ECOLOGICAL AND PUBLIC HEALTH ASPECTS OF STABLE FLIES (DIPTERA: MUSCIDAE): MICROBIAL INTERACTIONS

by

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B.S., University of Dar-es- Salaam, 1983

M.S., University of Florida, 2002

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 fly, *Stomoxys calcitrans* (L.), and house fly, *Musca domestica* L., are two major pests affecting both confined and pastured livestock in the United States. It costs livestock producers millions of dollars annually to reduce populations of these two pests. Control of stable flies and house flies based on chemical insecticides is only marginally effective and unsustainable in the long term due to the development of insecticide resistance. This has created a demand for alternative methods which are environmentally friendly and cost effective for the management of these pests. Information on stable fly and house fly oviposition behavior and the aggregation and segregation of their immatures may help in an integrated pest management control program for these pests.

This research identified specific bacterial species from the surface of stable fly eggs which are suspected of releasing chemical cues used to induce gravid females to oviposit at sites where eggs have been deposited and inhibit additional deposition of eggs in the same habitat when it is already colonized. My research also showed that stable fly and house fly larvae tend to be aggregated in distribution, even in apparently homogenous habitats, and to be spatially segregated from each other. Finally, I evaluated the vector competence of stable flies for an emerging food-borne pathogen, *Enterobacter sakazakii*, showing that this fly species is potentially a good vector for this pathogen.

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Dedication

I dedicate this work to my savior, counselor, comforter, strong tower and provider Jehovah father for his guidance, strength, and health. Without him I would have lost my direction in my studies. I also dedicate this work to my husband Wenceslaus Mramba, my children Wilfred, Freada, Juanita, and Flora for their love, prayer and understanding, without which I would not have managed to complete this work. I am also very grateful to my mother Anna Makishe, my brother Abdul Makishe and my sisters Miriam and Zaituni, for their love and moral support. Lastly I give special thanks to my friends, Samuel Ochieng, Newton Ochanda, Solange Uwituze, Yapo Nguessan, Dona Warren and my entire bible study group, who in one way or another, contributed to my personal intellectual challenges and successes during my years of study.

CHAPTER 1

Literature Review

Of the many insect pests that negatively impact the livestock industry, stable flies and house flies constantly draw man's attention. The stable fly, *Stomoxys calcitrans* (L.), and house fly, *Musca domestica* L., are two species in the family Muscidae that are of significant veterinary importance as major pests of both confined and pastured livestock. Both of these two species are largely cosmopolitan and native to Africa. Stable flies are members of the subfamily Stomoxynae (Zumpt 1973), while house flies belong to the subfamily Muscinae (Hewitt 1910).

Stable flies resemble house flies in appearance. However, a stable fly can be distinguished by an anterior, non re-tractable piercing /sucking long proboscis that points forward from under the head. This proboscis is composed of the labium, with a short labellum, the labrum, and the hypopharynx (Zumpt 1973), whereas a house fly has a sponging/sucking mouthparts consisting of a fleshy elbowed labium, the distal end of which is a large sponge-like labellum. The labellae are made up of many grooves, called pseudotracheae, which the fly uses to filter liquid food. The wing venation of stable fly is quite different from that of house fly as it has a slight bend upwards on vein M1+2, whereas that of house fly is slightly curved (Castro 1967, Foil and Hogsette 1994).

Adult stable flies are 5 - 6 mm in length, dark gray in color and possess four longitudinal black stripes on the thorax and a larger checkered abdomen with a distinct pattern of black spots, whereas the thorax of house flies bears four narrow black stripes. The abdomen is pale gray to

yellowish with dark midline and irregular dark markings on the sides (Service 1980, Hewitt 1910).

Both sexes of stable fly are hematophagous (i.e., blood feeders), which mostly draw blood from warm-blooded animals and feed to full capacity in less than 4 minutes (Harwood and James 1979). Adults of both sexes approach host, two to three times daily to feed (Schofield and Torr 2002). Stable flies are diurnal feeders and they have a bimodal pattern of feeding with peaks at 10 A.M. and 4 .00 P.M. under favorable environmental conditions (Hoffman 1968). The majority of adult stable flies prefer to feed on the lower side of large animals such as cattle, horses, pigs, sheep and donkeys (Dougherty et al. 1994). During summer, when large populations of stable flies occur, they may also feed on the sides or the backs of their hosts (Hogsette and Farkas 2000). Female stable flies are anautogenous, meaning they require several blood meals to complete their reproductive function (Jones et al. 1992). The males require at least one blood meal to produce seminal fluid and to stimulate sexual drive (Klowden 1996). Additionally, Jones et al. (1992) observed adult stable flies feeding on nectar from different flowers, but the nectar itself was insufficient for reproduction if a blood meal was not available (Jones et al. 1985).

Cattle under attack by stable flies bunch together, with each animal attempting to find a position within the bunch to protect their front legs, which are the favored feeding site of the flies. Considerable energy is expended by foot stamping, tail twitching, and throwing the head toward the front legs in an effort to dislodge the flies or prevent feeding. Stable flies can reduce weight gain, milk production, and feed efficiency both from their feeding and because of the bunching behavior of the cattle, which may induce or increase heat stress and hence reduce feed intake (Wieman et al. 1992). Bruce and Decker (1958) estimated stable flies to cause an average

production loss of 0.7% per fly per dairy cow. As a result of host's defensive behaviors, stable flies make numerous visits, biting repeatedly before obtaining a full meal. Economic losses caused by stable flies are mainly associated with reduction of feed efficiency, resulting in the reduction in weight gain. Weight gain reductions of 0.02 to 0.05 Kg per day and feed efficiency reduction of 11 to 13 percent have been documented in feedlots (Catangui et al. 1997). Several economic thresholds have been estimated. Mc Neal and Campbell (1981) used an economic threshold of 5 stable flies per cow's front leg, while Catangui et al. (1997) established an economic threshold of 7 per cow per leg.

Unlike stable flies, house flies feed on almost anything with nutritional value. Having such a broad food preference they may visit many different food sources and may come into contact with microbe-rich substrates (Axtell and Arends 1990). They feed on liquefied types of food. House flies cannot take up large particles of solid food. Ostrolenk and Welch (1942) found that the flies could not ingest particles larger than 0.045 mm. When a house fly feeds on dry substrates such as sugar, it first liquefies the substrate by salivary secretions, which flows into the oral pit and onto the substrates or moistening the substrate by the regurgitation of food from the crop. Regurgitation is one of the methods by which house flies that have fed on infected products continue to contaminate other substrates for days (Pospisil 1958). House flies have not been shown to reduce animal weight gain and feed efficiency, but are known to transmit pathogens of several animal diseases, especially those associated with enteric infections. The house fly mouthparts and feeding habits make the species highly efficient in transmitting bacterial and viral agents. Over one hundred pathogens that cause diseases in humans and animals have been recovered from house flies (Sukontason et al. 2000). Transmission generally

involves the mechanical transfer of the disease agent from the mouthparts or body of the fly to the animal host (Tan et al. 1997).

The life cycles of these two species are similar, consisting of eggs, three larval stages (maggots), pupa, and adult (Ross et al. 1982). During the summer months, the stable fly completes its life cycle in about 3 weeks while the house fly requires about 2 weeks. After a stable fly female has taken enough of a blood meal, she seeks out a suitable oviposition site and deposits eggs throughout the media. A female stable fly can lay between 40 and 60 eggs in each gonadotrophic cycle, and its life fecundity ranges between 30 and 700 eggs, depending on temperature (Lysyk 1998). Oviposition sites of stable fly females are composed of decomposing grass clippings, green chop, compost piles, spilled feed, manure and urine - contaminated hay or straw, and manure especially manure over 3 weeks old (Meyer and Petersen 1983). The eggs of stable flies are about 1 mm long and 0.2 mm wide and banana-like in shape (Harwood and James 1979). The eggs hatch between 12 and 24 hours after being laid. The 1st instar larvae requires 24 h for development, whereas the second instar lasts for 28 h and the third instar lasts for up to 7 days (Foil and Hogsette 1994).

Larval habitats of stable flies in confined livestock operations are well documented, and include spilled feed, stored manure, and silage (Meyer and Peterson 1983). It was demonstrated by Skoda et al. (1991) in a feedlot study that feed aprons yielded about 63% of the larvae of stable flies. However, information is limited on stable fly development in pastures. Hay wasted by cattle while feeding from large bales and mixed with manure may constitute the greatest medium for stable fly development in certain places (Foil and Hogsette 1994). It has been demonstrated that hay wasted by cattle during winter feeding mixed with manure and remaining in the field through early spring, can become excellent habitats for the development of stable fly

larvae (Broce et al. 2005). Other materials not related to livestock such as compost piles containing grass clippings, and poultry litter used as fertilizer in horticultural crop production have also been shown to be ideal habitats for stable fly development (Broce 1993; Cook et al. 1999).

Stable fly pupal development takes place inside the puparium, which is the hardened cuticle of the 3rd instar. Pupal development lasts between 5 and 26 days. In tropical areas, development of larvae and pupae is fast and continuous year round. However, in temperate areas, development of larvae and pupae is slower during cooler temperatures (Service 1980). Once an adult emerges, it elongates and the body turns dark within 30 minutes, the wings expands, the proboscis folds forward, and then the newly emerged fly is ready to fly (Castro 1967). Unlike stable fly, female house flies lay their eggs on a variety of decomposing materials such as animal manure, poultry dung, animal bedding, carcasses, decomposing organic materials found in rubbish dumps, household garbage and waste food from kitchens and hotels (Krafsur 1985). A female may lay eggs five or six times in her life time in batches of 100 - 150 eggs, deposited together or in separate batches.

Similar to stable fly larvae, house fly eggs are creamy-white, 1 - 1.2 mm long and banana-shaped in appearance (Lysyk 1993b). The eggs hatch into the first instar between 6 and 12 hrs. The 1st instars molt to 2nd instars within 24 hrs, before finally molting to 3rd instars after 36 hrs. Development of immature stages is highly dependent on temperature. Larval development requires 27 d at 16° C and 5 days at 35° C (Lysyk and Axtell 1987). The 3rd instar contracts and the larval skin harden, and after about six hours it turns into a black cylindrical pupa. Pupal development requires 16 d at 16° C and 4 d at 35° C. The adult house fly emerges

from the pupa to begin the next generation (Lysyk and Axtell 1987). Within 2 - 3 d flies become sexually mature and in 4 d after copulation the females deposit their first batch of eggs.

Stable flies are not considered one of the important vectors of animal or human diseases but their biting action makes livestock become more susceptible to diseases (Castro 1967). However, it has been stated that stable flies can serve as carriers of pathogens for cutaneous leishmaniasis, anthrax, brucellosis, equine infectious anemia and trypanosomiasis, and that they also play the role of intermediate hosts of nematode worms and some stomach parasites (Horsfall 1962; Greenberg 1971; Harwood and James 1979). Lameness in horses has also been reported to be due to the continuous stomping, and swelling; and stiff joints in other animals bitten by stable flies are common (Zumpt 1973). House flies differ from stable flies in that they constitute a major problem in a variety of industries such as poultry, pig and dairy farms (Hansens 1963, Axtell and Arends 1990). House flies have a broader preference for food causing them to visit many different food sources and come into contact with microbe-rich substrates such as manure or decaying corpses as well as livestock feed. In doing so, they transmit pathogens of several diseases. A wash from the surface of a house fly yielded total bacterial counts of 2.5×10^6 to 29.5×10^6 per fly, whereas in the digestive tract the counts were from 8.4×10^4 to 2.0×10^6 bacteria per fly (Osterolenk and Welch 1942). House flies may be contaminated with several different species of pathogenic bacteria at the same time (Sukontason et al. 2000) and are associated with incidences of diseases such as gastroenteritis, ulcers, dysentery, cholera and tuberculosis (Sulaiman et al. 2000, Olsen and Hammock, 2000, Fotedar 2001). Like stable flies, house flies cause annoyance to man and animals. High population densities of house flies in animal farms can be a nuisance to the extent that the animals avoid going to the feed bunks to feed (Schmidtmann 1985).

Several different pest management strategies have been employed to eliminate fly problems in and around livestock facilities. Sanitation is one of the most important aspects of fly management programs. To be effective, sanitation is normally supplemented with insecticide sprays and baited traps, as well as biological control agents such as parasitoids (parasitic wasps). Reduction of fly breeding areas in feedlots is dependent primarily on manure management and keeping the lots dry. A number of biological control agents such as pathogens, predators and parasites affecting stable flies have been identified (Petersen 1989). These biological agents are similar to those identified for control of house flies (Harwood and James 1979). The natural enemies identified so far include species of beetles and mites that prey on fly eggs and small larvae, and about 10 different species of pteromalid wasps that prey on stable fly and house fly pupae. The parasitic wasp kills a pupa by drilling the pupal case with her ovipositor in order to lay her own egg (Mullen and Durben 2004).

Mass release of sterile (e. g., irradiated) male stable flies and house flies has been considered as one of the possible control management strategy since both species can easily be reared in the laboratory and the females are monogamous. However, the sterile insect technique is not a suitable strategy against stable flies because both sexes are blood feeders and releasing large numbers of stable fly and house fly males, even though sterile, would increase the nuisance level on livestock (Buschman and Patterson (1981). Various kinds of traps have been used in an attempt to control both fly species. The box trap has proved effective in catching stable flies on sandy beaches. The discovery of adhesive alsynite fiberglass has greatly improved the catch of stable flies to the traps. Williams trap, a translucent alsynite fiberglass sticky panel, has also been found to be highly effective for monitoring stable flies (Williams 1973). Broce (1988) developed a new cylindral alsynite plastic traps that uses a cheap and thin plastic with less adhesive material

than the old Williams trap. Broce (1988) found that his new trap was equal to the Williams trap in catching house flies, but caught fewer stable flies; possibly due to the smaller surface area. However, Hogsette and Ruff (1990) found that the Broce's cylindrical trap captured fewer total numbers of flies but more flies per cm^2 than any of the Williams traps used in their experiments.

Several traps with various volatile compounds such as acetone, octenol, CO_2, propanoids, and others have been added in attempt to increase the capture of stable flies and house flies. For example, Hoy (1969) found that Malaise traps baited with CO_2 caught 3 times as many stable flies compared to Malaise traps alone. Cilek (1999) used the alsynite cylindrical traps with various volatile substances such as dry ice, acetone, and octenol, and found that CO_2 from the dry ice was a very powerful attractant for collecting stable flies. The only drawback to these kinds of traps is the use of dry ice as a CO_2 source, which can be very costly. Several traps and baits containing sugar or other substances house flies feed on have been tried over the years with variable success. The simplest traps are sticky surfaces onto which flies get caught (Williams 1973). Most recently, traps have been used to disintegrate insects with a high-voltage electrocutor grid. These traps have been effective for house flies, but the disadvantage is that they are not good hygienically as they release bacteria and viruses into the air during operation (Urban and Broce 2000). Natural product attractant baits have also been tried in house fly traps, but they proved inconvenient and are not economical due to the high frequency of bait-replacement and trap maintenance (Ashworth and Wall 1994). Although traps and odors used for surveillance do remove some stable flies and house flies from the environment, most traps do not control flies in sufficiently large numbers, neither do they reduce significantly the biting and nuisance level of these two fly species (Eldridge and Edman 2004).

Numerous commercial chemical products are available for the control of stable flies and house flies. The most common ones are those, which are water-soluble formulations of pyrethroids such as permethrin, which offer a quick knockdown of adult flies (Mock and Greene 1989). In the case of house flies, selective chemical insecticides with short residual activity such as pyrethrins have been used to reduce fly populations with minimal impact on natural enemies (Geden et al.1992). Direct spraying on animals has also been used to reduce both stable flies and house flies (Foil and Hogsette 1994). Spraying of pesticides with residual action on fly resting sites such as building walls, bunks, and shelters also provide good fly control, but the insecticidal action only lasts for a short period of time (Campbell 1993). The historical approach of depending on chemicals for stable fly and house fly control has been demonstrated to be only marginally effective and unsustainable for the long term. Extensive use of pesticides has lead to the development of insecticide resistance in fly populations, which eventually limits efficacy of the insecticides (Cilek and Greene 1994). Many insecticides used for the control of these flies have been removed from the market while few new ones have been developed (Hogsette 1999).

The prevailing situation as described above has created a great demand for alternative methods that are environmentally friendly for the control of these most important livestock pests. In several Dipteran species of medical and veterinary importance, it has been recorded that females aggregate during oviposition. This is common in some mosquitoes, sand flies (*Lutzomyia longipalpis*), black flies (*Simulium damnosum*) and some species of screwworm (McCall et al. 1994, El-Naiem and Ward 1991). It has been shown that females of these species significantly oviposited more on substrates already containing eggs or volatiles from freshly laid eggs than on control substrates (McCall et al. 1994, El-Naiem and Ward 1991, Catts and Mullen 2002). McCall et al. (1994) recorded *Simulium damnosum* laying eggs communally and

depositing huge masses of eggs by the thousands onto selected individual substrates. Coupland (1991) showed that *S. damnosum* preferred to oviposit on substrates already containing eggs and the preference was mediated by the eggs themselves. In addition, he found the attraction decreased as the eggs aged. Stadlier et al. (1994) found that *Rhagoletis cerasi* (Diptera: Tephritidae) deposited some pheromones during oviposition which discourage further oviposition. Extensive work has also been done on the olfactory stimuli for the primary screwworm, *Cochliomyia hominovorax*, causative agent of obligatory myiasis (Catts and Mullen 2002). Catts and Mullen (2002) showed that screwworm females oviposited on blood inoculated with bacteria that had been isolated from screwworm infested animal wounds. Their study also suggested that volatiles from the bacteria could be the oviposition stimulant for gravid flies. Due to the above reasons, therefore, aggregation of insects during oviposition may potentially be manipulated and used as an environmentally friendly and effective tool for catching gravid females, and thus drastically reduce the numbers of insects in a population

Much is already known about the habitats of stable fly larvae, but little is known about whether gravid stable flies oviposit in communal aggregation like other dipterans and if so, what are the factors responsible for this behavior? Answers to these questions may provide new perspectives of controlling these pests without adversely affecting the environment. Stable flies often lay their eggs in clusters. It is speculated that ovipositing eggs in clusters may be advantageous to both the eggs and the larvae. Egg clustering may decrease the exposed surface, thereby reducing accessibility to parasitoids and predators in addition to reducing desiccation, and thus increasing the likelihood of the offspring's survival. Females utilize a variety of oviposition sites on the farm, primarily on the decomposing animal feces mixed with hay and other forages (Broce and Haas 1999, Skoda et al. 1996). Similar to that of other muscoid flies,

stable fly larval development depends in part on environmental factors and a live microbial community in the natural habitat (Lysyk et al. 1999, Carlson et al. 2000). Females are capable of selecting an oviposition site based on the microbially derived stimuli that indicates the suitability of the substrate for larval development (Romero et al. 2006). Further studies in the field have shown that stable fly and house fly larvae graze their habitat in separate aggregations, even though they are commonly found in a similar environment. Nothing is known about the causes of such behaviors, whether it is the female fly's oviposition pattern or the larval behavior. Information on stable fly and house fly oviposition behavior, and knowledge on aggregation and segregation of their immature stages may be used as an effective tool in an integrated pest management control program of these two species. The association between stable flies and bacteria with regard to pathogen transmission is not well known. Such is the case for *Enterobacter sakazakii*, an emerging food borne pathogen that causes meningitis and sepsis (Nazarowec et al. 1997). Several studies have failed to identify the natural reservoir, extent of environmental contamination and the mode of transmission of this bacterium. My study of *E. sakazakii*'s association with stable flies and that of vector competence have shown that stable flies are potential vectors of this pathogen.

Objectives

Evidence in the literature strongly suggests that bacteria play a significant role in mediating oviposition behavior in various Dipterans; thus the first objective of the research project herein described was:

(1) To elucidate the role of microbes as stimulants and arrestants of oviposition of gravid stable flies

Stable fly and house fly larvae are found in separate species-specific aggregations while colonizing what appears to be a homogeneous larval habitat; whether this segregation / aggregation behavior is female or larval mediated is not known. Thus, the second objective of this study was:

(2) To determine if the observed segregation and aggregation distributions of stable fly and house fly larvae are larval- or maternal-mediated

Although stable flies are hematophages, they have been implicated as vectors of relatively few pathogens; their potential vectoring capability might be greater in relation to this fly's development in animal feces. Thus, the third objective was

(3) To evaluate the role of stable flies in the ecology of an emerging human pathogen *Enterobacter sakazakii.*

Reference cited

Ashworth, J.R. and R. Wall, 1994. Responses of the sheep blowflies *Lucilia sericata* and *L. cuprina* to odours and the development of semiochemical baits. Med. Vet. Entomol. 8 : 303-309.

Axtell, R.C. and J.J. Arends. 1990. Ecology and management of arthropod pests of poultry. Ann. Rev. Entomol. 35: 101-126.

Broce, A.B. 1988. An improved Alsynite trap for stable flies, *Stomoxys calcitrans* (Diptera : Muscidae). J. Med. Entomol. 25: 406-409

Broce, A.B. 1993. Dispersal of house flies and stable flies. In: G.D. Thomas and S.R. Skoda, Eds. Rural Flies in the Urban Environment? Agricultural Research Division Institute of Agriculture and natural Resources. University of Nebraska-Lincoln. P: 50-60.

Broce, A.B. and M.S. Haas. 1999. Relation of cattle manure age to colonization by stable fly and house fly (Diptera: Muscidae). J. Kans. Entomol. Soc.72: 60-72.

Bruce, W.N. and G.C. Decker. 1958. The relationship of stable fly abundance to milk production in dairy cattle. J. Econ. Entomol. 51: 269-274.

Buschman, L.L. and R.S. Patterson. 1981. Assembly, mating, and thermoregulating behavior of stable flies under field conditions. Environ. Entomol. 10: 16-21.

Campbell, J.B. 1993. The economics of the fly problem. In G.D. Thomas and S.R. Skoda. Rural flies in the urban environment? Agricultural Research Division Institute of Agriculture and natural Resources. University of Nebraska-Lincoln. 34-39.

Carlson, D. A., R. A. Alzogaray and J. A. Hogsette. 2000. Behavioral response of *Stomoxys calcitrans* (L.)(Diptera: Muscidae) to conspecific feces and feces extracts. J. Med. Entomol. 37: 957-961.

Castro, J.D. 1967. Chemical sterilization of the stable fly, *Stomoxys calcitrans* (Linneus), with Metepa and Hempa. Ph.D. dissertation, University of Florida, Gainesville, FL.

