The procurement, transmission, and abundance of bacteria in and among house flies (*Musca domestica* L.)

by

Jessica Lynne Thomson

B.S., Kansas State University, 2014

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Entomology
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2019

Abstract

Microorganisms are a necessary component of house fly (*Musca domestica* L.) development. Animal manure and urban garbage are rich in microbes and may include human pathogens such as *Salmonella enterica* serovar Typhimurium. *S.* Typhimurium is a common food-borne pathogen shed by livestock which can potentially be vectored by adult house flies. Because the interaction of male and female house flies with cattle manure may differ due to differences in sex-specific nutritional requirements and behavior, the first objective of this dissertation was to determine if male and female house flies differ in bacteria acquired over time from cattle manure in the presence and absence of an alternative food source. Mated male and female adult house flies were exposed to cattle manure inoculated with either *S.* Typhimurium or non-pathogenic *Escherichia coli* in the presence (assay with both manure and sugar water provided) and absence (assay with manure only provided) of an alternative food source. Overall, female flies harbored more bacteria than males after exposure to *S.* Typhimurium or *E.* coli inoculated cattle manure; however, differences in abundance were only significant at 4 h and 12 h time points. Male and female house flies only differed in number of colony forming units (CFU) of *E. coli* at 4 h and 12 h time points in assays when both manure and sugar were provided likely due to sex-specific nutritional and behavioral differences; however, they differed in CFU of *S.* Typhimurium at 4 h and 12 h time points in both manure assays. Observations of the fly alimentary canal from manure-sugar assays supported these initial differences especially at 4 h where females held manure and fly food, while males held only sugar water. The initial differences in CFU *S.* Typhimurium at early time points regardless of the presence or absence of sugar may also have been attributed to differences in *S.* Typhimurium excretion rates. Because *S.* Typhimurium can survive and grow on fruits, such as cantaloupe, the second objective was to

determine if house flies can transfer *S.* Typhimurium to and from cantaloupe and if cantaloupe facilitates the transfer of *S.* Typhimurium between flies. Adult female house flies (mated, 5-7 days old) were given *ad libitum* sugar water and were exposed to manure inoculated with sterile PBS (ST-) or *S.* Typhimurium (ST+) for 12 h. After manure exposure, to test for survival of *S.* Typhimurium, the ST+ flies were placed individually in empty jars and bacterial abundance was monitored over 24 h. To monitor the transmission of *S.* Typhimurium for 24 h after manure exposure, the flies were placed into jars containing either (1) a single ST+ fly with fresh cantaloupe (fly to food transmission), (2) four ST- flies with *S.* Typhimurium-inoculated cantaloupe (food to fly transmission), or (3) a single ST+ fly with four ST- flies with or without fresh cantaloupe (fly to fly transmission, with or without food). In all experiments, flies and cantaloupe (if present) were processed and cultured at 0, 6, 12, and 24 h after experimental setup for GFP *S.* Typhimurium abundance. *S.* Typhimurium survived in ST+ flies but decreased in total abundance over time even with cantaloupe present indicating that the flies were digesting the *S.* Typhimurium. The abundance of *S.* Typhimurium increased in both, inoculated cantaloupe and ST- flies, over time indicating that the *S.* Typhimurium was growing in the cantaloupe and flies were picking up *S.* Typhimurium from the cantaloupe. Additionally, in fly to fly transmission experiments, more ST- flies were positive for *S.* Typhimurium when cantaloupe was present. Therefore, presence of a shared food source likely facilitates fly-to-fly transfer. Because house flies have sex-specific behavioral and nutritional requirements and bacteria and coliform abundance differs across habitats, the final objective was to determine if house fly sex and habitat affect bacterial abundance and coliform abundance in house flies. Male and female house flies were collected from 3 different habitats (urban, sub-urban, agricultural) to determine if fly sex and location affected the total bacterial abundance and coliform abundance.

Overall, house flies collected from the sub-urban site had the greatest mean CFU/fly of bacteria possibly due to males and females having equal access to microbe-rich substrates, while house flies collected from the urban site had the lowest mean CFU/fly of coliforms likely due to there being no immediate access to animal waste. Females were consistently greater in the mean CFU/fly of bacteria and coliforms than males within all sites, except for at the sub-urban site. Furthermore, females did not differ across sites in mean CFU/fly of bacteria and coliforms, most likely a result from females spending more time interacting with microbe-rich substrates, while males were highest at the sub-urban sites and lowest at the urban site suggesting that differences in male activity likely drive differences in bacterial and coliform abundance across habitats. Therefore, house fly sex and habitat affect the mean bacterial and coliform abundance in house flies. Results from all three objectives indicate that because house flies can acquire, harbor, and transmit *S.* Typhimurium to other flies in the presence of a food source, they have vector competence for *S.* Typhimurium. Furthermore, fly sex, habitat, and food source all should be considered in fly pest management programs to effectively reduce abundance of bacteria in and dissemination of pathogens by house flies, since house flies are key players in food safety and human and animal health.

The procurement, transmission, and abundance of bacteria in and among house flies (*Musca domestica* L.)

by

Jessica Lynne Thomson

B.S., Kansas State University, 2014

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Entomology
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2019

Approved by:
Major Professor
Yoonseong Park

Copyright

Abstract

Microorganisms are a necessary component of house fly (*Musca domestica* L.) development. Animal manure and urban garbage are rich in microbes and may include human pathogens such as *Salmonella enterica* serovar Typhimurium. *S.* Typhimurium is a common food-borne pathogen shed by livestock which can potentially be vectored by adult house flies. Because the interaction of male and female house flies with cattle manure may differ due to differences in sex-specific nutritional requirements and behavior, the first objective of this dissertation was to determine if male and female house flies differ in bacteria acquired over time from cattle manure in the presence and absence of an alternative food source. Mated male and female adult house flies were exposed to cattle manure inoculated with either *S.* Typhimurium or non-pathogenic *Escherichia coli* in the presence (assay with both manure and sugar water provided) and absence (assay with manure only provided) of an alternative food source. Overall, female flies harbored more bacteria than males after exposure to *S.* Typhimurium or *E.* coli inoculated cattle manure; however, differences in abundance were only significant at 4 h and 12 h time points. Male and female house flies only differed in number of colony forming units (CFU) of *E. coli* at 4 h and 12 h time points in assays when both manure and sugar were provided likely due to sex-specific nutritional and behavioral differences; however, they differed in CFU of *S.* Typhimurium at 4 h and 12 h time points in both manure assays. Observations of the fly alimentary canal from manure-sugar assays supported these initial differences especially at 4 h where females held manure and fly food, while males held only sugar water. The initial differences in CFU *S.* Typhimurium at early time points regardless of the presence or absence of sugar may also have been attributed to differences in *S.* Typhimurium excretion rates. Because *S.* Typhimurium can survive and grow on fruits, such as cantaloupe, the second objective was to

determine if house flies can transfer *S.* Typhimurium to and from cantaloupe and if cantaloupe facilitates the transfer of *S.* Typhimurium between flies. Adult female house flies (mated, 5-7 days old) were given *ad libitum* sugar water and were exposed to manure inoculated with sterile PBS (ST-) or *S.* Typhimurium (ST+) for 12 h. After manure exposure, to test for survival of *S.* Typhimurium, the ST+ flies were placed individually in empty jars and bacterial abundance was monitored over 24 h. To monitor the transmission of *S.* Typhimurium for 24 h after manure exposure, the flies were placed into jars containing either (1) a single ST+ fly with fresh cantaloupe (fly to food transmission), (2) four ST- flies with *S.* Typhimurium-inoculated cantaloupe (food to fly transmission), or (3) a single ST+ fly with four ST- flies with or without fresh cantaloupe (fly to fly transmission, with or without food). In all experiments, flies and cantaloupe (if present) were processed and cultured at 0, 6, 12, and 24 h after experimental setup for GFP *S.* Typhimurium abundance. *S.* Typhimurium survived in ST+ flies but decreased in total abundance over time even with cantaloupe present indicating that the flies were digesting the *S.* Typhimurium. The abundance of *S.* Typhimurium increased in both, inoculated cantaloupe and ST- flies, over time indicating that the *S.* Typhimurium was growing in the cantaloupe and flies were picking up *S.* Typhimurium from the cantaloupe. Additionally, in fly to fly transmission experiments, more ST- flies were positive for *S.* Typhimurium when cantaloupe was present. Therefore, presence of a shared food source likely facilitates fly-to-fly transfer. Because house flies have sex-specific behavioral and nutritional requirements and bacteria and coliform abundance differs across habitats, the final objective was to determine if house fly sex and habitat affect bacterial abundance and coliform abundance in house flies. Male and female house flies were collected from 3 different habitats (urban, sub-urban, agricultural) to determine if fly sex and location affected the total bacterial abundance and coliform abundance.

Overall, house flies collected from the sub-urban site had the greatest mean CFU/fly of bacteria possibly due to males and females having equal access to microbe-rich substrates, while house flies collected from the urban site had the lowest mean CFU/fly of coliforms likely due to there being no immediate access to animal waste. Females were consistently greater in the mean CFU/fly of bacteria and coliforms than males within all sites, except for at the sub-urban site. Furthermore, females did not differ across sites in mean CFU/fly of bacteria and coliforms, most likely a result from females spending more time interacting with microbe-rich substrates, while males were highest at the sub-urban sites and lowest at the urban site suggesting that differences in male activity likely drive differences in bacterial and coliform abundance across habitats. Therefore, house fly sex and habitat affect the mean bacterial and coliform abundance in house flies. Results from all three objectives indicate that because house flies can acquire, harbor, and transmit *S.* Typhimurium to other flies in the presence of a food source, they have vector competence for *S.* Typhimurium. Furthermore, fly sex, habitat, and food source all should be considered in fly pest management programs to effectively reduce abundance of bacteria in and dissemination of pathogens by house flies, since house flies are key players in food safety and human and animal health.

Table of Contents

List of Figures

List of Tables

Acknowledgements

I would like to thank my co-advisor, Dr. Dana Nayduch, and former major advisor, Dr. Ludek Zurek, for their patience, guidance, time, and support. I would also like to thank Dr. Yoonseong Park for stepping up close to the end of my program to take on the role of my official major advisor. I truly cannot thank you all enough. Thank you to both my committee members, Dr. Berlin Londono and Dr. Natalia Cernicchiaro, for providing me with the resources needed to make future employment connections and for guidance on analyzing my data for this dissertation, respectively, and to Dr. Rollie Clem for representing the graduate school as the outside chairperson on my committee. I also would like to sincerely thank Dr. John Ruberson and Dr. Brian McCornack for their unwavering support, guidance, advice, and encouragement throughout my program. Thank you both for always having an "open door policy", being there for me, and believing in my success even at the times when I did not. Thank you to all my former lab mates who helped me to complete my experiments. I would also like to thank all my friends and fellow graduate student colleagues for their ongoing support and companionship. They know who they are. I'm so glad I had you to hang out with, cry with, and/or laugh with during the not so great days. Thank you to my snapchat "bestie", Andrew Woolley. We reconnected at just the right time and you have been the perfect "good kind of distraction" during this final year of my program. I'm so thankful to have you in my life and can't wait to see what the future holds. Thank you to my brother, Anthony, for always making me laugh. I seriously could not have made it through last summer without you and all the fun adventures we had. I especially enjoyed our long walks through Konza Prairie and working out together a few days each week. You truly kept me going. Also, thank you to both sets of grandparents and my church family back home. Your prayers and support have been much appreciated. From the bottom of my heart, thank you

to my best friend in the world, Dinesh Erram. Nesh, no one will ever be able to understand and/or relate to me as well as you. We stuck it through this rough journey together and I don't know what I would have done without you. Thank you for always being my #1 fan from the very beginning of my program, for the late-night pep talks, for being my "calming stone", and for supporting and encouraging me all the way through to the end. We finally did it! We both have our doctorates! Finally, this last acknowledgement is specifically for my Grandma Joan. You have always been an inspiration to me and I'm so glad I could finally reach the milestone in academia that both you and I have always dreamed of achieving. Thank you for always encouraging me, sending me sweet letters and texts, and for believing in me.

Dedication

I dedicate this entire dissertation to my strong, beautiful and kind parents. Without their unwavering support and optimism none of this would have been possible. Thank you for always loving me, admiring the moon with me each night, and bringing a smile to my face even when I was far away. I love you both forever and always!

Chapter 1 - Introduction

The common house fly (*Musca domestica* L.), belongs to the family Muscidae and is generally referred to as a filth fly. Filth flies get their name from the filth they develop in which consists of a variety of organic substrates such as sewage, manure, rotting food, animal feed, or soiled animal litter (Moon 2019). Organic substrates suitable for filth fly survival and development are found in both urban and agricultural habitats. There are about 9,000 species of true flies in the family Muscidae which make up about 190 genera worldwide (Moon 2019). House flies belong to the subfamily Muscinae which also includes other commonly known filth flies, many of which are important veterinary pests: stable flies (*Stomoxys calcitrans*), horn flies (*Haematobia irritans)*, face flies (*Musca autumnalis*), and sweat flies (*Hydrotaea* spp.) (Moon 2019). Of the filth flies, house flies are considered important to both human and animal health because of their association with microbe-rich substrates, synanthropic nature and ability to thrive in a multitude of environments.

Worldwide, house flies are found in habitats where they coexist with humans (West 1951, Nayduch and Burrus 2017), especially when animals are nearby. A partial list of where house flies have been collected, surveyed, or studied in urban and agricultural habitats is given in Table 1.1. House flies can travel between agricultural and urban areas and therefore serve as a bridge for pathogens between animal and human environments (Greenberg 1971, 1973, Chakrabarti et al. 2010, Zurek and Ghosh 2014, Nayduch and Burrus 2017). Interestingly, the general dispersal distance for house flies is 3 km, but some studies have shown they can travel distances of up to 30 km (Baldacchino et al. 2017).

Life cycle

The house fly has a holometabolous life cycle, meaning it goes through drastic changes in morphology during its life cycle with four life stages: egg, larvae, pupae, and adult (Service 2012, Moon 2019). Four to eight days after mating, adult females deposit eggs either singly or in batches by extending their ovipositor directly into a substrate (West 1951). Up to 1000 eggs total, or 4-6 clutches (gonotrophic cycles), each with approximately 120-150 eggs per clutch, can be laid by one female adult in her lifetime (West 1951, LaBrecque et al. 1972). House fly eggs have a pearly white color, are oval shaped, and are about 1mm in total length. Eggs hatch within 12-18 h after oviposition and the newly emerged larvae then undergo 3 larval instars, molting twice.

The time flies spend in the larval stage is highly variable in length, due to factors such as temperature and larval substrate overcrowding. For example, Hogsette and Farkas (2000) stated that the larval stage only takes 3-4 days, while West (1951) stated that the first instar larval stage itself can take anywhere from 20 hours to 4 days before the first molt. The second instar stage requires 1-3 days before the second molt and then the third instar larvae will feed anywhere from 3-9 days before seeking a dry and cool place to begin pupation (West 1951). To commence pupation, the larva will contract itself and separate from its own integument, which will darken and harden to become the pupal case (puparium) (West 1951). Moon and Meyer (1985) stated that the pupal stage can last 3-10 days and within the first 48 hours most structures necessary for the adult fly are developed. Pupation is also temperature-dependent, and the length of this stage is inversely proportional to temperature. House flies can overwinter in several different stages. House flies most likely overwinter as immature stages (e.g. larvae or pupae; Dove 1916, Hewitt 2011), although adult house flies can overwinter in barns and other indoor structures as adults in

more temperate climates (Matthysse 1945). Overall, fly development from egg to adult ranges from 8-14 days, depending on the temperature and relative humidity (West 1951). More specifically, house fly development is faster with higher temperatures and lower humidity levels in the environment and slower with lower temperatures and higher humidity levels in the environment (Ngoen-klan et al. 2011).

Adult flies live for 10-14 days in the field (Hogsette and Farkas 2000). In order to eclose from the puparium, house flies break open the anterior end of the pupal case by inflating their ptilinum, a sac that expands out from the front part of the head above the antennae base, and then crawl outside leaving behind the nymphal sheath within the empty puparium (West 1951). Sclerotization or "tanning" of the adult cuticle and wing expansion occurs within the first couple hours after eclosion (Andersen 2012). An adult house fly is grayish black in color with black vertical stripes along the dorsal side of the thorax and measures 4-12 mm in total body length (West 1951, Baldacchino et al. 2017, Moon 2019). Eyes of adult males are narrowly spaced while adult females have a wider space between their eyes. House flies have 2 antennae on the head each consisting of 3 segments, with the longest region at the tip, called the arista, being the most prominent. The arista is a sensory organ used by flies to detect changes in temperature and moisture. (Service 2012). House flies also have sponging mouthparts which form a proboscis with a fleshy labellum at the end which is used to pull liquids up through the fine channels that line it called pseudotracheae (Service 2012). Nutrition and substrate population density during larval development causes variation in adult size. For example, reduced nutrient availability and increased larval population density in substrate negatively affects larval development and results in a decrease in mature adult size, while an increase in nutrient availability and decrease in larval

population density in substrate positively affects larval development and results in an increase in mature adult size (West 1951, Black and Krafsur 1985).

Mating and Oviposition

Water is a necessary component of the house fly diet that they cannot survive without for more than 48 h (Lodge 1918) and food is a necessary resource required by adults to maintain body processes and provide for complete maturation of sex organs. In particular, sugars or soluble starch are necessary for house fly longevity and protein or peptone is necessary for the maturation of the female ovaries and for production and oviposition of eggs, as females are anautogenous (Glaser 1923, 1924, Kobayashi 1934). After eclosion, males will not mate for at least 16 h and females will not mate for at least 24 h (Murvosh et al. 1964, Riemann et al. 1967). To initiate mating, a male will seize a female and remain on top facing the same direction in a vertical position (Lamb and Gardiner 1922). A permissive female will extend her ovipositor to contact the male genital atrium and the male and female genitals will remain held in close contact long enough to allow the spermatozoa to leave the male and enter the female where the sperm will then be stored in the spermatheca for future egg fertilization (West 1951, Murvosh et al. 1964). Murvosh et al. (1964) reported that mating occurs for 44-96 minutes while Sacca and Benetti (1960) reported mating can last as long as 136 minutes. However, complete sperm transfer from male into the female spermathecae only requires 10 minutes or less (Murvosh et al. 1964). A female that has already been inseminated will reject any future mating attempts by males since sperm is already stored in the spermatheca for future egg fertilization.

Oviposition occurs 4-8 days after mating. Oviposition substrates consist of decomposing organic matter such as spilled feed, haylage (Meyer and Petersen 1983), piles of manure (Meyer and Petersen 1983, Lysyk 1993a), decaying crops (Cook et al. 1999), and soiled bedding

(Schmidtmann 1988). Substrate odor is an important signal for oviposition behavior (De Bruyne and Baker 2008, Shah et al. 2016). In addition to its odor, manure serves as a suitable substrate for house fly oviposition because it provides a rich source of protein and other essential nutrients needed for larval development (Spiller 1964, Hanski 1987). House flies are most attracted to cattle manure odor (Ascher 1958, Larsen et al. 1966) and show oviposition preference for cattle manure over dung or manure from calves, horses, dogs, humans, chickens, swine, goats, donkeys, and sheep (Larsen et al. 1966, Larraín and Salas 2008, Shah et al. 2016). However, the eventual acceptance or rejection of a potential oviposition substrate by females relies on the contact cues from substrate, not just substrate odor (Lam 2010). Poultry manure was considered a suitable substrate for house fly oviposition because it increased house fly mating behavior, mating length, and fecundity (Larraín and Salas 2008, Khan et al. 2012). Poultry manure has a lower carbon/nitrogen ratio which enhances microbial activity in larval substrates (Moon et al. 2001).

Interestingly, adult female house flies prefer to oviposit near other fresh conspecific eggs (Lam 2010) because larvae closer in age help to warm and moisten the organic material (Bryant 1970, Barnard and Geden 1993) and inhibit the growth of competitive fungi (Zvereva 1986). Furthermore, Lam (2010) found that egg-associated bacteria isolated from house fly eggs were responsible for house fly oviposition behavior and suggested that it was the alkyl disulfide volatiles produced by the bacteria that attracted the flies. However, once a certain threshold density of egg-associated bacteria was reached on substrate, oviposition was inhibited. Specifically, *Klebsiella oxytoca* were responsible for the detection of egg age by other gravid females (Lam et al. 2007) and deterred gravid females from oviposition. Bacteria are also left behind on the egg surface by adult female house flies after oviposition to provide nutrition for fly

larvae survival upon hatching, regardless of how nutrient dense the substrate (Lam et al. 2009). In addition to the presence of fresh eggs and or specific bacterial species, female house flies are also stimulated by other complex stimuli such as egg-associated pheromones (Jiang et al. 2002), chemical compounds (Tang et al. 2016), and visual cues from conspecifics (Collins and Bell 1996) for oviposition.

Larval nutrition and digestion

Diverse bacterial communities are necessary for the complete development of house fly larvae (Ferrar 1987, Zurek et al. 2000) and serve as a direct source of larval nutrition. Substrates for development must be in a state where oxygen is available and pH is not too low for a variety of bacteria to grow and for larvae to survive (Zurek et al. 2000). Immediately after hatching, larvae begin feeding on and filtering bacteria and nutrients from microbe-rich, liquidized substrates via a sieve or "food channel" on their ventral side (West 1951, Dowding 1967). Ingested bacteria and nutrients then move through the esophagus and past the proventriculus into the larval midgut. The midgut is lined by the peritrophic matrix (PM) which is the primary physical barrier that protects flies from ingested bacteria and keeps gut contents contained and separated from the epithelium (Nayduch and Burrus 2017). The PM is secreted by a specific group of cells called the "cardia" next to the proventriculus, completely lines the midgut, and is comprised of chitin in a protein-carbohydrate matrix (Tellam 1996, Lehane and Billingsley 1996). This Type II PM is continuously produced in both larvae and adults regardless of the presence or absence of a food bolus. The PM in house fly larvae has two layers and is semipermeable which allows secreted digestive enzymes to flow inside the PM and break down microorganisms, while also allowing by-products from digestion to flow outside the PM and be absorbed by epithelial cells (Espinoza-Fuentes and Terra 1987). The PM also prevents

microorganisms from moving out of the PM into the "ecto-PM space" and contacting the gut epithelial cells (Tellam 1996, Nayduch and Burrus 2017).

House fly larvae rely on various enzymes to help digest important nutrients to utilize for nutrition and development. When house fly larvae begin feeding by sieving material through their "food channel", salivary amylase, the main digestive enzyme involved in starch digestion, is secreted from the salivary glands and gut caeca which liquifies the substrate before being ingested (Espinoza-Fuentes and Terra 1987). Once the ingested material moves through the esophagus and past the proventriculus into the midgut, the liquified starch is digested in the anterior midgut where some bacteria are destroyed as well. As the ingested material passes through the anterior midgut into the mid-midgut, which is the region of midgut with the highest acidity (Terra et al. 1988), secreted lysozymes and cathepsin D-like proteinases, in combination with a lower pH, digest or kill bacteria present in the ingested material (Terra and Ferreira 2012). Lysozymes are an important part of immune defense against bacteria and can cause bacterial cell lysis by catalyzing hydrolysis of 1,4 β-glycosidic linkage between molecules present in peptidoglycan (Terra and Ferreira 2012). Some of the lysozymes in the fly midgut resemble those secreted in gut-fermenting vertebrates (Lemos et al. 1993, Cançado et al. 2008, 2010). Any leftover undigested material from the killed bacteria is digested in the posterior portion of the midgut. In the posterior midgut there is a "counter-current flow" (Tellam 1996) where secretion of fluid takes place in the posterior midgut and then is reabsorbed back into the midgut to aid in digestion for the next "food bolus" (Terra and Ferreira 2012) or round of ingested material.

Metamorphosis

House flies are surrounded by bacteria throughout their larval development. Interestingly, bacterial abundance is highest in 3rd instar larvae, decreases throughout pupal development, and

is lowest upon eclosion as an adult (Greenberg 1959a, Radvan 1960). Nayduch and Burrus (2017) suggested bacterial loss in house fly development results from three different processes: (1) the purging of gut contents by wandering third-instar larvae, (2) the processes of pupation which destroys bacteria during larval gut histolysis, and (3) pupation which involves the adult fly leaving behind the puparium containing the old larval foregut and hindgut cuticular lining with bacteria. Although many bacteria are lost through larval metamorphosis, some species acquired by larvae survive and can be trans-stadially carried to the adult, including *Salmonella* Paratyphi, *Bacillus anthracis, Salmonella* Schottmulleri, *Shigella sonnei*, *Escherichia coli*, *Morganella* spp., *Providencia* spp., *Proteus* spp., *Alcaligenes faecalis*, and *Pseudochrobactrum* spp. (Greenberg 1959a, 1959b, Radvan 1960, Greenberg and Klowden 1972, Rochon et al. 2005, Su et al. 2010, Zurek and Nayduch 2016).

Adult interactions with bacteria

Acquisition

Bacteria in the gut of house flies are acquired directly through feeding or indirectly through grooming behaviors. While searching for food, house flies will use their proboscis to taste or test different surfaces. A suitable food substrate will consist of some form of sugar, soluble starch, and protein (Glaser 1923). Therefore, house flies find a variety of different foods very attractive, but they can survive on a diet as simple as milk soaked bread (Tischler 1931) since the fermentation of milk, protein, and natural sugars are enough to support longevity and reproductive health (West 1951). Adult house flies directly acquire bacteria through feeding using their proboscis which consists of sponging mouthparts. Bacteria and other food substrates are taken up through the pseudotracheae located on the ventral side of the labella when it is

pressed up against a liquidized food substrate and larger particles can be ingested and mixed or homogenized using prestomal teeth (West 1951) before entering the house fly alimentary canal.

The main purpose of grooming is to protect the fly against pathogen invasion (Zhukovskaya et al. 2013). However, incidental and indirect acquisition of bacteria occurs during the grooming process. For example, house flies completely covered with *Pseudomonas aeruginosa* and *Escherichia coli* removed the bacteria entirely from their body surface by grooming with their sponging mouthparts and legs (Jacques et al. 2017), suggesting that the bacteria may have been ingested during the process. Additionally, males spend more time grooming than females (Barber and Starnes 1949) indicating possible sex-specific differences in cleaning behavior and bacterial acquisition.

Digestion

Ingested bacteria enter the anterior region of the alimentary canal, known as the foregut, which consists of the mouth, pharynx, esophagus, crop, proventriculus and associated salivary glands (West 1951, Cantwell et al. 1976). Adults begin feeding within 24 h of emergence (Williams 2009) and once food material is acquired and swallowed, it is either temporarily stored in the crop where pre-digestion of starch occurs or goes through the proventriculus into the midgut to be digested. The crop is a chitinous structure that can expand to adjust to changes in volume of liquid food storage. Salivary amylase is secreted from the salivary glands and combined with ingested food which both pass to the crop to commence breakdown of nutrients (Terra and Ferreira 2012, Stoffolano and Haselton 2013). Stored liquid food in the crop is regurgitated onto substrate to help liquify the next meal for subsequent ingestion. Regurgitant in the form of vomit specks are also created during the "bubbling" process which consists of forming a droplet from crop contents onto the surface of the labella and using it to help evaporate

any residual liquid from substrate and enhance the nutrient concentration of substrate for digestion (Hendrichs et al. 1992, Stoffolano and Haselton 2013). Bacteria present in the crop can be transferred to substrate incidentally via "bubbling" or regurgitation.

Digestion of food continues when the food bolus passes through the proventriculus and enters the midgut. The midgut epithelium secretes amylases, lipases, proteinases, and lysozyme to help with the destruction of bacteria and the digestion of food material and is lined with microvilli to increase absorptive surface area (Terra et al. 1988, McGaughey and Nayduch 2009). Food material that enters the midgut is contained within the peritrophic matrix (PM). Adult house flies, just like larvae, have a type II PM which is continuously produced, double-layered, and lines the entire midgut protecting the epithelial cells of the gut from bacterial invasion (Lehane 1997). The PM allows digestive enzymes and immunity effectors secreted by epithelial cells to enter the midgut to digest food material and destroy bacteria (Richards and Richards 1977).

The hindgut is the posterior region consisting of the distal intestine, rectal valve, rectum, and anus (West 1951, Cantwell et al. 1976). Any leftover water and salts in the hindgut and rectum are reabsorbed and any food material not destroyed or digested in the midgut are secreted from the house fly anus into the environment. The rectum aides Malpighian tubules in osmoregulation and removal of waste in the form of uric acid (McGavin 2001). The foregut and hindgut, originating from invagination of ectoderm in the embryonic development, are covered with a layer of cuticle that serves as a mechanical barrier protecting and preventing invasion of microbes through the gut lining (West 1951).

Gut immunity

In addition to digestive processes, the house fly's innate immune response detects and kills bacteria in the midgut. Bacteria are recognized by receptors on the midgut cells that bind microbe-associated molecular patterns (MAMPs) such as peptidoglycan (Lemaitre and Hoffmann 2007, Nayduch and Burrus 2017). Peptidoglycan (PGN) is a polymer consisting of several amino acid and sugar components that forms a lattice-like structure and comprises part of the cell wall of bacteria, acting as a protective layer just outside of the plasma membrane (Madigan et al. 2014). The sugar component of the structure consists of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues that are β-1,4 linked. Short chains of amino acids are linked to *N*-acetlyuramic acid and are cross-linked to other peptide strands to form more of a mesh-like structure (Madigan et al. 2014).There are two main "types" of PGN that are detected by the insect innate immune response: meso-diaminopimelic acid (DAP)-type PGN and lysine (Lys)-type PGN (Madigan et al. 2014). If PGN is "DAP-type" it will have diaminopimelic acid in the third position of the peptide strand and if it is considered to be a "Lys-type" it will have lysine found in that same position instead (Schleifer and Kandler 1972, Lim et al. 2006). Examples of bacteria that have a DAP-type PGN are *Salmonella* spp., *E. coli*, *Pseudomonas aeruginosa,* and several other Gram-negative bacteria species (Schleifer and Kandler 1972). Some examples of Lys-type PGN bacteria include *Staphylococcus aureus*, *Streptococcus pyogenes,* and many other Gram-positive bacteria (Schleifer and Kandler 1972). After house flies recognize bacteria in the gut, the epithelium secretes antimicrobial peptides (AMPs) which diffuse across the PM and kill the bacteria therein (Lemaitre and Hoffmann 2007, Nayduch and Burrus 2017). These AMPs can specifically target and destroy bacteria present in the midgut, thus serving a dual purpose in digestion and defending the larvae and adult house flies from the bacteria they ingest.

