DIVERSITY AND EFFECT OF THE MICROBIAL COMMUNITY OF AGING HORSE MANURE ON STABLE FLY (*STOMOXYS CALCITRANS*) FITNESS

by

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M.V., Universidade de São Paulo, 2002
M.S., Universidade de São Paulo, 2007

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Entomology
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Abstract

Stable flies (*Stomoxys calcitrans* L.) are blood-feeding insects with a great negative impact on livestock resulting in annual losses around $2 billion in the USA alone. One of the main reasons for such an impact is because stable fly management is very difficult. Stable fly larvae develop primarily in animal manure and live bacteria were shown to be essential for stable fly development. I hypothesized that the microbial community of horse manure changes over time and plays an important role in stable fly fitness. Two-choice bioassays were conducted using 2 week old horse manure (control) and aging horse manure (fresh to 5 week old) to evaluate the effect of manure age on stable fly oviposition. The results showed that fresh manure did not stimulate oviposition and that the attractiveness increased as manure aged but started to decline after 3 weeks. Stable fly eggs artificially placed on 1, 2, and 3 week old manure resulted in significantly higher survival and heavier adults comparing to those developing in fresh, 4, and 5 week old manure. Analysis of the bacterial community of aging horse manure by 454-pyrosequencing of 16S rDNA revealed a major shift from strict anaerobes (e.g. *Clostridium, Eubacterium, Prevotella, Bacteroidales*) in fresh manure to facultative anaerobes and strict aerobes (e.g. *Rhizobium*, *Devosia*, *Brevundimonas*, *Sphingopyxis*, *Comamonas, Pseudomonas*) in 1-5 week old manure. Identified volatile compounds emitted from 2 and 3 week old horse manure included phenol, indole, p-cresol, and m-cresol. However, none of them stimulated stable fly oviposition in two-choice assays. In conclusion, the microbial community of 2 and 3 week old horse manure stimulates stable fly oviposition and provides a suitable habitat for stable fly development. Manure at this stage should be the main target for disrupting the stable fly life cycle. Volatile compounds acting as oviposition stimulants/attractants and their specific bacterial origin remain to be determined. Better understanding of stable fly microbial ecology is critical for development of novel management strategies based on alteration of the microbial community of stable fly habitat to generate a substrate that is non-conducive to fly oviposition and/or larval development.

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Table of Contents

List of Figures

List of Tables

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Dedication

I dedicate this work to my family, specially my grandparents and parents, who always supported me my entire life and in my time away to advance my studies.

With love,

Thais

Chapter 1 - Relevance of study, Objectives and Literature Review

Relevance of Study

Stable flies (*Stomoxys calcitrans*) are blood-sucking Diptera of great economic importance worldwide, negatively affecting many animals including people. Stable fly painful bites change animal behavior and consequently compromise weight gain, milk production, increase injuries, and in high densities, cause anemia. Annual economic losses are estimated at $2.2 billion in the United States alone. Stable fly management is very difficult. Insecticides applied directly on animals are not effective because stable flies approach a host just for a short blood meal and do not remain in contact with animals long enough to make insecticides efficient. Insecticides applied on stable fly resting sites such as walls, poles, and feed bunks are more effective but this is not an option for pastured animals. Furthermore, this approach requires repeated applications during the fly season and that increases chances of development and spread of insecticide resistance. Stable flies develop in decomposing organic substrates, especially animal manure mixed with hay or straw. This habitat is commonly available around livestock animals, both confined and pastured. Consequently, manure management is the most effective method for stable fly control because it targets larval development sites and disrupts the stable fly life cycle before they become adults. Unfortunately, very little is known about stable fly behavior and how the microbial community in animal manure affects stable fly oviposition and larval development. This study is focused on the assessment of temporal changes in diversity of the microbial community and associated volatile compounds of the horse manure and how that affects stable fly fitness.

Objectives

The objectives in this study were to:

1- Assess the effect of horse manure age on stable fly oviposition.

2- Evaluate the effect of horse manure age on stable fly larval development.

3- Monitor temporal changes in the microbial community structure of horse manure.

4- Identify volatile compounds emitted from the aged horse manure and evaluate their effects on stable fly oviposition.

Literature Review

General Biology

Stable flies (*Stomoxys calcitrans*) are blood-sucking insects from the order Diptera, family Muscidae. They are most commonly known as stable flies, but they have been called biting house flies, because of their similarity to house flies, barn flies, dog flies, and power mower flies. Stable fly distribution is cosmopolitan and they are most prevalent in tropical and temperate climates (Shipley, 1915). Studies analyzing stable fly population structure and genetic diversity using a PCR-based technique using a one-enzyme restriction fragment length polymorphism (RFLP) (in Canada and USA) (Szalanski et al., 1996), microsatellites markers (in La Reunion Island) (Gilles et al., 2007), and amplified fragment length polymorphism (AFLP) markers (in Gabon and France) (Dsouli et al., 2009) revealed a low genetic diversity among the studied populations, which made deciphering stable fly origin difficult. However, a study using mitochondrial cytochrome oxidase I (COI), and 16S rDNA (Marquez et al., 2007) showed a

greater genetic differentiation among populations from 11 different countries in 5 different zoogeographical regions. The Neartic, the Neotropical and the Paleartic regions showed the strongest genetic similarities, suggesting that stable flies in the New World have Paleartic (Old World) origins which corroborates an older theory (Brues, 1913). Thus, they are believed to have been introduced to the United States with horses, cattle, and hogs that Europeans brought during the colonial era (16^{th} and beginning of 17^{th} centuries). The earliest record attesting to stable fly presence in the United States was when Thomas Jefferson noted that the signing of the Declaration of Independence in 1776 was made more quickly because of the attack of biting flies, which, by the description, could only have been stable flies (Anonymous, 1927).

Stable flies are very similar to house flies (*Musca domestica*) in size and shape. However, stable flies have a hard and long piercing proboscis, which at rest stays parallel to their body and during feeding moves to a perpendicular position. Their thorax has four longitudinal black stripes, and ventral abdomen has a "checkerboard" pattern (Williams, 2010). Another feature that can help differentiate stable flies is that their fourth great wing vein (M1+2) is less angular than that on house flies (Shipley, 1915; Axtell, 1986). Another characteristic that is exclusive to stable flies is the fact that they rest and feed at usually no more than 1 to 5 feet from the ground, attacking more commonly the lower part of the cattle and horse belly and legs (Hall & Smith, 1986).

Stable flies have holometabolous (complete) metamorphosis, and their life cycle consists of eggs, three larval stages, pupa, and adult. It takes around 16 days to develop from egg to adult stage under optimum conditions (~ 28°C, above 70% humidity, and in a nutrients rich substrate), and one more week to start the egg laying. Great alterations from these conditions can accelerate,

delay, or stop their development, but on average, during the Summer months, stable flies complete their life cycle in about 3 weeks (egg to egg) (Hall & Smith, 1986; Williams, 2010).

Stable fly eggs are white and banana-like shaped, 1 to 2 mm in length and have a deep longitudinal groove in the thicker side, where the egg splits and allows the larva to escape (Shipley, 1915). The egg stage lasts for about one day. Eggs are very sensitive to desiccation, which could destroy them in a few hours, but are very resistant to water, usually surviving heavy Summer precipitation. Temperatures higher than 104°F (40°C) reduce the viability of eggs, which explains the decline in stable fly population during mid-Summer in the Midwest (Hall & Smith, 1986). All these factors lead to a very important aspect of the stable fly life cycle, the importance of selecting the oviposition site to increase the offspring chances for survival.

The first instar larva is white, translucent, and the anterior part is thinner than the posterior part. Stable fly larvae have two spiracles on the posterior end that can also be used to differentiate them from house flies (on stable flies, spiracles are further apart from each other, while on house flies they are side by side) (Axtell, 1986). Stable fly larvae need a very humid habitat to develop, they survive the temperature range from - 6°C to 32°C (Hall & Smith, 1986). Stable flies develop from first instar to third instar (~ 1cm in length) in about 7 days.

Pupae are formed from the outer cuticle of the third instar larvae, which forms the puparium and have a pill-like shape, about 5 mm long. In the beginning of pupation, the hard shell is the same color as the third instar larvae (white) and darkens with time to a dark brown color just before adult emergence. The pupa stage lasts around 8 days (Shipley, 1915).

Stable flies are strong fliers and were observed to fly 8 km in less than 2 hours (Eddy et al, 1962) and up to 225 km in few days (Hogsette & Ruff, 1985). However, in mark-release-recapture experiments, 90% of the stable flies were recaptured within 1.65 km (Gersabeck &

Merritt, 1985), indicating that flies in a given area likely emerged from the larval development sites within a 5 km radius of the animals (Taylor et al., 2010).

Both stable fly sexes feed on warm-blooded animals two to fifteen times a day (Schofield & Torr, 2002; Williams, 2010) and can achieve full capacity in less than 4 minutes if not disturbed (Harwood & James, 1979). Female stable flies require several blood meals to produce eggs (Jones et al. 1992) and males require at least one blood meal to produce seminal fluid and to stimulate the sexual drive (Hall & Smith, 1986; Klowden, 1996).

Stable fly oviposition occurs 1 or 2 days after mating and a single female can produce 10 to 11 batches of 35 eggs (Hall & Smith, 1986). According to Lysyk (1998), a female stable fly can lay between 40 and 60 eggs in each gonotrophic cycle, and the life fecundity ranges between 30 and 700 eggs, depending on temperature. Mello and Garcia (1988) stated that one female could oviposit 151 eggs in just one oviposition, with the number of ovipositions per female varying from 3 to 7 during 17 days, and that there was no relationship between the number of ovipositions and total number of eggs laid per female.

After blood meals, females seek out a suitable oviposition site and deposit eggs. Eggs are usually laid in grooves close to the surface of any decaying or fermenting organic material, such as grass clippings, compost piles, spilled feed, manure and urine-contaminated hay or straw, and aged manure (Meyer & Petersen, 1983; Broce & Haas, 1999).

Economic importance

Stable flies are an important pest of both, humans and animals, mainly because of the worldwide distribution, painful bites, and nuisance (Moon, 2002; Taylor et al., 2012). They can bite and suck blood of any warm-blooded animal and are considered one of the major pests of confined and pastured animals. Cattle on pasture cattle has become more affected by stable flies

after an increase in the use of large round hay bales to feed animals, where a significant amount of wasted hay mixed with manure creates an excellent habitat for larval development (Hall et al., 1982; Campbell et al., 2001). Manure around bales became a primary source of stable flies in late spring (May) in Kansas and Nebraska (Broce et al., 2005; Taylor & Berkebile, 2011), with an estimated production of 200,000 stable flies per hay feeding site (Taylor et al., 2012).

Stable flies also cause important economic losses in tourist areas, such as in the Great Lakes area (Newson, 1977), Florida coast (King & Lenert, 1936), and New Jersey seaboard (Hansens, 1951), where they were reported to attack people and sometimes causing complete evacuation of the area.

Many haematophagous insects from the order Diptera vector pathogens, such as mosquitoes (Culicidae) are responsible for transmission of malaria, yellow fever, dengue, and filariasis; sand flies (Phlebotominae) for leishmaniasis; horse flies (Tabanidae) for equine infectious anemia; tsetse (Glossinidae) for trypanosomiasis; and blackflies (Simuliidae) for onchocerciasis (Gibson & Torr, 1999). Accordingly, stable flies have been studied regarding transmission of several pathogens but are not considered important vectors of disease (Williams, 2010). Although they were shown to be able to mechanically transmit equine infectious anemia (Hawkins et al., 1973), African swine fever (Mellor et al., 1987), enzootic bovine leucosis (Weber et al., 1988), bovine diarrhea virus (Tarry et al., 1991), Rift Valley fever (Turell et al., 2010), and more recently, West Nile virus (Doyle et al., 2011), such studies were performed under the laboratory conditions, and natural transmissions have not been documented (Moon, 2002; Doyle et al., 2011).

Because of the number of cattle in the United States (around 98 million heads) (USDA cattle inventory, 2012), the major economic losses are in the livestock (Taylor et al., 2012).

These losses are caused by reduced milk production and decrease in weight gain (Taylor et al., 2012) caused by reduced grazing time when animals are trying to escape the painful bites and by higher energy losses due to defensive behavior (Mullens et al., 2006). Cattle also spend more time in water (ponds, lakes) or exhibit a bunching behavior. In this case, animals from outside of the bunch continuously try to move to the center where they are more protected from stable flies. This constant movement also increases the risk of injuries and decreases grazing time (Campbell et al., 2001; Moon, 2002).

Based on the latest report on economic importance of stable flies to livestock production, annual estimated losses were $360 million in dairy cattle, $1.268 billion in pastured cattle, $358 million in cow-calf herds, and $226 million for cattle in feedlots, accruing an annual loss for the cattle industry of $2.212 billion in the United States (Taylor et al., 2012).

Stable fly management

Stable flies are very difficult to control. Since 1915, the most efficient control method has been to destroy the larval developmental sites by burning, burying, or treating them with insecticides (Shipley, 1915). Currently, sanitation, or the elimination of the larval habitat, is the most important method of controlling stable flies. Insecticides applied to adult fly resting areas and biological control approaches using parasitic wasps are used as secondary measures (Broce, 2006).

Because stable flies can develop in various decaying organic materials on a farm such as spilled food, animal bedding, silage, and manure, all these have to be frequently removed, burned, or covered (Meyer & Petersen, 1983). Of these substrates, manure is the most abundant. In dairies and feedlots, manure should be scraped weekly and removed, including accumulated manure from under the fences and around feed bunks, and spilled feed around troughs and bunks

(Hogsette, 1986). Other locations that should be considered for stable fly control are drainage areas or ditches leading to runoff control facilities, which accumulate manure and other organic material, such as weeds, and stay wet the whole season (Gilbertson, 1986). And because stable flies are strong fliers and can also use the wind to migrate longer distances, areas further afield of the animals should be controlled for larval breeding sites, emphasizing the need for an area-wide control strategy (Hogsette, 1986).

In cattle being fed on pasture, a sanitation approach is to move hay bale feeders to different locations once a week to avoid accumulation of a deep layer of manure and spilled hay. Thinner accumulation of manure dries faster and becomes less attractive for stable flies to oviposit (Foil & Hogsette, 1994).

For biological control, the first attempt is usually to try to protect and/or improve the habitat of the natural enemies (Bellows & Fisher, 1999). However, on livestock farms, naturally occurring enemies rarely exist in levels that would achieve satisfactory fly control (Petersen, 1986). Therefore, augmentation of natural enemies is a better solution to improve biological control efficiency. It can be done by an early release of the enemies (inoculative release) that will proliferate and present early in the fly season or by continuous release during the whole fly season (inundative release) (Skovgard & Nachman, 2004). For stable flies, the parasitic pteromalid wasps of the fly pupae offer some potential for biological control. The female wasp lays an egg inside the stable fly puparium where the wasp offspring develops and kills the stable fly larva (Moon, 2002). Although some studies showed that parasitoid infestations by *Spalangia cameroni* and *Muscidifurax raptor* contributed to tolerable levels of stable flies (Greene, 1990; Skovgard, 2004), others studies did not confirm these findings and could not show reduction in

stable flies number with the use of parasitic wasps (Petersen et al, 1983; Greene & Guo, 1997; Skovgard & Nachman, 2004).

Insecticides for stable flies control can be of a great help in facilities with confined animal when used in combination with sanitation (Shugart, 1986). There are many products registered for this purpose, but all are based on only few active ingredients, which can lead to increased resistance (Romero et al., 2006). One of the most commonly used insecticides is permethrin, from the pyrethroids group, because of its residual effect and its quick fly knockdown (Moon, 2002). Permethrins are applied to fly resting areas, such as walls, fences, feedbunks, buildings, and vegetation surrounding animal lots, and they may be reapplied during the fly season (Shugart, 1986). Insecticides sprayed directly on the animal legs provide only a few days of relief because the residues are easily removed by vegetation and water (Campbell & Hermenussen, 1971). Insecticides sprayed to larval developmental sites are not effective because microbes in decomposing organic materials break down the active ingredients. Furthermore, this can lead to an increase in insect resistance because of the exposure to low doses of insecticide (Shugart, 1986). Other types of insecticide applications, such as dust bags, oilers, or ear tags are not effective against stable flies (Broce et al, 2005). Feed additives are not effective because stable flies prefer to lay their eggs on cattle manure older than 2 weeks and at this point larvicidal residues are already degraded (Broce & Haas, 1999).

Finally, traps are effective if used in indoor facilities. Outdoor traps are useful for estimating the fly population size and to optimize the application of control methods and they do not reduce stable fly populations to a level that would prevent economical losses (Moon, 2002; Eldridge & Edman, 2004).

General olfaction and oviposition

Olfaction is a critical sensory trait for insect survival, allowing them to acquire chemical information from the external world and translate into a specific behavior. It is an important tool that enables insects to find food, a mate, and the oviposition site (Zwiebel & Takken, 2004). Feeding and host seeking behavior are primarily affected by odors (Takken & Knols, 1999). In reproduction, olfaction is used to confirm the species identity, if the partners are in their reproductive period, if the female has mated already, and to assess the quality of the male (Vosshall, 2008). After mating, to succeed with reproduction, insects need to find a suitable habitat to lay eggs.

Specific sensory mechanisms including chemoreceptors (olfaction and contact perception) and mechanoreceptors (tactile perception) are the most used receptors for choosing an oviposition site. For example, the face fly locates fresh cattle manure by olfactory receptors first, and then, using mechanoreceptors and chemoreceptors on legs, labellum, and ovipositor, it determines the moisture content and physical constitution of the substrate (Elzinga, 1981).

In general, oviposition site selection is influenced by semiochemicals (chemical signals) released by the natural habitat (Bentley & Day, 1989). A microbial community in the habitat can be responsible for producing and releasing volatiles that affect insect oviposition behavior. For example, *Klebsiella oxytoca* on the surface of newly laid house fly eggs mediates immediate oviposition induction by other females. However, as this bacterium proliferates over time, it inhibits late-arriving females from laying their eggs in that specific location, avoiding overcrowding and/or presence of larvae of different ages, which could lead to cannibalism (Lam et al., 2007).

Bacteria were also shown to produce oviposition attractants for the primary screwworm (*Cochliomia hominovorax*). It was shown that these flies were attracted and stimulated to lay more eggs on blood inoculated with bacteria isolated from the screwworm-larvae-infested wounds (*Enterobacter cloacae*, *Enterobacter sakazakii*, *Klebsiella oxytoca*, *Proteus mirabilis*, *Proteus vulgaris*, *Providencia rettgeri*, *Providencia stuartii* and *Serratia liquefaciens*) than on the sterile blood (Chaudhury et al., 2002).

Stable fly oviposition

Stable flies develop in decomposing organic substrates including grass clippings, garden compost piles, silage, hay, spilled animal feed, stored manure, mixture of animal manure-vegetable matter, and fermenting marine vegetation, such as seaweed and sea grass deposits (Meyer & Petersen, 1983; Axtell, 1986; Skoda et al., 1991). It was suggested that some oviposition sites are more important than others, but very few studies have been conducted to address this subject (Broce & Haas, 1999; Jeanbourquin & Guerin, 2007; Romero et al., 2006). Consequently, not much is known about stable fly oviposition behavior, but studies with other insects show that olfaction plays an important role (Dougherty & Knapp, 1994; Collins & Blackwell, 2002; Pelletier et al., 2010; McCallum et al., 2011).

Broce and Haas (1999) showed that the number of visitations by female stable flies to cattle manure increased as the manure got older (> 2 weeks). However, stable fly visitation patterns did not correlate with manure temperature, pH, osmolality, and CO_2 or amonia production of aging cattle manure (Broce & Haas, 1999).

Jeanbourquin and Guerin (2007) analyzed the response of stable flies to odors from fresh horse and cattle manure in wind tunnel experiments and were able to show that both substrates attracted the flies. However, when these manures were used in a two-choice assay, flies clearly

laid more eggs on fresh horse manure. They also recorded higher levels of CO_2 released by the fresh horse manure, suggesting that this may be one of the reasons for the greater attraction to this type of manure (Jeanbourquin & Guerin, 2007).

Additionally, Romero et al. (2006) showed that bacteria play a crucial role in stable fly oviposition. They evaluated stable fly oviposition response to sterile or control horse manure, showing that stable flies laid more eggs on manure with a complex and active bacterial community. They also tested bacteria isolated from the natural larval habitat (horse manure collected around bale feeders) and showed that stable flies laid more eggs on the inoculated substrate than on the sterile one.

Overall, these studies show that stable flies use volatile cues for selection of oviposition sites and that bacteria are likely responsible for producing these cues (Romero et al., 2006; Jeanbourquin & Guerin, 2007).

Stable fly larval development

Several studies on stable fly breeding sites were conducted in cattle facilities to identify the most common larval habitats (Meyer & Petersen, 1983; Skoda et al., 1991; Gilles et al., 2008; Hogsette et al., 2012). Meyer and Petersen (1983) showed that fence lines, drainage ditches, and haylage were important in small feedlots, while spilled feed in large feedlot, and stored manure in the dairy farm. The differences in breeding sites were attributed to differences in the size of facilities, their design, and the farm management (Meyer & Petersen, 1983). In another study, the feed apron of cattle feedlots was the predominant larval breeding site followed by fence lines and mound (Skoda et al., 1991). In a recent study in a cattle feedlot, immature stable flies were most abundant in the drainage areas (Hogsette et al., 2012). The variability in

breeding site preferences shows the stable fly flexibility when selecting an oviposition site and that a specific assessment of each facility is important for stable fly control.

Nutritional requirements for stable fly larval development are not well understood. To maintain stable flies in a laboratory colony, a protein source (typically meat-bone or fish), fiber (alfalfa and/or wheat bran), water, and yeast in different ratios are commonly used (Campau et al., 1953; Champlain et al., 1954; Ashrafi, 1964; Bridges & Spates, 1983). Although more and better quality nutrients improve insect growth rate and body weight (Davidowitz & Nijhout, 2004), the specific types and proportions of required nutrients for stable flies are unknown.

Accumulated manure mixed with wasted hay around bale feeders can produce up to 200,000 stable flies per site in Kansas and Nebraska during the fly season (Broce et al., 2005; Taylor & Berkebile, 2011; Taylor et al., 2012). The physical and chemical characteristics of the natural substrate and relationship to larval development are not well understood mainly because these characteristics change considerably over time as a result of the growth of the microbial community and consequent decomposition of a specific substrate (Couteaux et al., 1995; Broce & Haas, 1999). Analyses of some of the biochemical properties (temperature, pH, moisture content, osmolality, and CO_2 and ammonia production) of cattle manure at different times of decomposition did not show any correlation to stable fly oviposition (Broce & Haas, 1999). In contrast, in a recent study, several physio-chemical characteristics of cattle manure around hay bales including ammonium concentration, electrical conductivity, total carbon concentration, pH, moisture level, and microbial respiration rate (CO_2) correlated with stable fly adult emergence (Wienhold & Taylor, 2012).

Gilles et al. (2008), studied the stable fly larval development in seven different natural larvae substrates (sugar cane leaves, sugar cane tops, Rhodes grass, Rhodes grass hay, Elephant

grass, Kikuyu silage, and old cattle manure) and suggested that substrates with higher cellulose content and with pH ranging from 5.6 to 6.0 such as sugar cane leaves, Rhodes grass and Elephant grass were most suitable for stable fly development.