Catangui, M. A., J.B. Campbell, G.D. Thomas and D. J. Boxler. 1997. Calculating economic injury levels for stable flies (Diptera : Muscidae on feeder heifers. J. Econ. Entomol. 90: 6-10.

Catts, E. P and G. R. Mullen. 2002. Myiasis (Muscoidea, Oestroidea), In*:* Medical and Veterinary Entomology, (G. R. Mullen and L. A. Durden, Eds.), pp. 318-348. Elsevier Science, San Diego, CA.

Cilek, J.E. and G.L. Greene. 1994. Stable fly (Diptera: Muscidae) resistance in Kansas cattle feedlots. J. Econ. Entomol. 87: 275-279.

Cilek, J.E. 1999. Evaluation of various substances to increase adult *Stomoxys calcitrans* (Diptera:Muscidae) collections on alsynite cylinder traps in north Florida. J. Med. Entomol. 36: 605-609.

Cook, D.F., I.R. Dadour, and N.J. Keals. 1999. Stable fly, house fly (Diptera:Muscidae), and other nuisance fly development in poultry litter associated with horticultural crop production. J. Econ. Entomol. 92: 1352-1357.

Coupland, J.B. 1991. Oviposition response of *Simulium reptans* (Diptera: Simuliidae) to the presence of conspecific eggs. Ecol. Entomol. 16: 11-15.

Dougherty, C.T., and F. W. Knapp. 1994. Oviposition and development of face flies in dung from cattle on herbage and supplemented herbage diets. Vet. Parasit. 55:115-127.

Eldridge, B.F. and J.D. Edman. 2004. Medical Entomology. A textbook on public health and veterinary problems caused by arthropods, Revised eds. Kluwer Academic publishers, Dordrecht, The Netherlands.

El-Naiem, D. A. and R.D. Wards 1991. Response of the sand fly *Lutzomyia longipalpis* to an oviposition pheromone associated with conspecific eggs. Med. Vet. Entomol. 5: 87-91.

Foil, L.D. and J.A. Hogsette. 1994. Biology and control of tabanids, stable flies and horn flies. Rev. Sc. Technol.13: 1125-1158.

Fotedar, R. 2001. Vector potential of house flies (*Musca domestica*) in the transmission of *Vibrio cholerae* in India. Acta Trop. 78: 31-34.

Geden, C.J., D. A. Rutz, J. G. Scott, and S. J. Long. 1992. Susceptibility of house flies (Diptera: Muscidae) and five pupal parasitoids (Hymenoptera: Pteromalidae) to abamectin and seven commercial insecticides. J. Econ. Entomol. 85: 435-440.

Hansens, E.J. 1963. Fly populations in dairy barns. J. Econ. Entomol. 56:842-844

Harwood, R.H. and M.T. James. 1979. Entomology in Human and Animal Health. Mac Millan Publishing Co., Inc. New York. Pgs. 248-296.

Hewitt, C.G. 1910. The house fly. A study of its structure, development, bionomics and economy. Edited by Sherratt and Hughes, University Press, Manchester.

Hoffman, R.A. 1968. Stable fly, *Stomoxys calcitrans* (L.): Biology and behavior studies. Ph.D. dissertation. Oklahoma State Univ., Stillwater.

Hogsette, J.A. and R. Farkas. 2000. Secretophagous and hematophagous higher diptera. Pages 769-792 in L.Papp and Darvas, editors. Manual of Palearctic Diptera, Science Herald, Budapest.

Hogsettee J.A.1999. Management of ectoparasites with biological control organisms. Int. J. Parasitol. 29: 147-151

Hogsette, J.A., and J.P. Ruff. 1990. Comparative attraction of four different fiberglass traps to various age and sex classes of stable fly (Diptera: Muscidae) adults. J. Econ. Entomol. 83: 883-886.

Horsfall, W.R. 1962. Medical Entomology. Arthropods and human disease. The Ronald Press Co. New York. Pg. 467.

Hoy, J.B. 1969. Trapping the stable fly by using CO_2 or CO as attractants. J. Econ. Entomol. 63: 792-795.

Jones, C. J., J.A.P. Hogsette, and D.E. Milne.1985. Effects of natural saccharides and pollen extract feeding on stable fly (Diptera: Muscidae) longevity. Environ. Entomol. 14: 223-227.

Jones, C.J., D. E. Milne, R.S. Patterson, E.T. Schreiber, and J.A. Milio. 1992. Nectar feeding by *Stomoxys calcitrans* (Diptera: Muscidae): Effect on reproduction and survival. Environ. Entomol. 21: 141-147.

Klowden, M. J. 1996. Vector behavior. In: The biology of disease vectors.University Press of Colorado. Niwot, Colorado. P. 44.

Krafsur, E.S. 1985. Age composition and seasonal phenology of house fly (Diptera: Muscidae) populations. J. Med. Entomol. 22: 515-523.

Lysyk, T. J. and R. C. Axtell. 1987. A simulation model of house fly (Diptera: Muscidae) development in poultry manure. Can. Entomol.119: 427- 437.

Lysyk, T. J. 1993. Adult resting and larval developmental sites of stable flies and house flies (Diptera: Muscidae) on dairies in Alberta. J. Econ. Entomol. 86:1746-753.

Lysyk, T.J. 1998. Relationships between temperature and life history parameters of *Stomoxys calcitrans* (Diptera: Muscidae) J. Med. Entomol.35: 107-119.

Lysyk, T.J., L. Kalischuk-Tymensen, L. B. Selinger , R. C. Lancaster, L. Wever and K. J. Cheng. 1999. Rearing stable fly larvae (Diptera: Muscidae) on an egg yolk medium. J. Med. Entomol. 36:382-388.

McNeal, C.D. and J.B. Campbell. 1981. Insect pest management in Nebraska feedlots and dairies: a pilot integrated pest management project. Nebraska Cooperative Extension Services and Institute of Agriculture & Natural Resources. Univ. Nebr. Dep. Entom. Res. Rep. No.10.

McCall, P., J., A.J. Tees, J.F. Walsh, and D.H. Molyneux, 1994. Aggregated oviposition in the *Simulium damnosum* complex is mediated by eggs in a laboratory bioassay. Med. Vet. Entomol. 8: 76-80.

Meyer, J. A. and J.J. Petersen. 1983. Characterization and seasonal distribution of breeding sites of stable flies and house flies (Diptera:Muscidae) on Eastern Nebraska feedlots and dairies. J. Econ. Entomol. 76: 103-108.

Mullen, G. and L. Durben. 2004. Medical and Veterinary Entomology. Academic Press, California.

Mock, D.E. and G.L. Greene. 1989. Current approaches in chemical control of stable flies In: J.J. Petersen and G.L. Greene, editors. Current status of stable fly (Diptera:Muscidae) research, Misc. Publ. Entomol. Soc. Am. 74.

Nazarowec-White, M., and J.M. Farber, 1997. *Enterobacter sakazakii*. J. Food Microbiol. 34: 103-113.

Olsen, A.R. and T.S. Hammack. 2000. Isolation of *Salmonella* spp. from the house fly, *Musca domestica* L., and the dump fly, *Hydrotaea aenescens* (Wiedemann) (Diptera: Muscidae), at caged-layer houses. J. Food Prot. 63: 958-960.

Ostrolenk, M. and H. Welch. 1942. The common house fly (*Musca domestica*) as a source of pollution in food establishments. *Food Research* 7: 192-200.

Parr, H.C.M. 1962. Studies of *Stomoxys calcitrans* (L.) in Uganda, East Africa. II. Notes on life-history and behaviour. Bull. Entomol. Res. 53: 437-443.

Petersen, J, J. 1989. Potential for biological control of stable flies associated with confined livestock. Pages 42-45. In J.J. Petersen and G.L. Greene Eds. Current status of stable fly (Diptera: Muscidae) Research. Misc. Publ. Entomol. Soc.Am. 74

Pospisil, J. 1958. Some problems of the smell of the saprophilic flies. Acta Soc. Ent. 55: 316-334.

Romero, A., A. Broce and L. Zurek. 2006. Role of bacteria in oviposition behaviour and larval development of stable flies. Med. Vet. Entomol. 20: 115-121.

Ross, H.H., C.A. Ross, and J.R.P. Ross. 1982. A textbook of entomology. John Wiley and Sons, New York.

Schofield, S., and S. J. Torr. 2002. A comparison of the feeding behavior of tsetse and stable flies. Med. Vet. Entomol. 16: 177-185.

Schimidtmann, **E.T. 1991**. Suppressing immature house and stable flies in outdoor calf hutches with sand, gravel, and sawdust bedding. J. Dairy Sci. 74: 3956-3960

Schmidtmann E .T. 1985. Arthropods pests of dairy cattle. In Livestock Entomology. R.E. Williams, R.D. Hall, A.B. Broce, and P.J. Scholl, ed. John Wiley and sons, Newyork, NY. P. 223-233.

Service, M. W. 1980. A guide to medical entomology. The Macmillan Press LTD, London and Basingstoke, Hong Kong, P:102-112.

Skoda, S. R., G.D. Thomas and J.B. Campbell. 1996. Comparison of core sampling and pupal traps for monitoring stable flies and house flies (Diptera: Muscidae) in beef feedlot pens. J. Econ. Entomol. 89: 428-434.

Skoda, S.R., G.D. Thomas, and J.B. Campbell. 1991. Developmental sites and relative abundance of immature stages of the stable fly (Diptera: Muscidae) in beef cattle feedlot pens in Eastern Nebraska. J. Econ. Entomol. 84: 191-197.

Stadlier, E., E. Beat, J. Hurter, and E. Boller, 1994. Tarsal contact chemoreceptors for the host marking pheromone of the cherry fruit fly, *Rhagoletis cerasi*: Responses to natural and synthetic compounds. Physiol. Entomol. 19:139-151.

Sukontason, K., Bunchoo, M., Khantawa, B., Piangjai, S. and Choochote, W. 2000 *Musca domestica* as a mechanical carrier of bacteria in Chiang Mai, north Thailand. J. Vector Ecol. 25: 114-117.

Sulaiman, S., M.Z. Othman, and A. H. Aziz. 2000. Isolations of enteric pathogens from synanthropic flies trapped in downtown Kuala Lumpur. J. Vector Ecol. 25: 90 - 93.

Tan, S.W., K.L.Yap, and H.L. Lee. 1997. Mechanical transport of rotavirus by the legs and wings of *Musca domestica* (Diptera: Muscidae). J. Med. Entomol. 34: 527-531.

Urban, J. E. and A. Broce. 2000. Flies and their bacterial loads in greyhound dog kennels in Kansas. Curr. Microbiol. 36:164-170

Wieman, G. A., J. B. Campbell, J.A, Deshazer, and I.L. Berry.1992. Effects of stable flies (Diptera: Muscidae) and heat stress on weight gain and feed efficiency of feeder cattle. J. Econ. Entomol. 85: 1835-1842.

Williams, D.F. 1973. Sticky traps for sampling populations of *Stomoxys calcitrans*. J. Econ. Entomol. 66: 1279-1280

Zumpt, F. 1973. The Stomoxyine biting flies of the world (Diptera: Muscidae) Stuttgart, Germany, Gustav Verlag, P 175.

CHAPTER 2

The effect of microbially derived stimulants on the oviposition behavior of stable flies

Abstract

The occurrence of aggregated oviposition in stable flies (Diptera: Muscidae) was investigated under laboratory conditions. Stable flies were allowed to lay eggs prior to the experiments and used in bioassays to measure different parameters of oviposition. A series of two-, three-, and five - choice tests demonstrated that gravid stable flies preferred to oviposit in the vicinity of conspecific freshly laid eggs (2 h old) than on substrates with old eggs (24 h old). The stimulant(s) originating from the eggs could be removed by hexane, surface sterilization with ethanol and sodium hypochlorite, or by water alone. In contrast, the 24 h old eggs did not enhance oviposition at all. The concentration of bacteria isolated from the surface of old eggs was 10 fold (10^6 CFU / egg) higher than that obtained from the surface of freshly oviposited eggs (10^5 CFU / egg). The increase of bacteria in old eggs most likely mediates inhibition of further oviposition. This is also supported by the results of assays using different concentrations of bacteria on the surface of sterilized eggs that demonstrated that low ($\leq 10^5$ CFU /egg) and high concentrations ($10^9 \geq$ CFU / egg) of bacteria did not stimulate oviposition. This study provides a basis upon which oviposition behavior of stable flies and stable fly-bacterial association could be developed into integrated pest control.

KEY WORDS: *Stomoxys calcitrans*; young; old; surface sterilized eggs; bacteria

Introduction

Stable fly (SF), *Stomoxys calcitrans* (L.), is a cosmopolitan blood - feeding insect and is one of the most important pests of confined as well as pastured livestock, primarily cattle and horses, in the United States. The biting actions of this insect cause considerable economic damage to the livestock industry (Hall and Smith 1986, Campbell et al. 1993, Campbell 2001), in addition to adversely affecting the tourism industry in areas such as Florida and the New Jersey coastline (Hogsette and Ruff 1985, Hansen 1951). Currently, however, there are no adequate management strategies effective for controlling this pest, especially on pastures. Control in confined operations relies solely on sanitary measures and on the selective use of chemical insecticides, which eventually might result in the development of resistant pest populations (Marcon et al. 1997, Campbell 2001).

Aggregation during oviposition is not uncommon behavior among many insect groups. It has been recorded in a number of Dipteran species of medical and veterinary importance. The most thoroughly described factors in oviposition site selection for *Musca domestica* L. (Young and Jiang 2002), *Lutzomyia longipalpis*, (El-Naiem and Ward 1991), and *Culex quinquefasciatus* (Laurence and Picketts 1985) are related to odor cues emanating from larval food resources (microorganisms) and water quality. Some female black flies, *Simulium damnosum*, have been shown to lay eggs communally. A large number of eggs were deposited on selected substrates within a few hours after selecting a substrate (McCall 1994, Walch 1984, Coupland 1991). It was also shown that *S. damnosum* prefer to oviposit on substrates already containing eggs. The attraction is possibly pheromone-mediated since freshly laid eggs were more attractive than older eggs. Some other insects such as *Rhagoletis cerasi* (Diptera: Tephritidae) have been shown to

deposit pheromones that discourage further oviposition by the same or other females into the same breeding sites (Stadlier et al. 1994).

Stable flies utilize a variety of oviposition sites on the farm, primarily decomposing animal feces mixed with hay and other forages (Broce and Haas 1999, Skoda et al. 1996). Similar to other muscoid flies, SF larval development and survival also depend on environmental factors and a live microbial community in the natural habitats (Lysyk et al. 1999, Carlson et al. 2000). Females are capable of selecting an oviposition site based on the microbially-derived stimuli that indicate the suitability of the substrate for larval development. Importantly it has been reported that the bacteria that support larval development of SF also stimulate SF oviposition (Romero et al. 2006). Furthermore, SF can utilize resource-limited habitats to prevent overcrowding of the resources and improve the food supply for their offspring. However, little is known about the effects bacterially- derived volatile compounds play in the choice of substrates upon which SF laid their eggs.

The main objective of this study was to determine the effect of oviposited SF eggs (fresh and old), and the associated microbial community, on oviposition behavior of SF. Investigation of substances inducing SF aggregation during oviposition in the field is an important tool for understanding SF ecology. Identification of attractants that can be used to lure gravid SF to common oviposition site can be a useful additional tool in both population monitoring and control.

Materials and Methods

Insect rearing: Stable flies were obtained from the Kansas State University rearing colony. Adults were reared at 25 ± 2^{o} C, 70 ± 10% RH and a photoperiod of 18: 6 (L: D). Flies were fed on citrated bovine blood supplied in saturated sanitary napkins. The newly emerged flies were

fed blood daily for 6 days at the end of which most of the females had full-developed eggs. On the 6-day, 30 gravid females were separated from males and held in glass cages in the rearing room conditions until used for the bioassays.

Oviposition bioassays: All two-choice tests, three-choice tests five-choice tests and seven choice tests were conducted in a rectangular plexi-glass wind tunnel (0.8x 0.8x 1.8 m) but with no air movement. The substrate used was a mixture of cattle manure and chopped hay (1:2 wt:wt) with a moisture content of 70%. Choice tests were carried out using the following treatments: 1) fresh eggs (FE) (≤ 2 h old); 2) old eggs (OE) (≤ 24 h old); 3) no eggs; 4) surface wash from FE placed on surface sterilized FE eggs; 5) surface wash from OE placed on surface sterilized OE eggs, and 6) surface sterilized FE eggs inoculated with three different bacterial isolates from the surface of stable fly eggs.

1. Two-choice tests using FE, no eggs, FE hexane washed, FE surface sterilized, FE distilled water washed treatments. The number of stable flies ovipositing on a substrate containing (60 eggs x 3) either as unwashed FE, hexane-washed FE, distilled water – washed FE, surface - sterilized FE, or a substrate with no egg were compared. Hexane washed FE were obtained by immersing 200 eggs in 1mL of HPLC grade hexane for 30 min. After 30 min., the hexane was decanted and the eggs were further rinsed with similar amounts of hexane followed by distilled water three times. In each experiment, ≤ 2 h fresh eggs were first obtained in a pre-bioassay by allowing several gravid stable flies to lay eggs between 0 and 2 h in a black wet cloth.

Four different experiments were performed in this study: a) Three Petri plates (60x15 mm size) with 60 fresh unwashed eggs and 20 g of manure that had been mixed with chopped hay at ratio of 1:2 and moisture content of 70% were set up. Three other Petri plates were set up with manure/hay mixture but with no eggs. The plates were placed in alternate order in a circle

separated by 3 cm. The second experiment (b) was set as described above with 3 plates containing unwashed FE and 3 plates containing hexane washed FE. The third experiment (c) was set as described above but 3 plates contained 2 h washed eggs and 3 plates contained distilled water washed eggs. Thirty gravid stable flies were released in the wind tunnel and allowed to oviposit for 3 hours under continuous top lighting after which the eggs in each Petri plate were counted. The bioassays were replicated 4 times.

2. Two-choice tests using OE, no eggs, FE, FE surface sterilized, and water wash from OE and FE treatments. The bioassay used in this oviposition set up was a simple two choice procedure as described in the 1st experiments but with 60 eggs that were 24 h old compared to substrates with no eggs, 24 h versus 2 h eggs, water wash from 24 h eggs compared with sterilized eggs; wash from 2 h eggs versus sterilized eggs and water wash from 2 h eggs versus water wash from 24 h eggs. Gravid flies were released in the wind tunnel and the number of eggs on each treatment was counted after three hours.

3. Three-choice tests using FE, OE, and no eggs treatments.

This bioassay was set up similar to experiment 1 except that the oviposition choices were made between fresh (FE), old (OE) and no egg substrates. Gravid stable flies were released in the wind tunnel and the number of eggs on each treatment was counted after three hours.

4. Two-choice tests using oviposition of stable flies (SF) and house flies (HF) in a substrate with SF eggs, HF eggs, no eggs, and surface sterilized (SS) HF and SF eggs. Bioassays were conducted as described in the first experiments. A comparison was made between substrates with a) 200 freshly laid eggs of the SF set in one Petri plate and the other was kept with no eggs (control); b) a similar experiment was repeated but with house flies; c) 200 SF eggs set as above but released gravid HF; d) 200 HF set as above but released gravid SF; e) 200 HF set in one Petri

plate and 200 SF set in the 2nd plate and released gravid SF; f) set as in (e); but released gravid HF; g) 200 SS SF and 200 SS HF were set as in (e); and released SF h) set as in (g) but released HF. All the treatments in the above setups were randomly set at an interval of 24 cm apart. Thirty gravid flies were released in the wind tunnel and allowed to oviposit for 3 hours under continuous top lighting after which the eggs in each Petri plate were counted. Each bioassay was replicated four times.

5. SF five-choice treatment using different concentrations of mixed and individual bacteria isolated from the surface of SF eggs were used. Five choice tests were performed with sterilized individual bacterium and a mixture of bacteria from an individual SF egg. These bacteria from the surface of fresh (≤ 2 h) and old (≤ 24 h) SF eggs were isolated by taking individual fresh and old egg, aseptically from the oviposition black wet cloth obtained from a laboratory colony and suspended in 1 ml of phosphate buffered saline (PBS) (MP Biomedical, Aurora, OH). Serial dilutions were plated onto trypticase soy broth agar (TSBA) (Difco, Detroit, MI, USA). Plates were incubated aerobically at 37° C. Three bacteria, namely *Staphylococcus saprophyticus, Proteus vulgaris* and S*erratia* spp., were characterized morphologically and identified by 16S rDNA. The bacteria were collected from plates and used in a mixture at a ratio of 3:2:1 depending on the proportion of the individual bacterium on substrates with sterilized eggs against substrates without bacteria. The best concentration was also used with sterilized SF eggs against substrates with surface sterilized eggs without bacteria. The predominant bacterium, *Staphylococcus saprophyticus* was cultured at a concentration of 10^8 CFU/ egg and used on the black wet cloth versus black wet cloth without bacterium and additionally was used with the sterilized HF eggs versus no bacterium.

6. SF two-choice oviposition bioassays using bacteria isolated from the surface of HF eggs placed on SF SS eggs versus SF SS eggs. A mixture of the three bacteria from HF at a concentration of 10^{10} was used on sterilized eggs of SF versus surface sterilized (SS) eggs without bacteria.

7. HF five-choice oviposition bioassays using different concentrations of individual and mixed bacteria isolated from HF eggs. Five choice tests with sterilized and mixed bacteria from individual eggs of HF with different concentrations were performed. The bacteria from the surface of fresh (<2 h) eggs of HF were isolated by taking individual fresh egg aseptically from the oviposition black wet cloth and processed as described in experiment no. 5 above. Plates were incubated aerobically at 28^{o} C for 24 hrs. Three bacteria, *Pantonea agglomerans*, *Pseudomonus* spp and HF (unidentified bacteria) were characterized morphologically and identified by 16S rDNA. The bacteria were collected from the plates and used in a mixture at a ratio of 4:2:1 depending on the proportion of the individual bacterium. The mixture was Re-suspended in sterile distilled water to concentrations of 10^{8} to 10^{12} CFU/ egg. The mixture of these bacteria was used on the substrate with HF sterilized eggs against substrate with HF sterilized eggs without bacteria. The best concentration was also used with sterilized HF eggs versus substrates without bacteria. The predominant bacterium, which was *Pantonea agglomerans* cultured at a concentration of 10^{10} CFU/ egg was used against surface sterilized HF eggs.

8. HF two -choice oviposition bioassays using *S. saprophyticus* isolated from SF eggs placed on HF SS eggs versus HF SS eggs. A two choice test between the SS HF eggs inoculated with *S. saprophyticus* versus surface sterilized HF eggs was performed in this experiment.