Relevance of adult house flies as vectors

A "vector" in the biological field is defined as an organism which harbors and can potentially transmit disease-causing pathogens to and from hosts. The two main ways vectors transmit pathogens is by (1) directly infecting a host with a pathogen or (2) contaminating the host environment with pathogens by dissemination of pathogens to fomites or food. Typically vectors that directly infect hosts fall into one of 3 different categories: (1) cyclodevelopmental, where the pathogen develops within the vector, but does not proliferate, (2) cyclopropagative, where the pathogen develops and proliferates within the vector, or (3) propagative, where the pathogen does not develop within the vector and only proliferates. Vectoring of bacterial pathogens by arthropods is always considered to be propagative since bacteria only increase or decrease in number and do not develop within the vector. Amazingly, over 200 different pathogenic species of microbes have been isolated from house flies alone (Nayduch and Burrus 2017) and just one house fly can carry up to 100 different pathogenic microbes (Greenberg 1973).

The family *Enterobacteriaceae* is a large family of widely distributed, gram-negative, non-spore forming, rod-shaped, facultative anaerobes that ferment glucose and produce acid and gas byproducts (Cordier 2006). Some bacteria in the family *Enterobacteriaceae* are considered coliforms and are found in human and animal digestive tracts. Coliforms are typically monitored by food manufacturers and are used to measure the effectiveness of good manufacturing and hygiene practices (Cox et al. 1988). Bacteria from the family *Enterobacteriaceae* have been isolated from house flies, many of which are serious human and animal pathogens (Nazni et al. 2005, Gupta et al. 2012). Some examples of bacterial species from the family *Enterobacteriaceae* isolated from house flies surveyed in the field include *Enterobacter*

aerogens (Gupta et al. 2012), *Enterobacter cloacae* (Rady et al. 1992), *Escherichia coli* O157:H7 (Buma et al. 1999, Iwasa et al. 1999, Moriya et al. 1999, Szalanski et al. 2004, Förster et al. 2009, Talley et al. 2009, Burrus 2010, Butler et al. 2010, Chaiwong et al. 2014, Burrus et al. 2017, Puri-Giri et al. 2017), *Klebsiella oxytoca* (Fotedar et al. 1992), *Klebsiella pneumonia* (Fotedar et al. 1992, Nmorsi et al. 2007, Gupta et al. 2012, Ranjbar et al. 2016), *Morganella morganii* (Rady et al. 1992, Gupta et al. 2012), *Proteus mirabilis* (Rady et al. 1992, Nmorsi et al. 2007, Gupta et al. 2012), *Proteus vulgaris* (Rady et al. 1992), *Providencia alcalifaciens* (Gupta et al. 2012), *Salmonella enterica* (Pava-Ripoll et al. 2015), *Salmonella typhi* (Béjar et al. 2006, Nmorsi et al. 2007, Chaiwong et al. 2014), *Shigella dysenteriae* (Rady et al. 1992, Butler et al. 2010), and *Yersinia pseudotuberculosis* (Rady et al. 1992, Zurek et al. 2001).

Transmission

Successful vectoring of bacteria by flies occurs when viable cells are dispersed into the environment. House flies contaminate the environment simply by harboring and transporting viable bacteria on their surface, mouthparts, legs, wings, which are then dispersed by physical contact, or through grooming behaviors where bacteria are physically "thrown off" their body surface (Yap et al. 2008, Jacques et al. 2017). Wild house flies collected in Thailand had several different known human pathogenic bacterial species including *Bacillus* sp., *Enterococcus* sp., *Escherichia coli* O157:H7, *Salmonella* sp., and *Shigella* sp. isolated from their body surface (Chaiwong et al. 2014) indicating that house flies have the potential to transfer this bacteria from their body to nearby surfaces. House flies are considered mechanical vectors for *Shigella* (Levine and Levine 1991) since their mouthparts remain contaminated with *Shigella* shortly after feeding on infected substrate and then pose a risk for directly contaminating other nearby substrates through feeding. Yap et al. 2008 demonstrated that *Vibrio cholerae* is carried on the

wings of house flies and decreases in abundance after house fly activities such as grooming and flying. However, *Vibrio cholerae* were only found on their legs or body 2-3 h after exposure to the bacteria and not on their wings. Additionally, Wasala et al. (2013) found that after house flies were exposed to a lawn of *Escherichia coli* O157:H7, viable cells were still detected and persisted on the house fly labellum and tarsus for up to 13 days after exposure to the bacteria indicating that house flies had the potential to directly transmit *E. coli* O157:H7 via their mouthparts and legs.

Ingested bacteria are harbored in the house fly crop and midgut temporarily before either being excreted or digested, respectively. The location and fate of the bacterium within the digestive tract is important to consider for vector potential. Some researchers have argued that house flies should not only be considered mechanical vectors but rather "bio-enhanced" vectors for certain bacteria that are not simply transported from one location to another, but instead survive for several days on body parts (such as mouth parts) and are successfully transmitted (Kobayashi et al. 1999).

Both persistence of bacteria and even propagative vectoring, where the ingested bacteria proliferate within the fly, have also been investigated in several studies. Nayduch et al. (2002) fed house flies *Aeromonas caviae* and demonstrated that the bacteria increased in abundance over the course of 2 days post-ingestion and that viable cells were present in excreta from the infected flies 2-3 days post-ingestion. Additionally, *A. caviae*-infected flies contaminated chicken and the chance of the chicken becoming contaminated by the infected flies increased with exposure time. Joyner et al. (2013) showed persistence of *Pseudomonas aeruginosa* in the crop, midgut, and rectum of house flies over 24 h as well as a significant increase in abundance from 12-24 h post-ingestion. Further, flies excreted *P. aeruginosa* throughout the 24 h

observation period. Regurgitation and defecation are both excretions from the crop and midgut, respectively, which contain viable bacteria (Kobayashi et al. 1999, Nazni et al. 2005, Wasala et al. 2013, Nayduch and Burrus 2017, Nayduch et al. 2018) and can pose a threat to both human and animal health. Fleming et al. (2014) demonstrated persistence of green fluorescent protein (GFP) expressing *Escherichia coli* O157:H7 in house flies for up to 12 h post-ingestion. Although there was a decrease in the abundance during the observation period, viable *E. coli* O157:H7 cells were still observed in the crop and rectum of flies which indicated transmission potential. The transmission of *E. coli* O157:H7 from house flies to spinach leaves was demonstrated by analyzing regurgitation spots by electron microscopy at 18 h, 4 days, and 8 days after exposure to infected flies (Wasala et al. 2013). Further, at 8 days post-fly exposure, viable cells of *E. coli* O157:H7 were still detected which indicated that the flies successfully transmitted *E. coli* to the spinach and that the bacteria persisted for several days. Viable GFP-expressing *Staphylococcus aureus* persist in the rectum of flies at 4 h post-ingestion, but the abundance of *S. aureus* had significantly decreased in the flies by 6 h (Nayduch et al. 2013). Excretion of *S. aureus* was highest at 2 h post-ingestion and continued until 4 h post-ingestion. Although, the house flies did not harbor *S. aureus* for an extended period, flies still effectively transmitted viable cells into the environment.

Salmonella enterica ser. Typhimurium, also survive and proliferate within the house fly digestive system (Chifanzwa and Nayduch 2018), and there appears to be a dose-dependent "fate" of bacteria, where bacteria in lower abundance proliferate more than those in high abundance. Fly sex appears to also alter bacteria fate (i.e., persistence, proliferation) as well as excretion. Nayduch et al. (2018) fed male and female house flies either a "low dose" or "high dose" of *S. enterica* ser. Typhimurium (*S.* Typhimurium) and determined that the bacteria

persisted for at least 16 h post-ingestion in all treatment groups, but there was both a dose and sex effect on bacterial persistence in flies. Using the same treatments in a separate experiment, excreta droplets were observed and collected between 6 and 12 h post-ingestion and bacteria were enumerated. Bacterial dose and fly sex affected both the number of "excretion events" and abundance of bacteria in excreta droplets. There was no difference in the total mean abundance of *S.* Typhimurium in excreta droplets and the proportion of droplets positive for *S.* Typhimurium shed from "high dose" males and females over the 6 h collection period. Interestingly, male flies fed the "high dose" of *S.* Typhimurium excreted a greater abundance of *S.* Typhimurium per droplet than any other treatment, while females had the most "excretion events" over the 6 h collection period. Therefore, house flies can effectively transmit *S.* Typhimurium and males may pose a higher risk of successfully transmitting the pathogen into the environment than females since males shed more *S.* Typhimurium per excreta droplet, but further research is needed to confirm this assumption.

Overall, the successful transmission, or vectoring, of bacterial pathogens by house flies relies on their ability to excrete viable bacterial cells into the environment. Bacteria must survive either on the surface or passage through the gut as flies move from one location to the next. Not all bacteria have the same "fate" within flies, and many factors—from bacterial species, to bacterial abundance or dose, to fly sex—have been shown to impact bacterial survival and transmission. Nonetheless, flies harbor and have the potential to transmit a large variety of microbes including many pathogens. A partial list of bacterial pathogens house flies can excrete and/or disseminate into the environment (via regurgitation or defecation) is provided in Table 1.2.

Significance of house flies in public health

House flies develop and thrive in many diverse habitats and bridge the gap between unsanitary habitats, such as agricultural livestock productions, and sanitary habitats where humans reside (Bahrndorff et al. 2017). When there is a lack of fly control, diarrheal diseases can increase in prevalence. In Bangladesh, a surge of the house fly populations in the spring correlated with the subsequent increase in children *Shigella* infections with diarrhea two months later (Farag et al. 2013). Interestingly, the control of flies at a military base decreased the fly populations by 64% and subsequently the number of clinic visits for shigellosis decreased by 85% (Cohen et al. 1991). Other house fly-associated outbreaks involved an epidemic of typhoid fever during the Spanish-American war from *Salmonella typhi* in military camps (Cirillo 2006), a colitis outbreak at a young children's school in Japan caused by enteropathogenic *E. coli* infections found to have come from a nearby animal farm (Moriya et al. 1999), and a dysentery outbreak caused by flies in a U.S. army camp (Kuhns and Anderson 1944).

The presence of flies exacerbates sanitation issues in places where human and animal excrement is close to human living spaces, as is prevalent in some developing countries. For example, the proximity of food and water sources to infective feces in addition to the presence of higher than average fly densities contributed to a 15% increase in the risk of diarrhea in humans from a study conducted in India (Collinet-Adler et al. 2015). Furthermore, flies were the likely cause of stored food contamination with enteropathogenic *E. coli* for children in Bangladesh since the food was improperly stored and nearby fecal matter (Doza et al. 2018). One effective way of reducing fly numbers and keeping fecal material separate from human food items is the proper use of latrines (Collinet-Adler et al. 2015). McCabe and Haines (1957) showed that over the 18 years after reconstructing outhouses in Boston to reduce house fly breeding in human

feces, the amount of *Shigella* infections in children decreased and the diarrheal disease rate for Boston was cut in half.

In the past, the chemical DDT was found to be effective in reducing house fly densities in Texas and at the same time reduced the prevalence of diarrheal diseases in kids under the age of 5 (Watt and Lindsay 1948) and the incidence of diarrheal disease was also lower in the children from towns in Pakistan where insecticides were used to control for flies (Chavasse et al. 1999). In addition, the prevalence of *Shigella* infections was significantly lower only during the time when an effective fly control program was carried out in select towns of rural Georgia (Lindsay et al. 1953). Thus, fly control is a necessary preventative measure to reduce diarrheal illness (Taylor and Greenough 1989).

The potential risk house flies may pose on human and animal health must always be considered in order to reduce the spread of disease in public areas. For example, house flies collected from wastewater management facilities carried antibiotic resistant *Enterococcus faecalis* and a few flies were found to carry these resistant bacteria in an RV park, a fast food restaurant, and an apartment complex close to some of the treatment facilities (Doud et al. 2014). Therefore, fly control should be regularly used to reduce the spread of antibiotic resistant bacteria between different habitats (Zurek and Ghosh 2014, Poudel et al. 2019). Preventing the spread of antibiotic resistant bacteria by flies is especially important in hospital environments where nosocomial infections are on the rise (Šrámová et al. 1992, Boulesteix et al. 2005). For example, house flies collected from hospitals and a nearby residential area were reported to carry bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Fotedar et al. 1992) which are common nosocomial pathogens.

House flies pose a threat to food safety

Food-borne illnesses are usually caused by ingestion of food contaminated with pathogens and the presence of house flies creates an opportunity for food contamination to occur. Several studies effectively demonstrated house fly transfer of bacterial pathogens to human food items. Macovei et al. (2008) collected wild house flies and exposed them to cooked hamburger patties from a fast food restaurant and within 30 minutes, enterococci were recovered from all the burger patties. Additionally, Nayduch et al. (2002) exposed sterile chicken to *Aeromonas caviae* infected flies in the laboratory, and all chicken pieces were contaminated after 8 h exposure. Interestingly, the chicken also became contaminated with *A. caviae* after 2-3 hours of exposure to wild flies at a dairy farm (Nayduch et al. 2002). Greenberg (1964) exposed flies for 3 hours to dog feces contaminated with *S.* Typhimurium. The flies were then exposed to Mexican drinks (containing milk and sugar) and after exposure to the flies, the drinks were given to 10 volunteers. After 15 hours, *S.* Typhimurium was recovered from 6/10 of the volunteer stool samples (Greenberg 1964) which confirmed that house flies successfully acquired a pathogen from a microbe-rich source (dog feces), contaminated a human food source (Mexican drinks), and that humans subsequently acquired the infection via ingestion.

Transfer of food-borne pathogens between flies and food items, such as fruits and vegetables, has long been a concern of the FDA. One report emphasized that human pathogens can survive in house flies for several days or even over the course of months and studies involving flies other than house flies have been reported to show successful transfer of common human foodborne pathogens to fruit (FDA 1999). Studies where house flies were found to carry *E. coli* O157:H7 most likely acquired from manure were also discussed. In the FDA report, fruit flies exposed to apple wounds were used as an example of fruit contamination. Infected fruit flies successfully contaminated apples with *E. coli* O157:H7 after exposure (Janisiewicz et al. 1999).

In earlier field studies, house flies in Hungary were found to be captured in greater abundance in traps that used decomposing or overripe fruit compared to traps that contained human feces (Lörincz and Makara 1936, Lörincz et al. 1936). Therefore, West (1951) emphasized that the handling and sales of fruit at markets can become a significant public health problem if house flies are nearby. In recent years, foodborne outbreaks of disease from contaminated produce have been more frequently reported and account for a larger proportion of disease outbreaks than meat and dairy products (Bennett et al. 2018). A summary table of house fly food-borne pathogen transmission studies and associated food sources is provided in Table 1.4.

Salmonella enterica

Salmonella enterica resides in most terrestrial vertebrate and human intestines and can be excreted in feces of infected hosts. Known modes of transmission for *S. enterica* include the consumption of contaminated food or water, contact with infected feces, and contact with infected animals, humans, or animal feed (Andino and Hanning 2014). *S. enterica* is a facultative anaerobe, about 0.5-1.5 µm wide and about 2-5 µm in length, has peritrichous flagella which allow high motility, and have thousands of different serovars (Hinshaw and McNeil 1951, Andino and Hanning 2014). Virulence genes that encode virulence factors for invasion are located in *Salmonella* pathogenicity islands which are essential for *S. enterica* to successfully invade hosts (Fàbrega and Vila 2013).

Salmonella is the leading food pathogen in the United States and there have been many outbreaks of disease originating from mammals and poultry, however, some food-borne outbreaks have been associated with produce (Andino and Hanning 2014). *Salmonella enterica* ser. Typhimurium is a serovar that is common in many animals and is one of the leading food-borne pathogens in human hospitalizations and deaths (Andino and Hanning 2014). The CDC

has reported illness, hospitalization, and death associated outbreaks with *S.* Typhimurium that ranges from food contamination, to pet exposure cases, and to teaching lab contamination incidents (Table 1.3; CDC). Many mammals and reptiles serve as reservoirs for *S.* Typhimurium and humans can get nontyphoidal salmonellosis from *S.* Typhimurium contaminated food or fecal contamination (Andino and Hanning 2014). People who are immunocompromised are more susceptible to nontyphoidal salmonellosis which can cause gastroenteritis and bacteremia. Symptoms of gastroenteritis consist of an acute onset of fever, cramping, and diarrhea which generally occurs 6-72 h after inoculation with *Salmonella* (Acheson and Hohmann 2001). Treatment for nontyphoidal salmonellosis typically consists of fluoroquinolones and/or cephalosporins (Fàbrega and Vila 2013), however, 5 percent of individuals with gastroenteritis will develop bacteremia which often results in death (Acheson and Hohmann 2001).

Salmonella enterica in house flies

In field surveys, house flies carried *Salmonella* spp. at California commercial poultry ranches and dairies (Mian et al. 2002), Washington caged layer houses (Olsen and Hammack 2000), Georgia feedlots and dairies (Xu et al. 2018), swine farms in Taiwan (Wang et al. 2011), and dual-purpose cattle farms in Venezuela (Fuenmayor et al. 2018). Additionally, *Salmonella enterica* was recovered at slaughter-aged beef cattle, dairy cattle, and sheep farms in Australia and serovar variability was the highest in dairy cattle (Vanselow et al. 2007). *Salmonella* spp. were also isolated from a university poultry farm and campus cafeteria in Malaysia (Choo et al. 2011).

As for the transmission of *Salmonella* spp., Holt et al. (2007) released house flies into a room full of hens that were challenged with *S. enterica* ser. Enteritidis and found that 50% of the flies released were contaminated after only 48 h of exposure to the hens. Furthermore, house

flies have been reported to harbor *S.* Typhimurium and transmit *S.* Typhimurium to humans in earlier studies (McNeil and Hinshaw 1944, Greenberg 1964). *S.* Typhimurium can be persistent in the house fly alimentary canal at all life stages, but survival in the alimentary canal is affected by the presence of other microbes (Greenberg 1959c, Greenberg et al. 1970). Recently, Chifanzwa and Nayduch (2018) found that the dose of *S.* Typhimurium fed to house flies impacted the bacteria survival in flies over time. Furthermore, Nayduch et al. (2018) demonstrated that the dose of *S.* Typhimurium fed to flies and the sex of flies affects the mean abundance of *S.* Typhimurium shed in excreta droplets and the number of droplets positive for *S.* Typhimurium over time.

Summary

House flies are holometabolous insects that go through 4 life stages during development. Adult females are anautogenous, require protein for egg development and prefer substrates that consist of decomposing organic matter for oviposition. Females rely on contact cues and substrate volatiles to determine whether a substrate is suitable for oviposition. Larval house flies require microbes in order to complete development. They have a Type II peritrophic matrix that lines the entire midgut and protects the midgut epithelium from bacterial invasion during digestion. Bacteria from the substrate can remain within the house fly midgut throughout larval development and may be trans-stadially carried from larval to adult life stages.

Adult house flies are synanthropic in nature, frequent a variety of different microbe-rich habitats, and serve as a bridge for microorganism dispersal between human and animal environments. Adult house flies can acquire bacteria directly through feeding or indirectly via grooming. Once acquired, bacteria enter the house fly alimentary canal where they will either be destroyed or digested in the midgut or transmitted via regurgitation or defecation. The house fly

immune system protects house flies from the bacteria they ingest and allows them to flourish in environments teeming with microbes. House flies are propagative vectors for some bacteria and field surveys indicate they carry over 200 different pathogenic microbes. Bacteria are only successfully vectored by a fly if viable cells are excreted or dispersed into the environment. Studies have effectively demonstrated that *Pseudomonas aeruginosa*, *E. coli* O157:H7, *Staphylococcus aureus*, *Aeromonas caviae*, and *Salmonella enterica* ser. Typhimurium can be successfully harbored and transmitted by house flies.

House flies can have a negative impact on public health when there is no fly control program implemented. More specifically, diarrheal disease outbreaks, such as shigellosis, typhoid fever, colitis, and dysentery can occur. Keeping fecal material isolated from human food items and reducing fly populations through use of insecticides has proved effective at lowering the incidence of diarrheal diseases worldwide over the years. Fly control is also imperative in hospital settings where the spread of antibiotic resistant bacteria has become a real issue. House flies also pose a threat to food safety. The transfer of food-borne pathogens between flies and food items, such as fruits and vegetables, is of current concern to the FDA.

Salmonella enterica resides in most vertebrates and is transmitted through consumption of food or water contaminated with feces or contact with infected animals. *S.* Typhimurium is one of the leading food-borne pathogens resulting in human hospitalizations and deaths. Surveys have isolated *S.* Typhimurium from house flies collected from livestock production facilities, farms, and a university campus cafeteria. House flies have also been reported to harbor *S. enterica* ser. Enteritidis around infected animals and have the capacity to acquire, harbor and excrete *S.* Typhimurium.

In the following chapters, the procurement, transmission, and abundance of bacteria in house flies was investigated. Because the interaction of male and female house flies with manure may differ due to differences in nutritional requirements and behavior, I first determined if male and female house flies differed in bacteria acquired over time from cattle manure in the presence and absence of an alternative food source (Chapter 2). Next, because *S.* Typhimurium survives and grows on fruits, such as cantaloupe (Chimbombi 2010, Bennett et al. 2018, Huang et al. 2019), I determined if house flies transferred *S.* Typhimurium to and acquired it from cantaloupe and if cantaloupe facilitated the transfer of *S.* Typhimurium between flies (Chapter 3). Lastly, because house flies have sex-specific behaviors and nutritional requirements and bacteria and coliform abundance varies across habitats, I determined if house fly sex and habitat affect bacterial abundance and coliform abundance in house flies (Chapter 4).

References Cited

Acheson, D., and E. L. Hohmann. **2001**. Nontyphoidal salmonellosis. Clin. Infect. Dis. 32: 263–269.

Andersen, S. O. 2012. Cuticular sclerotization and tanning, pp. 167–192. *In* Gilbert, L.I. (ed.), Insect Molecular Biology and Biochemistry. Academic Press, San Diego.

Andino, A., and I. Hanning. **2014**. *Salmonella enterica*: survival, colonization, and virulence differences among serovars. Sci. World J. 2015: 1–16.

Ascher, K. E. S. 1958. The attraction of the Levant housefly *Musca vicina* Macq. to natural breeding media. Acta Trop. 15: 1–14.

Bahrndorff, S., N. de Jonge, H. Skovgård, and J. L. Nielsen. **2017**. Bacterial communities associated with houseflies (*Musca domestica* L.) sampled within and between farms. PLoS ONE. 12: 1–15.

Baldacchino, F., M. Desquesnes, G. Duvallet, T. Lysyk, and S. Mihok. **2017**. 3. Veterinary importance and integrated management of Brachycera flies in dairy farms, pp. 55–90. *In* Pests and Vector-Borne Diseases in the Livestock Industry, Ecology and Control of Vector-Borne Diseases. Wageningen Academic Publishers.

Barber, G. W., and E. B. Starnes. **1949**. The activities of house flies. J. N. Y. Entomol. Soc. 57: 203–214.

Barnard, D. R., and C. J. Geden. **1993**. Influence of larval density and temperature in poultry manure on development of the house fly (Diptera: Muscidae). Environ. Entomol. 22: 971–977.

Barro, N., S. Aly, O. C. A. Tidiane, and T. A. Sababénédjo. **2006**. Carriage of bacteria by proboscises, legs, and feces of two species of flies in street food vending sites in Ouagadougou, Burkina faso. J. Food Protect. 69: 2007–2010.

Béjar, V. C., J. C. Chumpitaz, E. C. Pareja, E. B. Valencia, A. Huamán, C. A. Sevilla, M. B. Tapia, and G. F. Saez. **2006**. *Musca domestica* como vector mecánico de bacterias enteropatógenas en mercados y basurales de Lima y Callao. Rev. Peru Med. Exp. Salud Publica. 23: 39–43.

Bennett, S. D., S. V. Sodha, T. L. Ayers, M. F. Lynch, L. H. Gould, and R. V. Tauxe. **2018**. Produce-associated foodborne disease outbreaks, USA, 1998–2013. Epidemiol. Infect. 146: 1397–1406.

Black, W. C., and E. S. Krafsur. **1985**. Use of sticky traps to investigate seasonal trends in the spatial distribution of house flies and stable flies (Diptera: Muscidae). J. Med. Entomol. 22: 550–557.

Boulesteix, G., P. D. Le, B. Chevalier, M. Dieng, B. Niang, and B. Diatta. **2005**. Role of *Musca domestica* in the transmission of multiresistant bacteria in the centres of intensive care setting in sub-Saharan Africa. Ann. Fr. Anesth. Reanim. 24: 361–365.

Braverman, Y. E., A. Chizov-Ginzburg, A. H. G. Saran, and M. Winkler. **1999**. The role of houseflies (*Musca domestica*) in harbouring *Corynebacterium pseudotuberculosis* in dairy herds in Israel. Rev. Sci. Tech. OIE. 18: 681–690.

Bryant, E. H. 1970. The effect of egg density on hatchability in two strains of the housefly. Physiol. Zool. 43: 288–295.

Buma, R., H. Sanada, T. Maeda, M. Kamei, and H. Kourai. **1999**. Isolation and characterization of pathogenic bacteria, including *Escherichia coli* O157:H7, from flies collected at a dairy farm field. Med. Entomol. Zool. 50: 313–321.

Burrus, R. G. 2010. *Musca domestica* l. (diptera: muscidae) dispersal from and *Escherichia coli* O157:H7 prevalence on dairies in north-central Florida. Ph.D. Dissertation, University of Florida, Gainesville.

Burrus, R. G., J. A. Hogsette, P. E. Kaufman, J. E. Maruniak, A. H. Simonne, and V. Mai. **2017**. Prevalence of *Escherichia coli* O157:H7 from house flies (Diptera: Muscidae) and dairy samples in north central Florida. J. Med. Entomol. 54: 733–741.

Butler, J. F., A. Garcia-Maruniak, F. Meek, and J. E. Maruniak. **2010**. Wild Florida house flies (*Musca domestica*) as carriers of pathogenic bacteria. Fla. Entomol. 93: 218–224.

Cançado, F. C., J. A. R. G. Barbosa, and S. R. Marana. **2010**. Role of the triad N46, S106 and T107 and the surface charges in the determination of the acidic pH optimum of digestive lysozymes from *Musca domestica*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 155: 387–395.

Cançado, F. C., P. Chimoy Effio, W. R. Terra, and S. R. Marana. **2008**. Cloning, purification and comparative characterization of two digestive lysozymes from *Musca domestica* larvae. Braz. J. Med Biol. Res. 41: 969–977.

Cantwell, G. E., A. J. Nappi, J. G. Stoffolano, and U. S. A. R. Service. **1976**. Embryonic and postembryonic development of the house fly (*Musca domestica* L.). U.S. Dep. Agric. Tech. Bull. 1519.

Cao, X. M., F. L. Song, T. Y. Zhao, Y. D. Dong, C. X. Sun, and B. L. Lu. **2006**. Survey of deltamethrin resistance in house flies (*Musca domestica*) from urban garbage dumps in northern China. Environ. Entomol. 35: 1–9.

Chaiwong, T., T. Srivoramas, P. Sueabsamran, K. Sukontason, M. R. Sanford, and K. L. Sukontason. **2014**. The blow fly, *Chrysomya megacephala*, and the house fly, *Musca domestica*, as mechanical vectors of pathogenic bacteria in northeast Thailand. Trop. Biomed. 31: 336–346.

Chakrabarti, S., S. Kambhampati, and L. Zurek. **2010**. Assessment of house fly dispersal between rural and urban habitats in Kansas, USA. J. Kansas Entomol. Soc. 83: 172–189.

Chavasse, D., R. Shier, O. Murphy, S. Huttly, S. Cousens, and T. Akhtar. **1999**. Impact of fly control on childhood diarrhoea in Pakistan: community-randomised trial. Lancet. 353: 22–25.

Chifanzwa, R., and D. Nayduch. **2018**. Dose-dependent effects on replication and persistence of *Salmonella enterica* serovar Typhimurium in house flies (Diptera: Muscidae). J. Med. Entomol. 55: 225–229.

Chimbombi, E. 2010. Targeted inactivation of *Salmonella enterica* serovar Typhimurium in fresh cantaloupe flesh (*Cucumis melo* L.) using elctron beam irradiation. Ph.D. Dissertation. Texas A&M University, College Station.

Choo, L. C., A. A. Saleha, S. S. Wai, and N. Fauziah. **2011**. Isolation of *Campylobacter* and *Salmonella* from houseflies (*Musca domestica*) in a university campus and a poultry farm in Selangor, Malaysia. Trop. Biomed. 28: 16–20.

Cirillo, V. J. 2006. "Winged sponges": houseflies as carriers of typhoid fever in 19th- and early 20th-century military camps. Perspect. Biol. Med. 49: 52–63.

Cohen, D., M. Green, C. Block, R. Slepon, R. Ambar, S. S. Wasserman, and M. M. Levine. **1991**. Reduction of transmission of shigellosis by control of houseflies (*Musca domestica*). Lancet. 337: 993–997.

