manure-belt system (Xin et al. 2011). Water and feed are supplied through automatic equipment. Egg collection is mostly through automatic conveyer belts; however, manual egg collection is also practiced (Axtell and Arends 1990). The ventilation system inside the house is designed in such a way that fresh air enters into the house from the roof, passes down through the bird cages and hot air passing over the manure surface vents through exhaust fans (Koenig et al. 2005, Xin et al. 2011). Although some variations exist in housing structure, design, and management, high-rise and manure-belt are two common caged-layer systems for table egg production (Axtell 1999).

1.3.1.1. Manure-belt cage system

In this system, rows of birds’ cages are stacked on either sides of multiple aisles and manure accumulates on a conveyer belt beneath each cage row, which is emptied at the end of the house daily (Axtell 1999). The manure on the belt is dried either naturally by ventilation air or artificially by a forced-air stream. Depending on the method of manure drying and season, moisture content of manure on belts varies from 30-60% (Xin et al. 2011). Even though the manure-belt system is generally 50% higher in capital costs than a high-rise cage system, it provides considerable long-term benefits over the latter (Xin et al. 2011). Manure removal is less labor-intensive, and because of the semi-automatic and frequent manure removal, fly problems are low (Liang et al. 2005), and indoor air quality is much better than the high-rise cage system (Green et al. 2009).
1.3.1.2. High-rise cage system

This system is similar to the manure-belt cage system except manure storage is in the basement and the birds are upstairs in a two story barn. Manure falls from cages and accumulates either directly to the basement or onto dropping boards, which are later scraped down to the basement multiple times a day (North and Bell 1990, Axtell 1999). Manure drying in either case takes place when mechanical ventilation for the birds passes air over the manure, which is exhausted from the barn. However, drying fans can also be used in the basement to supplement the drying process (Xin et al. 2011). Manure removal is labor-intensive and less frequent generally coordinated with its land application as a fertilizer for crop production. In-house manure storage and handling operations create chemically and biologically complex indoor environments through manure decomposition and emission of air-borne pollutants (Axtell and Arends 1990). Sometimes instead of being confined to cages, birds are allowed to move freely over wooden or plastic slats on the second floor, and manure accumulated on the first floor is treated like the standard cage systems.

1.3.2. Littered-floor non-cage system for meat production and breeding

In this system, broiler breeders or grow-out birds (broilers and turkeys for meat production) are commonly reared on a littered-floor or partially littered-floor house, and water and feed are provided through automatic equipment (Axtell and Arends 1990). For breeders, one-third of the floor is covered with absorbent litter materials and two-thirds of the house is usually slatted (Axtell 1999). The slats are generally elevated 0.5-1.75 m above the floor, and egg collection is either manual or mechanical with an automatic belt system. Usually manure collects on the littered-floor or below the slatted areas and is typically removed at the end of the flock cycle (Parkhurst and Mountney 1988). Litter is mostly dry and friable because movement of the birds
and constant scratching in the litter makes it unattractive for fly oviposition and unsuitable for maggot development (Axtell 1986b). In breeder houses, any fly breeding generally occurs in the manure slats because manure is less disturbed and moisture is greater. Because of low stocking density, ventilation is usually lower and consequently ammonia accumulation increases. In addition, indoor air quality can have suspended particulate matter mainly due to constantly scratching in the litter.

1.4. House fly management in poultry production facilities

1.4.1. Cultural management

The primary cultural tools that help prevent the introduction and build-up of fly populations in barns are biosecurity, the housing system, waste management and monitoring procedures (Axtell 1999). Biosecurity practices, for instance, monitoring feed and other inputs for contamination; cleaning and disinfecting construction materials and equipment can prevent the introduction of flies and other pests into the barns (Axtell 1986b, Axtell 1999). Application of pesticides and disinfection of the barns and equipment in-between flock cycles will reduce hibernating stages of flies and other organisms. Regular monitoring and proper maintenance of the egg collection and feeding equipment helps to minimize fly population build-up as spilled feed and cracked eggs provide proteinaceous foods for flies. Leaky water systems and wet manure enhance these substrates as suitable breeding materials. Improved air ventilation through adjustment of fans and vents, and improvement of outdoor drainage systems are necessary to prevent manure moisture contamination and maintain a thermoneutral indoor environment (Axtell and Arends 1990). Manure moisture management is the key cultural practice that helps to prevent population build up because dry manure is unattractive for fly oviposition and unsuitable for larval development (Geden et al. 2001, Mullens et al. 2001). In high-rise systems, cleaning of basement manure
aisles and turning pupae back onto manure piles can reduce adult emergence. Cultural management is mostly preventive with the aim to reduce manure suitability as well as break development cycles in already established populations to form a good basis for other management strategies.

1.4.2. Fly monitoring

Monitoring of the fly population is an indispensable part of poultry integrated pest management (IPM). Several monitoring tools have been developed for adult and larval populations to enable farm managers to monitor for impending emergence of adult flies and provide a basis for timing and frequency of spray applications. Spot cards, sticky ribbons and baited jug traps are most widely used tools for monitoring of adult populations (Beck and Turner 1985, Turner and Ruszler 1989, Turner et al. 1992, Jacobs et al. 1993, Hogsette et al. 1993, Pickens et al. 1994, Kaufman et al. 2001b). Adults can be monitored by spot cards; small 7.5 x 12.5 cm index cards fastened in multiple locations within barns where a large number of flies are present (Rutz and Axtell 1979, Lysyk and Axtell 1986, Kaufman et al. 2001b). The number of fliespecks (vomit and excreta) on each card gives an indirect estimate of fly populations, and cards should be replaced weekly. Average fliespecks of 50-100 per card indicate a high fly activity and a need for control interventions (Stafford et al. 1988, Williams 2010). One advantage of using spot cards is they provide a long-term historical record of fly activity (Lysyk and Axtell 1985). This is an easy, economic and reliable method of adult monitoring; however, it provides no information on fly species or sex, and indoor temperature and strategic placement of cards are critical (Lysyk and Axtell 1986).

Sticky ribbons are tapes with sticky surfaces placed at different locations in barns and should be replaced weekly (Anderson and Poorbaugh 1964). The tapes can either be stationary or
an individual can walk them through the barn for monitoring purposes. The stationary tapes are 3-4 cm wide ribbons hung from beams, pillars and other structures, whereas moving sticky paper ribbons are 45 cm tapes fully unrolled, suspended about 5-7 cm off the floor and carried throughout the barn (Burg and Axtell 1984, Stafford et al. 1988, Kaufman et al. 2000b, Stafford 2008, Williams 2010). An average weekly count above 100 flies per stationary tape, or after walking 300 m in the barn in case of moving tapes is considered a high fly activity. One disadvantage of stationary sticky tapes is dust or heavy fly activity quickly overcome them (Stafford 2008). Additionally, the observer should use the same walking pattern at the same time of the day for more accuracy. Nonetheless, the tapes provide information on both fly species and sex as individual flies can be counted every week (Hogsette et al. 1993).

The use of baited jug traps is another popular passive method of adult monitoring, wherein a small one-gallon, plastic milk-jug with four holes on each upper side and insecticide-infused pheromone bait inside is hung in several locations inside barns and periodically assessed for the numbers of captured flies (Burg and Axtell 1984, Axtell 1999). An average count of 250 flies per jug trap per week indicates that control measures should be initiated (Watson et al. 1994). Baited jug traps give information on fly species and sex; however, fly counting is laborious and replacing the muscalure pheromone is expensive compared to other tools.

Scudder grid is another method of adult monitoring. It is a standard 60-cm square grid consists of 16-24 wooden slats, which is fastened at equal intervals to cover an area of approximately 0.8 square meters (FDA 2009). After a period of 30-60 seconds, the flies resting on the grid are quickly counted and recorded. The count is repeated 10 to 15 times in areas with higher fly numbers. Sampling should typically be carried out two to three times per week and counts should be carried out at times when flies are active, typically between 10.00 and 16.00 hrs.
(Scudder 1998). A count of less than 20 flies on a Scudder grill is likely to indicate satisfactory fly control (FDA 2009).

For livestock facilities, the Danish Pest Infestation Laboratory developed a visual fly index system to monitor adults on livestock. This fly infestation index has a 0-7 scale (0= 0-3 flies/animal and 7= 200-400 flies/animal) and the scale 7 indicates an economic threshold to initiate control measures (Kristiansen and Skovmand 1985).

In addition to adults, regular monitoring of larval populations is also very important to predict impending fly burst. Routine visual inspection of manure piles for potential hot spots of larval development by walking the length of manure aisles is required. Manure surfaces with a ‘coffee ground’ appearance indicate a high larval activity (Axtell and Arends 1990, Axtell 1999). Maggots can also be monitored by pupal traps or extracting immature from manure using Berlese funnels or floating them in 0.6 M sucrose solution (Stafford and Bay 1994, Tobin and Pitts 1999).

In addition to their monitoring function, traps such as mobile and stationary sticky tapes, light traps with electrocuting grids are frequently used to lure and kill adult flies as a mechanical control method (Rutz and Scoles 1988, Pickens et al. 1994). However, these practices are not very effective in reducing populations (Kaufman et al. 2000b), but are useful in trapping small populations in the egg processing areas or break rooms.

1.4.3. Chemical control

In line with crop production systems, the poultry industry relies heavily on broad-spectrum chemical pesticides for fly management, and chemical control is the only reliable tool to manage overwhelming populations (Tomberlin and Drees 2004). Pyrethroids, organophosphates and
carbamates as space or residual sprays and bait formulations are the most commonly used adulticides as well as for manure treatment and feed-through larvicides (Axtell 1986b, Axtell 1999, Malik et al. 2007). Space sprays of organophosphates, pyrethrins and pyrethroids are used to quickly knockdown adults. Misting fly resting surfaces with these chemicals is the most common way to suppress overwhelming populations with short residual actions (Axtell 1999, Kaufman et al. 2000b, Williams 2010). Pesticides in these categories can also be used as residual sprays for long-term population suppressions; however, risk of resistance development is high with such practices (Scott et al. 2000) and treated areas quickly get covered with dust particles making them less effective (Stafford 2008).

Organophosphates and carbamates are also used in bait formulations to supplement residual or space sprays (Axtell 1999). The bait formulations are very useful in trapping and killing adult flies at bird level in high-rise layer barns, but the bait stations should be far enough from birds’ cages to avoid food and water contamination. Methomyl, dichlorovos, imidacloprid and spinosad pesticides are commonly used in baiting materials (Geden et al. 2001). Organophosphates are also used as larvicides for hot-spot treatment in manure. Insect growth regulators such as cyromazine (larvadex) are used as feed-through larvicides, which after excretion makes the manure toxic and kills maggots or disrupts their development (Kaufman et al. 2000b, Williams 2010). The feed-additive larvicides are safe for birds and are effective in suppressing larval populations with no or minimal non-target impact on manure-inhabiting natural enemies.

House flies have developed behavioral as well as physiological resistance to almost all chemical insecticides commonly used against them (Kaufman et al. 2001c, Geden 2012), and
insecticide resistance is now a global problem (Scott et al. 2000). Resistance to spinosad (Shono and Scott 2003, Deacutis et al. 2006), imidacloprid and other neonicotinoid pesticides (Wen and Scott 1997, Kaufman et al. 2006, Kaufman et al. 2010a, b) has occurred within a few years of their release. A similar pattern of rapid resistance is evidenced in pyrethroid, organophosphate and carbamate insecticides (Boxler and Campbell 1983, Plapp 1984, Scott and Georghiou 1985, 1986, Price and Chapman 1987, Scott 1989, Butler et al. 2007, Kozaki et al. 2009, Memmi 2010). Flies have also developed resistance to larvicides such as diflubenzuron and cyromazine (Bloomcamp et al. 1987, Shen and Plapp 1990), which was most likely due to continued delivery system like feed-through larvicides (Geden 2012). Cross-resistance in flies is also common, for instance, flies resistance to phyrethrin also have a high level of resistance to abamectin (Scott 1989, Liu and Yue 2000). These increasing trends of fly resistance suggest rethinking the ways pesticides are being used in the industry as more frequent and haphazard applications of even less-toxic chemicals can quickly result in resistance. Appropriate fly monitoring tools should be used to evaluate fly pressure and make a decision on when, which type and how much insecticide is required. Chemical pesticides should be considered supplements to other management tools and used as a last resort. Indiscriminate use of broad-spectrum pesticides along with poor sanitation and manure management could promote fly resistance, which necessitates frequent applications of toxic and wide spectrum pesticides that could make the situations worse.

**1.4.4. Biological control**

Augmentation and conservation are two major biocontrol approaches used in poultry houses to manage fly populations. The conservation biocontrol includes practices such as provisioning for temporary manure-refuge of natural fly enemies, selective use of less toxic pesticides and
Manure moisture management at low levels, all aim to increase the efficiency of natural enemies (Kaufman et al. 2000a). Manure refuges are necessary during manure removal between flock cycles to preserve habitats and breeding sites for natural enemies (Mullens et al. 1996). Routine monitoring of manure for larval population and hot spot treatment with larvicides help minimize non-target impacts on natural enemies. The effectiveness of conservation biocontrol techniques depends predominantly on manure, flock and housing management practices; however, these alone are not enough to suppress fly populations. Therefore, the existing populations are periodically supported by augmentative release of mass-reared biocontrol agents to suppress impending emergence of adult flies. Predatory beetles and parasitoid wasps are commonly used with this approach (Crespo et al. 1998, Malik et al. 2007).

depends on manure management with better performance in dry manure (Rutz and Axtell 1981, Greene et al. 1989, Geden et al. 1992a). Depending on the production system, housing type and management practices, weekly release of 50-80 pupae per m² may satisfactorily suppress fly emergence (Skovgard and Nachman 2004).

Similarly, predatory beetles, *Carcinops* spp. and mites, *Macrocheles* spp. voraciously prey on eggs and early instars maggots and can reduce adult fly emergence (Geden 1990, Kaufman et al. 2000a, 2001a, Hinton and Moon 2003). These beetles are available commercially (http://www.rinconvitova.com/fly_control.htm) and are suggested to use in combination with parasitoid wasps to maximize the biocontrol outcome. Predators are manure-inhabitants and like parasitoids, can perform well at low moisture levels. Further, Dipteran larvae in the Genus *Hydrotaea* are facultative predators of house fly maggots available commercially in US (http://www.rinconvitova.com/fly_control.htm) and have been used for fly management in other parts of the world (Nolan and Kissam 1987, Turner and Carter 1990, Turner et al. 1992, Hogsette et al. 2002).

In addition to predators and parasitoids, several species of entomopathogenic nematodes from the families Heterorhabditidae and Steinernematidae have been extensively studied for their potential as biocontrol agents against flies, and one species, *Steinernema feltiae* from the family Steinernematidae is available commercially in US (http://www.rinconvitova.com/fly_control.htm). However, the majority of laboratory and semi-field evaluations have ended-up with inconsistent results (Renn et al. 1985, Geden et al. 1986, Belton et al. 1987, Mullens et al. 1987a, Renn 1995, Tylor et al. 1998). Chemically and biologically harsh environments presented by manure and poor persistence of infective juveniles in indoor environments make their use impractical (Renn 1995, 1998, Tylor et al. 1998, Renn and
Wright 2000). Further, a parasitic nematode, *Paraiotonchium muscacomesticae* can make female flies sterile by invading the ovaries and spreading into the populations through mock oviposition events (Coler and Nguyen 1994, Geden 1997), which with additional research on production, formulation, storage and field delivery systems, has potential in fly management (Geden 2012).

Insect disease-causing bacteria are promising biocontrol agents in controlling fly larvae and several studies have attempted to screen virulent isolates and to develop appropriate formulations and field application strategies (Burns et al. 1961, Miller et al. 1971, Rupes et al. 1987, Johnson et al. 1998, Zhong et al. 2000). The majority of these works use exotoxin-producing *Bacillus thuringiensis* (Bt); however, rapidly developed resistance and safety concerns of exotoxins to vertebrates have constrained their use (Harvey and Howell 1965, Wilson and Burns 1968, McClintock et al. 1995, Tsai et al. 2003). The emphasis has shifted to the use of endotoxin-producing Bt species, which have shown promising results both in laboratory and field evaluations (Indrasith et al. 1992, Johnson et al. 1998, Choi et al. 2000, Zhong et al. 2000, Labib and Rady 2001, Oh et al. 2004, Mwamburi et al. 2009, 2011).

Recently, Salivary Gland Hypertrophy Virus (MdSGHV), a double-stranded DNA virus that causes enlargement and blue-whitish discoloration of salivary glands in adults as early symptoms, has gained attention in fly management (Lietze et al. 2009, 2011, Geden 2012). The viral replication and morphogenesis occur in salivary glands, and after invading ovaries at early stages of fly development, the virus shuts down the female reproductive system and makes them sterile (Lietze et al. 2007, 2010). Infected flies are usually short-lived and show low mating success (Lietze et al. 2007). Very little is known about viral ecology and disease epidemiology and further studies to develop formulation and application strategies are needed to exploit its potential in fly management (Geden 2012).
Similarly, plant materials and plant-derived essential oils have been used since ancient times to repel or kill flies and have recently drawn a renewed interest for commercialization and use in poultry IPM (Malik et al. 2007, Geden 2012). A few plant-derived essential oil-products are also available commercially; however, effective formulations and field application techniques are still lacking (Geden 2012).

1.4.4.1. Entomopathogenic fungi as biocontrol agents

Insect disease-causing fungi are obligate or saprophytic parasites that cause fatal diseases in insects. More than 750 species are described from 100 genera and are associated with different insects living in diverse habitats (Shah and Pell 2003). The entomopathogenic fungi fall into four groups: the Oomycetes, the Zygomycetes, the Chytridiomycetes and the Deuteromycetes (Shah and Goettel 1999). The fungi in the first three groups are more specialized with narrow host ranges and have complex nutritional requirements for culture and mass production. The Deuteromycetes, however, are generalists with a wider host range and can be cultured and mass-produced in starch-rich media. The majority of the fungi currently used in biological control belong to either Entomophthorales in the Zygomycetes or the class Hyphomycetes in the Deuteromycetes (Shah and Pell 2003).