9. Color preference bioassay. Seven choice color preferences were made. The bioassays were performed with the Petri plates having a mixture of hay and manure as described above but the choices were made between six different colors and no color. The different colored art paper was cut into small rectangular pieces and in each substrate six pieces of different colors: white, blue, black, yellow, orange, red and no color were set in a wind tunnel (no air movement) at an interval of 3 cm apart. Thirty gravid SF were released for three hours and the number of eggs laid in each Petri plate was counted. The experiment was replicated four times. The voucher specimens used in this study are deposited in the KSU Museum of Entomological and Prairie Arthropod Research as voucher number 196.

Statistical analysis

Oviposition counts were calculated as the percentage number of eggs oviposited on the control and on each treatment. Percentage values were transformed (arcsines) to stabilize error variances and comparisons made using a paired t-test (P - value = 0.05) (SAS Institute 1999). Means for the 3, 5, and 7 choice tests were compared by carrying out the least-square means (LSMEANS) procedure (P> 0.05) of the general linear model (PROC GLM) (SAS Institute 2003).

Results

1. Two-choice tests using FE, no eggs, FE hexane washed, FE surface sterilized, FE distilled water washed: There were statistically significant preference for FE (69%) versus no eggs (31%) (t = 1.46, p = 0.03847); FE (78%) versus hexane washed FE (22%) (t = 1.60, P = 0.0318); FE (76%) versus distilled water washed FE (24%) (t = 3.13, P = 0.0204); and FE (72%) versus sterilized FE (28 %) (t = 15.36, P = 0.0001) (Fig. 2.1).

2. Two-choice tests using OE, no eggs, FE, FE surface sterilized, and water wash from OE and FE. Gravid SF females oviposited significantly fewer eggs in substrates with OE (27%) than

in substrates with no eggs (67%) (t = 0.59, P = 0.034) and in old eggs (20%) versus fresh eggs (80%) eggs (t = 2.73, P = 0.0340). Flies oviposited significantly more eggs in the water wash from fresh (86%) than in the water wash from old eggs (t = 15.36, P = 0.0001). The water wash from young eggs obtained significantly more eggs (61%) than surface sterilized eggs (t = 2.09, P = 0.021) and wash from old eggs (54%) did not elicit a significant attraction when compared with the sterilized eggs (46%) (t = 0.59, P = 0.5746) (Fig. 2. 2).

3. Three-choice tests between young, old and no eggs: Results from the three choice tests showed that the females laid significantly more eggs on the substrates with 2 h old eggs (63%) compared to all other substrates with 24 h (15%) and no eggs (22%). However the number of eggs laid in no eggs substrate and 24 h old eggs did not vary. F= 6.49, df = 2, P = 0.0180) (Fig. 2.3).

4. Two-choice tests between gravid SF and HF in fresh and sterilized eggs. The oviposition response of gravid SF and HF in choice experiments among the conspecific eggs on the wind tunnel (no air) and blank controls are shown in Fig. 2.4. When gravid SF were released, the plates with conspecific eggs of SF received more oviposited eggs (73%) than no eggs (27%) (t = 3.91, P = 0.0079). Similarly when HF were released, the plates with conspecific eggs produced more oviposited eggs (82%) than plates with no eggs (t = 2.64, P = 0.039). Gravid SF oviposited more in the substrate with no eggs (63%) than to the substrates with HF eggs (37%) (t = 2.66, P = 0.029). A similar response pattern was obtained when gravid HF were given a choice between substrates with no eggs (74%) and substrates with SF eggs (26%) (t = 3.61, P = 0.0112) (Fig. 2.4).

Using either HF or SF egg substrates and releasing gravid SF, significantly more eggs were oviposited on substrates with SF eggs (88%) than substrates with HF eggs (12%)

(t = 95.18, P< 0.0001). Significantly more eggs were laid on substrates with HF eggs (90%) than substrates with SF eggs (10%) (t = 50.83, P = 0.0004) when gravid HF were released (Fig. 2:4). No significant difference was obtained from the substrates with surface sterilized SF eggs (54%) versus substrates with surface sterilized HF eggs (46%) when SF were released (t = 0.43, P = 0.691). Similarly, when HF were released on substrates with surface sterilized HF eggs (51%) and surface sterilized SF eggs (49%) (t = 0.16, P = 0.88) (Fig. 2: 4).

5. SF five-choice oviposition bioassays using different concentrations of mixed and individual bacteria isolated from surface of SF eggs. The results showed that the concentration of bacteria on the surface of individual old eggs increased ten fold more ($7.8 \pm 2.5 \times 10^6$ CFU/egg), than that of the surface of the fresh eggs ($6.9 \pm 1.5 \times 10^5$ CFU/egg). Bioassays revealed that the 100 µl of the three mixed bacteria with concentration of 1.3×10^8 CFU/ egg gave a statistical significant different more than in concentrations of 1.2×10^5, 7.7×10^4, 1.6×10^9 CFU/ egg and no concentration (control) F = 5.98, df = 4, P = 0.0101 respectively. (Fig.2.5a). However substrates with sterilized eggs inoculated with the best concentration of 10^8 from mixed bacteria (73%) significantly obtained more eggs than substrates with SS eggs (27%) (t = 4.29, P = 0.0036) (Fig.2.5b).

The number of eggs is significantly higher in substrates with *S. saprophyticus* (71%) F = 14.16, df = 3, P = 0.0015 compared to all other bacteria. However the number of eggs laid in P. *vulgaris* (11%), *Serratia* (14%) or sterilized eggs (5%) did not vary (Fig. 2. 5c). A statistical significance was attained when gravid SF were released between wet black cloth inoculated with *S. saprophyticus* (90%) against black wet cloth without this bacterium (10%) (t = 5.90, P< 0.0011) (Fig. 2.5d).

6. SF two-choice oviposition bioassays using bacteria isolated from surface of HF eggs placed on SF SS eggs versus SF SS eggs. SF oviposited significantly less on the substrates inoculated with bacteria isolated from HF eggs (16%) than on the control substrates (84%) ($t = 3.06$, $P = 0.0223$) (Fig. 2. 6).

7. HF five-choice oviposition bioassays using different concentrations of mixed and individual bacteria isolated from HF eggs. The bioassays revealed that the 100 µl of the three mixed bacteria from individual HF egg with a concentration of 10^{10} CFU/ egg attained a statistical difference (51%) versus other concentrations of 10^{11} CFU/ egg (13%),10^{12} CFU/ egg (17%), 10^{8} CFU/ egg (4%) and no concentration (control) (5%) respectively ($F = 44.11$, $df = 4$, <0.0001) (Fig. 2. 7a). SS HF eggs inoculated with the best concentration of mixed bacteria (10^{10} CFU/ egg) (90%) significantly obtained more eggs than substrates with sterilized eggs (10%) ($t = 37.54$, and $P = 0.024$) (Fig. 2.7b). However no significant difference was attained from *Pantonea agglomerans, Pseudomonus*, and HF (Unidentified) isolated from a house fly egg versus SS eggs respectively ($F = 0.73$, $df = 3$ and $P = 0.552$) (Fig. 2. 7c).

8. HF choice oviposition bioassays using *S. saprophyticus* isolated from SF eggs placed on HF SS eggs versus HF SS eggs. Unlike SF, when *S. saprophyticus* was inoculated onto sterilized HF, the released HF oviposited significantly more on sterilized eggs (97%) than on sterilized eggs inoculated with this bacterium (3%) ($t = 3.32$, $P = 0.016$) (Fig. 2.8).

9. Color preference.

Significantly more oviposition occurred in yellow colored substrates compared to black, white, blue, orange, red and no color $F= 2.55$, $df = 6$, $P = 0.0319$ respectively. (Fig.2.9).

Discussion

Chemical cues:

Our results indicate that SF selection of oviposition sites is influenced by already oviposited con-specific eggs in the substrate and this is mediated by cues originating from the microbial community on the surface of eggs. This study demonstrated that gravid SF clearly prefers to oviposit in the vicinity of freshly laid eggs (FE). The stimulant(s) originating from the eggs can be removed by hexane, surface sterilization with ethanol and sodium hypochlorite, and by water alone. In contrast, 24 h old eggs do not enhance oviposition at all. In fact, SF preferred to oviposit onto a substrate with no eggs rather than onto the substrate with old eggs. Moreover, when given a choice, SF laid significantly more eggs onto the substrate with fresh eggs than on one with old eggs. In addition, the oviposition stimulant(s) could be transferred from the surface of fresh eggs to the surface of sterilized eggs in water solution as these eggs became more stimulating for oviposition than surface sterilized eggs. Water wash from old eggs placed on surface sterilized eggs did not stimulate stable flies oviposition. When comparing water wash from fresh eggs to that of old eggs, stable flies clearly laid significantly more eggs into the substrate with surface sterilized eggs with water wash from fresh eggs than that of old eggs. In the three-choice assays stable flies laid significantly more eggs on the substrate with fresh eggs than on the substrate with old eggs or no eggs. Numbers of eggs oviposited were not significantly different in substrates with old eggs and no eggs.

The concentration of bacteria isolated from the surface of OE was 10 fold (10^6 CFU /egg) higher than that obtained from the surface of fresh eggs (10^5 CFU /egg). The increase of bacteria in old eggs likely mediates inhibition of oviposition probably to prevent dis-synchronization of SF offspring. This is also supported by results of our bioassays using different concentrations of

bacteria on the surface of SS eggs showing that low ($\leq 10^5$ CFU /egg) and high concentrations ($10^9 \geq$ CFU /egg) of bacteria do not stimulate oviposition.

The bioassays designed to examine the specificity of the above results clearly demonstrated that the oviposition attractants/stimulants on the surface of stable flies are species specific. Stable flies laid significantly more eggs onto substrate with fresh eggs of stable flies than on those with eggs of house flies. When given a choice between house flies and no eggs, SF oviposited significantly more onto a substrate with no eggs. The same scenario was true for house flies as they clearly preferred to lay eggs on a substrate with fresh house fly eggs than that with SF eggs or no eggs. Also, the substrate with no eggs was preferred for oviposition compared to a substrate with SF eggs. However, neither SF nor HF was able to differentiate between surface sterilized eggs of con - specific and the other species and both laid comparable number of eggs onto substrates with SS HF and SS SF eggs.

Of the bacteria isolated from the surface of SF eggs, *Staphylococcus saprophyticus* clearly stimulated SF oviposition the most when used as the inoculum (1.2×10^8 CFU / SF egg) on SS SF eggs or even on a sterile substrate (a moist black cloth), indicating that neither the substrate (manure) nor the eggs are required to stimulate SF oviposition. The species specificity of the stimulant(s) produced by *S. saprophyticus* was also demonstrated.

The oviposition bioassays with HF demonstrated that the bacteria in a relatively high concentration (1.8×10^{10} / HF egg) on the surface of HF eggs are stimulating oviposition of HF but not SF and the mixture of three isolates, *Pantonea agglomerans*, *Pseudomonas* spp. and HF unidentified species was required to generate the oviposition stimulant. Results of this study are similar to those of Yong Jiang et al. (2002) who showed that chemical signals originating from the ovaries of gravid females of *M. domestica* attract ovipositing females to common egg-laying

substrates, which already contained eggs. It is possible that the bacteria and/or associated oviposition stimulants on the surface of SF and HF FE originate in ovaries but this remains to be investigated. Mc Call (1997) showed that significantly more black flies, *Simulium damnosum*, oviposited on substrates baited with freshly laid eggs than on control substrates and substrates baited with 12 h old eggs. Elnaiem and Ward (1991), however, showed that female sand flies, *Lutzomyia longipalpis,* do not oviposit on a substrate containing eggs that have been washed with hexane and water as opposed to untreated FE.

Campbell et al. (2001) showed that the presence of young egg masses hastens the onset of SF and HF in oviposition sites and that once one fly begins to oviposit, others appeared to be attracted to the same site and also began egg - laying within minutes. The same author also noted that flies preferred to oviposit on the edges of the substrate similar to the preferred oviposition sites on the field, mostly along the fence lines and areas behind feeding aprons and at the edges of mounds. Observations reported by Elnaiem et al. (1991) and Mc Call (1994) indicated that sand flies, black flies, and *Drosophila melanogaster* females oviposited preferentially in medium containing con-specific eggs or larvae.

It has been observed in different species of dipterans that oviposition is stimulated by bacterial odors, such as in some species of mosquitoes where gravid females oviposited in water containing chemicals produced by *Enterobacter aerogenes* (Ikeshoji et al. 1975). Romero et al. (2006) demonstrated that SF laid greater number of eggs on a substrate with an active microbial community than on a sterilized substrate. In addition, SF larvae could not develop in a sterilized natural or artificial substrate/medium. They isolated and identified bacteria from a natural stable fly oviposition/developmental habitat and assessed their individual effects on SF oviposition responses and larval development. Of nine bacterial strains evaluated in oviposition bioassays,

Citrobacter freundii stimulated oviposition to the most and also supported SF development. *Serratia marcescens* and *Aeromonas* spp. neither stimulated oviposition nor supported stable fly development. Emenns (1982) showed that *Lucilia cuprina* was attracted and stimulated to oviposit in lesions, which are associated with odors produced by different species of bacteria.

Visual cue: It is common for some gravid insects, for example black fly species, to be attracted to certain colored substrates (Golini 1975). Some mosquitoes such as *Aedes aegypti* prefer to oviposit in yellow and green containers (Laurence 1985). In our present study, the substrate with different colors influenced the oviposition preference of gravid SF. All tests performed in this experiment showed a significant oviposition of the gravid SF in yellow tagged substrates followed by black colored papers but less so to the substrates with blue, red, orange, white, and no color label, respectively. The reason may be because the gravid females in the field prefer to oviposit on substrates of manure mixed with hay. The color formed by this mixture is relatively close to yellowish brown. Possibly the yellow and black color may absorb more light across most of the visible spectrum than the other colors. This may suggest that the critical wavelength band for stable flies is between 300-600 nm where SF is able to differentiate yellow and black from the other colors. However, further studies of the visual physiology and ecology of SF are required.

This study shows a promising starting point of exploiting SF oviposition behavior and SF bacterial associations for development of novel approach for stable fly integrated control programs (IPM). The potential identification and isolation of oviposition attractants/stimulants involved will be of great benefit in monitoring and possibly establishing control programs for SF.

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References cited

Broce, A. B. and M. S. Haas. 1999. Relation of cattle manure age to colonization by stable fly and house fly (Diptera: Muscidae). J. Kan. Entomol. Soc.72: 60-72.

Carlson, D. A., R. A. Alzogaray, and J. A. Hogsette. 2000. Behavioral response of *Stomoxys calcitrans* (L.)(Diptera: Muscidae) to conspecific feces and feces extracts. J. Med. Entomol. 37: 957-961.

Campbell, J.B. 1993. The economics of the fly problem. In G.D. Thomas and S.R. Skoda. Rural flies in the urban environment? N. Cent. Reg. Res. Pub. 335: 34-39.

Campbell, J.B., S.R. Skoda, D.R. Berkebile, D.J. Boxler, G.D. Thomas, D.C. Adams and R. Davis. 2001. Effects of stable flies (Diptera: Muscidae) on weight gains of grazing yearling cattle. J. Econ. Entomol. 80: 117-119.

Dougherty, C.T. and F.W. Knapp. 1994. Oviposition and development of face flies. In dung from cattle on herbage and supplemented herbage diets. Vet. Parasit. 55: 115-127.

El-Naiem, D. A. and Wards. R.D. 1991. Response of the sand fly *Lutzomyia longipalpis* to an oviposition pheromone associated with conspecific eggs. Med.Vet. Entomol. 5: 87-91.

Emmens, E. L,and M.D. Murray. 1982. The role of bacterial odours in oviposition by *Lucilia cuprina* (Wiedemann) (Diptera: Calliphoridae), the Australian sheep blowfly. Bull. Ent. Res. 72: 367-375.

Gil, R., A. Latorre and Moya, A. 2004. Bacterial endosymbionts of insects: Insights from comparative genomics. Environ. Microbiol. 6: 1109-1122.

Golini, V.I. and D.M. Davies. 1975. Relative response to coloured substrates by ovipositing black flies (Diptera:Simuliidae) 1. Oviposition by *Simulium* (*Simulium verecundum*).

J.Zool. 53: 521-535.

Hall, R.D. and J.P. Smith. 1986. Life history and economic importance of stable flies. In: stable fly biology and control in cattle feedlots. Department Report,Garden City Branch Station. Garden City, Kansas P. 2-7.

Hansens , E.J. 1951. The stable fly and its effect on seashore recreational areas in New Jersey. J. Econ. Entomol. 44: 482- 487.

Hogsette, J.A., and J.P. Ruff. 1985. Stable fly (Diptera: Muscidae) migration in Northwest Florida. Environ. Entomol. Soc. Amer.74: 27-31.

Laurence, B.R. and J.A. Picketts. 1985. An oviposition attractant pheromone in *Culex quinquefasciatus* Say (Diptera: Culicidae). Bull. Entomol. Res.75: 283-290.

Lysyk, T.J., L. Kalischuk-Tymensen, L. B. Selinger , R. C. Lancaster, L. Wever and K. J. Cheng. 1999. Rearing stable fly larvae (Diptera: Muscidae) on an egg yolk medium. J. Med. Entomol. 36: 382-388.

Ikeshoji, T., K. Saito, and A. Yano, 1975. Bacterial production of the ovipositional attractants for mosquitoes on fatty acids substrates. App. Entomol. Zool. 10: 239-242.

Marcon, P., G.D. Thomas, B.D Siegfried, and J.B. Campbell, 1997. Susceptibility of stable flies (Diptera: Muscidae) from Nebraska beef cattle feedlots to selected insecticides and comparison of 3 bioassay techniques. J. Econ. Entomol. 90: 293-298.

Mc Call, P. J., A.J. Tees, J.F. Walsh, and D.H. Molyneux, 1994. Aggregated oviposition in the *Simulium damnosum* complex is mediated by eggs in a laboratory bioassay. Med. Vet. Entomol. 8: 76-80.

Romero, A., A. Broce, and L. Zurek, 2006. Role of bacteria in oviposition behavior and larval development of stable flies. Med. Vet. Entomol. 20:115-21.

SAS Institute. 1999. SAS user's guide. SAS Institute, Cary, NC.

Skoda, S. R., G.D. Thomas, and J.B. Campbell. 1996. Comparison of core sampling and pupal traps for monitoring stable flies and house flies (Diptera: Muscidae) in beef feedlot pens. J. Econ. Entomol. 89: 428-434.

Stadlier, E., E. Beat, J. Hurter, and E. Boller 1994. Tarsal contact chemoreceptors for the host marking pheromone of the cherry fruit fly, *Rhagoletis cerasi*: Responses to natural and synthetic compounds. Physiol. Entomol. 19: 139-151

Walsh, J.F. 1984. Aspects of the biology and control of *Simulium damnosum* (Diptera Simuliidae) in West Africa. Ph.D Thesis Salford University.

Young Jiang, Chao-liang Lei, Chang-Ying Niu, Yu- ling Fang, Chun Xial and Zhong-ning Zhang 2002. Semiochemcals from ovaries of gravid females attract ovipositing females house flies, *Musca domestica*. J. Ins. Physiol. 48: 945-950.

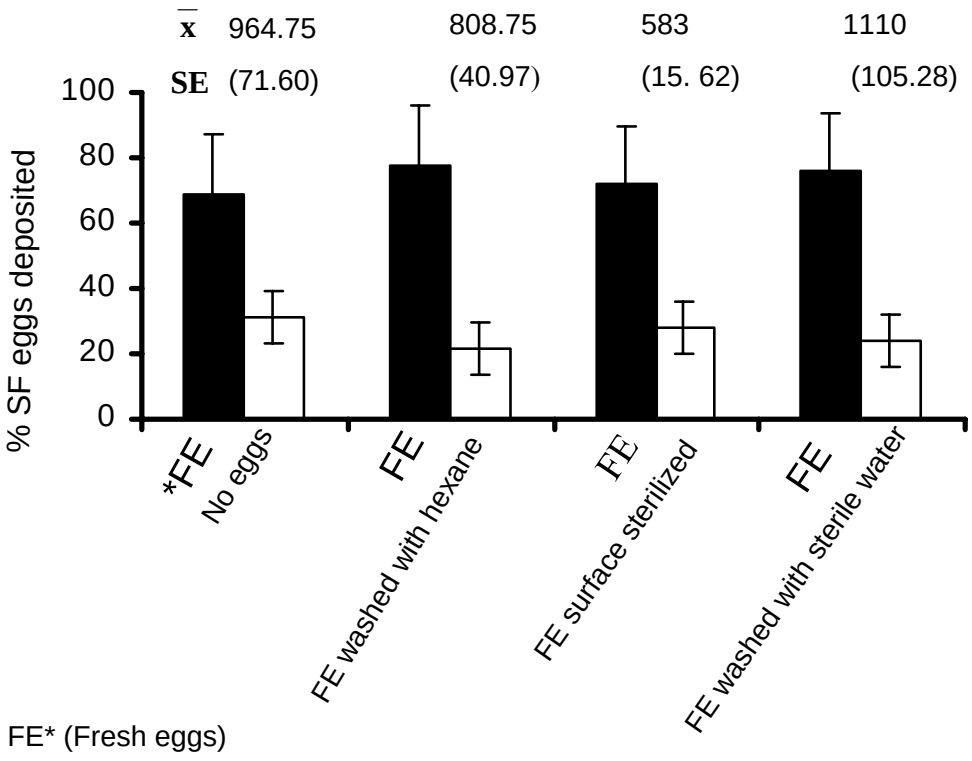


Figure 2.1 Mean % of stable fly (SF) eggs laid by 30 gravid females in two-choice assays on the substrates with fresh eggs (FE) (2 h old) versus FE with various treatments. The numbers above represents the mean of the oviposited eggs and the standard error (n = 4).