Bacteria in animal manure were shown to be essential for the development of immature stages of house flies (*Musca domestica*), face flies (*Musca autumnalis*), and stable flies (Hollis et al., 1985; Schmidtmann & Martin, 1992; Lysyk et al., 1999). Stable flies were unable to develop when their eggs were artificially placed on the sterile substrate (animal manure or artificial medium), showing that bacteria are critical for stable fly larval survival (Lysyk et al., 1999; Lysyk et al., 2002; Romero et al., 2006). However, not all bacteria are vital for stable fly development. *Acinetobacter* sp., *Empedobacter breve* and *Flavobacterium odoratum* alone or in different combinations supported larval development, whereas *Aeromonas* sp. or *Serratia marcescens* resulted in larval death at the first instar (Lysyk et al., 1999). Bacteria also greatly affect stable fly developmental time and larvae growing in mixed bacterial cultures developed faster than when they were growing in pure cultures (Lysyk et al., 1999). *Serratia fanticola* and *Citrobacter freundii* isolated from a mixture of horse manure and hay from the round hay bale feeders supported stable fly larval development and they also stimulated stable oviposition (Romero et al., 2006). Romero et al. (2006) findings corroborate Lysyk et al. (1999) results that bacteria are essential for stable fly larval development and a complex bacterial community supports stable flies better than individual bacterial isolates.

In the field study, a high concentration of fecal coliforms in hay feeding sites of pastured cattle was the only parameter that correlated with high stable fly emergence, while the substrate temperature, moisture, pH, and hay-manure ratio did not affect stable fly emergence (Talley et al., 2009). These findings together indicate the importance of bacteria for stable fly larval

development and are likely the main reason for stable fly seasonality around pastured cattle (Talley et al., 2009).

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Chapter 2 - Effect of the horse manure age on stable fly (*Stomoxys calcitrans*) oviposition

Introduction

Stable flies are common in rural and urban areas and a major cosmopolitan pest because both male and female flies bite animals and humans to feed on blood. Their bite is painful and the problem is greater on farms where the annoyance caused by stable fly bites keeps animals from feeding, consequently reducing weight gain and milk production. The annual economic losses in animal productions can rise up to $2.2 billion in the cattle industry in the United States alone (Taylor et al., 2012).

The ability to select a suitable oviposition site is critical for fitness of the offspring (Elzinga, 1981; Zwiebel & Takken, 2004). Stable flies are flexible when it comes to their oviposition site that includes decomposing grass clippings, garden compost piles, silage, hay, fermenting marine vegetation, spilled animal feed, mixture of animal manure and organic matter, and stored manure (Meyer & Petersen, 1983; Axtell, 1986; Skoda et al., 1991).

Animal manure is the most common breeding site because it is a nutritive substrate for larval development and because of large amounts of this material are produced and accumulate daily at large animal production facilities. At least 22 species of beetles (Coleoptera) and 23 species of flies (Diptera) can be found developing in and or using animal manure for a given part of their life cycle (Wall & Lee, 2010). Based on an animal weight (450 kg), a single cow can produce up to 39 kg of manure per day (dairy cattle) or 26 kg per day (beef cattle), while horses with the same weight could produce on average 23 kg of manure per day (ASAE, 2003). This means that on a farm with as few as 10 horses, the annual manure production is around 84 ton.

Storage and disposal of manure requires large spaces and is labor intensive. Consequently, on food animal farms, insects usually have no difficulties in finding a substrate for oviposition and in the case of adult stable flies, they are close to the hosts that they feed on.

Some flies that colonize manure/feces are specialists, such as *Musca sorbens* (bazaar fly) which oviposits on human feces (Hafez & Attia, 1958; Emerson et al., 2000), *Musca autumnalis* (face fly) and *Haematobia irritans* (horn fly) that oviposit in fresh cattle manure (Moon, 2002; Foil & Hogsette, 1994), while others, including *Musca domestica* (house fly) and *Stomoxys calcitrans* (stable fly) are more generalists, laying eggs in a much broader variety of substrates (Moon, 2002).

Insects attraction to oviposition sites relies greatly on the olfactory system (Dougherty & Knapp, 1994; Collins & Blackwell, 2002; Pelletier et al., 2010; McCallum et al., 2011). Some studies showed that stable fly oviposition preference is based on olfaction alone (Jeanbourquin & Guerin, 2007) or olfaction and tactile perception (Broce & Haas, 1999; Romero et al., 2006). The age of cattle manure is an important factor in stable fly oviposition preference (Broce & Haas, 1999). The number of gravid females visitation increased after the manure had aged for 2 weeks, showing that the attractiveness of cattle manure varies overtime. This variation was not correlated with manure pH, osmolality, CO_2, amonia production, and temperature (Broce & Haas, 1999).

In general, microbial populations are responsible for 95% of plant material decomposition and they can degrade cellulose, hemicellulose, and lignin (Berg & Laskowski, 2005). Differences in the bacterial community during manure decomposition could be behind the stable fly oviposition preference in a specific manure age. Bacteria, in addition of being a food source or nutritional complement (Rochon et al., 2004; Lam et al., 2009) can also be responsible

for producing cues for insect oviposition (Chapman, 1971). *Klebsiella oxytoca*, for example, regulates house fly oviposition behavior (Lam et al., 2007). This bacterium is found on the surface house fly eggs and induces immediate oviposition by other females. However, overtime *Klebsiella oxytoca* proliferate and inhibit further oviposition and prevent overcrowding and larval cannibalism (Lam et al., 2007). A mixture of eight bacteria isolated from wounds infested with the primary screwworm larvae (*Cochliomia hominovorax*) including *Klebsiella oxytoca*, *Enterobacter cloacae*, *Enterobacter sakazakii*, *Proteus mirabilis*, *Proteus vulgaris*, *Providencia rettgeri*, *Providencia stuartii*, and *Serratia liquefaciens*, was shown to increase this insect egg laying (Chaudhury et al., 2002).

Bacteria were also shown to play a crucial role in stable fly oviposition. Stable flies laid more eggs on manure with a complex bacterial community than on sterile manure (Romero et al., 2006). Individual bacteria cultured from natural larvae habitat (horse manure collected around bale feeders) were also more attractive for stable flies to oviposit than sterile substrates (Romero et al., 2006).

Horse manure was shown to be more attractive for stable flies than cattle manure (Jeanbourquin & Guerin, 2007). Plumes of both types of manure were attractive and elicited upwind flight in wind tunnel experiments. However, when both types of manure were offered together in a two-choice oviposition assay, flies clearly laid more eggs on fresh horse manure (Jeanbourquin & Guerin, 2007).

Stable fly control is difficult and relies primarily on sanitation and manure management (Broce, 2006). Therefore, it is important to identify and characterize stable fly oviposition site preference so manure management can be done in a more effective way. This knowledge could be also used for development of stable fly oviposition traps and/or repellents.

Objective of the study

To assess the effect of horse manure age on stable fly oviposition.

Hypothesis

Null hypothesis: Horse manure age does not affect stable fly oviposition.

Materials and Methods

Manure collection

The manure was collected from the Kansas State University Horse Facility, from a pen with 16 two year old horses on a diet consisting of 25% of grain (Purina Strategy-Professional formula GX®, Purina, Gray Summit, MO) and 75% of alfalfa hay. Horses did not receive any treatment (e.g., insecticides, dewormers, or antibiotics) for at least 2 weeks before the manure collection.

On the first day of the experiment, three 16 liters plastic buckets (24 x 24 x 36 cm) full of fresh manure (< 10 min) were collected from the pen floor. Only the top 2/3 of each manure patch were collected to avoid soil contamination. Buckets were cleaned with hot water and soap (Palmolive original detergent®, Colgate-Palmolive Company, New York, NY), sprayed with alcohol 70% and dried before use. Manure was then taken to the laboratory, mixed by stirring, and divided equally into five 16 liters plastic buckets (24 x 24 x 36 cm) and closed with the plastic lid with two meshed breathing holes (to keep the air flow). Buckets were then placed in an environmental chamber (26°C, 40% RH, 15:9 L/D) to be aged for up to 5 weeks.

Each week, one bucket was removed from the chamber and used for the experiment. Fresh manure was used immediately after collection from the horse pen floor.

In addition, one extra bucket of fresh manure was collected every week, for 6 consecutive weeks, to be used as a control. This was done two weeks before each bioassay as the age of the control substrate was 2 weeks. This manure was collected from the same horses, mixed, closed with the lid, and incubated for 2 weeks.

Manure preparation

On the first day, the control (2 weeks old) manure was removed from the incubator, the top dried layer (3 cm) was removed, and the rest of the manure mixed. Manure was then distributed into 23 Petri dishes (60 x 150 mm) (Fisher Scientific, Pittsburgh, PA) (20 for individual bioassays and 3 for the group bioassays) and labeled. Fresh manure (less than 2 hours old) was also placed into 23 small Petri dishes and labeled. On the following weeks, the 2 buckets (control and aging manure) were removed from the incubator and processed as described above. The manure moisture was determined by measuring the dry weight of 10 grams of each manure sample (placing it in the oven at 120°C for 48 hours). The moisture was then adjusted to 80% by adding sterile distilled water.

Flies

Stable flies used in the experiments were from the Kansas State University laboratory colony, maintained on a supersaturated solution of sugar water and citrated cattle blood. Flies were synchronized to lay eggs on the same day by selecting only flies that emerged in a period of 24h or less. After seven days of daily blood meals, synchronized flies were cooled down in a refrigerator for 15 min, transferred to a Petri dish, female flies were separated and transferred to

individual Petri dish plates (individual fly bioassays) or in a collective Petri dish (group fly bioassays) to recover from the cooling process.

Individual fly two-choice tests

Individual females were transferred to a plastic insect rearing cage (30 x 30 x 30 cm) (BugDorm®, MegaView, Taichung, Taiwan) and offered two manure types (control and aged) for oviposition. The experiment was conducted with 20 flies that were allowed to oviposit for 6 hours on day one and for an additional 6 hours the next day to maximize the probability of egg laying. After the first 6 hours, plates with the horse manure were removed and refrigerated for egg counting later. On the second day of the experiment, new plates were prepared and placed inside the cages for the second 6 hours of oviposition. The experiment was replicated twice. During the experiment, the flies did not receive any food or water.

Group two-choice tests

Groups of 30 females were transferred to a plastic insect cage (30 x 30 x 30 cm) (BugDorm®, MegaView*, Taichung, Taiwan) and offered the two manure types (control and aged) for oviposition. The experiment was conducted in 3 replicates and the flies were allowed to oviposit for 2 hours on day one and 2 additional hours on the next day to maximize egg laying. After the first 2 hours, the plates were removed and refrigerated. On the second day of the experiment, new plates were prepared and placed inside the cages for the second 2 hours of oviposition. The experiment was replicated twice. During the experiment flies did not receive any food or water.

Egg counting

After the oviposition assay was completed, manure from the Petri dish was transferred into a saturated solution of salt water to separate the eggs from the manure debris by floatation and counted.

Statistical analysis

The Chi-Square test was performed to analyze the data on the number of flies laying eggs in either aged manure, control manure, or both. This is represented as percentage of flies ovipositing (Fig 2.1). Student's t-test (StatPlus:mac, AnalystSoft Inc. 2009) was performed to determine significant differences between the mean number of eggs on aging and control manure (Figures 2.2 and 2.3).

Results

Assays with individual flies

The number of flies ovipositing on each manure type (aged manure vs. control vs. both) (Figure 2.1) showed when horse manure was fresh (fresh vs. control vs. both), 100% of stable flies oviposited on the control substrate (2 weeks old horse manure = 2WHM). On week 1 (1WHM vs. control vs. both), 12.9% of the flies laid the eggs on the aged manure, 38.2% on the control, and 48.9% on both substrates ($p=0.018$) (Figure 2.1, Table 2.1). On week 2 (2WHM vs. control vs. both), 23.4% of flies deposited eggs only on the aged manure, other 10.9% laid only on the control, and the majority (65.7%) on both substrates ($p=0.00015$) (Figure 2.1). From the confidence intervals (Table 2.1), it is clear that flies preferred to lay their eggs on both manure types (in this case, the same manure age). On week 3 of the experiment (3WHM vs. control vs. both), 20.8% of flies oviposited on the aged manure, 48.2% on control, and 31% on both manure

types (p=0.15189) (Figure 2.1; Table 2.1). During week 4 (4WHM vs. control vs. both), only 2.8% of flies laid eggs on the aged manure was while 57.8% preferred the control, and 39.4% chose to oviposit on both substrates (p=0.00031) (Figure 2.1; Table 2.1). On the last week of the experiment (5WHM vs. control vs. both), 3.8% of the flies oviposited only on the aged manure, 75.8% laid on the control manure, and 20.4% oviposited on both substrates (p=0.00001). At this time point, confidence intervals (Table 2.1) indicate that number of flies laying eggs on aged manure and on both substrates is not significantly different, but they are significantly different from flies ovipositing on the control manure (Figure 2.1).

The data regarding the number of eggs laid on each manure type showed that, when the horse manure was fresh, the mean number of eggs laid per fly on control (2WHM) was 63.2 (± 4.27) and no eggs were found in fresh manure (Figure 2.2). Females laid a mean number of eggs (12.9 ± 3.69) on the 1WHM and that was significantly different (p=0.00104) from the mean number of eggs laid on the control (2WHM) manure (31.54 ± 4.03). On the week 2, the mean number of eggs laid on the two manure types were not significantly different (p=0.66794) (17.0 ± 2.86) eggs on aged manure (2WHM) and (18.9 ± 3.50) on the control substrate (2WHM) (Figure 2.2). On the week 3, there was also no statistically significant preference (p=0.24442), with a mean of 9.69 (± 3.48) eggs laid on 3WHM and 16.31 (± 4.41) on the control (2WHM) (Figure 2.2). A mean number of 11.6 ± 3.2 eggs oviposited on 4WHM was significantly different (p=0.02858) from the eggs (20.91 ± 2.70) laid on the control substrate. When the aged manure was 5 weeks old, a significant difference (p=0.00001) was detected between the mean number of eggs laid on 5WHM (3.24 ± 1.71) and (40.0 ± 4.4) eggs on the control horse manure (Figure 2.2).

Assays with a group of flies

The response of stable flies in the two-choice assay showed that the mean number of eggs laid per fly on fresh horse manure (0.06 eggs ± 0.04), was significantly different (p=0.00001) from the eggs laid on the control manure (48.4 ± 3.1), which accounted for 99.87% of the eggs. When the aged manure was 1 week old (1WHM), the mean number of eggs laid was 15.39 ± 2.37 and it was also significantly different (p=0.02545) from the number of eggs deposited on the control manure (34.5 ± 6.9). From week 2 to week 5, no significant differences were seen in the mean number of eggs laid on each manure type (Figure 2.3). In the week 2, 18.5 ± 3.7 eggs were laid on the aged manure (2WHM), while 12.23 ± 3.2 eggs were oviposited on the control manure (also 2WHM) (p=0.22862). When the aged manure was 3 weeks old (3WHM), 16.12 ± 1.95 eggs were laid compared to 13.61 ± 5.33 eggs oviposited on the control substrate (p=0.66777). In the experiment conducted on in the week 4, 8.42 ± 2.35 eggs were laid on 4WHM and 18.88 ± 6.29 on the control manure (p=0.15074). In the last week, 15.86 ± 5.28 eggs were oviposited on 5WHM while 22.91 ± 3.09 were counted from the control manure (p=0.27588) (Figure 2.3).

Discussion

The assays with individual flies provided two perspectives on stable fly oviposition: qualitative (preference for the substrate type) and quantitative (number of eggs laid on a specific substrate). Laying eggs on more than one substrate within the same gonadotropic cycle has been attributed to: 1) egg density in the substrate; 2) prevention of intraspecific competition, and 3) minimizing the loss of all eggs in case of adverse events in one of the oviposition sites (Courtney, 1986).

Regarding the selection of the oviposition substrate, it was clear that fresh horse manure does not stimulate stable fly oviposition when compared to 2 weeks old manure. However, fresh horse manure is not repellent because in a case when this type of manure is the only substrate available, stable flies use it as an oviposition site (not shown). This situation is very unlikely in the field, however. Our data are supported by a study with cattle manure where there were no visitations by gravid females while the manure was fresh and visitations increased as the manure got older and the first stable fly larvae were found in two week old cattle manure (Broce & Haas, 1999).

In our study, as the age of the horse manure increased, the frequency of oviposition on aging manure also increased but only up to certain point. Once the oviposition choice was between four week old manure or older and the control (2 week old manure), very few stable flies laid eggs on older manure and the majority preferred the control manure or divided their eggs between both substrates with a greater tendency to oviposit on the control manure.

The same trend was seen from the quantitative perspective; no eggs were deposited on fresh manure and the number of eggs in aging manure increased as the manure aged; however, after 3 weeks of the aging process, significantly more eggs were oviposited on two week old manure compared to that of older manure.

The same trend was observed from the second type of the oviposition assays with a group of stable fly females. Almost no eggs were laid by a group of 30 females on fresh manure and as the manure aged from one to 3 weeks, the number of eggs deposited on aging manure increased. However, flies in the group assay, did lay some eggs on 4 and 5 weeks old manure possibly to avoid overcrowding. It is conceivable that the overcrowding would be more harmful for the flies than the manure quality itself. Stable flies in low densities were observed to lay the eggs

preferentially in spilled feed mixed with animal manure, but in high densities they spread the eggs on the entire bedding material (Skovgard & Nachman, 2004).

It is likely that after three weeks of decomposition, the nutrients in the manure become depleted and the diversity and activity of the microbial community are diminished. This is then probably reflected in emitted bacterial compounds that serve as cues for stable fly oviposition and signal that the substrate is too old and not suitable for larval development (addressed in the Chapter 3).

Group assays were conducted to detect possible group interference in stable fly oviposition behavior. It is known that for some insect species such as house flies, the group behavior is important when deciding where to lay the eggs (Lam et al., 2007). For example, it can help house flies to recognize a suitable area that was already assessed by another individual. It was shown that house flies rely on the presence of the egg associated bacteria *Klebsiella oxytoca* to induce oviposition of other females by signaling to other females to lay eggs in the same area for a short period of time and increase the number of larvae of the same age (Lam et al., 2007). However, as this bacterium proliferates over time, it inhibits further oviposition that might otherwise lead to overcrowding and cannibalism of younger larvae (Lam et al., 2007). Being in a group can also increase egg production, the tephritid fruit flies *Anastrepha ludens* and *Anastrepha oblique*, lay more eggs in the presence of other flies (Aluja et al., 2001). On the other hand, the cherry fruit fly (*Rhagoletis cerasi*) and the apple maggot fly (*Rhagoletis pomonella*) utilize pheromone after their oviposition to discourage new ovipositions in the same area to prevent overcrowding (Averill & Prokopy, 1987; Stadler et al., 1994). The age of a substrate is also an important characteristic for insect oviposition. The cabbage cluster caterpillar (*Crocidolomia pavonana*) prefers to oviposit on cabbage leaves that are 7 to 8 weeks old and this

preference was advantageous to larval growth. In contrast, compromised larval development was observed if the eggs were laid on the older leaves (Smyth et al., 2003). Three day old artificial medium of the screwworm (*Cochliomyia hominovorax*) with the larvae was more attractive for gravid females to lay eggs than the larval medium of other ages. Also, three day old larval medium without larvae was not attractive for females to oviposit (Chaudhury et al., 2012).

The stable fly oviposition preference for different ages of the same substrate shows that they use the olfaction/chemosensory system and are well equipped to select the suitable oviposition site. Identification of the most attractive natural substrate for oviposition is of great relevance for improving stable fly management. The preferred substrate (type and age) can help predict when adult flies are emerging and when control measures have to be applied. This study clearly shows that fresh and more than three weeks old manure do not stimulate stable fly oviposition and consequently, the stable fly management in terms of disrupting larval development should focus on two to four weeks old horse manure.

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Table 2.1 Oviposition (% of flies) of individual stable flies on aging (fresh to 5 weeks old) and 2 week old horse manure (control).

	% Flies (95% CI)*					
	Week 0	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**
Aging	0 (-2-12)^{b}	12.9 (5-27)^{a}	23.4 (13-40)^{a}	20.8 (8-39)^{a}	2.8 (-1-16) ^{a}	3.9 (-1-17)^{a}
Control	100 (88-102)^{a}	38.2 (25-54)^{ab}	10.9 (4-25)^{a}	48.2 (32-68)^{a}	57.8 (41-72)^{b}	75.8 (62-90)^{b}
Both plates	0 (-2-12)^{b}	49 (34-64)^{b}	65.8 (49-78)^{b}	31 (17-50)^{a}	39.4 (26-56)^{b}	20.4 (8-35)^{a}

Aging manure is fresh on week 0 and ages progressively to 5 weeks old. Different letters indicate significant differences ($p<0.05$) among the three different choices within the same time period. *CI = confidence interval.

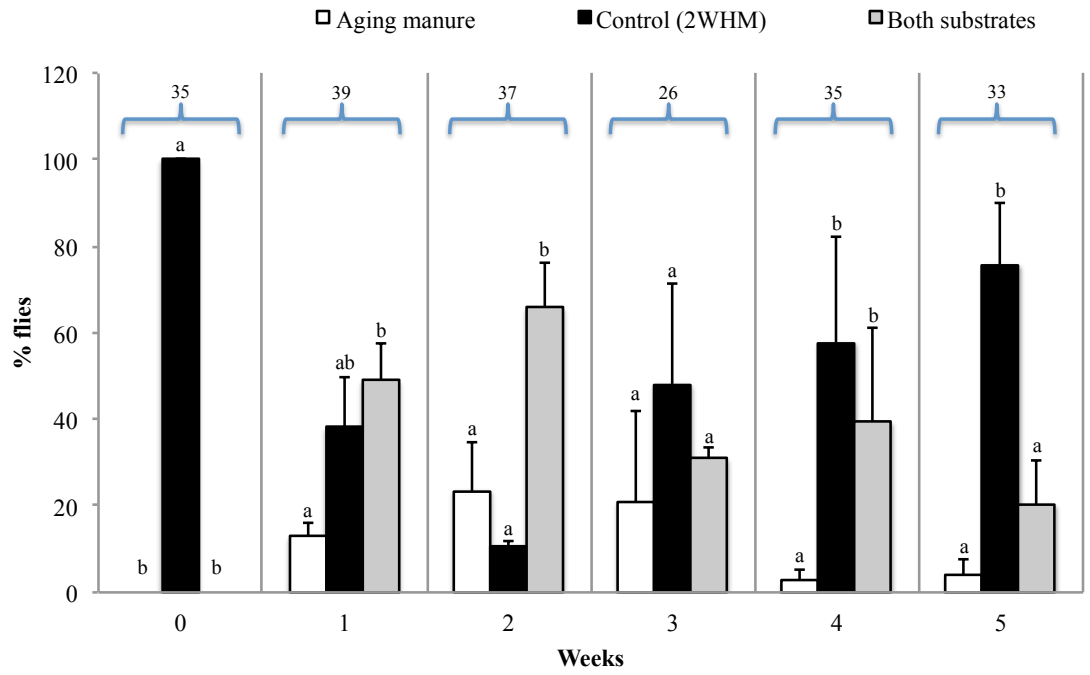

Figure 2.1 Oviposition (% of flies) of individual stable flies on aging (fresh to 5 week old) and 2 week old horse manure (control).

Aging manure (white bars) is fresh on week 0 and ages progressively to 5 weeks old. White bars show percentage of flies that oviposited only on aging manure, black bars represent the flies that laid only on the control (always 2 weeks old horse manure) and gray bars show the percentage of flies laying their eggs on both options. Different letters indicate significant differences ($p<0.05$) among the three different possibilities within a week. Numbers above bars are total number of flies that oviposited in the two experiment replicates. Error bars are SEM.