Collinet-Adler, S., S. Babji, M. Francis, D. Kattula, P. S. Premkumar, R. Sarkar, V. R. Mohan, H. Ward, G. Kang, V. Balraj, and E. N. Naumova. **2015**. Environmental factors associated with high fly densities and diarrhea in Vellore, India. Appl. Environ. Microbiol. 81: 6053–6058.

Collins, R. D., and W. J. Bell. **1996**. Enhancement of resource finding efficiency by visual stimuli in *Musca domestica* (Diptera: Muscidae). J. Kansas Entomol. Soc. 69: 204–207.

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.

Cordier, J.-L. 2006. Enterobacteriaceae, pp. 450–475. *In* Motarjemi, Y., Adams, M. (eds.), Emerging Foodborne Pathogens, Woodhead Publishing Series in Food Science, Technology and Nutrition. Woodhead Publishing.

Cox, L. J., N. Keller, and M. van Schothorst. **1988**. The use and misuse of quantitative determinations of Enterobacteriaceae in food microbiology. J. Appl. Bacteriol. Symp. Suppl. 65: 237S-249S.

De Bruyne, M., and T. C. Baker. **2008**. Odor detection in insects: volatile codes. J. Chem. Ecol. 34: 882–897.

Doud, C. W., H. M. Scott, and L. Zurek. **2014**. Role of house flies in the ecology of *Enterococcus faecalis* from wastewater treatment facilities. Microb. Ecol. 67: 380–391.

Doud, C. W., and L. Zurek. **2012**. *Enterococcus faecalis* OG1RF:pMV158 survives and proliferates in the house fly digestive tract. J. Med. Entomol. 49: 150–155.

Dove, W. E. 1916. Some notes concerning overwintering of the housefly, *Musca domestica*, at Dallas, Texas. J. Econ. Entomol. 9: 528-538.

Dowding, V. M. 1967. The function and ecological significance of the pharyngeal ridges occurring in the larvae of some cyclorrhaphous Diptera. Parasitology. 57: 371–388.

Doza, S., M. J. Rahman, M. A. Islam, L. H. Kwong, L. Unicomb, A. Ercumen, A. J. Pickering, S. M. Parvez, A. M. Naser, S. Ashraf, K. K. Das, and S. P. Luby. **2018**. Prevalence and association of *Escherichia coli* and diarrheagenic *Escherichia coli* in stored foods for young children and flies caught in the same households in rural Bangladesh. Am. J. Trop. Med. Hyg. 98: 1031–1038.

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. Insect Biochem. 17: 809–817.

Fàbrega, A., and J. Vila. **2013**. *Salmonella enterica* serovar Typhimurium skills to succeed in the host: virulence and regulation. Clin. Microbiol. Rev. 26: 308–341.

Farag, T. H., A. S. Faruque, Y. Wu, S. K. Das, A. Hossain, S. Ahmed, D. Ahmed, D. Nasrin, K. L. Kotloff, S. Panchilangam, J. P. Nataro, D. Cohen, W. C. Blackwelder, and M. M. Levine. **2013**. Housefly population density correlates with shigellosis among children in Mirzapur, Bangladesh: a time series analysis. PLoS Neglect. Trop. D. 7: 1-9.

FDA. **1999**. Potential for infiltration, survival, and growth of human pathogens within fruits and vegetables. https://www.fda.gov/food/hazard-analysis-critical-control-point-haccp/potential-infiltration-survival-and-growth-human-pathogens-within-fruits-and-vegetables

Ferrar, P. 1987. A guide to the breeding habits and immature stages of Diptera Cyclorrhapha. Entomonograph. 8: 222–226.

Fleming, A., H. V. Kumar, C. Joyner, A. Reynolds, and D. Nayduch. **2014**. Temporospatial fate of bacteria and immune effector expression in house flies fed GFP- *Escherichia coli* O157:H7. Med. Vet. Entomol. 28: 364–371.

Förster, M., K. Sievert, S. Messler, S. Klimpel, and K. Pfeffer. **2009**. Comprehensive study on the occurrence and distribution of pathogenic microorganisms carried by synanthropic flies caught at different rural locations in Germany. J. Med. Entomol. 46: 1164–1166.

Fotedar, R., U. Banerjee, S. Singh, Shriniwas, and A. K. Verma. **1992**. The housefly (*Musca domestica*) as a carrier of pathogenic microorganisms in a hospital environment. J. Hosp. Infect. 20: 209–215.

Fuenmayor, Y., A. Rodas-González, G. Carruyo, A. E. Hoet, T. Wittum, and C. Narváez-Bravo. **2018**. *Salmonella* prevalence and antimicrobial drug resistance in dual-purpose cattle operations in the eastern region of Zulia State, Venezuela. Foodborne Pathog. Dis. 16: 205–213.

Fukuda, A., M. Usui, C. Masui, and Y. Tamura. **2019**. Quantitative analysis of houseflies-mediated food contamination with bacteria. J. Food Safety. 7: 11–14.

Gerry, A. C., G. E. Higginbotham, L. N. Periera, A. Lam, and C. R. Shelton. **2011**. Evaluation of surveillance methods for monitoring house fly abundance and activity on large commercial dairy operations. J. Econ. Entomol. 104: 1093–1102.

Gill, C., S. Bahrndorff, and C. Lowenberger. **2016**. *Campylobacter jejuni* in *Musca domestica*: an examination of survival and transmission potential in light of the innate immune responses of the house flies. Insect Sci. 24: 584–598.

Glaser, R. W. 1923. The effect of food on longevity and reproduction in flies. J. Exp. Zool. 38: 383–412.

Glaser, R. W. 1924. Rearing flies for experimental purposes with biological notes. J. Econ. Entomol. 17: 486–496.

Graham, J. P., L. B. Price, S. L. Evans, T. K. Graczyk, and E. K. Silbergeld. **2009**. Antibiotic resistant enterococci and staphylococci isolated from flies collected near confined poultry feeding operations. Sci. Total Environ. 407: 2701–2710.

Greenberg, B. 1959a. Persistence of bacteria in the developmental stages of the housefly II. Quantitative study of the host-contaminant relationship in flies breeding under natural conditions. Am. J. Trop. Med. Hyg. 8: 412–416.

Greenberg, B. 1959b. Persistence of bacteria in the developmental stages of the housefly I. Survival of enteric pathogens in the normal and aseptically reared host. Am. J. Trop. Med. Hyg. 8: 405–411.

Greenberg, B. 1959c. Persistence of bacteria in the developmental stages of the housefly IV. Infectivity of the newly emerged adult. Am. J. Trop. Med. Hyg. 8: 618–622.

Greenberg, B. 1959. House fly nutrition. 1. Quantitative study of the protein and sugar requirements of males and females. J. Cell. Comp. Physiol. 53: 169–177.

Greenberg, B. 1964. Experimental transmission of *Salmonella typhimurium* by houseflies to man. Am. J. Hyg. 80: 149–156.

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

Greenberg, B. 1973. Flies and Disease: II. Biology and Disease Transmission. Princeton University Press, Princeton, NJ.

Greenberg, B., and M. Klowden. **1972**. Enteric bacterial interactions in insects. Am. J. Clin. Nutr. 25: 1459–1466.

Greenberg, B., J. A. Kowalski, and M. J. Klowden. **1970**. Factors affecting the transmission of *Salmonella* by flies: natural resistance to colonization and bacterial interference. Infect. Immun. 2: 800–809.

Gupta, A. K., D. Nayduch, P. Verma, B. Shah, H. V. Ghate, M. S. Patole, and Y. S. Shouche. **2012**. Phylogenetic characterization of bacteria in the gut of house flies (*Musca domestica* L.). FEMS Microbiol. Ecol. 79: 581–593.

Hanski, I. 1987. Nutritional ecology of dung-and carrion-feeding insects, pp. 837–884. *In* Nutritional Ecology of Insects, Mites, and Spiders. John Wiley & Sons Inc., New York, NY.

Hawley, J. E., L. R. Penner, S. E. Wedberg, and W. L. Kulp. **1951**. The role of the house fly, *Musca domestica*, in the multiplication of certain enteric bacteria. Am. J. Trop. Med. Hyg. 1: 572–582.

Hendrichs, J., S. S. Cooley, and R. J. Prokopy. **1992**. Post-feeding bubbling behaviour in fluid-feeding Diptera: concentration of crop contents by oral evaporation of excess water. Physiol. Entomol. 17: 153–161.

Hewitt, C. G. 2011. The house-fly: *Musca Domestica* Linn: its structure, habits, development, relation to disease and control. Cambridge University Press, UK.

Hinshaw, W. R., and E. McNeil. **1951**. *Salmonella* infection as a food industry problem., pp. 209–240. *In* Mrak, E.M., Stewart, G.F. (eds.), Advances in Food Research. Academic Press, Urbana, IL.

Hogsette, J. A., and R. Farkas. **2000**. Secretophagous and hematophagous higher Diptera. *In* Papp, L., Darvas, B. (eds.), Contributions to a Manual of Paearctic Diptera, General and Applied Dipterology. Science Herald, Budapest.

Hogsette, J. A., R. D. Jacobs, and R. W. Miller. **1993**. The sticky card: device for studying the distribution of adult house fly (Diptera: Muscidae) populations in closed poultry houses. J. Econ. Entomol. 86: 450–454.

Hogsette, J. A., R. Urech, P. E. Green, A. Skerman, M. M. Elson-Harris, R. L. Bright, and G. W. Brown. **2012**. Nuisance flies on Australian cattle feedlots: immature populations. Med. Vet. Entomol. 26: 46–55.

Holt, P. S., C. J. Geden, R. W. Moore, and R. K. Gast. **2007**. Isolation of *Salmonella enterica* serovar Enteritidis from houseflies (*Musca domestica*) found in rooms containing *Salmonella* serovar Enteritidis-challenged hens. Appl. Environ. Microbiol. 73: 6030–6035.

Huang, J., Y. Luo, B. Zhou, J. Zheng, and X. Nou. **2019**. Growth and survival of *Salmonella enterica* and *Listeria monocytogenes* on fresh-cut produce and their juice extracts: impacts and interactions of food matrices and temperature abuse conditions. Food Control. 100: 300-304.

Iwasa, M., S. I. Makino, H. Asakura, H. Kobori, and Y. Morimoto. **1999**. Detection of *Escherichia coli* O157:H7 from *Musca domestica* (Diptera: Muscidae) at a cattle farm in Japan. J. Med. Entomol. 36: 108–112.

Jacques, B. J., T. J. Bourret, and J. J. Shaffer. **2017**. Role of fly cleaning behavior on carriage of *Escherichia coli* and *Pseudomonas aeruginosa*. J. Med. Entomol. 54: 1712–1717.

Janisiewicz, W. J., W. S. Conway, M. W. Brown, G. M. Sapers, P. Fratamico, and R. L. Buchanan. **1999**. Fate of *Escherichia coli* O157:H7 on fresh-cut apple tissue and its potential for transmission by fruit flies. Appl. Environ. Microbiol. 65: 1–5.

Jiang, Y., C. Lei, C. Niu, Y. Fang, C. Xiao, and Z. Zhang. **2002**. Semiochemicals from ovaries of gravid females attract ovipositing female houseflies, *Musca domestica*. J. Insect Physiol. 48: 945–950.

Joyner, C., M. K. Mills, and D. Nayduch. **2013**. *Pseudomonas aeruginosa* in *Musca domestica* L.: Temporospatial examination of bacteria population dynamics and house fly antimicrobial responses. PLoS ONE. 8: 1-9.

Kaufman, P. E., D. A. Rutz, and S. Frisch. **2005**. Large sticky traps for capturing house flies and stable flies in dairy calf greenhouse facilities. J. Dairy Sci. 88: 176–181.

Kaufman, P. E., J. G. Scott, and D. A. Rutz. **2001**. Monitoring insecticide resistance in house flies (Diptera: Muscidae) from New York dairies. Pest Manag. Sci. 57: 514–521.

Keiding, J. 1965. Observations on the behaviour of the housefly in relation to its control. Riv. Parassitol. 26: 45–60.

Khan, H. A. A., S. A. Shad, and W. Akram. **2012**. Effect of livestock manures on the fitness of house fly, *Musca domestica* L. (Diptera: Muscidae). Parasitol. Res. 111: 1165–1171.

Kobayashi, H. 1934. The influence of foods on the fecundity of *Musca domestica*. Keio J. Med. 5: 36–67.

Kobayashi, M., T. Sasaki, N. Saito, K. Tamura, K. Suzuki, H. Watanabe, and N. Agui. **1999**. Houseflies: not simple mechanical vectors of enterohemorrhagic *Escherichia coli* O157:H7. Am. J. Trop. Med. Hyg. 61: 625–629.

Kuhns, D. M., and T. G. Anderson. **1944**. A fly-borne bacillary dysentery epidemic in a large military organization. Am. J. Public Health. Nations Health. 34: 750–755.

LaBrecque, G. C., D. W. Meifert, and D. E. Weidhaas. **1972**. Dynamics of house fly and stable fly populations. Fla. Entomol. 55: 101–106.

Lam, K. 2010. Oviposition ecology of house flies, *Musca domestica* (Diptera: Muscidae): competition, chemical cues, and bacterial symbionts. Ph.D. dissertation, Simon Fraser University, British Columbia.
Lam, K., D. Babor, B. Duthie, E. M. Babor, M. Moore, and G. Gries. **2007**. Proliferating bacterial symbionts on house fly eggs affect oviposition behaviour of adult flies. Anim. Behav. 74: 81–92.
Lam, K., C. Geisreiter, and G. Gries. **2009**. Ovipositing female house flies provision offspring larvae with bacterial food. Entomol. Exp. Appl. 133: 292–295.
Lamb, C. G., and J. S. Gardiner. **1922**. The geometry of insect pairing. Proc. R. Entomol. Soc. Lond. Ser B. Taxon. 94: 1–11.
Larraín, P. S., and C. F. Salas. **2008**. House fly (*Musca domestica* L.) (Diptera Muscidae) development in different types of manure. Chil. J. Agr. Res. 68: 192-197.
Larsen, J. R., R. E. Pfadt, and L. G. Peterson. **1966**. Olfactory and oviposition responses of the house fly to domestic manures, with notes on an autogenous strain. J. Econ. Entomol. 59: 610–615.
Lehane, M., and P. Billingsley. **1996**. Biology of the Insect Midgut. The University Press, Great Britain.
Lehane, M. J. 1997. Peritrophic matrix structure and function. Annu. Rev. Entomol. 42: 525–550.
Lemaitre, B., and J. Hoffmann. **2007**. The host defense of *Drosophila melanogaster*. Annu. Rev. Immunol. 25: 697–743.
Lemos, F. J. A., A. F. Ribeiro, and W. R. Terra. **1993**. A bacteria-digesting midgut-lysozyme from *Musca domestica* (diptera) larvae. Purification, properties and secretory mechanism. Insect Biochem. Molec. 23: 533–541.
Levine, O. S., and M. M. Levine. **1991**. Houseflies (*Musca domestica*) as mechanical vectors of Shigellosis. Rev. Infect. Dis. 13: 688–696.
Lim, J.-H., M.-S. Kim, H.-E. Kim, T. Yano, Y. Oshima, K. Aggarwal, W. E. Goldman, N. Silverman, S. Kurata, and B.-H. Oh. **2006**. Structural basis for preferential recognition of diaminopimelic acid-type peptidoglycan by a subset of peptidoglycan recognition proteins. J. Biol. Chem. 281: 8286–8295.
Lindsay, D. R., W. H. Stewart, and J. Watt. **1953**. Effect of fly control on diarrheal disease in an area of moderate morbidity. Public Health Rep. 68: 361–367.
Lodge, O. C. 1918. An examination of the sense-reactions of flies. B. Entomol. Res. 9: 141–151.
Lörincz, F., and G. Makara. **1936**. Investigations into the fly density in Hungary in the years 1934 and 1935. Quart. Bull. World Health Organ. LoN. 5: 219–227.
Lörincz, F., G. Szappanos, and G. Makara. **1936**. On flies visiting human faeces in Hungary. Quart. Bull. World Health Organ. LoN. 5: 228–236.
Lysyk, T. J. 1993a. Adult resting and larval developmental sites of stable flies and house flies (Diptera: Muscidae) on dairies in Alberta. J. Econ. Entomol. 86: 1746–1753.
Lysyk, T. J. 1993b. Seasonal abundance of stable flies and house flies (Diptera: Muscidae) in dairies in Alberta, Canada. J. Med. Entomol. 30: 888–895.
Machtinger, E. T., N. C. Leppla, and J. A. Hogsette. **2016**. House and stable fly seasonal abundance, larval development substrates, and natural parasitism on small equine farms in Florida. Neotrop. Entomol. 45: 433–440.
Macovei, L., B. Miles, and L. Zurek. **2008**. Potential of houseflies to contaminate ready-to-eat food with antibiotic-resistant enterococci. J. Food Protect. 71: 435–439.

Macovei, L., and L. Zurek. **2006**. Ecology of antibiotic resistance genes: characterization of enterococci from houseflies collected in food settings. Appl. Environ. Microbiol. 72: 4028–4035.

Madigan, M. T., J. M. Martinko, K. S. Bender, D. H. Buckley, and D. A. Stahl. **2014**. Microbial cell structure and function, pp. 49–96. *In* Brock Biology of Microorganisms. Pearson, USA.

Marçon, P. C. R. G., G. D. Thomas, B. D. Siegfried, J. B. Campbell, and S. R. Skoda. **2003**. Resistance status of house flies (Diptera: Muscidae) from southeastern Nebraska beef cattle feedlots to selected insecticides. J. Econ. Entomol. 96: 1016–1020.

Matthysse, J. G. 1945. Observations on housefly overwintering. J. Econ. Entomol. 38: 493–494.

McCabe, L. J., and T. W. Haines. **1957**. Diarrheal disease control by improved human excreta disposal. Public Health Rep. 72: 921–928.

McGaughey, J., and D. Nayduch. **2009**. Temporal and spatial fate of GFP-expressing motile and nonmotile *Aeromonas hydrophila* in the house fly digestive tract. J. Med. Entomol. 46: 123–130.

McGavin, G. 2001. Essential entomology. Oxford University Press, UK.

McNeil, E., and W. R. Hinshaw. **1944**. Snakes, cats, and flies as carriers of *Salmonella* Typhimurium. Poultry Sci. 23: 456–457.

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.

Mian, L. S., H. Maag, and J. V. Tacal. **2002**. Isolation of *Salmonella* from muscoid flies at commercial animal establishments in San Bernardino County, California. J. Vector Ecol. 4: 82-85.

Moon, R. D. 2019. Chapter 17 - Muscid Flies (Muscidae), pp. 345–368. *In* Mullen, G.R., Durden, L.A. (eds.), Medical and Veterinary Entomology, 3rd ed. Academic Press, USA.

Moon, R. D., J. L. Hinton, S. D. O'Rourke, and D. R. Schmidt. **2001**. Nutritional value of fresh and composted poultry manure for house fly (Diptera: Muscidae) larvae. J. Econ. Entomol. 94: 1308–1317.

Moon, R. D., and H. J. Meyer. **1985**. Nonbiting flies, pp. 65–82. *In* Williams, R.E., Hall, R.D., Broce, A.B., Scholl, P.J. (eds.), Livestock Entomology. John Wiley & Sons Inc., New York, NY.

Moriya, K., T. Fujibayashi, T. Yoshihara, A. Matsuda, N. Sumi, N. Umezaki, H. Kurahashi, N. Agui, A. Wada, and H. Watanabe. **1999**. Verotoxin-producing *Escherichia coli* O157:H7 carried by the housefly in Japan. Med. Vet. Entomol. 13: 214–216.

Mullens, B. A., and J. A. Meyer. **1987**. Seasonal abundance of stable flies (Diptera: Muscidae) on California dairies. J. Econ. Entomol. 80: 1039–1043.

Murvosh, C. M., R. L. Fye, and G. C. Labrecque. **1964**. Studies on the mating behavior of the house fly, *Musca Domestica* L. Ohio J. Sci. 64: 264–271.

Nayduch, D., and R. G. Burrus. **2017**. Flourishing in filth: house fly–microbe interactions across life history. Ann. Entomol. Soc. Am. 110: 6–18.

Nayduch, D., H. Cho, and C. Joyner. **2013**. *Staphylococcus aureus* in the house fly: temporospatial fate of bacteria and expression of the antimicrobial peptide defensin. J. Med. Entomol. 50: 171–178.

Nayduch, D., G. P. Noblet, and F. J. Stutzenberger. **2002**. Vector potential of houseflies for the bacterium *Aeromonas caviae*. Med. Vet. Entomol. 16: 193–198.

Nayduch, D., K. Zurek, J. L. Thomson, and K. M. Yeater. **2018**. Effects of bacterial dose and fly sex on persistence and excretion of *Salmonella enterica* serovar Typhimurium from adult house flies (*Musca domestica* L.; Diptera: Muscidae). J. Med. Entomol. 55: 1264–1270.

Nazni, W. A., B. Seleena, H. L. Lee, J. Jeffery, T. A. R. Rogayah, and M. A. Sofian. **2005**. Bacteria fauna from the house fly, *Musca domestica* (L.). Trop. Biomed. 22: 225–231.

Ngoen-klan, R., K. Moophayak, T. Klong-klaew, K. N. Irvine, K. L. Sukontason, C. Prangkio, P. Somboon, and K. Sukontason. **2011**. Do climatic and physical factors affect populations of the blow fly *Chrysomya megacephala* and house fly *Musca domestica*? Parasitol. Res. 109: 1279–1292.

Nmorsi, O. p. g., G. Agbozele, and N. c. d. Ukwandu. **2007**. Some aspects of epidemiology of filth flies: *Musca domestica*, *Musca domestica vicina*, *Drosophila melanogaster* and associated bacteria pathogens in Ekpoma, Nigeria. Vector-Borne Zoonotic Dis. 7: 107–117.

Nurita, A. T., and A. A. Hassan. **2013**. Filth flies associated with municipal solid waste and impact of delay in cover soil application on adult filth fly emergence in a sanitary landfill in Pulau Pinang, Malaysia. Bull. Entomol. Res. 103: 296–302.

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

Pace, R. C., J. L. Talley, T. L. Crippen, and A. C. Wayadande. **2017**. Filth fly transmission of *Escherichia coli* O157:H7 and *Salmonella enterica* to lettuce, *Lactuca sativa*. Ann. Entomol. Soc. Am. 110: 83–89.

Pava-Ripoll, M., R. E. G. Pearson, A. K. Miller, B. D. Tall, C. E. Keys, and G. C. Ziobro. **2015**. Ingested *Salmonella enterica*, *Cronobacter sakazakii*, *Escherichia coli* O157:H7, and *Listeria monocytogenes*: transmission dynamics from adult house flies to their eggs and first filial (F1) generation adults. BMC Microbiol. 15: 1-12.

Poudel, A., T. Hathcock, P. Butaye, Y. Kang, S. Price, K. Macklin, P. Walz, R. Cattley, A. Kalalah, F. Adekanmbi, and C. Wang. **2019**. Multidrug-resistant *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus* spp. in houseflies and blowflies from farms and their environmental settings. Int. J. Environ. Res. Public Health. 16: 1-12.

Puri-Giri, R., A. Ghosh, J. L. Thomson, and L. Zurek. **2017**. House flies in the confined cattle environment carry non-O157 Shiga toxin-producing *Escherichia coli*. J. Med. Entomol. 54: 726–732.

Radvan, R. 1960. Persistence of bacteria during development in flies. Folia Microbiol. 5: 1–50.

Rady, M. H., N. Abdel-Raouf, I. Labib, and A. I. Merdan. **1992**. Bacterial contamination of the housefly *Musca domestica*, collected from 4 hospitals at Cairo. J. Egypt. Soc. Parasitol. 22: 279–288.

Ranjbar, R., M. Izadi, T. T. Hafshejani, and F. Khamesipour. **2016**. Molecular detection and antimicrobial resistance of *Klebsiella pneumoniae* from house flies (*Musca domestica*) in kitchens, farms, hospitals and slaughterhouses. J. Infect. Public Health. 9: 499–505.

Richards, A. G., and P. A. Richards. **1977**. The peritrophic membranes of insects. Ann. Rev. Entomol. 22: 219–240.

Riemann, J. G., D. J. Moen, and B. J. Thorson. **1967**. Female monogamy and its control in houseflies. J. Insect Physiol. 13: 407–418.

Rochon, K., T. J. Lysyk, and L. B. Selinger. **2005**. Retention of *Escherichia coli* by house fly and stable fly (Diptera: Muscidae) during pupal metamorphosis and eclosion. J. Med. Entomol. 42: 397–403.

Sacca, G., and M. P. Benetti. **1960**. Ricerche sperimentali sulla maturità e sul comportamento sessuale di *Musca domestica* L.(Diptera, Muscidae). Rend. Ist. Superiore Sanita. 23: 423–432.

Sasaki, T., M. Kobayashi, and N. Agui. **2000**. Epidemiological potential of excretion and regurgitation by *Musca domestica* (Diptera: Muscidae) in the dissemination of *Escherichia coli* O157: H7 to food. J. Med. Entomol. 37: 945–949.

Savage, E. P., and H. F. Schoof. **1955**. The species composition of fly populations at several types of problem sites in urban areas. Ann. Entomol. Soc. Am. 48: 251–257.

Schleifer, K. H., and O. Kandler. **1972**. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. 36: 407–477.

Schmidtmann, E. T. 1988. Exploitation of bedding in dairy outdoor calf hutches by immature house and stable flies (Diptera: Muscidae). J. Med. Entomol. 25: 484–488.

Service, M. 2012. Houses flies and stable flies (Muscdidae) and latrine flies (Fanniidae), pp. 139–155. *In* Medical Entomology for Students. Cambridge University Press, UK.

Shah, R. M., F. Azhar, S. A. Shad, W. B. Walker, M. Azeem, and M. Binyameen. **2016**. Effects of different animal manures on attraction and reproductive behaviors of common house fly, *Musca domestica* L. Parasitol. Res. 115: 3585–3598.

Shane, S. M., M. S. Montrose, and K. S. Harrington. **1985**. Transmission of *Campylobacter jejuni* by the housefly (*Musca domestica*). Avian Dis. 29: 384–391.

Spiller, D. 1964. Nutrition and diet of muscoid flies. Bull. World Health Organ. 31: 551–554.

Šrámová, H., M. Daniel, V. Absolonová, D. Dědičová, Z. Jedlièková, H. Lhotová, P. Petráš, and V. Subertová. **1992**. Epidemiological role of arthropods detectable in health facilities. J. Hosp. Infect. 20: 281–292.

Stoffolano, J. G., and A. T. Haselton. **2013**. The adult dipteran crop: a unique and overlooked organ. Ann. Rev. Entomol. 58: 205–225.

Su, Z., M. Zhang, X. Liu, L. Tong, Y. Huang, G. Li, and Y. Pang. **2010**. Comparison of bacterial diversity in wheat bran and in the gut of larvae and newly emerged adult of *Musca domestica* (Diptera: Muscidae) by use of ethidium monoazide reveals bacterial colonization. J. Econ. Entomol. 103: 1832–1841.

Szalanski, A. L., C. B. Owens, T. Mckay, and C. D. Steelman. **2004**. Detection of *Campylobacter* and *Escherichia coli* O157:H7 from filth flies by polymerase chain reaction. Med. Vet. Entomol. 18: 241–246.

Talley, J., Greta Schuster, David Parker, Bill Clymer, and Carl Patrick. **2002**. Monitoring population trends of house flies and stable flies (Diptera: Muscidae) on Texas high plains feedlots. *In* 2002 Chicago, IL July 28-31, 2002. Am. Soc. Ag. Biol. Eng. Presentation.

Talley, J. L., A. C. Wayadande, L. P. Wasala, A. C. Gerry, J. Fletcher, U. DeSILVA, and S. E. Gilliland. **2009**. Association of *Escherichia coli* O157:H7 with filth flies (Muscidae and Calliphoridae) captured in leafy greens fields and experimental transmission of *E. coli* O157:H7 to spinach leaves by house flies (Diptera: Muscidae). J. Food Protect. 72: 1547–1552.

Tang, R., F. Zhang, N. Kone, J.-H. Chen, F. Zhu, R.-C. Han, C.-L. Lei, M. Kenis, L.-Q. Huang, and C.-Z. Wang. **2016**. Identification and testing of oviposition attractant chemical compounds for *Musca domestica*. Sci. Rep. 6: 1–9.

Taylor, C. E., and W. B. Greenough. **1989**. Control of diarrheal diseases. Annu. Rev. Public Health. 10: 221–244.

Tellam, R. L. 1996. The peritrophic matrix, pp. 86–114. *In* Biology of the Insect Midgut. The University Press, Great Britain.

Terra, W. R., F. P. Espinoza-Fuentes, and C. Ferreira. **1988**. Midgut amylase, lysozyme, aminopeptidase, and trehalase from larvae and adults of *Musca domestica*. Arch. Insect Biochem. Physiol. 9: 283–297.

Terra, W. R., and C. Ferreira. **2012**. 11 - Biochemistry and Molecular Biology of Digestion, pp. 365–418. *In* Gilbert, L.I. (ed.), Insect Molecular Biology and Biochemistry. Academic Press, San Diego, CA.

Tischler, N. 1931. A satisfactory nutriment for adult houseflies. J. Econ. Entomol. 24: 1–559.

Urech, R., R. L. Bright, P. E. Green, G. W. Brown, J. A. Hogsette, A. G. Skerman, M. M. Elson-Harris, and D. G. Mayer. **2012**. Temporal and spatial trends in adult nuisance fly populations at Australian cattle feedlots. Aust. J. Entomol. 51: 88–96.