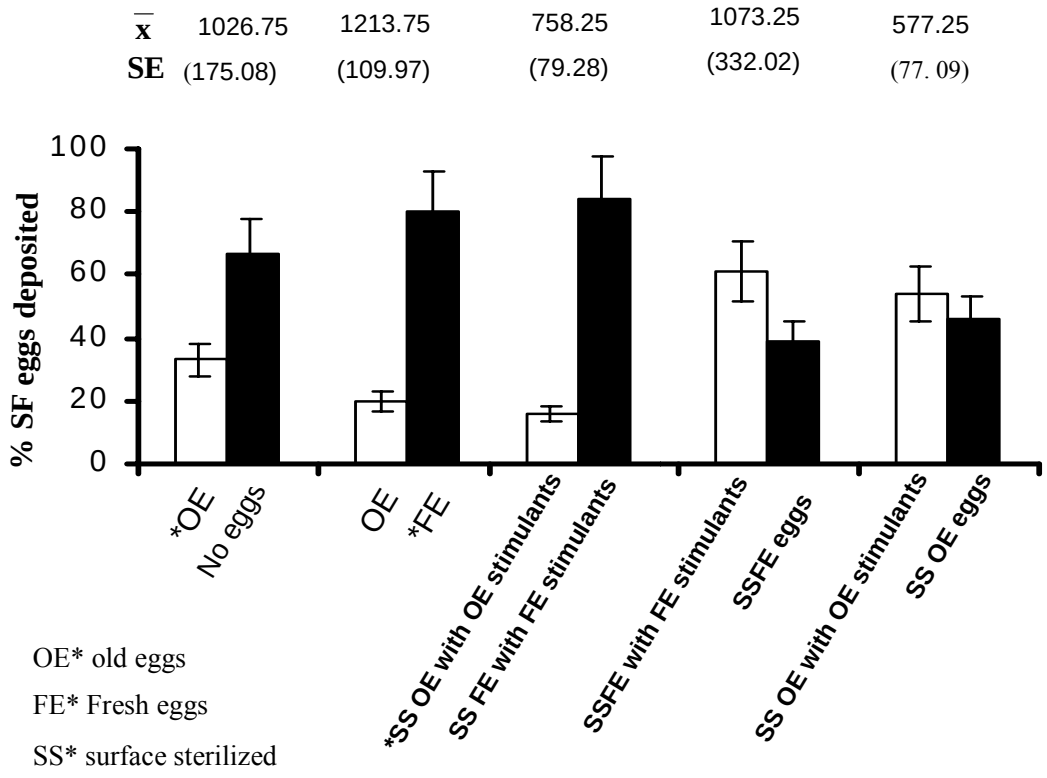


Figure 2. 2. Mean % of SF eggs laid on the substrate with old eggs (24h old) or on substrate with no eggs, fresh sterilized eggs and wash from old and young eggs. The numbers above represents the mean of the oviposited eggs and the standard error (n = 4).

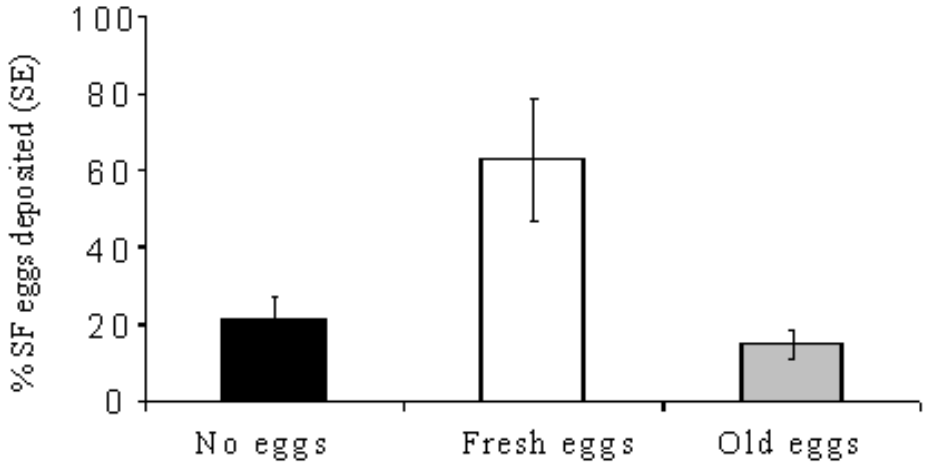


Figure 2. 3 Mean % of SF eggs laid in substrates with either young, old, or no eggs (n = 4)

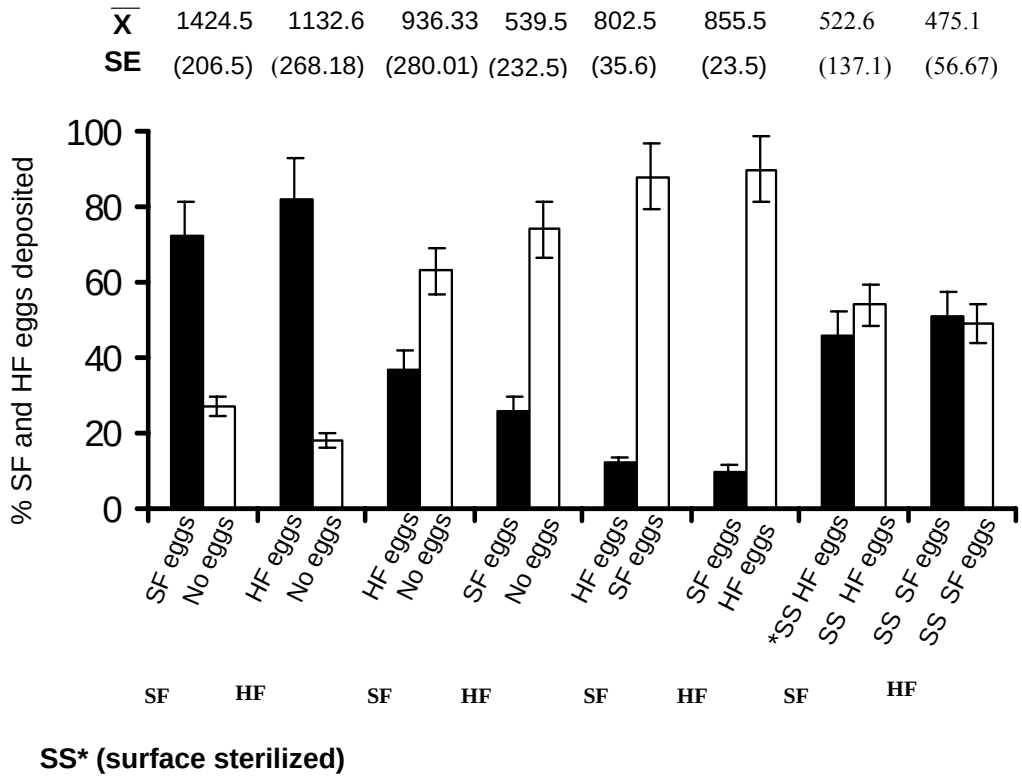


Figure 2. 4 Oviposition preferences of gravid SF and HF in fresh and surface sterilized eggs. The letters HF and SF below the graphs indicate the type of females released. The numbers above represents the mean of the oviposited eggs and the standard error (n = 4).

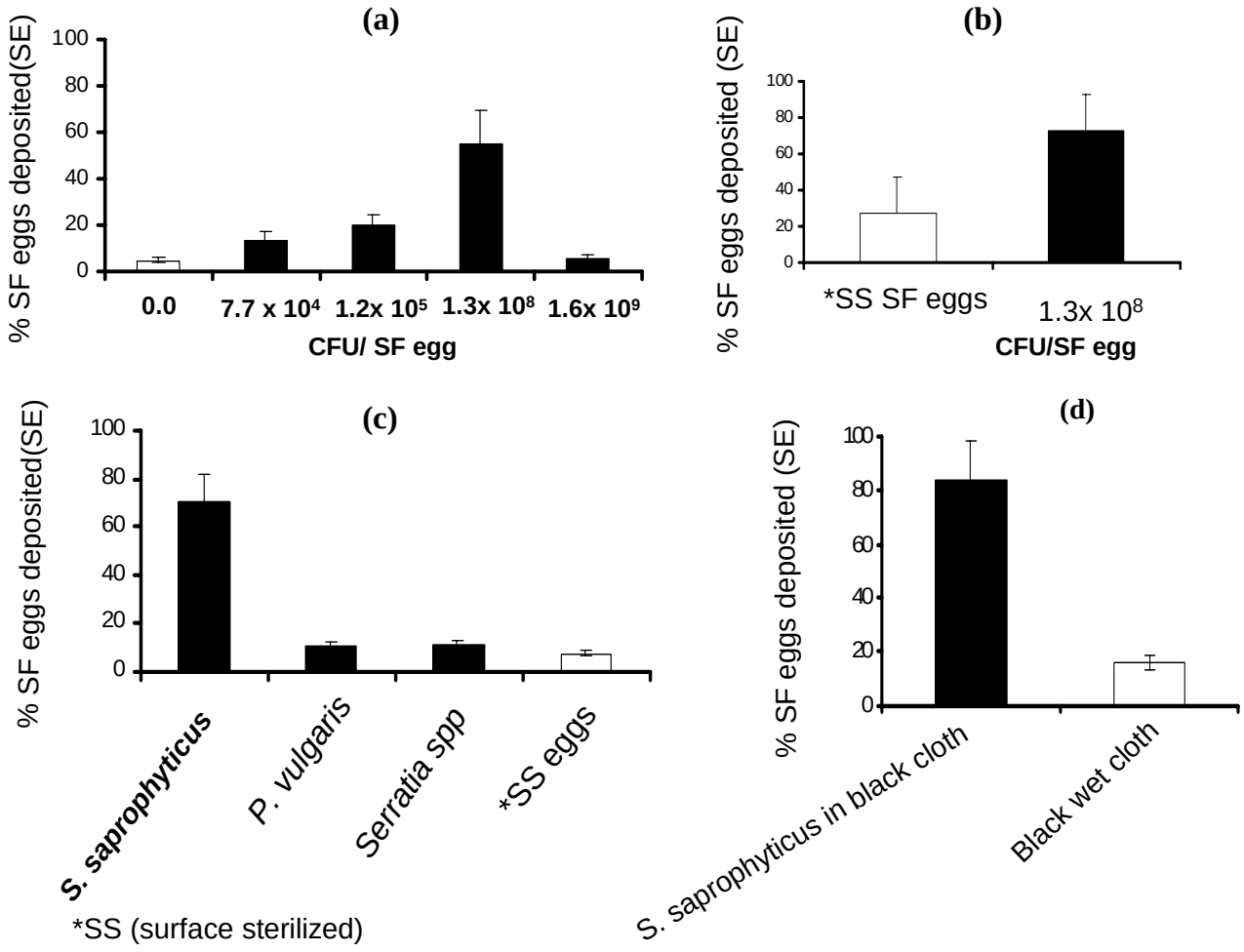


Figure 2. 5. Mean % of SF eggs oviposited on substrates with SF eggs inoculated (a) mixture of colonies obtained from individual SF egg with different concentrations; b) with the best concentration (10^8) from the mixed colonies versus surface sterilized SF eggs; c) with *Staphylococcus saprophyticus, Proteus vulgaris, Serratia spp* and control and d) *Staphylococcus sapropyticus* in black cloth with black cloth as a control (n = 4)

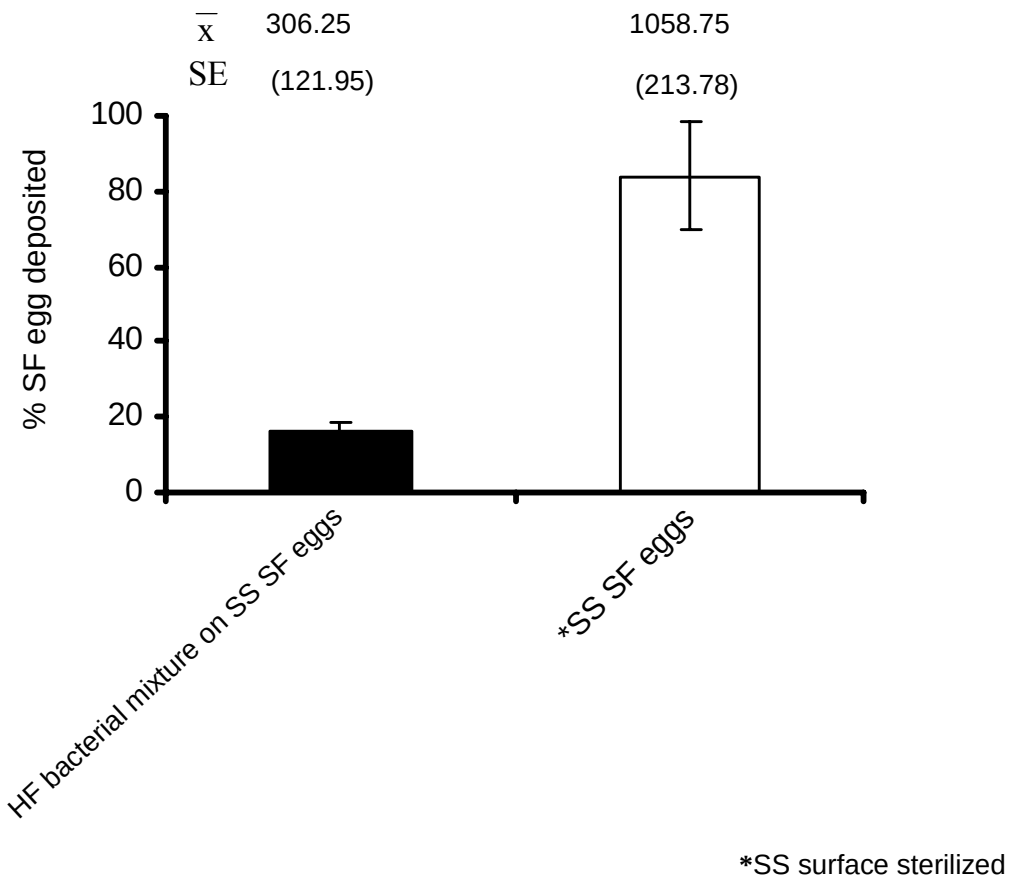


Figure 2. 6 Mean % of SF eggs oviposited on substrates with either sterilized SF eggs inoculated with mixed bacteria from HF or on sterilized SF eggs. The numbers above represents the mean of the oviposited eggs and the standard error (control) (n = 4)

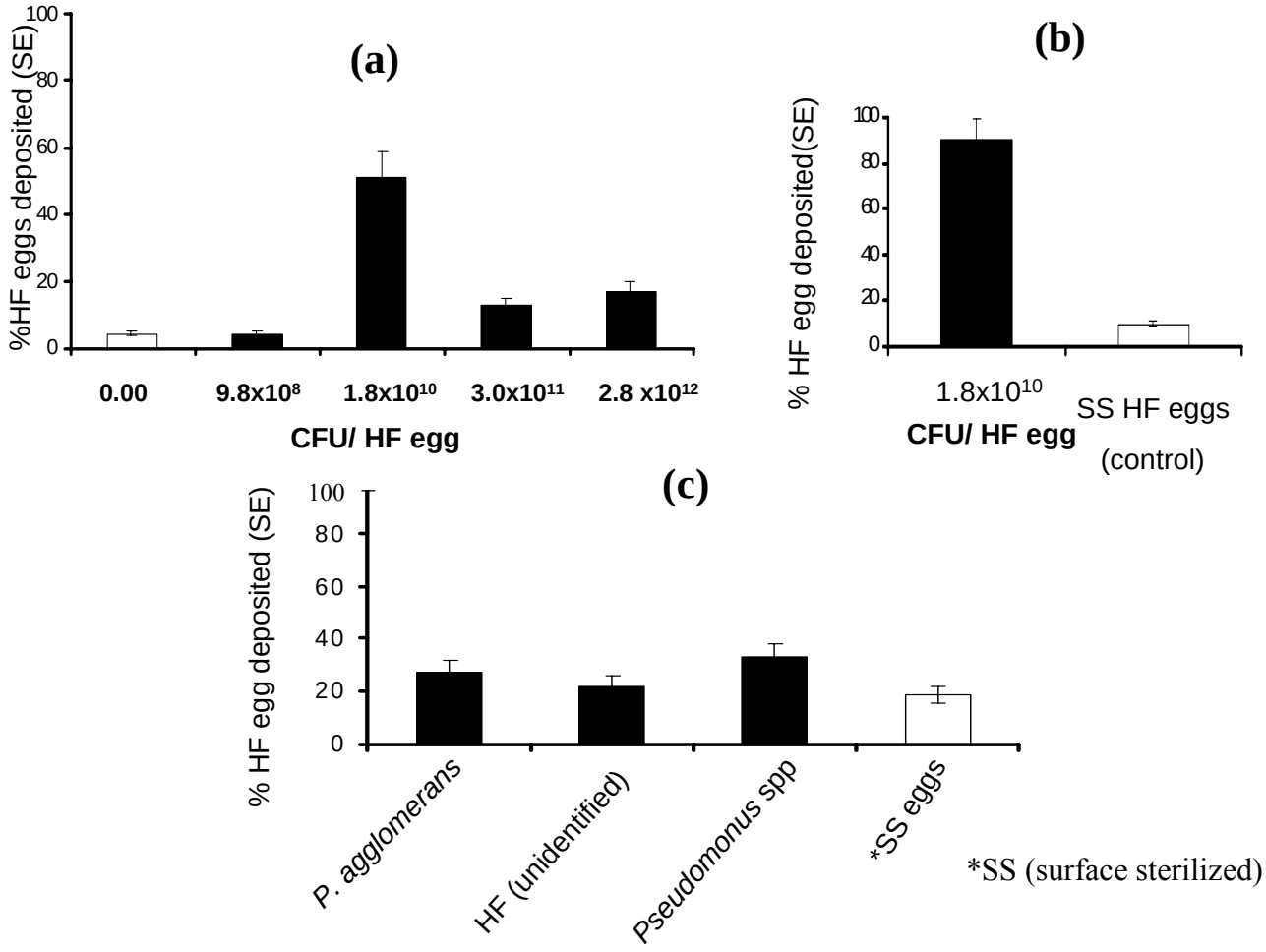
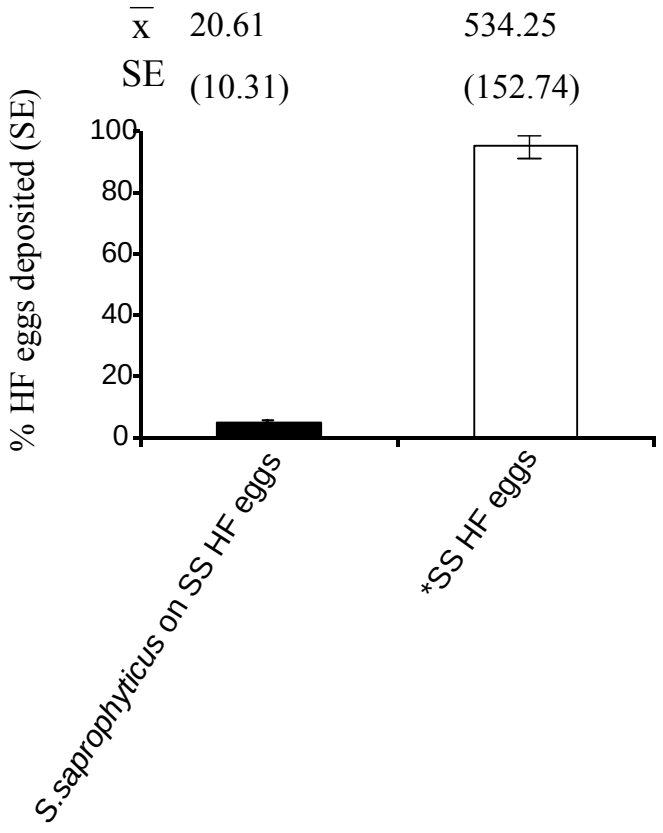


Figure 2.7. Mean % of HF eggs oviposited on (a) mixed bacteria at different concentrations; (b) the best concentration versus surface sterilized eggs (control); and (c) individual colonies obtained from HF eggs (n = 4)



*SS (Surface sterilized)

Figure 2. 8. Mean % of HF eggs oviposited on sterilized HF eggs inoculated with *Staphylococcus saprophyticus* at a concentration of 4.7 x 10^8 CFU/ HF egg versus sterilized eggs without bacterium. The numbers above represents the mean of the oviposited eggs and the standard error (n = 4).

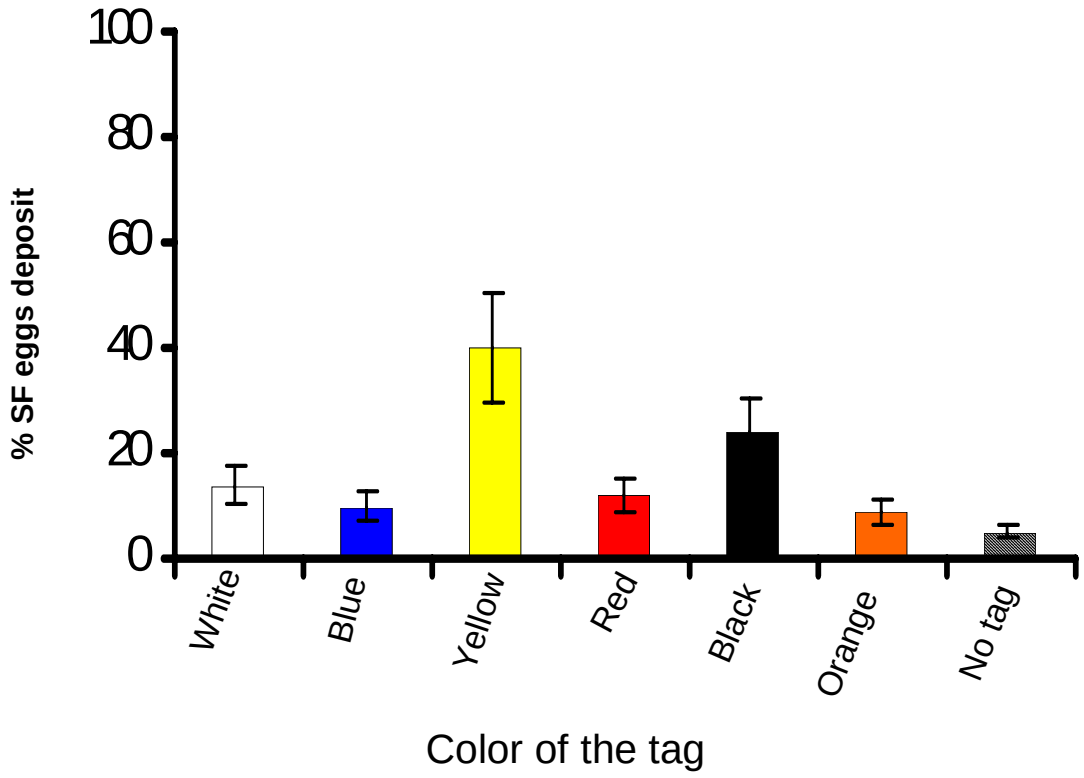


Figure 2.9 Mean % of SF eggs oviposited in different colored substrates (n = 4).