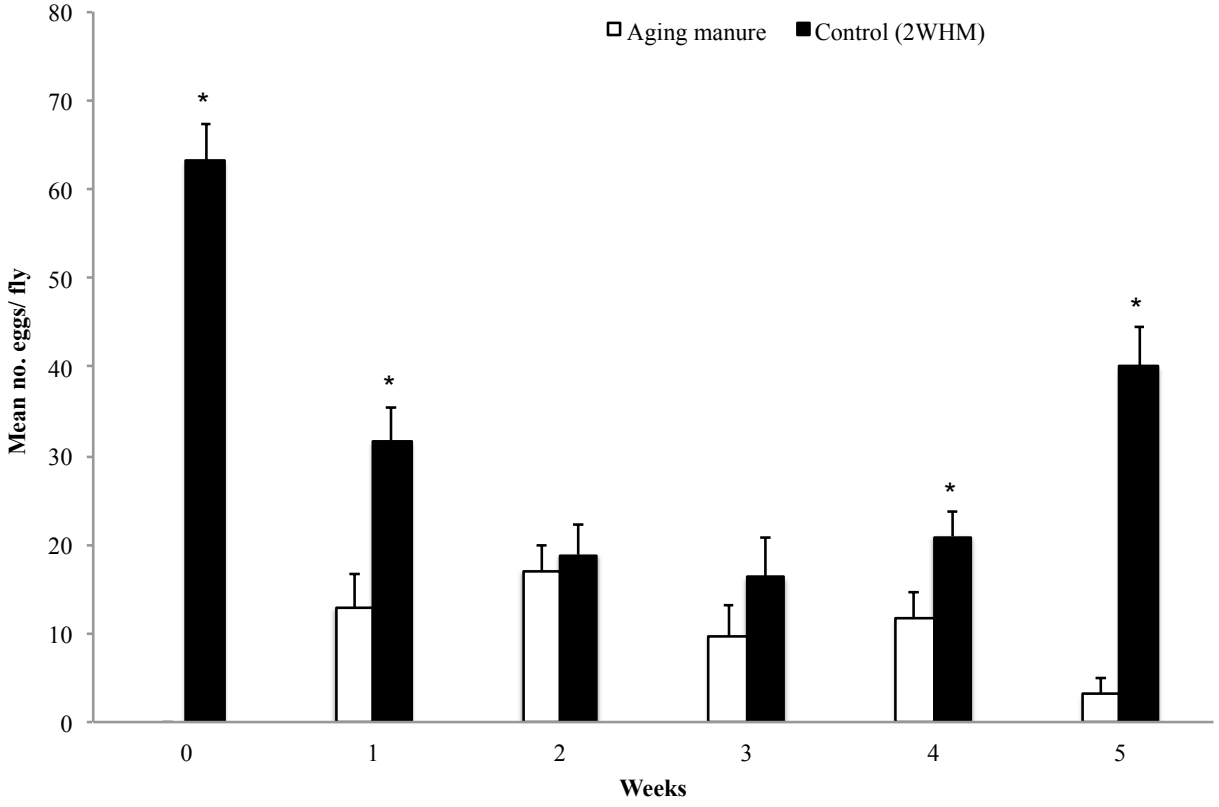

Figure 2.2 Oviposition (number of eggs) of individual stable flies on aging and 2 week old horse manure (control).

Aging manure (white bar) is fresh on week 0 and ages progressively to 5 weeks old on week 5. White bars show mean number of eggs per fly laid on aging manure, black bars represent the mean number of eggs oviposited on the control (always 2 weeks old horse manure). Stars indicate significant differences ($p<0.05$) between the two choices within a week. n=35, 39, 37, 26, 35, and 33, respectively. Error bars are SEM.

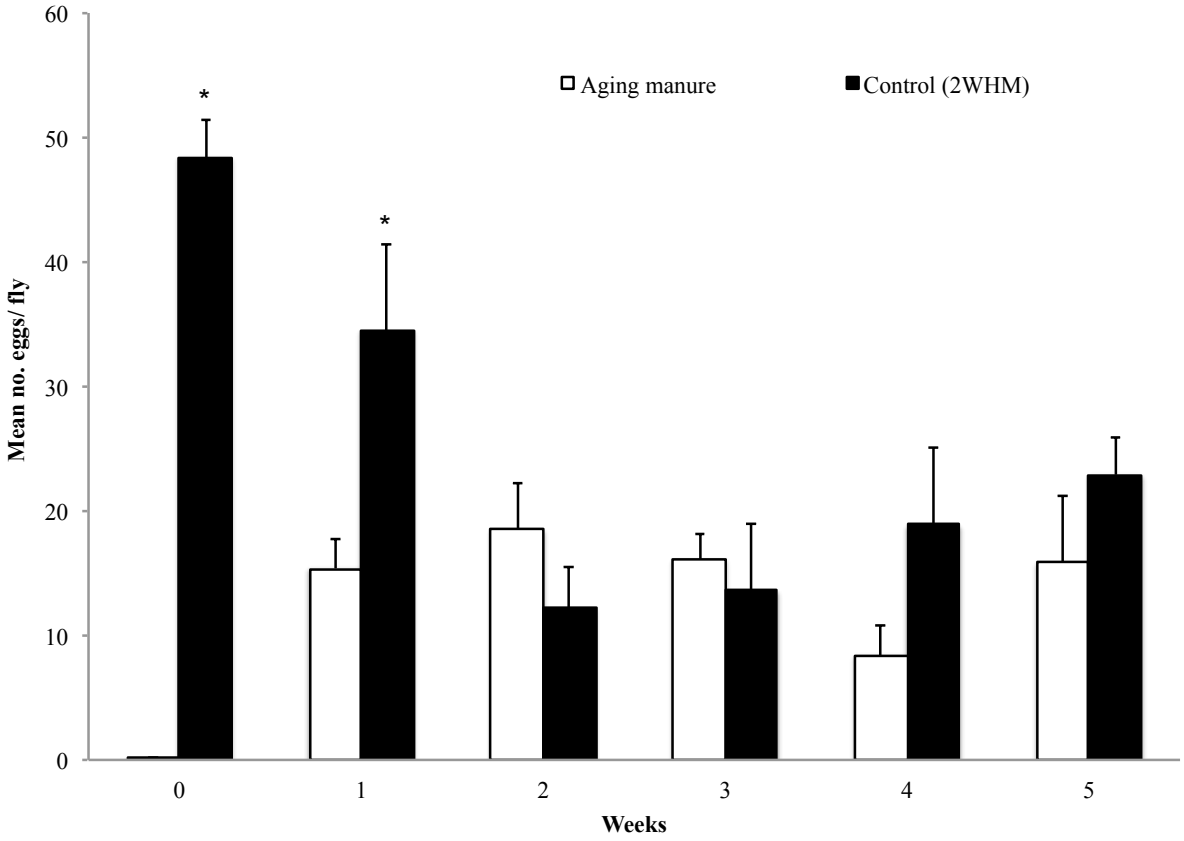

Figure 2.3 Oviposition (number of eggs) of a group of stable flies on aging and 2 week old horse manure (control).

Aging manure (white bar) is fresh on week 0 and ages progressively to 5 weeks old on week 5. White bars show mean number of eggs per fly laid on aging manure, black bars represent the mean number of eggs oviposited on the control (always 2 weeks old horse manure). Stars indicate significant differences ($p<0.05$) between the two choices within a week. n=180. Error bars are SEM.

Chapter 3 - Effect of horse manure age on stable fly (*Stomoxys calcitrans*) larval development

Introduction

Stable flies in animal production facilities cause great economic damage. Annual losses in cattle farms are estimated at $2.21 billion in the United States alone (Taylor et al., 2012). This loss is mainly due to stable fly painful bites that stress the animals and conversion of feed to milk or meat is reduced (Moon, 2002; Taylor et al., 2012). Control of stable flies is very difficult and depends most importantly on sanitation to minimize larval breeding sites (Broce, 2006). Stable fly larvae can develop in various types of organic decomposing substrates including hay, silage, other crop residues, and animal manure.

Cattle operations can contain numerous sites for stable fly larval development (Meyer & Petersen, 1983; Skoda et al., 1991; Gilles et al., 2008; Hogsette et al., 2012). Meyer and Petersen (1983) evaluated different breeding sites on a dairy farm, four small feedlots, and one large feedlot, and classified areas as haylage, fence line manure, potholes, drainage ditches, spilled feed, old corn silage, empty lots, stored manure, soiled straw bedding, feed aprons, oat silage, fresh corn silage, manure under feedbunks, feed in feedbunks, and feed under feedbunks. The results of their study showed fence lines, drainage ditches, and haylage as the most common breeding sites of stable flies in the small feedlots, spilled feed in the large feedlot, and stored manure on the dairy farm. These differences in breeding sites were attributed to the differences in size of facilities, design, and management. In another study, Skoda et al. (1991) determined the feed apron of cattle feedlots to be the predominant larval breeding site, followed by fence lines and mound. And in a more recent study, conducted in three feedlots with similar numbers

of animals, animal breed, feed type, feeding practices, management systems, and isolation from other feedlots in Australia by sampling selected fly breeding sites, Hogsette et al. (2012) showed that immature stable flies were most abundant in the drain areas. These differences in preference for breeding locations show that a specific assessment of each facility is important for stable fly control.

Animal manure is of special importance because it is a very nutritious substrate for many insects including stable flies and because of large amounts of this material is produced and accumulated daily on animal production facilities. A single cow produces up to 39 kg of manure daily, while a same sized horse produces 23 kg (ASAE, 2003). This translates into one horse alone producing up to 8.4 ton of manure in one year. Interestingly, horse manure was shown to be more attractive for stable fly oviposition than cattle manure (Jeanbourquin & Guerin, 2007) although overall, cattle manure is much more common and available for stable fly development.

The age of cattle manure influenced stable fly oviposition preference and females visitation increased in piles that had aged for 2 weeks (Broce & Haas, 1999). The aging process is caused mainly by the microbial decomposition (Berg & Laskowski, 2005). Stable fly nutritional requirements for larval development are not well understood. Several studies made it feasible to rear stable flies under laboratory conditions (Campau et al., 1953; Champlain et al., 1954; Ashrafi, 1964; Bridges & Spates, 1983). These studies designed diets with a protein source (meat-bone or fish), fiber (alfalfa and/or wheat bran), water, and in some cases yeast, all in different proportions.

Furthermore, very little is known about the characteristics of the natural larval substrates of stable flies. Gilles et al. (2008) studied the chemical properties of seven different stable fly natural larval breeding sites and their influence on larval development including sugar cane

leaves, sugar cane tops, Rhodes grass, Rhodes grass hay, Elephant grass, Kikuyu silage, and old cattle manure. Of these seven, sugar cane leaves, Rhodes grass and Elephant grass, substrates with higher cellulose content and with pH ranging from 5.6 to 6.0, were more suitable for stable fly development. Broce and Haas (1999) studied the relation of cattle manure age and its colonization by stable flies and analyzed some of the characteristics of the manure at different ages (temperature, pH, moisture content, osmolality, and CO_2 and ammonia production) but did not find any correlation between these and gravid female visitation. However, adult emergence was demonstrated to correlate with moisture content, ammonium concentration, electrical conductivity, total carbon concentration, pH, and microbial respiration rate (CO_2) (Wienhold & Taylor, 2012) showing that these characteristics are indeed important for stable fly larval development.

Clearly, physical characteristics of a substrate can change considerably over time most likely because warm temperatures and humidity influence bacterial community growth and consequently decomposition and nutrient availability in the specific substrate (Couteaux et al., 1995; Broce & Haas, 1999).

Bacteria have been shown to be important for the development of the immature stages of muscoid flies including house flies (*Musca domestica*), face flies (*Musca autumnalis*), and stable flies (Hollis et al., 1985; Schmidtmann & Martin, 1992; Lysyk et al., 1999). Moreover, microbial activity was proven crucial for stable fly larval development (Lysyk et al., 1999; Lysyk et al., 2002; Romero et al., 2006). In particular, Lysyk et al. (1999) studied the influence of five species of bacteria, *Acinetobacter* sp., *Aeromonas* sp., *Empedobacter breve*, *Flavobacterium odoratum*, and *Serratia marcescens*, previously isolated from the fly colony artificial medium. Bacterial isolates were inoculated on the egg yolk medium and used to assess larval development.

Acinetobacter sp., *Empedobacter breve* and *Flavobacterium odoratum* alone or in different combinations supported larval development, while larvae died in the first instar on plates inoculated with *Aeromonas* sp. or *Serratia marcescens.* Uninoculated plates could not sustain larval development, confirming the importance of bacteria for larval survival. Also, developmental time was faster when larvae were growing in mixed bacterial cultures than in a pure culture (9.5 days from eggs to pupae, compared to 11.3 days) (Lysyk et al., 1999).

The influence of individual bacterial isolates from the natural larval habitat (a mix of hay and horse manure collected around the bale feeder) on stable larval development was also examined in study of Romero et al. (2006). Specifically, *Citrobacter freundii*, *Pseudomonas* sp., *Serratia fanticola*, *Serratia marcescens*, *Aeromonas* sp., and *Enterococcus* sp. were individually inoculated on trypticase soy egg yolk agar to test larval survival. *Serratia fanticola* and *Citrobacter freundii* cultures supported stable fly larvae development with a pupation rate of 70 and 48 % respectively, but to a lesser extent than natural substrate (84%), with a complex bacterial community. *Serratia marcescens* and *Aeromonas* sp. did not support larval development to the pupa stage, in accordance with Lysyk et al. (1999) findings. Overall, developmental time from eggs to pupae was also faster when larvae were growing in natural substrate (8.5 days) than on any tested bacterial isolate (10.4 days or more).

In natural conditions, manure around round bale feeders is a great source of adult flies (200,000 stable flies / bale site) in Kansas and Nebraska during the fly season (Broce et al., 2005; Taylor & Berkebile, 2011; Taylor et al., 2012). Higher concentrations of fecal coliforms around hay feeding sites for pastured cattle in Kansas were shown to correlate with the high number of emerged flies (Talley et al., 2009). Meanwhile, other parameters such as substrate temperature, moisture, pH, and thickness of the hay-manure layer were constant and did not correlate with

stable fly emergence. These findings indicate the importance of bacteria for stable fly development and may be the main factor responsible for the stable fly seasonality on pastured cattle (Talley et al., 2009).

Better understanding of the effect of the aging process of the natural developmental substrate such as horse manure on stable fly survival and development may result in improvement of management practices and could lead to more focused, less expensive and time consuming approach for stable fly control.

Objective of the study

Assess the effect of horse manure age on stable fly larval development and adult body weight.

Hypothesis

Null hypothesis: Larvae of stable flies develop equally in horse manure of different ages.

Materials and Methods

Manure collection

The manure was collected from the Kansas State University Horse Facility, from a pen with 16 two year old horses on a diet consisting of 25% of grain (Purina Strategy-Professional formula GX®, Purina, Gray Summit, MO) and 75% of alfalfa hay. Horses did not receive any treatment (e.g., insecticides, dewormers, or antibiotics) for at least 2 weeks before the manure collection.

On the first day of the experiment, three 16 liters plastic buckets (24 x 24 x 36 cm) full of fresh manure (< 10 min) were collected from the pen floor. Only the top 2/3 of each manure patch were collected to avoid soil contamination. Buckets were cleaned with hot water and soap (Palmolive original detergent®, Colgate-Palmolive Company, New York, NY), sprayed with alcohol 70% and dried before use. Manure was then taken to the laboratory, mixed by stirring, and divided equally into five 16 liters plastic buckets (24 x 24 x 36 cm) and closed with the plastic lid with two meshed breathing holes (to keep the air flow). Buckets were then placed in an environmental chamber (26°C, 40% RH, 15:9 L/D) to be aged for up to 5 weeks.

Each week, one bucket was removed from the chamber and used for the experiment. Fresh manure was used immediately after collection from the horse pen floor.

In addition, one extra bucket of fresh manure was collected every week, for 6 consecutive weeks, to be used as a control. This was done two weeks before each bioassay as the age of the control substrate was 2 weeks. This manure was collected from the same horses, mixed, closed with the lid, and incubated for 2 weeks.

Larval substrates preparation

Every week, on the day of the eggs inoculation, six plastic bottles were washed with hot water and soap, sprayed with alcohol 70%, and dried. Three bottles were filled with aging manure (from fresh to 5 weeks old) and the other 3 with the stable fly larval medium (control).

For the aging manure, one bucket was removed from the incubator, the top dried layer was removed and the rest of the manure mixed, and 500g were placed in each bottle. As a control, 500g of stable fly larval medium were placed in each of 3 bottles (Figure 3.1). The moisture on different ages of horse manure was measured by determination of dry weight of 10g of manure and adjusted to 80%.

The stable fly larval artificial medium was a mixture of 130g of cattle feed (Calf Manna multi species performance supplement®, Manna Pro, Chesterfield, MO), 170g of vermiculite, 500g of wheat bran (Wingold Bakers Bran®, Bay State Milling Company, Wichita, KS) and 1.8L of water.

Stable fly egg collection and inoculation

One day prior each assay, an egg-collecting jar was placed inside of the stable fly colony cage. On the day of the assay, five hundred eggs were then placed on top of each substrate in 6 bottles, and closed with a paper towel and rubber band (Figure 3.1). After the eggs inoculation, all bottles were placed in the environmental chamber (26°C, 40% RH, 15:9 L/D).

Bottles were monitored daily for larval development and adult emergence. Adult flies were transferred to a plastic container, cooled down, and weighted. This procedure was repeated until there was no fly emergence for at least 7 consecutive days.

Correlation between stable fly wing size and body weight

The wing size of 150 wild stable flies collected at the horse facility (n=50), dairy farm (n=50) and feedlot area (n=50) of Kansas State University was measured. Another 140 stable flies from the laboratory colony had their wing size (mm) and fresh adult body weight (mg) measured. The wing size was measured from the base of the wing basicosta to the farthest point (close to the 4th vein) (Figure 3.2) using a Leica MZ APO microscope. Linear regression was used to assess the correlation between fresh body weight and wing size (SAS 9.2- proc reg).

Results

Stable fly survival to adult stage

Survival to adult stage when larvae developed in one week old horse manure (84.13%) and that was not significantly different from that of two (79.33%, p= 0.399) and three week old manure (73.13%, p=0.0627) (Figure 3.3). However, mortality was very high in 4 and 5 week old manure, where only 14.93% and 2.13% of larvae respectively, developed into the adult stage. Interestingly, fresh manure supported larval development (61.27%) although into a lesser extent than that of 1 to 3 week old manure (Figure 3.3.). Fly survival to adult stage in the control artificial medium was 66.38% and not significantly different (p=0.2752) from that of fresh and three week old horse manure (Figure 3.3).

Larval developmental time

Larval development time was usually shorter when flies were developing in fresher horse manure compared to that of more aged manure (Figure 3.4). Stable fly development time from the first instar to the adult stage was shortest in one week old manure (19.4 ± 0.07 days) and as manure aged the length of the development time was extended (Table 3.1). Significant differences were detected among one, two and three week old manure ($p<0.0001$); however the development time remained relatively stable around 24 days and not significantly different in three, four and five week old manure (Table 3.1). Interestingly, larval development in fresh manure was significantly longer comparing to that of 1 week old manure ($p<0.0001$). Larvae in the artificial medium developed the fastest (19.08 ± 0.04 days) (Table 3.1).

Stable flies started emerging from the fresh manure 15 days after the eggs inoculation with the peak of emergence on day 20 (mean = 44 flies). The majority (76.9%) of the flies

emerged between days 17 and 23 (Figure 3.5). The last fly emerged on day 38. When the manure was one week old, stable fly emergence was between day 15 and day 32, with the majority of flies (94.9%) emerging between days 17 and 22 and peaking on day 18 (mean = 98.7 flies) (Figure 3.5). Flies from 2WHM started emerging on day 17, peaking on day 21 (mean = 62.7 flies), and the majority (85.6%) of flies emerged between days 19 and 25. The last fly emerged on day 38 (Figure 3.5). When the eggs were placed on 3WHM, adult stable flies started to appear on day 18 with high numbers emerging between days 21 and 27 (peak on day 23, 69.7 flies) and the last fly emerged on day 38 (Figure 3.5). When flies started developing in 4WHM, the first flies emerged on day 21, most of them emerged between days 22 and 24 (peak of 18.3 flies on day 23), and the last fly emerged on day 33 (Figure 3.5). Very few flies emerged from 5 week old manure (Figure 3.5). Flies developing in the control media started to emerge on day 16, the majority (92.5%) emerged between days 17 to 21, they peaked on day 18 with a mean of 104.4 flies and the last fly emerged on day 32 (Figure 3.5).

Stable fly body weight upon emergence

There was a strong negative correlation between the stable fly body weight and the age of the substrate (r^2=-0.82), with lighter flies emerging from older substrates (Figure 3.6). The mean body weight of all flies emerging from the same substrate showed that heavier flies emerged from the artificial medium (control) with the overall mean body weight of 10.05 mg (Figure 3.7), followed by flies that emerged from 1 week old horse manure (7.14 mg) (Figure 3.7). Larvae that developed in fresh manure and 3WHM resulted on flies weighing on average 4.80 mg and 4.87 mg respectively with no statistically significant difference (p=0.1610) (Figure 3.7). Flies emerging from 4WHM and 5WHM were very light, with the overall mean body weight of 3.38 mg and 3.36 respectively, with no statistically significant difference (p=0.9167) (Figure 3.7).

The fresh adult body weight upon emergence was typically higher for the stable flies that emerged earlier and declined for the adults emerging later when developing in the same type of substrate (Figure 3.8). The exception was the fresh manure, where flies started emerging on day 15 weighing 4.5 ± 0.2 mg and ceased emergence on day 38 weighing 4.8 ± 0.6 mg (Figure 3.8). Stable flies developing in the control (artificial medium) started emerging on day 16 with the mean weight 10.1 ± 0.2 mg and the last fly emerged on day 32 weighing only 7.0 mg (Figure 3.8). Stable flies developing in 1 week old horse manure (1WHM) started emerging on day 15 after eggs inoculation with the mean adult body weight 7.8 ± 0.1 mg and this declined to 5.1 mg on the day of the last emerging adult (day 32) (Figure 3.8). When the manure was 2 weeks old, the fly emergence began on day 17 with a fly weighing 7.8 mg and lasted until day 34 with the mean of 3.1± 0.2 mg (Figure 3.8). For the larvae that started developing in 3 weeks old horse manure, it took 18 days for the first fly to emerge with 6.2 mg body weight and emergence lasted until day 38 with a fly weighing 2.1 mg (Figure 3.8). Emergence of stable flies developing in 4 weeks old horse manure started on day 21 with 4.2 ± 0.2 mg of the mean body weight and ceased on day 33 with the last fly weighing 2.6 mg (Figure 3.8). When the horse manure was 5 weeks old, first flies emerged on day 20 with the mean body weight 4.3 ± 0.5 mg and the last fly emerged on day 28 with the body weight of 3.5 mg (Figure 3.8).

Wing size and fresh adult body weight

The analysis of the fresh adult body weight and the wing size of the laboratory colony stable flies revealed a strong positive correlation ($r^2 = 0.8416$) (Figure 3.9), and this made it possible to predict the fresh adult body weight of the wild flies collected from various animal facilities. The formula used was: Predicted weight (mg) = -13.87199 + 4.54316 x wing size (mm). The predicted fresh adult body weight of the wild flies was 9.12 mg (±0.11) and they were

significantly heavier ($p<0.0001$) than the flies emerging from the 1 week old horse manure (7.14 ± 0.02 mg) (Figure 3.10).

Combined results of oviposition assays and larval development assays are represented in Figure 3.10.

Discussion

The best substrates for larval development are considered those where larvae develop fast and adults are big (Gilles et al., 2008). This is the first study that focuses on the stable fly larval development in the same breeding natural substrate of different ages. Larval development time, survival to adult stage, and adult body weight were different when larvae were reared in horse manure of six different ages. In general, with the exception of fresh manure (< 1hr old), stable flies developed faster, were heavier, and had higher survival when developing in horse manure of 1, 2 and 3 weeks old compared to that of the ones developing in 4 and 5 weeks old horse manure.

The adult emergence from the horse manure was high when the manure was 1, 2, and 3 weeks old (84%, 79%, and 73%, respectively), and greatly declined in 4 and 5 weeks old manure. This is likely due depletion of nutrients during manure decomposition and decline in the microbial community that are required for stable fly larval development (moisture was maintained constant in all the substrates). Interestingly, fresh manure (< 1hr old) supported larval development well (61% survival to the adult stage), but to a lesser extent than 1-3 weeks old manure. That is possibly because the microbial community of fresh manure is not yet adequate for the larval development as it contains mainly anaerobic bacteria (addressed in Chapter 4).