The group Entomophthorales contains more than 220 species, of which about 70 infect and kill arthropods such as human disease vectors, spider mites, thrips, aphids, plant and leafhoppers, locusts, grasshoppers, several lepidopterans and dipterans. *Entomophthora muscae, E. schizophora, E. maimaiga, E. aciculae, and E. grylli* are the most important ones that attack muscid flies, gypsy moth, crickets and other important insect pests (Watson et al. 1993, Hajek and St. Leger 1994, Roy et al. 2006). They are very efficient natural regulators and can reduce host populations dramatically within a short period causing epizootics under ideal cool, humid
environments (Kramer and Steinkraus 1987, Mullens et al. 1987b, Geden et al. 1993, Steinkraus et al. 1993, Watson and Petersen 1993a). In and around animal rearing facilities, more than 60-77% epizootics have been recorded in natural fly populations (Mullens et al. 1987b, Steinkraus et al. 1993, Watson and Petersen 1993a, Six and Mullens 1996). *E. muscae* and *E. schizophora* have been extensively studied for their potential as biocontrol agents against flies (Mullens 1985, Mullens and Rodriguez 1985, Mullens et al. 1987b, Geden et al. 1993, Watson and Petersen 1993a, b, Krasnoff et al. 1995); however, their commercial development has been constrained by lack of effective culture media and mass-production techniques (Kramer and Steinkraus 1981).

*Beauveria bassiana* and *Metarhizium anisopliae* from the class Hyphomycetes are the two most widely used entomopathogenic fungi to manage agricultural and forest insect pests (Roberts 1989, Shah and Goettel 1999, Butt et al. 2001) and offer good potential as pesticide alternatives to control house flies in the indoor environments of animal production facilities. Although field populations of house flies usually have low rates of natural infections with these fungi (Steinkraus et al. 1990, Skovgaard and Steenberg 2002), they show promising results in laboratory and semi-field or field evaluations using various formulations (Steinkraus et al. 1990, Kuramoto and Shimazu 1992, Barson et al. 1994, Geden et al. 1995, Watson et al. 1995, Renn et al. 1999, Darwish and Zayed 2002, Kaufman et al. 2005, Lecuona et al. 2005, Malik et al. 2007, Geden 2012). Vegetable and mineral oils can be combined with spores to protect spores from rapid drying and aids in germination at low humidity (Bateman et al. 1993). These fungi are highly compatible with chemical pesticides such as spinosad, larvadex and biocontrol agents such as predators, parasitoids and other entomopathogens (Geden et al. 1995, Quintela and McCoy 1998, Kaufman et al. 2005, Nielsen et al. 2005, Mwamburi et al. 2009, Farenhorst et al. 2010, Sharififard et al. 2011a, b).
1.4.4.1. Mode of action

Entomopathogenic fungi have a contact mode of action (Charnley 1989) and are suitable to apply as residual sprays on fly resting surfaces. Fungi usually take five to seven days to kill host insects; however, infected insects can live for much longer, depending on host type, dose and environmental conditions (Thomas and Read 2007). The fungi reproduce by spores, also called conidia that infect hosts by penetrating the cuticle. The outer layers of fungal conidia are composed of interwoven fascicles of hydrophobic rodlets. It is the non-specific hydrophobic forces imposed by the rodlets that facilitate adhesion to insect cuticle (Boucias et al. 1988, Boucias and Pendland 1991). Additional proteins such as lectins and adhesins aid the binding of spores to insect cuticle. Once conidia have attached to the cuticle, molecular and nutritional cues as well as compatible microenvironments containing high humidity and/or moisture in the immediate airspace above the cuticle cause them to germinate.

Conidia germination is greatly influenced by availability of water, nutrients, oxygen, the correct pH and temperature and the presence of microbes and anti-fungal substances at the cuticular surface (Sandhu 1995, Bouamama et al. 2010). Germinating conidia form appresoria, which exert physical pressure on the cuticle, and penetration is usually through thinner areas of cuticle such as intersegmental folds and body openings (Hajek and St. Leger 1994). Cuticle invasion involves both enzymatic degradation and mechanical pressure (Clarkson and Charnley 1996). A range of extracellular enzymes that can degrade major components of cuticle including chitinases, lipases, esterases and proteases are thought to be associated with the penetration process with the outcome of such enzymatic degradation providing nutrients for fungal growth (Hajek and St. Leger 1994, Shah and Pell 2003). Once inside the hemocoel, fungal cells multiply and produce blastospores by budding and spread through hemolymph. The developing fungus
sequesters nutrients from the hemolymph and internal tissues and blocks the free flow of hemolymph within the hemocoel. Host death is due to combined actions of nutrient sequestration, tissue degradation and production of toxic metabolites (Samuels et al. 1988, Vey and Quiot 1989, Clarkson and Charnley 1996). Under favorable environmental conditions, the fungus will grow out of the cadaver and produce aerial conidia over the host surface (Feng et al. 1994), which eventually disseminate into the surrounding environments under influence of wind, water and other factors.

Starting from conidial attachment to the cuticle, the fungus is subjected to insect immune responses. The cuticle itself is a heavily sclerotized structure consisting of three different layers and presents the primary physical barrier for penetration. Cuticle surfaces have several microbes and chemicals and other antifungal substances, which interfere with conidia attachment and the germination processes (Hajek and St. Leger 1994, Shah and Pell 2003). Even if the conidia are able to germinate and penetrate the host cuticle under favorable environments, fungal cells have to face several cellular and humoral immune responses. Once in the hemolymph, fungal cells secret toxic metabolites that inhibit both cellular as well as humoral immune responses. Some of immunosuppressive responses include inhibition of hemocyte pseudopod formation, retention of phenoxidase expression (Huxham et al. 1989, Hung and Boucias 1992, Hung et al. 1993, Pendland et al. 1993, Vilcinskas et al. 1997) and disruption of eicosanoid biosynthesis, a primary defense compound in insects (Miller et al. 1994, Howard et al. 1998).

1.4.4.1.2. Use of fungi in fly management

*Entomophthora muscae*, *E. schizophorae*, *Metarhizium anisopliae* and *Beauveria bassiana* are the most widely studied entomopathogenic fungi as fly biocontrol agents. Early studies of fungi against flies before and during 1990s mostly focused on *E. muscae* and *E. schizophorae* to
understand their ecology and epidemiology, evaluate their biocontrol potential and to develop appropriate culture media and mass production techniques. *Entomophthora muscae* and *E. schizophorae* usually take four to six days to cause mortality, while speed of kill depends on host age, dose and environmental conditions (Mullens 1985). Flies infected with these fungi usually die in exposed places such as top of plants, fixed on walls or plant parts, which facilitates spore dispersal. Typical infection symptoms in flies include a distended abdomen, legs spread and wings out-stretched with the body attached to substrates by mouthparts (Maitland 1994). After host death, fungi produce sexual spores called zygospores on cadaver surfaces, which are forcibly discharged under ideal environmental conditions (Kalsbeek et al 2001a). Conidia discharged from cadavers infect other flies in natural populations, and the duration and intensity of conidia discharge and conidia survival and germination are temperature and humidity-dependent (Mullens and Rodriguez 1985, Krasnoff et al. 1995, Six and Mullens 1996, Madeira 1998, Kalsbeek et al. 2001a).

In natural populations, *E. muscae* and *E. schizophorae* epizootics usually occur during fall in temperate regions where infection rates commonly exceed 50% (Mullens et al. 1987b, Steinkraus et al. 1993, Watson and Petersen 1993a, Six and Mullens 1996). As these fungi have complex nutritional requirements for culture and mass production, previous studies have used conidia shower, cadaver bait or mass-release of infected flies or sporulated cadavers to contaminate populations for evaluating their control potential (Kramer and Steinkraus 1987, Geden et al. 1993, Steinkraus et al. 1993, Watson and Peterson 1993b, Six and Mullens 1996). Although the fungi have very good insecticidal properties, their commercial development is constrained by short-lived conidia, intolerance to high temperature, complex nutritional requirements, difficulty in mass production and poor conidia storage and stability (Kramer and
Steinkraus 1981, Geden 2012). Moreover, high fly populations are needed to sustain epizootics in natural conditions (Geden et al. 1993) and flies have developed the ability to reduce virulence and efficacy through behavioral fevering (thermoregulatory behavior) (Watson et al. 1993, Kalsbeek et al. 2001b). Therefore, interests have shifted to *B. bassiana* and *M. anisopliae*, which are easier to mass-produce with good conidia stability and shelf life, and have good commercial potential (Blanford et al. 2012).

Relatively more focus has been given to *B. bassiana* as a fly biocontrol agent, while studies evaluating control potential of *M. anisopliae* against flies are limited. Exposure to linseed oil-formulated *M. anisopliae* has successfully prevented adult emergence and produced almost 100% mortality in adults within three to six days depending on formulation and dose (Barson et al. 1994). Spores formulated in sugar-based baits caused 100% mortality within 10 days, and housing of uninfected flies with fungal-infected cadavers produced more than 90% mortality under semi-field setting in a plastic enclosure suggesting that horizontal spore transmission can contribute to control (Renn et al. 1999). Temperature and humidity are crucial for optimal infections, and temperature-dependent dose response was observed in *M. anisopliae* infection (Carswell et al. 1998). At 25 and 30°C, fewer than 25 conidia were sufficient to kill a fly within nine days, whereas more than 5000 conidia needed for the same impact at 20°C. Likewise, Rizzo (1977) demonstrated that *M. anisopliae* isolates are slower to kill flies than *B. bassiana* and fly age has no significant effect on virulence of fungal species; however, speed of kill varies with isolates and species. Fernandes et al. (2013) tested an aviary-derived isolate of *M. anisopliae* against larvae. Depending on the isolate, fungal infections caused up to 60% larval mortality 10 days post-application. Subsequent field testing of the most virulent isolate together with a *Metarhizium*-based commercial product indicated that spores sprayed on manure reduced adult
populations by two-fold compared to controls; however, non-target impacts on manure-inhabiting natural enemies, long-term spore persistence and sustainability of the manure application technique still remain questionable.

Although *B. bassiana* has been successfully used against several insect pests in outdoor agricultural settings (Kooymian et al. 1997, Burges 1998, Wraight et al. 2000, Wraight and Ramos 2002), it gained attention in fly management only after Steinkraus et al. (1990) first reported its natural occurrence in fly populations. Since then, several studies have evaluated its virulence and efficacy against larvae and adults using different formulations as well as application methods under laboratory conditions (Rizzo 1977, Geden et al. 1995, Watson et al. 1995, Carswell et al. 1998, Lecuona et al. 2005, Kaufman et al. 2008, Cova et al. 2009, Mwamburi et al. 2010, Anderson et al. 2011, Mishra et al. 2011, Shariffard et al. 2011a, b, Lopez-Sanchez et al. 2012, Mishra and Malik 2012) and semi-field or field settings (Watson et al. 1996, Renn et al. 1999, Kaufman et al. 2005, Cova et al. 2009, Mwamburi et al. 2009, Mishra et al. 2011, Fernandes et al. 2013). Dust formulations of fly-derived *B. bassiana* isolates applied on plywood were more effective than aqueous, sugar-baits or fungus-contaminated water or food formulations and caused 100% adult mortality within six days (Geden et al. 1995). Similarly, *B. bassiana* spores mixed with wheat-flour produced significantly higher mortality than a Tween-80 plus aqueous formulation in a dose-dependent manner (Waston et al. 1995). A similar dose-dependent pattern of mortality was observed with another *B. bassiana* isolate tested against different ages of adults, yet mortality was not different among age groups (<1, 3, 7 and 14 days post-eclosion) (Kaufman et al. 2008). Fly exposure to paper substrates treated with different concentrations of *B. bassiana* and *M. anisopliae* (10⁶, 10⁸, 10⁹ spores/ml) produced a dose-dependent mortality (Anderson et al. 2011). Within two weeks post-exposure, 100% fly mortality
was achieved after exposure to a high dose (10^9 spores/ml) compared to 60-70% mortality in three weeks with a low dose exposure (10^6 spores/ml). Based on isolates, *B. bassiana* caused more than 90% mortality in adults within two days; however, it was only marginally effective against larvae (Mwamburi et al. 2010). *B. bassiana* and *M. anisopliae* caused 28-100% mortality in larval and adult populations depending on isolates, where adults were more susceptible to fungal infections depending on formulation and method of application (Sharififard et al. 2011a). Mishra and Malik (2012) demonstrated a similar pattern of higher fungal susceptibility in adults under laboratory conditions where the infections caused 72-100 and 36-72% mortality in adults and larvae, respectively, depending on isolates. In a similar study, *B. bassiana* isolates caused significantly higher mortality in adults than larvae and pupae (Lecuona et al. 2005). Both *B. bassiana* and *M. anisopliae* tested against house flies and stable flies in laboratory settings demonstrated a great variation in efficacy among isolates, and the fungal infections caused 20-91% mortality within one week post-exposure (Lopez-Sanchez et al. 2012). The study also demonstrated temperature is a key factor that shapes vegetative and reproductive growth characteristics in fungal pathogens with an optimal temperature for fungal biomass production and sporulation at 27°C.

Semi-field or field evaluation reports of *B. bassiana* using different formulations and application methods are limited. Sawdust bedding in calf hutches supported lower numbers of fly larvae than straw bedding, and conidia sprayed on walls of calf hatches caused about 50% mortality in adult populations (Watson et al. 1996). Water-based *B. bassiana* formulations used as residual sprays in high-rise, caged-layer production barns substantially reduced adult populations comparable to pyrethrin treatments (Kaufman et al. 2005). Fly exposure to mass production residues of *B. bassiana* formulated as sugar-based baits has shown up to 90% adult
mortality within 15 days in semi-field settings (Lecuona et al. 2005). Weekly misting or fogging of *B. bassiana* formulations for three weeks produced desired fly control in poultry barns; however, it requires frequent re-treatments afterward for sustained population suppression (Cova et al. 2009). Spores of *B. brongniartii* and *B. bassiana* applied inside poultry barns @ 9 x 10^7 conidia/ml caused a 14-100 and 36-100% reduction in adult populations, respectively (Cova et al. 2009). *M. anisopliae* has shown 100% fly mortality within four to five days under laboratory and simulated field settings and was found to be more effective than *B. bassiana* (Mishra et al. 2011). Likewise, *B. bassiana* in combination with Bt and a commercial larvicide (larvadex) has substantially reduced fly populations compared to *B. bassiana*, Bt or larvadex alone suggesting that the fungus is compatible with and can work synergistically with other biopesticide products and insect growth regulators (Mwamburi et al. 2009). *M. anisopliae* applied with sub-lethal doses of a chemical insecticide, spinosad caused more than 95% adult mortality compared to only 72% alone indicating that the fungus can be used synergistically with biorational pesticides (Sharififard et al. 2011b).

1.4.4.1.3. Post-application persistence in field settings

The effectiveness of fungal biopesticides depends on persistence of the product in treated environments (Inyang et al. 2000, Jackson et al. 2010), and post-application longevity is influenced by several biotic and abiotic factors and their interactions (Jackson et al. 2010, Jaronski 2010). Persistence of spray residues is an important field performance attribute that optimizes application parameters as well as economizes reapplications. There are two ways that hosts acquire infective propagules- direct contact, conidia directly sprayed over the hosts and indirect contact, secondary attachment of conidia indirectly through contact of treated surfaces after spray applications (Jaronski 2010). The latter is the most common route of conidia
acquisition following field applications, hence good coverage and persistence of infective propagules on treated surfaces and/or in treated environments is necessary for effective and sustained control (Meekes et al. 2000).

Spores are usually short-lived under natural outdoor environments. The decline of conidia persistence and infectivity on plant surfaces largely depends on solar radiation, temperature, humidity, rainfall, leaf surface chemistry, phylloplane microbiota and interactions among them (Inglis et al. 1993, 1995, Fargues et al. 1996, Jaronski and Goettel 1997, Moore et al. 1997, Costa et al. 2001). Solar radiation, particularly the ultraviolet component, and temperature are two key drivers that determine the fate of spray residues in epigeal habitats (Jaronski 2010). In open outdoor settings, the half-life of conidia following application ranges from few hours to few days, and UV-A (320-400 nm) and UV-B (280-320 nm) components are considered damaging and mainly responsible for conidia death and delayed germination (Moore et al. 1993, Braga et al. 2001). Persistence reduction by solar radiation is also affected by location of spray application, formulations and fungal species or isolates. For example, conidia applied on the lower plant canopy and/or lower leaf surface are relatively long-lived than those on the upper canopy and/or upper leaf surfaces due to shading effects (Jaronski 2010). Spores formulated in oils tend to have longer persistence, as oils can protect spores from rapid drying and aid in germination at low humidity (Bateman et al. 1993). Metarhizium acridum conidia were more resistant to UV radiation than B. bassiana and M. anisopliae, and a significant difference exists among isolates of the same species. Further, rainfall washes off sprayed inoculum from the treated surfaces especially in aboveground applications depending on formulation type and reduces the numbers of effective propagules needed to infect hosts. Oil-formulated conidia, for
instance, were more stable and less likely to be washed away by rainwater compared to aqueous and other wettable and emulsifiable formulations (Inglis et al. 1999, Inyang et al. 2000).

Temperature is another important abiotic factor that determines conidia germination, penetration and overall disease progress (Fargues et al. 1997). In outdoor settings, fungal efficacy depends on ambient temperature and season. For instance, *B. bassiana*, isolate GHA efficacy in the field against *Lygus Hesperus* was much lower in July compared to June of the same year (Noma and Strickler 1999). The temperature regimes within a plant canopy keep on fluctuating, mostly exceed 30°C during day and are much cooler at night. Temperatures below 16°C increasingly slow germination and growth rate of most fungi and influence efficacy of spray residues (Inglis et al. 1999, Ihara et al. 2008). The daily ambient temperature regime is mostly beyond 30°C and insects such as house flies can raise their body temperature through behavioral fevering and sun basking activities (Watson et al. 1993, Kalsbeek et al. 2001b, Anderson et al. 2013a, b), whereas night temperature is cold enough to slow germination within insects. Moreover, some isolates within the same species have better tolerance to the upper or lower thermal limits depending on origin; therefore, selection of right isolates according to climatic condition is critical (Fargues et al. 1996, Bugeme et al. 2009). Thermoregulatory behaviors in insects may slow down germination and penetration and sometimes hosts can clear infection (Quedraogo et al. 2004, Anderson et al. 2013a, b). Temperature also has indirect effects on host immune response as high temperature has shown to enhance humoral immune response in *Galleria mellonella* (Wojda et al. 2009). *Galleria* larvae exposed to 38°C before injecting blastopores increase their survival time compared with non-heat shocked control larvae. Most fungal entomopathogens have thermal optima within 23-30°C, and germination and growth is severely affected above 30°C. In addition, humidity and moisture also play an important role
during conidia germination and sporulation after host death. Moisture and humidity particularly at the microspace above insect cuticle affects conidia germination and host penetration. Oil-based formulations can cover conidia and prevent excessive moisture loss thereby helps provide moist conditions for germination (Bateman et al. 1993).