CHAPTER 3

Interspecific larval aggregation behavior of stable flies (*Stomoxys calcitrans*) and house flies (*Musca domestica*).

Abstract

The spatial aggregation and segregation of early third instars of stable fly and house fly larvae emerging from clumped or evenly distributed eggs in an apparently homogeneous substrate was studied. The spatial distributions of 3rd instar of both SF and HF larvae that emerged from clumped eggs and clumped 2nd instars were highly aggregated compared to those emerged from evenly distributed as eggs or larvae. A significant dissociation was obtained from both species of larvae set as clumped or evenly distributed. Survival to 3rd instars was greater from clumped eggs or 2nd instars than those of the larvae that emerged from evenly distributed eggs or 2nd instars. A choice test between compacted and loosened manure showed that stable fly larvae preferred to aggregate in the compacted manure than in the loosened manure, possibly because of higher moisture and pH in the compacted manure.

Key words: *Stomoxys calcitrans*, *Musca domestica*, SADIE, aggregation, segregation

Introduction

Stable flies (SF), *Stomoxys calcitrans* (L.), and house flies (HF), *Musca domestica* L., are major pests of livestock in feedlots, dairies and pastures (Schmidtmann 1985, Lysyk 1993). The damage caused by stable flies involves painful bites, especially on the legs of animals, which result in production losses (Schmidtmann 1985, Campbell et al. 1987). The economic losses caused by house flies are much more difficult to quantify as they are capable of carrying pathogens of more than 65 diseases affecting humans and animals (Greenberg 1971). The most common control methods employed against these pests include the use of less toxic insecticides, elimination of larval developmental habitats by frequent sanitation, use of biological control agents such as pupal parasitoids, and the use of traps in fly resting sites (Pickens and Miller 1987, Petersen 1989, Schmidtmann 1991, Miller et al. 1993a). But, these control strategies are just marginally effective. However, an effective control strategy may be developed by taking into consideration the behavior of SF and HF larvae in their habitats. Both SF and HF larvae use manure or manure mixed with vegetative materials as larval developmental habitats (Campbell et al. 1987). Even though a lot of data are available concerning suitable larval habitats for both species, little is known about larval behavior in these habitats. For instance, although HF and SF larvae are found aggregated in clumps in what may appear to be homogeneous substrates, these aggregations are seldom made of larvae of mixed species. Whether this segregation of larvae by species is a function of the mother's oviposition behavior or the result of larval behavior is not known. Implementation of successful control strategies aimed at the larvae requires an understanding of their spatial and temporal distributions patterns in both large and microhabitat scales.

Methods for analyzing spatial patterns have been developed recently in a wide range of ecological disciplines. Dixon (1994) and Perry (1998) developed the Spatial Analysis by Distance IndicEs, SADIE, software, which provides a means to measure the spatial association or dissociation of two set counts created in identical conditions. It has been reported from several insect species that larvae emerging from eggs laid in clusters are more active and survive better than those from singly -laid eggs (Stamp 1980). It is unknown whether the same applies to SF and HF larvae. To study this behavior, a study on survival of larvae emerged from clumped and evenly distributed eggs were conducted and the same 2nd instar larvae that were set as clumped and evenly distributed larvae.

A variety of studies have classified larval habitats in dairy cattle and feedlots in terms of development media of SF and HF. Campbell and Mc-Neil (1979) have identified several breeding habitats for SF and HF. Broce and Haas (1999) discovered that SF larvae prefer older manure 12 - 20 days while HF prefers fresher manure of up to 2 d old to 30 days. However no information about whether the preference is caused by compaction or looseness of medium. The objectives of this study were to describe the aggregation and segregation behaviors of early third instars of both SF and HF larvae in an assumed homogeneous substrate (pH, moisture and temperature). In addition, the effect that compaction of manure has on larval aggregation was evaluated.

Materials and Methods

Fly larvae.

All stable fly and house fly larvae used in this study were from laboratory colonies maintained at 25 ± 2^{o} C, 70 ± 10% RH and a photoperiod regime of 18:6 (L: D). Adult stable flies were fed daily on citrated bovine blood and by the 6th day, most females were fully gravid. House flies were fed granulated sucrose and egg protein and after 3 days they had fully developed eggs. Gravid females of both species were separated from males one day prior to use and held in screened clean cages at the same environmental conditions. The eggs were collected from the colony prior to the experiment and divided into two parts: one part was transferred as eggs into the larval medium while the other portion was left to hatch and the 2nd instars were transferred to the surface of the medium.

Larval aggregation tests.

Round metal pan (fig. 3.1), 42 cm diameter and 8 cm deep were filled to a depth of 3 cm with a medium of manure: chopped prairie hay at a 1:2 ratio. The manure was obtained from cows that had not received any pesticide treatment recently at the Kansas State University Purebred Beef Teaching Research Center. Manure was frozen (-20° C) to kill any fly larvae present. Hay was obtained from the Kansas State University Cow/Calf Unit and consisted of native brome grass hay. Hay was chopped and sieved through a No.10 sieve (W.S. Tyler Company, Mentor, OH) for uniformity. Approximately 1.5 L of distilled water was added to all media samples to provide adequate moisture content (70%) for larval development. For recording larval distribution, the medium in each pan was divided into 102 equal grid cells of 3 × 3 × 3 cm each (Fig. 3.1).

Aggregation vs. segregation of larvae

To determine if aggregation and segregation of SF and HF larvae are mediated by the mother's oviposition or larval behavior, the following experiments were conducted:

(a) Clumped eggs: Each pan was set with 300 eggs of either SF or HF in two clumps on the center of the medium and separated by 1.2 cm. Controls consisted of 300 eggs of just one species per arena.

(b) Uniform egg distribution: Equal number of eggs as in (a) were placed on the medium and distributed evenly of which each cell with 2-3 eggs of either SF or HF set in alternate pattern. Controls consisted of 300 eggs of just one species per arena.

(c) Clumped larvae: Same as in (a) but using 2nd instar larvae in lieu of eggs. Controls were as in (a) but using larvae instead of eggs.

(d) Uniform larval distribution: Same as in (b) but using 2nd instar larvae in lieu of eggs. Controls were as in (b) but using larvae instead of eggs.

Each pan was covered with a clear plastic wrapping with ten small holes for aeration. Substrates were kept at 25 – 26° C, 70% RH, and 24 h lighting. Temperature and pH were recorded at the beginning and at the end of each experiment using a portable data logger (Acorn® pH 6 series, Omni Controls Inc., Tampa, FL).

Treatments with both egg and 2nd instars were monitored when larvae were in the 3rd instar. Stable fly larvae when near the end of their larval development purge their gut and initiate larval wandering (McPheron and Broce 1999). As a result of this movement, larvae follow the base of the walls of the rearing pans and prefer to aggregate at the inside corners. To avoid the confounding effects of this wandering behavior, we used round metal pans and larvae positions in the 102 cells were recorded before the larvae reached the anterior retraction stage in which they attain a barrel shape. Larvae locations were identified by thoroughly disrupting individual

cells. The number of larvae of each species in each cell was recorded. Survival rate of each species that emerged from a clumped versus evenly distributed eggs and second instar larvae were also recorded. Each test was replicated four times. The voucher specimens used in this study are deposited in the KSU Museum of Entomological and Prairie Arthropod Research as voucher number 196.

Analysis of interspecific larvae aggregation:

The data was analyzed by the Spatial Analysis by Distance IndicEs (SADIE Shell Software (Perry 1998), a package which measures the spatial association of two set counts created in identical conditions and measures clusters of units with positive association or negative dissociation. Three SADIE indices were used to measure the spatial patterns in two sets of counts: a) Index of aggregation which measure the complete crowding of the individuals (I_a); b) patch clusters, v_i; which considers counts larger than the sample mean with an expectation value of 1 and gap clusters, v_j; which is comprised of those counts smaller than the mean, with an expectation value of -1; c) probability of association or dissociation between the two species. For a SADIE two tail test, the probability is considered significant association when the P level is < 0.025 and dissociation when the level is > 0.975. If v_i; >1 then it is indicative of local association, and if v_j is < -1 then it is indicative of local dissociation. When I_a and v_i are >1, the aggregation is considered strong. Bubble plots were used to visualize the data; bubble dimensions being a function of larval counts. A paired t- test was used to measure survival rate of 3rd instars of SF between (a) Clumped, evenly distributed eggs and controls; and (b) clumped, evenly distributed of 2nd instar larvae and control.

Distribution of stable fly larvae in loosened and compacted manure.

A choice test experiment was set with the mixture of manure and chopped hay as described above, with one half of the pan as compacted medium and the other as loosened medium. Compaction of manure medium was accomplished by placing another same-size pan over the medium and adding 10 kg of sand (the load of manure mixed with hay 7.2g / cm^2). Sand was stirred for 5 min to an even distribution over the pan. The pan with the sand was then removed and the manure medium divided into two equal parts. One side was left compacted and divided into 51 cell grids. The other half was loosened with a spatula and also divided into 51 cell grids. Three hundred SF eggs were clumped in the center of the pan, between the loosened and compacted media. Temperature, moisture, and pH of the medium on each side were recorded before placing the eggs and when the larvae reached the 3rd instar stage. This experiment was replicated four times. The results were analyzed by paired t - test.

Results

Aggregation versus segregation of larvae

a) and b) Clumped vs evenly distributed eggs. The distribution of 3rd instar HF and SF larvae that emerged from clumped eggs had an index of aggregation (I_a) of 1.344 and patch clusters (v_i) of 1.860, whereas larvae that emerged from eggs evenly distributed had an *Ia* of 1.014 and patch clusters of 0.921 (Table 3.1; Fig. 3.2 and 3.3). The gap clusters value (v_j) of larvae from clumped eggs was - 1.350 and larvae from evenly distributed eggs, - 0.952.

c) and d) Clumped vs evenly distributed 2nd instar larvae: The distribution of 3rd instar larvae when placed on the medium as clumped 2nd instar larvae had an I_a value of 1.070 and a v_i of 1.090 while those from evenly distributed larvae were 1.047 and 0.993, respectively. The gap

clusters value (v_j) of larvae from clumped larvae was - 1.110 and - 0.925 for evenly distributed instars.

A t - test at the 5% level showed a significant dissociation of P = 0.993 in larvae emerged from clumped eggs and 0.988 from evenly distributed eggs. The P values for larvae from the clumped and the evenly distributed 2nd instars were 0.998 and 0.993, respectively. All P values showed a significant dissociation, as their probabilities were above 0.975 (table 3.1).

Both SF and HF larvae had significantly higher survival when they emerged from the clumped eggs (151 HF and 145 SF) than from the evenly distributed eggs (84 HF and 80 SF) (t = 4.51; P = 0.0107) (Fig. 3.6). Similar results were observed from larvae placed as clumps (199 HF and 170 SF) compared to those evenly distributed (92 HF, and 76 SF) respectively (t = 5.27; P = 0.0062) (Fig. 3.7).

Distribution of stable fly larvae in loosened and compacted manure.

Stable fly larvae aggregated significantly more in compacted manure than in the loosened manure (t = 2.64; P = 0.0385) (Fig. 3.8). The moisture content in compacted manure was significantly higher (56%) than in loose manure (30%) (t = 3.19; P = 0.0189). The pH of compacted manure (7.53) was significantly also different from that of loosened manure (8.93) (t = 18.18; P = <.0001), whereas the temperature of compacted manure (26.5º C) was not significantly different from that of loosened manure (26.3º C) (t = 0.75; P = 0.4792).

Discussion

Third instar SF and HF larvae placed as clumps of either eggs or 2nd instars on the manure: hay arenas demonstrated higher levels of aggregation than the larvae emerged from evenly distributed pattern over the arenas. Larval aggregation may be advantageous to the group in overcoming adverse environmental conditions such as parasitism or predation or may be

advantageous in creating and preserving a humid environment to avoid desiccation. An intensity of aggregation may increase when humidity decreases, suggesting that grouping might induce a reduction of net water loss per individual by the formation of local microclimates around the larvae (Manning 1979; Dambach and Goelem 1999). The present study has shown that SF and HF larvae aggregated separately with minimal overlapping, possibly due to the reasons mentioned above but also mainly to maintain coexistence by avoiding excluding each other, similar to what has been reported in fruit breeding dipteran species (Atkinsons 1984).

Atkinsons (1984) reported that aggregation is important because coexistence depends on independent aggregation. Aggregation of the larvae of these two species may be due to oviposition behavior. It was noted from my earlier studies (Mramba unpublished) that gravid females of HF and SF laid eggs preferentially in substrates already containing conspecific eggs. Additionally, both gravid SF and HF oviposited in clusters and preferred to oviposit at the edges of Petri plates. A similar trend was noted from this study, that the larvae of these two species aggregate separately with insignificant overlap. This process could give rise to independent aggregation of immature stages of their own species as was noted by Atkinsons and Shorrocks (1981) who suggested that “two species of insect breeding in discrete sites could coexist in spite of strong competition between the larvae for as long as their eggs were aggregated and distributed independently”. They also found that the aggregation resulted mainly from the flies laying their eggs in clutches whose distribution was separate and independent which they recommended as a strong condition for coexistence. This may be the underlying reason why aggregation is common to most dipteran insects, which use common areas for breeding such HF and SF.

This study also showed that the survival of the larvae that emerged from clumped eggs and 2nd instar larvae was significantly higher than from those that emerged from evenly distributed eggs or 2nd instar larvae. The study also showed that stable flies preferred aggregating in the substrates with compacted manure mixed with hay than in loosened substrates. The reasons for the preference may be associated with higher moisture content and pH that was noted in the compacted side in contrast to conditions in the loose substrate. This supports the results observed by McPheron and Broce (1999) who obtained more pupae in the medium with higher moisture level (71%) and the pH of 9.3 when given a choice than in medium with low moisture and pH. In my study I found that the pH in the compacted side was 7.5, which was lower than in the loose side (8.9), but this is appropriate pH for SF larvae survival as was recommended by Rasmussen and Campbell (1981) and Broce and Haas (1999). The temperature in this particular study was not an issue as both sides had equal temperatures (26^{o}C). McPheron and Broce (1999) noted that temperature variations influence survival of SF larvae. They noted that temperatures of 26^{o}C resulted in higher survival of SF larvae than temperatures of 21^{o}C and 32.5^{o}C.

Acknowledgement

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References cited

Atkinsons, W.D. and B. Shorrocks. 1981. Competition on a divided and ephemeral resource: A simulation model. J. Anim. Ecol. 50: 461-471.

Atkinsons, W.D. and B. Shorocks, 1984. Aggregation of larval Diptera over discrete and ephemeral breeding sites: The implications for coexistence. Amer. Natural. 124: 3.

Campbell, J.B., I.L. Berry, D.J. Boxler, R.L. Davis, D.C. Clanton, and G.H. Deutscher. 1987. Effects of stable flies (*Diptera*: *Muscidae*) on weight gain and feed efficiency of feedlot cattle. J. Econ. Entomol. 80: 117-119.

Dambach, M. and B. Goehlen, 1999. Aggregation density and longevity correlate with humidity in first instar nymphs of cochroaches , (*Blattaria germanica* L., Dictyoptera). J. Ins. Physiol. 45: 423-429.

Dixon, P.M. 1994.Testing spatial segregation using a nearest- neighbor contingency table. Ecol. 75: 1940-1948.

Early, M. 1986. Arthropods succession patterns in exposed carrion on the island of O'ahu, Hawaiian Islands, USA. J. Med. Entomol.5: 520-531.

Greenberg, B. 1971. Flies and Disease. Ecology, Classification and Biotic Associations, Princeton University Press, Princeton, NJ.

Mc Pheron, L.J. and A. B. Broce. 1999. Environmental components of pupariation- site selection by the stable fly (Diptera: Muscidae). Environ. Entomol. 25: 665-671.

Miller, R.W., D.A. Rutz, L.G. Pickens and C.J. Gedens. 1993a. Evaluation of traps and parasitoids *Muscidifurax raptor* Girault and Sanders to manage house flies and stable flies on dairy farms. J. Agric. Entomol. 10: 189-196.

Perry, J.N. 1998. Measures of spatial pattern association for counts of insects. In: J. Baumgartner, P. Brandmayr & B.F.J. Manly (eds). Population and community ecology for insect management and conservation. Balkema, Rotterdam. Proceeding of the Ecology and Population Dynamics Section of the 20th Perry & Dixon: Spatial association of tupelos. P. 21-33

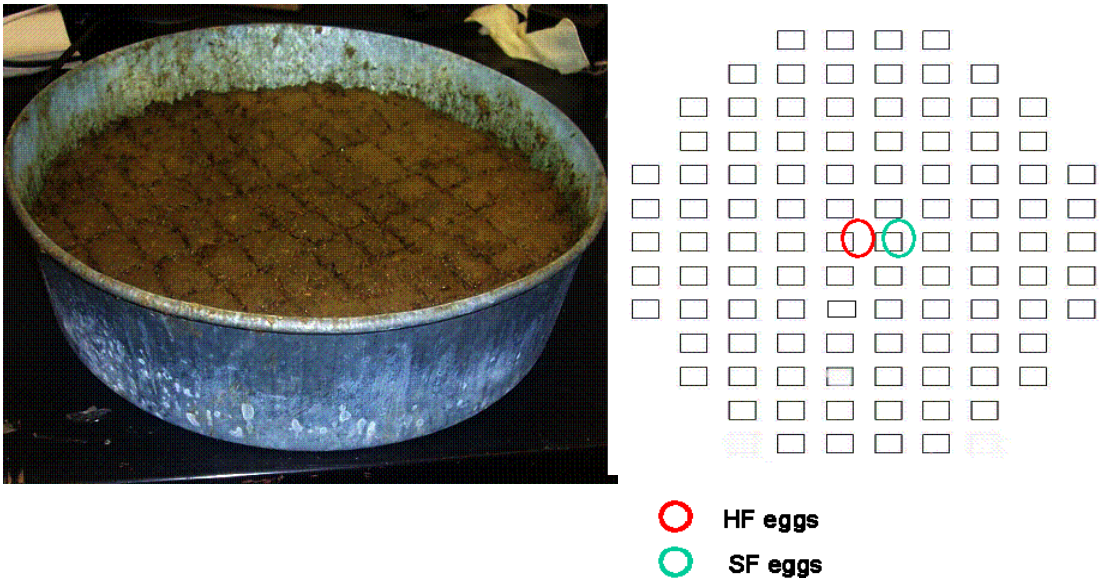
Petersen, J.J. 1989. Potential for biological control of stable flies associated with confined livestock. Misc. Publ. Entomol. Soc. Am. 74: 41- 45.

Pickens, L.G., and R.W. Miller, 1987. Techniques for trapping flies in dairy farms. J. Agric. Entomol. 4: 305-313.

Rasmussen, R. L., and B. Campbell. 1981. Investigation of environmental factors and their relationship to populations of the stable fly, *Stomoxys calcitrans* (L). Environ. Entomol. 10: 798-800.

Schmidtmann, E.T. 1991. Suppressing immature house and stable flies in outdoors cow hutches with sand, gravel, and sawdust bedding. J. Dairy Sci.74: 3956-3960.

Schmidtmann E .T. 1985. Arthropods pests of dairy cattle. In Livestock Entomology. R.E. Williams, R.D. Hall, A.B. Broce, and P.J.Scholl, ed. John Wiley and sons, Newyork, NY. P. 223-233.