Vanselow, B. A., M. A. Hornitzky, K. H. Walker, G. J. Eamens, G. D. Bailey, P. A. Gill, K. Coates, B. Corney, J. P. Cronin, and S. Renilson. **2007**. *Salmonella* and on-farm risk factors in healthy slaughter-age cattle and sheep in eastern Australia. Aust. Vet. J. 85: 498–502.

Wang, Y.-C., Y.-C. Chang, H.-L. Chuang, C.-C. Chiu, K.-S. Yeh, C.-C. Chang, S.-L. Hsuan, W.-H. Lin, and T.-H. Chen. **2011**. Transmission of *Salmonella* between swine farms by the housefly (*Musca domestica*). J. Food Protect. 74: 1012–1016.

Wasala, L., J. L. Talley, U. DeSilva, J. Fletcher, and A. Wayadande. **2013**. Transfer of *Escherichia coli* O157:H7 to spinach by house flies, *Musca domestica* (Diptera: Muscidae). Phytopath. 103: 373–380.

Watt, J., and D. Lindsay. **1948**. Diarrheal disease control studies; effect of fly control in a high morbidity area. Public Health Rep. 63: 1319–1333.

West, L. S. 1951. The housefly: its natural history, medical importance, and control, 1st ed. Comstock Publishing Company Inc., Ithaca, NY.

Williams, R. E. 2009. Veterinary Entomology : Livestock and Companion Animals. CRC Press.

Xu, Y., S. Tao, N. Hinkle, M. Harrison, and J. Chen. **2018**. *Salmonella,* including antibiotic-resistant *Salmonella*, from flies captured from cattle farms in Georgia, U.S.A. Sci. Total Environ. 616: 90–96.

Yap, K. L., M. Kalpana, and H. L. Lee. **2008**. Wings of the common house fly (*Musca domestica* L.): importance in mechanical transmission of *Vibrio cholerae*. Trop. Biomed. 25: 1–8.

Zhukovskaya, M., A. Yanagawa, and B. T. Forschler. **2013**. Grooming behavior as a mechanism of insect disease defense. Insects. 4: 609–630.

Zurek, K., and D. Nayduch. **2016**. Bacterial associations across house fly life history: evidence for transstadial carriage from managed manure. J. Insect. Sci. 16: 1-4.

Zurek, L., S. S. Denning, C. Schal, and D. W. Watson. **2001**. Vector competence of *Musca domestica* (Diptera: Muscidae) for *Yersinia pseudotuberculosis*. J. Med. Entomol. 38: 333–335.

Zurek, L., and A. Ghosh. **2014**. Insects represent a link between food animal farms and the urban environment for antibiotic resistance traits. Appl. Environ. Microbiol. 80: 3562–3567.

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.

Zvereva, E. L. 1986. Peculiarities of competitive interaction between larvae of the house fly (*Musca domestica*) and microscopic fungi. Russ. J. Zool. 65: 1517–1525.

Table 1.1. Partial list of house fly collections/surveys in agricultural and urban habitats.

Habitat type	Collection site	Location	References
Agricultural	Dairies	California	Mullens and Meyer 1987, Gerry et al. 2011
		Canada	Lysyk 1993a, 1993b
		New York	Kaufman et al. 2001, 2005
		Iowa	Black and Krafsur 1985
		Denmark	Keiding 1965
	Feedlots	Texas	Talley et al. 2002
		Nebraska	Marçon et al. 2003, Puri-Giri et al. 2017
		Australia	Hogsette et al. 2012, Urech et al. 2012
	Poultry houses	Maryland	Hogsette et al. 1993, Graham et al. 2009
		Florida	Hogsette et al. 1993
	Equine farms	Florida	Machtinger et al. 2016
Urban	Residential houses	Mediterranean	Keiding 1965
	RV Park/Apartments	Kansas	Doud et al. 2014
	Restaurants	Kansas	Macovei and Zurek 2006, Doud et al. 2014
	City Dumpsters	Kansas	Savage and Schoof 1955
		Arizona	Savage and Schoof 1955
		Florida	Butler et al. 2010
	Landfills	Malaysia	Nurita and Hassan 2013
		China	Cao et al. 2006
		Michigan	Savage and Schoof 1955
		New York	Savage and Schoof 1955
	Street vendors	Burkina Faso	Barro et al. 2006

Table 1.2. Partial list of studies that investigated house fly transmission of bacterial pathogens.

Pathogen	Extent of dissemination	References
Aeromonas caviae	Contaminate environment	Nayduch et al. 2002
Aeromonas hydrophila	Viable in excreta	McGaughey and Nayduch 2009
Campylobacter jejuni	Viable in excreta	Gill et al. 2016
	Contaminate environment	Shane et al. 1985
Corynebacterium pseudotuberculosis	Viable in excreta	Braverman et al. 1999
Enterococcus faecalis	Contaminate environment	Doud and Zurek 2012
Escherichia coli O157:H7	Viable in excreta	Sasaki et al. 2000, Fleming et al. 2014
	Contaminate environment	Wasala et al. 2013
Pseudomonas aeruginosa	Viable in excreta	Joyner et al. 2013
Salmonella Typhimurium	Viable in excreta	Chifanzwa and Nayduch 2018
Salmonella Schottmullerris	Viable in excreta	Hawley et al. 1951
Shigella dysenteriae	Viable in excreta	Hawley et al. 1951
Staphylococcus aureus	Viable in excreta	Nayduch et al. 2013
Yersinia pseudotuberculosis	Contaminate environment	Zurek et al. 2001

Table 1.3. Hosts and sources of *S.* Typhimurium during outbreaks as reported by the Centers for Disease Control*.

Year	Source
2019	Hedgehogs
2018	Dried coconut, Chicken salad
2017	Live poultry, Teaching micro lab exposure
2014	Teaching micro lab exposure
2013	Live poultry, Ground beef
2012	Hedgehogs, Cantaloupe
2011	Ground beef, African Dwarf Frogs, Teaching micro lab exposure
2009	Peanut butter
2006	Tomatoes

*Source: https://www.cdc.gov/salmonella/outbreaks.html

Table 1.4. Food-borne pathogens transmitted to or from food by house flies.

Pathogen	Food type	References
Aeromonas caviae	Various	Nayduch et al. 2002
enterococci	Cooked hamburger patty	Macovei et al. 2008
Escherichia coli O157:H7	Spinach	Wasala et al. 2013
	Lettuce	Pace et al. 2017
Antimicrobial-resistant *E. coli*	Various	Fukuda et al. 2019
Salmonella enterica	Mexican drink	Greenberg 1964
	Lettuce	Pace et al. 2017

This table only includes studies that have specifically described food-borne pathogens and food sources and demonstrated house flies may have an impact on food safety.

Chapter 2 - Abundance and accumulation of *Escherichia coli* and *Salmonella* Typhimurium procured by male and female house flies (Diptera: Muscidae) exposed to cattle manure

Published in *Annals of the Entomological Society of America*, 110(1), 2017, 37-44

J. L. Thomson[1]
K. M. Yeater[2]
L. Zurek[1]
D. Nayduch[1,3]

[1]Department of Entomology, Kansas State University, 123 W. Waters Hall, Manhattan, KS 66506
[2]USDA-ARS-PA-NRRC, Office of the Director, 2150 Centre Avenue, Building D, Suite 300, Fort Collins, CO 80526
[3]USDA-ARS, Center for Grain and Animal Health Research, 1515 College Ave, Manhattan, KS 66502

Abstract

House flies, *Musca domestica* L. develop within and feed upon microbe-rich substrates such as manure, acquiring and potentially disseminating pathogenic bacteria. Because adult female flies frequent manure due to oviposition or nutrition requirements, we hypothesized females would consume more manure than males even in the presence of additional food sources (e.g. sugar), resulting in measurable differences in bacterial load between sexes. House fly acquisition of bacteria from manure inoculated with GFP-expressing *E. coli* or *Salmonella* sp. was examined for both sexes over 24 h in assays where (1) inoculated manure was the only food source and (2) both inoculated manure and sugar water were provided. We conducted assays with mated male and female flies separately to determine sex-specific effects on bacterial acquisition. Over 24 h, bacterial abundance increased in manure inoculated with *S.* Typhimurium, but not *E. coli*. In flies, bacterial abundance increased within sex only in *S.*

Typhimurium assays. Overall, female flies harbored more bacteria than males; however, differences in abundance were only significant at early time points. In the *E. coli* manure-sugar assays, male and female CFU abundance differed at 4 h and 12 h, while CFU abundance differed at 4 and 12 h in all *S.* Typhimurium assays. Fly digestive tract observations from manure-sugar assays supported these initial differences especially at 4 h where females contained manure and fly food, while males contained only sugar water. Identifying sex-specific effects on house fly acquisition and carriage of bacteria from manure facilitates risk assessment of pathogen transmission on farms.

Keywords: *Musca domestica*, bacteria acquisition, vector potential

As larvae, house flies (*Musca domestica* L.) require a microbe-rich substrate such as manure to complete development (Zurek et al. 2000). In a typical commercial dairy farm setting, adult female house flies can be observed investigating both managed manure piles and fresh pats or slurries of manure through contact, touch, taste, and ingestion. While no studies, to our knowledge, have specifically described the reasons for this behavior, we infer this attraction to manure is both for oviposition and nutritional purposes. Ruminant manure is rich in undigested carbohydrates, proteins, vitamins, minerals, and additional nutrients, making it an ideal food source for coprophilous insects (Hanski 1987). Being polyphagous, both male and female flies opportunistically ingest nutrient-rich manure, although coprophagy is not obligate (West 1951). Like other muscid flies, female house flies are anautogenous (Glaser 1923) and can use manure, animal secretions and other bodily fluids to provide the necessary proteins for egg development (Moon 2009). Ingestion of manure or other substrates is not always a result of direct feeding;

house flies spend a considerable amount of time grooming (Barber and Starnes 1949), where they may inadvertently ingest organic matter, including bacteria, present on their surfaces (Nayduch and Burrus, 2017).

Due to their life-long association with substrates rich with microbes, as well as their high mobility and gregariousness, adult house flies have been implicated as potential vectors for various pathogens (West 1951, Graczyk 2001, Gupta et al. 2012). *Escherichia coli* O157:H7 and *Salmonella enterica*, commonly present in fecal matter from infected hosts, are among several pathogens transmitted from house flies to animals or vice versa (Ahmad et al. 2007, Holt et al 2007). Ingestion of pathogens likely enhances survival and dissemination, especially if microbes proliferate and are excreted (Sasaki et al. 2000; McGaughey and Nayduch 2009; Chifanzwa 2011, Joyner et al. 2013, Nayduch and Burrus 2017). For example, *Pseudomonas aeruginosa*, has been shown to proliferate and grow in high numbers inside the house fly digestive tract. Additionally, the fly enables its dissemination into the environment by excreting the pathogen for at least 24 hours post ingestion (Joyner et al. 2013). House flies can also disseminate bacteria by harboring them on their external body parts, although microbe survival may be affected by drying during flight (Yap et al. 2008).

Male and female house flies may differentially interact and associate with manure due not only to oviposition interest, but also nutritional preferences/requirements. Subsequently, this would lead to differences in bacterial accumulation in male and female flies over time, where more manure contact (e.g. through grooming, feeding) would result in an increased abundance of bacteria acquired by female house flies over males. Further, due to oviposition interest and the nutritional requirements of anautogeny (i.e. necessary Ftrav intake), we hypothesized that female house flies would consume more manure compared to male house flies, even when presented

with an alternate food source. Therefore, the major goals of this study were to determine if males and females differentially accumulated bacteria from exposure to manure only, to determine if they still predominantly acquired bacteria from manure when presented with an alternate food source (sugar) free of microbes, and to determine whether or not there were differences in sex in relation to the amount of time they were exposed. Assessing the potential for male and female house flies to differentially obtain bacteria from manure, especially when additional nutrient sources are present, will help in determining whether there is a fly sex-effect on the acquisition and potential dissemination of bacteria.

Materials and methods

House flies. House flies used for this study were established from the main laboratory colony at Kansas State University and reared separately on fresh cattle manure collected from the dairy unit, which served as larval substrate. Sugar cubes and water were given *ad libitum* to adult flies and egg yolk powder was provided when flies were six days old, the day before they were used for an experiment. These 7-day old flies had all mated prior to initiation of the experiment and all females were gravid, to allow for oviposition as a reason for manure investigation. Flies not selected for experiments were kept to maintain the colony for subsequent experiments. Specimens used in this research were deposited as voucher number 257 in the KSU Museum of Entomological and Prairie Arthropod Research.

Culture of green fluorescent protein (GFP) expressing *Escherichia coli* (*E. coli*) and *Salmonella enterica* ser. Typhimurium (*S.* Typhimurium). Non-pathogenic GFP *E. coli* dH5α was transformed with a pGFPuv plasmid (Clontech, Mountain View, CA, USA) that encoded resistance to kanamycin and ampicillin (Kumar and Nayduch 2016). GFP *S.* Typhimurium was a

pathogenic SR-11 strain also transformed with the same plasmid, as previously described (McGaughey and Nayduch 2009). Both GFP *E.coli* and *S.* Typhimurium cultures were maintained on Ampicillin-Kanamycin (Amp/Kan) tryptic soy broth (TSB) or agar (TSA) (50.0 µg/ml w/v each of ampicillin sodium and kanamycin sulfate; Fisher Scientific, Atlanta, GA, USA) and incubated at 37°C before inoculating into manure. For GFP *E. coli* experiments, bacteria were cultured in 50.0 ml Amp/Kan TSB at 37°C in a rotating incubator at 60 rpm for 17 h. Then 75.0 µl culture was transferred to 15.0 ml Amp/Kan TSB and incubated at 37°C at 100 rpm for 3 h to an approximate concentration of $4.38 \pm 1.12 \times 10^7$ colony forming units (CFU)/ml. For GFP *S.* Typhimurium experiments, bacteria were cultured in 50.0 ml TSB Amp/Kan at 37°C at 60 rpm for 19 h and then 75.0 µl was transferred to 15.0 ml Amp/Kan TSB and incubated for 1 h at 100 rpm in 37°C to an approximate concentration of $1.16 \pm 0.287 \times 10^7$ CFU/ml. Both *E. coli* and *S.* Typhimurium cultures were plated on TSB Amp/Kan agar and incubated at 37°C to confirm target concentrations for each experiment were reached for manure inoculation (described below for manure preparation). These species have been previously isolated from house flies in surveys (Mian et al. 2002; Giri 2015) and the concentration of bacteria used in our study was within the range of CFU previously shown to naturally persist in manure (Losinger et al. 1995; Hancock 1997; Himathongkham et al. 1999).

Manure collection and preparation. In November 2014, manure was collected from a managed manure pile at the Kansas State University dairy unit and frozen for later use. Manure was autoclaved at 121°C for 30 min on liquid setting at least 2 times to ensure that all contaminants were eliminated and then stocks were refrozen. Manure was thawed to room temperature just before the start of an experiment. A manure sample (1.0 g) was re-suspended in 19.0 ml phosphate buffer saline (PBS) and plated on a TSB agar plate to ensure sterility. To

inoculate manure, 1.0 ml of either GFP *E. coli* or *S.* Typhimurium culture was centrifuged at 10,000 rpm for 15 min or 14,000 rpm for 30 seconds, respectively, and supernatant was removed. Five-hundred µl of phosphate buffer saline (PBS) was added to gently re-suspend the bacterial pellet and this suspension was evenly distributed on the surface of 30.0 g autoclaved manure in a sterile 60 × 15 mm plastic petri dish (Fisherbrand). A sterile wooden tongue depressor was used to evenly mix manure and bacterial suspension.

Experimental design: Manure-only assay. To determine whether female flies acquired more bacteria in comparison to males, flies were exposed to manure inoculated with GFP *E. coli* or *S.* Typhimurium. Half-gallon plastic containers (Mainstays 2-Qt Canister, Walmart) were used for all treatments: male flies, female flies, and control with no flies. The cap of each container had a hole cut out of the end and a piece of 100 × 15 mm filter paper (Fisherbrand) taped over to permit air flow while minimizing contamination. Thirty grams of prepared manure (approximately 10^6 CFU/g of either GFP-expressing *E. coli* or *S.* Typhimurium) was placed inside each container in a small petri dish. Mated flies approximately 7-9 days old were chilled down to be sorted and separated into a group of 25 female flies and 25 male flies to be used for each treatment. Flies (n = 25/treatment) were knocked down again with CO_2 to transfer to new clean containers set up with manure. The control consisted of a container only containing the inoculated manure, with no flies to determine that presence of flies did not affect bacterial concentration in manure over time. Each assay was repeated 4 times.

Experimental design: Manure-sugar assay. To determine whether the presence of an additional substrate free of microbes affected the amount of bacteria acquired from manure, flies were exposed to inoculated manure and an additional food source. One gallon plastic containers

(Rubbermaid 1-Gal. Canister) were used for both treatments (25 male flies, 25 female flies) and the control (no flies). These containers were twice the size of those used for the manure-only assays in order to provide space for an additional food option (sugar droplet). Stocks of 1.0 ml of 10% sucrose ("sugar") mixed with one drop of green food coloring (Assorted Food Colors, Kroger Co., Cincinnati, OH) were made to be used as an additional food source. From these stocks, a 60.0 µl droplet of 9.9% sucrose mixed with the food coloring was placed in a small petri dish on a piece of Parafilm® and placed on the end furthest from the canister opening. This was replenished every 4 h through a small hole made on the end of the container. Additionally, 30.0 g of prepared manure (approximately 10^6 CFU/ g of either GFP-expressing *E. coli* or *S.* Typhimurium) in a small petri dish was placed at the end nearest the canister opening, approximately 10.5 cm apart from the petri dish with the sucrose droplet. Flies were added to each treatment in the same way as the manure-only assays, and control containers had manure and sucrose mixture, but no flies. Each assay was repeated 4 times.

Enumeration of bacteria from flies and manure. All flies were immobilized with CO_2 at 4, 12, 24 h post-exposure and at each time, 5 flies and approximately 1.0 g of manure were removed from each treatment for bacterial enumeration. Manure was re-suspended in 19.0 ml sterile PBS and vortexed for 3 min. Flies were individually washed by agitating in 1.0 ml PBS for recovery of external bacteria and then individually surface sanitized by sequential 1 min washes in 1.0 ml (each) 10% sodium hypochlorite, 70% ethanol, and sterile deionized H_2O. Each fly was homogenized in 500 µL PBS and brought to a final volume of 1.0 ml homogenate. Manure suspensions, fly external washes, and fly homogenates were 10-fold serially diluted and 100 µl of each sample was plated in duplicate on Amp/Kan TSB agar and incubated at 37°C for 24 h for CFU enumeration. All enumerations of CFU on selective media were made under UV

light to positively identify the GFP bacterial colonies. Manure samples also were cultured on TSB agar without antibiotics to screen for contaminants.

Microscopy of house fly digestive tract. To visually observe distribution of ingested food *in situ* in the fly alimentary canal, (e.g. manure (brown) and/or sugar (green food coloring)) a separate replicate was performed for each experiment exactly as above. At each time point (4, 12, 24 h post-exposure) flies were knocked down with CO_2 and 5 flies were removed. Flies were dissected in PBS to remove the entire digestive tract (crop, midgut, hindgut, rectum). The alimentary canals were observed using a stereomicroscope (Nikon model SMZ745T), and detailed observations were noted for each specimen. A representative picture was taken for each treatment (1 for males and 1 for females) at each time point using a mounted Tucsen TCA 9.0C camera (Tucsen Photonics Co., Ltd., Fujian, PR China) attached to the C mount of the stereomicroscope. Dissected guts were then homogenized in 1.0 ml PBS, 10-fold serially diluted, and 100 µl from each were plated in duplicate on Amp/Kan TSB agar to confirm that the bacterium of interest was present in each house fly.

Statistical analyses. To determine whether house fly sex significantly affected bacterial accumulation over time, we analyzed the enumeration data from the flies at each independent time point using the GLIMMIX procedure in SAS/STAT 13.2, SAS Version 9.4. The response measures of CFU counts were log transformed and the linear model included fixed factors gender and time main effects and their interaction. For each of the analyses, the statistical model is as follows:

$$Y_{ijkl}=u+A_i+G_j+T_k+GT_{jk}+E_{(ijk)l}$$

Where Y_{ijkl} is the observed response,

u is the grand mean,

A_i is the random effect of the i^{th} Assay (no interactions with Assay are included as all were equal to 0 variance),

G_j is the fixed effect of the j^{th} Gender,

Ti_k is the fixed effect of the k^{th} Time Point,

GT_{jk} is their interaction.

The LSMEANS option within this procedure was used to determine which specific time points were statistically significant for differences in CFU between male and female flies (using the 'at' and 'diff' options). To determine if CFU counts in manure increased over time and remained consistent across genders for each experiment, a quadratic regression model was developed to investigate change in CFU (log transformed) within linear and quadratic time factors using JMP Version 12. To determine if the number of flies positive for bacteria on their body surface was different between males and females, we compared data using the Fisher's Exact test option in Minitab 17 software.

Results

House fly procurement of GFP *E. coli*: Manure-only assay. There was no significant change in total bacterial concentration in manure over 24 h in any of the treatment groups (Fig. 2.1a). Bacterial abundance ranged from 4.18×10^4 to 1.04×10^6 CFU/g manure across 4-24 h and there was no effect of time on *E. coli* growth in manure (F = 2.79; df = 1,1; P = 0.1045). Additionally, there was no significant difference in bacterial concentration in manure across the treatments at each time point (F = 0.21, df = 2, 2; P = 0.8112).

In house flies, the amount of *E. coli* acquired did not change over time within each sex (Fig. 2.1a; F = 3.10; df = 1, 17; P = 0.0961). Similarly, there was no significant effect of fly sex

on *E. coli* procurement ($F = 0.06$; df = 1, 17; $P = 0.8019$). Although *E. coli* CFU counts in females were consistently higher than the male CFU counts over time, there was no significant difference between male and female flies at any given time point (Fig. 2.1a; 4 h: $t = 0.02$, df = 17, $P = 0.9847$; 12 h: $t = 1.05$, df = 17, $P = 0.3103$; 24 h: $t = 1.59$, df = 17, $P = 0.1303$). It was observed that *E. coli* was recovered from body surfaces of female flies more frequently than male flies, although none significantly different (Fisher's exact test; $P > 0.05$; Table 2.1). Also, of the house flies that were positive for bacteria on their body surface, abundance of GFP *E. coli* was greater from the external surface of females.

To assess qualitative differences in ingestion between male and female flies, we compared the gross morphology of dissected alimentary canals. At 4 h, white material, i.e. retained fly food from feedings prior to experiment setup, was observed in the posterior portion of the female fly digestive tract (Fig. 2.2). At the 12 and 24 h time points, the fly food was no longer observed and the entire digestive tract of the female appeared to be filled with ingested cattle manure, which appeared as dense brown material. Male house flies did not appear to have as much manure in the digestive tract which was more obvious at 24 h (Fig. 2.2). Overall, the female house flies appeared to acquire and retain more manure in the digestive tract over the 24 h period. Bacteria were cultured from all dissected guts on Amp/Kan TSB agar.

House fly procurement of GFP *E. coli*: Manure-sugar assay. Flies were exposed to inoculated manure, as above, and a separate additional sugar droplet (manure-sugar) containing green food coloring. There was a significant increase in total bacterial concentration over time in the manure, ranging from 4.39×10^4 to 6.98×10^4 CFU/g at 4 h to 6.07×10^6 to 1.39×10^7 CFU/g at 24 h (Fig. 2.1b; $F = 25.68$, df = 1, 1; $P < 0.0001$). The growth of *E. coli* in the manure was

consistent across treatments, with no significant difference in concentration at each time point (F = 0.36; df = 2, 2; P = 0.7021).

Male and female house flies did not show a significant increase in *E. coli* acquisition over time within sex (Fig. 2.1b; F = 2.83; df = 1, 17; P = 0.1106), but there was a significant overall effect of fly sex on bacterial procurement (F = 4.76; df = 1, 17; P = 0.0435). Additionally, there was a significant difference in *E. coli* CFU counts between sexes at 4 h, with females having $1.27\pm0.492\times10^4$ CFU and males having $3.69\pm1.52\times10^2$ CFU (t = 2.42; df = 17; P = 0.0272), and at 12 h, with females having $3.38\pm1.42\times10^4$ and males having $2.50\pm1.08\times10^4$ CFU (t = 2.62; df = 17; P = 0.0177). Furthermore, at each time point, it was observed that more female flies had *E. coli* on their external surfaces than males, especially at 12 h where statistically the numbers of males and females positive were shown to be different(Fisher's exact test; P = 0.0197; Table 2.1), and only one male fly at each time point had bacteria on its surface (118 CFU at 4 h, 10 CFU at 12 h, and 690 at 24 h). Additionally, females had more GFP *E. coli* on their body surfaces at each time point even though there was high variation in CFU counts (486 ± 285.5 CFU at 4h, 76.3 ± 52.3 CFU at 12 h, and 42675 ± 41450.4 CFU at 24 h).

When we observed dissected male and female guts at the 4 h, the posterior gut of females was full with leftover fly food and the midgut was packed with manure (Fig. 2.2). In contrast, the anterior portion of the gut in male flies contained green liquid (sugar water). At 12 h, female flies retained green sugar water in the posterior region of the gut and manure in the anterior region, while the male flies had only manure in their gut (Fig. 2.2). At 24 h, both male and female flies appeared to primarily have green sugar water in their digestive tracts. All flies were culture positive for bacteria.

House fly procurement of GFP *S.* Typhimurium: Manure-only assay. The GFP *S.* Typhimurium concentration in manure increased over time from 9.17×10^{6} to 1.58×10^{7} CFU/g at 4 h to 7.16×10^{9} to 1.02×10^{10} CFU/g at 24 h (Fig. 2.1c; $F = 28.86$; df = 1,1; $P < 0.0001$). There was no significant difference in *S.* Typhimurium concentration in manure across treatments at each time point ($F = 0.21$; df = 2, 2; $P = 0.8113$).

In house flies, the amount of *S.* Typhimurium procured by males and females increased over time (Fig. 2.1c; $F = 55.15$; df = 1, 20; $P < 0.0001$). Also, there was a significant difference between the amount of *S.* Typhimurium acquired by females and males ($F = 5.15$; df = 1, 20; $P = 0.0056$). More specifically, there was a significant difference between sexes at 4 h, with females having $1.75\pm0.986\times10^{5}$ CFU and males having $1.06\pm0.383\times10^{4}$ CFU, ($t = 2.60$; df = 20; $P = 0.0172$) and 12 h, with females having $1.16\pm0.412\times10^{6}$ CFU and males having $4.04\pm1.71\times10^{4}$ CFU ($t = 3.10$; df = 20; $P = 0.0056$). Additionally, more females had *S.* Typhimurium on their external surface than males, even though not statistically different, (Fisher's exact test; $P > 0.05$; Table 2.1) and CFU counts from positive females were much higher and variable than those from males at each time point. Male fly CFU recovery on the surface was 0 CFU at 4 h, 80 CFU at 12 h, and 175 ± 165 CFU at 24 h in comparison to female fly CFU recovery of 10 CFU at 4 h, 1070 ± 915.6 CFU at 12 h, and 936088 ± 547042.3 CFU at 24 h.

Microscopy observations of dissected male and female digestive tracts revealed that both the anterior and mid regions of the gut of female flies were packed with manure while the hindgut contained leftover fly food at the 4 h time point; in contrast, the male fly guts contained no apparent ingested materials (Fig. 2.2). At 12 h, both male and female flies had large amounts of manure in the midgut, but at 24 h, both male and female digestive tracts contained only small

amounts of manure that was evenly dispersed throughout the gut. Subsequent cultures of dissected alimentary canals revealed all flies had recoverable bacteria.

House fly procurement of GFP *S.* Typhimurium: Manure-sugar assay. The concentration of *S.* Typhimurium in manure significantly increased over time in all treatments, ranging from 1.86×10^7 to 1.88×10^7 CFU/g at 4 h and 3.45×10^9 to 4.14×10^9 CFU/g at the 24 h (Fig. 2.1d; $F = 119.58$; df = 1, 1; $P < 0.0001$). There was no significant difference in *S.* Typhimurium concentration in manure across treatments at each time point ($F = 0.49$; df = 2, 2; $P = 0.6194$).

In house flies, there was a significant increase of *S.* Typhimurium procurement within sex over time (Fig. 2.1d; $F = 53.94$; df = 1, 20; $P < 0.0001$). Additionally, there was an overall significant effect of sex on the amount of *S.* Typhimurium found in females and males ($F = 5.52$; df = 1, 20; $P = 0.0292$). Significant differences in acquisition between males and females were evident at 4 h, with females having $4.34\pm1.44\times10^4$ CFU and males having $1.62\pm0.461\times10^3$ CFU ($t = 2.79$; df = 20; $P = 0.0114$) and 12 h, with females having $6.38\pm2.88\times10^5$ CFU and males having $1.60\pm0.769\times10^5$ CFU ($t = 3.65$; df = 20; $P = 0.0016$). Furthermore, the number of flies observed positive for *S.* Typhimurium on their body surface was higher in females except for at the 4 h time point where only one male and one female were positive (Table 2.1). At 24 h there was a statistical difference in the number of females positive for bacteria on their surface compared to males (Fisher's exact test; $P = 0.0202$). Additionally, females still had higher CFU counts on their surface than male flies.

At 4 h, microscopy showed that the female digestive tracts had leftover fly food in the posterior region and manure in the anterior region, while males had predominately green sugar water throughout the gut (Fig. 2.2). At 12 h, male flies retained manure in the crop in addition to

green sugar water, which was observed in the gut of both males and females. At 24 h, both sexes retained green sugar water in the crop, while females had a mixture of both green sugar water and manure in the gut and male guts contained predominately green sugar water. All flies were positive for bacteria by culture, even those where manure was no longer present.