Studies examining post-application persistence of conidia in indoor settings are limited. One study on house flies examined the persistence of *B. bassiana* spores formulated in Tween 80 and water and demonstrated that spores remained infective for about four weeks on plywood exposed inside animal barns and caused up to 97% adult mortality (Watson et al. 1995). Another unpublished report showed that spores could survive for more than seven weeks in poultry litter and more than three weeks on sprayed surfaces (Arends and Black 2000, [http://www.rinconvitova.com/balance%20fly%20control.htm](http://www.rinconvitova.com/balance%20fly%20control.htm)). Other studies examining conidia persistence following application in protected environments (i.e. not open field) in both agriculture and human health contexts have shown highly variable results. Persistence studies in protected crop settings, such as soil inoculation and green houses, have shown that spores can persist for months or even years (O’Callaghan 1998, Vanninen et al. 2000, Costa et al. 2001, Milner et al. 2003, Pliz et al. 2011). More recently, oil-formulated conidia of *B. bassiana* were shown to remain infective to *Anopheles* mosquitoes for five to seven months on clay tiles. Nonetheless, persistence lasted for only about two to three months on concrete and wood tiles (Blanford et al. 2012). In other mosquito studies, pathogenic effects of *B. bassiana* applied on mud panel, black cloth and polyester netting caused 73-80% mortality in 14 days with overall mortality being substrate-dependent (Mnyone et al. 2009, 2010). In addition, fungal isolates can also show considerable variations in their ability to remain viable after application, particularly *Metarhizium* spp. showing little germination after three weeks, whereas other *B. bassiana*
isolates maintained ≥ 50% viability in 14 weeks after spray application (Darbro and Thomas 2009).

1.5. Current scenarios of fly management in poultry production units

Current fly management strategies in the poultry production facilities involve a combination of cultural, biological and chemical tactics (Axtell 1986, Malik et al. 2007). Manure management and other cultural practices aimed at keeping manure dry to disrupt fly developmental cycles can prevent population build up, but are not always effective (Axtell and Arends 1990, Hinton and Moon 2003). Biocontrol agents such as predators and parasitoids can also play an important role in fly management (Axtell 1986a, Crespo et al. 1998, Geden and Hogsette 2006, Geden 2012), but most agents target juvenile stages necessitating multispecies releases, and their efficacy is influenced by concurrent use of broad-spectrum chemical pesticides and insufficient manure moisture management (Geden et al. 1992b, Axtell 1999, Rutz and Scott 1999). Organophosphate, carbamate and pyrethroid-based sprays and baits can contribute to control (Axtell 1986, Kaufman et al. 2005), but flies have developed resistance to almost all commonly used chemical insecticides and resistance is now a global problem (Scott et al. 2000, Kaufman et al. 2001c, Geden 2012). In addition, the poultry industry continues to lose many currently registered organophosphate and carbamate pesticides for fly management due to regulatory constraints (Food Quality Protection Act, Egg Safety Rule; Kaufman et al. 2005, FDA 2009). Further, development of new chemistries is costly and time-consuming and effective chemicals are increasingly becoming dearth on the market. Thus, biologically based, integrated fly management as part of an overall IPM is needed, and the poultry industry is readily adopting such an approach for sustainable management of arthropod pests including filth flies (Sheppard...
et al. 1992, Axtell 1999, Kaufman et al. 2005). As a part of this strategy, biopesticides containing naturally occurring entomopathogenic fungi such as *Beauveria bassiana* and *Metarhizium anisopliae* offer potential alternatives to chemical pesticides for fly management in poultry production facilities.

Several studies evaluated the potential for strains of *B. bassiana* to infect and kill house flies through a range of simple laboratory, semi-field and field evaluations (see Section 1.4.4.1.2). Much of this fly research focuses on direct mortality effects of fungi on flies, with relatively little emphasis on other operationally relevant properties such as infectivity and persistence. Currently, just one *B. bassiana*-based biopesticide product, balEnce™ (Terregena Inc., USA) is commercially available to treat manure and fly-resting surfaces as a larvicide and adulticide in animal production units; however, there is limited information available on its persistence on treated surfaces and/or in the interior environments of the poultry houses. Conidia sprayed on the manure surface are also likely to succumb to complex biological and chemical conditions (saprophytic microbes, heat, humidity, nitrogenous waste product, toxic gases etc.), and can threaten non-target species, especially other natural enemies associated with the manure (Geden et al. 1995, Geden 2012). Fungal application without knowing the fate of the inoculum in treated environments unnecessarily increases treatment costs making the technology less appealing to poultry growers. Further, an important element in the successful development of a biopesticide product is an efficient delivery system. The commercial product has been recommended to apply three to four times per week depending on fly population pressure (http://terregena.com/balEnce/product-info); however, its current formulation and delivery methods add unnecessary water onto the manure and structural substrates (Kaufman et al. 2005). These practices with additional water promote fly population build-up through increasing
manure moisture for oviposition and larval development. The balEnce™ strategy is costly, labor-intensive and creates additional challenges for manure moisture management. In this context, the poultry industry desperately needs an ecofriendly and user-friendly field delivery strategy that could improve the efficiency of available products as well as economize application parameters and give long-term fly suppression in a cost-effective manner.

Considering these shortcomings, this dissertation reports on a series of laboratory and field studies designed to address several questions related to field application, performance and persistence of fungal biopesticides, particularly Beauveria bassiana, one of the leading candidates for development as a fly biopesticide in the poultry houses. The specific objectives of the study were:

- To evaluate the potential for biocontrol of house flies using fungal biopesticides, Beauveria bassiana and Metarhizium anisopliae,

- To evaluate persistence and efficacy of a B. bassiana biopesticide against the house fly on typical structural substrates of poultry houses,

- To assess the impact of fly population on persistence and efficacy of a B. bassiana biopesticide under laboratory conditions, and

- To evaluate persistence and efficacy of a B. bassiana biopesticide against house flies in a commercial high-rise layer house.

1.6. Dissertation outline

The dissertation is divided into the following sections:
Chapter 2. Potential for biocontrol of house flies using fungal biopesticides, *B. bassiana* and *M. anisopliae*

This chapter evaluates the potential of barrier application technique for fungal biopesticides in plastic containers whereby newly emerged adult flies are targeted via the application of oil-formulated spores to inexpensive contractor-grade plastic sheeting, fixed to the lower section of basement walls. Specifically, the section tests population control potential of the fungal biopesticides through lethal and sub-lethal impacts on flies under laboratory conditions.

Chapter 3. Persistence and efficacy of a *B. bassiana* biopesticide against the house fly, *Musca domestica*, on typical structural substrates of poultry houses

This chapter investigates the persistence and infectivity of an oil-based formulation of *B. bassiana* applied to cinder block (painted or left bare), cement board and wood that constitute the primary wall materials found in poultry production facilities. Fungal persistence and efficacy are assessed on contractor-grade plastic sheeting as potential wall covering materials prior fungal application.

Chapter 4. Impact of fly population on persistence and efficacy of a *B. bassiana* biopesticide under laboratory conditions

The chapter investigates impacts of long-term exposure of different fly densities on conidia persistence and efficacy under laboratory conditions.

Chapter 5. Persistence and efficacy of a *B. bassiana* biopesticide against the house fly, *Musca domestica* in a commercial high-rise layer house

In this chapter, conidia persistence and efficacy against house flies are evaluated in a commercial high-rise barn at two different seasons using contractor-grade plastic sheets as a
fungal carrier substrate. Conidia longevity and efficacy are also evaluated under laboratory conditions against flies.

**Chapter 6. Conclusions and broader implications**

In this chapter, salient findings from all aforementioned projects and their implications using entomopathogenic fungal biopesticides in managing house fly populations in poultry houses are presented with current pitfalls and future directions.
1.7. References


Barson, G., Renn, N., and Bywater, A. F. (1994). Laboratory evaluation of six species of entomopathogenic fungi for the control of the house fly (*Musca domestica* L.), a pest of intensive animal units. *Journal of Invertebrate Pathology, 64, 107-113.*


Dipeolu, O. O. (1982). Laboratory investigations into the role of *Musca vicina* and *Musca domestica* in the transmission of parasitic helminth eggs and larvae. *International Journal of Zoonoses, 9*(1), 57-61.


Hillerton, J. E., and Bramley, A. J. (1985). Carriage of *Corynebacterium pyogenes* by the cattle nuisance flies *Hydrotaea irritans* (Fallen) and *Musca autumnalis* (De Geer). *Veterinary Parasitology, 18*, 223-228.


Miller, R. W., Pickens, L. G., and Potts, W. E. (1993a). Comparison of traps and an integrated program to manage house flies (Diptera, Muscidae) and stable flies (Diptera, Muscidae) on dairy farms. *Journal of Agricultural Entomology, 10*, 189-196.


Rizzo, D. C. (1977). Age of three Dipteran hosts as a factor governing the pathogenicity of *Beauveria bassiana* and *Metarhizium anisopliae*. *Journal of Invertebrate Pathology, 30*, 127-130.


Williams, R. E. (2010). Livestock and poultry: Control of poultry pests. Purdue Extension, Purdue University. 7p.


Chapter 2: Potential for biocontrol of house flies, *Musca domestica*, using fungal biopesticides

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Abstract

Chemical control of house flies in poultry production facilities is becoming increasingly difficult due to insecticide resistance and regulatory constraints. Biopesticides based on entomopathogenic fungi could provide an alternative approach. Here we evaluated population control potential of two fungal pathogens, *Beauveria bassiana* and *Metarhizium anisopliae*. Cohorts of adult flies were established in large plastic boxes in the laboratory and were exposed to residues of oil-formulated fungal conidia sprayed on strips of plastic sheeting attached to the box walls. Exposure to the biopesticide barrier treatments caused 100% mortality in adult populations within 8-16 days, depending on the fungal species. In contrast, control flies survived until 96-110 days. Additionally, fungal infections caused 13-20% reduction in egg viability and >70% reduction in fecundity of flies prior to death. The combined lethal and pre-lethal impacts resulted in 21- to 26-fold reduction in basic reproductive rate in the fungus-exposed populations relative to controls. Based on these promising proof-of-principle results, further research is currently underway to determine the feasibility of developing a biopesticide product for operational use.

*Key words*: Poultry production facilities, insecticide resistance, entomopathogenic fungi, *Beauveria bassiana, Metarhizium anisopliae, Musca domestica*
2.1. Introduction

House flies, *Musca domestica* L., are important pests in poultry production facilities. High fly populations have been shown to reduce both egg production and quality, and can potentially transmit a number of human and poultry diseases (Axtell, 1986, 1999; Forster et al., 2007; Malik, Singh, & Satya, 2007; Olsen & Hammack, 2000; Wanarata, Panyim, & Pakpinyo, 2011). Although cultural, mechanical and biological tactics have potential in preventing and suppressing fly populations (Crespo, Lecuona, & Hogsette, 1998; Malik et al., 2007), use of broad-spectrum chemical pesticides is currently the only option for managing overwhelming populations in poultry barns (Axtell, 1986; Axtell & Arends, 1990). However, house flies have developed resistance to almost all commonly used chemical insecticides and resistance is now a global problem (Geden, 2012; Kaufman, Scott, & Rutz, 2001; Scott, Alefantis, Kaufman, & Rutz, 2000). In addition, the poultry industry continues to lose many currently registered organophosphate and carbamate pesticides for fly management due to the implementation of the Food Quality Protection Act (Kaufman, Reasor, Rutz, Ketlizis, & Arends, 2005). These challenges create a demand for alternatives to conventional chemical pesticides.

Several previous studies have evaluated the potential for strains of entomopathogenic fungi, such as *Beauveria bassiana*, to infect and kill house flies (e.g. Anderson, Bell, Blanford, Paaijmans, & Thomas, 2011; Cova et al., 2009; Geden, Rutz, & Steinkraus, 1995; Lecuona, Turica, Tarocco, & Crespo, 2005; Mishra, Kumar, Malik, & Satya, 2011; Watson, Geden, Long, & Rutz, 1995). However, beyond infection and virulence, an important element in successful development of a biopesticide product is an efficient delivery system. In poultry barns, newly hatched adult flies crawl from the litter to the nearest vertical surface to rest while their cuticle
and wings harden. Further, high densities of adult flies tend to be found in darker areas of the poultry house, often within 0.5-1 meter of the manure floor (Jacobs, Hogsette, & Miller, 1993). We propose a delivery system whereby these adult flies are targeted via the application of oil-formulated conidia to inexpensive contractor-grade plastic sheeting, fixed to the lower section of the basement walls. Plastic sheeting is an inert substrate with good conidia-transfer characteristics, so we anticipate that flies resting or walking over the treated plastic will pick up conidia and become infected. Direct residual spraying of existing structural substrates such as concrete and wood is an option, but it has been shown previously that these substrates can influence infection rates and conidia persistence (Blanford et al., 2012). Additionally, the targeted barrier application strategy should minimize the surface area for treatment compared with conventional premise sprays and provides the option of either washing or replacing the sheeting prior to re-application.

This publication describes the use of large plastic boxes with fungal treated walls to evaluate the potential of two candidate fungal pathogens to reduce fly populations through impacts on survival and lifetime reproductive output. Our approach simulates the targeted residual spray delivery system with flies exposed to barrier treatments of treated plastic following adult emergence.

### 2.2. Materials and methods

#### 2.2.1. Fly rearing

Insecticide susceptible house flies were obtained from Cornell University (Schwardt Lab) and maintained in an environmental chamber at 26-27°C, 50-60% RH with a 12:12 light: dark photoperiod. Fly eggs were collected by placing a clear plastic cup (266 ml) containing
Kleenex™ tissue paper saturated with skim milk powder, sugar and water into a fly stock cage for 2-3 h. To initiate a fly cohort, eggs were washed from the tissue into another plastic cup with room temperature tap water, and the cup was then sealed with a lid and gently shaken to break up egg clumps. The eggs were allowed to settle for few minutes, and roughly 1.5 ml of settled eggs were added to 3 L of larval medium comprising wheat bran (2 L), Manno-Pro™ calf protein supplement (1 L), baker’s yeast (150 ml) and warm water (1.5 L), in a 35 x 30 x 15 cm plastic box. Larval development was monitored daily, and on formation, pupae were collected by gently shaking them from the surface of the larval medium into plastic cups. Harvested pupae were either used for the experiments, or to continue the colony. In the latter case, cups containing pupae were placed in clean stock cages until eclosion ceased. Adult flies were provided access to food, consisting of a 1:1 ratio of powdered milk and granulated sugar, and water ad libitum.

2.2.2. Fungal production, formulation and application

Dry conidia powder of *Beauveria bassiana* sensu lato (*Bb*), isolate 193-825 and *Metarhizium anisopliae* sensu lato (*Ma*), isolate ESF1 (Envera, PA) were produced using our standard two stage process (Jenkins, Heviefo, Langewald, Cherry, & Lomer, 1998), and stored in a refrigerator at 7°C until use. Prior to spray application, conidia powder was suspended in oil formulation containing 4:1 Isopar M and Ondina oil (Blanford et al., 2011). The concentration was enumerated with an Improved Neubauer Hemocytometer and adjusted to $1 \times 10^9$ conidia/ml. Prior to application, conidia were checked for viability by plating on Sabouraud Dextrose Agar, and assessed for germination at 400x under a microscope after 20 h incubation at 25°C (conidia were confirmed to be greater than 90% viable at the beginning of all experiments). Pieces of contractor-grade plastic sheet were fixed inside a 0.25 m² spray area on the rear wall of a reverse flow laminar cabinet, and sprayed with an artist’s airbrush at a volume application rate of 20
ml/m². A blank formulation was used for the control, using the same volume application rate as for the fungal formulation. The sprayed substrates were removed from the wall of the cabinet, and used to line the lower two-thirds of the inner walls of each plastic box and allowed to dry at room temperature overnight. Translucent plastic storage boxes (41 cm long, 28 cm wide and 23 cm high) with fitted lids were used to establish artificial fly colonies. The lids were cut to accommodate a ventilation screen made of fly proof mesh (18 x 16 cm), together with an open-ended mesh sleeve to enable access into the boxes during the experiments.

2.2.3. Establishment of fly colonies in boxes

A cohort of 300, ready to emerge, mature pupae was placed at the bottom centre of each box, and allowed to emerge. Eight boxes, four fungus-treated replicates and four controls, were maintained for each experiment under the same conditions as the main fly colony. Flies were provided access to a 1:1 mixture of skim milk power and sugar, and water ad libitum for the duration of the experiments. For *B. bassiana*, we conducted two experimental runs using different batches of the fungus produced approximately two months apart (batches identified as batch 1 and batch 2). For *M. anisopliae* we conducted one experimental run only (although with appropriate internal replication) as parallel studies on persistence (to be reported elsewhere) showed spray residues of this isolate to have a half-life just a few days, making it a poor candidate for ultimate operational use.

2.2.4. Fly survival

Starting from the first day of emergence and daily thereafter, dead flies were removed from the boxes, counted, sexed and allowed to dry at room temperature (26-27°C, 50-60% RH with a
12:12 light: dark photoperiod) for five to seven days. To confirm mycosis, cadavers were subsequently transferred into 9 cm petri dishes containing water-saturated filter papers, and sealed with parafilm. After five to seven days, the cadavers were examined for external sporulation using a dissection microscope at 15x magnification.

2.2.5. Egg collection, enumeration and viability test

Beginning two to three days after the first adults emerged, fecundity was measured daily by collecting the eggs from each replicate box. To collect eggs, a small clear plastic cup (266 ml) containing a Kleenex™ tissue paper saturated with milk powder and sugar mixture was placed into each box for 8 h (8 am to 3 pm) every day. Eggs from each oviposition cup were rinsed from the Kleenex™ paper into another plastic cup (266 ml) with room temperature tap water. The cup was sealed with a lid and gently shaken to break up egg clumps. Egg counting and viability tests were carried out following the protocol as outlined by Anderson, Branford, & Thomas (2013) with slight modifications. The water containing the eggs was poured into a petri dish, which was placed under a dissection microscope. One hundred individual eggs were removed using a plastic pipette, and placed into a petri dish containing a 9 cm circle of black filter paper (Whatman, Inc.). These dishes were sealed with parafilm and kept at 26-27°C and 50-60% RH. After 48 h, empty chorions were counted using a dissecting microscope, and any eggs that had not hatched by this time were considered non-viable. The remaining eggs from the collection were quantified volumetrically using a graduated 0.5 ml plastic insulin syringe. The tip of the syringe was removed using a razor blade, and covered by several layers of stretched nylon mesh. The eggs in water were drawn up into a plastic pipette and transferred into the syringe barrel. Water drained out through the mesh and the settled egg volume was measured using the graduations on the side
of the syringe. To calibrate the egg number-to-volume relationship, five replicates of 200, 400, 600 and 800 eggs were counted directly under the microscope, and measured using the volumetric method. The egg number-to-volume relationship was described by the following regression equation: Total eggs = (11.984*volume (µl)) + 88.063; R² = 0.975. Daily egg production per female was calculated by dividing the total number of eggs produced by the number of living females remaining in each box.