Previous studies utilizing different types of fresh manure showed stable fly survival of 85% in pig manure, 83% in donkey manure, 70% in sheep manure, 67% in horse and goat manure, 55% in cattle manure, and no survival in chicken manure (Sutherland, 1978). Some of

these substrates such as goat and sheep manure resulted in relatively high fly survival because the fecal pellets were broken down and water was added to make it more suitable for stable fly development. Under natural conditions, these substrates dry quickly and are not suitable for stable fly development. A 3% survival rate was shown for stable flies growing in old cattle manure by Gilles et al (2008), but the exact age of that manure was not specified. Romero et al (2006) reported the stable fly survival of 79% when developing in an active larval habitat (aged horse manure collected around bale feeders), but the age of this substrate was also not specified. However, since horses were constantly defecating around the feeders it was probably a mixture of manure of various ages.

In our study, flies could develop in fresh horse manure, as reported by Sutherland (1978), and the development time (~20 days) was slightly faster than when growing in 2 weeks old horse manure (~22 days). Stable fly survival in the fresh horse manure was relatively high (~60%) confirming that fresh manure could be a breeding site for stable flies. However, it is clear from our oviposition assays (Chapter 2) that stable flies do not lay any eggs on fresh horse manure when 2 weeks old horse manure is available. This is likely because the microbial community of the fresh manure releases cues that make stable flies avoid this breeding site. On pastures, fresh manure is commonly colonized by large populations of horn flies and face flies (Moon, 2002; Williams, 2010). The oviposition behavior of stable flies might reflect a strategy to avoid competition with larvae of these two fly species. However, the effect of larvae of horn flies and face flies on development of stable flies remains to be evaluated.

Considering the development time and horse manure age, it was shown that larvae took longer to develop from eggs to adult stage, as manure became older. The development time from when the horse manure was 3 weeks old and older (4 and 5 weeks of age) was not significantly

different, but the number of stable flies that developed to the adult stage was greatly reduced, suggesting that nutrients in these old substrates were depleted. Increased nutrition improves insect growth rate and body weight (Davidowitz & Nijhout, 2004); however, no data are available on the nutritional composition of horse manure of any age.

The relationship of the stable fly fresh body weight and horse manure age was also analyzed and the results showed that the adults were heavier when developing in younger ages of horse manure. The overall adult body weight was 7.14 mg when the eggs were inoculated in 1 week old horse manure and got as low as 3.36 mg when larvae started developing in 4 and 5 week old manure. This decrease in the body weight also points to a reduced amount of nutrients available in the older manure. *Drosophila melanogaster* and *Manduca sexta* have to reach a critical body weight to initiate the metamorphosis. The quality of the larval diet consumed during the 24h period after the critical weight is achieved and before the molting hormone ecdysone is released are what determines the insect adult body weight (Mirth & Riddiford, 2007; Davidowitz et al., 2005). If this is the case for stable flies, all flies that emerged reached their critical body weight to start the metamorphosis but the quality or quantity of these nutrients were greatly reduced when the manure was older. This would also explain why flies that emerged earlier from the same manure type were heavier than those that emerged later.

Combining all three factors including survival, development time, and adult body weight, it is concluded that 1, 2 and 3 week old horse manure is the most preferred and suitable horse manure for stable fly development. Results of the oviposition assays showed that stable flies laid more eggs on horse manure that had been aging for 2 and 3 weeks, making these two manure types the most appropriate for oviposition.

Analyses of the body weight of wild stable flies collected from dairy, feedlot, and horse facilities revealed that wild flies were heavier than those developing in the horse manure in our bioassays. The mean weight of wild flies was 9.12 mg comparing to 7.14 mg of the heaviest adults from the horse manure suggesting that wild flies developed in more nutritious substrate than horse manure. Wild flies have a broad variety of substrates available and it is likely that horse manure is not the most suitable substrate for their larval development. On the other hand, it was shown that when given a choice stable flies laid more eggs to fresh horse manure than fresh cattle manure (Jeabourquin & Guerin, 2007). However, our study showed that fresh manure is not a preferred oviposition substrate for stable flies and consequently assays comparing aged horse and cattle manure need to be conducted.

The fact that stable flies can successfully develop in horse manure of different ages makes it more difficult to develop an effective management strategy. Weather conditions also affect stable fly development and temperatures well above 26°C likely result in faster decomposition of horse manure allowing stable flies to oviposit on fresher manure. In addition, higher temperatures also accelerate stable fly development time. Temperatures well below 26°C will have the opposite effect of manure decomposition and larval development. Consequently, timing of the larval breeding site management has to be adjusted to variations in temperature. The temperature 26°C was selected for the assays in this study because it is the most common daily average temperature during the peak of the stable fly population in the spring in Kansas.

Currently, control of stable flies in animal facilities relies on sanitation, primarily reducing the larval breeding sites (Broce, 2006). The schedule of this sanitation is not well established. A fortnightly cleaning schedule in Nebraska feedlots during the stable fly season showed 50% reduction in stable fly populations (Thomas et al., 1996). In dairy farms and

feedlots, it is suggested weekly manure removal of all possible breeding sites. Then, manure should be piled and covered with a heavy-duty plastic sheets or spread on agricultural fields (Hogsette, 1986). This practice is very laborious and time consuming and therefore not commonly adopted.

In conclusion, based on this study, at temperatures close to 26°C, horse manure management should be done twice a month to break the stable fly life cycle and to prevent a build-up of large populations of stable flies.

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Table 3.1 Developmental time (mean ± SEM) of stable flies reared in different ages of horse manure (fresh to 5 weeks old) and the artificial medium (control).

Substrate	Developmental time (days)
Artificial Medium	19.08 ± 0.04^{a}
Fresh	20.60 ± 0.08^{c}
1WHM	19.35 ± 0.07^{b}
2WHM	22.01 ± 0.07^{d}
3WHM	24.20 ± 0.07^{e}
4WHM	24.30 ± 0.15^{e}
5WHM	23.56 ± 0.41^{e}

Means followed by different letters are significantly different.

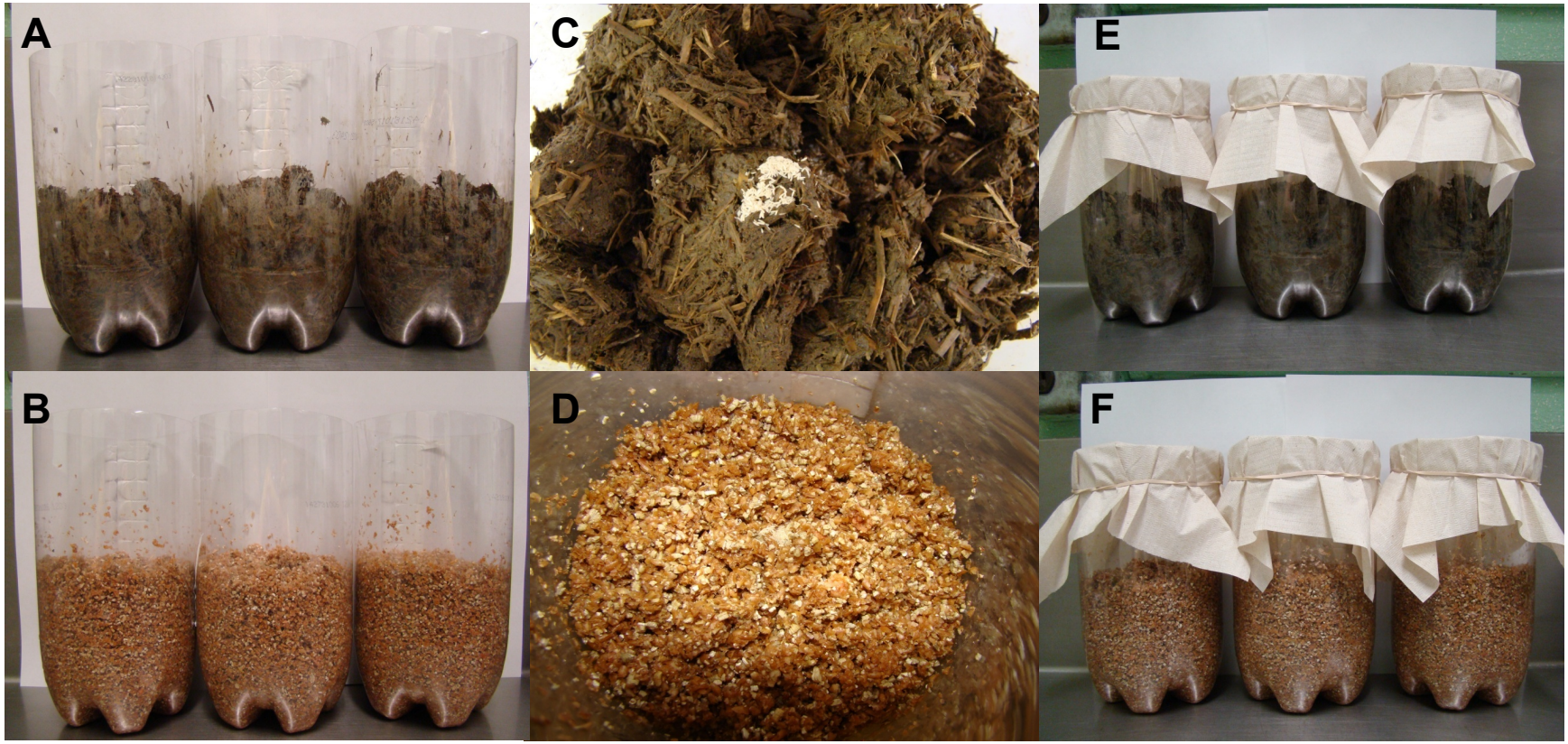

Figure 3.1 Aged horse manure and artificial medium (control) used for larval development assay.

Bottles with 500g of horse manure (A) and artificial medium (B). Eggs placed on top of horse manure (C) and artificial medium (D). Bottles were covered with paper towel and held in place with a rubber band (E, F).

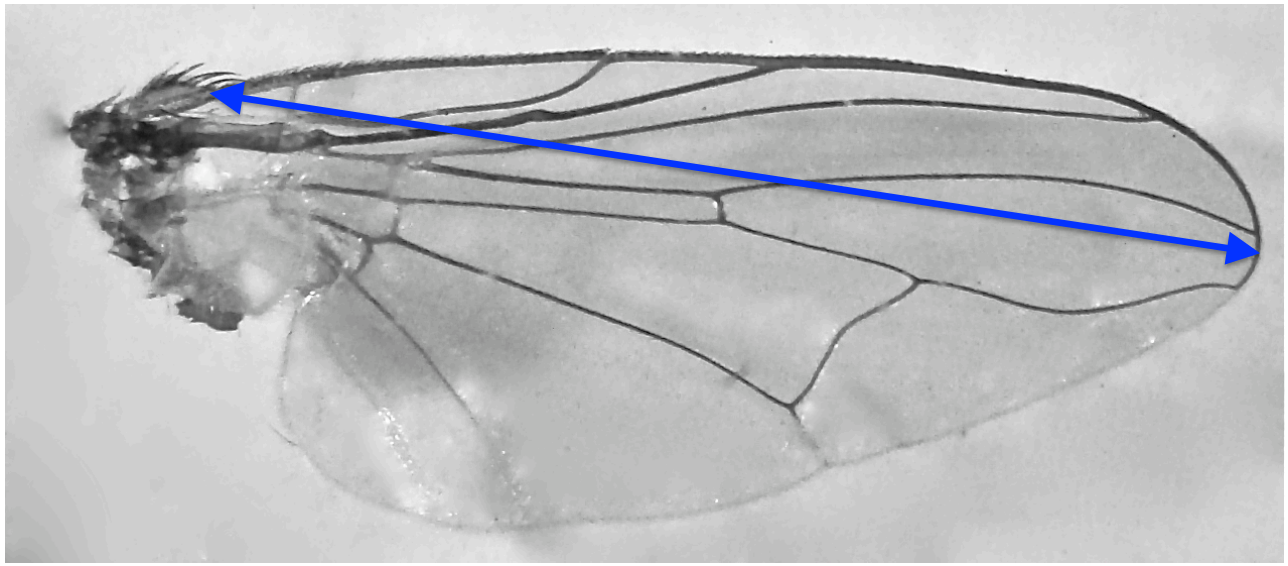

Figure 3.2 Stable fly wing size measurement.

Blue line depicts the distance used to measure the wing size.

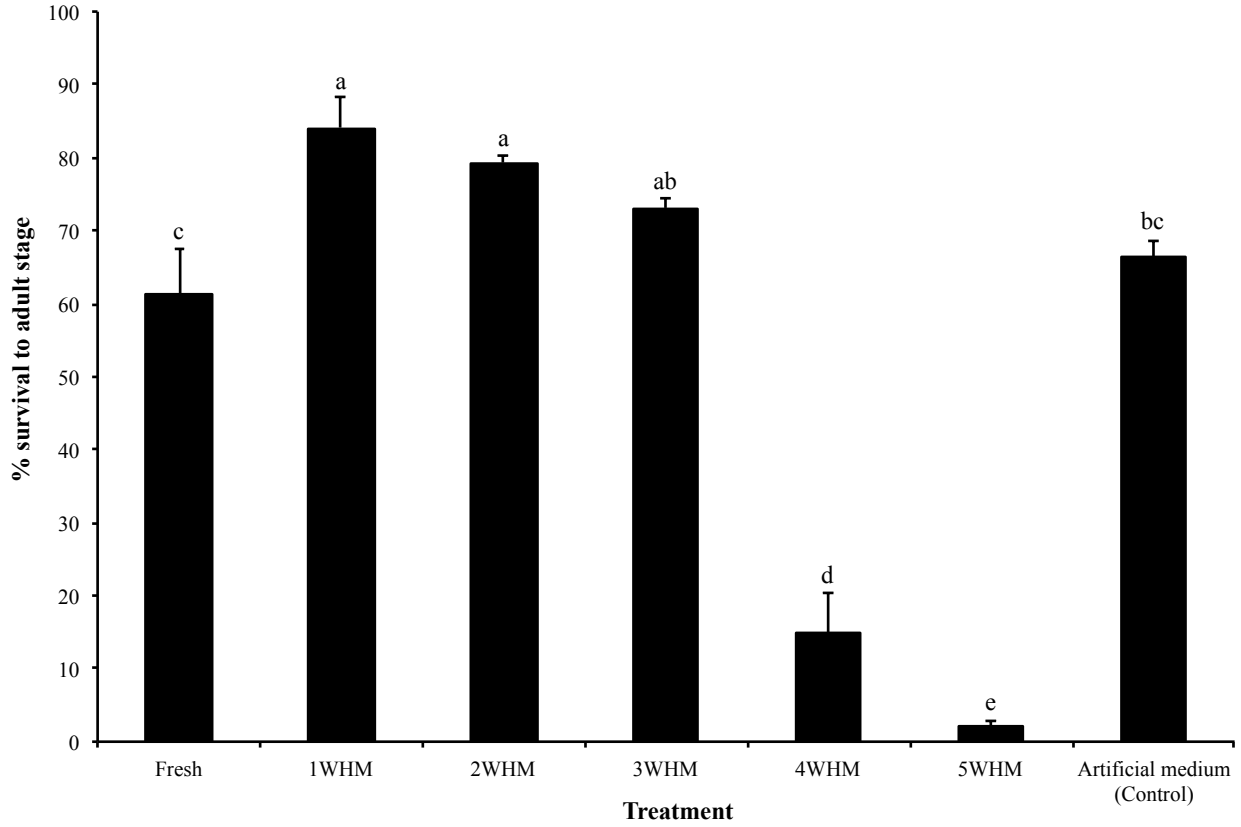

Figure 3.3 Survival of stable flies from eggs to the adult stage in different ages of horse manure and in the artificial medium (control).

Different letters indicate significant differences ($p<0.05$) among all treatments. n=1500. Error bars are SEM.

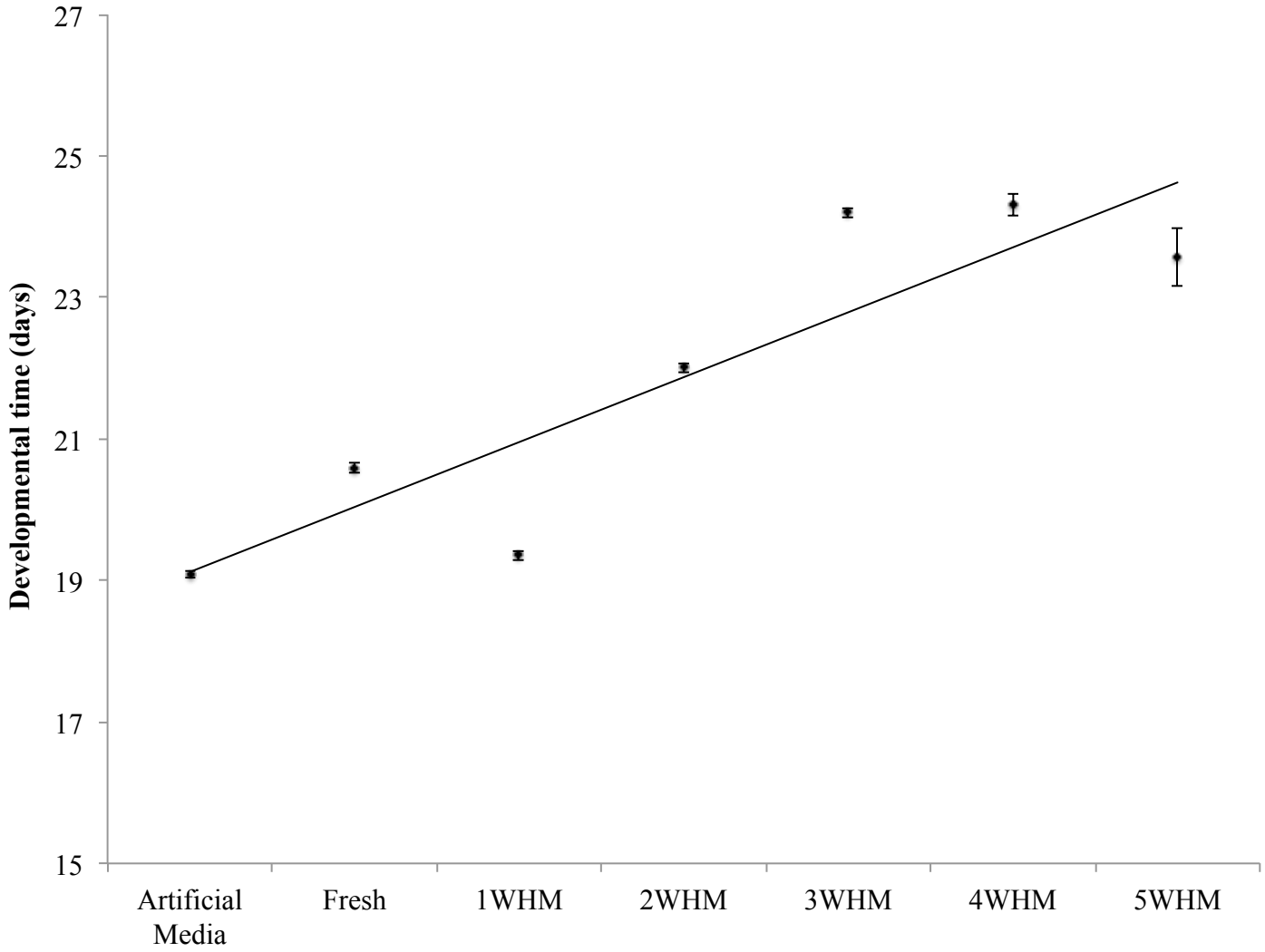

Figure 3.4 Correlation between stable fly development time and the substrate age.

Bars are standard error of mean (SEM). r^2= 0.88. n=2987, 919, 1262, 1190, 1097, 224, and 32, respectively.

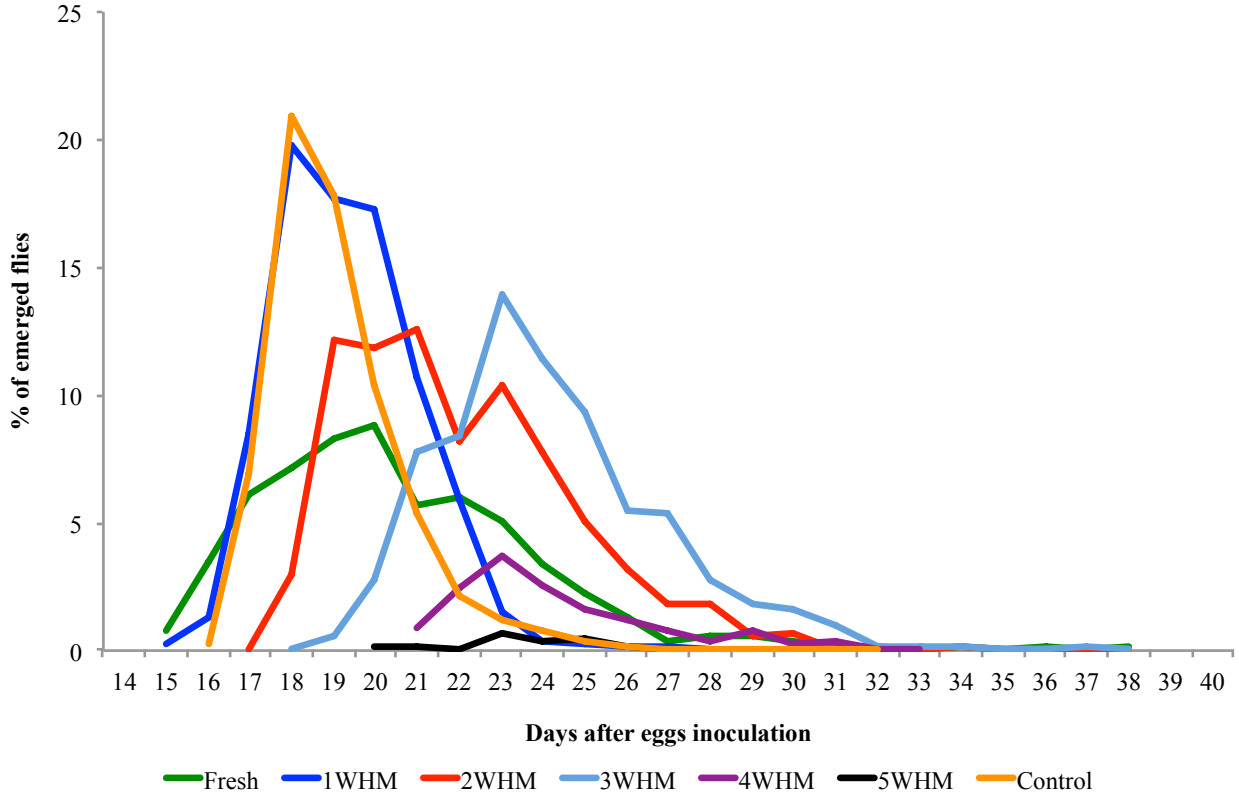

Figure 3.5 Stable fly daily emergence from the horse manure of different ages and artificial medium (control).

n=1500.