Discussion

A viable microbial community has been shown to be necessary for house fly larval development (Zurek et al. 2000), and cattle manure acts as an optimal substrate (West 1951; Hanski 1987) as it provides essential nutrients and microbes such as bacteria. Although adults do not have a nutritional requirement for bacteria, they often associate with microbe-rich habitats for breeding purposes (Meyer and Shultz 1990). The combined effects of adult fly mobility and the presence of potential pathogens in manure pose a health concern to nearby areas housing domestic animals or livestock (Greenberg and Klowden 1972; Graczyk et al. 2001; Smallegange and den Otter 2007; Butler et al. 2010). A greater understanding of how house flies disseminate microbes to the surrounding environment involves identifying biological factors that influence the amount of bacteria they acquire and carry. One such factor may include fly sex, as female house flies would presumably associate more frequently with manure due to oviposition interest and have different nutritional requirements (West 1951; Larsen et al. 1966; Khan et al. 2012; Shah et al. 2016). Our study aimed at determining differences in bacteria acquisition by male and female house flies exposed to cattle manure inoculated with either *E. coli* or *S.* Typhimurium. We monitored the concentration of bacteria both in manure and house flies over 24 h and found that female house flies harbored higher amounts of bacteria over time than male flies both internally and externally, except for in the *E. coli* manure-only assay where there was no

difference in bacterial accumulation between males and females. This suggests that differences in the amount of manure ingested, the presence of an additional food source, and the species of bacteria present in the manure may influence whether females harbor more bacteria than males.

The amount of GFP *E. coli* in flies did not change across the 24 h exposure period despite *ad libitum* access to manure in both the manure-only and manure-sugar assays (Fig.1). A previous study demonstrated that starting at 4 h post ingestion, this same strain of GFP *E. coli* was enclosed and immobilized in food boluses in the house fly gut, and lysis of cells was observed in many flies (Kumar and Nayduch 2016). In our study, *E. coli* abundance inside the fly may reach stasis due to combined effects of lysis/immobilization and peristalsis along with subsequent ingestion (replacement) from manure. Despite no change in GFP *E. coli* over time within fly sex, there was a significant difference in bacterial abundance between males and females, but only in the manure-sugar assay. This suggests that female flies accumulate more bacteria than male flies even when there was second, sugar-rich food option. Studies indicate that female oviposition underlies behavioral differences in attraction to manure via olfaction (West 1951; De Bruyne and Baker 2008; Shah et al. 2016). This innate behavior in females leads to increased contact with manure while searching for the best site to deposit eggs. Our enumeration data was supported by comparing the distribution of material in male and female guts (Fig. 2.2), where manure was present in both males and females in the manure-only assay (Fig. 2.2), but is less evident in the manure-sugar assay (Fig. 2.2), where in males the anterior of fly digestive tracts primarily contained green sugar water and in females the anterior gut contained manure. This observation substantiates the significant difference in bacterial accumulation in male and female house flies in the manure-sugar assay, particularly at 4 h (Fig. 2.1b).

In contrast to GFP *E. coli*, there was a significant change in CFU of GFP *S.* Typhimurium cultured from flies over time within sex in both the manure-only and manure-sugar assays (Fig. 2.1). Persistence of GFP *S.* Typhimurium in house flies can be due to persistence combined with additive effect of subsequent ingestions and/or proliferation of bacteria in the gut. In a previous study, this same strain of GFP *S.* Typhimurium was found to persist and proliferate in the house fly gut for 24 h post-ingestion (Chifanzwa 2011). Survival and proliferation of GFP *S.* Typhimurium in the house fly gut was attributed to its resistance to an immune response involving antimicrobial peptides (AMPs). Resistance to or evasion of an epithelial immune response in *S.* Typhimurium, but not *E. coli*, is particularly interesting since both species are Gram-negative bacteria and have similar DAP-type peptidoglycan in their cell wall which should induce the dipteran Imd-pathway associated AMPs (Lemaitre and Hoffman 2007; Broderick et al. 2009). Maintenance of motility by *S.* Typhimurium, but not by *E. coli*, in the house fly alimentary canal may allow *S.* Typhimurium to evade AMPs, other immune effectors or digestive processes. Additionally, differences in the growth rate of *E. coli* and *S.* Typhimurium could have attributed to the difference in CFU recovery from flies over time. However, it is not known if the growth rate of *E. coli* and *S.* Typhimurium would be different at 28°C for flies like that observed for bacterial culture growth in 37°C.

There were differences in the abundance of *S.* Typhimurium CFU recovered from males and females in both manure only and manure-sugar assays, which was most evident at 4 and 12 h (Fig. 2.1), indicating that in both the presence and the absence of an alternate food source, females acquired more bacteria. A previous field study sampling flies from a dairy farm found that female flies more frequently carried *Salmonella* and also had higher bacterial abundance (Mian et al. 2002). As with our *E. coli* assays, visual comparison between the dissected guts of

male and female flies supported our enumeration observations (Fig. 2.2). At 4 h in the manure-only assays, female flies retained manure in the anterior portion of the gut while males did not (Fig. 2.2). At 24 h, however, the distribution of material in the gut appeared similar between males and females, both being less full of manure; at this time point, there was no significant difference in bacterial abundance between fly sexes. This suggests that regardless of what bacteria species was present, both males and females were ingesting the manure and eventually accumulated the same amount of bacteria. The initial time points could have yielded differences in bacterial abundance between sexes due to the amount of manure ingested early on. In the manure-sugar assay, females retained manure in the gut at 4 h, while male guts contained green sugar water (Fig. 2.2); likewise, male guts contained little to no apparent manure across 24 h. This implies that the behavior of house flies, e.g. their eating habits, influenced the amount of *S.* Typhimurium ingested.

Although it was not possible to compare statistically, bacterial accumulation appeared greater in flies exposed to *S.* Typhimurium compared to flies exposed to *E. coli*. These results could be related to the abundance and proliferation of *S.* Typhimurium in manure (Fig. 2.1), but also may indicate a difference in preference or attraction of adult house flies to the presence of specific microorganism species. Different bacterial microorganism odors, such as those produced by *Klebsiella oxytoca* found on house fly egg surfaces, have been demonstrated to attract or detract house flies for oviposition on animal manure (Lam et al. 2007) and provide information to help choose where to lay their eggs (Lam et al. 2010). Therefore, the difference in the concentration of *E. coli* and *S.* Typhimurium in manure over the 24 h period, although originally inoculated at the same concentration (approx. 10^6 CFU/g manure), could have influenced the

attractiveness to flies, especially females. Whether *S.* Typhimurium is more attractive to house flies than *E. coli* remains to be determined.

In our study, bacteria carried on the surface of flies also were enumerated. Although the CFU data were not statistically compared, due to small numbers of flies being positive for bacteria, we more often observed female flies with bacteria on their surface than males, especially at 12 h in the *E. coli* manure-sugar assays and at 24 h in the *S.* Typhimurium manure-sugar assays (Fisher's exact test; $P < 0.0202$; Table 2.1). This may be due to differences in contact with manure between male and female flies, and therefore is congruent with our whole fly enumeration results (Fig. 2.1). Interestingly, this may also suggest differences in grooming habits between male and female flies. Male house flies spend 8.6-50.9% of their activities grooming compared to 4.8-42.3% for female flies (Barber and Starnes 1949). Since males spend more time grooming compared to females, this could explain the lower number of males that tested positive for the presence of either bacterial species on their body surfaces. In *Drosophila melanogaster*, grooming behaviors can be initiated by activating chemoreceptors on the body surface using bacterial compounds (Yanagawa et al. 2014). While grooming, debris is removed from the surface and deposited into the surrounding environment (Zhukovskaya et al. 2013). House flies also have been shown to ingest material present groomed from their external surfaces (Nayduch and Burrus 2017). However, because viable bacteria such as *E. coli* O157:H7 can persist on the surface of house flies for up to 13 days after exposure to inoculated manure (Wasala 2010), fly grooming may not remove all bacteria from the body surface.

Although female house flies used for this study were gravid, there were only a few cases where eggs were observed within 24 h indicating reasons for manure visitation were not entirely attributable to oviposition attraction. Manure may have been of interest to female flies for

nutritional purposes, as it can serve as a source of protein and other essential nutrients (Hanski 1987). Thus, female flies would seek this food source to attain protein required for the next gonotrophic cycle (Spiller 1964; Moon 2009), irrespective of the species of bacteria present.

Overall, we determined that female house flies harbored greater amounts of bacteria than male flies when exposed to manure inoculated with two different bacterial species at early time points, except for when exposed to manure inoculated with *E. coli* in the manure-only assay. These results suggest that, for some bacteria species, female flies potentially may pose a higher risk of pathogen dissemination into nearby environments than male flies and that the presence or absence of an additional nutrient source may influence whether a difference in risk exists. Further research is needed to determine differences in subsequent excretion of these bacteria between male and female flies which would bolster dissemination potential. Since our study was conducted under laboratory conditions, it would be helpful to see if these sex-specific differences in bacteria acquisition also exist in wild flies naturally exposed to manure. Our findings emphasize the importance of considering fly sex in assessing risk for bacterial carriage which may help inform pest management strategies on farms where flies have access to manure.

Acknowledgments

We would like to say a very special thank you to D. Erram, E. Stricker, and K. Zurek for helping with manure collection and experimental house fly colony maintenance, making large amounts of media, and assisting with microscopy pictures respectively. This work was funded by USDA-ARS National Program 104, project number 3020-32000-007. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of

Agriculture. Any opinions, findings, conclusion, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture.

References Cited

Ahmad, A., T. G. Nagaraja, and L. Zurek. 2007. Transmission of *Escherichia coli* O157: H7 to cattle by house flies. Prev. Vet. Med. 80: 74-81.

Barber, G. W., and E. B. Starnes. 1949. The activities of house flies. J. N. Y. Entomol. Soc. 57: 203-214.

Butler, J.F., A.G. Marunaki, F. Meek, and J.E. Marunaki. 2010. Wild Florida house flies (*Musca domestica*) as carriers of pathogenic bacteria. Fla. Entomol. 93: 218-223.

Broderick, N. A., Welchman, D. P., and B. Lemaitre. 2009. Recognition and response to microbial infection in *Drosophila*, pp. 13-33. *In* J. Rolff and S. E. Reynolds (eds.), Insect infection and immunity: evolution, ecology, and mechanisms. Ox. Univ. Pr. NY.

Chifanzwa, R. 2011. House fly (*Musca domestica* L.) temporal and spatial immune response to *Streptococcus pyogenes* and *Salmonella typhimurium*: role of pathogen density in bacterial fate, persistence and transmission. M. S. thesis, Georgia Southern University, Statesboro.

De Bruyne, M. and T. C. Baker. 2008. Odor detection in insects: volatile codes. J. Chem. Ecol. 34: 882-897.

Giri, R. P. 2015. Role of muscoid flies in the ecology of shiga toxin-producing *Escherichia coli* (STEC) in confined cattle environments. M. S. thesis, Kansas State University, Manhattan.

Glaser, R. W. 1923. The effect of food on longevity and reproduction in flies. J. Exp. Zool. 38: 383–412.

Graczyk, T.K., R. Knight, R. H. Gilman, and M. R. Cranfield. 2001. The role of non-biting flies in the epidemiology of human infectious diseases. Microbes Infect., 3: 231–235.

Greenberg, B., and M. Klowden. 1972. Enteric bacterial interactions in insects. Amer. J. Clin. Nutr. 25: 1459-1466.

Gupta, A. K., D. Nayduch, P. Verma, B. Shah, H. V. Ghate, M. S. Patole, and Y. S. Shouche. 2012. Phylogenetic characterization of bacteria in the gut of house flies (*Musca domestica* L.). FEMS Micro. Ecol. 79: 581-593.

Hancock, D. D., D. H. Rice, D. E. Herriott, T. E. Besser, E. D. Ebel, and L. V. Carpenters. 1997. Effects of farm manure-handling practices on *Escherichia coli* O157 prevalence in cattle. J. Food Prot. 4: 349-453.

Hanski, I. 1987. Nutritional ecology of dung- and carrion-feeding insects, pp. 837–884. *In* F. Slansky Jr. and J. G. Rodriguez (eds.), Nutritional ecology of insects, mites, and spiders. John Wiley & Sons Inc. New York, USA.

Himathongkham, S., S. Bahari, H. Riemann, and D. Cliver. 1999. Survival of *Escherichia coli* O157: H7 and *Salmonella typhimurium* in cow manure and cow manure slurry. FEMS Micro. Letters. 178: 251-257.

Holt, P. S., C. J. Geden, R. W. Moore, and R. K. Gast. 2007. Isolation of *Salmonella enterica* serovar Enteritidis from houseflies (*Musca domestica*) found in rooms containing *Salmonella* serovar Enteritidis-challenged hens. Appl. Environ. Microbiol. 73: 6030-6035.

Joyner, C., M. K. Mills, and D. Nayduch. 2013. *Pseudomonas aeruginosa* in *Musca domestica* L.: Temporospatial examination of bacteria population dynamics and house fly antimicrobial responses. PLoS ONE. 8: e79224–9.

Khan, H. A. A., S. A. Shad, and W. Akram. 2012. Effect of livestock manures on the fitness of house fly, *Musca domestica* L. (Diptera: Muscidae). Parasitol. Res. 111: 1165-1171.

Kumar, N. H. V., and D. Nayduch. 2016. Dose-dependent fate of GFP-expressing *Escherichia coli* in the alimentary canal of adult house flies. Med.Vet. Entom. 30: 218-228.

Lam, K., D. Babor, B. Duthie, E. M. Babor, M. Moore, and G. Gries. 2007. Proliferating bacterial symbionts on house fly eggs affect oviposition behavior of adult flies. Anim. Behav. 74: 81-92.

Lam, K., M. Tsang, A. Labrie, R. Gries, and G. Gries. 2010. Semiochemical-mediated oviposition avoidance by female house flies, *Musca domestica*, on animal feces colonized with harmful fungi. J. Chem. Ecol. 36: 141-147.

Larsen, J. R., R. E., Pfadt, and L. G. Peterson. 1966. Olfactory and oviposition responses of the house fly to domestic manures, with notes on an autogenous strain. J. Econ. Entomol. 59: 610-615.

Lemaitre, B., and J. Hoffmann. 2007. The host defense of *Drosophila melanogaster*. Annu. Rev. Immunol. 25: 697-743.

Losinger, W. C., S. J. Wells, L. P. Garber, H. S. Hurd, and L. A. Thomas. 1995. Management factors related to *Salmonella* shedding by dairy heifers. J. Dairy Sci. 78: 2464-2472.

McGaughey, J. and D. Nayduch. 2009. Temporal and spatial fate of GFP-expressing motile and nonmotile *Aeromonas hydrophila* in the house fly digestive tract. J. Med. Entomol. 46: 123-130.

Meyer, J., and T. Shultz. 1990. Stable fly and house fly breeding sites on dairies. Cal. Ag. 44: 28-29.

Mian, L. S., H. Maag, and J. V. Tacal. 2002. Isolation of *Salmonella* from muscoid flies at commercial animal establishments in San Bernardino County, California. J. Vect. Ecol. 27: 82-85.

Moon, R. D. 2009. Muscid flies (*Muscidae*), pp. 275-306. *In* G. Mullen and L. Durden (eds.), Medical and Veterinary Entomology. Academic Press, San Diego, CA.

Nayduch, D., and R. G. Burrus. 2017. Flourishing in filth: house fly-microbe interactions across life history. Ann. Entomol. Soc. Am. (in press, this issue)

Sasaki, T., M. Kobayashi, and N. Agui. 2000. Epidemiological potential of excretion and regurgitation by *Musca domestica* (Diptera: Muscidae) in the dissemination of *Escherichia coli* O157: H7 to food. J. Med. Entomol. 37: 945-949.

Shah, R. M., F. Azhar, S. A. Shad, W. B. Walker III, M. Azeem, and M. Binyameen. 2016. Effects of different animal manures on attraction and reproductive behaviors of common house fly, *Musca domestica* L. Parasitol. Res. 115: 3585-3598.

Smallegange, R. C., and C. J. den Otter. 2007. Houseflies, annoying and dangerous, pp. 241-260. *In* W. Takken and B. G. J. Knols (eds.), Emerging pests and vector-borne diseases in

Europe: ecology and control of vector-borne diseases, vol. 1. Wageningen Academic Publishers, The Netherlands.

Spiller, D. 1964. Nutrition and diet of muscoid flies. Bull. Wld. Hlth. Org., 31: 551-554.

Wasala, L. P. 2010. Dissemination of *Escherichia coli* O157: H7 to the Spinach, *Spinacea oleraceae* Phylloplane by House Flies, *Musca domestica* L. M. S. thesis, Oklahoma State University, Stillwater.

West, L. S. 1951. The housefly: its natural history, medical importance, and control. Comstock Publishing Company Inc., Ithaca, NY.

Yanagawa, A., A. M. Guigue, and F. Marion-Poll. 2014. Hygienic grooming is induced by contact chemicals in *Drosophila melanogaster*. Front. Behav. Neurosci. 8: 1-9.

Yap, K. L., M. Kalpana, and H. L. Lee. 2008. Wings of the common house fly (*Musca domestica* L.): importance in mechanical transmission of *Vibrio cholerae*. Trop. Biomed. 25: 1-8.

Zhukovskaya, M., A. Yanagawa, and B. T. Forschler. 2013. Grooming behavior as a mechanism of insect disease defense. Insects. 4: 609-630.

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 2.1. The proportion of flies positive for presence of bacteria on their external body surface and total colony forming units (CFU) of bacteria (Mean ± SEM values) recovered.

	E. coli				*S.* Typhimurium			
	Female		Male		Female		Male	
Assay/time	# Pos. (%)	Mean ± SEM Range	# Pos. (%)	Mean ± SEM Range	# Pos. (%)	Mean ± SEM Range	# Pos. (%)	Mean ± SEM Range
Manure-only								
4 h	3 (15)	116.7 ± 86.7 30 – 290	0 (0)	0 ± 0 -	1 (5)	10 ± 0 10	0 (0)	0 ± 0 -
12 h	5 (25)	446 ± 376.8 10 – 1950	2 (10)	90 ± 30 60 – 120	3 (15)	1070 ± 915.6 100 – 2900	1 (5)	80 ± 0 80
24 h	4 (20)	550 ± 286.7 10 – 1070	0 (0)	0 ± 0 -	6 (30)	936088 ± 547042.3 130 – 2470000	2 (10)	175 ± 165 10 – 340
Manure-sugar								
4 h	5 (25)	486 ± 285.5 40 – 1530	1 (5)	118 ± 0 118	1 (5)	10 ± 0 10	1 (5)	20 ± 0 20
12 h	8 (40)	76.3 ± 52.3 10 – 440	1 (5)	10 ± 0 10	3 (15)	1216.7 ± 1102.5 190 – 3420	0 (0)	0 ± 0 -
24 h	4 (20)	42675 ± 41450.4 50 – 167000	1 (5)	690 ± 0 690	6 (30)	1044232 ± 1031173 30 – 6200000	0 (0)	0 ± 0 -

Only flies that were positive for bacteria on their surface were included in the mean and range of surface bacteria CFU counts.

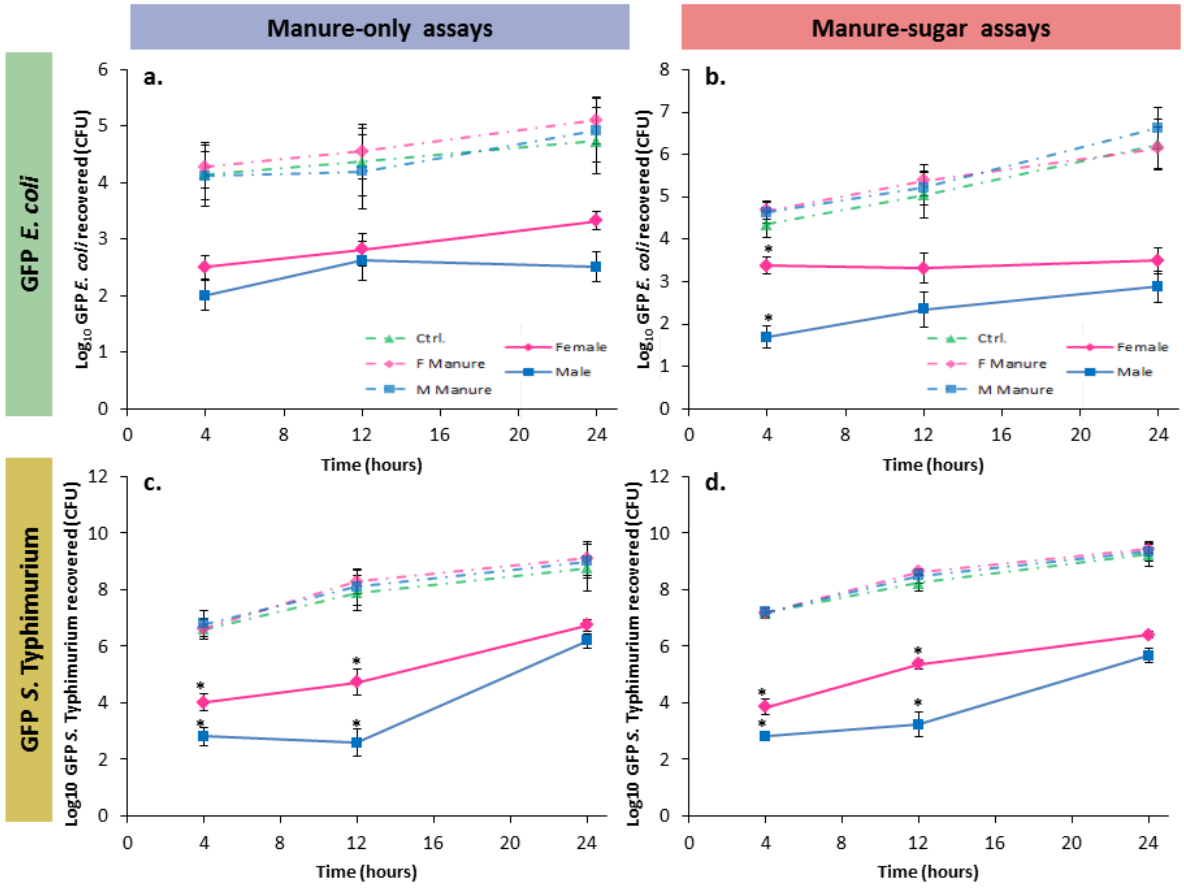

Figure 2.1. Enumeration of GFP *E. coli* or GFP *S.* Typhimurium from manure and house flies.

Manure-only assays are represented in panels a and c and manure-sugar assays are represented in panels b and d. Bacteria (GFP *E. coli* or GFP *S.* Typhimurium) were inoculated into manure and flies were exposed for 24 h. Flies (n = 5) and manure (1.0 g) were collected at 3 time points, and colony forming units (CFU) were enumerated on selective media; mean CFU are shown for 4 biological replicates. Error bars represent SEM of CFU of bacteria from flies at each time point. Bacterial abundance increased ($P > 0.05$) in manure (dashed lines) over time in all treatments (b-d) except the manure-only *E. coli* assay (a). There was no difference in the bacterial abundance from manure in containers that contained male, female or no flies (blue, pink and green dashed lines, respectively). There was a significant difference in bacterial abundance from flies (solid lines) over time, within sex, across all treatments ($P < 0.05$; b-d) except for manure-only *E. coli* assay ($P > 0.05$; a). Pairwise comparisons of CFU abundance at each time point in male (blue) and female (pink) flies revealed significant differences, especially at early time points ($P < 0.05$, indicated by *). Bacterial growth in manure was compared using a regression model in JMP and CFU abundance in flies was compared using the GLIMMIX procedure in SAS.

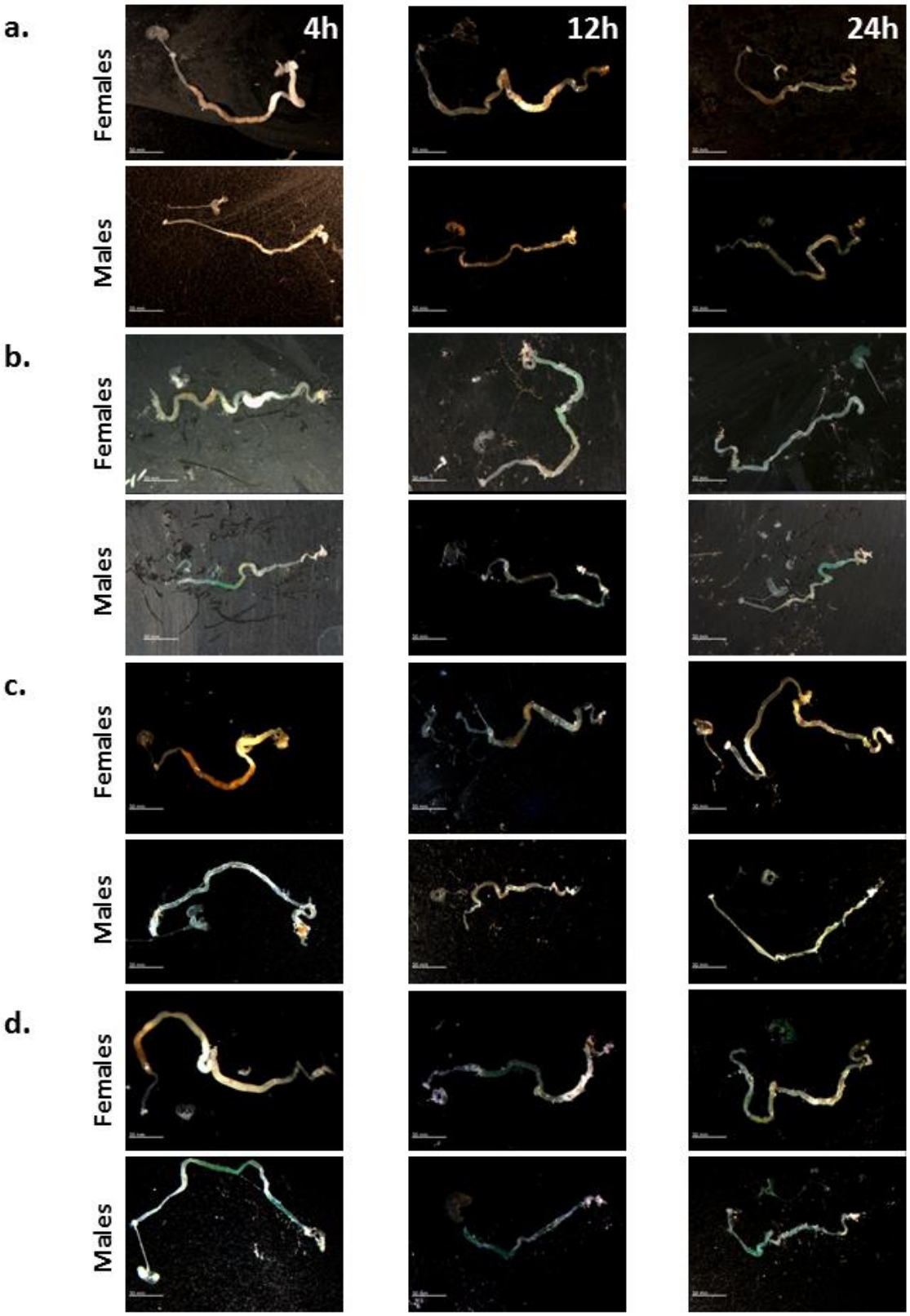
a.
b.
c.
d.
4h
12h
24h
Females
Males
Females
Males
Females
Males
Females
Males

Figure 2.2. Alimentary canals of male and female house flies exposed to manure with GFP *E. coli* or GFP *S*. Typhimurium.

Male and female flies were exposed to manure inoculated with bacteria, either GFP *E. coli* or GFP *S.* Typhimurium, in assays where only manure was present (top panel) or both manure and sugar water with green dye were present (bottom panel). Each picture is a representative view of the appearance of male (n = 5) or female (n = 5) house flies at 4, 12, and 24 h after exposure. Anterior of gut (i.e. crop and proventriculus) is oriented on the left of each image, and the remainder continues to the right as foregut, midgut, hindgut and rectum. Food can be discriminated by color: manure (brown), fly food (white) and sugar water (green). Arrows provided give example of colors to look for. Scale bar = 5 mm.

Chapter 3 - Cantaloupe facilitates *Salmonella* Typhimurium survival within and transmission among adult house flies (*Musca domestica* L.)