### 2.2.6. Statistical analysis

House fly survival data from each experiment were analyzed separately using Kaplan-Meier survival analysis (SPSS, software version 22) with differences in median survival time between treatments (fungus vs. control) compared using the log-rank test. Life-time fecundity per female and viable eggs produced per female were log-transformed and analyzed separately for each experiment using a general linear model incorporating infection status (fungus vs. control) as a main factor. The life-time fecundity per female in the cohort was measured as the total number of eggs divided by the initial number of females present in each replicate at the start of the experiments, and the viable eggs produced per female was estimated in the similar way. Cohort life tables were constructed for each experiment and basic reproductive rates were calculated for treated and control populations (Begon, Harper, & Townsend, 1996). All statistical analyses were performed in SPSS (version 22) and significance for all statistical tests was set at P < 0.05.
2.3. Results

2.3.1. Fly survival

Both *B. bassiana* and *M. anisopliae* infections caused significant reductions in fly survival compared to control groups (Figure 2-1A-C). House fly survival was significantly different between fungal-treated and control populations in all experiments ($\chi^2=2483.04$, $P<0.001$ for *Bb* batch 1; $\chi^2=2582.85$, $P<0.001$ for *Bb* batch 2 and $\chi^2=2557.95$, $P<0.001$ for *Ma*). The *B. bassiana* treatments had median survival times of 6 days ($6 \pm 0.02$ for batch 1; $6 \pm 0.03$ for batch 2), with 100% mortality in both runs by day 8. *M. anisopliae* was less virulent, with median survival time of 9 ($\pm 0.01$) days and 100% mortality by day 16. Flies in the control treatments, on the other hand, survived for up to 96-110 days, with median survival times of 26-34 days. There were significant differences in survival between male and female flies in all experiments (for *Bb* batch 1 experiment, $\chi^2=552.69$, $P<0.001$ for control and $\chi^2=123.06$, $P<0.001$ for fungus; for *Bb* batch 2 experiment, $\chi^2=714.89$, $P<0.001$ for control and $\chi^2=3.89$, $P=0.049$ for fungus; and for *Ma* experiment, $\chi^2=231.42$, $P<0.001$ for control and $\chi^2=360.80$, $P<0.001$ for fungus), with females surviving longer than males in the control treatments but males surviving longer than females in the fungus treatments. Wherever significant mortality occurred in the fungal treatments, > 90% of cadavers showed external signs of mycosis.

2.3.2. Fecundity and egg viability

Egg laying patterns in both control and treatment groups were similar across all experiments. In control groups, a higher number of eggs were produced during the first weeks after emergence, and this decreased slowly with age. The egg production in all control groups lasted for about two
months. Conversely, treated populations only produced eggs for the first two to five days after emergence and stopped after day six (Figure 2-2A-C).

Both fungal species caused more than 90% reduction in lifetime fecundity as well as about 13-20% reduction in egg viability (Figure 2-3A and B). There was a significant difference in life-time fecundity per female and number of viable eggs produced per female between fungal-treated and control populations in all experiments (for life time fecundity: $F_{1,6} = 316.38$, $P<0.001$ for $Bb$ batch 1; $F_{1,6} = 500.76$, $P<0.001$ for $Bb$ batch 2; $F_{1,6} = 898.29$, $P<0.001$ for $Ma$). For viable egg production: $F_{1,6} = 715.89$, $P<0.001$ for $Bb$ batch 1; $F_{1,6} = 488.95$, $P<0.001$ for $Bb$ batch 2; $F_{1,6} = 825.18$, $P<0.001$ for $Ma$). Overall, the basic reproductive rates (essentially the average number of viable offspring produced across the lifetime of an individual fly) for flies were 8.4 in the population treated with $B. bassiana$ batch 1 and 17.3 for those treated with $B. bassiana$ batch 2, compared with 225 and 367 for the respective controls. Similarly, for the $M. anisopliae$ experiments, the basic reproductive rates were 11.4 for fungus-exposed flies compared with 265 in the controls (Figure 2-3C).

2.4. Discussion

This study demonstrated the potential for residual spray treatments of fungal pathogens to suppress housefly populations by increasing adult fly mortality and reducing lifetime reproductive output (partly due to shorter lifespan, but also reductions in per capita fecundity and egg viability).

$B. bassiana$ treatments resulted in 100% mortality in fly populations within 8 days irrespective of production batch, compared to 16 days in the experiment with $M. anisopliae$. This
difference in speed of kill could be due to simple variation in virulence between isolates. However, the female flies exposed in the *M. anisopliae* experiment died at more or less the same rate as the flies exposed to *B. bassiana*, so the longer survival at the population level was driven by reduced virulence against the males only. Interestingly, the male flies in the *B. bassiana* experiments also died more slowly than the females. This sex-difference in susceptibility contrasts with the baseline patterns of survival in the control flies where, consistent with previous studies (Anderson et al., 2013; Rockstein & Lieberman, 1958), females lived longer than males. The reasons for this greater susceptibility of female flies are unknown. Female flies tend do have a larger body size, which could result in more conidia being picked up from the sprayed surface, increasing effective dose. Additionally physical (e.g. persistent courtship or coercion) and physiological stresses (e.g. seminal fluid proteins) exerted by copulatory males could impact susceptibility (Fowler & Partridge, 1989; Lew, Morrow, & Rice, 2006; Partridge, Fowler, Trewitt, & Sharp, 1986; Wigby & Chapman, 2005). Post-mating reduction in immune defence is common in female insects (Rolff & Siva-Jothy, 2002; Short & Lazzaro, 2010; Siva-Jothy, Tsubaki, & Hooper, 1998) possibly due to reallocation of resources from immunological requirements to reproduction (Sheldon & Verhulst, 1996).

In addition to mortality, both fungal pathogens significantly reduced the reproductive fitness of fly populations. In all experiments, the infections caused more than 90% reduction in per capita lifetime female fecundity. Similar impacts on female reproductive output have been documented in several other insect-fungus systems (Afity & Mattler, 1969; Fargues, Delmas, Auge, & Lebrun, 1991; Mathews, Smith, & Edwards, 2002; Santiago-Alvarez & Vargas-Osuna, 1988). While the impact on lifetime reproduction clearly derived in part from reduced female lifespan, per capita fecundity of infected females over the first few days of the experiment also
showed a 70-80% reduction in egg production compared to control populations over the same period. The fungal pathogens also reduced egg viability by 13-20%. These combined lethal and pre-lethal impacts resulted in 21- to 26-fold reduction in basic reproductive rate in the fungus-exposed populations relative to controls. Other anticipated side effects of fungal infection, such as reduced flight capacity (Blanford et al., 2011), should further add to control by limiting fly dispersal capacity.

Overall, the current study provides encouraging proof of principle that residual treatments of oil-formulated fungi can infect adult house flies shortly following emergence and could have substantial impacts on fly densities and population growth rates in intensive animal units such as poultry houses. The notion of using a barrier treatment above the litter layer is attractive as it would likely minimize non-target impact on manure-inhabiting natural enemies and enable a substantial reduction in total treatment area compared to current methods wherein the entire basement and manure surface tend to be treated with insecticide formulations (Kaufman et al., 2005). Moreover, the use of cheap, non-toxic contractor-grade plastic sheeting to cover part of the wall surfaces prior fungal application would reduce the potential impact of different structural surfaces on conidial viability and availability (Branford et al., 2012). That said, the M. anisopliae isolate used in the current study decayed to zero viability within one to two days after application so represents a poor candidate for field use. Further research is currently underway to investigate the impact of a suite of abiotic and biotic factors on persistence and likely efficacy of the candidate B. bassiana isolate in high-rise layer production facilities.
2.5. Acknowledgements

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2.6. References


Figure 2-1. Cumulative proportional survival (mean ±SE) of adult house fly populations exposed to residual spray treatments of A) *B. bassiana* I93-825 (batch 1), B) *B. bassiana* I93-825 (batch 2), and C) *M. anisopliae* ESF1 in plastic boxes under laboratory conditions.
Figure 2-1. Continued
Figure 2-2. Average daily egg laying pattern of female flies (A, B and C) in fungal-treated and untreated-control plastic boxes. Data show mean (± SE) number of eggs per live female per day.
Figure 2-3. Impact of fungal infections on A) total number of eggs per female from the original cohort, B) viable eggs per female from the original cohort, C) basic reproductive rate relative to uninfected control house fly populations in plastic boxes under laboratory conditions.
Chapter 3: Persistence and efficacy of a *Beauveria bassiana* biopesticide against the house fly, *Musca domestica*, on typical structural substrates of poultry houses

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Abstract

Entomopathogenic fungi, such as *Beauveria bassiana*, offer potential for use as biopesticides for control of house flies in poultry production facilities. This study evaluates persistence and efficacy of oil-formulated *B. bassiana* conidia against adult house flies on a range of structural substrates commonly found in poultry houses. Exposure of flies to fungal-treated surfaces produced high levels of infection leading up to 100% mortality in 6-10 days. However, the infectivity of the spray residues declined rapidly within one or two weeks following repeated fly exposures. Investigations showed that, in the absence of flies, conidia remained viable on test surfaces for up to three months regardless of substrate type, application method or fungal production batch. Rather, it was the presence of flies themselves that was responsible for reducing persistence. The exact mechanisms remain unclear but involve a combination of physical removal and chemical deactivation, with decay rates increasing at higher fly densities. While the rapid decay could pose a challenge for operational use, the results suggest it might be possible to tailor treatment frequencies to fly densities with, for example, weekly applications at high fly densities and longer intervals when populations decline. Further research is needed to determine persistence in semi-field and field settings and to quantify the influence of fly densities under natural exposure conditions.

Key words: *Beauveria bassiana*, persistence, residual sprays, pesticide resistance, biocontrol, *Musca domestica*
3.1. Introduction

Entomopathogenic fungi have been successfully used against agricultural pests in a variety of crop settings (Anderson & Lewis, 1991; Faria & Wraight, 2001; Milner, Soper, & Lutton, 1982; Rombach, Aguda, Shepard, & Rober, 1986; Wraight & Ramos, 2002), and recent research has identified the potential to extend use for control of various pests in indoor environments (Barbarin, Jenkins, Rajotte, & Thomas, 2012; Blanford et al., 2011; Cherry, Abalo, & Hell, 2005; Hidalgo, Moore, & Patourel, 1998). Several studies have investigated the potential use of fungal pathogens, especially isolates of Beauveria bassiana, for control of house flies, which are important pests and disease-vectors in poultry production facilities (Geden, 2012; Malik, Singh, & Satya, 2007). Studies range from simple laboratory assays (e.g. Anderson, Bell, Blanford, Paaijmans, & Thomas, 2011; Cova et al., 2009; Geden, Rutz, & Steinkraus, 1995; Lecuona, Turica, Tarocco, & Crespo, 2005; Mishra, Kumar, Malik, & Satya, 2011; Watson, Geden, Long, & Rutz, 1995) through to semi-field and field evaluations (Cova et al., 2009; Kaufman, Reasor, Rutz, Ketzis, & Arends, 2005; Mishra et al., 2011; Mwamburi, Laing, & Miller, 2010; Watson, Rutz, & Long, 1996). The focus of much of this house fly research has been on direct mortality effects of fungi on larvae and adults, with relatively little emphasis on other operationally relevant properties such as infectivity and persistence.

Currently just one B. bassiana-based biopesticide product is commercially available for control of flies in animal production facilities. However, its current formulation and delivery methods in poultry production barns require intensive re-applications, with additional water sprayed onto the manure (Kaufman et al., 2005). Such a strategy is costly, labor-intensive and may create additional challenges for manure moisture management. Conidia sprayed on the
manure surface are also likely to succumb to complex biological and chemical conditions (saprophytic microbes, heat, humidity, nitrogenous waste product, toxic gases etc.; Xin et al., 2011) and can threaten non-target species, especially other natural enemies associated with the manure (Geden et al., 1995).

High densities of adult flies tend to be found in darker areas of the poultry house, within 0.5-1 meter of the floor (Jacobs, Hogsette, & Miller, 1993). Further, newly emerged adult flies congregate on the walls and pillars adjacent to the manure layer to harden their cuticle and expand their wings (Axtell & Arends, 1990; Watson et al., 1995). Treating these surfaces with a biopesticide residual spray could provide an opportunity to target teneral as well as older adult flies. The cost effectiveness of such an approach is likely to depend, in part, on the infectivity and persistence of conidia after treatment.

To date there has been limited research on post-application persistence of conidia in interior environments and/or on realistic substrates. One study on house flies examined persistence of conidia of *B. bassiana* formulated in Tween 80 and water and showed conidia to remain infective for 28 days following application to plywood (Watson et al., 1995). Other studies examining persistence of oil-based fungal sprays being developed for control of mosquitoes showed conidia of *B. bassiana* to remain infective for two to seven months after treatment, depending on the test substrate (Blanford et al., 2012 and see also Darbro & Thomas, 2009). These results are encouraging. However, fungal persistence will depend on the specifics of the isolate, formulation, substrate and the environment. Here we investigated the persistence and infectivity of oil-based formulations of an isolate of *B. bassiana* applied to cinder block, cement board and wood (painted or left bare) that constitute the primary wall materials found in high-rise layer production facilities. We also evaluated persistence on contractor-grade plastic
sheeting as a possible substrate to line sections of the basement walls of poultry houses prior to application.

3.2. Materials and methods

3.2.1. Fly rearing

Insecticide-susceptible house flies (Musca domestica L.) were obtained from the Department of Entomology (Schwardt Lab), Cornell University and maintained in an environmental chamber at 26-27°C, 50-60% RH with a 12:12h (L: D) photoperiod. Fly eggs were collected by placing a small clear plastic cup (18 cm bottom and 28 cm top diameter, 266 ml) into a fly stock cage for 2-3 h. The cups contained Kleenex™ paper saturated with skim milk powder, sugar and water. To initiate a fly cohort, roughly 1.5 ml of eggs were placed into 3 L of larval medium comprising wheat bran (2 L), Manno-Pro™ calf protein supplement (1 L), baker’s yeast (150 ml) and warm water (1.5 L), in a 35 x 30 x 15 cm plastic box, and larval development was monitored daily. Pupae were collected by gently shaking them from the surface of the diet into plastic cups (266 ml). Cups containing pupae were placed in clean stock cages until eclosion ceased. Adult flies had access to food, consisting of a 1:1 ratio of powdered milk and granulated sugar, and water ad libitum.

3.2.2. Efficacy of spray residue on different structural surfaces over 30 days with repeated fly exposures (I)

3.2.2.1. Conidia spray application

A dry conidia powder of Beauveria bassiana sensu lato (isolate I93-825 production batch PSU 31) was produced using a standard two stage process (Jenkins, Heviefo, Langwald, Cherry, &
Lomer, 1998) and stored in a refrigerator at 7°C until use. Prior to spray application, conidia powder was suspended in an oil formulation containing 80% Isopar M and 20% Ondina (Blanford et al., 2011). Concentration was enumerated with an Improved Neubauer Hemocytometer and adjusted to 1.8 x 10⁹ conidia/ml. Conidia were checked for viability by plating on Sabouraud dextrose agar (SDA), and germination rates were assessed at a magnification of 400x under a microscope after 20 h incubation at 25°C (conidia were greater than 93% viable at the beginning of all assays). Test substrates included untreated cinder block, cinder block coated with oil-based or water-based latex paint, raw wood, pressure-treated wood and cement board. Blocks of each substrate (15 x 15 cm) were fixed inside a 0.25 m² spray area on the rear wall of a reverse flow laminar cabinet and sprayed using an artist’s airbrush at a volume application rate of 20 ml formulation per m², giving a coverage on each surface of 1.5 x 10⁶ conidia/cm² (verified by extracting sprayed conidia from a representative substrate and counting using a hemacytometer). Spray application of conidia was carried out in batches of six (containing one replicate of each substrate type) with each substrate type replicated four times. A blank formulation was used for the control and sprayed onto each substrate at the same volume and application rate as for the fungal formulations. The exposed substrates were removed from the wall of the laminar flow cabinet and allowed to dry at room temperature overnight.

3.2.2.2. Exposing flies to spray residue

Three- to five-day-old adult house flies were removed from stock colonies using a battery-powered insect aspirator (BioQuip Inc.) and anesthetized by placing them at -20°C for three to four minutes. Forty adult flies of mixed sex were randomly selected by placing them on a 9 cm petri dish lid with a block of dry ice suspended above to maintain the cold temperature. Selected
flies were placed on each substrate, covered with a 9 cm diameter petri dish bottom and allowed to revive and move freely within the enclosure (Anderson et al., 2011). After 4 h, the flies were released into translucent plastic rearing boxes (35.6 cm long, 20.3 cm wide and 11.7 cm deep) fitted with nylon mesh on top and open-ended nylon mesh sleeve on one side and monitored for mortality daily for three weeks. Flies had access to water and a 1:1 mixture of skim milk powder and granulated sugar ad libitum. Dead flies were removed and a fungal mycosis confirmed by drying cadavers for five to seven days before transferring them to a humid environment and monitoring external sporulation under a dissecting microscope at 15x magnification over the subsequent seven days. The treated substrates were stored under the same conditions as the caged flies (26-27°C and 50-60% RH) and repeat exposures using naïve flies were performed 15 and 30 days after the initial exposures to monitor changes in infectivity of the spray residues.

3.2.3. Conidia viability test on ‘inert’ glass slides and plastic sheet comparing two production batches and two application techniques

Viability of conidia can vary depending on the substrate (Blanford et al., 2012) and also production batch. Two batches of B. bassiana (I93-825), PSU 31, and a batch of the same fungal isolate produced two months later (PSU 37) were applied to either glass slides or non-porous, chemical-free plastic sheeting materials to test persistence independent of any potential physical or chemical effects of structural substrates using the methodology of Darbro & Thomas (2009). Oil-formulated conidia with a concentration of 1 x 10⁹ conidia/ml were either sprayed using the technique described in Section 3.2.2.1, or painted onto the surface of glass slides and plastic sheet using a soft nylon paintbrush at 20 ml/m². Treated substrates were allowed to air-dry overnight and then maintained at 26-27°C and 50-60% RH for the duration of the experiment.
Conidial viability was determined every week by washing the conidial residue from the glass slides or cutting a 3-cm² swatch from the plastic sheeting and suspending conidia in 3 ml of Isopar M. A single drop of the resulting conidial suspension was spread over the surface of SDA in a 5 cm diameter petri dish and incubated for 20 h at 25°C. Conidia were examined under a microscope at 400x magnification to determine the proportion of germinating and non-germinating conidia. A total of 300 conidia were examined from each of three replicate samples, and the data were used to calculate percent viability of the conidia.