(a) **(b)**

Figure 3.1. (a) Division of grids in individual pan. (b) Diagram showing the grids with clumps of stable fly and house fly eggs

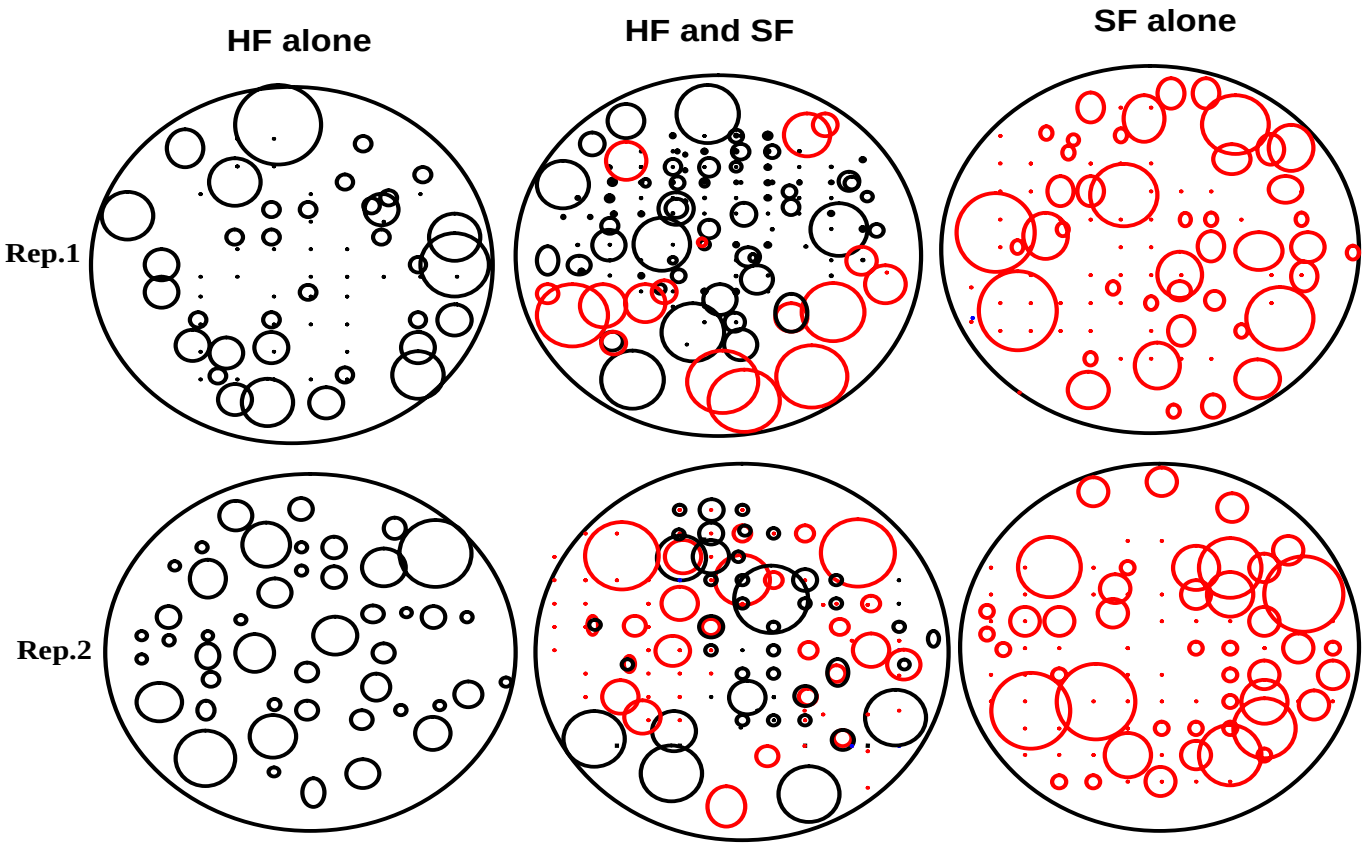


Figure 3.2. Examples of bubble plots of two typical replicates of the distribution of house fly (HF) and stable fly (SF) 3rd instar larvae after starting as clumped eggs at the center on the arena (42 cm diameter and 8 cm deep)

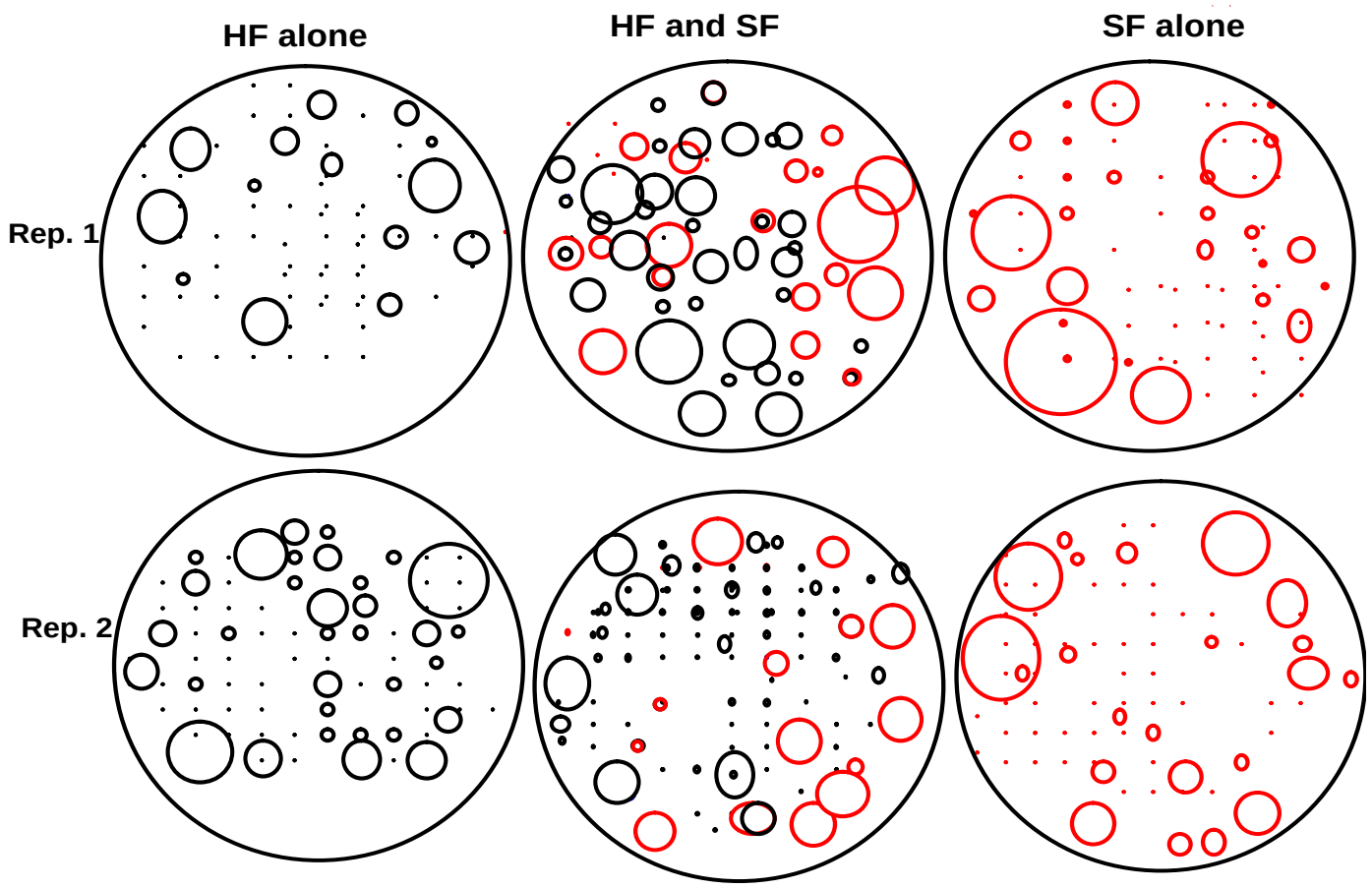


Figure 3.3. Examples of bubble plots of two typical replicates of the distribution of house fly (HF) and stable fly (SF) 3rd instar larvae after starting as evenly distributed eggs of the arena (42 cm diameter and 8 cm deep)

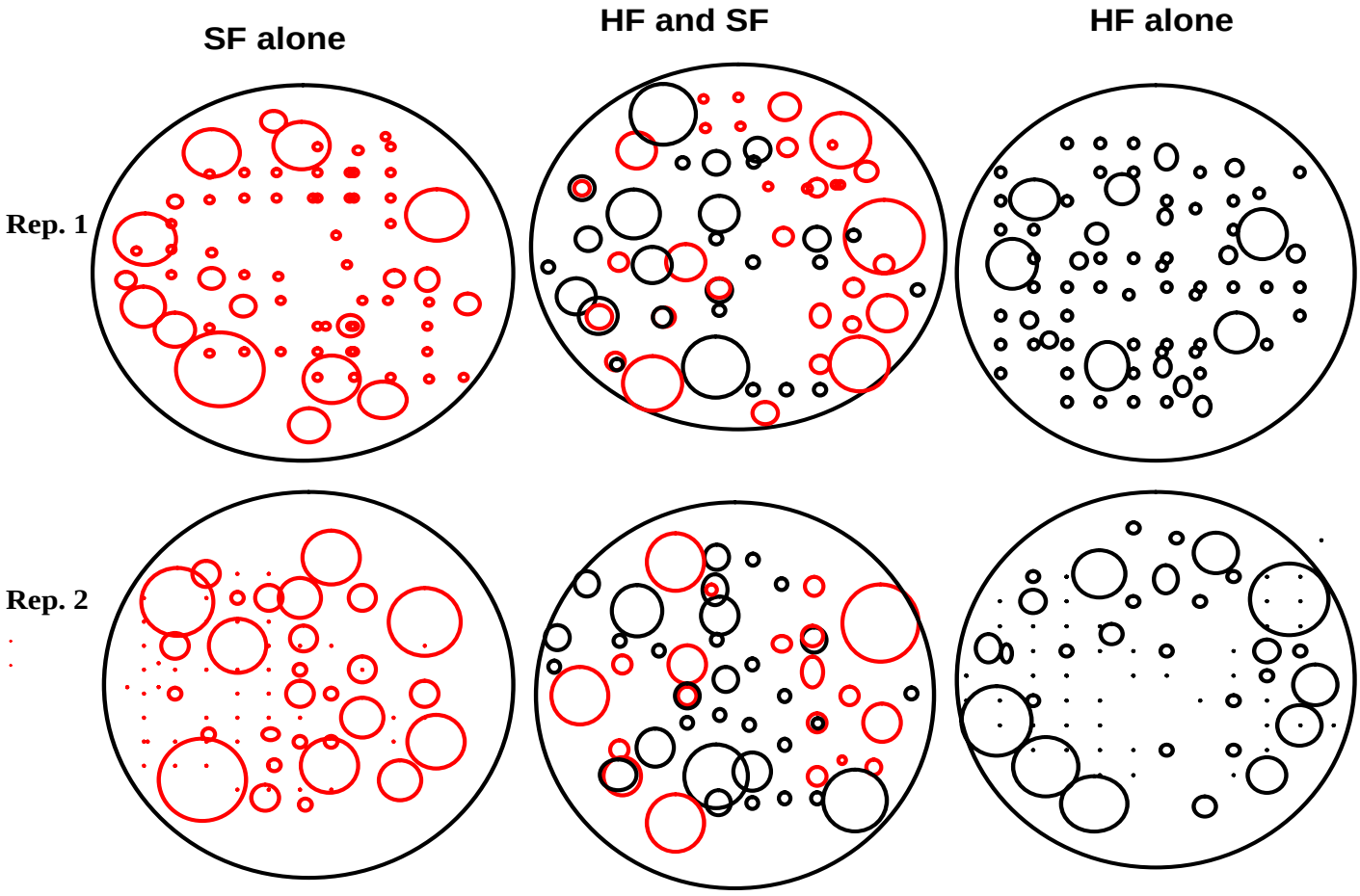


Figure 3. 4. Examples of bubble plots of two typical replicates of the distribution of house fly (HF) and stable fly (SF) 3rd instar larvae after starting as clumped 2nd instar at the center of the arena (42 cm diameter and 8 cm deep)

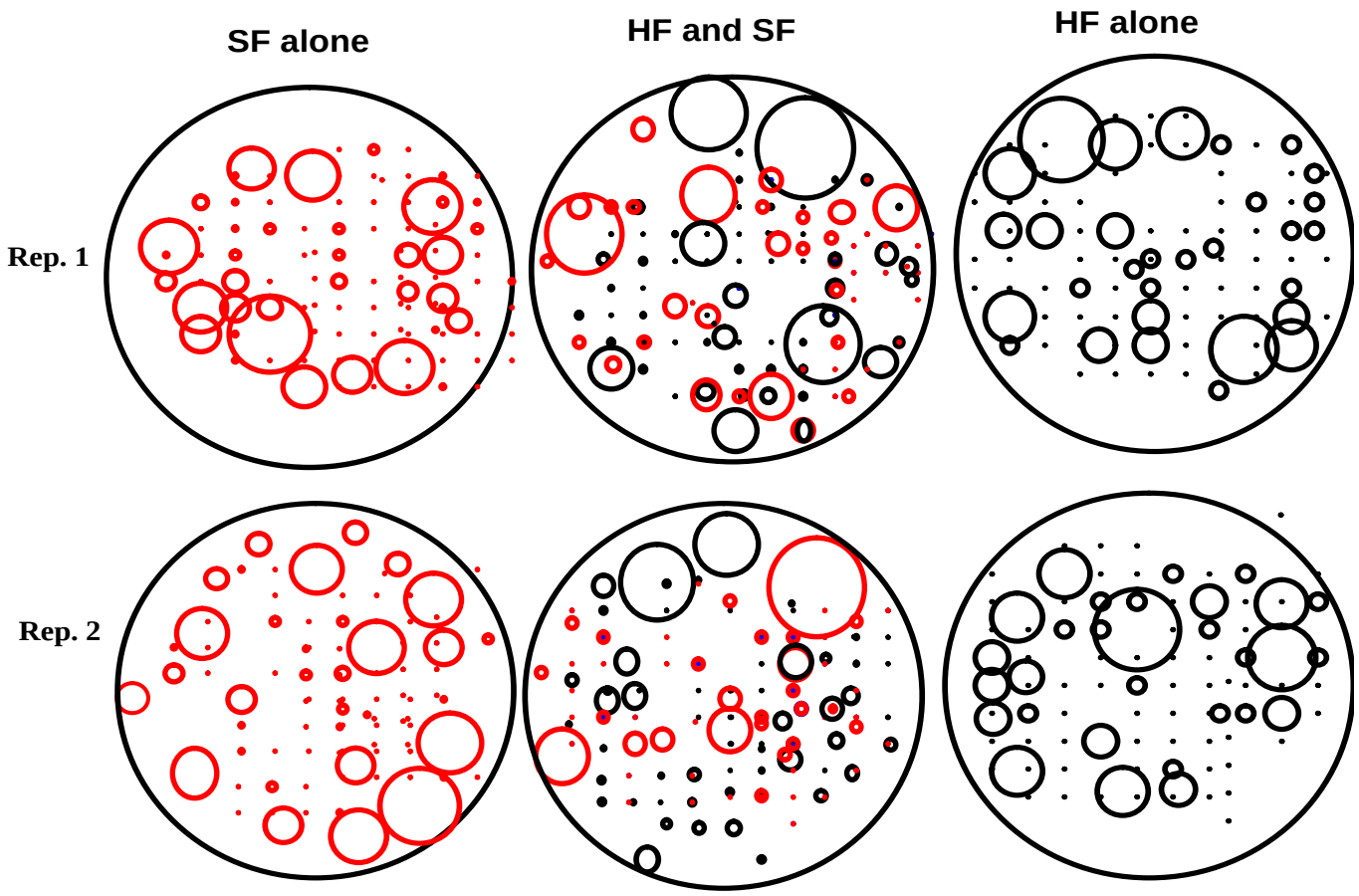


Figure 3.5. Examples of bubble plots of two typical replicates of the distribution of house fly (HF) and stable fly (SF) 3rd instar larvae after starting as evenly distruted 2nd instar of the arena (42 cm diameter and 8 cm deep)

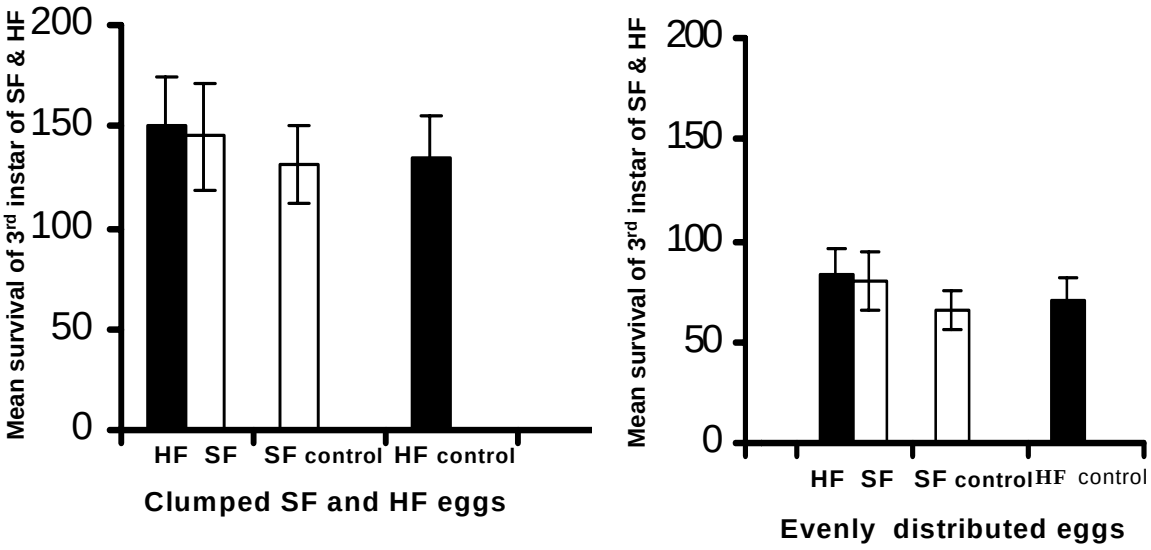


Figure 3.6 Survival of 3rd instar larvae emerged from clumped and evenly distributed eggs

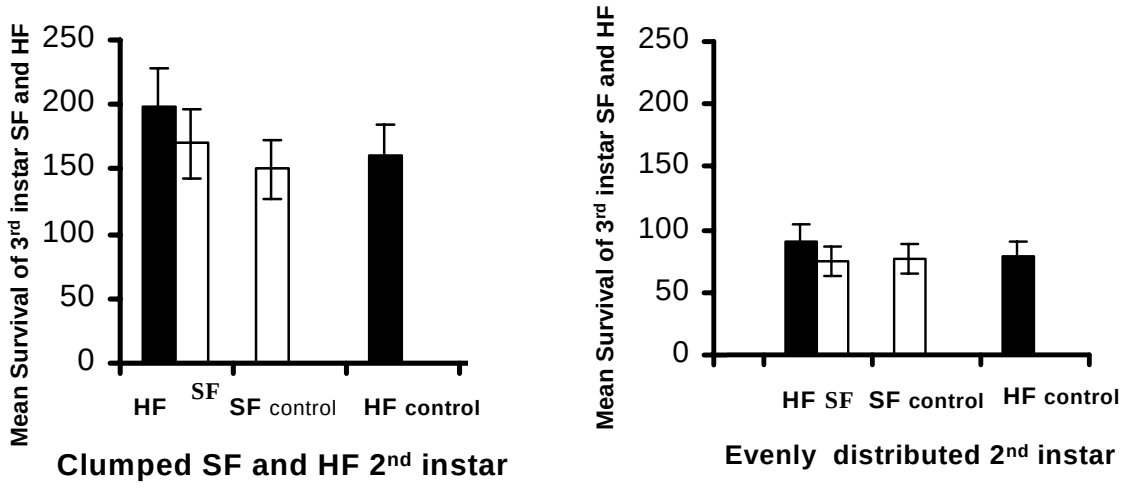


Figure 3.7 Survival of 3nd instar from clumped and evenly distributed 2nd instars

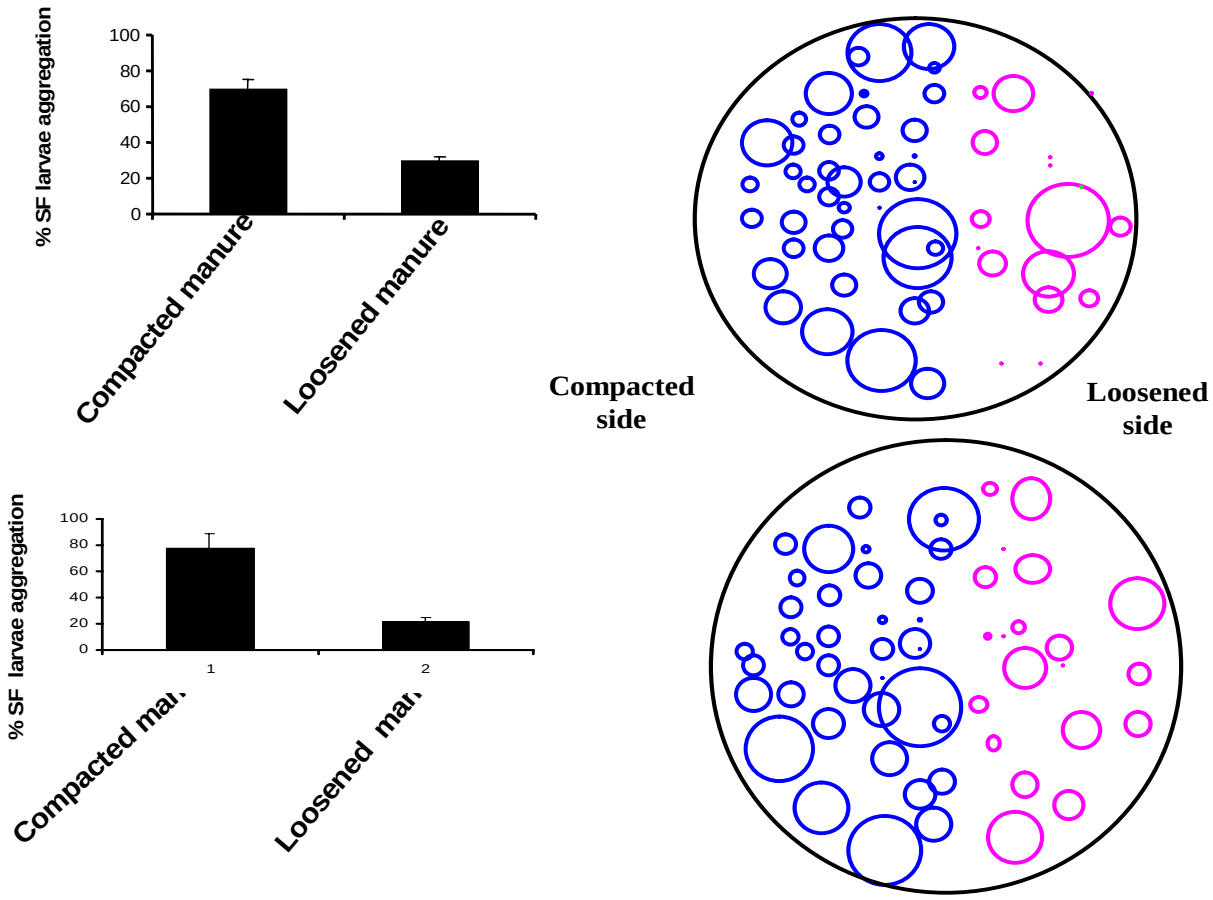


Figure 3.8 Bar graph for replicates 1 and 2 and bubble plots showing the 3rd instar larvae. Aggregation of the stable flies in compacted side and in loosened manure substrates

Table 3.1. SADIE analysis of the spatial- distribution of 3rd instars of both clumped and evenly distributed HF and SF larvae.

Groups and Treatments	Patch clusters (v_i)	Gap clusters (v_j)	Association or Dissociation	Index of Aggregation (I_a)	P- value
HF and SF egg clumped	1.860	-1.350	0.993*	1.344	0.385
HF and SF evenly distributed	0.921	-0.952	0.988*	1.014	0.055
2nd Instars of HF and SF clumped	1.090	-1.110	0.998*	1.070	0.153
2nd Instar larvae of HF and SF evenly distributed	0.925	-0.918	0.993*	1.047	0.076

* Significant dissociations >0.975

CHAPTER 4

Isolation of *Enterobacter sakazakii* from wild stable flies (Diptera: Muscidae).

Abstract

Enterobacter sakazakii is an emerging opportunistic food-borne pathogen causing meningitis, enterocolitis, and sepsis, primarily in immunocompromised infants. Previously, it was suggested stable flies, *Stomoxys calcitrans*, are a vector/reservoir of this pathogen. In this study, using a culturing approach combined with 16S rDNA PCR-RFLP-sequencing, we screened 928 individual stable flies collected in Kansas and Florida. Two stable flies (0.2%) were found positive for *E. sakazakii*. In addition, 411 (46%) stable flies carried bacteria forming red colonies (presumably enterics) on a violet red bile glucose agar (VRBGA) (mean concentration: 6.4 x 10^4 CFU per fly); and 120 (13%) stable flies carried fecal coliforms (mean concentration: 8.7 x 10^3 CFU per fly). Sequencing of 16S rDNA revealed that enterics from VRBGA were represented by several genera, including *Escherichia, Shigella, Providencia, Enterobacter, Pantoea, Proteus, Serratia,* and *Morganella*. Our study shows that stable flies carry bacteria typically present in animal manure (a developmental site of stable fly larvae) that indicates that the natural reservoir of *E. sakazakii* is the digestive tract and/or manure of domestic animals. The low prevalence of *E. sakazakii* associated with stable flies suggests that stable flies do not play a major role as a reservoir and/or vector of this pathogen.