Soon to be submitted for publication in *Foodborne Pathogens and Disease*

Abstract

Salmonella enterica ser. Typhimurium (*S.* Typhimurium) is a pathogen harbored by livestock that causes food-borne illness in humans. We have previously demonstrated that both male and female adult house flies acquire and harbor *S.* Typhimurium (ST) after exposure to inoculated cattle manure. Factors facilitating acquisition, survival and transmission of *S.* Typhimurium, and fly to fly transmission have not been determined. We investigated the transmission of green fluorescent protein (GFP) *S.* Typhimurium from infected flies to cantaloupe, from inoculated cantaloupe to flies, and from infected (ST+) to uninfected (ST-) flies in the presence and absence of cantaloupe. Mated adult female house flies (5-7 d old) were exposed to manure inoculated with either sterile PBS or GFP *S.* Typhimurium resuspended in PBS for 12 h and then used in 4 different experiments. Experiment 1: To test for survival of GFP *S.* Typhimurium, flies were placed individually in jars and bacterial abundance in flies was monitored over 24 h. Experiment 2: To assess fly to food transmission of GFP *S.* Typhimurium, a single ST+ fly was placed in a jar containing fresh cantaloupe and transmission to fruit was monitored over 24 h. Experiment 3: To test for food to fly GFP *S.* Typhimurium transmission, four ST- flies were placed in jars containing GFP *S.* Typhimurium-inoculated cantaloupe, and acquisition of bacteria by flies was monitored over 24 h. Experiment 4: To assess fly to fly

transmission of GFP *S.* Typhimurium in the presence and absence of fresh cantaloupe, a single ST+ fly and four ST- flies were placed in jars either with or without fresh cantaloupe and transmission of bacteria among the flies was monitored over 24 h. In all experiments, flies were processed and cultured at 0, 6, 12, and 24 h after experimental setup for GFP *S.* Typhimurium abundance and in experiments 2-3, cantaloupe was processed in a Stomacher®400 Circulator and cultured at 6, 12, and 24 h after experimental setup to measure GFP *S.* Typhimurium concentration. Our results revealed that GFP *S.* Typhimurium survived in ST+ flies but that the abundance of GFP *S.* Typhimurium in ST+ flies decreased between 0 and 6 h after experimental setup when cantaloupe was absent (Experiment 1) and decreased between 0 and 6 h and 6 and 24 h after experimental setup when cantaloupe was present (Experiment 2). The food to fly GFP *S.* Typhimurium transmission examined in Experiment 3 showed that ST- flies acquired GFP *S.* Typhimurium from inoculated cantaloupe and that bacterial abundance increased in both cantaloupe and flies from 6 - 24 h after experimental setup. Experiment 4 demonstrated that more ST- flies were positive for GFP *S.* Typhimurium when cantaloupe was present for 24 h than when cantaloupe was absent. We infer that flies successfully transfer GFP *S.* Typhimurium to, and become infected from, cantaloupe and that the presence of a shared food source facilitates fly to fly transfer of bacteria. Understanding the dynamics of fly bacterial acquisition and transmission of bacteria between flies and food can help in determining the risk flies pose to food safety and human health.

Keywords: House flies, *Salmonella enterica*, pathogen transmission, cantaloupe

House flies are synanthropic nuisance pests that pose a risk to human and animal health worldwide (Moon 2019). Adult flies associate with animal excrement and feed upon human food items which raises concern for public food safety (West 1951, Graczyk et al. 2001, Gupta et al. 2012, Nayduch and Burrus 2017). House flies can also fly distances as far as 30 km (Baldacchino et al. 2017) which allows them to serve as long-range bridges between sanitary and unsanitary habitats.

Salmonella enterica ser. Typhimurium (*S.* Typhimurium) is a bacterial pathogen that can cause mild to severe illness in humans (Andino and Hanning 2015) and manifest as nontyphoidal salmonellosis. This pathogen also colonizes the alimentary canal of livestock and is often shed in their dung (Himathongkham et al. 1999, Holt et al. 2007). We have previously shown that *S.* Typhimurium can survive in the house fly digestive tract for at least 24 h post-ingestion and can be acquired by house flies from inoculated cattle manure (Chifanzwa and Nayduch 2017, Thomson et al. 2017). Furthermore, we have demonstrated that viable *S.* Typhimurium is excreted by house flies, thus, implicating them as potential vectors (Nayduch et al. 2018).

Flies harbor bacteria externally on their body surfaces and internally by ingestion either via direct feeding or indirectly via grooming behaviors (Jacques et al. 2017, Nayduch and Burrus 2017). Ingested bacteria can be excreted from flies by regurgitation or defecation (Nayduch et al. 2018). The gregarious nature of house flies and behaviors such as trophallaxis, allo-coprophagy, and co-feeding, provides opportunities for transmitting pathogens from house flies to human food items and both between and among flies (West 1951, Hanski 1987). Several studies have already described the potential of house flies to contaminate dairy and meat products with food-borne pathogens (Nayduch et al. 2002, De Jesús et al. 2004; Macovei et al. 2008, Pace et al.

2017), but very few have focused on their potential to contaminate fresh produce, more specifically fruit. Fruits are very attractive food sources to house flies due to their high sugar content (Greenberg 1959, Moon 2019). Because *S.* Typhimurium can survive and grow on fruits such as cantaloupe (Chimbombi 2010, Bennett et al. 2018, Huang et al. 2018), the presence of fruit may facilitate bacterial acquisition from and transmission between house flies.

Our study objectives were to determine if GFP *S.* Typhimurium (1) persists in house flies, (2) can be transferred from house flies to cantaloupe, (3) is acquired from cantaloupe by flies, and (4) is transferred between house flies in the absence and presence of a shared food source (cantaloupe). We hypothesized that GFP *S.* Typhimurium would survive in house flies and that flies harboring GFP *S.* Typhimurium would transfer the GFP *S.* Typhimurium to cantaloupe. Furthermore, we hypothesized that cantaloupe would serve as a suitable substrate for GFP *S.* Typhimurium growth and that flies not harboring GFP *S.* Typhimurium would acquire GFP *S.* Typhimurium from contaminated cantaloupe. We also hypothesized that GFP *S.* Typhimurium would be transferred to other flies regardless of cantaloupe presence.

Materials and methods

House flies. Flies used in this study came from a colony that uses cattle manure as larval substrate (established September 2014). Flies used to create this manure-reared colony originated from the parent Kansas State University colony (Thomson et al. 2017). Mixed-sex adult flies were provided sugar cubes and water *ab libitum*. Egg powder was given to the adult flies the day prior to an experiment to induce egg development in mated females. All flies used in the experiments below were 5-7 d old gravid females. Specimens used in this research are deposited as voucher number 257 in the KSU Museum of Entomological and Prairie Arthropod Research.

GFP *Salmonella enterica* Ser. Typhimurium (*S.* Typhimurium). A recombinant green fluorescent protein (GFP) expressing strain of *S.* Typhimurium SR-11 conferring kanamycin- and ampicillin-resistance was used in this study (Chifanzwa and Nayduch 2017). Bacterial cultures were incubated at 37˚C in either tryptic soy broth (TSB) or agar (TSA) containing antibiotics (50 µg/ml w/v each of ampicillin sodium (Amp) and kanamycin sulfate (Kan); Fisher Scientific, Atlanta, GA, USA) for plasmid maintenance and selective culture. Seed culture was prepared by inoculating a single GFP *S.* Typhimurium colony into 100 µl of TSB Amp/Kan and incubating for 19 hours with 60 rpm rotation. For each experiment, GFP *S.* Typhimurium inoculum was prepared by transferring 75 µl of the seed culture into 15 ml TSB Amp/Kan and incubating for 1 h at 37°C with 100 rpm rotation. Bacterial inoculum was then transferred to manure as described in the pre-experimental setup below. GFP *S.* Typhimurium concentration was determined by serial dilution, duplicate plating on TSA, 24 h incubation at 37˚C, and total GFP colony enumeration.

Cantaloupe preparation. Pre-cut cantaloupe was purchased from a local grocery store chain the day before each assay and was kept at 4°C overnight. Cantaloupe was cut into pieces weighing approximately 10 g each before experimental use in Experiments 2, 3 and 4, below.

Pre-experimental setup: Exposure of house flies to manure. Cattle manure was collected from a manure slurry pile at the Kansas State University dairy unit on November 16, 2016. Approximately 1600 g of manure were sterilized by autoclaving, then individual 200 g aliquots were frozen for later use. For each assay, flies were exposed to 30 g autoclaved manure that was inoculated with either 500 ml sterile PBS (for control flies) or 500 ml of $5.25 \pm 0.72 \times 10^5$ CFU GFP *S.* Typhimurium resuspended in PBS (for *S.* Typhimurium flies) as described in Thomson et

al. 2017. For fly exposure, flies were placed in half-gallon plastic canisters containing a 60 × 15mm plastic petri dish (Fisherbrand) with inoculated manure (as above) and an additional 150 µl droplet of 10% sucrose containing green food coloring on parafilm (provided as an alternate food source, to remain consistent with our previous study; Thomson et al. 2017). Twenty-five adult female flies were added to the canister and allowed to feed *ad libitum* for 12 h at room temperature (25°C - 27°C).

Experiment 1: GFP *S.* Typhimurium survival in house flies. Twenty-five female flies were exposed to manure inoculated with GFP *S.* Typhimurium, as described in the pre-experimental setup. After the 12 h exposure, 1 g of manure and five flies were removed for processing and bacterial culture. To determine the GFP *S.* Typhimurium concentration in the manure (colony forming units (CFU)/g), a 1 g sample was suspended in 19 ml of sterile PBS and homogenized by vortexing for 3 min. To determine the concentration of GFP *S.* Typhimurium in the house flies after manure exposure, flies were individually homogenized in 1 ml PBS. Serial dilutions of manure or fly homogenate were plated in duplicate on TSA Amp/Kan and incubated at 37°C for 24 h, after which CFUs of GFP *S.* Typhimurium were enumerated.

To determine the survival of GFP *S.* Typhimurium in flies over time, 15 of the remaining flies were removed from the canister and individually placed in separate 32 oz. polypropylene jars. Jars were secured with sealing wrap (with small air holes provided) and maintained at room temperature (25°C - 27°C) until processing. At 6, 12, and 24 h after removal from manure, 5 jars were chilled and flies (n=5) were removed, individually homogenized, and processed for GFP *S.* Typhimurium culture and enumeration as described above. In addition, to investigate GFP *S.* Typhimurium survival in house fly excrement, the number of excreta droplets left in each jar

after fly removal was recorded and the inside of each jar was swabbed and streaked on TSA Amp/Kan. The experiment was replicated three times.

Experiment 2: Transmission of GFP *S.* Typhimurium from flies to cantaloupe. As in Experiment 1, 25 female flies were exposed to manure inoculated with GFP *S.* Typhimurium and after 12 h manure (1 g) and flies (n=10) were removed and processed to culture and enumerate GFP *S.* Typhimurium. The remaining flies (n=15) were placed into individual 32 oz. polypropylene jars and maintained at room temperature. A 10 g piece of cantaloupe was placed in a small petri dish in each jar. As in Experiment 1, flies (n=5) were removed and processed at three time points (6, 12, 24 h). At each time point, the cantaloupe pieces (n=5) were also removed to culture GFP *S.* Typhimurium. Cantaloupe pieces were individually processed by placing in a Stomacher®400 Classic closure bag (177 × 305mm, 80 - 400 ml; Seward Limited, Roman Way, United Kingdom) with an equivalent ml/g ratio of sterile PBS and homogenizing in a Stomacher®400 Circulator (Seward Limited, Roman Way, United Kingdom) at 260 rpm for 10 min until samples were liquefied. Cantaloupe homogenate was serially diluted and cultured in the same manner as flies and manure samples from Experiment 1. Excreta droplets in the jar were enumerated and collected with a sterile swab for GFP *S.* Typhimurium culture. The experiment was repeated three times.

Experiment 3: Acquisition of GFP *S.* Typhimurium by flies from inoculated cantaloupe. Fifty female house flies (two control canisters with n=25 flies in each) were exposed to manure inoculated with PBS and after 12 h, flies (n=5) and manure (1 g) were processed from both canisters as in Experiment 1, to verify absence of GFP *S.* Typhimurium. A subset of the remaining control flies (n=24 equally selected from both canisters) were placed in six 32-oz polypropylene jars (n=4 flies/jar) with a 10 g piece of cantaloupe inoculated with a mean of 165

± 11 CFU GFP *S.* Typhimurium in a 2 µl droplet placed on the surface. This number of bacteria was selected because it fell within the range of GFP *S.* Typhimurium that flies excrete (Nayduch et al. 2018) and was determined by suspending 2 µl from the GFP *S.* Typhimurium inoculum used in 100 µl PBS and then culturing on TSA Amp/Kan as described above. To monitor bacterial growth on cantaloupe, six jars were setup with inoculated cantaloupe alone (no flies) which served as controls. At 6, 12, and 24 h post-inoculation two control and two treatment jars were processed as in Experiment 2 to culture GFP *S.* Typhimurium from flies, cantaloupe, and jar swabs. The experiment was repeated three times.

Experiment 4: Transmission of GFP *S.* Typhimurium between flies with and without cantaloupe. Twenty-five female house flies were exposed to manure inoculated with GFP *S.* Typhimurium (1 canister) and fifty females were exposed to manure inoculated with PBS (2 control canisters with n=25 each). After 12 h, manure (1 g) and flies (n=10) were removed and processed from each canister to either culture and enumerate GFP *S.* Typhimurium or verify absence of GFP *S.* Typhimurium. One GFP *S.* Typhimurium-exposed (ST+) fly and 4 PBS-exposed (ST-) flies were placed into each of six 32 oz. polypropylene jars and maintained at room temperature. A 10 g piece of cantaloupe was placed on a petri dish in 3 of the 6 jars while the other 3 jars contained only the ST+ and ST- flies (no cantaloupe present). After 24 h exposure, all flies, cantaloupe, and jar swabs were processed for GFP *S.* Typhimurium culture, as described above. The experiment was repeated 3 times.

Statistical analyses. A generalized linear mixed model (GLMM; Proc GLIMMIX in SAS) with a Gaussian distribution, identity link and restricted pseudo-likelihood estimation was used to determine if the abundance or concentration of GFP *S.* Typhimurium in flies or cantaloupe varied over time in Experiments 1 and 2, respectively. The outcome consisted of the log10

transformed colony forming units (CFU) of GFP *S.* Typhimurium in flies or the log10 CFU/g of GFP *S.* Typhimurium in cantaloupe. Time (0, 6, 12 and 24 h) was included as a fixed effect and trial as a random intercept.

For Experiment 3, a similar model was built to determine the effect of the concentration of GFP *S.* Typhimurium in cantaloupe over time with the abundance of GFP *S.* Typhimurium in flies. The outcome consisted of the log10 of CFU of GFP *S.* Typhimurium in flies whereas independent variables included the concentration of GFP S. Typhimurium in cantaloupe (in log10 CFU/g), time (6, 12, and 24 h) and a two-way interaction term between concentration of GFP *S.* Typhimurium in cantaloupe and time. Additionally, a GLMM with a Gaussian distribution, identity link and restricted pseudo-likelihood estimation was used to determine whether the presence or absence of flies affected the GFP *S.* Typhimurium concentration on cantaloupe over time. The outcome consisted of the log10 CFU/g of GFP *S.* Typhimurium in cantaloupe and fixed effects consisted of time, cantaloupe type (with or without flies), and an interaction term between time and cantaloupe type. Trial was included as a random effect.

For all experiments, a Chi-square test was employed (Proc FREQ and compprop macro in SAS version 9.4 (SAS Institute Inc., Cary, NC); Zarr 1999) to compare raw proportions (number of flies positive for GFP *S.* Typhimurium/total number of flies tested or the number of cantaloupe pieces positive for GFP *S.* Typhimurium/total number of cantaloupe pieces tested). GLMMs were used to determine the effect of time on: (1) the proportion of flies positive for GFP *S.* Typhimurium and (2) the proportion of cantaloupe positive for GFP *S.* Typhimurium (both outcomes are described below). A binomial distribution, logit link and restricted pseudo-likelihood estimation were used. Time (categorical: 0, 6 and 24 h) was included as an

independent variable, except for Experiment 4 where time was not a variable, and a random intercept for trial was incorporated to account for clustering of observations within trial.

In Experiments 1 and 2, a linear mixed model with a negative binomial distribution, logit link and restricted pseudo-likelihood estimation was fitted in SAS to determine the effect of time on the number of excreta droplets recovered from the jars. The outcome consisted of the mean number of excreta droplets counted from jars. Time (0, 6, 12 and 24 h) was included as a fixed effect and trial as a random intercept.

The Tukey-Kramer procedure was used to adjust for multiple comparisons in all linear mixed models. Mean log10 CFU, mean proportions, mean excreta droplets, and their 95% confidence intervals were computed. *P*-values < 0.05 were used to determine statistical significance. For experiments where jars were swabbed and cultured to determine presence or absence of GFP *S.* Typhimurium, no statistical analyses were performed.

Results

Experiment 1: GFP *S.* Typhimurium survival in house flies. The abundance of GFP *S.* Typhimurium in flies significantly decreased from $2.18 \pm 0.31 \times 10^5$ CFU/fly at 0 h to $2.54 \pm 0.31 \times 10^1$ CFU/fly at 6 h (Fig. 3.1a; $P < 0.001$). Over the 24 h period, there was no significant difference between bacterial abundance in flies at 6 h ($2.54 \pm 0.31 \times 10^1$ CFU/fly) and 12 h ($1.96 \pm 0.31 \times 10^1$ CFU/fly; P=0.432) as well as between flies at 12 h ($1.96 \pm 0.31 \times 10^1$ CFU/fly) and 24 h ($6.17 \pm 0.31 \times 10^0$ CFU/fly; P=0.653). Overall, we did not observe any effect of time on the proportion of flies positive for GFP *S.* Typhimurium (Fig. 3.1a; P=0.580), but there was a significant difference in the proportion of flies positive for GFP *S.* Typhimurium at 0 h (15/15) compared to flies from other collection times (P=0.001). Also, there was no significant

difference in the number of excreta droplets collected from the jars over time. (Table 3.1; *P*=0.6765). Interestingly, no GFP *S.* Typhimurium was recovered from excreta droplets from the jars except for 1/15 jars at 12 h (Table 3.2).

Experiment 2: Transmission of GFP *S.* Typhimurium from flies to cantaloupe. The abundance of GFP *S.* Typhimurium in flies decreased from $2.63 \pm 0.33 \times 10^5$ CFU/fly at 0 h to $1.08 \pm 0.33 \times 10^3$ CFU/fly at 6h ($P < 0.001$) and again decreased from $1.08 \pm 0.33 \times 10^3$ CFU/fly at 6 h to $8.00 \pm 0.33 \times 10^1$ CFU/fly at 24 h (Fig. 3.1b; *P*=0.022), however there was no significant decrease in GFP *S.* Typhimurium abundance between 6 h ($1.08 \pm 0.33 \times 10^3$ CFU/fly) and 12 h ($1.79 \pm 0.33 \times 10^2$ CFU/fly; *P*=0.183). Additionally, there was no significant difference between bacterial abundance at 12 h ($1.79 \pm 0.33 \times 10^2$ CFU/fly) and 24 h ($8.00 \pm 0.33 \times 10^1$ CFU/fly; *P*=0.798). While there was no overall significant effect of time on the proportion of flies positive for GFP *S.* Typhimurium (Fig. 3.1b, *P*=0.7845), there was a significant difference in the proportion of flies positive for GFP *S.* Typhimurium at 0 h (15/15) compared to flies at 24 h (9/15; *P*=0.018). The total concentration of GFP *S.* Typhimurium in cantaloupe increased from $0.00 \pm 0.18 \times 10^0$ CFU/g at 0 h to $1.45 \pm 0.25 \times 10^1$ CFU/g at 12 h (Fig 3.1b; *P*=0.002), but there was no significant difference in bacterial abundance between 0 h ($0.00 \pm 0.18 \times 10^0$ CFU/g) and 6 h ($0.24 \pm 0.25 \times 10^0$ CFU/g; *P*=0.584), between 6 h ($0.24 \pm 0.25 \times 10^0$ CFU/g) and 12 h ($1.45 \pm 0.25 \times 10^1$ CFU/g; *P*=0.133) or between 12 h ($1.45 \pm 0.25 \times 10^1$ CFU/g) and 24 h ($1.88 \pm 0.45 \times 10^1$ CFU/g; Fig. 3.1b; *P*=0.989). Although time did not significantly affect the proportion of cantaloupe positive for GFP *S.* Typhimurium overall (Fig. 3.1b; *P*=0.245), there was a significant difference in the proportion of cantaloupe positive for GFP *S.* Typhimurium at 0 h (0/15) compared to 12 h (8/15, *P*=0.001) and 0 h (0/15) compared to 24 h (9/15, *P*=0.001). The mean number of excreta droplets collected from jars differed over time (Table 3.1; $P < 0.001$).

More specifically, there was an increase in the mean number of excreta droplets between 6 and 12 h (P=0.012), 12 and 24 h (P=0.001), and 6 and 24 h ($P < 0.001$). GFP *S.* Typhimurium was recovered from excreta droplets of 5/15 jars at both 6 and 12 h time points and only 3/15 jars at 24 h (Table 3.2).

Experiment 3: Acquisition of GFP *S.* Typhimurium by flies from inoculated cantaloupe.

The abundance of GFP *S.* Typhimurium in flies increased from $2.70 \pm 0.31 \times 10^0$ CFU/fly at 6 h to $1.99 \pm 0.31 \times 10^1$ CFU/fly at 12 h (Fig 3.1c; P=0.004), from $1.99 \pm 0.31 \times 10^1$ CFU/fly at 12 h to $9.87 \pm 0.31 \times 10^1$ CFU/fly at 24 h (P=0.024), and from $2.70 \pm 0.31 \times 10^0$ CFU/fly at 6 h to $9.87 \pm 0.31 \times 10^1$ CFU/fly at 24 h ($P < 0.001$). Overall, time influenced the proportion of flies positive for GFP *S.* Typhimurium (Fig. 3.1c; P=0.011). The proportion of flies positive for GFP *S.* Typhimurium increased from 6/24 flies at 6 h to 21/24 flies at 24 h (P=0.10). There was no significant difference in the proportion of flies positive for GFP *S.* Typhimurium between 6 and 12 h (P=0.097) and between 12 and 24 h (P= 0.256). There was a significant effect of time on the concentration of GFP *S.* Typhimurium in cantaloupe ($P < 0.001$), but no significant effect of the presence or absence of flies (P=0.465). The concentration of GFP *S.* Typhimurium in cantaloupe without flies present increased from $3.35 \pm 0.17 \times 10^2$ CFU/g at 6 h to $5.53 \pm 0.17 \times 10^3$ CFU/g at 12 h (Fig. 3.1c; $P < 0.001$) and increased from $5.53 \pm 0.17 \times 10^3$ CFU/g at 12 h to $1.04 \pm 0.17 \times 10^5$ CFU/g at 24 h ($P < 0.001$). The concentration of GFP *S.* Typhimurium in cantaloupe with flies present increased from $4.69 \pm 0.13 \times 10^2$ CFU/g at 6 h to $4.64 \pm 0.10 \times 10^3$ CFU/g at 12 h ($P < 0.001$) and increased from $4.64 \pm 0.10 \times 10^3$ CFU/g at 12 h to $5.16 \pm 0.10 \times 10^4$ CFU/g at 24 h ($P < 0.001$). However, pairwise comparisons within each time point revealed that there was no significant difference between the CFU/g GFP *S.* Typhimurium in cantaloupe with flies and cantaloupe without flies present at 6 h (P=0.965), 12 h (P=0.998), and 24 h

(*P*=0.564). Furthermore, both time and the concentration of GFP *S.* Typhimurium in cantaloupe positively affected the abundance of GFP S. Typhimurium in house flies (Fig. 3.1c; *P*=0.001). GFP *S.* Typhimurium was recovered from excreta droplets collected from 0/4 jars at 6 h, 3/4 jars at 12 h, and 4/4 jars at 24 h (Table 3.2). The number of excreta droplets was not recorded since more than one fly was present in each jar.

Experiment 4: Transmission of GFP *S.* Typhimurium between flies with and without cantaloupe. At 24 h, there was no significant effect of treatment (cantaloupe presence or absence) on the abundance of GFP *S.* Typhimurium in ST+ flies (Fig. 3.1d; *P*=0.1845). However, treatment significantly affected the abundance of GFP *S.* Typhimurium in ST- flies, where ST- flies without cantaloupe had $1.14 \pm 2.27 \times 10^0$ CFU/fly while ST- flies with cantaloupe present had $5.85 \pm 2.27 \times 10^2$ CFU/fly ($P < 0.001$). There was no difference in the proportion of ST+ flies positive for GFP *S.* Typhimurium regardless of the presence or absence of cantaloupe (Fig. 3.1d; *P*=0.9780). However, only 2/36 ST- flies were positive for GFP *S.* Typhimurium in the absence of cantaloupe compared to 32/36 when cantaloupe was present (Fig. 3.1d; *P*=0.001). No GFP *S.* Typhimurium was recovered from excreta swabs from the jars without cantaloupe, while excreta from 7/9 jars with cantaloupe present were culture-positive for GFP *S.* Typhimurium (Table 3.2). The number of excreta droplets was not recorded since more than one fly was present in each jar.

Discussion

We investigated the acquisition and transmission of GFP *S.* Typhimurium between house flies and cantaloupe in four different experiments. For Experiment 1, we investigated the survival of GFP *S.* Typhimurium acquired from inoculated cattle manure in house flies. The

abundance of GFP *S.* Typhimurium and the proportion of flies positive for GFP *S.* Typhimurium decreased in flies over time (Fig. 3.1a). However, we did not observe a significant overall effect of time on the proportion of flies positive for GFP *S.* Typhimurium which most likely resulted from having too small of a sample size (n=15 flies) for proportion comparison over time. The decrease in GFP *S.* Typhimurium CFU/fly between 0 and 6 h may have resulted from bacterial digestion and/or excretion, which could have been exacerbated due to starvation (no food source was provided after removal from manure). In contrast to our results, bacterial persistence and even multiplication was observed in flies that ingested GFP *S.* Typhimurium suspended in culture broth (Chifanzwa and Nayduch 2017). Such differences in bacterial survival and proliferation may be attributable to the substrate within which the bacteria were suspended (manure vs. culture media). Additionally, Chifanzwa and Nayduch (2017) demonstrated that the ingested "dose" or abundance of bacteria affected survival, where high doses of bacteria were shown to proliferate to a lesser degree than low doses. In our study, the dose ingested by each fly was difficult to assess due to the nature of our mode of exposure (*ad libitum* access to manure inoculated with bacteria) making comparisons to this other study difficult.

In Experiment 1, at 0 and 6 h, no excreta collected from jars were culture positive for GFP *S.* Typhimurium and there was no change in the number of excreta droplets recovered from jars (Table 3.1) However, in this same time interval, there was a significant change in GFP *S.* Typhimurium abundance in flies (Fig. 3.1a), suggesting that GFP *S.* Typhimurium was digested by the flies rather than being shed in excreta. Previous studies have shown that some bacterial species are shed by house flies in excreta (McGaughey and Nayduch 2009, Nayduch et al. 2013, Fleming et al. 2014). In particular, GFP *S.* Typhimurium was shown to proliferate and increase in abundance within flies at 6 h post-ingestion of inoculated broth (Chifanzwa and Nayduch

2018); however, in our study GFP *S.* Typhimurium decreased in abundance in flies 6 h post-ingestion of inoculated manure. Perhaps the broth provided enough nutrients for the GFP *S.* Typhimurium to survive and grow in abundance within the fly alimentary canal all the way through to excretion, while the manure was insufficient for bacteria survival which furthers the assumption that the fate of GFP *S.* Typhimurium may depend on the substrate from which it was acquired (i.e. manure).

Experiment 2 assessed the transfer of GFP *S.* Typhimurium from flies to cantaloupe by measuring the abundance and concentration of GFP *S.* Typhimurium in flies and cantaloupe, respectively, over time. There was a decrease in both the abundance of GFP *S.* Typhimurium in flies and the proportion of flies positive for GFP *S.* Typhimurium over time (Fig. 3.1b). In contrast, the proportion of cantaloupe pieces positive for GFP *S.* Typhimurium increased over time indicating that the flies were successfully transferring bacteria to the cantaloupe (Fig. 3.1b). Furthermore, concentration of the GFP *S.* Typhimurium on positive cantaloupe increased from $0.00x10^0$ CFU/g to $1.88x10^1$ CFU/g within 24 h. Whether the increase in both the proportion of positive cantaloupe pieces and the CFU concentration recovered from cantaloupe is attributable to repeated inoculations from flies or growth of bacteria on the cantaloupe (or a combination of both phenomena) could not be determined. Cantaloupe flesh is a suitable growth substrate for *S.* Typhimurium due to its low acidity and high sugar content (Chimbombi 2010). Chimbombi (2010) found that *S.* Typhimurium growing on cantaloupe flesh had a lag phase of 7.76 hours, with peak growth occurring at 30 hours. Similarly, in our study (Experiment 3) the control cantaloupe (without flies) had a slow increase in GFP *S.* Typhimurium concentration to 3.35×10^2 CFU/g within the first 6 hours after which the concentration increased nearly 1000-fold to 1.04×10^5 CFU/g at 24 h. Because the abundance of GFP *S.* Typhimurium on the cantaloupe

without flies did not differ from the abundance of GFP *S* Typhimurium on the cantaloupe with flies over time, we infer that flies did not significantly contribute to GFP *S.* Typhimurium growth on the cantaloupe although they successfully inoculated the fruit (Fig. 3.1b).

Abundance of GFP *S.* Typhimurium in flies from Experiment 2 (Fig. 3.1b) did not appear to decrease as quickly between 0 and 6 h compared to the abundance in flies from Experiment 1 (Fig. 3.1a), possibly because cantaloupe was readily available as a food source for Experiment 2 flies but absent in Experiment 1. The additional nutrients from the cantaloupe in the fly gut may slow the initial digestion of the GFP *S.* Typhimurium since there is more to be digested at once. Furthermore, in Experiment 2, 75% of flies (12/15) were still positive for GFP *S.* Typhimurium at 12 h whereas less than half (7/15) of flies without cantaloupe in Experiment 1 were still harboring GFP *S.* Typhimurium at that same time point. Therefore, the presence of cantaloupe affects the vector potential of *S.* Typhimurium in house flies, possibly by enhancing bacterial survival in the fly gut.

Experiment 3 assessed the transfer of GFP *S.* Typhimurium from inoculated cantaloupe to flies and there was an increase in abundance of GFP *S.* Typhimurium in both flies and cantaloupe over time (Fig. 3.1c). Increase in bacterial abundance in flies and proportion of flies positive for GFP *S.* Typhimurium over time may be attributable to bacterial proliferation on the cantaloupe followed by repeated ingestion of viable bacteria from this source by flies, as we demonstrated in Experiment 3 that bacteria proliferate several orders of magnitude on cantaloupe in this time period. Additionally, the proliferation of GFP *S.* Typhimurium on the cantaloupe may produce volatiles that are attractive to the flies (Lam et al. 2007) and thus, increase their propensity for contacting the cantaloupe and feeding. Proliferation of GFP *S.* Typhimurium on

cantaloupe likely was not associated with re-inoculation by the flies, since there was no difference in the CFU recovered from cantaloupe in the presence or absence of flies (Fig. 3.1c).