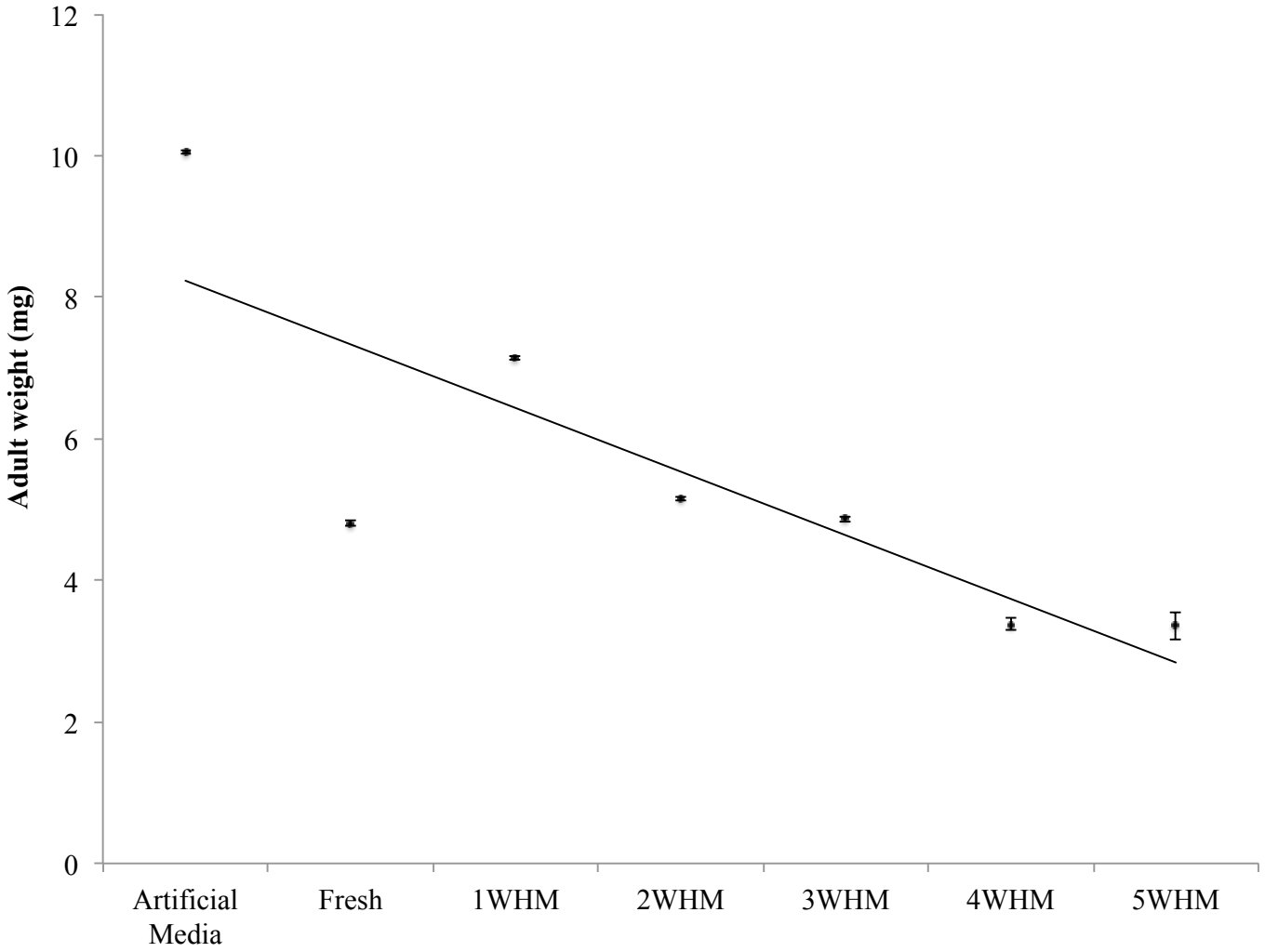

Figure 3.6 Correlation between stable fly fresh adult body weight (mg) and the substrate age.

Bars are standard error of mean (SEM). r^2= -0.82. n=2987, 919, 1262, 1190, 1097, 224, and 32, respectively.

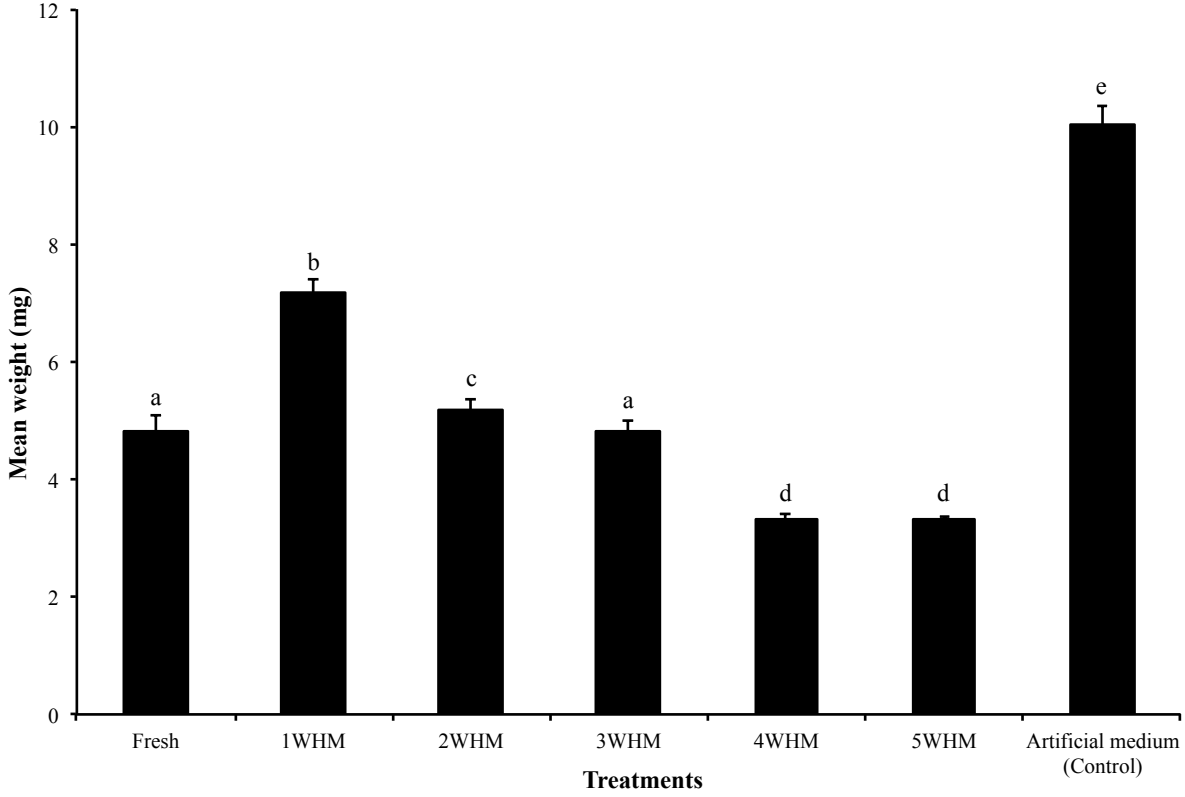

Figure 3.7 Mean body weight of newly emerged adult stable flies that developed in different ages of horse manure and in the artificial medium (control).

Different letters indicate significant differences ($p<0.05$) among all treatments. n=919, 1262, 1190, 1097, 224, 32, and 2987, respectively. Error bars are SEM.

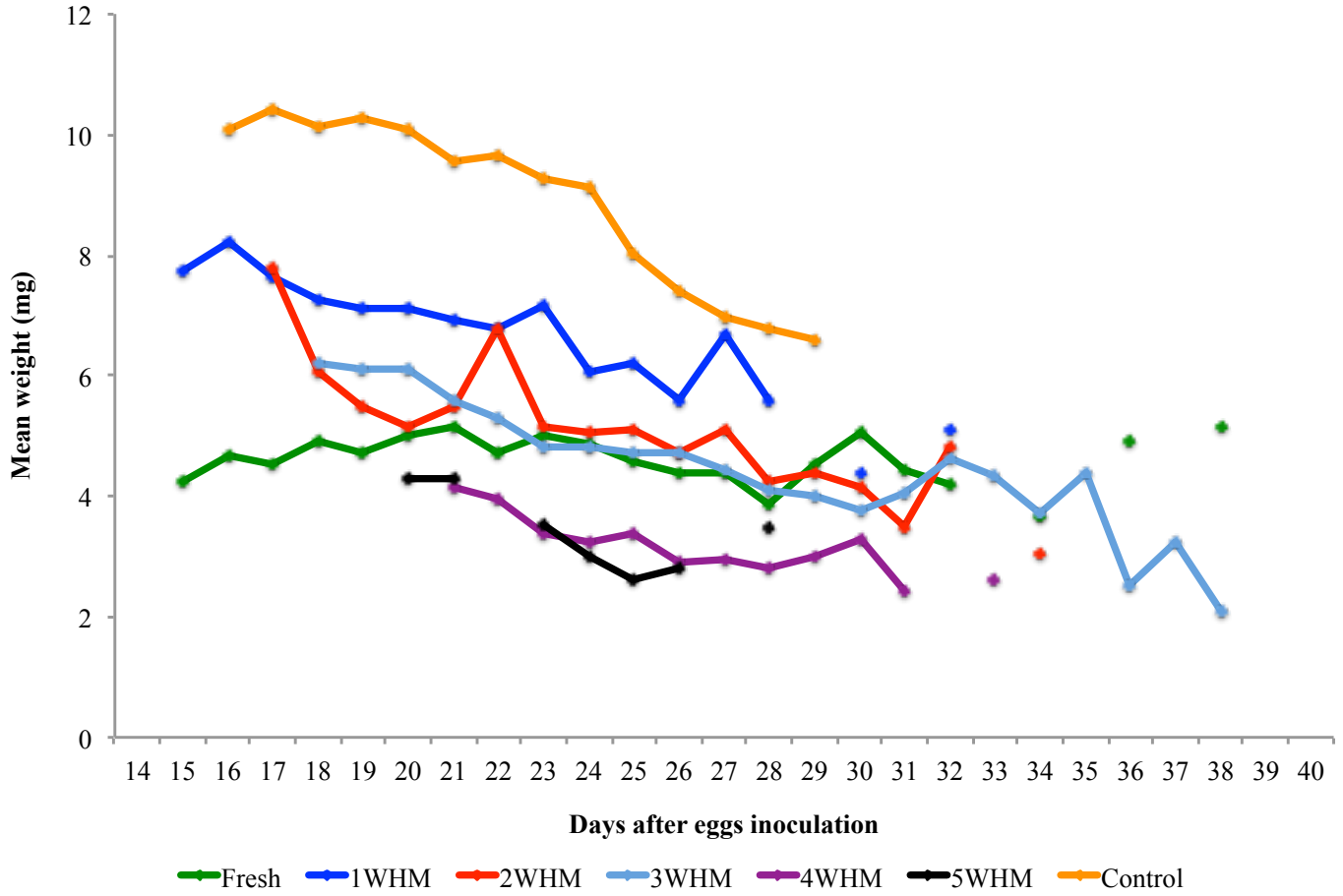

Figure 3.8 The daily mean weight of stable flies freshly emerged from horse manure of different ages and from the artificial medium (control).

n=919, 1262, 1190, 1097, 224, 32, and 2987, respectively.

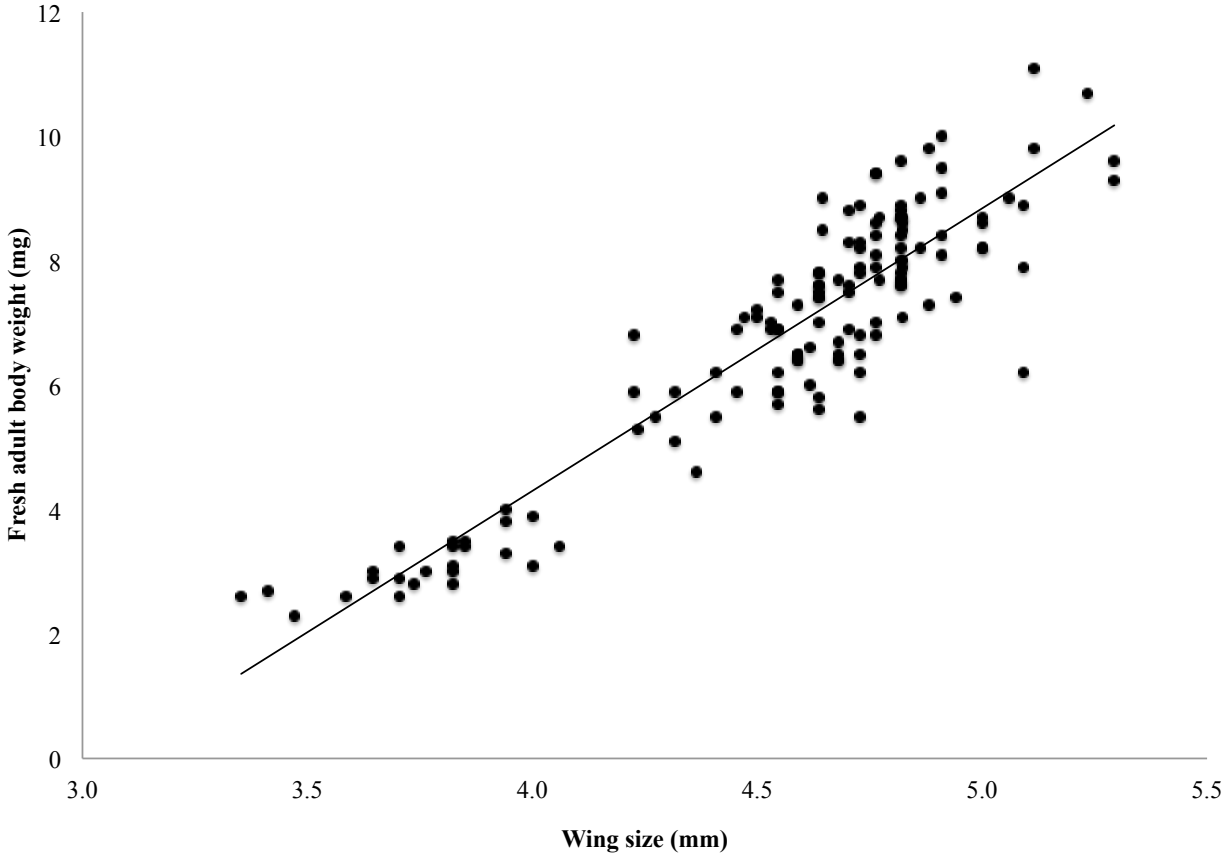

Figure 3.9 Correlation of the stable fly wing size (mm) and the fresh adult body weight (mg).

n= 140. r^2= 0.8416.

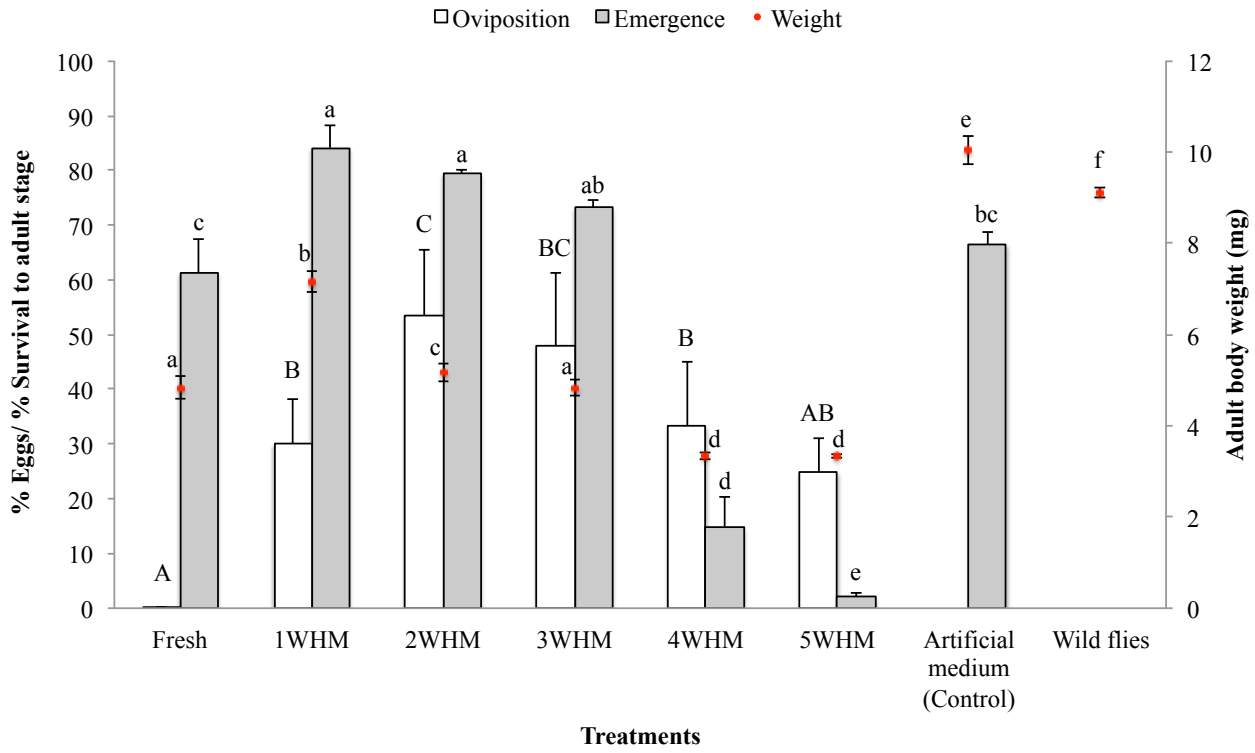

Figure 3.10 Stable fly oviposition (% eggs) and development (% survival to adult stage and fresh adult body weight) in the aging horse manure compared to artificial medium (control) and wild flies.

Different letters indicate significant differences ($p<0.05$) among same treatments. n=3. Error bars are SEM.

Chapter 4 - Microbial community structure of aging horse manure

Introduction

Studies on animal-bacterial associations and their importance for the host organism are abundant (Abe et al., 2000; Backhed et al., 2004; Dethlefsen et al., 2007; Mateos et al., 2006; O'Hara & Shanahan, 2006; Lam et al., 2007; Feldhaar, 2011). Recently, a new hologenome theory was proposed where the host and the symbiont are considered as a "superorganism" and to study evolutionary processes, the sum of the genetic information of all partners in the symbiosis should be taken into account (Zilber-Rosenberg & Rosenberg, 2008).

Symbiosis can be divided into three categories: parasitism (beneficial for one partner and detrimental for the other), commensalism (beneficial for one partner and with a little or no cost for the other) and mutualism (beneficial for all partners) (Lim, 1998). However, to classify these interactions is not always straightforward. For instance, if the ratio of bacteria per host is altered, what previously was regarded as a mutualistic interaction can become parasitic or pathogenic. An example are Clostridia that have been observed to influence animal health both negatively, reducing protein availability in fresh forage (Reilly & Attwood, 1998), and positively, acting as a beneficial probiotic and improving cellulose digestion (Wielen et al., 2002).

Economically, the most important example of animal-microbe symbiosis is that of the ruminants. Rumen bacteria primarily ferment cellulose to be absorbed by the animal (Tortora, 2004). Horses also depend on a fermentative digestion which is similar to that in the cattle rumen (AlJassim & Andrews, 2009). A horse fermentative chamber is the hindgut (cecum and colon), which harbors a complex microbial population. In addition to cellulose degradation, these microorganisms play an important role in immune responses, pathogen exclusion, and

detoxification (Shepherd et al., 2012). Hindgut bacterial fermentation enables herbivores to digest high-fiber diets (Shepherd et al., 2012) and more than 50% of horse daily energy originates from the microbial degradation of fiber (Argenzio et al., 1974). *Ruminococcus flavefaciens* and *Fibrobacter* spp. are among the most common cellulolytic bacteria in the horse digestive tract (Julliand et al., 1999; Lin & Stahl, 1995). Equine hindgut microbiota has received less attention comparing to that of rumen and culture-independent methods characterizing the equine hindgut bacterial community are scarce (Daly et al., 2001; Daly & Shirazi-Beechey, 2003; Yamano et al., 2008; Shepherd et al., 2012). The resident microbial community of the horse hindgut comprises the phyla *Firmicutes*, *Verrucomicrobia*, *Proteobacteria*, *Bacteroidetes*, and *Spirochaetes* (Daly et al., 2001; Shepherd et al., 2012). Studies on how the horse fecal bacterial community changes overtime and how that impacts fitness of insects that develop in horse manure are lacking.

In general, microbes are important for nutrition of many insect species (Rochon et al., 2004; Lam et al., 2009) and bacterially-mediated volatiles can be used as cues for insects to locate new oviposition sites (Chapman, 1971). *Klebsiella oxytoca*, commonly present on the surface of house fly eggs, induces new ovipositions when present in low concentrations but acts as an oviposition repellent in high concentrations. Hence, bacteria regulate the number of house fly eggs deposited in a specific habitat (Lam et al., 2007). A group of bacteria, including *K. oxytoca*, *Enterobacter cloacae*, *Enterobacter sakazakii*, *Proteus mirabilis*, *Proteus vulgaris*, *Providencia rettgeri*, *Providencia stuartii*, and *Serratia liquefaciens*, isolated from infested wounds by the primary screwworm larvae (*Cochliomia hominovorax*), was shown to increase oviposition in females (Chaudhury et al., 2002).

Bacteria were also shown to play a crucial role in the stable fly (*Stomoxys calcitrans*) biology (Lysyk et al., 1999; Lysyk et al., 2002; Romero et al., 2006; Gilles et al., 2008). Stable fly larvae cannot develop beyond the first instar in sterilized substrates (Lysyk et al., 1999; Lysyk et al., 2002; Romero et al., 2006;). Stable flies also prefer to lay eggs on substrates containing a live bacterial community rather than on sterile substrates, and the same bacterial isolates that stimulated oviposition, such as *Serratia fanticola* and *Citrobacter freundii*, also supported larval development (Romero et al., 2006). *Acinetobacter sp*., *Empedobacter breve* and *Flavobacterium odoratum* supported stable fly larval development but were not tested for their effect on stable fly oviposition (Lysyk et al., 1999). Moreover, it was suggested that the fluctuations in the concentration of fecal coliforms around round hay bale feeders used in pastured cattle were responsible for the seasonality of stable flies (Talley et al., 2009).

The attractiveness of animal manure for stable flies to oviposit was shown to vary overtime (Broce & Haas, 1999; Chapter 2). The decomposition of cattle manure also influenced stable fly oviposition preference, with increase in gravid stable fly visitation on cattle manure that had aged for 2 weeks (Broce & Haas, 1999). Interestingly, the fresh horse manure was reported to be more attractive for stable fly oviposition when compared to that of fresh cattle manure (Jeanbourquin & Guerin, 2007). However, fresh manure does not reflect the natural oviposition substrate for stable flies. Differences in the bacterial community during manure decomposition may likely explain stable fly oviposition preference for 2 and 3 weeks old horse manure (shown in Chapter 2) and the most successful larval development in 1 to 3 weeks old horse manure (shown in Chapter 3).

Analyzes of the animal manure microbial community are typically conducted to assess an animal health. This is the first study focused on the temporal changes in the horse manure

bacterial community and their implications for stable fly fitness. The differences in bacterial community at different ages of horse manure decomposition may offer new insites on stable fly oviposition behavior and larval development and consequently lead to new management strategies. The comprehensive characterization of the microbial community of aging horse manure is critical for better understanding of insect colonization and can help identify bacteria that release volatiles that could be used as attractants in oviposition traps or as repellents in new insecticide formulations.

Objective of the study

To assess the changes in the microbial community structure of aging horse manure.

Hypothesis

Null hypothesis: The microbial community of horse manure does not change overtime.

Materials and Methods

Manure samples

Samples (10 g) of aging horse manure (from fresh to 5 weeks old) used in the oviposition assays (Chapter 2) were collected weekly (on the day of the two-choice experiment) in 60 x 15 mm Petri dishes (Fisher Scientific, Pittsburgh, PA) and frozen in -80°C freezer for later analysis.