3.2.4. Efficacy of spray residue on different structural surfaces over 30 days with repeated fly exposures (II)

This persistence experiment was designed as a repeat of Section 3.2.2 considering a sub-set of the original substrates (bare cinder block and cinder block painted with water-based latex paint) and plastic sheeting (as used in viability test in Section 3.2.3); the latter having potential as a temporary surface that could be attached to the poultry manure room walls prior to spray application. All conidia used in this experiment were from batch PSU 37. Conidia were formulated and applied as in Section 3.2.2, at 1 x 10⁹ conidia/ml (1.5 x 10⁶ conidia/cm²). Germination rate at application was greater than 90%. All other procedures were identical to Section 3.2.2. Fly exposures were conducted on day 1, 7, 15 and 30 post-spray to better monitor the short-term infectivity of the spray residue.

3.2.5. Effect of fly density on efficacy and viability of spray residue on plastic sheet

Conidia from production batch PSU 37 were formulated and sprayed onto plastic sheeting disks following the same procedure and concentrations as mentioned in Section 3.2.4 above. Sprayed
disks were dried overnight and placed in the lids of 9 cm diameter plastic petri dishes. Either 25 or 50 temporarily anesthetized adult flies were added per disk and removed after 4-h exposure. A new batch of flies at the same density was exposed to the same substrates on day 2, and this procedure repeated again on day 3. In addition, some substrates were exposed only on day 2 or day 3 to separate time effects from prior exposure. Six replicate disks were used for the low-density (25 flies) treatment and four replicates were used for the high-density treatment. Flies were maintained and monitored as described in Section 3.2.2.2. Viability of the conidia in the spray residue was evaluated prior to the first exposure (93.9%) and immediately after each exposure event using the methods described in Section 3.2.3.

3.2.6. Effect of fly density on viability and concentration of conidia in spray residue on plastic sheet

Once again, conidia from batch PSU 37 were formulated and sprayed on to plastic sheeting and allowed to dry as described in Section 3.2.5 above. In this experiment, we exposed flies to the spray residue at lower densities (5, 10 and 20 flies per 9 cm exposure arena, four replicate plastic disks for each density). Fly populations were allowed to remain on the treated surface for 4 h and then removed. Flies were not monitored for mortality, but destructive samples were taken from each exposed surface to evaluate viability and total number of conidia remaining following each exposure. Repeated exposures using the same density of flies were made on day 2 and 3 after spraying (total of three repeated exposures). Due to the destructive nature of the sampling, 12 exposure disks were used for each population density on day 1, with four being destructively sampled at each subsequent time point.
3.2.7. Statistical analysis

Survival data were analyzed using Kaplan-Meier survival analysis (SPSS v. 20) with differences in median survival time (MST) among treatments compared using the log-rank test. The proportions of conidia germinating from Section 3.2.3 were analyzed using a general linear model (SPSS v. 20) with substrate type and application method as treatment factors. The proportion of germinating conidia from Section 3.2.5 was square-transformed and analyzed using a general linear model (SPSS v. 20) with sampling time as a treatment factor. The conidia count and proportional germination data from Section 3.2.6 were square root and square-transformed, respectively and analyzed using a general linear model (SPSS v. 20) with fly density and sampling time as treatment factors.

3.3. Results

3.3.1. Efficacy of spray residue on different structural surfaces over 30 days with repeated fly exposures (I)

*Beauveria bassiana* spray residues on all structural surfaces were highly effective 1 day after spray application and resulted in 100% fly mortality within 7-10 days following a 4-h exposure (Figure 3-1A). Fly survival was significantly lower than control survival at this time point (Table 3-1), with no clear effects of substrate type. By day 15, the infectivity of the spray residue had reduced substantially (Figure 3-1B). Most treatments still showed reduced survival relative to controls (Table 3-1), but none resulted in 50% mortality by the end of the monitoring period. By day 30, the fungal spray residues had no obvious effects on fly survival with only one treatment showing reduced survival relative to controls (Figure 3-1C; Table 3-1). Wherever significant
mortality occurred, 80-100% of cadavers in the fungal-exposed treatments showed external signs of mycosis.

3.3.2. Effect of application method on conidia viability on ‘inert’ substrates

Because infectivity declined so rapidly in our initial assays (Section 3.3.1), we sought to determine whether this was because the test substrates were universally bad, or possibly due to another common factor such as damage to conidia during the spray procedure, or the fungus production batch. Thus, we tested conidia viability using inert substrates (glass slides and plastic sheet), comparing spray application versus a paintbrush, and then examined a separate production batch of fungus. The half-life of conidia from batch PSU 31 varied between 24 and 45 days regardless of application methods (spray or paintbrush), with measurable viability up to 90 days in certain treatments (Figure 3-2A). There was no significant effect of substrate type and application method on conidia viability ($F_{1, 176} = 3.358$, $P=0.69$ for substrate; $F_{1, 176} = 0.127$, $P=0.722$ for method) or their interaction ($F_{1, 176} = 0.790$, $P=0.375$). Similarly, conidia of *B. bassiana* PSU 37 showed a half-life of 35-49 days, with measurable viability up to 112 days (Figure 3-2B). There was no significant effect of substrate type on viability ($F_{1, 106} = 1.585$, $P=0.211$).

3.3.3. Efficacy of spray residue on different structural surfaces over 30 days with repeated fly exposures (II)

Having determined that fungal viability was not overtly affected by application method or production batch, we returned to examine persistence on different substrates comparing painted and unpainted cinder block (both showing poor persistence as measured by fly mortality in
Section 3.3.1) with plastic sheeting (good long-term viability as in Section 3.3.2). The patterns of fly mortality 1 day after spray application were similar to those observed in Section 3.3.1, with 90% mortality occurring in the treated groups within five to eight days irrespective of substrate type (Figure 3-3A). Mortality was slightly slower on the latex painted cinder block relative to other substrates, but all treatments were significantly different to controls (Table 3-2). By day 7, infectivity of all treatments had once again fallen substantially (Figure 3-3B). There were still significant effects on survival relative to controls (Table 3-2), but no treatment (including plastic sheeting) could achieve 50% mortality. On days 15 and 30, the fungal treatments became increasingly indistinguishable from controls (Figure 3-3C and D; Table 3-2). Wherever significant mortality appeared, 80-100% of cadavers in the fungal-exposed treatments showed external signs of mycosis.

3.3.4. Effect of fly density and repeat exposure on efficacy and viability of fungal spray residue on plastic sheeting

The results thus far indicated that fungal conidia can remain viable for several weeks after treatment, with no obvious effect of substrate, application method or fungus batch. However, when flies are exposed to the treated surfaces, infectivity of the fungal residues appears to decline rapidly. To investigate the influence of flies, we compared different fly densities and single vs. multiple exposures of fly populations over consecutive days. A first exposure to treated plastic one day after application caused 100% mortality of flies within 7-10 days (Figure 3-4A), with no significant difference between high (50 flies per exposure disk) and low (25 flies per exposure disk) density treatments (Table 3-3). Exposure of fresh batches of flies to the same substrates just one day later revealed a significant decline in infectivity, with MSTs increasing by
2 days relative to day 1 (Table 3-3; Figure 3-4B). Mortality rate was further reduced following another exposure to the same substrates on day 3 (Figure 3-4C). There was also an emergent density effect with high-density treatments showing significantly greater increases in MSTs compared with the low-density treatments (Table 3-3). When flies were exposed to treated sheeting on day 2 and 3 that had not experienced prior exposure to flies, patterns of mortality were more or less identical to the day 1 exposures (Figure 3-4 D and E) indicating that the decline in infectivity was due to the presence of flies on the substrate. This effect was further confirmed by measures of conidial viability on sheeting from the high-density repeat exposure regime, and conidia germination was significantly different among the sampling times ($F_{3, 11}=77.69; P<0.001$). Just one exposure reduced viability to about 50%, with two further exposures reducing this by another 20% (Figure 3-4F). Following repeated fly exposures, it also became increasingly difficult to obtain sufficient conidia from the substrate samples to quantify germination suggesting apparent removal of conidia by the flies during the exposure period.

### 3.3.5. Effect of fly density on viability and concentration of conidia in spray residue on plastic sheet

To partition deactivation of conidia from physical removal of conidia, we repeated the short-term exposure study using a more discriminating range of fly densities (5, 10 and 20 flies per 9 cm exposure plastic disk). Exposure to 10 or 20 flies reduced viability more rapidly than 5 flies (Figure 3-5A). There were significant effects of fly density ($F_{2, 23}=9.12, P=0.001$) and exposure number ($F_{3, 23}=118.57, P<0.0001$) on germination of conidia as well as a significant interaction between the two ($F_{6, 23} = 4.08, P=0.006$). Germination remained at approximately 70% following three, four-hour exposures to 5 flies, whereas it declined to 40-50% with the higher fly densities.
Flies also clearly removed conidia from the substrate. High-density exposures of 10 and 20 flies removed about half of the conidia after a single exposure, while the 5 fly treatment required three repeated exposures to reduce conidia density to 50% (Figure 3-5B). With three exposures of the highest fly density, available conidia were reduced from $1.5 \times 10^6$ to $5 \times 10^4$ conidia/cm$^2$. There was a significant difference in number of conidia/cm$^2$ between fly density treatments ($F_{2, 24}=53.36$, $P<0.001$) and number of exposure ($F_{3, 24}=166.33$, $P<0.001$) with a significant treatment-exposure interaction ($F_{6, 24}=12.18$, $P<0.001$).

3.4. Discussion

Exposure of adult house flies to a range of substrates freshly treated with an oil-based formulation of *B. bassiana* (isolate I93-825) produced high levels of infection leading to 100% mortality in 6-10 days. This pattern of mortality is similar to that reported previously with this isolate (Anderson et al., 2011; Anderson, Branford, & Thomas, 2013a; Anderson, Branford, Jenkins, & Thomas, 2013b). Given that adult flies can live for several weeks and produce many thousands of eggs over their lifetime (Fletcher, Axtell, & Stinner, 1990), this relatively rapid mortality suggests considerable potential for use of a fungal biopesticide in integrated fly management (Acharya, Rajotte, Jenkins, & Thomas, 2015).

Recent work investigating the use of *B. bassiana* as an indoor residual spray for control of mosquitoes showed that conidia could remain infective for several months after treatment (Blanford et al., 2012). Effective persistence varied between substrate types, with clay/mud showing persistence up to seven months. Persistence on wood and concrete (also used in the current study) was less, but it was still possible to infect mosquitoes on these substrates up to two to three months after spraying. In light of these findings, the results of our persistence assays
showing limited infection of house flies beyond 15 days after spraying were surprising. Systematic investigations of possible factors contributing to the more rapid decline showed no effects of application method, production batch or substrate. We cannot completely discount possible direct negative effects of certain test substrates, but we showed plastic sheeting to be a relatively benign substrate for conidial viability, yet the effective persistence on plastic still declined within days once flies were included in the assays. Rather, it appears flies themselves play a substantial role in reducing effective persistence.

Fly populations removed substantial numbers of conidia from exposed substrates, with higher densities of flies removing conidia more rapidly. House flies are covered with fine hairs, have large feet (tarsi) comprising gripping hairs, and groom extensively (Graczyk, Knight, Gilman, & Cranfield, 2001; Hedges, 1990; Sukontason et al., 2006). Further, the flies in our assays were maintained in constrained environments, which tended to promote constant movement as individuals disturbed one another. These factors appear to lead to rapid removal or displacement of the fine layer of conidia retained on the sprayed substrate. Studies using quantitative PCR to enumerate transfer of conidia to house flies from treated substrates indicate that individual flies might pick up $10^4$ to $10^5$ conidia in 4 h (Anderson et al., 2011).

In addition to physical removal, flies also deactivated conidia on the substrate, again in a density dependent manner. The exact mechanism of conidia deactivation is unknown, but high numbers of fecal spots were seen on the substrates at the end of exposure period. House flies can defecate up to ten times per hour (Sasaki, Kobayashi, & Agui, 2000), and their feces contain several types of pathogenic microbes (Forster et al., 2007; Sasaki et al., 2000) that could have fungistatic effects. Uric acid and other organic waste in feces could also have inhibitory effects on conidia viability as previous studies suggested that nitrogenous compounds such as ammonia
have fungistatic properties on plant pathogenic and saprophytic fungi (Bacon, 1986; Schippers, Meijer, & Liem, 1982). House flies also secrete saliva as part of a pre-digestive feeding process (Graczyk et al., 2001), and this too could impact on the fungus. In the desert locust, conidia passed through the gut or re-treated with gut-content have substantially reduced viability (Dillon & Charnley, 1986). Further, the distal tarsomeres of house flies comprise a small sack-like structure called the ‘pulvillus’, which is coated with hundreds of tiny oily hairs (Barro, Aly, Tidiane, & Sababenedjo, 2006; Sukontason et al., 2006), and it is possible that secretions from these glandular hairs have fungistatic effects. Whatever the mechanism, just 4-h exposure to the highest fly density was sufficient to reduce viability to 50%.

The significance of our findings for development of a biopesticide product depends on a number of factors. Persistence will influence the economics and logistics of operational use. In principle, a requirement to retreat at approximately weekly intervals would seem prohibitive, but certain residual (premise) insecticide sprays are already applied every one to two weeks (Axtell, 1986; Stafford, 2008). Moreover, persistence is affected by fly density, or perhaps more correctly, fly contact rate. Our lab assays followed a previous study (Anderson et al., 2011) in using high fly densities up to 0.63 flies/cm² (equivalent to more than 6000 flies/m²) with flies constrained in direct contact with the substrate for 4 h. How this equates to exposure in poultry houses is unclear. Fly density inside poultry facilities varies with season, production system, and housing and farm management practices (Axtell, 1986-1999). Field population assessments are made by placing ‘spot cards’ (7.5 x 12.5 cm file cards) in barns, and pesticide application generally commences when fecal spots on these cards exceeds 50-100 per week (which is equivalent to approximately 300-350 flies/m³ at 25°C; Lysyk & Axtell, 1985) depending on the animal production systems (see Stafford, 2008). Fecal spots on our test substrates often exceeded
100 after 4 h, especially in the high-density treatments (40 flies/9 cm exposure arena). It seems likely, therefore, that fly densities in the field will generally be much lower than in our test set-up and so we would expect deactivation rate of conidia to be reduced accordingly. Furthermore, given decline in persistence is influenced by density, it is possible that a spray regime could be developed in which application frequency scales with density (i.e. high spray frequency when populations exceed a certain threshold and then reduced frequency as and when populations decline). There is the possibility that the negative effects of the fly exudates could have residual action rendering (re)treatments less effective. Moreover, several indoor biotic and abiotic factors such as temperature and humidity fluctuations, toxic gas emission and dust particles with free-living microbes may have negative effects on post-application longevity and efficacy in fields. Studies investigating these factors to determine persistence in real poultry houses and quantify the influence of fly densities under natural exposure conditions are currently underway.
3.5. Acknowledgements

We thank the Schwardt Lab, Department of Entomology, Cornell University for providing fly pupae, and Dr Robert D. Anderson for suggestions on rearing flies. The authors also thank Dr Simon Blanford for advice on statistical analysis and editorial suggestions to an initial draft of the manuscript. The study is supported by US Agency for International Development (USAID cooperative agent No: EPP-A-00-0400016-00) and in part by Animal Health and Diagnostic Commission (AHDC), Pennsylvania Department of Agriculture.
3.6. References


Forster, M., Klimpel, S., Mehlhorn, H., Sievert, K., Messler, S., & Pfeffer, K. (2007). Pilot study on synanthropic flies (e.g. *Musca, Sarcophaga, Calliphora, Fannia, Lucilia, Stomoxys*)
as vectors of pathogenic microorganisms. *Parasitology Research, 101*(1), 243-246. doi:10.1007/s00436-007-0522-y


Table 3-1. Analysis of house fly survival following exposure to a range of *B. bassiana*-treated structural substrates relative to controls

<table>
<thead>
<tr>
<th>Substrate/Day post exposure</th>
<th>Day 1</th>
<th>Day 15</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinder block</td>
<td>$\chi^2=247.63; P&lt;0.001$</td>
<td>$\chi^2=29.244; P&lt;0.001$</td>
<td>$\chi^2=1.722; P=0.189$</td>
</tr>
<tr>
<td>Cinder block with water-based latex paint</td>
<td>$\chi^2=179.07; P&lt;0.001$</td>
<td>$\chi^2=13.960; P&lt;0.001$</td>
<td>$\chi^2=4.450; P=0.035$</td>
</tr>
<tr>
<td>Cinder block with oil-based latex paint</td>
<td>$\chi^2=255.99; P&lt;0.001$</td>
<td>$\chi^2=2.2130; P=0.137$</td>
<td>$\chi^2=2.692; P=0.101$</td>
</tr>
<tr>
<td>Wood raw</td>
<td>$\chi^2=237.89; P&lt;0.001$</td>
<td>$\chi^2=10.120; P=0.010$</td>
<td>$\chi^2=1.397; P=0.237$</td>
</tr>
<tr>
<td>Wood treated</td>
<td>$\chi^2=303.87; P&lt;0.001$</td>
<td>$\chi^2=11.243; P=0.010$</td>
<td>$\chi^2=2.119; P=0.146$</td>
</tr>
<tr>
<td>Cement board</td>
<td>$\chi^2=184.89; P&lt;0.001$</td>
<td>$\chi^2=0.988; P=0.320$</td>
<td>$\chi^2=0.551; P=0.458$</td>
</tr>
</tbody>
</table>
Table 3-2. Analysis of house fly survival following exposure to a range of *B. bassiana*-treated substrates relative to controls

<table>
<thead>
<tr>
<th>Substrate/time after exposure</th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 15</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinder block</td>
<td>$\chi^2=215.51; P&lt;0.001$</td>
<td>$\chi^2=9.49; P=0.002$</td>
<td>$\chi^2=22.96; P&lt;0.001$</td>
<td>$\chi^2=1.69; P=0.193$</td>
</tr>
<tr>
<td>Cinder block with water-based latex paint</td>
<td>$\chi^2=241.31; P&lt;0.001$</td>
<td>$\chi^2=5.05; P=0.025$</td>
<td>$\chi^2=0.66; P=0.420$</td>
<td>$\chi^2=3.26; P=0.071$</td>
</tr>
<tr>
<td>Plastic sheet</td>
<td>$\chi^2=160.31; P&lt;0.001$</td>
<td>$\chi^2=19.76; P&lt;0.001$</td>
<td>$\chi^2=1.89; P=0.169$</td>
<td>$\chi^2=0.48; P=0.492$</td>
</tr>
</tbody>
</table>
Table 3-3. Comparative survival of house flies after exposure to *B. bassiana*-treated plastic sheeting with different exposure histories