We assessed the transfer of GFP *S.* Typhimurium between one ST+ fly and 4 ST- flies in the presence and absence of cantaloupe over a 24 h period in order to determine whether a food substrate facilitated fly to fly transmission of bacteria. The presence or absence of cantaloupe did not affect the abundance of GFP *S.* Typhimurium recovered from ST+ flies or the proportion of ST+ flies positive for GFP *S.* Typhimurium (Fig. 3.1d). Interestingly, the abundance of GFP *S.* Typhimurium in ST+ flies was greater than ST+ flies used in Experiment 1, where cantaloupe also was not present. The difference between those two experiments was the presence of other flies in the container in Experiment 4. We surmise that the presence of the ST- flies may have affected the ST+ fly behavior in a way which ultimately impacted their digestion or excretion of GFP *S.* Typhimurium. House flies spend most of their time either resting or regurgitating when they are alone (Barber and Starnes 1949), however, due to their gregarious nature, when other flies are present these behaviors may be interrupted. Because of the important role regurgitation, or "bubbling", plays in digestion (Stoffolano Jr. and Haselton 2013), it follows that in the presence of other flies, GFP *S.* Typhimurium may not have been digested as rapidly in ST+ flies that had other flies present in the container.

The objective of Experiment 4 was to determine whether food facilitated the transfer of bacteria from ST+ flies to ST- flies. Our results demonstrated that both abundance of GFP *S.* Typhimurium in ST- flies and proportion of ST- flies positive for GFP *S.* Typhimurium was greater when cantaloupe was present than when it was absent. (Fig. 3.1d). ST+ flies likely contaminated the cantaloupe with GFP *S.* Typhimurium, which then served as a source of GFP *S.* Typhimurium for the ST- flies when they fed upon the cantaloupe. When cantaloupe was absent,

only 2/32 ST- flies became positive for GFP *S.* Typhimurium after 24 h compared to nearly all the ST- flies being positive for GFP *S.* Typhimurium when cantaloupe was present (32/36). Interestingly, the 4 ST- flies that were not positive for GFP *S.* Typhimurium after 24 h were all from the same jar/replicate and although the ST+ fly was positive for GFP *S.* Typhimurium, the cantaloupe was not. This observation lends support to the role of contaminated cantaloupe in GFP *S.* Typhimurium transmission between flies.

Fly excreta may also serve as an indirect source for transmission of GFP *S.* Typhimurium among flies, but only when cantaloupe is present. There was no change in the number of excreta droplets recovered from jars over time when cantaloupe was not present (Experiment 1; Table 3.1); however, when cantaloupe was present, more excreta droplets were present in the jars (Experiment 2; Table 3.1), likely due to fly feeding activity or the ingestion of cantaloupe which induces peristalsis and subsequent excretion (Moon 2009). In all experiments, more jars were culture positive for GFP *S.* Typhimurium when cantaloupe was present than jars without cantaloupe, which suggests the presence of cantaloupe facilitates transmission (viable GFP *S.* Typhimurium in excreta) of GFP *S.* Typhimurium among house flies. For example, more jars tested positive for GFP *S.* Typhimurium in Experiment 2 compared to Experiment 1 (Table 3.2) indicating that the presence of cantaloupe not only provides nutrition for bacterial survival in the fly gut, but also enhances excretion of viable bacteria. Additionally, in Experiment 3, all 4 jars containing cantaloupe inoculated with GFP *S.* Typhimurium were positive for GFP *S.* Typhimurium by 24 h (Table 3.2), bolstering the assumption that the presence of cantaloupe promotes excretion of viable GFP *S.* Typhimurium. Furthermore, in Experiment 4, 0/ 9 jars were positive for GFP *S.* Typhimurium when there was no cantaloupe compared to 7/9 jars when cantaloupe was present (Table 3.2).

This study explored the survival of a human pathogenic bacterium in house flies and the potential for flies to acquire bacteria from contaminated fruit, to transmit bacteria to fruit and to transfer bacteria to each other via a shared food source. Our results emphasize the importance of considering house flies in food safety. House flies harbored and excreted viable GFP *S.* Typhimurium, and can potentially transfer pathogens to food, each other and can disseminate bacteria from the source to the surrounding environment. We demonstrated that the presence of both flies and food creates a potential public health issue whereby flies inoculate food with pathogens and not only contaminate the food and facilitate food-borne illness, but also create a source of bacteria for other flies. In practical sense, flies present in garbage bins (presumed sources of bacteria) near human dining areas may amplify the risk of food-borne pathogen contamination, especially if other flies are present and food is available for *ab libitum* consumption and contact by flies, for example at buffets, picnics or salad bars.

In order to fully assess the role of house flies in food safety, further research is needed to determine the acquisition and transmissibility of other bacterial pathogens from house flies to cantaloupe as well as other types of food. Because pathogens have distinct growth and nutrition requirements, some food items may be more susceptible to pathogen contamination and subsequent bacterial acquisition by house flies. The attractiveness of fruits to house flies also varies depending on each fly's nutritional state and reproductive requirements. It would be interesting to assess the effects of fly age and sex on pathogen survival and transmission potential across various food sources. Food type, pathogen species, fly age, and fly sex should be included as variables in designing future studies aimed to determine house fly pathogen acquisition and transmission to food.

Acknowledgements

We would like to thank A. Cooper for her helpful feedback on this manuscript. This manuscript was funded by USDA-ARS National Program 104, project number 3020-32000-007-00D. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. Any opinions findings, conclusion, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture.

References cited

Andino A and Hanning I. *Salmonella enterica*: Survival, colonization, and virulence differences among serovars. Sci World J 2015; 1-16.

Baldacchino, F., Desquesnes M., Duvallet G., Lysyk T., and Mihok S. Veterinary importance and integrated management of Brachycera flies in dairy farms, pp. 55–90. In: *Pests and Vector-Borne Diseases in the Livestock Industry, Ecology and Control of Vector-Borne Diseases.* Wageningen Academic Publishers, 2017.

Barber GW and Starnes EB. The activities of house flies. J. N. Y. Entomol Soc 1949; 57: 203-214.

Bennett SD, Sodha SV, Ayers TL, Lynch MF, Gould LH, Tauxe RV. Produce-associated foodborne disease outbreaks, USA, 1998–2013. Epidemiol Infect 2018; 1-10.

Chifanzwa R and Nayduch D. Dose-dependent effects on replication and persistence of *Salmonella enterica* serovar Typhimurium in house flies (Diptera: Muscidae). J Med Entomol 2017; 55: 225-229.

Chimbombi EM. Targeted inactivation of *Salmonella enterica* serovar Typhimurium in fresh cantaloupe flesh (*Cucumis melo* L.) using electron beam irradiation. Ph.D. Dissertation 2010; Texas A&M University, College Station.

De Jesús AJ., Olsen AR, Bryce JR, Whiting RC. Quantitative contamination and transfer of *Escherichia coli* from foods by houseflies, *Musca domestica*, L. (Diptera: Muscidae). Int J Food Microbiol 2004; 93: 259-262.

Fleming A, Kumar HV, Joyner C, Reynolds A, Nayduch D. Temporospatial fate of bacteria and immune effector expression in house flies fed GFP-*Escherichia coli* O157: H7. Med Vet Entomol 2014; 28: 364-371.

Graczyk TK, Knight R, Gilman RH, Cranfield MR. The role of non-biting flies in the epidemiology of human infectious diseases. Micro Infect 2001; 3: 231–235.

Greenberg B. House fly nutrition: I. Quantitative study of the protein and sugar requirements of males and females. J Cell Comp Physio 1959; 53: 169-177.

Gupta AK, Nayduch D, Verma P, Shah B, Ghate HV, Patole MS, Shouche YS. Phylogenetic characterization of bacteria in the gut of house flies (*Musca domestica* L.). FEMS Micro Ecol 2012; 79: 581-593.

Hanski I. Nutritional ecology of dung- and carrion-feeding insects. pp. 837–884. In: *Nutritional ecology of insects, mites, and spiders.* Slansky Jr. F and Rodriguez JG (eds.). New York: John Wiley & Sons Inc., 1987.

Himathongkham S, Bahari S, Riemann H, Cliver D. Survival of *Escherichia coli* O157: H7 and *Salmonella typhimurium* in cow manure and cow manure slurry. FEMS Microbiol Letters 1999; 178: 251-257.

Holt PS, Geden CJ, Moore RW, Gast RK. Isolation of *Salmonella enterica* serovar Enteritidis from houseflies (*Musca domestica*) found in rooms containing *Salmonella* serovar Enteritidis-challenged hens. Appl Environ Microbiol 2017; 73: 6030-6035.

Huang J, Luo Y, Nou X. Growth of *Salmonella enterica* and *Listeria monocytogenes* on fresh-cut cantaloupe under different temperature abuse scenarios. J Food Protect 2015; 78: 1125-1131.

Jacques, BJ., Bourret TJ., Shaffer JJ. 2017. Role of fly cleaning behavior on carriage of *Escherichia coli* and *Pseudomonas aeruginosa*. J Med Entomol 2017; 54: 1712–1717.

Lam K, Babor D, Duthie B, Babor E, Moore M, Gries G. Proliferating bacterial symbionts on house fly eggs affect oviposition behaviour of adult flies. Anim Behav 2007; 74: 81-92.

Macovei L, Miles B, Zurek L. Potential of houseflies to contaminate ready-to-eat food with antibiotic-resistant enterococci. J Food Protect 2008; *71*: 435-439.

McGaughey J and Nayduch D. Temporal and spatial fate of GFP-expressing motile and nonmotile *Aeromonas hydrophila* in the house fly digestive tract. J Med Entomol 2009; 46: 123-130.

Moon, RD. Muscid flies (*Muscidae*), pp. 345–368. In: *Medical and Veterinary Entomology* Mullen GR and Durden LA (eds.). San Diego, CA: Academic Press, 2019.

Nayduch, D, Pittman Noblet G, Stutzenberger FJ. Vector potential of houseflies for the bacterium *Aeromonas caviae*. Med Vet Entomol 2002; 16:193-198.

Nayduch D, Cho H, Joyner C. *Staphylococcus aureus* in the house fly: Temporospatial fate of bacteria and expression of the antimicrobial peptide *defensin*. J Med Entomol 2013; 50: 171-178.

Nayduch D and Burrus RG**.** Flourishing in filth: House fly–microbe interactions across life history. Ann Entomol Soc Amer 2017; 110: 6-18.

Nayduch D, Zurek K, Thomson JL, Yeater, KM. Effects of bacterial dose and fly sex on persistence and excretion of *Salmonella enterica* serovar Typhimurium from adult house flies (*Musca domestica* L.; Diptera: Muscidae). J Med Entomol 2018; 55: 1264-1270.

Pace RC, Talley JL, Crippen TL, Wayadande AC. Filth fly transmission of *Escherichia coli* O157: H7 and *Salmonella enterica* to Lettuce, *Lactuca sativa*. Ann Entomol Soc of Amer 2017; 110: 83-89.

Stoffolano Jr. JG and Haselton AT. The adult Dipteran crop: A unique and overlooked organ. Ann Rev Entomol 2013; 58: 205-225.

Thomson JL, Yeater KM, Zurek L, Nayduch D. Abundance and accumulation of *Escherichia coli* and *Salmonella* Typhimurium procured by male and female house flies (Diptera: Muscidae) exposed to cattle manure. Ann Entomol Soc Amer 2017; *110*: 37-44.

West LS. *The Housefly: Its Natural History, Medical Importance, and Control*, 1st edition. Ithaca, NY: Comstock Publishing Company Inc., 1951.

Yap KL, Kalpana M, Lee HL. Wings of the common house fly (*Musca domestica* L.): Importance in mechanical transmission of *Vibrio cholerae*. Trop Biomed 2008; 25: 1-8.

Zar JH. *Biostatistical Analysis*, 4th edition p 564, Prentice Hall, 1999.

Table 3.1. The mean (± SEM) number of fly excreta droplets recovered from jars.

Time	Experiment 1	Experiment 2
6 h	17 ± 1.48	18 ± 2.17[a]
12 h	19 ± 3.21	27 ± 2.51[b]
24 h	20 ± 2.38	44 ± 3.11[c]

Means were calculated from n=15 jars at each time point and letters represent significant differences between time points within an experiment (a vs. b P=0.012; b vs. c P=0.001; a vs. c $P < 0.001$). There was no significant difference between mean number of excreta droplets collected at the three time points in Experiment 1.

Table 3.2. Proportion of jars swabbed that were positive for GFP *S*. Typhimurium.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	
Time				**No cantaloupe**	**Cantaloupe**
6 h	0/15	5/15	0/4	N/A	N/A
12 h	1/15	5/15	3/4	N/A	N/A
24 h	0/15	3/15	4/4	0/9	7/9

Each proportion is represented as the number of jars that tested positive for GFP *S.* Typhimurium from fly excreta swabs out of the total number of jars sampled at each time point. No statistical analyses were performed on these data.

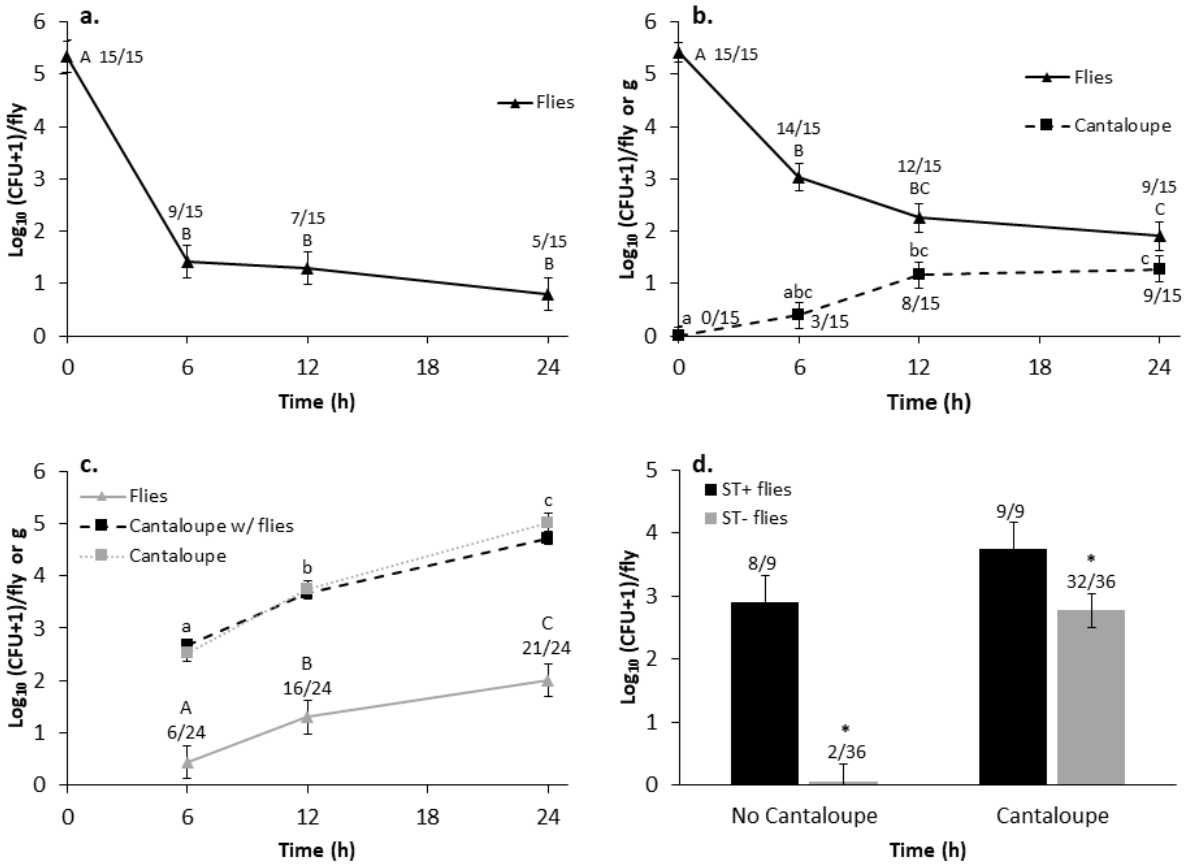

Figure 3.1. Recovery of GFP *S*. Typhimurium from flies and cantaloupe over time.

Each panel displays results from an experiment: **a.** Experiment 1: GFP *S.* Typhimurium survival in house flies, **b.** Experiment 2: transmission of GFP *S.* Typhimurium from flies to cantaloupe, **c.** Experiment 3: acquisition of GFP *S*. Typhimurium by flies from inoculated cantaloupe, and **d.** Experiment 4: transmission of GFP *S*. Typhimurium between flies with and without cantaloupe. Mean Log_{10} CFU (colony forming units) ± SEM of GFP *S.* Typhimurium and proportions from 3 biological replicates are shown. Proportions represent the number of flies positive for GFP *S.* Typhimurium out of the total flies sampled. Abundance of GFP *S.* Typhimurium in flies and cantaloupe was compared between time points in all experiments using the GLIMMIX procedure with LS means Tukey-Kramer option in SAS. A similar model was used to compare proportions in all experiments as well. In all panels, different letters represent statistical significance in abundance and proportions over time and asterisks represent statistical difference between treatments (panel d.; $P < 0.05$).

Chapter 4 - Bacterial abundance and diversity in male and female house flies (Diptera: Muscidae) surveyed from urban, sub-urban, and agricultural habitats

Abstract

House flies flourish in microbe-rich environments, such as rotting food or animal manure, which they utilize for development, nutrition, and oviposition. Adults acquire bacteria, including coliforms, by feeding on decaying matter and can disseminate microbes into the surrounding environment, causing animal and public health concerns. Male and female house flies differ in their nutritional requirements which affect their interaction with microbe-rich substrates and habitats where flies reside vary depending on food and oviposition site availability. This study assessed whether fly sex and habitat affects bacterial abundance in adult house flies. Wild adult house flies were collected from 3 different habitats (urban, sub-urban, and agricultural) and mean colony forming units (CFU) of bacteria and coliforms were enumerated from individual male and female flies. We determined that location and sex of house flies affect mean bacterial and coliform abundance in flies. Irrespective of sex, flies collected from the sub-urban site had the greatest abundance of bacteria, while the flies collected from the urban site had the least abundance of coliforms. Females had a greater abundance of bacteria and coliforms than males at the agricultural and urban sites. Furthermore, females did not differ in bacteria and coliform abundance across sites, while males had the greatest bacterial abundance at the sub-urban site and lowest coliform abundance at the urban site. Therefore, female house flies likely harbor a consistent bacterial load, regardless of habitat, due to their nutritional requirements and

oviposition behaviors, while male flies vary in bacterial load and drive differences in overall sex effects on bacterial abundance within and across different habitats. Identifying variables that may contribute to differences in coliform and bacterial abundance in house flies is important to assess the risk house flies pose in procuring, harboring, and disseminating bacteria from different habitats.

Keywords: *Musca domestica*, field survey, bacterial abundance

House flies (*Musca domestica* L.) are common, cosmopolitan pests that can live in a variety of different environments. Bacteria are an essential component of house fly larval development sites which may consist of decaying matter, such as animal dung, rotting food, or spoiled animal feed (Zurek et al. 2000, Moon 2019). These developmental sites can exist in urban areas (e.g. restaurants and city housing), sub-urban areas (e.g. residential housing and businesses), and agricultural areas (e.g. farms and livestock facilities) (West 1951, Moon 2019). Adult house flies may acquire bacteria from these environments trans-stadially or from direct contact (Zurek and Nayduch 2016, Nayduch and Burrus 2017). Differences in fly nutritional requirements, such as female requirements for protein in order to initiate vitellogenesis, are likely to influence which substrates males and females are more attracted to for feeding (Greenberg 1959d, Spiller 1964). Additionally, females are attracted to and inspect bacteria-rich substrates in the interest of potential oviposition sites (Lam et al. 2007).

While house flies have been shown to harbor a large variety of bacterial taxa, members of the *Enterobacteriaceae* are largely represented in house fly surveys, most likely due to the prevalence of these bacteria in the typical habitat of house flies, which is often contaminated

with dung or manure (Gupta et al. 2012, Bahrndorff et al. 2017, Khamesipour et al. 2018, Moon 2019). In this family are coliforms, which are Gram-negative, lactose-fermenting, rod-shaped bacteria many of which originate from fecal matter (Forstinus et al. 2016). Coliforms are important indicators of fecal contamination that have been isolated from plants, insects, and water (Geldreich et al. 1964) and their presence and concentration are assessed in food safety inspections and water quality assessments (Soares et al. 2013, Mishra et al. 2018). Coliforms have been recovered from house flies that were collected from areas such as feed bunks containing steam-flaked corn, dumpsters with rotting food, spinach in agricultural fields, wastewater treatment facilities, and other areas where animal or human waste is prevalent (Nurita and Hassan 2013, Wasala et al. 2013, Doud et al. 2014, Ghosh and Zurek 2015, Puri-Giri et al. 2017, Pohlenz et al. 2018). The presence of coliforms in house flies raises a public health concern, due to the increased risk for disease transmission where house flies may disseminate pathogens into the surrounding environment (Zurek and Ghosh 2014, Forstinus et al. 2016).

We have previously demonstrated that female house flies acquire a higher abundance of bacteria from cattle manure than males over time in an experimental setting (Thomson et al. 2017); however, sex effects on bacterial acquisition and carriage in the field setting remains unknown. Additionally, while many house fly surveys have isolated bacteria from flies collected from different habitats, the effect of geographical location on bacterial carriage has not been thoroughly investigated. Our study aimed to determine if adult house fly sex and habitat affected bacterial and coliform abundance in house flies collected from three different environments. Male and female house flies were surveyed from three different habitats in the field (urban, sub-urban, and agricultural) in order to enumerate and compare mean bacteria and coliform abundance. We hypothesized that both habitat and house fly sex would affect the bacterial and

coliform abundance recovered from house flies and that these variables would be important factors to consider in the assessment of house flies as potential disease vectors.

Materials and methods

House fly field collection. Wild house flies (n = 10 male and 10 female) were collected from 3 different habitats (urban, sub-urban, agricultural; Fig. 4.1 and 4.2) in Manhattan, KS one day per week for n=5 weeks in late August through the end of September in the year 2018. The urban collection sites were in the downtown area of Manhattan, KS and flies were collected from municipal dumpsters in the back alley of restaurants (Fig. 4.1). The sub-urban site was a local farmer's cooperative (co-op) that was close to a residential neighborhood and was located between an urbanized restaurant/business area and an agricultural area that included field crops and nearby pullet poultry production facilities. Flies were collected from around the grain bin and dumpster at this sub-urban location. The agricultural collection site was at the Kansas State University beef cattle feedlot and flies were collected from the feed bunks (Fig. 4.1). Flies were collected by sweep net and anesthetized on site by ice exposure in a cooler, confirmed as *M. domestica*, aseptically sorted by sex, and individually placed in sterile 1.5 ml microcentrifuge tubes (Fisher Scientific) for transport. Racks of tubes with individual flies were kept in a cooler on ice during transport. Dry weight (mg) of each fly was recorded using a scale (Adam Equipment, Oxford, CT) before processing (data not shown).

Bacteria recovery from house flies. Flies were sexed and were individually homogenized in 1 ml sterile phosphate buffered saline (PBS). Homogenate was serially diluted and plated in duplicate on Tryptic Soy Agar (TSA; Remel, Lenexa, KS) and Violet Red Bile Agar (VRBA; Sigma-Aldrich, Milwaukee, WI) to measure culturable bacterial abundance and

coliforms, respectively. TSA plates were incubated at 26˚C for 48 h and VRBA plates were incubated at 37˚C for 24 h after which colonies were enumerated and recorded for each fly. All colonies were counted on TSA plates while only pink or reddish pink colonies were recorded for VRBA plates (indicating lactose fermentation by coliforms; Fig. 4.3). Pink or red colonies (3-5/plate) appearing to have different morphologies were randomly picked from VRBA plates, mixed in 100 µl nuclease-free water, and stored at -20˚C for future sequence analysis and taxonomic identification (details not shown).

Statistical Analyses. A generalized linear mixed model (GLMM; Proc GLIMMIX in SAS 9.4) with a Gaussian distribution, identity link and restricted pseudo-likelihood estimation was used to determine if mean bacterial abundance varied between house fly sex and collection site. The outcome consisted of the $\log_{10}$ CFU of mean bacteria/fly. Site (urban, sub-urban, agricultural) and sex (male, female), and a two-way interaction term between site and sex were included as fixed effects and week (1-5) as a random effect. A similar model was built to determine the effect of sex and site on the mean CFU coliforms recovered from flies. The only difference was that the $\log_{10}$ mean CFU/fly of coliforms was used as the outcome variable in the model instead of $\log_{10}$ mean CFU/fly of bacteria. The Tukey-Kramer procedure was used to adjust for multiple comparisons in all linear mixed models. Mean $\log_{10}$ CFU, SEM, and their 95% confidence intervals were computed. *P*-values (< 0.05) were used to determine statistical significance.

Results

Comparison of mean bacterial and coliform abundance between male and female house flies. Overall, female flies had a greater abundance of bacteria ($5.36 \pm 0.07 \times 10^5$ CFU/fly)

than males ($8.46 \pm 0.09 \times 10^4$ CFU/fly; Fig. 4.4; $t = 8.04$, df = 290, $P < 0.001$). Additionally, females had a greater overall mean abundance of coliforms ($1.28 \pm 0.07 \times 10^4$ CFU/fly) than males ($2.39 \pm 0.11 \times 10^3$ CFU/fly; Fig. 2b; $t = 5.86$, df = 290, $P < 0.001$). However, males ($2.94 \pm 0.10 \times 10^5$ CFU/fly) and females ($8.12 \pm 0.10 \times 10^5$ CFU/fly) did not significantly differ in mean bacterial abundance ($t = 2.55$, df = 290, $P = 0.113$) at the sub-urban site, while females had greater bacterial abundance than males at both the urban (females: $4.42 \pm 0.15 \times 10^5$ CFU/fly, males: $2.41 \pm 0.17 \times 10^4$ CFU/fly; $t = 7.31$, df = 290, $P < 0.001$) and agricultural (females: $4.28 \pm 0.10 \times 10^5$ CFU/fly, males: $8.53 \pm 0.13 \times 10^4$ CFU/fly; $t = 4.06$, df = 290, $P = 0.001$) sites. Females also had a greater abundance of coliforms than males at both the urban (females: $7.14 \pm 0.15 \times 10^3$ CFU/fly, males: $7.93 \pm 0.17 \times 10^2$ CFU/fly; $t = 4.44$, df = 290, $P = 0.001$) and agricultural (females: $2.00 \pm 0.09 \times 10^4$ CFU/fly, males: $2.51 \pm 0.20 \times 10^3$ CFU/fly; $t = 4.18$, df = 290, $P = 0.001$) sites, while they did not significantly differ in coliform abundance at the sub-urban site (females: $1.46 \pm 0.13 \times 10^4$ CFU/fly, males: $6.82 \pm 0.17 \times 10^3$ CFU/fly; $t = 1.54$, df = 290, $P = 0.640$).

Comparison of mean bacterial and coliform abundance in house flies across sites.

The mean abundance of bacteria in all flies collected from the sub-urban site was $4.88 \pm 0.07 \times 10^5$ CFU/fly which was significantly different than the urban ($1.03 \pm 0.13 \times 10^4$ CFU/fly; $t = -5.52$, df = 290, $P < 0.001$) and agricultural sites ($1.91 \pm 0.09 \times 10^4$ CFU/fly; $t = 3.34$, df = 290, $P = 0.001$), respectively (Fig. 4.4). There was no significant difference in bacterial abundance in flies between the urban and agricultural sites ($t = -2.19$, df = 290, $P = 0.075$). Overall, the urban collection site had a significantly lower coliform abundance ($2.38 \pm 0.12 \times 10^3$ CFU/fly) compared to the sub-urban (9.98×10^3 CFU/fly; $t = -4.09$, df = 290, $P = 0.001$) and agricultural sites (7.08×10^3 CFU/fly; $t = -3.11$, df = 290, $P = 0.006$), respectively (Fig. 4.4). There was no

significant difference in coliform abundance between the sub-urban and agricultural sites (t = 3.34, df = 290, P = 0.003).

Interestingly, the effect of sex on the bacterial abundance recovered from house flies (n = 300 flies) depended on the habitat (F = 5.92, df = 2, 290, P = 0.003). Mean bacterial abundance in female flies did not significantly differ in all site-by-site pairwise comparisons (Fig. 4.4; urban vs. sub-urban: t = -1.53, df = 290, P = 0.648; sub-urban vs. agricultural: t = 1.61, df = 290, P = 0.593; urban vs. agricultural: t = 0.08, df = 290, P = 1.000). However, bacterial abundance in male flies was significantly different across all sites (urban vs. sub-urban: t = -6.29, df = 290, P < 0.001; sub-urban vs. agricultural: t = 3.11, df = 290, P = 0.025; urban vs. agricultural: t = -3.17, df = 290, P = 0.021). Further, male flies collected at the sub-urban site had the greatest bacterial abundance ($2.94 \pm 0.10 \times 10^5$ CFU/fly), followed by $8.53 \pm 0.13 \times 10^4$ CFU/fly from flies at the agricultural site, and only $2.41 \pm 0.17 \times 10^4$ CFU/fly from flies collected from the urban site.

