PERSISTENCE AND SIGNIFICANCE OF *E. COLI* IN HOUSE FLIES (*MUSCA DOMESTICA*) AND STABLE FLIES (*STOMOXYS CALCITRANS*)

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ABSTRACT

The persistence of *Escherichia coli* in the larval, pupal and adult stages of both house

flies, *Musca domestica* (L.), and stable flies, *Stomoxys calcitrans* (L.) was examined.

Abundance of *E. coli* declined over time in immature house flies, but remained constant

in immature stable flies, suggesting house fly larvae digest *E. coli* but stable fly larvae do

not. Survival of house fly and stable fly larvae averaged 62% and 25% respectively when

reared on pure *E. coli* cultures. *E. coli* load in pupae decreased significantly one day

before emergence of adult house flies, but remained constant until stable fly emergence.

Nevertheless, *E. coli* was detected in 78% of emerging house flies and in 28% of

emerging stable flies. House flies are more important *E. coli* vectors as adults, whereas

stable flies may be overlooked vectors of *E. coli* during immature development.

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CHAPTER 1. INTRODUCTION

Concern for food safety is increasing as food production becomes more intensive in industrialised countries. The emergence of new pathogens and the growing number of immune-compromised individuals intensify the need for safer food supplies. Nevertheless, outbreaks of food borne and waterborne pathogens are increasingly reported and communicated in North America. The bacterium, *Escherichia coli* serotype O157:H7, is of particular concern to the public in relation to such outbreaks. Infections with *E. coli* O157:H7 have mostly been associated with consumption of undercooked ground beef (Doyle 1991), but also with other products such as fresh vegetables (Abdul-Raouf et al. 1993, Ackers et al. 1998), unpasteurised apple cider (Besser et al. 1993, Miller and Kaspar 1994), and sausages (Duffy et al. 2000).

Beef and dairy cattle are asymptomatic carriers of *E. coli* O157:H7 (Zhao et al. 1995), which makes detection of the bacterium difficult for producers. Animals are mostly infected during the summer months, and shed bacteria in their faeces sporadically (Besser et al. 1997). This species can reach populations of 10^5 colony-forming units (CFU)/g of faeces and survive for weeks under natural conditions (Wang et al. 1996).

The association between flies and enteric bacteria and the importance of flies as vectors of pathogenic bacterial strains have been studied extensively. Studies continue to link morbidity and mortality from diarrhoeal diseases to the abundance of synanthropic flies (Cohen et al. 1991, Levine and Levine 1991, Chavasse et al. 1994). House flies and stable flies are important pests of dairy and beef cattle in Alberta, and they are also associated with faeces at several points in their life cycle. Adult house flies feed on

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manure and are associated with many pathogens that may be transmitted mechanically through regurgitation and excretion (Greenberg 1973, Graczyk et al. 1999). Sasaki et al. (2000) observed that *E. coli* O157:H7 is harboured in the digestive tract of adult house flies after feeding, and is excreted for up to four days. Both house fly and stable fly adults oviposit on manure and can become infected on their surfaces. Furthermore, larvae of both species develop in cattle manure and other potentially contaminated matter, and feed on bacteria in the environment. In spite of this, relatively limited work has been done in recent years to examine the persistence of bacteria throughout the life cycle of flies. This is probably because initial studies concluded that bacteria were destroyed during metamorphosis, either by the maggot or by events during metamorphosis (Greenberg 1959d, Radvan 1960a). The amount of bacteria remaining on the adult was assumed to have no epidemiological significance. However, these studies were conducted using unknown flora. Bacterial digestion and destruction by larvae can vary among bacterial species (Perotti et al. 2001), and *E. coli* may not be destroyed during metamorphosis. There is a need to examine insect-bacteria interaction in a case-by-case basis.

This study examined the persistence of a non-pathogenic *E. coli* strain during part of the life cycle of house flies and stable flies. Larvae were fed *E. coli* suspensions for 20 minutes and the presence of the bacterium in the gut was monitored for 48 hours. High and low doses of bacteria were used to determine possible differences in the pattern of persistence with the lower experimental dose being more representative of what the larvae would likely contact in the environment (Zhao et al. 1995, Wang et al. 1996, Shere et al. 1998). The effect of rearing larvae on *E. coli* was examined to provide a possible explanation for the observed persistence patterns. The current study also examined the

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fate of *E. coli* through metamorphosis of house flies and stable flies. Third instar larvae of both species reared on *E. coli* plates were left to pupate and were processed at different intervals throughout the pupal stage to determine *E. coli* infection. Finally, the presence of the bacterium in and on emerging adults was assessed.

CHAPTER 2.   REVIEW OF LITERATURE

2.1. Life history

2.1.1. Eggs and larvae

Both house fly and stable fly females lay eggs periodically, following a cycle of

egg maturation and oviposition. For both species, eggs are laid on moist organic matter

which also serves as growth medium for the larvae. Development sites for larval house

flies in confined cattle systems include silage mounds and indoor bedding material

(Meyer and Shultz 1990, Lysyk 1993b). Stable fly larvae occur close to fence lines and in

manure mounds (Lysyk 1993b), as well as close to feed aprons (Skoda et al. 1991).