Assessment of the bacterial diversity by 454 pyrosequencing

Total genomic DNA extraction of 1.0 g of each horse manure sample was performed using the FastDNA® SPIN Kit for soil (MP Biomedicals, Solon, OH) following manufacturer's

instructions. DNA quantification was done using the Nanodrop spectrophotometer (Nyxor Biotech, Paris, France). The bacterial tag-encoded FLX 16 rDNA amplicon pyrosequencing (bTEFAP) and post sequencing processing were carried out at the Medical Biofilm Research Institute (Lubbock, TX). The DNA from all samples was adjusted to 100 ng/ µl and 100 ng (1 µl) aliquot used for a 50 µl PCR reactions. The primers used to amplify the 600 bp region of 16S rRNA genes were the 16S universal Eubacterial primers 530F (5'-GTG CCA GCM GCN GCG G) and 1100R (5'- GGG TTN CGN TCG TTG). HotStarTaq Plus Master Kit (Quiagen, Valencia, CA) was used for PCR under the following conditions: 94°C for 3 minutes followed by 32 cycles of 94°C for 30 seconds; 60°C for 40 seconds and 72°C for 1 minute; and a final elongation step at 72°C for 5 minutes. A secondary PCR was performed for FLX (Roche, Nutley, New Jersey) amplicon sequencing to incorporate tags and linkers. This was done under the same conditons described above and using 1.0 µl of the original PCR reaction as a template. The tag is a sample-specific sequence to identify, from the bulk reaction, which sequence came from which sample. They were designed using a custom script written in the C# programming language (Microsoft, Seattle, WA) that generates all possible combinations of 10-mer oligonucleotide tags with GC content between 40% and 60%. From this pool we utilized 6 individual tags. The primers were the same used above and the linker was the standard FLX sequencing specific sequence. After this secondary PCR, all amplicon products from different samples were mixed in equal volumes and purified using Agencourt Ampure Beads (Agencourt Bioscience, Beverly, MA).

Before FLX sequencing (Roche, Nutley, NJ), concentration and size of the DNA fragments were accurately measured using DNA chips under a Bio-Rad Experion Automated Eletrophoresis Station (Bio-Rad Laboratories, CA, USA) and a TBS-380 Fluorometer (Turner

Biosystems, CA, USA). A sample containing 9.6 E + 06 double-stranded DNA molecules/ µl and an average size of 625 bp was combined with 9.6 million DNA capture beads, and amplified by emulsion PCR. After bead recovery and bead enrichment, the bead-attached DNAs were denaturated with NaOH, and annealed with sequencing primers. A two-region 454 sequencing run was performed on a 70x75 GS PicoTiterPlate (PTP) using a Genome Sequencer FLX System following manufacturer's instructions (Roche, Nutley, NJ). Quality trimmed sequences obtained from the FLX sequencing run were processed using a custom scripted bioinformatics pipeline. Sequences shorter than 150 bp after quality trimming were not considered for further analysis.

Data were analyzed and interpreted using Sequencher 4.8 (Genes Codes) for alignment and sequencing editing, MOTHUR (www.mothur.org) for diversity and richness, and BLAST2GO for the NCBI Gen-Bank search and identification.

Results

The number of trimmed sequences and richness and diversity of the bacterial community of each sample of horse manure of different ages is depicted in Table 4.1. The number of good quality sequences was above 5000 for all samples with the exception of that of one week old manure (1WHM). The number of operational taxonomic units (OTUs) with a difference of 3% (genus level) showed that the bacterial community in the fresh manure is more diverse (1458 OTUs) than that of all older manure (all with less than 800 OTUs). Another diversity estimator, such as Shannon index (H') (considering richness and abundance together), and statistical richness estimators, such as ace and Chao1 (that account for the number of different organisms), are useful to compare the relative complexity of communities (Schloss & Handelsman, 2005). The Shannon (H'), Chao1, and ace indices also indicated greater bacterial diversity (H') and

richness (Chao1 and ace) of the fresh manure when compared to that of the aged manure (Table 4.1).

Overall, in all samples of the horse manure, a total of 421 bacterial genera and 917 species were detected. The bacterial diversity of the horse manure on the genus level is shown in Figure 4.1 and Table 4.2 (strict anaerobes) and Figure 4.2 and Table 4.3 (facultative anaerobes and aerobes). Only bacterial taxa that were represented by ≥ 1% of identified sequences in at least one horse manure sample are reported. The fresh manure was clearly dominated by strictly anaerobic bacteria including *Clostridium* (38.5%), *Eubacterium* (17.9%), *Bacteroides* (9.7%), *Prevotella* (8.3%), *Parabacteroides* (5.6%), and *Roseburia* (4.2%). Spirochete*s* were more common when the horse manure was 4 weeks old (33.42%) and *Turicibacter* when the manure was 5 weeks old (24.13%) (Table 4.2) comparing to that of the fresh manure. A shift from strictly anaerobic bacteria in fresh manure to strict aerobes or facultative anaerobes in 1WHM and older was evident (Figure 4.1, 4.2).

Only few facultative anaerobic and aerobic bacterial taxa were detected when the manure was fresh (Table 4.3). Overall, *Rhizobium* (40.20%), *Devosia* (34.69%), *Sphingopyxis* (33.26%), *Brevundimonas* (28.54%), and *Comamonas* (20.09%) were the five most common genera in aging manure (1 - 5WHM) (Table 4.3). 2WHM and 3WHM (that stimulated stable fly oviposition the most) was dominated by genera *Rhizobium* (21.38%), *Devosia* (19.46%), *Brevundimonas* (14.47%), *Sphingopyxis* (10.42%) and *Sphingobium* (8.41%) (Table 4.3).

Overall on the species level (1% difference among sequences), *Spirochaeta stenostrepta* (25.94%), *Turicibacter sanguinis* (18.05%), and *Clostridium symbiosum* (10.55%) were most common strict anaerobes (Table 4.4), and *Sphingopyxis witflariensis* (26.49%), *Devosia limi* (18.11%), and *Comamonas aquatica* (16.29%) were most common among the facultatively

anaerobic and strictly aerobic bacteria (Table 4.5). The genus *Clostridium* was the most diverse and represented 55 species with *Clostridium symbiosum* and *Clostridium xylanolyticum* detected most frequently. *Devosia limi* (9.93%), *Rhizobium giardinii* (8.22%), *Brevundimonas diminuta* (7.48%), and *Sphingopyxis witflariensis* (7.29%) were the most commonly detected bacterial species in 2 and 3 weeks old manure (Table 4.5).

Discussion

The gastrointestinal tract of horses is colonized by microorganisms that impact the host physiology profoundly (AlJassim & Andrews, 2009) as horses depend on bacteria to digest fiber, which provides more than 50% of the energy (Argenzio et al., 1974).

The pyrosequencing approach to assess the bacterial diversity of the horse manure over time revealed high diversity of bacterial genera and species. The number of operational taxonomic units (OTUs) in fresh manure was higher than that from any other manure age reflecting the conditions in the horse digestive tract conducive for a large and very diverse bacterial community.

The species richness (Chao 1 and ace indices) was highest when the manure was fresh, declined after one week and remained at the same level for up to 5 weeks. This is most likely because strictly anaerobic bacteria, abundant in the horse hindgut, starts to die after exposure to oxygen. In contrast, strictly aerobic and facultative anaerobic bacteria that were present in a very low level in the fresh manure took advantage of oxygen exposure and replaced strict anaerobes in 1WHM and older. In our study, the fresh horse manure was composed mainly by *Firmicutes* (61.6%), *Bacteroidetes* (20.8%), *Proteobacteria* (2.9%), *Spirochaetes* (1.6%), and 13.2% of unclassified bacteria. *Firmicutes* was also the most abundant bacterial group identified in Shepherd et al. (2012) study on the horse fecal microbiota (43.7% of total bacterial sequences),

followed by *Verrucomicrobia* (4.1%), *Proteobacteria* (3.8%), *Bacteroidetes* (3.7%), and 38.1% of unclassified bacteria. However, the abundance of *Firmicutes* in these two studies is lower than that (72%) reported by Daly et al. (2001). Differences in the horse feed and number of sequences analyzed likely account for the differences in the hindgut bacterial community among different studies. Moreover, animal-to-animal variations in the fecal microbial diversity was observed in beef cattle with similar physiological characteristics and following the same diet for 2 months prior manure sampling (Durso et al., 2010). The *Firmicutes* also dominated the fecal bacterial community of other mammals including people (56%), pigs (55%), dairy cattle (81.3%) and beef cattle (62.8% and 70%), indicating that this bacterial group likely plays an important role in the gut physiology of many animal species (Lamendella et al., 2011; Ozutsumi et al., 2005; Durso et al., 2010). In dairy cattle, *Firmicutes* represented 81.3% of the sequences, *Bacteroidetes* (14.4%), *Actinobacteria* (2.5%), and *Proteobacteria* (1.4%) (Ozutsumi et al., 2005). In beef cattle, *Firmicutes* accounted for 62.8% of the sequences, *Bacteroidetes* (29.5%), *Proteobacteria* (4.4%), *Actinobacteria* (0.79%), *Tenericutes* (0.63%), *Verrucomicrobia* (0.05%) and other bacteria (1.8%) (Durso et al., 2010).

The fecal bacterial diversity of the fresh manure in our study (Shannon index H'= 6.24) was comparable to that (6.7) observed in Shepherd et al. (2012) and higher than in other species; H'= 4.9 in beef cattle feces (Durso et al., 2010), H'= 3.2 in pig manure (Lamendella et al., 2011), and H'= 4.0 in humans (Andersson et al., 2008). This difference in diversity is possibly because of the high fiber diet for horses (Shepherd et al., 2012).

On the genus level, the microbiota of the fresh feedlot cattle manure is composed of *Bacteroidetes* including *Prevotella* and *Bacteroides*; the *Firmicutes* such as *Faecalibacterium*, *Ruminococcus*, *Roseburia*, and *Clostridium*; and the Proteobacterium *Succinovibrio* (Durso et al.,

2010). In the fresh dairy cattle manure *Clostridium*, *Bacteroides*, *Porphyromonas*, *Ruminococcus*, *Alistipes*, *Lachnospiraceae*, *Prevotella*, *Lachnospira*, *Enterococcus*, *Oscillospira*, *Cytophage*, *Anaerotruncus*, and *Acidaminococcus* were most common (Dowd et al., 2008).

In this study, the most abundant genus in the fresh horse manure was *Clostridium*, representing 38.5% of all sequences. This is in contrast to dairy and beef manure where clostridia represented only 20% and 1.5% of the microbiota, respectively (Dowd et al., 2008, Durso et al., 2010). *Eubacterium* was the second most prevalent genus detected in the fresh horse manure (17.9%) followed by *Bacteroides* (9.7%) and *Prevotella* (8.3%). *Prevotella* was the most common genus in the beef cattle manure representing 24% of the total number of sequences (Durso et al., 2010), while in dairy cattle this genus represented only 5.5% of the microbiota (Dowd et al., 2008).

Overall, in our study, 240 species corresponding to 106 genera were detected in the fresh horse manure. Dowd et al. (2008) reported 274 bacterial species corresponding to 142 genera in their study with fresh dairy cattle manure.

Studies of the temporal assessment of the bacterial community of any animal manure are lacking. After exposure to air, a shift from anaerobic bacteria to aerobic and facultative anaerobic bacteria was observed. When the manure was 2 and 3 weeks old (most attractive ages for stable fly oviposition), the most common genera were represented by Alphaproteobacteria including *Rhizobium*, *Devosia, Brevundimonas*, *Sphingopyxis*, and *Sphingobium*. *Rhizobium* is a common soil bacterium capable of fixing nitrogen and found in association with plant roots, especially in the family Leguminosae (Brenner et al., 2005). The diet of horses used in this study included alfalfa hay that is most likely the source of *Rhizobium* spp. detected in the horse manure. *Devosia*

spp. are gram negative rods, also commonly found in soil (Brenner et al., 2005; Yoo et al., 2006). *Devosia* spp. are not able to hydrolyze gelatin or starch but they can degrade urea and poorly reduce nitrate to nitrite (Brenner et al., 2005). *Brevundimonas* are aquatic bacteria (Brenner et al., 2005), non-fermenting gram-negative bacilli that have been reported to be rare opportunistic pathogens of immunocompromised hosts (Lee et al., 2011); all species can grow utilizing pyruvate, and most isolates can also use the amino acids (glutamate and proline), and the organic acids (acetate, butyrate, fumarate, and succinate). All *Brevundimonas* spp. can use glucose, galactose, maltose, and starch (Brenner et al., 2005). *Devosia* spp. and *Brevundimonas* spp. do not produce indole as a result of their metabolism (Brenner et al., 2005).

Bacteria from the phylum Proteobacteria (mainly Gammaproteobacteria and Alphaproteobacteria) were the most abundant groups of bacteria (50% of the sequences) identified in water samples collected after *Aedes aegypti* and *Aedes albopictus* oviposition (Ponnusamy et al., 2008a,b), indicating that some of these bacteria may be releasing volatiles that attract mosquitoes for oviposition. Moreover, eight volatile compounds were identified and tested as oviposition stimulants, and three of them, nonanoic acid, tetradecanoic acid, and methyltetradecanoate, were highly effective at inducing mosquito egg laying (Ponnusamy et al., 2008b). *Rhizobium* and *Brevundimonas* were among detected genera indicating that they may be responsible for production of mosquito oviposition stimulants or attractants. Therefore, since these two genera were common in 2 and 3 weeks old horse manure, it is possible that they play a role in stable fly oviposition behavior although this remains to be evaluated.

This is the first study analyzing the bacterial community in the horse manure of different ages. The preference of stable flies to oviposit on 2 and 3 weeks old horse manure and the successful larval development in this manure (Chapters 2 and 3) suggest that the bacterial

community in 2 and 3 weeks old horse manure generates the cues for stable fly oviposition and is also important for the larval development. However, the identification of the semiochemical cues and their specific bacterial origin as well as the basis of the larval dependence on bacteria remain to be evaluated. Future investigations of the stable fly - microbial associations will lead to development of novel approaches for management of this insect pest based on the modification of the bacterial community of the horse manure and/or development of novel attractants and repellents and/or paratransgenic approach.

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Table 4.1 Diversity and richness of horse manure microbial community at different ages.

Sample	no. of trimmed seqs	OTU 3%	H' 3%	ace 3%	chao 3%
Fresh	6102	1458	6.24	2252.0	2145.51
1WHM	3500	330	4.08	632.05	621.38
2WHM	5470	625	5.18	861.17	854.29
3WHM	5569	767	5.50	1100.39	1143.79
4WHM	5127	685	4.63	959.56	880.85
5WHM	6101	796	5.28	1131.01	1115.81

OTU: operational taxonomic units observed with 3% cutoff (genus level) in distance units for describing and comparing communities.
H': nonparametric Shannon diversity index with 3% cutoff.
ace: nonparametric richness abundance-coverage estimator with 3% cutoff.
chao1: nonparametric richness estimator signifies the estimated minimum number of OTUs with a 3% cutoff.

Table 4.2 Genera of anaerobic bacteria (%) detected in different ages of the horse manure.

genus (no. of species)	Fresh	1W	2W	3W	4W	5W	Total
***Clostridium* (55)**	38.47	0.13	1.93	0.15	1.36	5.62	**47.66**
***Spirochaeta* (4)**	0.01	0.00	0.00	6.18	33.42	4.68	**44.30**
***Turicibacter* (1)**	0.00	0.00	0.18	0.00	0.03	24.13	**24.33**
***Eubacterium* (20)**	17.93	0.13	0.89	0.03	0.17	2.65	**21.80**
***Bacteroides* (17)**	9.70	0.26	0.35	0.11	1.02	0.08	**11.52**
***Prevotella* (17)**	8.27	0.36	0.44	0.51	0.44	0.15	**10.17**
***Ruminococcus* (7)**	2.56	0.00	0.11	0.00	0.00	3.46	**6.13**
***Parabacteroides* (2)**	5.64	0.00	0.03	0.03	0.03	0.00	**5.72**
***Roseburia* (2)**	4.16	0.10	0.05	0.00	0.00	1.12	**5.43**
***Opitutus* (1)**	0.00	0.00	0.03	0.06	1.24	1.86	**3.18**
***Sporobacter* (1)**	2.37	0.00	0.03	0.05	0.00	0.21	**2.66**
***Treponema* (8)**	1.80	0.12	0.03	0.17	0.39	0.10	**2.60**
***Porphyromonas* (5)**	0.03	0.00	2.55	0.00	0.00	0.00	**2.58**
***Pseudobutyrivibrio* (1)**	2.42	0.00	0.14	0.00	0.00	0.00	**2.56**
***Fusobacterium* (4)**	0.01	0.43	1.54	0.00	0.00	0.43	**2.41**
***Anaerovorax* (1)**	1.84	0.00	0.16	0.00	0.00	0.00	**2.01**
***Allobaculum* (1)**	0.00	0.00	1.72	0.00	0.00	0.14	**1.86**

Numbers are percentage of identified sequences.

Table 4.3 Genera of facultative and aerobic bacteria (%) detected in different ages of the horse manure.

genus (no. of species)	Fresh	1W	2W	3W	4W	5W	Total
***Rhizobium* (8)**	0.00	5.24	8.56	12.81	6.17	7.41	**40.20**
***Devosia* (3)**	0.00	6.53	6.46	13.01	4.42	4.28	**34.69**
***Sphingopyxis* (5)**	0.00	19.62	5.53	4.89	2.05	1.16	**33.26**
***Brevundimonas* (5)**	0.00	6.56	5.76	8.71	4.62	2.88	**28.54**
***Comamonas* (5)**	0.00	13.33	2.98	2.08	1.05	0.65	**20.09**
***Pseudomonas* (26)**	0.03	4.16	3.24	3.69	3.23	2.65	**17.00**
***Stenotrophomonas* (4)**	0.00	6.99	3.02	3.35	1.55	1.29	**16.20**
***Sphingomonas* (13)**	0.00	0.65	1.29	5.89	5.06	2.96	**15.86**
***Massilia* (3)**	0.00	9.36	2.76	1.03	0.90	0.60	**14.65**
***Sphingobium* (5)**	0.00	1.00	3.21	5.20	2.83	2.12	**14.36**
***Caulobacter* (4)**	0.00	0.00	2.70	3.30	5.20	1.88	**13.08**
***Rhodoferax* (3)**	0.00	2.57	3.12	3.16	0.75	1.25	**10.84**
***Flavobacterium* (20)**	0.00	0.81	2.04	2.99	2.05	2.33	**10.22**
***Haemophilus* (4)**	0.18	0.33	9.18	0.00	0.00	0.00	**9.69**
***Rhodobacter* (2)**	0.00	5.92	0.93	1.03	0.53	0.26	**8.68**
***Phenylobacterium* (4)**	0.00	0.20	1.27	2.16	2.32	1.55	**7.50**
***Phyllobacterium* (3)**	0.00	0.00	0.09	2.50	2.43	2.22	**7.24**
***Rubrivivax* (1)**	0.00	0.00	0.14	0.34	1.99	4.64	**7.11**
***Pedobacter* (8)**	0.09	1.60	0.93	1.93	0.93	1.05	**6.54**
***Dyadobacter* (3)**	0.00	0.00	1.03	2.54	1.27	1.03	**5.86**
***Wautersiella* (1)**	0.00	0.48	0.09	2.79	1.55	0.93	**5.83**
***Terrimonas* (1)**	0.00	0.00	0.11	1.10	1.89	2.33	**5.42**
***Paracoccus* (9)**	0.00	3.28	0.66	0.78	0.41	0.15	**5.29**
***Janthinobacterium* (2)**	0.00	4.08	0.48	0.18	0.00	0.32	**5.06**
***Algoriphagus* (8)**	0.26	0.16	0.70	1.28	1.34	1.18	**4.92**

***Alysiella* (1)**	0.00	0.00	4.78	0.00	0.00	0.00	**4.78**
***Streptococcus* (14)**	1.02	0.54	2.51	0.00	0.58	0.04	**4.69**
***Novosphingobium* (6)**	0.00	0.57	0.93	1.32	1.14	0.72	**4.67**
***Lactobacillus* (8)**	0.13	0.03	0.90	0.05	1.65	1.81	**4.58**
***Acinetobacter* (6)**	0.00	2.86	1.18	0.32	0.05	0.15	**4.56**
***Moraxella* (1)**	3.06	0.00	0.65	0.03	0.14	0.17	**4.05**
***Mesorhizobium* (5)**	0.00	0.00	0.25	1.53	0.97	1.25	**4.00**
***Ochrobactrum* (5)**	0.00	0.13	0.36	1.67	0.74	0.68	**3.59**
***Xanthomonas* (5)**	0.00	1.32	0.69	0.55	0.41	0.21	**3.18**
***Chloroflexus* (1)**	0.00	0.00	0.05	0.23	1.14	1.61	**3.02**
***Actinobacillus* (4)**	0.00	0.00	2.90	0.00	0.00	0.00	**2.90**
***Neisseria* (5)**	0.00	0.00	2.68	0.00	0.03	0.00	**2.71**
***Chromohalobacter* (1)**	0.00	0.00	0.03	0.26	0.53	1.62	**2.43**
***Gemella* (1)**	0.00	0.00	2.22	0.00	0.00	0.00	**2.22**
***Conchiformibius* (1)**	0.00	0.12	1.88	0.00	0.00	0.00	**2.00**
***Bergeyella* (1)**	0.00	0.00	1.57	0.00	0.00	0.00	**1.57**

Numbers are percentage of identified sequences.

Table 4.4 Species diversity of strictly anaerobic bacteria (%) representing ≥ 1% of identified sequences in ≥ 1 age of horse manure.

name	Fresh	1WHM	2WHM	3WHM	4WHM	5WHM	Total
Bacteroidales oral	5.18	0.17	0.07	0.07	0.09	0.03	**5.61**
Bacteroides capillosus	1.61	0.06	0.04	0.00	0.04	0.00	**1.76**
Clostridium algidixylanolyticum	1.19	0.00	0.00	0.00	0.00	0.00	**1.19**
Clostridium bolteae	2.21	0.00	0.02	0.00	0.00	0.05	**2.28**
Clostridium celerecrescens	1.43	0.00	0.00	0.02	0.00	1.07	**2.52**
Clostridium hathewayi	2.35	0.00	0.00	0.00	0.02	0.03	**2.41**
Clostridium jejuense	1.04	0.00	0.02	0.00	0.02	0.00	**1.08**
Clostridium methylpentosum	1.92	0.00	0.02	0.04	0.00	0.00	**1.98**
Clostridium nexile	1.08	0.00	0.00	0.00	0.00	0.00	**1.08**
Clostridium orbiscindens	1.25	0.00	0.02	0.00	0.00	0.01	**1.28**
Clostridium saccharolyticum	1.01	0.00	0.00	0.00	0.00	0.31	**1.32**
Clostridium symbiosum	10.13	0.03	0.21	0.02	0.04	0.12	**10.55**
Clostridium xylanolyticum	5.33	0.00	0.00	0.00	0.00	0.00	**5.33**
Eubacterium rectale	2.16	0.00	0.00	0.00	0.00	0.00	**2.16**
Eubacterium ruminantium	9.12	0.09	0.07	0.02	0.11	0.24	**9.66**
Eubacterium siraeum	2.73	0.03	0.00	0.00	0.02	0.00	**2.78**
Parabacteroides goldsteinii	4.60	0.00	0.02	0.02	0.02	0.00	**4.66**
Prevotella bivia	3.09	0.03	0.00	0.02	0.00	0.00	**3.14**
Prevotella bryantii	2.04	0.11	0.07	0.14	0.09	0.05	**2.51**
Roseburia intestinalis	3.52	0.09	0.04	0.00	0.00	0.80	**4.45**
Ruminococcus obeum	0.15	0.00	0.09	0.00	0.00	2.23	**2.47**
Spirochaeta americana	0.00	0.00	0.00	0.98	6.14	0.76	**7.88**
Spirochaeta stenostrepta	0.00	0.00	0.00	3.83	19.49	2.61	**25.94**
Turicibacter sanguinis	0.00	0.00	0.14	0.00	0.02	17.88	**18.05**

Numbers are percentage of identified sequences.