Overall, the interaction between sex and site on coliform abundance was not significant (F = 2.58, df = 2, 290, P = 0.078), but only fell slightly above the alpha (P < 0.05) standard. Females did not differ in mean coliform abundance across sites (Fig. 4.4; urban vs. sub-urban: t = -1.45, df = 290, P = 0.697; sub-urban vs. agricultural: t = -0.63, df = 290, P = 0.989; urban vs. agricultural: t = -2.08, df = 290, P = 0.303), whereas males from the urban site had lower coliform abundance ($7.93 \pm 0.17 \times 10^2$ CFU/fly) than males at the sub-urban site ($6.82 \pm 0.17 \times 10^3$ CFU/fly; t = -4.34, df = 290, P = 0.001). Males from the agricultural site ($2.51 \pm 0.20 \times 10^3$ CFU/fly) were not significantly different in coliform abundance from males at both the sub-urban (t = 2.02, df = 290, P = 0.336) and urban sites (t = -2.33, df = 290, P = 0.186).

Comparison of mean bacterial and coliform abundance in house flies across weeks. Overall, flies collected from week 1 had a greater bacterial abundance compared to flies

collected from weeks 2 (t = 4.30, df = 270, P = 0.001), 3 (t = 4.88, df = 270, P < 0.001), 4 (t = 5.36, df = 270, P < 0.001), and 5 (t = 4.79, df = 270, P < 0.001; Fig. 4.5a) and flies collected from week 5 had a greater coliform abundance compared to flies collected from weeks 1 (t = -4.14, df = 270, P = 0.001), 2 (t = -2.80, df = 270, P = 0.044), and 3 (t = -3.03, df = 270, P = 0.022; Fig. 4.5b).

Discussion

The purpose of this study was to determine if house fly sex and habitat affected bacterial carriage by flies. We collected adult house flies from 3 different habitats and measured abundance of culturable aerobic bacteria and abundance of coliforms in male and female flies. Overall, the greatest abundance of culturable aerobic bacteria was associated with house flies collected from the sub-urban site (Fig. 4.4). The house flies from the sub-urban site were collected from a dumpster that was consistently full of spoiled feed and the location sits in close proximity to private chicken farming operations, both of which provide opportunities for increased house fly exposure to bacteria. Even though the urban and agricultural sites both had different types of substrates available for bacterial growth (i.e., garbage and manure, respectively), the house flies collected from both sites had a lower abundance of culturable aerobic bacteria compared to the sub-urban site due. Surprisingly, the agricultural site did not differ from the urban site in fly bacterial abundance even though manure was always accessible to flies at the feedlot. However, flies harboring the lowest abundance of coliforms were collected from the urban site (Fig. 4.4) which may have resulted from flies being collected from dumpsters and there being no nearby livestock or access to animal waste (typically associated with coliforms).

Overall, our study demonstrated that female house flies harbored a greater abundance of both culturable aerobic bacteria and coliforms than male flies. We previously demonstrated that female house flies harbored a greater abundance of bacteria than males over time when experimentally exposed to sterilized cattle manure containing either *Escherichia coli* or *Salmonella enterica* ser. Typhimurium and an alternative sugar source, and deduced that female flies likely spend more time ingesting and interacting with the manure than male flies (Thomson et al. 2017). Their frequent interaction with oviposition sites as well as sex-specific behaviors and nutritional requirements likely contribute to a greater abundance of bacteria and coliforms in female house flies. Female flies are attracted to and spend time visiting and inspecting microbe-rich oviposition sites (Greenberg 1959d, Shah et al. 2016) which may result in them procuring and harboring more bacteria than males. In contrast to females, male house flies spend a large percentage of their active time walking, flying, and grooming (Barber and Starnes 1949) which may detract from time spent interacting with microbe-rich substrates. Females require protein to support egg development (Greenberg 1959d, Moon 2019) and can acquire protein and other essential nutrients by ingesting animal manure (Hanski 1987) or by feeding on animal secretions, both of which may harbor abundant microbes.

Within collection sites, females harbored a greater abundance of culturable aerobic bacteria and coliforms than males at the urban and agricultural sites, but there was no difference in culturable aerobic bacteria and coliform abundance between the sexes at the sub-urban site (Fig. 4.4). We observed that house fly populations were more abundant and concentrated in one spot (inside the feed dumpster) at the sub-urban site compared to the urban and agricultural sites where house flies appeared to be more dispersed. Female house flies were consistently observed interacting with waste inside dumpsters at the urban site or in feed bunks near the animals at the

agricultural site, while the males were typically observed resting outside of dumpsters or on the inner walls of feed bunks and nearby structures. Because female house flies likely spend more time interacting with microbe-rich substrates than males at these sites, they have a greater chance of acquiring and harboring microbes. At the sub-urban site, however, both males and females were observed and captured from inside the dumpster, and likely had equal opportunities to acquire microbes, although this was not determined in this study.

Mean bacteria and coliform abundance in female flies did not differ across collection sites (Fig. 4.4). Gerry et al. (2011) observed that more female house flies were caught in fly traps located near the cattle at a dairy and more male house flies were collected from traps set further away from the animals. Therefore, because females stay near animals who serve as a source of microbes (dung, secretions, wounds) and inhabit microbe-rich oviposition sites it follows that they would harbor a steady abundance of bacteria and coliforms in habitats with livestock present. In our study, females from agricultural and urban settings harbored a consistent abundance of bacteria and coliforms which may indicate that, regardless of habitat, females consistently seek out and contact microbe-rich substrates.

In contrast to females, male house flies harbored the greatest culturable aerobic bacterial abundance at the sub-urban site (Fig. 4.4a) and carried the lowest coliform abundance at the urban site (Fig. 4.4b). Males may have had the greatest culturable aerobic bacterial abundance at the sub-urban site because of their observed presence inside the dumpster, as discussed above. Lack of access to animals and manure in an urban habitat, and propensity to rest outside dumpsters rather than inside, likely resulted in the low abundance of coliforms recovered from males collected from the urban site. We infer that male house fly interactions with microbe-rich substrates are inconsistent across sites and are rather specific to each habitat. Therefore, because

bacterial abundance in female house flies does not differ across sites, male house flies likely drive the sex-specific differences we observed within sites.

There are some limitations to using only a culture-based approach to assess house fly carriage of bacteria. For example, microaerophilic, slow-growing and non-culturable bacteria were not reflected in total bacterial abundance calculations because our study quantified only culturable aerobic microbes on TSA. In the future, pairing culture-based with sequence-based approaches could help improve estimates of fly-associated bacteria. Additionally, we quantified total culturable aerobic bacteria on TSA and coliforms on VRBA, but we did not quantify bacterial abundance at lower taxonomic levels and therefore do not know species abundance, richness or diversity. Future research is aimed at determining sex and site effects on abundance of bacterial species carried by flies, especially human and animal pathogens. Such studies will help in determining how fly sex and habitat impacts risk for pathogen procurement and transmission. Determination of seasonal effects on bacterial abundance in flies across various collection sites is also of interest in future studies. Because bacterial and coliform abundance varied across weeks (Fig. 4.5), we accounted for this variability by incorporating week (biological replicates) as a random effect in our statistical model. However, it would be interesting to more thoroughly explore the effect of seasonal effects (e.g. temperature and humidity) on abundance of bacteria and coliforms in house flies.

Overall, we determined that house fly sex and habitat affected mean culturable aerobic bacterial abundance and coliform abundance in house flies. Depending on the habitat, females may harbor greater loads of bacteria than males which may implicate them in dissemination potential. Female flies harbor and potentially disseminate coliforms (and presumably other enteric bacteria) in agricultural settings, and therefore serve an important role in animal health in

livestock operations where there is open access to both manure and animals. From a public health perspective, our study indicated that the sub-urban habitat, which sat between agricultural and residential areas, and where flies harbored the greatest bacterial abundance, may be more at risk for house fly bacterial dispersal irrespective of fly sex. In contrast, at the urban habitat, where restaurants and businesses are nearby, female flies may pose a greater risk in harboring and disseminating microbes from dumpsters. Therefore, a sex-targeted fly control management approach may be more effective at reducing bacterial carriage by flies than a more generalized pest control approach in urban and agricultural habitats where female house flies harbor greater loads of bacteria than males. Overall, our results emphasize that fly sex and habitat are two important factors to be considered in fly control and management approaches.

Acknowledgments

We would like to thank R. Hammel from USDA SPIERU for lending us his data logger for field collections and magnetic bar stirrers for media preparation for this project. Furthermore, we would like to thank C. McCoy and H. Meier for their hard work and assistance with media preparation, fly collection and processing, microbial culture and data collection. This work was funded by USDA-ARS National Program 104, project number 3020-32000-007. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. Any opinions, findings, conclusion, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture.

References Cited

Bahrndorff, S., N. de Jonge, H. Skovgård, and J. L. Nielsen. **2017**. Bacterial communities associated with houseflies (*Musca domestica* L.) sampled within and between farms. PLoS ONE. 12: 1–15.

Barber, G. W., and E. B. Starnes. **1949**. The activities of house flies. J. N. Y. Entomol. Soc. 57: 203–214.

Doud, C. W., H. M. Scott, and L. Zurek. **2014**. Role of house flies in the ecology of *Enterococcus faecalis* from wastewater treatment facilities. Microbiol. Ecol. 67: 380–391.

Forstinus, N., N. Ikechukwu, M. Emenike, and A. Christiana. **2016**. Water and waterborne diseases: a review. Int. J. Trop. Dis. Health. 12: 1–14.

Geldreich, E. E., B. A. Kenner, and P. W. Kabler. **1964**. Occurrence of coliforms, fecal coliforms, and *Streptococci* on vegetation and insects. Appl. Environ. Microbiol. 12: 63–69.

Ghosh, A., and L. Zurek. **2015**. Fresh steam-flaked corn in cattle feedlots is an important site for fecal coliform contamination by house flies. J. Food Protect. 78: 567–572.

Greenberg, B. 1959. House fly nutrition. 1. Quantitative study of the protein and sugar requirements of males and females. J. Cell. Comp. Physiol. 53: 169–177.

Gupta, A. K., D. Nayduch, P. Verma, B. Shah, H. V. Ghate, M. S. Patole, and Y. S. Shouche. **2012**. Phylogenetic characterization of bacteria in the gut of house flies (*Musca domestica* L.). FEMS Microbiol. Ecol. 79: 581–593.

Hanski, I. 1987. Nutritional ecology of dung-and carrion-feeding insects, pp. 837–884. *In* Nutritional Ecology of Insects, Mites, and Spiders. John Wiley & Sons Inc., New York, NY.

Khamesipour, F., K. B. Lankarani, B. Honarvar, and T. E. Kwenti. **2018**. A systematic review of human pathogens carried by the housefly (*Musca domestica* L.). BMC Public Health. 18: 1–15.

Lam, K., D. Babor, B. Duthie, E.-M. Babor, M. Moore, and G. Gries. **2007**. Proliferating bacterial symbionts on house fly eggs affect oviposition behaviour of adult flies. Anim. Behav. 74: 81–92.

Mishra, M., A. P. Arukha, A. K. Patel, N. Behera, T. K. Mohanta, and D. Yadav. **2018**. Multi-drug resistant coliform: water sanitary standards and health hazards. Front. Pharmacol. 9: 1-8.

Moon, R. D. 2019. Chapter 17 - Muscid Flies (Muscidae), pp. 345–368. *In* Mullen, G.R., Durden, L.A. (eds.), Medical and Veterinary Entomology, 3rd ed. Academic Press, USA.

Nayduch, D., and R. G. Burrus. **2017**. Flourishing in filth: house fly–microbe interactions across life history. Ann. Entomol. Soc. Am. 110: 6–18.

Nurita, A. T., and A. A. Hassan. **2013**. Filth flies associated with municipal solid waste and impact of delay in cover soil application on adult filth fly emergence in a sanitary landfill in Pulau Pinang, Malaysia. Bull. Entomol. Res. 103: 296–302.

Pohlenz, T. D., K. Zavadilova, A. Ghosh, and L. Zurek. **2018**. Prevalence of Shiga-toxigenic *Escherichia coli* in house flies (Diptera: Muscidae) in an urban environment. J. Med. Entomol. 55: 436–439.

Puri-Giri, R., A. Ghosh, J. L. Thomson, and L. Zurek. **2017**. House flies in the confined cattle environment carry Non-O157 Shiga Toxin-Producing *Escherichia coli*. J. Med. Entomol. 54: 726–732.

Shah, R. M., F. Azhar, S. A. Shad, W. B. Walker, M. Azeem, and M. Binyameen. **2016**. Effects of different animal manures on attraction and reproductive behaviors of common house fly, *Musca domestica* L. Parasitol. Res. 115: 3585–3598.

Soares, K., J. García-Díez, A. Esteves, I. Oliveira, and C. Saraiva. **2013**. Evaluation of food safety training on hygienic conditions in food establishments. Food Control. 34: 613–618.

Spiller, D. 1964. Nutrition and diet of muscoid flies. Bull. World Health Organ. 31: 551–554.

Thomson, J. L., K. M. Yeater, L. Zurek, and D. Nayduch. **2017**. Abundance and accumulation of *Escherichia coli* and *Salmonella* Typhimurium procured by male and female house flies (Diptera: Muscidae) exposed to cattle manure. Ann. Entomol. Soc. Am. 110: 37–44.

Wasala, L., J. L. Talley, U. DeSilva, J. Fletcher, and A. Wayadande. **2013**. Transfer of *Escherichia coli* O157:H7 to spinach by house flies, *Musca domestica* (Diptera: Muscidae). Phytopath. 103: 373–380.

West, L. S. 1951. The housefly: its natural history, medical importance, and control, 1st ed. Comstock Publishing Company Inc., Ithaca, NY.

Zurek, K., and D. Nayduch. **2016**. Bacterial associations across house fly life history: evidence for transstadial carriage from managed manure. J. Insect. Sci. 16.

Zurek, L., and A. Ghosh. **2014**. Insects represent a link between food animal farms and the urban environment for antibiotic resistance traits. Appl. Environ. Microbiol. 80: 3562–3567.

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.

Figure 4.1. House fly field collection sites.

House flies were collected from three different habitats in Kansas: a. K-State feedlot agricultural area, b. Downtown Manhattan urban area and c. Co-op sub-urban area.

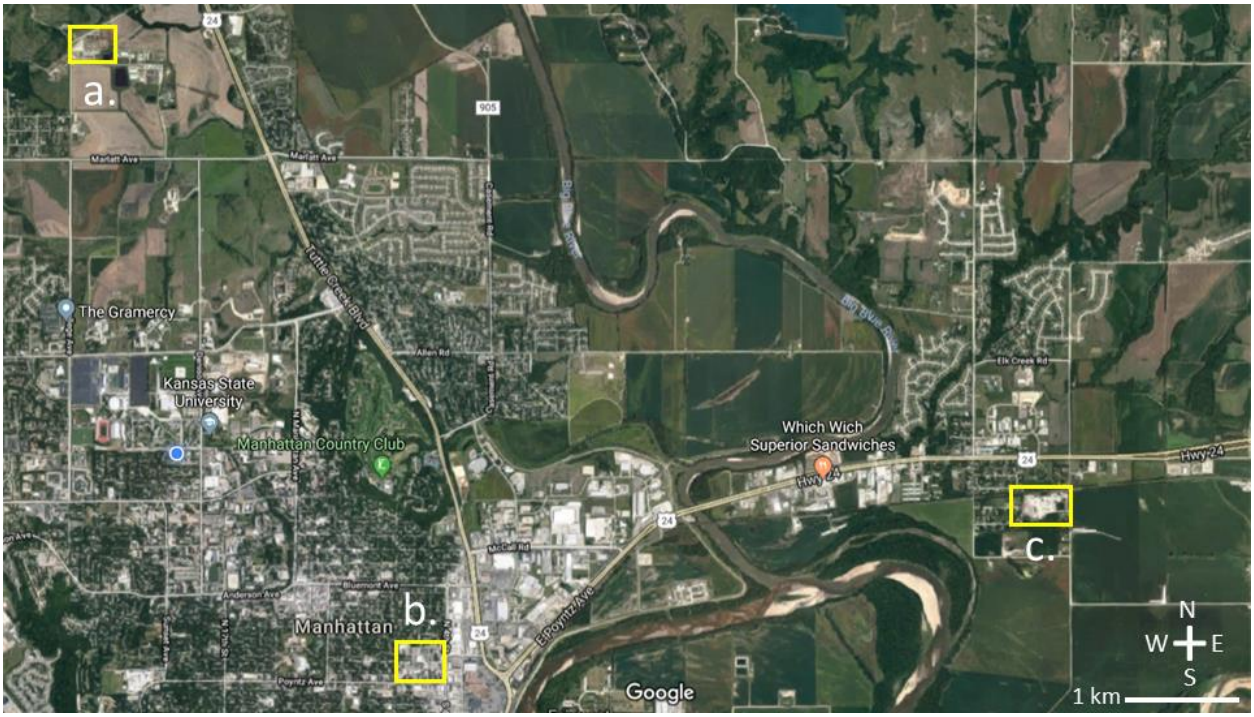

Figure 4.2. Map of field collection sites.

This figure shows the exact location of each collection site in vicinity of Manhattan, Kansas. The estimated distances between sites are as follows: a-c = 8.75 km, b-c = 5.75 km, a-b = 5.25 km.

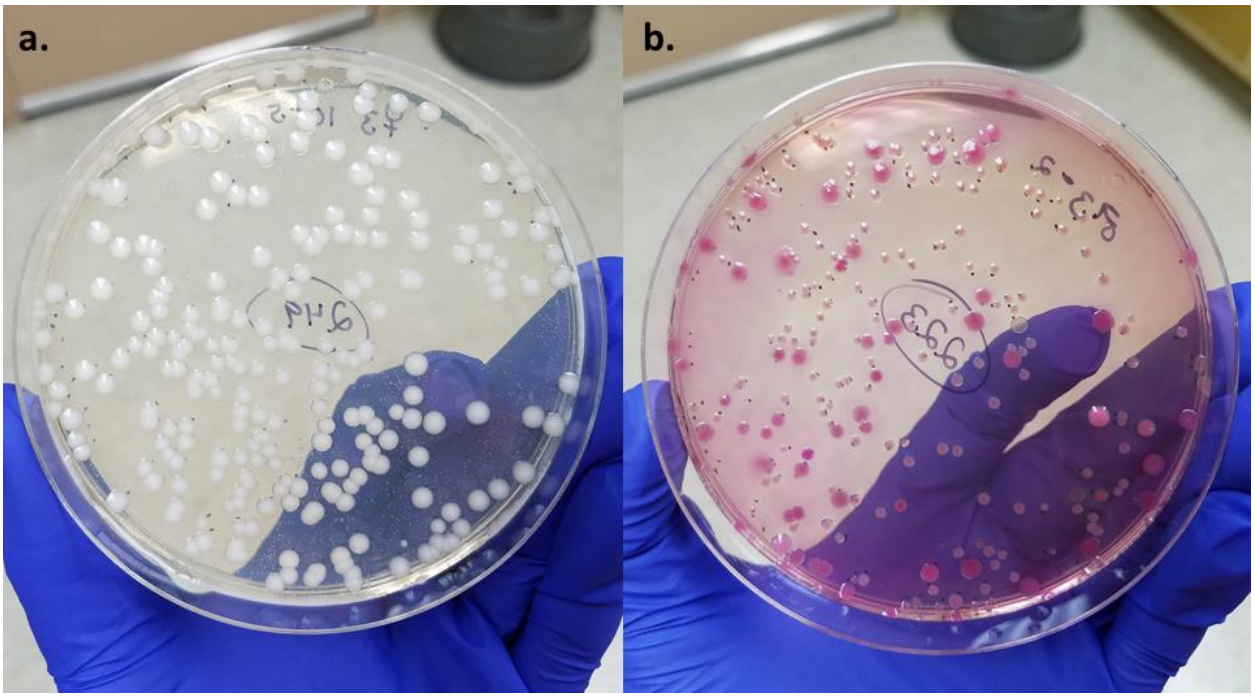

Figure 4.3. Bacteria morphotypes enumerated for each media type.

House flies were processed and cultured on Tryptic Soy Agar (TSA; a) and Violet Red Bile Agar (VRBA; b). All colonies were enumerated on TSA to determine abundance of culturable aerobic bacteria (colony forming units (CFU)/fly; a). Only red and pink colonies were enumerated on VRBA to determine coliform abundance (CFU/fly; b). Isolates (n=3-5/plate) were picked from VRBA plates and stored at -20˚C for further identification.

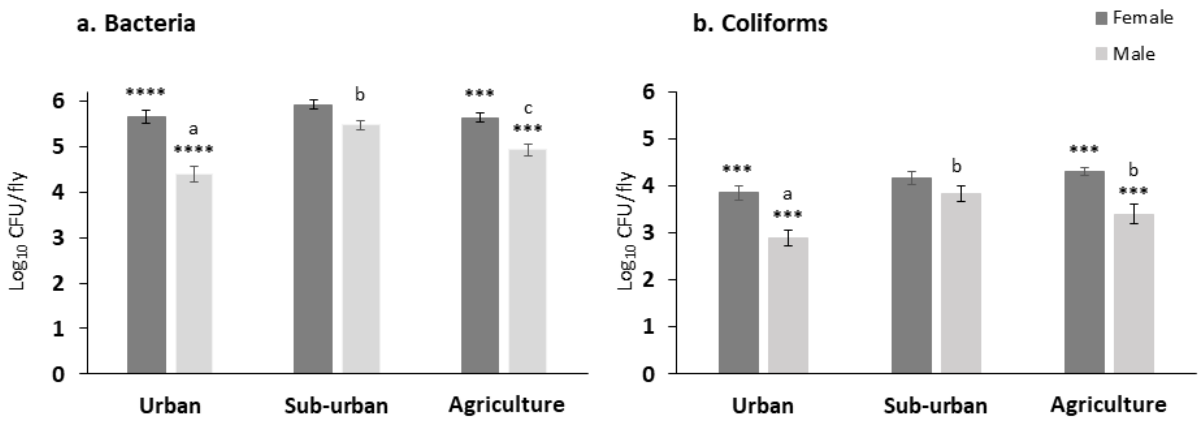

Figure 4.4. Mean bacteria and coliform abundance in house flies.

Male (n=10) and female (n=10) house flies were collected from 3 different sites in Manhattan, KS (urban, sub-urban, and agricultural) once a week for 5 weeks (from late August through end of September 2018) and processed to determine abundance (colony forming units; CFUs) of culturable aerobic bacteria (a.) and coliforms (b.) using two different media (Fig. 4.3). Mean ± SEM aerobic bacterial and coliform abundance recovered from flies are shown (each bar represents n=50 flies). Lowercase letters represent statistical significance across site within sex ($P < 0.05$) and asterisks denote statistical significance within site between sex (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$).

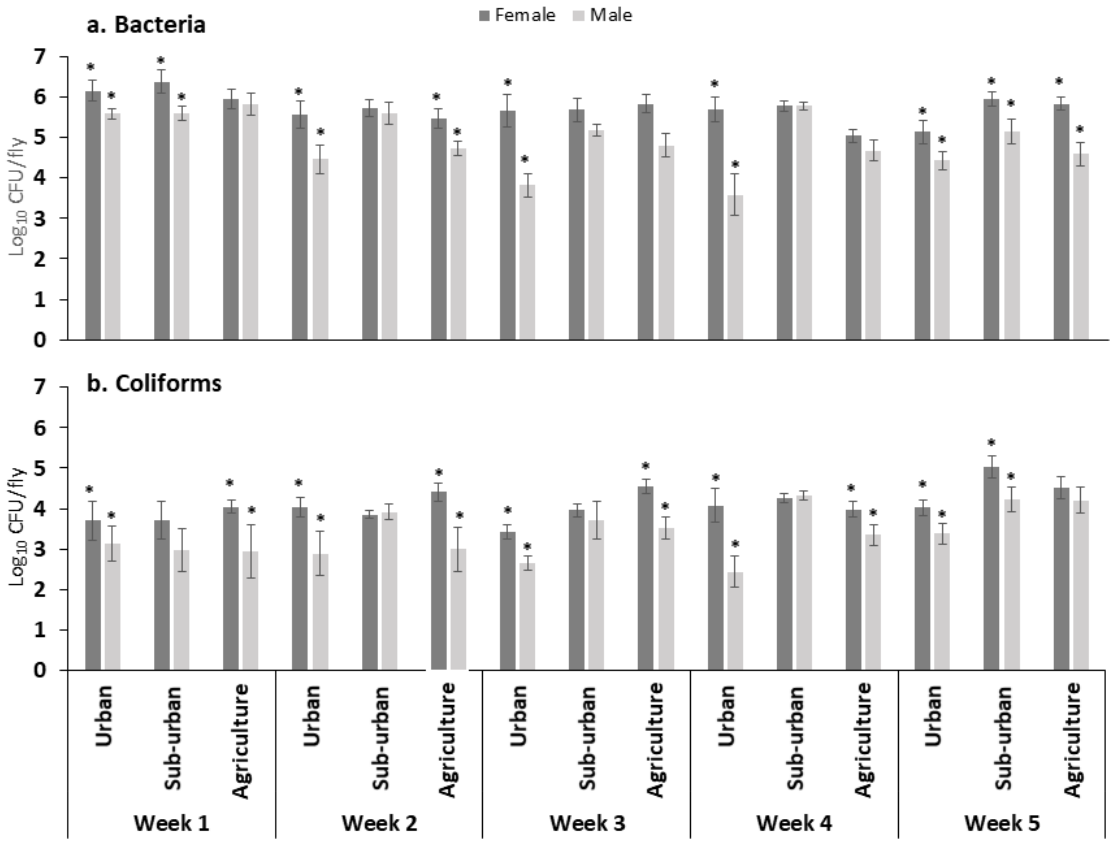

Figure 4.5. Mean bacteria and coliform abundance in house flies by week.

Male (n=10) and female (n=10) house flies were collected from 3 different sites in Manhattan, KS (urban, sub-urban, and agricultural) once a week for 5 weeks (from late August through end of September 2018) and processed to determine abundance (colony forming units; CFUs) of culturable aerobic bacteria (a.) and coliforms (b.) using two different media (Fig. 4.3). Mean ± SEM aerobic bacterial and coliform abundance recovered from flies are shown (each bar represents n=10 flies). Asterisks denote significantly differences in bacterial abundance between male and female flies within each site by week ($P < 0.05$).

Chapter 5 – Conclusions

The purpose of this dissertation research was to investigate the potential risk house flies pose to human and animal health by (1) determining if male and female house flies differed in their acquisition of bacteria from cattle manure in the presence and absence of an alternative food source, (2) assessing *S.* Typhimurium survival in house flies, acquisition from food by flies, transfer to food by flies and transfer between flies in the presence and absence of food, and (3) measuring the abundance of bacteria and coliforms in wild male and female adult house flies collected from different habitats.

Female house flies acquired and harbored a greater abundance of bacteria than males, presumably due to more time spent interacting with and ingesting substrate containing bacteria. Therefore, female flies may pose a greater risk of disseminating bacteria than males on livestock facilities where open access to manure, and the microbes therein, exist. Thus, sex-specific fly management strategies should be considered to reduce bacterial dissemination on farms. Additionally, females acquired a greater abundance of *E. coli* from manure than males only when an alternative food source was present indicating that males and females differ in their nutritional requirements. Therefore, the presence of an alternative food source directly affects sex-specific nutritional preferences and should be considered in risk assessment.

House flies are vector competent for *S.* Typhimurium because they (1) directly acquired *S.* Typhimurium from cattle manure and cantaloupe, (2) harbored *S.* Typhimurium in their gut and (3) transferred *S.* Typhimurium to cantaloupe and to other flies via a shared food source. Thus, if house flies have access to a source of *S.* Typhimurium, such as manure or food, they can acquire the bacteria and potentially transmit *S.* Typhimurium to other food and/or nearby flies. The presence of cantaloupe facilitated the transfer of *S.* Typhimurium between flies. Preventing

flies from having access to food will inhibit bacterial acquisition from food and reduce transmission of bacteria from food to other flies. Effective fly control and fresh produce storage in public food settings is extremely important in order to prevent flies from contaminating food with bacterial pathogens.

House fly sex and habitat affected the bacterial abundance and coliform abundance in house flies. Females had a greater bacterial abundance of aerobic bacteria than males at the urban and agricultural sites. Sex-specific behaviors and additional nutritional requirements needed for oviposition likely contributed to an overall greater abundance of aerobic bacteria in female house flies. Females seek out microbe-rich environments for oviposition regardless of the habitat which will continue to result in greater loads of aerobic bacteria and coliforms than males. Therefore, females serve as more significant reservoirs and potential disseminators of bacteria than males. Interestingly, male and female house flies did not differ in aerobic bacterial abundance at the sub-urban site. Habitats where males and females congregate in one space (e.g. dumpsters at the sub-urban site) result in similar bacterial abundance between sexes. However, female house flies did not differ from other females in aerobic bacterial and coliform abundance across all sites, while males did differ from other males. Therefore, females consistently seek out and contact microbe-rich substrates regardless of habitat and males likely drive overall differences in aerobic bacterial and coliform abundance in house flies from different habitats. It is important to consider fly sex and habitat type both in assessing bacterial transmission risk and in designing mitigation strategies. For example, effective fly pest management programs should rely more on generalized fly control approaches (e.g. bait traps, sticky traps, natural enemy release) in habitats where male and female aerobic bacterial abundance does not differ and more

on sex-targeted fly control methods (e.g. fly sex attractant traps that use oviposition pheromones) in habitats where females harbor a greater abundance of aerobic bacteria than males.

In summary, fly sex, habitat and food source availability should all be considered when developing and implementing effective fly pest management programs to reduce dissemination of pathogens that pose a risk to human and animal health. Otherwise, house flies will continue to be successful in contaminating food items and the environment with pathogens.