<table>
<thead>
<tr>
<th>Exposure treatment</th>
<th>Fly density</th>
<th>MST in days (95% CI)</th>
<th>Log rank statistic (significance compared to control)</th>
<th>Log rank statistic (25 vs. 50 density treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>25 flies/disk</td>
<td>4 (3.77-4.25)</td>
<td>$\chi^2=110.073; P&lt;0.001$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 flies/disk</td>
<td>4 (3.71-4.30)</td>
<td>$\chi^2=245.745; P&lt;0.001$</td>
<td>$\chi^2=0.094; P=0.760$</td>
</tr>
<tr>
<td>Day 2 (with 1 prior exposure)</td>
<td>25 flies/disk</td>
<td>6 (5.59-6.42)</td>
<td>$\chi^2=168.513; P&lt;0.001$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 flies/disk</td>
<td>6 (5.19-6.82)</td>
<td>$\chi^2=169.725; P&lt;0.001$</td>
<td>$\chi^2=19.180; P&lt;0.001$</td>
</tr>
<tr>
<td>Day 3 (with 2 prior exposures)</td>
<td>25 flies/disk</td>
<td>9 (7.80-10.21)</td>
<td>$\chi^2=158.299; P&lt;0.001$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 flies/disk</td>
<td>17 (13.81-20.20)</td>
<td>$\chi^2=28.6180; P&lt;0.001$</td>
<td>$\chi^2=25.907; P&lt;0.001$</td>
</tr>
<tr>
<td>Day 2 (without prior exposure)</td>
<td>25 flies/disk</td>
<td>4 (3.82-4.19)</td>
<td>$\chi^2=246.953; P&lt;0.001$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 flies/disk</td>
<td>4 (3.81-4.20)</td>
<td>$\chi^2=449.223; P&lt;0.001$</td>
<td>$\chi^2=0.103; P=0.749$</td>
</tr>
<tr>
<td>Day 3 (without prior exposure)</td>
<td>25 flies/disk</td>
<td>5 (4.86-5.16)</td>
<td>$\chi^2=252.389; P&lt;0.001$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 flies/disk</td>
<td>4 (3.81-4.21)</td>
<td>$\chi^2=378.064; P&lt;0.001$</td>
<td>$\chi^2=15.292; P&lt;0.001$</td>
</tr>
</tbody>
</table>
Figure 3-1. Cumulative proportional survival of fly populations exposed to *B. bassiana* 193-825 (batch PSU 31) conidial spray residue on different structural substrates 1 day (A); 15 days (B); and 30 days (C) after spray application.
Figure 3-1. Continued
Figure 3-2. Long term viability of *B. bassiana* I93-825 (batch PSU 31) on glass slides and contractor plastic sheeting following application with an airbrush sprayer or paintbrush (A); viability of *B. bassiana* I93-825 (batch PSU 37) applied with an airbrush sprayer to either glass slides or plastic (B)
Figure 3-3. Cumulative proportional mortality of fly populations exposed to *B. bassiana* I93-825 (batch PSU 37) spray residue on different structural substrates 1 day (A); 7 days (B); 15 days (C); and 30 days (D) after spray application.
Figure 3-3. Continued
Figure 3-4. Cumulative proportional survival of fly populations exposed to *B. bassiana* I93-825 (batch PSU 37) conidial spray residue on contractor plastic sheeting (9 cm disk) at 1 day (A), 2 days (B) and 3 days (C) after spray application; and without prior exposure of fly populations at 2 days (D), and 3 days (E) after spray application; and (F) viability of oil-formulated conidia of *B. bassiana* I93-825 (batch PSU 37) on plastic sheeting following repeat exposures to high (50) fly densities at 26-27°C, 50-60% RH.
Figure 3-5. A) Viability, and B) density of oil-formulated conidia of *B. bassiana* 193-825 (batch PSU 37) on plastic sheeting following sequential exposure of different densities of house flies.
Chapter 4: Impact of fly population on persistence and efficacy of a *Beauveria bassiana* biopesticide under laboratory conditions

Abstract

Previous chapter demonstrates exposure of fungal-treated surfaces to high fly densities (800-7000/m²) rapidly reduced persistence and infectivity through deactivation and physical removal of conidia in a density-dependent manner. This chapter evaluates the impacts of realistic fly population densities on *Beauveria bassiana* persistence under laboratory conditions. Long-term exposure of flies on fungal-treated plastic surfaces for 11 days demonstrates 17-60% reduction in viability and 50 to > 90% reduction in conidia concentration, with higher fly densities and greater cumulative exposure hastening the decline. Nonetheless, effective persistence reduced slowly and overall efficacy was minimally affected since very low densities of viable conidia were still able to cause rapid mortality, suggesting the potential for relatively long re-treatment intervals as fly populations are controlled. The results provide some basic information necessary to optimize application parameters; however, further studies to assess impacts of indoor biotic and abiotic factors including fly population are still needed in the poultry barns to develop operational spray guidelines and better predict the outcome of biocontrol.
4.1. Introduction

Fungal biopesticides, such as *Beauveria bassiana*, offer alternatives to chemical pesticides for sustainable house fly management in poultry production facilities and can be applied as residual spray treatments acting as contact pesticides to suppress populations by increasing adult mortality and reducing lifetime reproductive output (Acharya et al. 2015a). The effectiveness of fungal biopesticides varies with the longevity of spray residue in treated environments, which is influenced by several biotic and abiotic factors including surface type (Jenkins and Thomas 1996, Thomas et al. 1997, Inyang et al. 2000, Darbro and Thomas 2009, Jackson et al. 2010, Jaronski 2010, Blanford et al. 2012, Acharya et al. 2015b). Previous chapter shows that host population density also affects post-application persistence and infectivity. Exposure of fungus-treated substrates to high fly densities (1500-7000 flies/m²) rapidly reduced persistence and efficacy through deactivation and physical removal of conidia. However, the reductions were far less significant at lower, more realistic fly densities (800 flies/m² treated surface) (Acharya et al. 2015b). In poultry barns, fly populations rarely reach these critical densities since control tactics are usually initiated once fliespecks on population-monitoring spot cards exceed 50-100, which is equivalent to more than 300 flies/m³ depending on the temperature (Lysyk and Axtell 1985, Axtell 1999, Stafford 2008). Further, spore transfer in natural conditions is inefficient relative to forced exposure procedure used in laboratory conditions. In poultry barns, flies have unrestrictive movement and contact the treated surfaces randomly in a momentary fashion; each landing on a surface could last only for a few seconds or minutes. Such unconstrained movement may allow them to remove only a few conidia and leave minimal conidia-deactivating exudates on the resting surfaces. No-choice exposure in typical laboratory bioassays does not represent natural exposure and surface contact rate, and accordingly deactivation and removal of conidia
from sprayed surfaces is likely to be lower under field conditions. This chapter therefore examines the impacts of different fly densities on viability and concentration as well as the infectivity of conidial spray residues of an isolate of *Beauveria bassiana* following simulated field exposure procedure.

### 4.2. Materials and methods

#### 4.2.1. Fly rearing

Insecticide-susceptible house flies (source: Cornell University) maintained on artificial diets under laboratory conditions (26-27°C, 50-60% RH with a 12:12 light: dark photoperiod) as of Chapter 3 were used for all experiments. Larvae were reared on a mixture of wheat bran, MannoPro™ calf protein supplement, baker’s yeast and warm water, and adults were maintained on a mixture of powdered milk and granulated sugar (1:1), and water *ad libitum*.

#### 4.2.2. Fungal production, formulation and application

Dry conidia powder of *B. bassiana* (isolate I93-825, production batch PSU 42) was produced using our standard two-stage process (Jenkins et al. 1998), and stored in a refrigerator at 7°C until use. Prior to spray application, conidia powder was suspended in an oil formulation (4:1 Isopar M and Ondina oil; Blanford et al. 2011) and the concentration was adjusted to 1 x 10⁹ conidia/ml with an Improved Neubauer Hemocytometer. Before application, conidia were checked for viability by plating on Sabouraud dextrose agar (SDA), and assessed for germination at 400x under a microscope after 20 h incubation at 25°C (conidia viability was greater than 90%).

Conidia were delivered by applying oil formulations to plastic sheeting and exposing flies to the spray residues. This exposure method simulates the planned delivery system of applying
conidia to plastic sheeting attached to the poultry house basement walls adjacent to the litter layer (Acharya et al. 2015a). For each experiment, thirty pieces of trapezoid-shaped contractor-grade plastic sheets, each 327.6 cm$^2$, were fixed inside a 0.25 m$^2$ spray area on the rear wall of a reverse flow laminar cabinet and sprayed with a fungal formulation in batches of five with an artist’s airbrush at a volume application rate of 20 ml/m$^2$ (Acharya et al. 2015b). The sprayed substrates were removed from the wall of the cabinet, and taped on the lower 3/5th of the inner walls of 1 L foam cups with the bottom removed @ one sheet per cup and allowed to dry at room temperature overnight. The cups were covered with nylon mesh on the top and bottom.

4.2.3. Impact of fly density on conidia density and viability

Spore persistence and infectivity after long-term exposure of various fly densities was evaluated in the laboratory using a cup bioassay. Six replicate experimental cups were set up for each of four fly densities, equivalent to 31, 92, 153, and 214 flies/m$^2$ treated surface by adding one, three, five or seven, adult flies of mixed sex, respectively, into individual experimental cups. The flies had access to a 10% sugar solution during the experiment and were maintained under the same conditions as the main fly colony (26-27°C, 50-60% RH). Cups with no flies served as controls. Starting one-day post fly release into the cups and every other day thereafter (days 3, 5, 7, 9 and 11), one experimental cup was randomly selected from each treatment density for destructive sampling. The flies in the remaining cups were removed and replaced with the same number of fresh flies at each sampling point to ensure that fly activity was not reduced due to infection or mortality.

The number and viability of the conidia was evaluated by removing the plastic liner from the cup and two 9 cm plastic disks were cut from the sheet. Each disk was cut into small pieces (approximately 0.5-1 cm$^2$) and suspended in 2-4 ml Isopar M in a 20 ml glass bottle. The bottles
were then vortexed and sonicated for one minute to wash conidia from the surface of the plastic and break up any conidial clumps. A single drop of the resulting conidia suspension was spread over the surface of SDA in a 5 cm diameter petri dish (three dishes per suspension) and incubated for 20 h at 25°C. A total of 300 conidia were examined from each of three replicate petri dishes per plastic disk under a microscope at 400x magnification to determine the proportions of germinating and non-germinating conidia, and the data were used to calculate percent viability of the conidia. The remainder of the conidia suspension was stored in a refrigerator at 7°C, and conidial concentration was determined later using an Improved Neubauer Hemocytometer. The number of conidia per cm² was calculated taking into account the volume of Isopar M used to suspend the plastic pieces; three replicate suspensions were counted per sample. The entire experiment was repeated four times.

4.2.4. Impact of fly density on infectivity of conidial spray residue

The impact of long-term exposure of different fly densities on infectivity of conidial spray residues was evaluated by preparing twenty cups as described in Section 4.2.2 and 4.2.3 above. The same four fly densities were created by adding one, three, five or seven flies of mixed sex to each of four replicate cups. Four experimental cups with blank oil sprayed plastic sheets as inner walls served as no-fungus control. Flies were maintained in the cups under the same conditions as above for a total of 11 days, again replacing the flies every two days with fresh flies. At the end of the exposure period, the flies were removed and discarded and the plastic sheet removed from the cups. Two, 9 cm disks were cut from each plastic liner and used for either infectivity bioassay or determination of conidial concentration following the same procedure as above. For the bioassay, a cohort of fresh flies was exposed to treated plastic disks following a forced exposure protocol (Anderson et al. 2011). Briefly, a 9 cm plastic petri dish was inverted and the
lid lined with the experimental plastic sheet. Fresh, three to five day-old adult flies of mixed sex were aspirated from the stock colonies and placed in a freezer at -20°C for three to four minutes to anesthetize them. Forty flies were placed on each plastic disk and covered with the petri dish bottom before the flies revived and began to move freely within the enclosure. After 4h exposure, flies were transferred into foam rearing cups (1 L) with nylon mesh on top and monitored daily for mortality for three weeks. Flies had access to 10% sugar solution, and dead flies were removed, counted and a fungal mycosis was confirmed by drying cadavers for five to seven days before transferring them to a humid environment for seven days and monitoring for external sporulation under a dissecting microscope at 15x magnification.

4.2.5. Statistical analysis

Conidia germination and enumeration data were analyzed using a general linear model (SPSS v. 22, IBM Inc. 2014) with fly density and sampling time as the main treatment factors. The proportional germination data were cubed-transformed before analysis to meet normality and homoscedasticity assumptions. Fly-days per unit area was estimated by multiplying fly densities (in m²) by each sampling day (1, 3, 5, 7, 9 and 11). Effective conidia persistence for each fly density was estimated by multiplying numbers of conidia at each sampling date by germination percent for that day (expressed as 10⁵/cm²), square-root transformed and regressed against fly-days per m² (SPSS v. 22, IBM Inc. 2014). Survival data were analyzed using Kaplan-Meier survival analysis (SPSS v. 22, IBM Inc. 2014) with differences in median survival time (MST) among treatment densities compared using the log-rank test.
4.3. Results

4.3.1. Impact of fly density on conidia density and viability

Conidia viability and removal were correlated with fly density. Exposure of fly populations to treated-plastic sheets affected conidia viability in a density-dependent manner (Figure 4-1A). Viability was significantly different among treatment densities and sampling times with no significant interactions between densities and sampling times ($F_{4,105}=13.19$, $P<0.001$ for fly density; $F_{6,105}=56.23$, $P<0.001$ for sampling time; $F_{24,105}=1.27$, $P=0.21$ for interaction). Low fly density (equivalent to 31 flies/m$^2$) had no significant effect on the viability of conidia on sprayed surfaces relative to the control (0 flies). However, the presence of medium fly densities (equivalent to 92 and 153 flies/m$^2$) resulted in 30% decline in conidial viability over the 11-day exposure period, while the highest density (equivalent to 214 flies/m$^2$) led to >60% reduction.

A similar density-dependent pattern was observed with conidia removal from the surfaces (Figure 4-1B). The numbers of conidia removed by flies were significantly different among treatment densities and sampling times with some significant interactions between densities and sampling times ($F_{4,105}=27.86$, $P<0.001$ for fly density; $F_{6,105}=26.41$, $P<0.001$ for sampling time; $F_{24,105}=1.75$, $P=0.031$ for interaction). Sprayed substrates exposed to fly densities of 214 flies/m$^2$ over an 11-day period lost approximately $1 \times 10^6$ conidia/cm$^2$, equivalent to greater than 90% of the conidia applied. Even at lower fly densities, between 75 and 80% of the conidia were removed by the end of the exposure period. Figure 4-1C uses the combined data from figure 4-1A and 4-1B to illustrate the ‘effective persistence’ of conidia by fly-days per unit area. This non-linear negative relationship is described by the regression function $y = 2.9743e^{-0.001x}$ ($F_{1,94}=265.42$; $P<0.001$; $R^2=0.739$)
4.3.2. Impact of fly density on infectivity of conidial spray residue

The impact of exposure of different fly densities over 11 days on subsequent infectivity of the spray residue was evaluated by exposing naïve flies to the 11-day-old surfaces. Fly survival in treatment groups was significantly different than controls ($\chi^2 = 336.59; P < 0.001$) and residue on the surfaces caused about 90% mortality between 5 and 11 days post-exposure, depending on treatment densities (Figure 4-2A). Survival in the fungal-treated surfaces that received treatment densities of 31 flies/m$^2$ (MST 4± 0.14), 92 flies/m$^2$ (MST 5± 0.16) and no-fly control (MST 5± 0.13) was similar, but significantly different to survival of flies exposed to the surfaces from the higher fly densities of 153 flies/m$^2$ (MST 6± 0.15) and 214 flies/m$^2$ densities (MST 7± 0.39). Fungal infectivity (measured by fly mortality) was inversely related to concentration of conidia on the surfaces (Figure 4-2B). Of note, a density of $< 1 \times 10^5$ conidia/cm$^2$ was still sufficient to produce extensive and relatively rapid mortality in flies.

4.4. Discussion

Long-term exposure of different fly population densities to fungal-treated plastic surfaces for 11 days reduced effective persistence in a density-dependent manner, with reduction slightly more attributable to physical removal of conidia than deactivation as seen previously (Acharya et al. 2015b). With the highest density exposure (equivalent to 214 flies/m$^2$), conidia numbers on the treated surfaces declined from $11 \times 10^5$ to $1 \times 10^5$ conidia/cm$^2$. Lower fly densities resulted in increasingly smaller reductions in conidial numbers. An earlier study using a bait formulation of fungus demonstrated that one or two flies could pick up an average of $4 \times 10^4 - 1 \times 10^5$ conidia in thirty minutes (Renn et al. 1999). Another study using quantitative PCR to enumerate transfer of conidia to house flies from treated substrates indicated that individual flies might pick up $10^4 - 10^5$ conidia during 4 h constant exposure (Anderson et al. 2011). In spite of these removal rates,
the fungal spray residues still caused extensive and relatively rapid mortality of flies 11 days post treatment at the highest fly density.

As shown previously in Chapter 3, artificially high fly population densities rapidly removed and deactivated conidia from spray residue after only 4 h, resulting in severe reductions in efficacy of the spray residue. However, these fly densities were equivalent to 6000 flies/m² (40 flies per 9 cm diameter petri dish), clearly far higher than anything that could reasonably be expected in a field situation. In the current study, even when flies were constantly present in experimental cups for 11 days, the rate of reduction in effective persistence was much slower compared to Acharya et al. (2015b). These results indicate that the impacts of realistic field population densities are far less detrimental than previously reported. Fly control interventions are usually initiated once fecal spots on spot cards exceed 50-100 per card, which is equivalent to more than 300 flies/m³ depending on temperature (Lysyk and Axtell 1985, Axtell 1999, Stafford 2008). It is difficult to determine how this action threshold based on flies per cubic meter translates to flies per square meter on a treated surface, but it is likely that in many settings, the density of flies will be much lower than our highest density treatment, lessening the impact of flies on effective persistence (see also Acharya et al. 2015b). Since poultry barns are much larger than our experimental settings and daily flight activity and movement behaviors vary with resource availability, temperature and other biotic, abiotic and management factors (Hindle and Merriman 1914, Sacca 1963, Murvosh and Thaggar 1966), surface contact rate and hence conidia removal and deactivation due to flies are likely to be much lower in the field conditions. Nevertheless, fungal conidia adhere readily to fly cuticle, and even a single contact with a sprayed surface could be sufficient to acquire the lethal dose.
The deactivation of conidia by the presence of flies might be attributed to pathogenic bacteria (Sasaki et al. 2000, Forster et al. 2007) as well as organic waste in feces such as uric acid. Digestive juices and saliva secreted by flies as a part of pre-digestive feeding process (Graczyk et al. 2001) and secretions from oily, glandular hairs of tarsomeral pulvilli (Barro et al. 2006, Sukontason et al. 2006) could also impact on the fungus. In desert locusts, conidia that passed through gut or were re-treated with gut-contents had substantially reduced viability (Dillon and Charnley 1986). With respect to removal of conidia from the sprayed surfaces, house flies are covered in fine hairs, have large feet (tarsi) comprised of gripping hairs (Hedges 1990, Sukontason et al. 2006), and these structures together with their excessive mobility and self-grooming behavior allow them to pick up substantial numbers of conidia and distribute them throughout the body surfaces.