Table 4.5 Species diversity of facultatively anaerobic bacteria and strictly aerobic bacteria (%) representing ≥ 1% of identified sequences in ≥ 1 age of horse manure.

name	Fresh	1WHM	2WHM	3WHM	4WHM	5WHM	Total
Brevundimonas diminuta	0.00	5.90	3.61	3.86	1.23	0.77	**15.38**
Brevundimonas nasdae	0.00	0.03	0.96	1.81	1.43	0.73	**4.96**
Caulobacter vibrioides	0.00	0.00	2.02	2.26	3.65	1.25	**9.18**
Comamonas aquatica	0.00	11.23	2.24	1.60	0.75	0.47	**16.29**
Devosia limi	0.00	4.62	3.56	6.37	1.93	1.62	**18.11**
Devosia riboflavina	0.00	1.22	1.61	3.06	1.01	0.63	**7.52**
Flavobacterium mizutaii	0.00	0.06	0.24	1.12	0.22	0.23	**1.85**
Massilia timonae	0.00	8.43	2.24	0.82	0.68	0.43	**12.60**
Pseudomonas lini	0.00	2.04	0.92	0.53	0.17	0.07	**3.74**
Rhizobium etli	0.00	0.20	0.43	1.77	1.37	1.36	**5.12**
Rhizobium giardinii	0.00	3.72	4.32	3.91	1.08	1.65	**14.68**
Rhizobium huautlense	0.00	0.14	1.80	4.05	1.98	2.04	**10.01**
Rhodoferax ferrireducens	0.00	2.32	2.55	2.47	0.60	0.92	**8.86**
Sphingobium herbicidovorans	0.00	0.90	2.18	3.40	2.07	1.39	**9.94**
Sphingomonas koreensis	0.00	0.03	0.18	1.90	2.35	1.53	**5.99**
Sphingopyxis witflariensis	0.00	17.68	4.39	2.90	0.90	0.62	**26.49**
Stenotrophomonas rhizophila	0.00	4.86	1.91	2.17	0.79	0.68	**10.41**

Numbers are percentage of identified sequences.

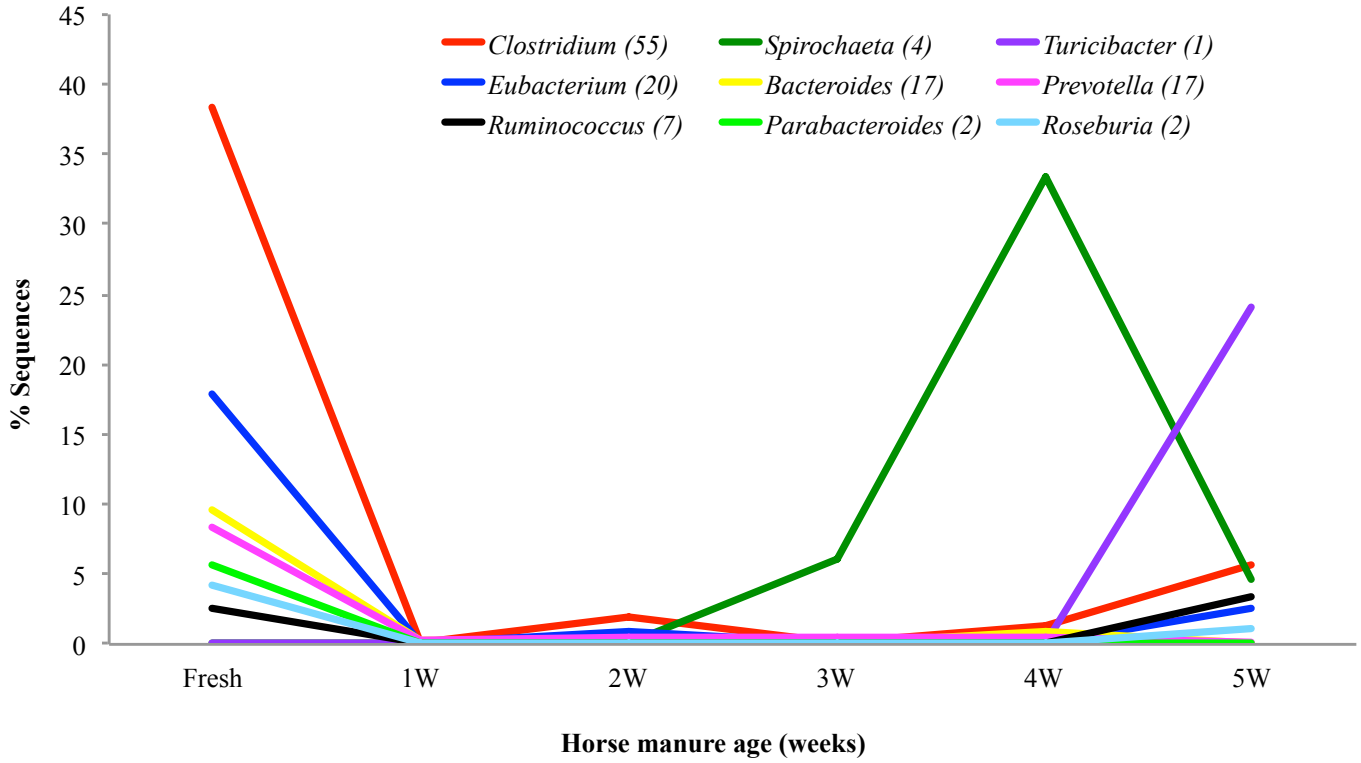

Figure 4.1 Dominant strictly anaerobic bacterial genera in different ages of the horse manure.

Genera (n=9) with more than 1% of identified sequences in at least one age of manure.

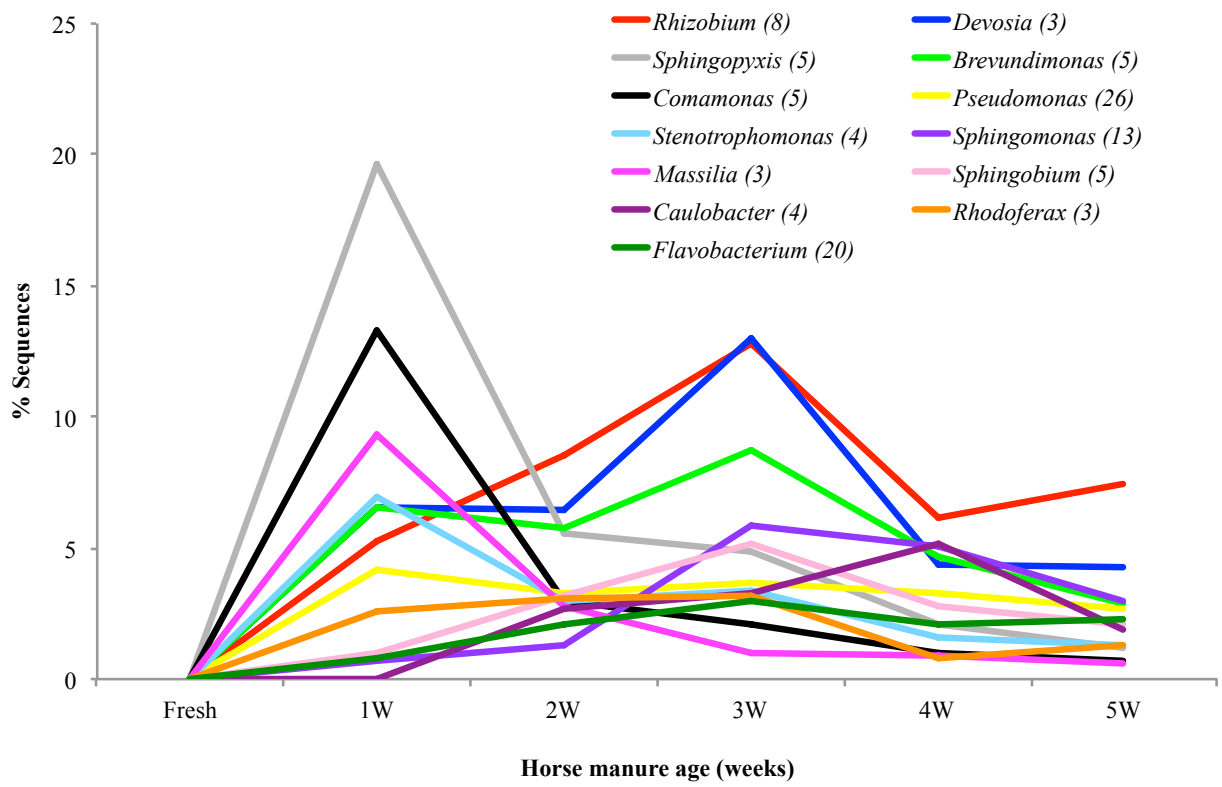

Figure 4.2 Dominant facultatively anaerobic and strictly aerobic bacterial genera in different ages of the horse manure.

Genera (n=13) with more than 1% of identified sequences on at least one age of manure.

Chapter 5 - Identification of volatile compounds emitted from the aged horse manure and their effect on stable fly (*Stomoxys calcitrans*) oviposition

Introduction

Insects have specialized sensors for detection of different external stimuli including visual, acoustic, tactile, and chemical signals that include olfaction and taste. The olfactory system allows the insect to recognize volatile cues and taste allows detection of and discrimination of non-volatile chemostimulants (Sanchez-Gracia et al, 2009). The primary contact with chemical signals occurs in porous chemosensory hairs (the sensilla) on the antennae by the uptake of signal molecules from the external environment. These are then transported by the odorant-binding proteins (OBPs) through the hemolymph of the sensory hair and bind with the chemoreceptor (Hildebrand & Shepherd, 1997; Stengl et al., 1999). The odor molecules are detected when they bind to a cognate trans-membrane receptor located on the dendrites of olfactory receptor neurons (ORNs). This activates a G-protein signaling cascade, producing an action potential that travels along the axon and converges onto glomeruli in the antennal lobe in the brain (Jefferis, 2005). Experiments using single-cell electrophysiology, an extracellular recording technique to examine the response of a single olfactory receptor (OR), have shown that different ORs from moths, mosquitoes, flies, and honey bees respond to different semiochemicals (Hallem et al., 2006).

Stable flies are important pests of animals especially important in cattle where annual economic losses are estimated at $2.2 billion per year in the United States alone (Taylor et al., 2012). Stable fly management relies primarily on sanitation (Shipley, 1915; Meyer & Petersen,

1983; Broce, 2006). Animal manure was shown to be an important substrate for stable fly larval development (Jeanbourquin & Guerin, 2007a). Moreover, the age of horse manure was shown to influence oviposition preference, with 2 and 3 week old manure attracting/stimulating stable flies the most (Chapter 2).

Microorganisms in animal manure were shown to start manure decomposition by fermenting starch (Miller & Varel, 2002). Once starch is depleted, fermentation continues by the use of other carbohydrates including cellulose and proteins (Miller & Varel, 2002). Manure odors contain a complex mixture of chemicals and feces from different animals have very different chemical composition profiles (Nibaruta et al., 1980). The major constituents of cattle manure are volatile fatty acids (VFA), alcohols, aromatic compounds, sulfides, and amines/ammonia. Ketones, esters, and aldehydes are also present, but in low concentrations (Miller & Varel, 2011). Volatiles released from fresh horse manure included alcohols, phenols (cresol) and amines (skatole) (Kimura, 2001).

Over the past decade, chemical ecology of livestock pests has been studied more intensively in attempts to generate alternative methods for insect pest management. In a study by Birket et al. (2004), 23 volatile compounds were identified from heifers and 2-day old cattle urine, showed that individual heifers generated different semiochemical profiles and these differences correlated with a level of infestation by horn flies. Phenol, p-cresol, and m-cresol were found to be released from all heifers but in lower concentrations from animals with a high horn fly infestation. Propylbenzene, styrene, camphene, 2-heptanone and propyl butanoate were unique to heifers with a very low horn fly population indicating their potential repellent activity (Birkett et al., 2004).

Electroantennogram (EAG) studies showed that stable flies responded to host semiochemicals collected from heifers and their urine. Significant EAG responses were elicited from antennae of stable flies to p-cresol, 2-methoxyphenol, (Z)-3-hexen-1-ol, 2-heptanone, naphthalene, 1-octen-3-ol, 3-octanol, 2-decanol, 1-nonanol, 6-methyl-5-hepten-2-one, linalool, tsetse lure and citronellol (Birkett et al., 2004). Indole, 1-octen-3-ol, phenol, and p-cresol (all at 10μg) elicited strong responses from both male and female stable flies in EAG experiments (Tangtrakulwanich et al., 2011). Consistent and significant EAG responses to a number of host kairomones such as octenol, 3-methylphenol, and various alcohols were also observed (Schofield et al., 1995). However, EAG results only detect the intensity of response and cannot distinguish between compounds with attractant and repellent activities.

Stable flies are also attracted by expired human breath, carbon dioxide, and acetone (Warnes & Finlayson, 1985). Field studies showed that traps baited with 1-octen-3-ol significantly increased the catch of *Stomoxys* spp. (Holloway & Phelps, 1991; Mihok et al., 1996); however, other studies showed that there was no significant response to octenol (Vale & Hall, 1985; Mullens et al., 1995). Furthermore, ammonium was shown to be related to stable fly attraction to biosolid cake (Dadour & Voss, 2009).

The identification of volatile compounds responsible for stable fly attraction to oviposition sites is a great tool for developing new control strategies.

Objective of the study

To identify volatile compounds emitted from 2 and 3 week old horse manure and to test their effect on stable fly oviposition.

Hypothesis

Null hypothesis: The semiochemicals emitted from 2 and 3 weeks old horse manure do not stimulate stable fly oviposition.

Materials and Methods

Manure collection

The fresh horse manure was collected from the floor of a pen with seven groups of horses and stored in plastic buckets (16 liters) (Table 5.1). Only the top 2/3 of each manure patch was collected to avoid soil contamination. Buckets were sterilized with hot water and soap (Palmolive original detergent®, Colgate-Palmolive Company, New York, NY), rinsed with clean water, sprayed with 70% ethanol, and dried. The manure was then taken to the laboratory, mixed by stirring, divided into 2 buckets filling 2/3 of each bucket, and closed with the plastic lid with two meshed breathing-holes (to keep the air flow). Buckets were then kept in an environmental chamber at 26°C to age for 2 and 3 weeks until being tested.

Preparation of manure samples for analysis and collection of volatile compounds

For chemical analysis, an aged-manure was removed from the incubator, the top dried layer of manure was removed, and only the moist manure was used for volatile collection. Three samples (3.0 g each) of the manure were placed into 3 pre-cleaned glass vials (15 ml) (rinsed with acetone, and let dry for 30 minutes in a fume hood).

The sample vials were closed with a silicone lid. Solid phase microextraction (SPME) portable field sampler with a Carboxen/PDMS fiber (75 mm carboxen/polydimethylsiloxane, Supelco, Bellefonte, Pennsylvania, USA) was used for volatile collection. The new SPME

portable field sampler was preconditioned for 1 hour at 300°C in the injection port of a gas chromatograph. During the collection, the Carboxen/PDMS fiber was exposed approximately 2–3 cm above the sample. Vials were incubated in a 37°C water bath, and the volatile collection lasted 18 hours. Three SPME fibers inserted into three empty vials were used as controls. After collection, SPME fibers were analyzed by a Perkin-Elmer GC-MS system (Waltham, MA). Details on the horse manure samples and volatile collections are shown in Table 5.2.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The identification of volatile compounds absorbed by the SPME fiber was done using the Perkin-Elmer gas chromatograph (GC) with a Clarus 500 Mass spectrometry (Perkin-Elmer, Waltham, MA). The SPME fiber was thermo-desorbed at 250ºC and analyzed through a DB-5 column (30m x 0.25 mm diameter, with 0.25µm film thickness, J&W Scientific, Folsom, CA). Helium at a flow rate maintained at 1.5 mL/min was used as the carrier gas. Samples were injected under the paused-splitless mode. The temperature program for the GC analyses was 60ºC for 1 min, rising by 5ºC/min to 120ºC, and then increased 20ºC/min up to 250ºC. Compounds were identified by comparisons of retention times and mass spectra with those of synthetic standards using PE MS Library Database (Perkin-Elmer, Waltham, MA). After each analysis, the SPME fiber was cleaned by exposing it in the heated GC injection port for 5 min. The cleaned fiber was analyzed once more in the GC to ensure no material left from previous analyses prior to next collection.

Synthetic standards of the identified volatile compounds from the aged horse manure were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), and the purity of those chemicals was >95%.

Stable flies

Stable flies used in the two-choice assays were from the laboratory colony maintained on a supersaturated solution of sugar water and citrate bovine blood. Flies were synchronized to lay eggs on the same day by selecting only flies that emerged within a period of 24h. After seven days of blood meals, the cage with the synchronized flies was cooled down in a refrigerator for 15 min, flies were transferred to a Petri dish, and kept cold for selection of females. Females were then allocated into groups of four per Petri dish and 20 dishes were used for each assay.

Two-choice oviposition assays

The oviposition site offered for the stable flies was a black cloth placed on a cotton pad in a Petri dish (Figure 5.1). Prior to each assay, 40 glass dishes (10 cm diameter) (Pyrex) were prepared with a circle of cotton (5.5 cm diameter, Swisspers – US Cotton) moistened with 9 ml of distilled water. A piece (13 x 4.5 cm) of black cloth (global crink type, 100% cotton) was placed on the top of the cotton circle, folded in half, and moistened with distilled water (1ml) (Figure 5.1). The plates were then closed with a glass lid and autoclaved for 30 min, and then cooled down overnight in a room temperature. Then, 10µl of a mixture of the synthetic compounds diluted in ethanol were used on each plate (20 autoclaved plates total), Selected compounds and concentrations are shown in Table 5.3. Another 20 plates were used as controls with 10µl of ethanol on each.

For oviposition, four stable fly females were transferred to a plastic rearing cage (30 x 30 x 30 cm - BugDorm®, MegaView*, Taichung, Taiwan) and offered one plate with the synthetic compound(s) and one with ethanol (control). The assay was conducted with 20 cages and the flies were allowed to oviposit for 6 hours per 3 consecutive days, receiving new prepared plates

each day to maximize the probability of egg laying. After each 6-hour period, plates were removed and refrigerated for egg counting.

Egg count

The eggs on each plate (treated and control) were counted manually. The total number of eggs per each cage was considered to be the sum of eggs from the three days of each assay.

Statistical analysis

Student's t-test (StatPlus:mac, AnalystSoft Inc. 2009) was performed to determine significant differences between the mean number of eggs laid on treated and control plates.

Results

Identification of volatile compounds

Volatile compounds identified were phenol, m-cresol, p-cresol and indole by comparisons of their mass spectra and retention indices with those of synthetic standards (retention times were shown at 4.43 for phenol, 6.42 for p-cresol and m-cresol, and 11.85 indole) (Figure 5.2). Overall, the results of the analysis of the headspace of the various horse manure samples (2 and 3 week old) showed various peaks with the same retention time although varying in size (Figures 5.3 and 5.4). In general, the peaks from 2 week old manure were greater than those from 3 week old manure. A more detailed analysis of the peaks, not considering their intensity, is shown in Table 5.4. A group of peaks within the retention time 13.78 - 16.25 minutes were consistently present in different samples from horse manure of both ages (Figures 5.3 and 5.4). Peaks with the retention times of 13.78 to 13.81 and 14.89 to 14.90 were present in more than 90% of all samples; peaks with retention times 14.20 to 14.21 and 14.44 to 14.46 were

present in ≥ 67% of the samples. Unfortunately, these compounds were not identified due to lack of the reference standard spectra available.

Oviposition assays

The mean number of eggs per fly laid on the control plates (14.03 ± 2.5) was significantly higher (p=0.00685) than that on plates with the synthetic compounds mix (5.72 ± 1.3) at the highest concentration tested (Mix 1 - 4.3µg phenol, 1.4µg p-cresol, 1.4µg m-cresol and 2.9µg indole) (Figure 5.5). Lower concentrations of the compounds (Table 5.3) resulted in the mean number of eggs laid not significantly different from the control (ethanol) (p=0.34897 for Mix 1/5, p=0.60939 for Mix 1/10, and p=0.45324 for Mix 1/100) (Figure 5.5).

Furthermore, stable flies laid significantly more eggs on the control plates (19.91 ± 2.1 and 29.03 ± 4.0, respectively) than on the plates with 4.3µg of phenol (13.15 ± 2.5) (p=0.04922) or 10mg of phenol (3.3 ± 1.4) (p=0.00001) (Figure 5.5).

Discussion

The identified compounds emitted from 2 and 3 week old horse manure in this study were phenol, p-cresol, m-cresol and indole. Phenol, p-cresol, and indole were also detected from the fresh horse manure and were shown to stimulate stable fly antennal responses in EAG experiments (Jeanbourquin & Guerin, 2007). However, whether they act as attractants or repellents is unknown. The fact that these compounds were detected from the fresh horse manure, which is not a natural oviposition site for stable flies (Chapter 2), indicates that they may not act as oviposition stimulants/attractants. Differences in ratios/concentrations of these compounds as the horse manure ages could also be a reason for difference in stable fly oviposition behavior. On the other hand, these 4 compounds were detected in only a half of the

samples of 2 and 3 week old manure suggesting that they are not the most important volatile compounds affecting stable fly oviposition.

Other two compounds, detected in more than 93% of the horse manure samples (with retention times between 13.78 and 13.81, and between 14.89-14.9), are potential candidates stimulating stable fly oviposition. However, these compounds were all eluted at a temperature above 120ºC, which may indicate their non-volatile status, and need to be further identified and tested for their effect on stable fly oviposition. These two compounds were also observed in 2 and 3 week old cattle manure (data not shown), ages that cattle manure were shown to be attractive for stable flies (Broce & Haas, 1999).

Further studies comparing volatiles emitted from fresh manure (non-stimulating stable flies oviposition) to those from 2-3 week old manure will be useful and would allow to focus on the candidate peaks influencing stable fly behavior.

Phenol, cresol, and indole were identified from different oviposition substrates for several mosquitos such as *Culex tarsalis, Culex quinquefasciatus, Aedes aegypti, and Aedes albopictus* (Beehler et al., 1994; Millar et al., 1994; Allan & Kline, 1995; Du & Millar, 1999; Mboera et al., 2000). Phenol and p-cresol are products of tyrosine fermentation, while indole is the end product of tryptophan metabolism (Macfarlane & Macfarlane, 1995). Tyrosine and tryptophan are amino acids metabolized in animal intestine by anaerobic bacteria, such as *Bacteroides*, *Clostridium*, *Lactobacillus*, and *Bifidobacterium* (Mackie et al., 1998). *Bacteroides* and *Clostridium* were among the main anaerobic genera identified in the fresh horse manure (Chapter 4). Further degradation of amino acids occurs only under aerobic conditions (Mackie et al., 1998). The main aerobic genera found in the horse manure stimulating stable fly oviposition (2 and 3 weeks old) were *Devosia* and *Brevundimonas* (Chapter 4). However, *Devosia* and *Brevundimonas* do not

produce indole (Brenner, 2005), which was confirmed by SPME collection of volatile compounds produced by these two bacteria (data not shown). This suggests that if indole is an important cue for stable fly oviposition, it is generated by other aerobic bacteria.

A diverse bacterial community in animal manure generates a complex mixture of volatile compounds that were shown to function as stable fly oviposition attractants/stimulants (Romero et al., 2006; Jeanbourquin & Guerin, 2007). However, to identify the specific compounds and their mixture and concentrations that positively influence the stable fly behavior is a major challenge. In this study, the mixture of phenol, indole, p-cresol and m-cresol at different concentrations was tested and shown not to stimulate stable fly oviposition. The ratio of the compounds used in our study was based on the proportion of these compounds emitted from 2 and 3 week old horse manure and on the concentration of phenol that elicited strong antennal response in EAG (Tangtrakulwanich et al., 2011). Phenol alone, in the two different tested concentrations, was also shown to be not attractive for the stable flies.

It is possible that stable flies are first attracted to an area with animals as they cue on host-associated odors and then they search for an oviposition site (animal manure). Rumen volatiles including p-cresol were shown to be important for stable fly host location (Jeanbourquin & Guerin, 2007b). The fact that p-cresol was detected from rumen as well as from fresh cattle and horse manure (Jeanbourquin & Guerin, 2007a; Jeanbourquin & Guerin, 2007b) and elicited upwind flight by stable flies is an indication that flies first find the host using olfactory system and then search for an appropriate oviposition site using relying on less volatile or contact compounds.