KEY WORDS: stable fly, *Enterobacter sakazakii*, reservoir, manure, food - borne pathogen

Introduction

Enterobacter sakazakii (formerly yellow pigmented *E. cloacae*) is a Gram-negative, yellow-pigmented gamma-proteobacterium in the family Enterobacteriaceae (Farmer et al. 1980). It is considered an opportunistic food-borne pathogen that can cause meningitis, necrotizing enterocolitis, and sepsis (Lehner and Stephan 2004). Although infections of adults have been reported, newborns and infants are the most susceptible, especially those that are immunocompromised (Lehner and Stephan 2004). Mortality rates range between 40 and 80% and in many cases survivors suffer from severe neurological disorders and retarded neural development (Farmer et al. 1980). Powdered infant formula was linked in several cases with the outbreak of the disease (Van Acker et al. 2001, Weir 2002, Lehner and Stephan, 2004); however, the source of contamination and natural reservoir(s) of *E. sakazakii* are unknown (Lehner and Stephan 2004). Recently, *E. sakazakii* was isolated from the gut of Mexican fruit flies (Kuzina et al. 2001) and from the gut of stable fly larvae (Hamilton et al. 2003) suggesting that insects serve as vector and /or reservoir of this pathogen. However, both reports were based on the isolation from insects kept in the laboratory colony and the prevalence of *E. sakazakii* in wild insects, including stable flies, is not known.

To evaluate the role of stable flies in the ecology of *E. sakazakii*, we collected 928 wild stable flies from pastured and confined cattle environments in Kansas and Florida and screened them individually for *E. sakazakii*, concentration of fecal coliforms, and diversity of enteric bacteria.

Materials and Methods

A total 928 stable flies (SF) were analyzed; 827 SF were collected from fifteen sites with pastured cattle in Kansas and 101 SF were collected from one site with dairy cattle in Florida.

Flies were collected with a sweep net or Alsynite traps and processed on the same day. Flies from Florida were shipped overnight on ice on the same collection day and processed on the next (arrival) day. Individual flies were homogenized in phosphate buffered saline (PBS) (MP Biomedicals, Aurora, OH), serially diluted, and plated on a Violet Red Bile Glucose Agar (VRBGA) (Oxoid, Basingstoke, England), a selective agar used for isolation of *E. sakazakii* (Nazarowec-White and Farber 1997), and on a Membrane Fecal Coliforms agar (mFC) (Oxoid, Basingstoke, England) for isolation of fecal coliforms; and incubated aerobically at 37 and 44.5^{o}C, respectively, for 24 to 48 hours. One to three colonies per sample with the typical *E. sakazakii* on VRBGA (purple colonies surrounded by purple halo of precipitated bile acids) morphology were isolated on Trypticase Soy Broth Agar (TSBA, Becton Dickinson, Sparks, MD) and incubated at 37°C for 24 to 48 hours. Individual yellow-pigmented, oxidase-negative, and catalase-positive colonies were analyzed further.

One to three colonies per isolate were used for DNA extraction by boiling and 1 µl of the supernatant was used as a template for PCR amplification of 16S rDNA with eubacterial universal primers (Barbieri et al. 2001) following the protocol reported previously (Barbieri et al. 2001). The PCR product was purified by the GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience, UK) according to manufacturer's instruction and visualized by agarose (1%) gel electrophoresis with 0.05% of ethidium bromide. The PCR products were further screened by the restriction fragment length polymorphism (RFLP) approach using three restriction enzymes *Hae*III, *Alu*I, and *Mbo*I (all Promega, Madison, WI) following manufacturer's recommendations. Digested PCR products were visualized on a 3% agarose gel (3:1 Amresco, Solon, OH). The type strain of *Enterobacter sakazakii* ATCC 29544 was used as a positive control. Isolates with the same digest profile as that of the type strain as well as

representatives of those with different profiles (to assess the diversity of enteric isolates) were selected for sequencing of 16S rDNA. Sequencing was done using the same eubacterial universal primers on an ABI 3700 DNA Analyzer (Applied Biosystems USA) at the K-State DNA Sequencing Facility. Sequences were analyzed for similarity to known sequences in the GenBank database using BLAST (Basic Local Alignment Search Tool) (Altschul et al. 1990). Manual alignment was done with CodonCode Aligner (version 1, 3, 4) (CodonCode Corporation, Dedham, MA). To estimate the concentration of fecal coliforms and bacteria capable of growth on VRBGA (mainly enterics), all red and pink colonies growing on mFC agar and all red colonies on VRBGA were counted and expressed as the number of CFU / fly.

Results and Discussion

In total, 411 (46%) of the stable flies carried bacteria that grew on VRBGA and formed red colonies. The mean concentration of these bacteria was 6.4 x 10^4 CFU per fly. Of these, 363 isolates were selected (based on the colony morphology similar to that of *E. sakazakii* ATCC 29544) for PCR amplification of 16S rDNA using universal eubacterial primers and then subjected to restriction digest using three restriction enzymes. Two isolates (0.2%) from two different flies from Kansas showed the same restriction profile as that of the positive control (Fig. 4:1). Sequencing of the full gene of 16S rDNA confirmed that these isolates were *E. sakazakii* (Table 4.1). None of the 101 stable flies from Florida tested positive for *E. sakazakii*. Fifteen isolates from VRBGA with different restriction profiles from the positive control and from each other were also selected for sequencing of 16S rDNA. Sequencing confirmed that most of these were enterics and represented several genera, including *Escherichia, Providencia, Enterobacter, Shigella, Proteus, Serratia,* and *Morganella*. Two non-enteric genera, *Aeromonas* and *Pseudomonas*, were also detected from VRBGA (Table 4.1). In addition, the counts from

mFC agar showed that 120 (13%) stable flies carried fecal coliforms (mean: 8.7 x 10^3 CFU / fly). These data show that stable flies carry bacteria typically present in animal manure (a developmental site of stable flies). This indicates that the reservoir of *E. sakazakii* is most likely feces/manure and/or digestive tract of domestic animals. The natural reservoir of *E. sakazakii* is not known; the powder infant formula has been reported as the main source of infant infections (Van Acker et al. 2001; Weir 2002, Lehner and Stephan, 2004, Kandhai et al. 2004). Previously, *E. sakazakii* was isolated from the larvae of stable flies in laboratory colony and it was suggested that this insect is a reservoir of this pathogen (Hamilton et al. 2003). Our data show that wild stable flies carry *E. sakazakii,* although the prevalence is very low. Stable flies can build very large populations around domestic animals, usually cattle and horses. It is very likely that the microbes in the stable fly gut and on the body surface originate from animal manure where stable fly larvae developed. This indicates that the natural reservoir of *E. sakazakii* is the digestive tract and/or feces of domestic animals including cattle and horses, and future studies should focus on examination of these habitats as reservoirs of *E. sakazakii*. *E. sakazakii* does not survive the heat treatment during milk pasteurization (Nazarowec-White and Farber 1997), therefore contamination of milk by stable flies directly would have to take place after the pasteurization treatment. In summary, our study shows that stable flies carry bacteria typically present in animal manure/feces, a developmental habitat of stable fly larvae. The finding of *E. sakazakii* associated with stable flies indicates that animal manure is a potential natural reservoir of this pathogen. The significance of stable flies as a vector of *E. sakazakii* pathogens remains to be investigated; however, other manure-borne insects such as house flies are more likely to play a role as a vector of this pathogen due to their mode of feeding (regurgitation) and attraction to residential areas.

Acknowledgement

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References cited

Altschul, S.F., W. Gish, W.E.W. Muller, and D.J. Leipman. 1990. Basic Local Alignment Search Tool. J. Mol. Biol. 215:403-410.

Barbieri, E., B.J. Pasteur, D. Hughes, L. Zurek, D.P. Moser, A. Teske, and M.L. Sogin. 2001. Phylogenetic characterization of epibiotic bacteria in the accessory nidamental gland and egg capsules of the squid *Loligo pealei* (Cephalopoda: Lolinidae). Environ. Microbiol. 3: 151-167.

Farmer, J.J. III., M.A. Asbury, F.W. Hickman, DJ. Brenner, and The Enterobacteriaceae Study Group. 1980. *Enterobacter sakazakii*: A new species of Enterobacteriaceae isolated from clinical species. Int. J. Syst. Bacteriol. 1980; 30: 569-84.

Hamilton, J.V., M.J. Lehane, and H.R. Braig. 2003. Isolation of *Enterobacter sakazakii* from the midgut of *Stomoxys calcitrans*. Emerg. Inf. Dis. 9:1355-356.

Kandhai, M.C., M.E. Reij, L.G.M. Gorris, O. Guillaume-Genti, and M. van Schothorst. 2004. Occurrence of *Enterobacter sakazakii* in food production environments and household. Lancet 363: 39-40.

Kuzina, L.V., J.J. Peloquin, D.C. Vacek, and T.A. Miller. 2001. Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens* (Diptera: Tephritidae). Curr. Microbiol. 42: 290-294

Lehner, A. and R. Stephan. 2004. Microbiological, epidemiological, and food safety aspects of *Enterobacter sakazakii*. J. Food. Prot. 67: 2850-2857.

Nazarowec-White M, Farber J M. 1997. *Enterobacter sakazakii:* a review. Int. J. Food Microbiol. 34: 103-13

van Acker, J., F. de Smet, G. Muyldermans, A. Bougatef, A. Naessens, and S. Lauwers. 2001. Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. J. Clin. Microbiol. 39: 293-297.

Weir, E. 2002. Powdered milk formula and fatal infection with *Enterobacter sakazakii*. CMAJ 166: 1570-570.

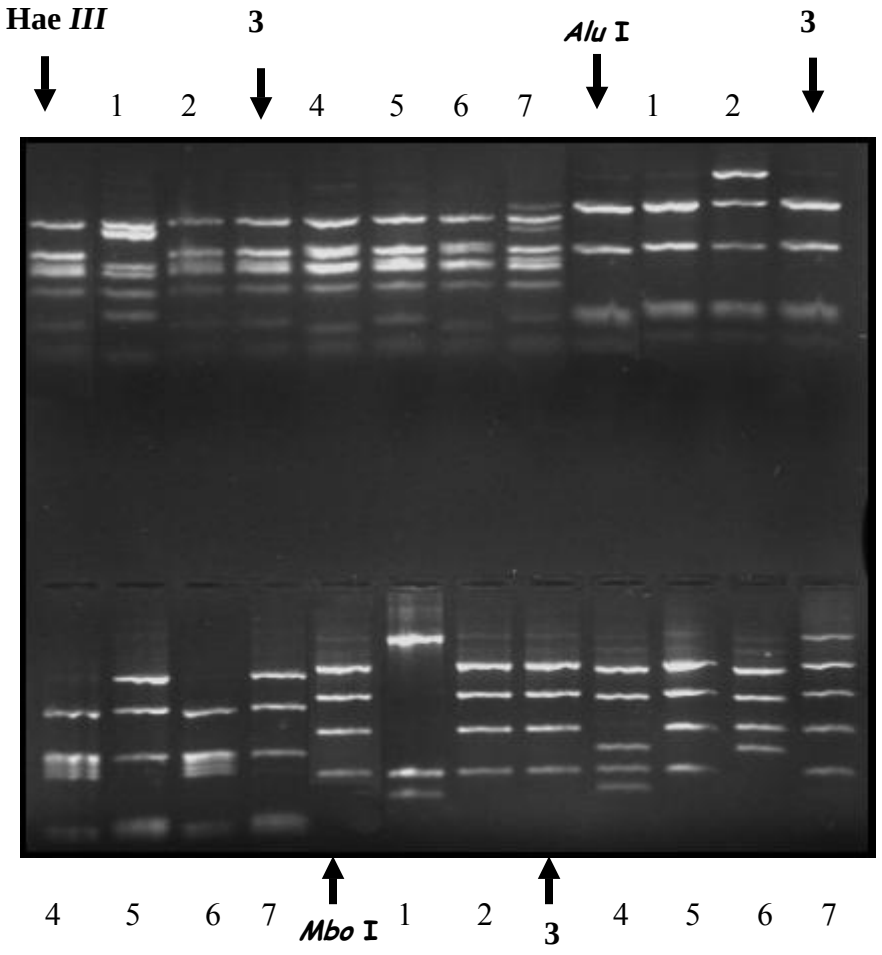

Figure 4.1. Profile of the digested PCR products of 16S rDNA of seven isolates using three restriction enzymes (*Hae*III, *Alu*I, and *Mbo*I). Lines with an enzyme designation indicate the digest of *E. sakazakii* ATCC 29544 (positive control). Sample no. 3 shows the same profile from all three enzymes as the type strain and was confirmed by sequencing as *E. sakazakii*.

Table 4.1. Identification of isolates from stable flies based on analysis of 16SrDNA sequences

Isolate	**Identification**	**Similarity (%)**
Sc 45	*Enterobacter cloacae*	98
Sc 44	*Serratia marcescens*	99
Sc 64	*Enterobacter* spp.	97
Sc 94	*Pantoea agglomerans*	95
Sc 86	*Enterobacter aerogenes*	97
Sc 18	***Enterobacter sakazakii***	99
Sc 97	*Shigella boydii*	98
Sc 91	*Morganella morganii*	99
Sc 108	*Pseudomonas spp.*	97
Sc 61	***Enterobacter sakazakii***	98
Sc 131	*Proteus penneri*	97
Sc 99	*Providencia* spp.	96
Sc 85	*Providencia rettgeri*	99
Sc 121	*Aeromonas* spp.	97
Sc 100	Unidentified	78
Sc 124	*Serratia* spp.	95
Sc 2	*Escherichia coli*	98

CHAPTER 5

Vector competence of stable flies, *Stomoxys calcitrans* (Diptera: Muscidae), for *Enterobacter sakazakii*

Abstract

Enterobacter sakazakii is an opportunistic food-borne pathogen causing meningitis, enterocolitis, and sepsis, primarily in immunocompromised infants. Previously, it was suggested that stable flies, *Stomoxys calcitrans* (L.), are a vector/reservoir of this pathogen. In this study, I assessed a) the vector competence of adult stable flies (SF) for *E. sakazakii*; b) the effect of *E. sakazakii* on SF development and; the c) survival of *E. sakazakii* during SF development and colonization of the digestive tract of newly emerged flies. My data show that the colony of adult SF can maintain *E. sakazakii* for at least 20 days regardless of the food source (blood or sugar) and contaminates the food source. The concentration of the pathogen per individual SF ranged from 1.8×10^5 to 6.4×10^6 CFU/ fly. *E. sakazakii* supported SF development in sterilized cattle manure and sterilized artificial medium with 78.0% and 77.5% SF survival to the adult stage, respectively. *E. sakazakii* also survived SF development and colonized the gut of adult SF, however, only when SF larvae were maintained on sterilized cattle manure inoculated with *E. sakazakii* (12% prevalence in adult SF) and on the sterile artificial medium with *E. sakazakii* (21% prevalence in adult SF). *E. sakazakii* was not recovered (from flies or the substrate) when larvae were reared on cattle manure with a complex microbial community (non-sterilized) with the *E. sakazakii* inoculum. This study shows that SF adults have a potential to carry *E. sakazakii* for an extended period of time. *E. sakazakii* supports SF development; and can survive during SF development and colonize the gut of newly emerged flies.

KEY WORDS: stable fly, *Enterobacter sakazakii*, development, vector, transmission, food - borne pathogen

Introduction

Enterobacter sakazakii is a motile gram-negative bacillus formerly known as yellow-pigmented *Enterobacter cloacae* (Farmer et al. 1980). It can cause neonatal meningitis and sepsis in people with a mortality range of 40 to 80%. In addition, in many cases, survivors suffer from severe neurological disorders and retarded neural development (Farmer et al. 1980, Gurtler et al. 2005). Newborns and full term infants are most susceptible, especially those with predisposing medical conditions (WHO 2004). Reconstituted dry infant formula has been linked to the outbreaks (Lehner and Stephen 2004, Kandhai et al. 2006, Bowen et al. 2006). Moreover, *E. sakazakii* has been isolated from infant incubators, birth canal, and blenders in kitchens where powdered infant formula are reconstituted (Nazarowec-White, M. and J.M. Farber 1997) as well as from different food products including, cheese, meat, vegetables, bread, herbs, and spices (Iversen and S.J. Forsythe 2004) and from the biofilm on enteral feeding tubes (Kim et al. 2006) However, the sources of contamination and the natural reservoir of *E. sakazakii* remain to be identified. Insects have been implicated as a possible vector/reservoir of this pathogen. Kuzina et al. (2001) reported *E. sakazakii* from a laboratory colony of Mexican fruit flies and Hamilton et al. (2003) isolated *E. sakazakii* from laboratory - reared larvae of stable flies (SF). *E. sakazakii* was also isolated from adult SF collected from pastured cattle (Mramba et al. 2006), however, nothing is known about the vector competence of SF and other insects for this pathogen.

SF are blood-feeding insects, they develop in decaying organic substrates such as animal manure (e.g. cattle, horses) and animal feces mixed with straw, soil, hay, silage, or grain (Broce et al. 2005). SF are intermediate hosts for nematodes including, *Setaria cervi,* a parasite of cattle as well as for several *Habronema* spp. - stomach parasites of horses (Greenberg 1973, Zumpt 1973). In addition, SF can transmit *Trypanosoma evansi* that causes fatalities in horses and mules

and serious symptoms in dogs and camels (Harwood and James 1979). Furthermore, SF cause serious economic losses primarily in the cattle and horse industry due to the painful bites affecting animal behavior and they are also an important nuisance factor for people (Broce et al. 2005, Mullens et al. 2006).

In this study the vector competence SF for *E. sakazakii*, effect of *E. sakazakii* on SF development, and survival of this pathogen during SF development were investigated.

Materials and Methods

Vector competence of adult SF for *E. sakazakii:*

Several hundred SF pupae were transferred from the laboratory rearing colony at K-State Entomology Dept. and held in clean cages for eclosion. The cages were maintained at room temperature (25 ± 2^{o}C), 70 ± 10% RH and a photoperiod of 18:6 (L: D). The newly emerged flies were divided into two main groups (appr. 300 flies each) and kept in cages with nylon screening on top to provide access for feeding. One group of flies was fed on bovine citrated blood inoculated with *E. sakazakii* ATTC (3.1 x 10^{7} CFU per ml) while the second group was used as a control (blood without *E. sakazakii*). Blood was offered on saturated sanitary napkins (soaked in blood) and placed on the top screen of the cage. The experimental flies were offered the inoculated blood for 3 hours and then continued feeding the regular blood for 2 days alternating with 2 days of feeding honey water solution (25: 75%). Each bioassay was replicated 4 times. For screening for *E. sakazakii*, 0.1 ml of blood or honey was aseptically squeezed from a sanitary napkin after each feeding (each fly group) and then serial diluted in phosphate buffered saline (PBS) (MP Biomedicals, Aurora, OH), 100 µl spread plated on R & F *Enterobacter sakazakii* chromogenic plating medium (ESCPM) (R&F Laboratories, Downers Grove, IL) and incubated aerobically at 35^{o}C for 24 hrs. Black colonies were counted and re-streaked on

Trypticase Soy Broth Agar (TSBA, Becton Dickinson, Sparks, MD), incubated at 37°C for 24 to 48 hours and individual yellow-pigmented colonies were further identified by PCR

In addition, periodically (3x per week), 10 SF were randomly picked from each group and examined for *E. sakazakii*. The selected flies were surface sterilized (Zurek et al. 2000), individually homogenized and serially diluted in PBS, and plated and cultured as described above. Single black colonies were counted, streaked, and isolated on Trypticase Soy Broth Agar (TSBA, Becton Dickinson, Sparks, MD) and incubated at 37°C for 24 to 48 hours. Individual yellow-pigmented colonies were further analyzed by PCR.

One to three colonies per positive sample were selected for species identification by PCR amplification and sequencing of the 16S rRNA gene. Genomic DNA was extracted by boiling 1µl of the supernatant was used as a template for polymerase chain reaction (PCR) using universal eubacterial primers UF: 5'AGA GTT TGA TYM TGGC 3' and UR: 5' GYT ACC TTG TTA CGA CTT 3'in a total volume of 25µl of a PCR mixture containing 25mM $MgCl_2$ 10x buffer (100mM Tris-HCL pH 8.3, 25mM of dNTP 50 pmol of each primer and 0.25 µl of Taq Gold (Applied Biosystems, Foster City, CA, USA) following the protocol reported previously (Barbieri et al. 2001). The PCR product was purified by the GFX PCR DNA and Gel Band Purification Kit (Amersham Bioscience, UK) according to manufacturer's instruction and visualized by agarose (1%) gel electrophoresis with 0.05 % of ethidium bromide. The type strain of *E. sakazakii* ATCC 29544 was used as a positive control. Sequencing was done using the same eubacterial universal primers used for PCR sequences analyzed and compared with the sequences in the NCBI Gen Bank database using BLAST (Basic Local Alignment Search Tool) (Altschul et al. 1997). Sequences were manually aligned and edited with CodonCode aligner version 1.3.4 (Codon Code Corporation, Dedham, MA. USA).

SF development with and without of *E. sakazakii*:

Stable fly eggs were obtained from the laboratory colony, surface sterilized with 0.05 % sodium hypochlorite and 70% ethanol as described above. Twenty eggs were transferred with a sterile camel hair brush onto individual conical flasks with 40 g of cattle manure mixed with hay at a ratio of 1:2 and 70% moisture. Treatments consisted of non-sterilized mixture of cattle manure and hay inoculated with *E. sakazakii* at a concentration of 4.89 x 10^7 CFU/mg of manure and sterilized cattle manure (autoclaved at 121^{o}C for 30 minutes) inoculated with *E. sakazakii* at a concentration of 5.41 x 10^7 CFU/mg of manure. In addition, Trypticase Soy Egg Yolk Agar (TSEYA) plates were inoculated with *E. sakazakii* at a concentration of 6.42 x10^7 CFU/mg of manure (Watson et al. 1993). The non-sterilized cattle manure, sterilized manure, and TSEYA plates without *E. sakazakii* were used as negative controls (pre-screened for *E. sakazakii*). The conical flasks and the TSEYA plate were incubated at 28^{o}C and monitored for larval pupation, pupal weight (pupae formed within 24 hrs were removed weighed and surface sterilized as described above and placed individually in sterile plastic Petri plates), and adult emergence.