Overall, the study provides some basic insights into the impact of fly populations on fungal biopesticides following simulated field exposure procedure, which with additional field studies, can be used to optimally adjust application parameters. The study constructs an effective persistence index combining conidia removal and conidia deactivation (reduction in viability) and associates this combination with an index, fly-days, which integrates fly densities with the length of time that flies are present. The effective persistence calculation can be used to track spore decay rate and determine optimal spray frequency on the basis of population density, duration of exposure and viable spores on the treated surfaces (Figure 4-1C). For example, even if 214 flies challenged one meter square treated surfaces for 11 days (2354 fly-days), there were still $0.2 \times 10^5$ to $0.5 \times 10^5$ viable spores per cm$^2$ surfaces, which would be sufficient to produce the desired fly mortality depending on fungal species/isolate, formulation and temperature as evidenced previously (Barson et al. 1994, Carswell et al. 1998) and also shown in bioassays.
(Figure 4-2A and 4-2B). The index indicates the possibility of tailoring spray frequency with one to two weeks based on spore persistence; however, further studies to determine how to calibrate standard fly spot card counts with our metric of ‘fly-days per m${^2}$’ will be needed in order to develop appropriate density-dependent spray thresholds and retreatment schedules. Additionally, potential impacts of individual indoor factors (air borne pollutants, free-living microbes; Hong et al. 2012) should be considered in the index.
4.5. References


IBM Corp. Released 2014. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, USA.


Figure 4-1. A) Viability, and B) density of oil-formulated conidia of *B. bassiana* I93-825 (batch PSU 42) on plastic sheets exposed to fly populations of different densities, and C) relationship between effective persistence (= density of viable conidia) and fly-days per unit area (= density of flies x cumulative days of exposure). Plastic sheeting was maintained in the laboratory at 26-27°C, 50-60% RH.
Figure 4-1. Continued
Figure 4-2. A) Cumulative proportional survival of house flies following exposure to Beauveria-treated plastic sheets (isolate I93-825, batch PSU 42) maintained for the previous 11 days in contact with fly populations of different densities at 26-27°C, 50-60% RH, and B) the relationship between the density of conidia remaining after the 11-day fly exposure treatments and median survival time (MST) of naïve flies exposed to the treated plastic at day 11.
Chapter 5: Persistence and efficacy of a *Beauveria bassiana* biopesticide against the house fly, *Musca domestica*, in a commercial high-rise layer house

Abstract

Studies suggest the potential for use of oil formulations of the entomopathogenic fungus, *Beauveria bassiana* as residual sprays for control of house flies in poultry production facilities. The effectiveness of the biopesticide treatment varies with longevity of spray residues in treated environments, which is influenced by several biotic and abiotic factors. This chapter investigates the impacts of indoor factors of the poultry houses on biopesticide persistence and efficacy. We found that fungal spray treatments remained viable for up to 13 weeks under laboratory conditions. Periodic exposure of flies to the spray residue showed high levels mortality, with very little decline in mortality rate over time. Equivalent treatments placed in a commercial poultry house showed much more rapid decline. One trial at the end of summer showed conidia to remain viable up to seven weeks. However, repeats during the winter months revealed decay in one to two weeks, with fly mortality rates influenced accordingly. The exact reasons for the more rapid decay remain unclear but could be linked to high concentrations of ammonia in the basement areas, especially during winter when ventilation is minimal. The results suggest the potential for adaptive treatment regimes with weekly spray intervals in conditions with very high ammonia levels and/or high fly populations, and potentially monthly spray intervals when ammonia levels and fly populations are reduced. However, further field studies to quantify impacts of individual indoor factors are still needed prior full field considerations.
5.1. Introduction

Post-application longevity and efficacy of fungal biopesticides can be influenced by a range of factors including formulation, substrate, fungal isolate, UV radiation and the prevailing abiotic conditions (Jenkins and Thomas 1996, Thomas et al. 1997, Inyang et al. 2000, Darbro and Thomas 2009, Jackson et al. 2010, Jaronski 2010, Blanford et al. 2012, Acharya et al. 2015). In enclosed poultry houses, temperature is relatively well controlled and UV radiation is minimal, so these factors might have small effects on fungal persistence relative to typical outdoor settings. On the other hand, chemically and biologically harsh indoor environments (air borne pollutants, free living microbes etc.) presented by on-site manure storage and handling operations (Green et al. 2009, Hong et al. 2012) as well as seasonal variations in thermal environments and air quality regimes (Axtell 1999, Liang et al. 2005, Xin et al. 2011) could play a significant role. For example, manure decomposition generates different toxic gases such as ammonia (Hong et al. 2012) and high ammonia concentrations reduce spore viability in several plant pathogenic and saprophytic fungi (e.g. Bacon 1986). Dust particles generated within poultry facilities are known to be carriers of several pathogenic microbes (Hong et al., 2012; Nonnenmann et al. 2010), and could have negative effects on the fungus. Similarly, indoor temperature and humidity levels will vary with season (Axtell and Arends 1990, North and Bell 1990, Green 2009) and such fluctuations even for brief time can affect fungal performance attributes (Anderson et al. 2013, Keyser et al. 2014). Further, fly populations in the field vary with season and management factors (Axtell 1986, Axtell and Arends, 1990), which impact fungal efficacy as it was shown recently that flies themselves can affect fungal persistence by both deactivating and physically removing conidia, with effects greater at higher fly densities (Acharya et al. 2015).
Currently, one *Beauveria*-based product is commercially available to use in animal rearing facilities against flies (Kaufman et al. 2005); however, there is limited information available on its longevity and efficacy in interior environments. One study on house flies examined the persistence of *B. bassiana* spores formulated in Tween 80 and water and demonstrated that spores remain infective for about four weeks on plywood exposed inside animal barns (Watson et al. 1995). Another unpublished report showed that spores could survive for more than seven weeks in poultry litter and more than three weeks on sprayed surfaces (Arends and Black 2000, http://www.rinconvitova.com/balance%20fly%20control.htm).

This chapter examines the impact of actual conditions in a commercial high-rise layer barn on persistence and efficacy of *Beauveria bassiana* spray residue against house flies. The results will contribute to the development of informed use strategies and spray intervals to enable the implementation of *Beauveria*-based biopesticides as part of an integrated pest management system for house fly control in poultry houses.

### 5.2. Materials and methods

#### 5.2.1. Fly rearing

Insecticide-susceptible house flies, *Musca domestica* L. (source: Cornell University) maintained on artificial diets under laboratory conditions (26-27°C, 50-60% RH with a 12:12 light: dark photoperiod) as of Chapter 4 were used for all experiments.

#### 5.2.2. Fungal production, formulation and application

Dry conidia powder of *B. bassiana*, isolate I93-825 (production batch PSU 46) was produced using our standard two-stage process (Jenkins et al. 1998), and formulated in 4:1 Isopar M and Ondina oil (Blanford et al. 2011). The concentration was enumerated with an Improved Neubauer
Hemocytometer and adjusted to $1.38 \times 10^9$ conidia/ml. Before application, conidia were checked for viability by plating on Sabouraud dextrose agar (SDA), and assessed for germination at 400x under a microscope after 20 h incubation at 25°C. Conidia germination rates were greater than 90%. Eighteen 0.25 m$^2$ pieces of contractor-grade plastic sheet were fixed inside a 0.25 m$^2$ spray area on the rear wall of a reverse flow laminar cabinet and sprayed with a fungal formulation, one sheet at a time, with an artist’s airbrush at a volume application rate of 20 ml/m$^2$. The sprayed sheets were removed from the wall of the cabinet and allowed to dry at room temperature overnight before used for the experiment.

5.2.3. Persistence and efficacy of conidial spray residue in a working high-rise layer house

In order to evaluate the impact of indoor biotic and abiotic factors on persistence, fungal-treated plastic sheets were affixed to the basement walls a half meter above the manure level at six equidistant locations with three sheets per location in a high-rise layer barn (183 m long x 19 m wide, Bedford County, PA) in September 2013. The house was a two-story building with egg laying hens housed on the second story over metal slats floor that allowed droppings to fall through and accumulate in the basement. Six of the sheets, one from each location, were covered with fine muslin cloths to prevent both large dust particles and flies from contacting the treated plastic. Another six sheets were similarly covered with large-sized mosquito netting to prevent flies from contacting the plastic, but allowing large dust particles. The remaining six sheets were left uncovered. Starting from the first week and weekly thereafter, one destructive sample of 9 cm diameter disk was taken from each treated sheet and checked for conidial viability. To check conidia viability, each disk was cut into small pieces (approximately 0.5-1 cm$^2$) and placed in 2-4 ml Isopar M in a 20 ml glass bottle. The bottles were then vortexed and sonicated for one minute.
to wash conidia from the surface of the plastic and break up any conidial clumps. A single drop of the resulting conidia suspension was spread over the surface of SDA in a 5 cm diameter petri dish (three dishes per suspension) and incubated for 20 h at 25°C. A total of 300 conidia were examined from each of three replicate petri dishes per plastic disk under a microscope at 400x magnification to determine the proportions of germinating and non-germinating conidia, and the data were used to calculate percent viability of the conidia. The fly density was monitored indirectly by placing 7.5 x 12.5 cm spot cards on the basement wall next to each plastic sheet, one card per location, which was replaced weekly (Kaufman et al. 2001). Humidity and temperature in the basement was monitored by Omega™ data logger 62 set to record at hourly intervals (Omega Engineering, Inc.).

In January 2014, a second replication of the field exposure was conducted to evaluate winter conditions in the same poultry barn, except that only one plastic sheet was fixed per location and none was covered with muslin or netting. Four, equivalent conidia-treated sheets were also stored under laboratory conditions (26-27°C, 50-60% RH). In this experiment, separate samples were taken for evaluation of viability and efficacy of the conidial spray residue using the destructive sampling methods and viability described above. For the infectivity bioassay, a cohort of fresh flies was exposed to treated plastic disks under laboratory conditions following a forced exposure protocol (Anderson et al. 2011). Briefly, a 9 cm plastic petri dish was inverted and the lid lined with the experimental plastic sheet. Fresh, three to five day-old adult flies of mixed sex were aspirated from the stock colonies and placed in a freezer at -20°C for three to four minutes to anesthetize them. Forty flies were placed on each plastic disk and covered with the petri dish bottom before the flies revived and began to move freely within the enclosure. After 4h exposure, flies were transferred into foam rearing cups (1 L) with nylon mesh on top
and monitored daily for mortality for three weeks. Flies had access to 10% sugar solution, and dead flies were removed, counted and a fungal mycosis was confirmed by drying cadavers for five to seven days before transferring them to a humid environment for seven days and monitoring for external sporulation under a dissecting microscope at 15x magnification. A set of plastic sheets sprayed with blank oil formulation served as controls for each of the two environments. The fly populations, as well as temperature and humidity regimes were monitored as above.

Finally, in March (2014), a third persistence experiment (evaluating conidial viability only) was repeated exactly as the January experiment, except ammonia gas concentration in the ambient air was also measured at daily intervals for the first week of the experiment by attaching passive Gastec ammonia dosimeter tubes (Zefon International, Inc.) to the basement walls next to the plastic sheets.

5.2.4. Statistical analysis

The proportions of conidia germinating from the persistence experiment in September (2013) were Log (x+1) transformed and analyzed using a general linear model (SPSS v. 22, IBM Inc. 2014) with cover treatment as a main factor. Conidia viability data from the field and laboratory persistence experiments (January and March 2014) were described and compared qualitatively. Fly survival data from the field and laboratory bioassays were analyzed separately at each sampling date using Kaplan-Meier survival analysis (SPSS v. 22, IBM Inc. 2014) with differences in median survival time among treatments (fungus vs. control) compared using Log-rank test.
5.3. Results

5.3.1. Persistence of conidial spray residue in a working high-rise layer house

In the September 2013 trial, the fungal treated-plastic sheets which were covered with different sized mesh to differentiate impact of natural fly populations from other factors (dust, dander etc.) indicated that, regardless of cover treatments, conidia germination on the surfaces fell to 80% within one week of placement and approached zero percent by week six or seven (Figure 5-1), giving a half-life of 1.8-2.3 weeks. There was no significant effect of cover treatments on conidia viability ($F_{2,101}=0.34$, $P=0.72$). Daily mean, maximum and minimum temperatures during this period were 24.15, 25.98 and 22.34°C, respectively, with the relative humidity varying between 50-73%.

In the January and March 2014 trials, the decline in viability of conidia in the spray residues in the layer house was more precipitous, falling to < 5% within one week (Figure 5-2). These field data contrast with those from parallel laboratory studies, which showed conidia to remain viable for >13 weeks, with a half-life of about six weeks post-application (Figure 5-2). The daily mean, maximum and minimum temperatures during January were 17.67, 19.11 and 16.26°C, respectively, with the relative humidity varying between 75-100%. For March, the daily mean, maximum and minimum temperatures were 17.68, 18.98 and 16.43°C, respectively, with the relative humidity varying between 73-100%. Additionally, the ammonia gas concentration in the basement of the layer house during the March trial was extremely high (>100 ppm), which exceeds the Occupational Safety and Health Administration permissible human ‘time weighted average’ exposure of 25 ppm for an 8 hr day (CDC 2007). The natural fly populations in the poultry house were negligible during all monitoring periods so the data are not presented.
5.3.2. Infectivity of conidial spray residue against house flies under the high-rise layer house in winter

The infectivity of spray residues on the treated plastic surfaces left in the layer house for one, two, three and four weeks in the January trial was evaluated by bioassay in the laboratory. The spray residue on plastic sheets after one week of exposure to layer house conditions resulted in about 70% fly mortality by the end of the 21-day monitoring period (Figure 5-3) and was significantly different from the control (Table 5-1). Infectivity of the spray residue further declined with each week of exposure to layer house conditions, but mortality remained significantly different to the control population even after four weeks exposure to layer house conditions (Table 5-1). However, corresponding plastic sheets that had been kept under laboratory conditions resulted in 96-100% fly mortality for thirteen weeks (Figure 5-4). Fly survival in treated groups was significantly different from control survival for all weeks in the January test (Table 5-2). Speed of kill decreased as the spray residues aged (Table 5-2) but the change was modest and even flies exposed at week 13 when conidial viability was approaching zero percent, had median survival times of six days only. Whenever significant mortality occurred, 80-90% cadavers in the fungal-exposed treatments showed external signs of mycosis (in all assays).

5.4. Discussion

The current study examined the persistence of fungal spray residues in laboratory and field (poultry house) settings considering the influence of a range of indoor factors. Compared to more than 13 weeks of persistence and efficacy under laboratory conditions, oil-formulated conidia of *B. bassiana* survived from one to seven weeks under the commercial high-rise layer barn
depending on season. Spore survival was significantly longer in fall than winter. Analysis of conidial spray residues from the poultry houses showed a rapid decline in infectivity against flies and hardly produced 20-70% mortality over a four-week evaluation period in winter.

Fly populations were negligible in the poultry facility during our study and so our field-based assessments focused on abiotic factors alone. In September, conidia remained viable for up to seven weeks in the poultry house, with no significant effect of air-borne particulates on persistence. Temperature and humidity during this period ranged from 23-30°C and 50-73%, respectively. Both temperature and humidity are known to affect spore viability (Fargues et al. 1997, Hong et al. 1999, Fargues and Luz 2000, Bouamama et al. 2010, Keyser et al. 2014) and these more variable conditions might explain the more rapid decay in the poultry house than in our equivalent laboratory studies, which demonstrated effective persistence for 13 weeks.

The field studies in January and March revealed much more rapid decay, with persistence of one to two weeks only. This decline was surprising given that temperatures during these periods were cooler (14-22°C), which should favor persistence. While the higher relative humidity (73-100%) could have countered this positive effect, other studies have shown that fungal spray residues can remain viable for many weeks under equivalent conditions (e.g. Darbro and Thomas 2009, Blanford et al. 2012). These results suggest an additional factor could be playing a role.

Ammonia has been shown to reduce spore viability in several plant pathogenic and saprophytic fungi (e.g. Schippers et al. 1982, Bacon 1986). We found that in the layer house, the majority of the automatic exhaust fans were not running in winter due to cold weather. This left the house poorly ventilated and the ammonia concentration at the basement was >100 ppm (note that although this exceeds the toxic threshold for egg production (CDC 2007, UEP 2010), our
measurements represent only a snap shot and were recorded in the basement and not where the birds are housed). Ammonia is a principal gas of manure decomposition (Reece et al. 1979, Xin et al. 2011) and even though emission rate is higher in summer, the concentration tends to be lower as a result of increased ventilation during warmer periods (Reece et al. 1979, Liang et al. 2005). We did some pilot experiments under laboratory conditions, where different amounts (0.5-1.5 kg) of two to three weeks old litter were stored into individual plastic containers (35.6 cm long, 20.3 cm wide and 11.7 cm deep) with B. bassiana-treated plastic sheets attached to their inner walls. The treated plastic sheets were destructively sampled on a daily basis and the spray residues were evaluated for conidia germination. Spore viability declined to almost zero percent within a few days in closed containers, where the ammonia concentration was recorded up to 600 ppm. However, in open containers with lids off, the conidia viability was greater than 60% one week post-application and the ammonia concentration varied between 20-60 ppm.