Manure can support development of both house fly and stable fly larvae (Meyer and

Shultz 1990) largely because this is rich in bacteria that larvae use as a food source.

Numerous studies have demonstrated the importance of bacteria to larval growth and

development. House fly larvae failed to develop on an artificial growth medium sterilised

immediately after preparation, but reached the adult stage on the same medium incubated

for two days prior to sterilisation (Greenberg 1954). This indicates larvae need bacterial

growth to develop properly but can develop on lysed bacteria. House fly and stable fly

larvae reared on agar-based diets did not grow on sterile media, but completed

development when selected bacterial strains were present (Schmidtmann and Martin

1992, Watson et al. 1993, Lysyk et al. 1999).

Bacterial diversity varies between larval guts and the environment the larvae

inhabit (Zurek et al. 2000, Perotti et al. 2001). Bacteria vary in their suitability to support

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larval growth (Schmidtmann and Martin 1992, Lysyk et al. 1999, Perotti et al. 2001).

Bacteria isolated from larval guts may not support immature development. Perotti et al.

(2001) found that larval survival on a specific strain of bacteria was inversely correlated

with the strain's relative abundance in the gut, but survival was highest on the strains

more abundant in the environment. This suggests bacterial strains remaining in the gut

represent a poor food source because they are indigestible, while other species are

digested and used as nutrients. This study also indicated that muscoid larvae vary in their

ability to use specific bacteria because survival among horn fly, house fly and stable fly

larvae was very different when reared on the same bacterial isolates (Perotti et al. 2001).

Physiological adaptations of house fly and stable fly larvae demonstrate evolution

towards the use of bacteria. The presence of pharyngeal ridges is an adaptation to

concentrate particles of a certain size from liquefied media, and may be further evidence

that larvae use bacteria as a food source (Dowding 1967). The digestive tract of house fly

and probably stable fly maggots has a highly acidic region (pH 3.0-3.2) in the midgut

where bacteria are lysed (Greenberg 1965, Espinoza-Fuentes and Terra 1987). This

region, the mid-midgut, is also the site of lysozyme activity and pepsin secretion

(Espinoza-Fuentes and Terra 1987). Lysozymes catalyse the hydrolysis of

peptidoglycans, a major component of the bacterial cell wall, and are present in the

haemolymph of many insects as part of the many defences against bacterial infections

(Dunn 1986). The midgut lysozymes isolated from house fly larvae are different from

other insect lysozymes because they have an optimal activity at a pH of 3.5 (Espinoza-

Fuentes and Terra 1987). Small amounts of lysozymes are also found in the salivary

glands of house fly larvae, but have higher activity at pH 6.0 (Lemos and Terra 1991).

Other characteristics also make these midgut lysozymes similar to the ones found in ruminant stomachs (Lemos et al. 1993), where they are digestive enzymes, not antimicrobial peptides.

Larvae pass through three instars in their larval habitat. Development time of house fly and stable fly larvae is dependent on temperature. House fly larvae reared in poultry manure will reach pupation in about eight days at 25°C, but will require five days at 35°C (Lysyk and Axtell 1987). Complete immature development in house flies requires 222 degree-days when using a threshold of 10°C (Lysyk 1993a). Stable fly larvae also develop faster as temperatures increase, up to 35°C (Lysyk 1998), and immature development is completed in 232 degree-days (Lysyk 1993a).

2.1.2. Pupae

Metamorphosis of third instar larvae of both species begins with a pulse of steroid hormone ecdysone, which causes cessation of feeding and roaming (Jiang et al. 1997). Larvae find a place to pupate that is generally cooler and drier than the larval habitat. Prepupae contract into a barrel shape within their own integument, which will harden and darken to become the puparium (West 1951, Thomas 1985). Complete pupal development of house flies takes about five days under natural conditions, but can take less than four days at 35°C (Greenberg 1959d, Lysyk and Axtell 1987). Stable fly pupae develop in six to ten days under favourable conditions (Lysyk 1998). Within 48 h, important structures of the newly formed adult house fly and stable fly are distinguishable (Radvan 1960b, Thomas 1985). Histolysis of larval tissues and histogenesis of adult tissues occur at the same time (West 1951, Jiang et al. 1997). As the

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new tissues are formed, the old ones degenerate and are destroyed or cast aside within the puparium. This is the case with the midgut, shed during larval-pupal ecdysis, to the space between the forming adult and the pupal case (Thomas 1985). Studies conducted on *Drosophila* metamorphosis indicate larval tissues are probably destroyed through hormone triggered apoptosis throughout pupal development (Jiang et al. 1997).

2.1.3. Adults

Adult house flies and stable flies emerge from the puparium, dry their wings, harden their cuticle, and begin their adult life. In Alberta, house fly populations are relatively high from July through September, while stable fly populations peak in August and September (Lysyk 1993a). Adult house flies feed on manure and other decaying organic matter. Females will mate once and lay about 120 eggs per cycle (Lysyk 1991). Adult stable flies will feed on vertebrate blood, and will attack cattle. Females mate once, and require a blood meal to produce an average of 80-90 eggs per gonotrophic cycle (Lysyk 1998).