Survival of *E. sakazakii* during SF development:

Newly emerged adult flies were individually homogenized and serially diluted in PBS, and screened for *E. sakazakii* as described above. The number of *E. sakazakii* was recalculated to CFU per SF. The voucher specimens used in this study are deposited in the KSU Museum of Entomological and Prairie Arthropod Research as voucher number 196.

Statistical analysis:

Length of larval development, pupal weight, length of pupal stage, and bacterial counts were analyzed by one-way ANOVA and means were compared using the least square means

(LSMEANS) procedure (P-value = 0.05) (PROC GLM) SAS Institute. Data for the larvae that reached pupation and proportion of pupae that reached adult stage were transformed by the arcsine square root (arcsine√ percent pupation/100 or arcsine square root (arcsine√ percent adult emergence/100 to stabilize error variance.

Results

Vector competence of adult SF for *E. sakazakii*:

Although the number of SF with positive *E. sakazakii* in the inoculated colony declined over time, a high percentage (55%) of SF carried this pathogen at 20 days after the oral inoculation (Table 5:1). No *E. sakazakii* - positive SF were detected in the control colony. Individual SF carried in the digestive tract high concentrations of *E. sakazakii*, ranging from 8.5 x 10^5 to 6.4 x 10^6 CFU per SF. Interestingly, although the prevalence of positive SF declined over time, the concentration of *E. sakazakii* per SF remained relatively constant with the highest concentration detected on day 20 after the exposure (Table 5.1). However, no significant differences in *E. sakazakii* concentration were detected during the screening period (F = 1.68; df = 8 and P = 1.161) (Table 5.1).

SF were capable of contaminating both diets, blood and honey water, during feeding (physical contact was allowed just for the mouth parts) and the concentration of the pathogen was high ranging from 9.9 x 10^4 to 3.6 x 10^6 CFU/ ml of blood and honey water (Table 5.1). PCR amplification and sequencing of 16S rDNA confirmed that the *E. sakazakii* was the strain ATCC 29544.

SF development with and without *E. sakazakii*:

Results showed significant effects of *E. sakazakii* on stable fly development. Length of the larval stage was shortest in the non-sterilized natural substrate (hay and manure mixture)

followed by the artificial medium (TSEYA) with *E. sakazakii*, non-sterilized natural substrate with *E. sakazakii* and sterilized manure with *E. sakazakii* (Table 5.2) (F = 69.33; df = 3; P = 0.0001). Sterilized natural substrate without *E. sakzakii* and sterile TSEYA did not support SF development confirming the necessity of bacteria for SF development (Lysyk et al.1999, Romero et al. 2006) (Table 5.2).

Highest survival of larvae to the pupal stage was observed in non-sterilized natural substrate with *E. sakazakii* (86.6%) and non-sterilized natural substrate (83.3%). Sterilized natural substrate with *E. sakazakii* also supported SF development to pupation (71.6% pupation) (F = 16.39; df = 3, P = 0.0103). (Table 5.2). The heaviest pupae (15.0 mg) were found in the non-sterilized natural substrate inoculated with *E. sakazakii.* Weight of pupae from non-sterilized manure, sterilized manure with *E. sakazakii* and artificial substrate with *E. sakazakii* were not significantly different (F = 4.29; df = 3; P = 0.0077) (Table 5.2).

No significant differences were detected in the length of the pupa stage between pupae from non-sterilized manure with or without *E. sakazakii.* The longest pupal period (8.3 days) was observed in the artificial medium (F = 8.33, df = 3, P = 0.019) (Table 5.2). The highest survival of SF to adult stage was in the non-sterilized natural substrate with and without *E. sakazakii* (83.5 and 81.0%, respectively) followed by that from sterilized natural substrate and TSEYA both with *E. sakazakii* (78.0 and 77.5%) and (F = 16.39, df = 3, P = 0.0103) respectively (Table 5.2).

Survival of *E. sakazakii* during SF development*:*

Result showed that *E. sakazakii* can survive in SF during fly development including pupation and can colonize the digestive tract of newly emerged flies. However, the pathogen was detected in SF adults from sterilized natural substrate (12% prevalence) and artificial substrate (21% prevalence) both with *E. sakazakii* inoculum. No *E. sakazakii* was detected from adult SF

from non-sterilized manure inoculated with *E. sakazakii* and also from control colonies without the inoculum (Table 5.3).

Discussion

This study showed that adult SF have a potential to retain and carry *E. sakazakii* for an extended period of time (at least 20 days after the intial exposure) and can contamine the food source (blood and sugar). Clearly, this study design allowed the cross-contamination of SF in the colony through the common food source that became contaminated during SF feeding. Nevertheless, these results indicate that the digestive tract of SF provides suitable conditions for *E. sakazakii* survival and multiplication considering that the concentration of the pathogen per single SF was very high (8.5×10^5 to 6.4×10^6 CFU/ fly). In addition, *E. sakazakii* alone supported SF development. It has been demonstrated previously that the larval development of SF and other muscoid flies including house flies and face flies strictly depends on a live microbial community in the natural substrate (e.g. manure and other decaying organic substrates) and bacterial isolates greatly differ in the degree into which they support the fly development (Lysyk et al. 1999, Hollis et al. 1985, Zurek et al. 2000, Romero et al. 2006). For SF, it has been shown that not only are specific bacterial strains required for successful SF larval development but the same strains also affect behavior of adult flies and stimulate oviposition (egg laying) of SF females (Romero et al. 2005). This study showed that *E. sakazakii* provides the nutrients or conditions in the artificial and natural media that support SF larval development.

More importantly, from the public health perspective, *E. sakazakii* can survive during SF development and colonize the digestive tract of newly emerged flies. Considering that SF can fly long distances (Broce et al. 2005) and have the potential to contaminate food sources, SF may play an important role in the ecology and dissemination of this human pathogen. On the other

hand, *E. sakazakii* was detected only in SF adults that developed as larvae in the sterilized natural substrate or artificial medium inoculated with *E. sakazakii*. I did not recover *E. sakazakii* from the natural substrate (manure and hay mixture) (and SF developed in this substrate) with the complex microbial community indicating that *E. sakazakii* did not compete well with other microbes in this substrate. However, it is important to keep in mind that the *E. sakazakii* type strain (ATCC 29544) has been kept on artificial bacterial media for a long time and was only artificially added to the mixture of manure and hay and therefore this design does not represent the natural conditions. Wild strains of *E. sakazakii* isolated directly from the environment may be better adapted to the conditions in substrates with large and diverse microbial communities.

The natural reservoir of *E. sakazakii* is not known. It has been isolated from the reconstituted dry infant formula that has been linked to the outbreaks of newborns and full term infants (Lehner and Stephen 2004, Bowen et al. 2006, Kandhai et al. 2006). In addition, *E. sakazakii* has been reported from birth canal, infant incubators, (Nazarowec-White M. and J.M. Farber 1997) as well as from different food products including, cheese, meat, vegetables, bread, herbs, and spices (Iversen and Forsythe 2004, Soriano et al. 2001, Jung et al. 2006). Kim et al. (2006) reported that *E. sakazakii* can form a biofilm on enteral feeding tubes and stainless steel (Kim et al. 2006).

Previously, *E. sakazakii* was isolated from the larvae of SF in laboratory colony (Hamilton et al. 2003) as well as from wild SF (Mramba et al. 2006). SF can build very large populations around domestic animals, usually cattle and horses. It is likely that the microbes in the stable fly gut and on the body surface originate from animal manure where SF larvae developed. This indicates that the natural reservoir of *E. sakazakii* is the digestive tract and/or feces of domestic

animals including cattle and horses, and future studies should focus on examination of these habitats as reservoirs of *E. sakazakii*.

In conclusion, this study assessed the vector competence of SF for *E. sakazakii*. It showed that adult SF can potentially be good vectors for *E. sakazakii*; SF can contaminate their food sources with this pathogen and *E. sakazakii* supports SF development and can colonize the gut of newly emerged flies.

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References cited

Altschul, S.F., W. Gish, W.E., W. Muller, and D.J. Leipman. 1997. Basic local alignment search tool. J. Mol. Biol. 215: 403- 410.

Barbieri, E., B.J. Paster, D. Hughes, L. Zurek, D.P. Moser, A. Teske, M. L. Sogin. 2001. Phylogenetic characterization of epibiotic bacteria in the accessory nidamental glands and egg of the squid *Loligo pealei* (Cephalopoda:Loliginidae). Environ. Microbiol. 3: 151-67.

Broce, A.B., J. Hogsette, and S. Paisley. 2005. Winter feeding sites of hay in round bales as major developmental sites of *Stomoxys calcitrans* (Diptera: Muscidae) in pastures in spring and summer. J. Econ. Entomol. 98: 2307-2312.

Bowen, A.B., C.R. Braden. 2006. Invasive *Enterobacter sakazakii* Diseases in Infants. Emerg. Inf. Dis. 12: 8-2

Espinoza-Fuentes, F. P., and W.R. Terra. 1987. Physiological adaptations for digesting bacteria Water fluxes and distribution of digestive enzymes in *Musca domestica* larval midgut. Ins. Biochem. 17: 809-817.

Farmer, J.J. III. M.A. Asbury, F.W., Hickman, D.J., Brenner, and The Enterobacteriaceae Study Group. 1980. *Enterobacter sakazakii*: A new species of Enterobacteriaceae isolated from clinical species. Int. J. Syst. Bacteriol. 30: 569-584.

Greenberg, B. 1973. Flies and disease. Vol. II. Ecology, Classification and Biotic Associations. Princeton Univ. Press, Princeton

Gurtler, J.B., J.L. Konacki,, L.R. Beuchat. 2005. *Enterobacter sakazakii*. A coliform of increased concern of infant health. Int. J.Food Microbiol. 104:1-34

Hamilton, J.V., M. J. Lehane, and H.R. Braig. 2003. Isolation of *Enterobacter sakazakii* from the midgut of *Stomoxys calcitrans*. Emerg. Infect. Dis. 9: 1355-1356.

Harwood, R.F. and M.T. James. 1979. Entomology in human and animal health. Mac Millan Publishing, New York.

Hollis, J.H., F.W. Knapp, and K.A. Dawson. 1985. Influence of bacteria within bovine feces on the development of the face fly (Diptera: Muscidae). Environ. Entomol. 14: 568-571.

Iversen, C. and S. J. Forsythe 2004. Isolation of *Enterobacter sakazakii* and other Enterobacteriaceae from powdered infant formula and related products. J. Food Microbiol. 21: 771-776.

Jung, M.K., and J.H. Park. 2006. Survival of *Enterobacter sakazakii* on fresh produce as affected by temperature, and effectiveness of sanitizers for its elimination. *Int. J.* Microbiol. 1:134-143

Kandhai, M.C., M.W. Reij, C. Grognou, M. Van Schothorst, L.G. M.Gorris and M.H. Zwietering, 2006. Effects of preculturing conditions on Lag time and Specific Growth Rate of *Enterobacter sakazakii* in reconstituted Powdered Infant Formula. App. Environ. Microbiol.72: 2721-2729.

Kim, H., J.H., Ryu, L.R. Beuchat. 2006. Attachment of and biofilm formation by *Enterobacter sakazakii* on stainless steel and enteral feeding tubes. Appl. Environ. Microbiol. 72:5846-5856.

Kuzina, L.V., J.J. Peloquin, D.C. Vacek, and T.A. Miller. 2001. Isolation and Identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens* (Diptera: Tephritidae). Curr. Microbiol.42: 290-294.

Lehner, A. and R. Stephan. 2004. Microbiological, epidemiological, and food safety aspects of *Enterobacter sakazakii*. J. Food. Prot. 67: 2850-2857.

Lysyk, T. J., L Kalischuk-Tymensen, L.B. Selinger, R.C Lancaster., L.Wever, and K. J. Cheng. 1999. Rearing stable flies larvae (Diptera: Muscidae) on an egg Yolk medium. J. Med. Entomol. 36: 382-388.

Mramba, F., A. Broce, and L. Zurek. 2006. Isolation of *Enterobacter sakazakii* from stable flies, *Stomoxys calcitrans* L. (Diptera: Muscidae). J. Food Prot., 69: 671-673.

Mullens, B.A., K.S. Lii, J.A. Mao, N.G. Meyer,. 2006. Behavioral responses of dairy cattle to the stable fly, *Stomoxys calcitrans* in an open field environment. Med. Vet. Entomol. 20:122-137.

Nazarowec- White, M. and J. M. Farber. 1997. *Enterobacter sakazakii:* a review. Int. J. Food. Microbiol. 34:103 - 113.

Romero, A., A. Broce, and L. Zurek. 2006. Role of bacteria in oviposition behaviour and larval development of stable flies. Med. Vet. Entomol. 20: 115-121.

SAS Institute. 2003. SAS online Doc® 9.1. Cary, NC: SAS Institute Inc.

Soriano, J. M., H. Rico, . J. C. Molt, and J. Manes, 2001. Survival and growth of *Enterobacter sakazakii* on fresh-cut fruits and vegetables and in unpasteurized juices as affected by storage temperature J. Food Prot. 68: 2541-2552.

Watson, D., W. Martin, and E.T. Schmidtmann. 1993. Egg yolk and bacteria growth medium for Musca domestica (Diptera: Muscidae). J. Med. Entomol.30: 820-823.

World Health Organisation, 2004. *Enterobacter sakazakii* and other microorganism in powdered infant formula: meeting report. Microbiological risk assessment series, no 6. (Online) http://www.who.int/food safety/publications/micro/mra6/en.

Zumpt, .F. 1973. The Stomoxyine biting flies of the world. Gustav Fischer Verlag. Stuttgart. 137-152.

Zurek, L., C. Schal, and D.W. Watson, 2000. Diversity and contribution of the intestinal bacterial community to the development of *Musca domestica* (Diptera: Muscidae) larvae. J. Med. Entomol. 37: 924-928.

Table 5.1.Temporal prevalence of *E. sakazakii* in a colony of adult stable flies

Day after exposure	No. Positive/Total (%)	CFU / SF* (mean ± SEM)	Diet	CFU / ml of diet
1	36/40 (90)	$1.0 \pm 0.3 \times 10^{6}$ [a]	Blood	2.0×10^{6}
3	32/40 (80)	$2.3 \pm 0.9 \times 10^{6}$ [a]	Honey	1.6×10^{6}
5	30/40 (75)	$1.8 \pm 0.1 \times 10^{5}$ [a]	Blood	3.6×10^{6}
8	30/40 (75)	$8.5 \pm 3.5 \times 10^{5}$ [a]	Honey	3.0×10^{5}
10	28/40 (70)	$2.6 \pm 0.7 \times 10^{6}$ [a]	Blood	8.9×10^{5}
12	30/40 (75)	$1.3 \pm 0.3 \times 10^{6}$ [a]	Honey	2.2×10^{6}
15	26/40 (65)	$1.9 \pm 0.4 \times 10^{6}$ [a]	Blood	9.9×10^{4}
18	24/40 (60)	$1.0 \pm 0.3 \times 10^{6}$ [a]	Honey	5.9×10^{5}
20	22/40 (55)	$6.4 \pm 0.2 \times 10^{6}$ [a]	Blood	9.9×10^{5}

Numbers in the same column followed by different letters are significant different ($P < 0.05$)
* Colony forming units per stable fly

Table 5. 2. Developmental parameters (mean ± SEM^) of stable flies reared with and without *Enterobacter sakazakii*

Substrate/bacteria	Length of larval stage (days)	Pupation %	Pupal weight (mg)	Length of pupa stage (days)	Adult emergence (%)
With *E. sakazakii*					
Non-sterilized manure	12.7 ± 0.33^{b}	86.6 ± 5.0^{a}	15.0 ± 0.3^{a}	7.7 ± 0.31^{ab}	83.5 ± 2.45^{a}
Sterilized manure	14.7 ± 0.34^{a}	71.6 ± 2.6^{b}	10.5 ± 0.3^{b}	6.7 ± 0.33^{b}	78.0 ± 1.01^{b}
TSEYA*	10.7 ± 0.33^{c}	81.6 ± 3.5^{a}	10.0 ± 0.4^{b}	8.3 ± 0.34^{a}	77.5 ± 2.50^{b}
Control	9.3 ± 0.35^{d}	83.3 ± 4.60^{a}	11.5 ± 1.8^{ab}	6.7 ± 0.32^{b}	81.0 ± 1.0^{a}
Non-sterilized manure	NA[#]	NA	NA	NA	NA
Sterilized manure					
TSEYA	NA	NA	NA	NA	NA

Numbers in the same column followed by different letters are significantly different ($P < 0.05$)

* Trypticase Soy Egg Yolk Agar

[#] Not applicable

^ Standard error of mean

Table 5. 3. Survival of *E. sakazakii* during stable fly development: Prevalence in adult flies.

Medium	No. positive/total (%)	Concentration CFU/fly
With *E. sakazakii*		
Non-sterilized manure	0/57	0.0
Sterilized manure	6/52 (12%)	2.96×10^4
TSEYA*	10/48 (21%)	1.36×10^5
Control		
Non-sterilized manure	0/56	0.0
Sterilized manure	N.A.[#]	N.A.
TSEYA	N.A.	N.A.

* Trypticase Soy Egg Yolk Agar

[#] Not applicable

Summary and conclusions

The stable fly, *Stomoxys calcitrans,* is a blood feeding dipteran insect that preys primarily on cattle and horses. It is the most important insect pest of confined, as well as pastured, livestock in the U.S.A. Currently, there is no effective method for controlling this pest in pastures. Control in confined operations relies heavily on the use of chemical insecticides, which might eventually result in the development of resistant fly populations. Controlling stable flies (SF) at the immature stage could be a more efficient way than removal of adult individuals. In particular, the use of oviposition stimuli at suitable locations in the fly's habitat should offer new possibilities for an environmentally safe strategy against this pest.

The first study examined the effect of oviposited eggs (fresh and old), and the associated microbial community upon subsequent oviposition by females in a series of choice tests. Results showed that SF prefers to oviposit in the vicinity of freshly laid eggs (2 h old maximum). It was also shown that whatever the oviposition stimulus from eggs might be, it is hexane-soluble and that it can be removed by egg surface sterilization with ethanol and sodium hypochlorite. In contrast, 24 h old eggs do not enhance oviposition; in fact, SF actually prefers to oviposit on substrates with no eggs rather than on substrates with older eggs. Bioassays designed to examine the species-specificity of the above results demonstrated that oviposition attractants/stimulants on the surface of SF eggs are in fact species-specific. SF laid significantly more eggs on substrates with extracts from SF fresh eggs than on eggs of house flies (HF). When given a choice between substrates with HF eggs and no eggs, SF oviposited significantly more on substrates with no eggs. The same scenario was true in the converse experiment using SF eggs and gravid house flies.

The concentration of bacteria isolated from the surface of old eggs was 10 fold (10^6 CFU per egg) higher than that from the surface of fresh eggs (10^5 CFU per egg). The increase of bacteria in old eggs may mediate inhibition of oviposition to prevent further oviposition so that stable flies offspring are not subjected to competition. This hypothesis was supported by results of our bioassays using different concentrations of bacteria on the surface of sterilized eggs, which show that low ($\leq 10^5$ CFU per egg) and high concentrations ($10^9 \geq$ CFU per egg) of bacteria do not stimulate oviposition. The bacterium *Staphylococcus saprophyticus* was isolated from the surface of SF eggs, identified, and characterized. It was demonstrated that *S. saprophyticus* stimulated SF oviposition when used as the inoculum on surface-sterilized SF eggs or on a sterile substrate (moist black cloth), suggesting that neither the substrate (manure) nor the eggs were required to stimulate SF to oviposit. The collection, isolation and identification of the active semiochemical compounds emanating from SF fresh eggs and from *S. saprophyticus* cultures should be the next steps taken towards the use of these chemicals in strategies for managing SF populations.

The second objective of this study aimed to determine the behaviors responsible for the observed aggregating/segregating distribution of immature SF and HF over seemingly homogeneous larval habitats. The spatial distribution of 3rd instar larvae of each species after they were placed on the arena when eggs or 2nd instars as clumps or evenly distributed over the arena confirmed that populations of third instar SF and HF are aggregated separately with minimal overlapping, possibly to maintain coexistence by reducing inter-specific competition. This spatial distribution was achieved regardless of whether they started in clumps (due to oviposition behavior) or evenly distributed. However, the degree of aggregation and segregation

was more pronounced in larvae that started as clumped eggs or larvae than in those that started distributed evenly over the medium.

Larval aggregation may be another behavior for overcoming adverse environmental conditions such as parasitism or predation by other organisms or a means for modifying the larval medium into a more suitable larval habitat.

Aggregation may be common to species of insects such as houseflies and stable flies, which exploit the same developmental habitat. Results showed that aggregation of the larvae of these two species are species specific. Stable flies laid significantly more eggs on substrates with stable fly eggs than onto substrates with house fly eggs. The same scenario was true for house flies; they clearly preferred to lay eggs onto substrates with house fly eggs than that with stable fly eggs or no eggs. Additionally, oviposition was preferentially along the edges of the substrate; this behavior is also observed in the field as they oviposit mostly along fence lines, areas behind feeding aprons and at the edge of mounds. Larvae of both species aggregated separately, with the majority aggregating along the edges of the pans.

The information gained during this study on the aggregation/segregation of larvae of these species should be applicable to the design of strategies for their control. Sampling for immatures as a component of integrated pest management programs will certainly use this information on their species-specific spatial distribution. But to get to that point, more research will be required in identifying species-specific chemical cues involved in intraspecific recognition.

Because stable flies are hematophagous as adults, they have been implicated as vectors of a few pathogens. Their potential vectoring capability might be enhanced by their development in animal feces. The third objective evaluated the association of SF and the bacterium *Enterobacter*

sakazakii, an emerging human pathogen. Results of this study showed that wild stable flies have a potential to carry this bacterium and demonstrated the significance of stable fly as a vector of *E. sakazakii* by showing that they could retain *E. sakazakii* for a period of up to 20 days after the intial exposure and could still contaminate a food source. This study also showed that the digestive tract of stable flies does provide suitable conditions for the survival and multiplication of *E. sakazakii*. This was demonstrated by the high concentration of this pathogen per stable fly. Since stable flies can build very large populations around domestic animals, usually cattle and horses, it is likely that the microbes in the stable fly gut and on the body surface originate from animal manure where stable fly larvae develop. Because stable flies can fly long distances and have the potential to contaminate the feeding source, it is possible to conclude that stable flies may play an important role in the ecology and dissemination of this human pathogen. However, other manure-inhabitants such as house flies are also likely playing a role as a vector of this pathogen due to their mode of feeding (regurgitation) and attraction to residential areas.