Data presented in this chapter are very preliminary and further identification of non- or semi-volatile compounds emitted from the 2 and 3 week old horse manure and their effect on

stable oviposition are needed. This is critical for development of a synthetic blend of compounds that could be used as an attractant or repellent for management of this insect pest.

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Table 5.1 Horse manure samples.

Sample	Horses	Collected	Diet
1	14 mares	Manhattan-KS	75% alfalfa + 25% grains (Purina strategy GX)
2	6 f mares	Manhattan-KS	75% alfalfa + 25% grains (Purina strategy GX)
3	2 males	Manhattan-KS	75% alfalfa + 25% grains (Purina strategy GX)
4	8 mares	Manhattan-KS	75% alfalfa + 25% grains (Purina strategy GX)
5	2 males	Lincoln-NE	80% hay + 20% grains (60% oats + 40% corn)
6	2 males	Lincoln-NE	80% hay + 20% grains (60% oats + 40% corn)
7	2 males	Lincoln-NE	80% hay + 20% grains (60% oats + 40% corn)

Table 5.2 Horse manure volatile collection.

Manure samples	Sub-samples	Volatiles collected	Temperature
2WHM1	9	Overnight	37°C
2WHM2	6	Overnight	37°C
2WHM3	14	Overnight	37°C
2WHM4	6	Overnight	37°C
2WHM5	6	Overnight	37°C
2WHM6	6	Overnight	37°C
3WHM1	6	Overnight	37°C
3WHM3	6	Overnight	37°C
3WHM4	6	Overnight	37°C
3WHM5	6	Overnight	37°C
3WHM6	3	Overnight	37°C
3WHM7	3	Overnight	37°C

Table 5.3 Concentrations of synthetic compounds used in two-choice oviposition assays.

	Phenol (µg)	Indole (µg)	m-cresol (µg)	p-cresol (µg)
Mix 1	4.3	2.9	1.4	1.4
Mix 1/5	0.86	0.58	0.28	0.28
Mix 1/10	0.43	0.29	0.14	0.14
Mix 1/100	0.043	0.029	0.014	0.014
Phenol 1	4.3			
Phenol 2	10 mg			

Table 5.4 Peaks (%) at each retention time.

Retention time (min)	no. peaks (%)		
	Control	2WHM	3WHM
1.21 - 1.24	74	87	80
1.26 - 1.28	19	6	17
1.28 - 1.31	45	85	30
1.32 - 1.36	0	15	17
1.37 - 1.40	29	64	70
1.45	0	6	10
1.50 - 1.51	7	17	20
1.55	0	9	17
1.60	0	17	40
1.65	7	36	27
1.70	0	0	7
1.71 - 1.79	43	66	80
1.80 - 1.85	12	19	47
1.87 - 1.90	0	11	33
1.92 - 1.98	24	26	20
2.00	0	6	0
2.10 - 2.15	7	47	50
2.20 - 2.30	33	28	13
2.35	5	0	3
2.40	0	9	0
2.47 - 2.48	2	6	0
2.50 - 2.55	7	6	10
2.60	0	6	0
2.65	0	6	3
2.70	2	0	0
2.77 - 2.80	0	30	20
2.81 - 2.85	0	6	0
3.05 - 3.10	7	15	23
3.13 - 3.20	5	21	30
3.25	0	6	0
3.38 - 3.41	5	0	0
3.41 - 3.45	2	9	3
3.50	2	0	0
3.55 - 3.60	0	21	3
3.61 - 3.67	0	15	40
3.75 - 3.78	2	9	0
3.79 - 3.85	0	32	3
3.92	2	4	0
4.05	0	9	3
4.10 - 4.15	0	23	33
4.19	7	0	0
4.20	0	0	10
4.25 - 4.27	0	19	13
4.28 - 4.32	7	6	7
4.39 - 4.49 ❖	**0**	**47**	**27**
4.45	5	0	0
4.50 - 4.55	5	17	7
4.60 - 4.66	0	36	17
4.68 - 4.72	2	21	40
4.75 - 4.78	0	26	10
4.80 - 4.82	38	19	27
4.85	0	0	3
4.95 - 5.00	2	9	3
5.07 - 5.15	2	34	7
5.15 - 5.20	0	11	20
5.25 - 5.35	0	28	33

5.40 - 5.45	0	17	3
5.55 - 5.60	0	21	33
5.65	0	0	3
5.70 - 5.75	0	4	3
5.80 - 5.82	0	17	20
5.85 - 5.90	0	2	7
6.00 - 6.10	2	17	7
6.15 - 6.19	2	19	10
6.20	0	0	20
6.25	0	6	7
6.30	2	0	13
6.35 - 6.40	0	0	7
6.42 - 6.45 ◆	**0**	**11**	**30**
6.48 - 6.55	2	53	33
6.60 - 6.64	5	0	7
6.65	2	0	0
6.70	5	0	0
6.75 - 6.78	0	6	0
6.80	0	0	10
6.88 - 6.95	2	9	17
7.05 - 7.10	2	15	17
7.12 - 7.15	0	15	27
7.19 - 7.20	2	6	13
7.40 - 7.45	0	19	23
7.50	0	4	3
7.54 - 7.60	5	26	13
7.65 - 7.67	2	6	7
7.70 - 7.75	0	6	10
7.80 - 7.85	0	45	23
7.95 - 8.05	0	13	10
8.06 - 8.10	0	40	37

8.15	0	2	3
8.25	0	15	10
8.40	0	2	3
8.45 - 8.50	71	53	60
8.55 - 8.60	0	17	53
8.70 - 8.74	0	4	13
8.80	0	2	3
8.84 - 8.90	0	17	13
9.05 - 9.10	0	6	20
9.15	0	4	3
9.35	0	4	3
9.40	0	6	7
9.53 - 9.55	0	6	10
9.60	0	0	3
9.70	0	4	3
9.80 - 9.86	2	19	20
9.90	0	6	3
10.05 - 10.10	0	13	10
10.11 - 10.15	0	51	50
10.20 - 10.25	0	17	10
10.30 - 10.36	0	47	53
10.40 - 10.45	0	40	37
10.50	0	6	0
10.60	0	4	0
10.70	0	6	23
10.80	0	19	33
10.95	2	0	0
11.00	0	4	0
11.15	0	6	0
11.40 - 10.41	0	13	7
11.58 - 11.60	2	11	0

11.83 - 11.86 ✤	**0**	**49**	**30**
11.90	2	0	7
11.95 - 11.96	5	6	3
12.20	0	6	0
12.45	0	0	3
12.65	0	0	3
12.70	0	6	0
12.80	0	2	0
12.92 - 12.95	67	60	80
13.15 - 13.20	0	60	40
13.30	0	6	0
13.35	0	2	0
13.40 - 13.42	0	30	23
13.50	0	6	0
13.55	0	2	0
13.58 - 13.60	0	19	3
13.65	0	0	10
13.78 - 13.81 ★	**5**	**94**	**93**
13.95	0	15	20
14.00 - 14.05	0	13	23
14.10 - 14.11	0	26	33
14.15	0	6	10
14.20 - 14.21	0	74	77
14.25	0	21	7
14.30	0	15	23
14.35	0	23	0
14.37 - 14.40	2	15	43
14.44 - 14.46	0	77	67
14.49 - 14.50	0	17	43
14.52 - 14.55	2	4	23
14.60 - 14.62	0	34	67
14.65 - 14.69	0	62	37
14.70	0	0	37
14.71 - 14.77	0	66	60
14.80	0	21	50
14.85	0	6	10
14.89 - 14.90 ★	**0**	**94**	**97**
14.95 - 14.97	0	36	37
15.00	0	19	50
15.05	0	23	3
15.10	0	13	0
15.15	2	36	60
15.20	0	0	27
15.22 - 15.25	0	34	40
15.30	0	0	3
15.34 - 15.36	0	64	23
15.38 - 15.40	69	60	70
15.45	0	13	17
15.50 - 15.52	7	26	33
15.55	0	19	37
15.60	0	34	43
15.65	0	15	3
15.70	0	6	23
15.75 - 15.76	2	6	10
15.80 - 15.83	0	68	43
15.85	0	0	7
15.90 - 15.93	0	19	30
15.95	0	6	0
16.00 - 16.05	0	13	20
16.10	0	4	7
16.15	0	0	7
16.19 - 16.25	10	81	57

16.29 - 16.35	0	11	13
16.40 - 16.42	0	11	0
16.45 - 16.50	0	4	10
16.50 - 16.55	7	19	10
16.60	0	6	13
16.65	0	4	7
16.70 - 16.75	31	45	27
16.75 - 16.76	0	6	3
16.80	0	4	3
16.85	0	4	3
16.90	0	11	10
16.93	0	4	0
17.00	0	0	3
17.15	0	0	3
17.20 - 17.25	2	2	17
17.30 - 17.35	0	2	7
17.40	0	2	0
17.45	0	4	7
17.50 - 17.55	0	0	7
17.60	0	6	0
17.70	5	4	7
17.75	0	0	3
17.78 - 17.80	0	23	20
17.90 - 17.95	2	15	37
18.00	2	6	0
18.10	0	0	7
18.20	2	2	3
18.24 - 18.26	0	13	30
18.30	2	0	0
18.35	0	4	0
18.45	0	4	0
18.50 - 18.55	5	0	7
18.58 - 18.60	5	0	3
18.65	5	0	0
18.95 - 18.96	2	19	7
19.05	2	0	0
19.26	2	0	0
19.55	2	0	0
19.80 - 19.83	5	0	0
20.05	2	0	0
20.21	2	0	0

❖Phenol
◆p-cresol or m-cresol
✜Indole
★non identified candidates compounds

Figure 5.1 Oviposition plate.

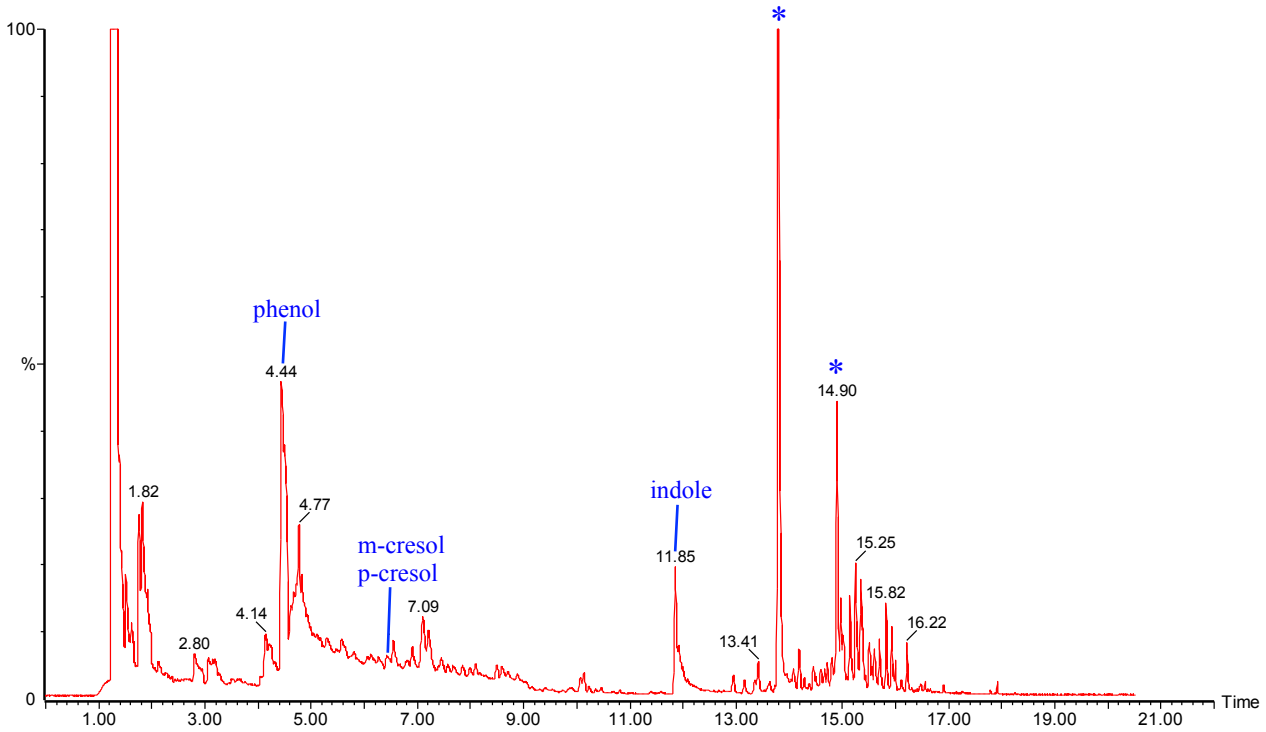

Figure 5.2 Representative graph showing peaks of phenol, m-cresol, p-cresol, indole and peaks most found in 2 and 3 weeks old horse manure.

* Indicates the peaks found in more than 93% of the samples of both horse manure ages (2WHM and 3WHM).

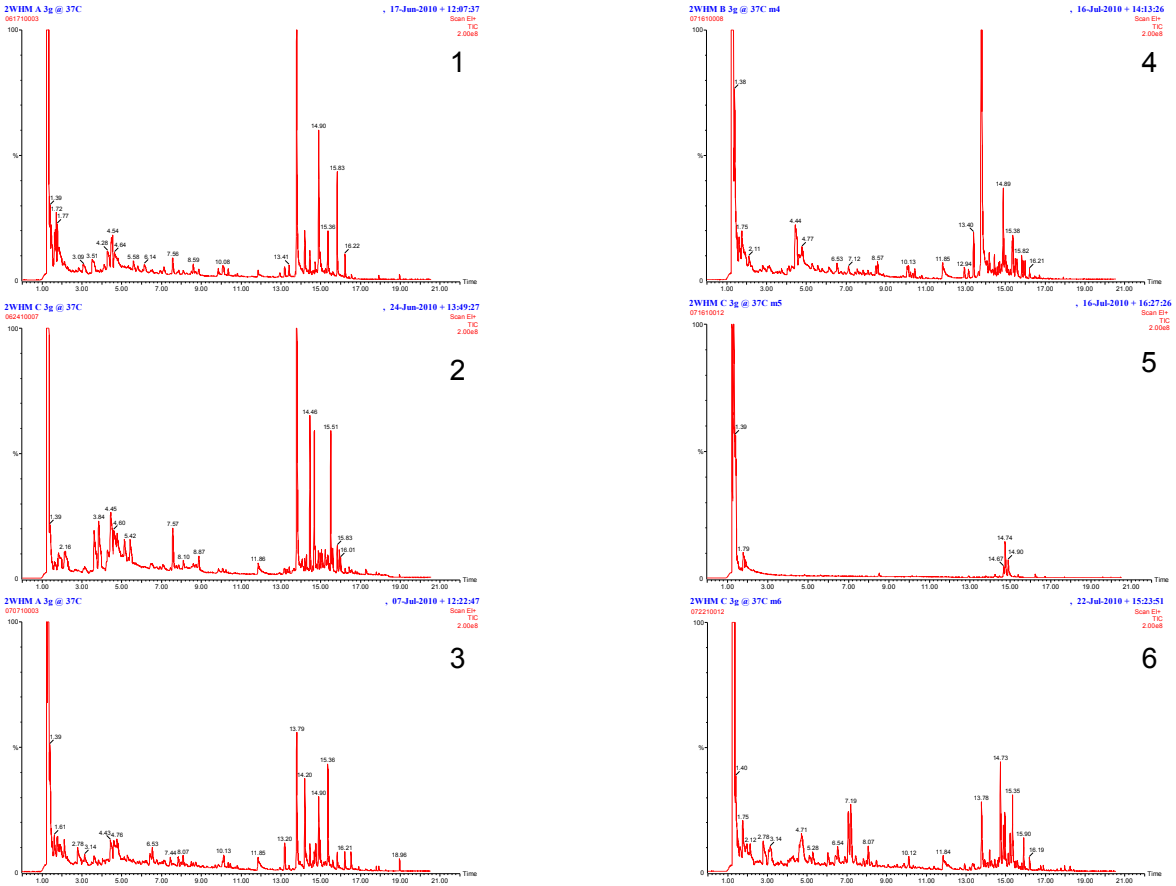

Figure 5.3 Profile of volatile compounds emitted from 2 week old horse manure (6 replicates).

Numbers on the top right corner refer to manure sample.

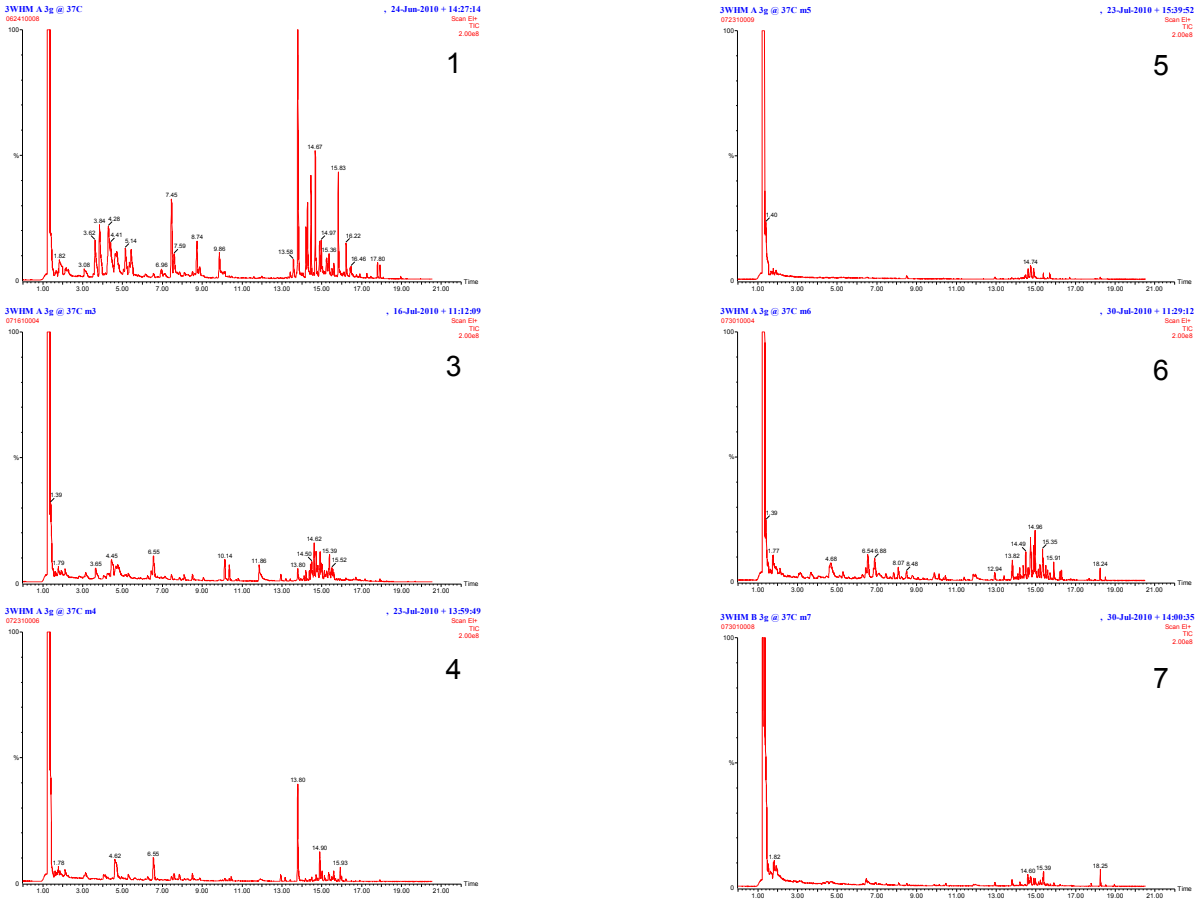

Figure 5.4 Profile of volatile compounds emitted from 3 week old horse manure (6 replicates).

Numbers on the top right corner refer to manure sample.

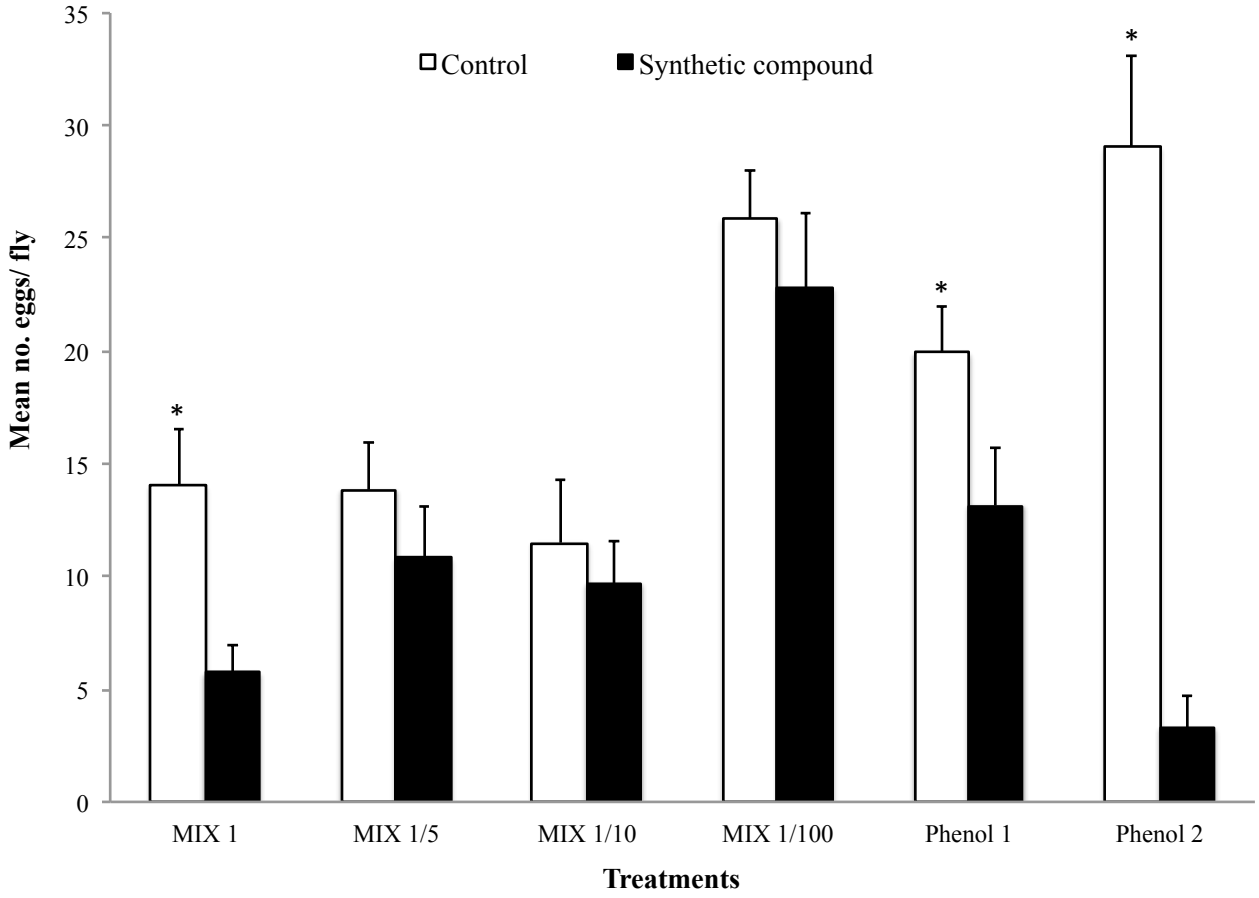

Figure 5.5 Mean number of eggs per fly oviposited on black cloth with different concentrations of a mix of phenol + indole + m-cresol + p-cresol or different concentrations of phenol. Control = ethanol.

Mix 1= 4.3µg of phenol, 2.9µg of indole, 1.4µg of m-cresol, and 1.4µg of p-cresol.
Mix 1/5 = Mix 1 diluted 5 times. Mix 1/10=Mix 1 diluted 10 times. Mix 1/100 = Mix 1 diluted 100 times.
Phenol 1 = 4.3µg of phenol and Phenol 2 = 10mg of phenol.