Overall, our study suggests the potential for development of adaptive spray regimes wherein treatment schedules are adjusted to local biotic and abiotic conditions. With very high fly populations, a spray treatment could be required every one to three weeks. At low population densities, retreatment frequency could extend to once every one to two months. The results suggest that retreatment frequencies would need to be highest during the winter months, possibly due to effects of ammonia. Treatments every week would be in line with spray frequencies of certain chemical insecticides already in use (Axtell 1986, Stafford 2008). However, fly populations themselves tend to be lower in the winter so this might not be an operational issue if densities remain below the action threshold. Regular population monitoring would be an essential element in determining spray frequency, both to prevent population outbreak and account for density dependent conidia removal and deactivation (Acharya et al. 2015). Further,
housing and manure management operations to improve indoor air quality and create thermally stable environments could increase the persistence of biopesticide treatments. It is also highly likely that with further research, novel formulations of fungal pathogens such as microencapsulation as well as additives or adjuvants could also be used to protect conidia against adverse factors and enhance biological activity (Stock 1997, Perrin 2000, Ravensberg 2011). Further research is needed to better characterize the influence of ammonia concentrations on persistence in order to develop operational spray guidelines.
5.5. References


Table 5-1. Median survival times of house flies following exposure to fungal-treated and control plastic sheets maintained in a high-rise layer house for up to four weeks

<table>
<thead>
<tr>
<th>Time after treated-plastic sheets placed in poultry barn</th>
<th>Treatment</th>
<th>Median survival time days (95% C.I.)</th>
<th>Log rank statistic (significance compared to control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td>Control</td>
<td>&gt;21*</td>
<td>χ²=128.1; P &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>*B. bassiana</td>
<td>13.0 (11.41-14.60)</td>
<td></td>
</tr>
<tr>
<td>Week 2</td>
<td>Control</td>
<td>&gt;21*</td>
<td>χ²=21.74; P &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>*B. bassiana</td>
<td>&gt;21*</td>
<td></td>
</tr>
<tr>
<td>Week 3</td>
<td>Control</td>
<td>&gt;21*</td>
<td>χ²=11.052; P = 0.001</td>
</tr>
<tr>
<td></td>
<td>*B. bassiana</td>
<td>&gt;21*</td>
<td></td>
</tr>
<tr>
<td>Week 4</td>
<td>Control</td>
<td>&gt;21*</td>
<td>χ²=6.392; P = 0.011</td>
</tr>
<tr>
<td></td>
<td>*B. bassiana</td>
<td>&gt;21*</td>
<td></td>
</tr>
</tbody>
</table>

* >21 days indicates that 50% mortality was not reached before the end of the 21-day monitoring period.
Table 5-2. Median survival times of house flies following exposure to fungal-treated and control plastic sheets maintained under laboratory conditions (26-27°C, 50-60% RH) for up to 13 weeks

<table>
<thead>
<tr>
<th>Time after fungal application</th>
<th>Treatment</th>
<th>Median survival time days (95% C.I.)</th>
<th>Log rank statistic (significance compared to control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>Control</td>
<td>&gt;21*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. bassiana</td>
<td>4.0 (3.81-4.19)</td>
<td>$\chi^2=241.61; P &lt; 0.001$</td>
</tr>
<tr>
<td>Week 1</td>
<td>Control</td>
<td>&gt;21*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. bassiana</td>
<td>4.0 (3.84-4.17)</td>
<td>$\chi^2=132.97; P &lt; 0.001$</td>
</tr>
<tr>
<td>Week 2</td>
<td>Control</td>
<td>13.0 (11.39-14.61)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. bassiana</td>
<td>5.0 (4.91-5.10)</td>
<td>$\chi^2=201.69; P &lt; 0.001$</td>
</tr>
<tr>
<td>Week 3</td>
<td>Control</td>
<td>&gt;21*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. bassiana</td>
<td>5.0 (4.89-5.12)</td>
<td>$\chi^2=246.99; P &lt; 0.001$</td>
</tr>
<tr>
<td>Week 4</td>
<td>Control</td>
<td>&gt;21*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. bassiana</td>
<td>5.0 (4.84-5.17)</td>
<td>$\chi^2=253.78; P &lt; 0.001$</td>
</tr>
<tr>
<td>Week 5</td>
<td>Control</td>
<td>&gt;21*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. bassiana</td>
<td>5.0 (4.83-5.18)</td>
<td>$\chi^2=237.53; P &lt; 0.001$</td>
</tr>
<tr>
<td>Week 6</td>
<td>Control</td>
<td>&gt;21*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. bassiana</td>
<td>5.0 (4.77-5.24)</td>
<td>$\chi^2=281.15; P &lt; 0.001$</td>
</tr>
<tr>
<td>Week 7</td>
<td>Control</td>
<td>&gt;21*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. bassiana</td>
<td>4.0 (3.83-4.18)</td>
<td>$\chi^2=232.12; P &lt; 0.001$</td>
</tr>
<tr>
<td>Week 8</td>
<td>Control</td>
<td>&gt;21*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. bassiana</td>
<td>5.0 (4.79-5.22)</td>
<td>$\chi^2=310.72; P &lt; 0.001$</td>
</tr>
<tr>
<td>Week 9</td>
<td>Control</td>
<td>&gt;21*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. bassiana</td>
<td>6.0 (5.84-6.18)</td>
<td>$\chi^2=344.76; P &lt; 0.001$</td>
</tr>
<tr>
<td>Week 10</td>
<td>Control</td>
<td>&gt;21*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. bassiana</td>
<td>5.0 (4.84-5.17)</td>
<td>$\chi^2=338.43; P &lt; 0.001$</td>
</tr>
</tbody>
</table>

* >21 days indicates that 50% mortality was not reached before the end of the 21-day monitoring period.
Table 5-2. Continued

<table>
<thead>
<tr>
<th>Week</th>
<th>Treatment</th>
<th>Mortality</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 11</td>
<td>Control</td>
<td>&gt;21*</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>B. bassiana</em></td>
<td>6.0 (5.85-6.16)</td>
<td>$\chi^2=270.46; P &lt; 0.001$</td>
</tr>
<tr>
<td>Week 12</td>
<td>Control</td>
<td>&gt;21*</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>B. bassiana</em></td>
<td>6.0 (5.64-6.37)</td>
<td>$\chi^2=237.41; P &lt; 0.001$</td>
</tr>
<tr>
<td>Week 13</td>
<td>Control</td>
<td>&gt;21*</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>B. bassiana</em></td>
<td>6.0 (5.6.0-6.41)</td>
<td>$\chi^2=298.76; P &lt; 0.001$</td>
</tr>
</tbody>
</table>

* >21 days indicates that 50% mortality was not reached before the end of the 21-day monitoring period.
Figure 5-1. Long-term viability of oil-formulated conidia of *B. bassiana* isolate I93-825 (batch PSU 46) on plastic sheeting placed in a high-rise layer house in September 2013, with different cover treatments (fine mesh muslin or more open mosquito netting) placed over the plastic to manipulate exposure to airborne particulates
Figure 5-2. Long-term viability of oil-formulated conidia of *B. bassiana* isolate 193-825 (batch PSU 46) on plastic sheeting without cover treatments placed in the poultry house in January and March 2014, or maintained in the laboratory at 26-27°C, 50-60% RH from January through March.
Figure 5-3. Cumulative proportional survival of house flies exposed periodically to oil-formulated conidia of *B. bassiana* I93-825 (batch PSU 46) applied on plastic sheets that were maintained in a high-rise layer house for four weeks in January 2014.
Figure 5-4. Cumulative proportional survival of house flies exposed periodically to oil-formulated conidia of *B. bassiana* I93-825 (batch PSU 46) applied on plastic sheets that were maintained in the laboratory for 13 weeks at 26-27°C, 50-60% RH
Figure 5-4. Continued
Figure 5-4. Continued
Chapter 6: Conclusions and future implications

House flies are economically and medically important insect pests in poultry production facilities. Standard, chemical pesticide-based control options for flies are with issues due to insecticide resistance and regulatory constraints. Biopesticides based on naturally occurring fungal pathogens offer alternatives to chemical pesticides for sustainable fly management. Through a series of laboratory and field experiments, this study addressed several issues related to field application, persistence and performance of fungal biopesticides against flies. The major objectives of the study were (1) to evaluate the potential for biocontrol of house flies using fungal biopesticides, *Beauveria bassiana* and *Metarhizium anisopliae*; (2) to evaluate persistence and efficacy of *B. bassiana* against house flies on typical structural substrates of poultry houses; (3) to assess the impact of fly population on persistence and efficacy of *B. bassiana* under laboratory conditions; and (4) to evaluate persistence and efficacy of *B. bassiana* against house flies in a commercial high-rise layer barn.

An important element in the successful development and implementation of a biopesticide product is an efficient delivery system. One *Beauveria*-based commercial product, balEnce™ is available for fly management in poultry production facilities, but its current formulation and application methods have several limitations. By exploiting post-eclosion behaviors, we developed a cost-effective field delivery system whereby teneral adult flies are targeted via the application of oil-formulated spores to plastic sheeting fixed to the lower portions of basement walls where flies momentarily rest to harden themselves and expand their wings following emergence from puparia. Indoor residual sprays of fungal formulations with the barrier application technique could sustainably suppress populations by influencing fly densities
and population growth rates through sub-lethal impacts on reproductive output (Chapter 2). We also examined the potential of inexpensive, non-toxic, contractor-grade plastic sheets as a wall-covering material prior fungal application to support long-term spore viability. Pre-sheeting of the basement sections appears costly initially; however, reductions in spray area, formulation volume and other indirect cost savings could offset initial inputs in long run and make the technology cost-effective and competitive. A simple cost/benefit analysis of the plastic sheeting application technique showed approximately 40-50% reduction in total treatment costs compared to the current application methods for balEnce™. However, the plastic sheeting effectiveness as well as economic and operational feasibility need to be validated through field testings, where several biotic, abiotic and management factors may affect fungal outcome. Further studies to characterize post-eclosion resting behaviors and their spatial distributions across the basement walls would be needed to optimally adjust dimension of spray patch for enhanced spore-transfer efficiency. Additionally, the poultry industry has been readily adopting IPM strategies to manage arthropod pests including filth flies; therefore, this technique could be part of their holistic approach for fly control. Poultry growers might need field demonstration events for wider acceptance of the technique.

Although fungal biopesticides offer good potential for fly management, economics and logistics of their operational use depend on a number of factors including persistence of spray residues on treated surfaces and/or indoor environments. Hence, we evaluated the persistence and efficacy of \textit{B. bassiana} against flies on typical structural substrates of poultry houses where the spores are being applied as residual sprays. Spore infectivity was poor on these surfaces because flies chemically deactivated and physically removed substantial numbers of spores from the surfaces, with higher fly densities and greater cumulative exposure hastening the decline
(Chapter 3). Nonetheless, effective persistence reduced slowly and overall efficacy was minimally affected under simulated field exposure procedure since very low densities of viable conidia were still able to cause rapid mortality (Chapter 4). Fly density inside poultry facilities is expected to be far below than used in laboratory studies and flight and other movement behaviors could vary thus lowering surface contact rates. Hence, detrimental effects are likely to be minimal at realistic population densities under field conditions. Given the decline in persistence is influenced by fly density, it is possible to replenish spores by tailoring spray regimes to accommodate different densities, for example, high spray frequency with a high rate and dose during peak fly seasons and then reduced frequency when populations decline.

Development of a biopesticide tool necessitates complete assessment of field performance attributes, which aids in understanding and optimizing the outcome of biocontrol. The study therefore assessed persistence and efficacy of B. bassiana spray residue against flies in actual conditions in a commercial high-rise layer barn. We found that fungal spray treatments remained viable for up to 13 weeks under laboratory conditions. Periodic exposure of flies to the spray residue showed high levels mortality, with very little decline in mortality rate over time. Equivalent treatments placed in a commercial poultry house showed much more rapid decline. One trial at the end of summer showed conidia to remain viable up to seven weeks. However, repeats during the winter months revealed decay in one to two weeks, with fly mortality rates influenced accordingly (Chapter 5). While chemical (e.g. ammonia) and thermal (e.g. high humidity) environments pose a challenge for operational use, the fungal biopesticides can still be used in fly management making necessary adjustments in current housing and manure management to improve indoor air quality and create stable thermal environments. This technology could contribute to the overall IPM program. Additional studies to identify the impact
of individual indoor factors including ammonia, humidity and temperature levels and natural fly populations are required to increase the long-term efficacy of the biopesticide treatments.

The effective persistence index developed under laboratory conditions can be used to track spore decay rate following field applications and, with further field studies, to determine optimal spray frequency based on population density, duration of exposure and viable spores on the treated surfaces (Chapter 4). The laboratory and field experimental results suggested the potential for adaptive treatment regimes with weekly spray intervals in conditions with very high fly populations and/or high ammonia levels, and potentially monthly spray intervals when fly populations and ammonia levels are reduced. Regular population monitoring would be an essential element in determining spray frequency, both to prevent population outbreaks and account for density dependent conidia removal and deactivation.

Several possibilities exist to reduce ammonia emission and accumulation in the poultry houses through dietary manipulation, in-house composting, use of manure and litter amendments and biofilters (Reece et al. 1979, Moore et al. 1996, Bottcher et al. 1999, Koenig et al. 2005, Liang et al. 2005, Li et al. 2008, Patterson and Adrizal 2005, Xin et al. 2011). Preventing manure moisture contamination through regular maintenance of problematic drainage and leaky watering systems can reduce ammonia emission rate (Groot Koerkamp 1994). Lime treatments of swampy manure areas not only control manure moisture, but also interrupt larval development. Providing additional drying fans in basements can help reduce ammonia build-up to high concentrations. High ammonia concentrations negatively affect bird health and performance (Cotterill and Nordsog 1954, Charles and Payne 1966, Nagaraja et al. 1983, Charlile 1984, Deaton et al. 1984); therefore, manure management is essential for bird health and efficiency and should be an
integral part of the overall production system. It is likely that with further research, novel formulations of fungal pathogens such as microencapsulation as well as additives or adjuvants could be used to protect conidia against adverse environmental factors and enhance biological activity (Stock 1997, Perrin 2000, Ravensberg 2011). Temperature and humidity optima for spore persistence may vary among different fungal isolates and selection of appropriate isolates that are more resistant to adverse indoor factors would be needed.

There still remains some questions related to long-term field application and performance of the fungal biopesticides that must be addressed in future research. House flies have minute sensory hairs on their tarsi, which could detect fungal spores and behaviorally avoid them under heavy biopesticide pressure since past studies have shown that Anthocorid bugs have behaviorally avoided B. bassiana-treated surfaces (e.g. Meyling and Pell 2006). Insecticide-resistant flies have a thicker cuticle than susceptible ones as shown previously (Keiding 1976), which could affect cuticle penetration by the conidia. Under heavy and continuous biopesticide pressure for long periods, physical, behavioral and transgenerational resistance may develop and reduce the fungal efficiency. Thus, further research may need to evaluate the impact of long-term heavy biopesticide use on fly behaviors and immunoeconomy. For example, the progeny from immune elicitor-challenged Tenebrionid larvae and bumblebees have shown some resistance to elicitors (Rolff and Siva-Jothy 2003, Moret 2006). As the speed of kill is highly variable among fungal isolates and host species as shown in the Chapter 2 and in several previous studies (Rizzo 1977, Davidson and Chandler 2005, Lecuona et al. 2005, Anderson et al. 2011, Blanford et al. 2012), selection of the most virulent isolates as future biopesticide products is necessary to maximize the outcome of the biocontrol program.
The US poultry industry spends about $20 million dollars on chemical pesticides for fly management per year (Geden et al. 2001). The new delivery system as described in Chapter 2 would contribute to wider adoption of balEnce™ as well as promote commercial development of other fungal species/isolates, thereby reducing pesticide consumption in the industry and generate pesticide-free poultry products. It provides an ecofriendly, sustainable fly management tool for both organic and inorganic growers which, with further research, can also be deployed in management of structural pests such as hide beetle and lesser mealworm that cause physical damage to insulation and structural members in the barns (Geden and Steinkraus 2003). Similarly, annual production loss due to insects, mites and ticks in the US cattle industry is estimated to be more than two billion dollars and, with further research, the biopesticides might be used to manage these susceptible ecotoparasites in cattle as well as swine production facilities (Watson et al. 1995, Geden et al. 2001, Perinotto et al. 2012).

Every year approximately 79,000 cases of food borne illnesses and 30 deaths are caused by consumption of Salmonella-contaminated eggs, and flies are considered as one of the vectors for the spread of Salmonellosis across layer farms (FDA 2009). Fungal infections have shown to decrease excretion frequency in flies (Anderson 2011) and feeding and flight capability in mosquitoes (Blanford et al. 2011). The biopesticides can be used to reduce vectoral capacity and disease transmission potential and, with further research on formulation and application strategy, can be integrated into vector management programs. As shown previously by Barson et al. (1994), spores could disseminate horizontally within populations through mating and courtship behaviors, which offer potential to use infected flies as well as mycosed cadavers as a low-cost field delivery technique similar to horizontal transmission of Beauveria among bedbugs (Barbarin et al. 2012); however, detailed investigations will be needed to document this
potential. Insecticide-resistant mosquitoes have higher susceptibility to fungal infections (Howard et al. 2010), and with further research, the biopesticides can be extensively used against insecticide-resistant house flies as the pesticide resistance is a rampant problem in the poultry industry. Additionally, *B. bassiana* works synergistically when combined with Bt, imidacloprid, larvadex and other soft chemical insecticides as shown in house flies (Mwumbari et al. 2009) and mosquitoes (Farenhorst et al. 2010), and can be concurrently used in IPM with other control tactics. Use of multiple tactics in a rotation with rational doses and rates may reduce potential selection pressure on populations and risks of resistance development.

The modern poultry production system in the US is highly sophisticated and organized and is largely managed by integrators who contract with individual farms to provide chickens and eggs. Thus, integrators can provide wider dissemination of the technology to client farms. However, good coordination and cooperation between the production companies, government and land-grant university extension, and individual contract farmers or their associations would be necessary. Many global companies now have joint ventures with local production industries in developing countries. Such worldwide expansion will increase the adoption of the technology, and the involvement of large international companies in poultry production might facilitate technology transfer around the world.

Commercial poultry production is expanding rapidly to meet the dietary demand of growing populations, and per capita egg and meat consumption is increasing accordingly. Such temporal and spatial expansion in demand of poultry products requires more integrated and intensive production system that generates large amount of waste materials, and consequently filth flies such as house flies will continue to be a biological threat as pests and disease-vectors
continue to demand more attention in pest management. In large-scale production operations, even very small losses due to flies (in terms of feed conversion, weight gain, egg production etc.) could have large economic impacts on the companies and individual growers. The situation would be worse if the companies had to face lawsuits and actions under public health laws due to fly nuisance caused by invading populations into nearby residential areas, which can result in significant economic loss and even farm closure. Thus, fly threats will be a constant challenge for the poultry industry and need to be taken seriously. Reliable, non-toxic, ecofriendly IPM-compatible tools that can sustainably keep the populations low are necessary. Because the development of new pesticides is costly, time-consuming and most of existing products have reduced functionality due to resistance and regulatory constraints, effective chemical pesticides are becoming increasingly rare in the market. In this context, demands for the fungal-based biopesticides will continue to grow in the poultry industry as pesticide alternatives.
6.1. References


Rizzo, D. C. (1977). Age of three Dipteran hosts as a factor governing the pathogenicity of *Beauveria bassiana* and *Metarhizium anisopliae*. *Journal of Invertebrate Pathology, 30*(2), 127-130.


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