Both house flies and stable flies are strong fliers capable of dispersal over long distances, although many remain relatively close to their origin. Unsanitary areas are used as feeding and breeding sites for both house flies and stable flies, and are therefore preferred for aggregation (Lysyk and Axtell 1986). An estimated 60% of adult house flies remain within 50 m of their origin (Lysyk and Axtell 1986). Adult house flies rest mostly indoors, while adult stable flies tend to rest outside on feed aprons and on east- and south-facing barns and fence surfaces (Lysyk 1993b).

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2.2. Importance of house flies and stable flies as pests

House flies and stable flies have biological properties that make them potential mechanical vectors. They live in close association with bacteria, they enter farm buildings and homes and adults disperse from one area to another. House flies and stable flies are termed synanthropic because they live in close proximity to humans and domestic animals, and they use the human environment for shelter and food. Adult flies and larvae are associated with the human environment and feed on excreta of humans or domestic animals, decaying organic matter, and possibly on vertebrate blood. They share the human environment because of its buildings or farmyards, which satisfy their requirements for shelter better than natural environments. These trophic and ecological requirements are also used to evaluate the epidemiological significance of flies (Greenberg 1971).

Flies can be an annoyance to people and animals, as well as pose a health threat. They are often in contact with highly contaminated substrates and are known vectors of numerous pathogens (Greenberg 1973, Schmidtmann 1985). Large fly populations at the feed bunks can also make animals reluctant to feed (Wieman et al. 1992). Stable flies have painful bites that cause irritation and can damage tissues and hide. Large fly numbers can also cause behaviour changes in cattle attempting to avoid bites, and lead to reductions in weight gain and milk production (Campbell et al. 1987). Stable flies are also persistent biters, and can transmit pathogens by interrupted feeding (Straif et al. 1990).

2.3. Disease transmission

The transmission of pathogens by flies and other arthropods may be *biological* or *mechanical*. For biological transmission to occur, the pathogen must undergo developmental changes within the vector. Biologically transmitted pathogens have evolved in close association with their arthropod vector and infection of the arthropod is a necessary step in the completion of the pathogen lifecycle. The pathogen develops to its infective state and multiplies in the vector, which then serves to infect a new host. Parasites such as *Plasmodium* spp. are transmitted biologically because without its mosquito vector, the protozoan would not be able to develop to it infective form, and could not be transmitted from one host to another. Arthropod borne viruses are considered biologically vectored because they require propagation within the arthropod host, where they attack the tissues and multiply (Gray and Banerjee 1999). Arthropods that are not susceptible to infection by a virus cannot act as its biological vector, but non-specific transmission is possible through contamination of mouthparts.

In mechanical transmission, the vector essentially transports the pathogen. The pathogen may be carried externally on the body surface or the mouthparts of the fly and then transmitted through simple body contact, or carried internally in the gut, and transmitted through regurgitation or defecation (Dipeolu 1982, Glass et al. 1982, Sasaki et al. 2000). Many types of pathogens are known to be spread this way, from viruses (Greenberg 1973, Tan et al. 1997) and bacteria (Bidawid et al. 1978, Cohen et al. 1991, Levine and Levine 1991, Sasaki et al. 2000), to protozoa (Straif et al. 1990, Fotedar et al. 1992, Graczyk et al. 1999) and even helminth eggs and larvae (Dipeolu 1982, Sulaiman

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et al. 1988).

Flies are good indicators of the micro organisms present in the environment. House flies trapped within a hospital had higher bacterial counts and carried more pathogens than flies captured in a residential area (Fotedar et al. 1992). Attenuated polioviruses used for vaccination were isolated from house flies, demonstrating humans may actually be responsible for contaminating flies (Greenberg 1973). House flies placed in contact with chicks infected with *Campylobacter jejuni* were contaminated with the pathogen five days later. These flies subsequently infected pathogen-free chicks with *C. jejuni* within eight days (Shane et al. 1985).

The association between the house flies and faeces is an important issue in disease transmission. The house fly was implicated in the transmission of numerous enteric pathogens such as *Shigella* spp. (Lindsay and Scudder 1956, Levine and Levine 1991), *Salmonella* spp. (Greenberg 1964), and *E. coli* (Moriya et al. 1999). House flies may also be important carriers of *Vibrio cholerae* during outbreaks (Fotedar 2001). Cohen et al. (1991) demonstrated that the implementation of control measures to reduce the fly population significantly decreased the number of diarrhoeal cases reported.

2.4. Acquisition and dissemination of pathogenic organisms by flies

The ability of house flies and stable flies to mechanically transmit pathogenic agents is influenced by their nutritional requirements and feeding habits. These characteristics are related to the morphology of the fly's mouthparts, the kinds of pathogens that can be transmitted, as well as the infection route taken by these pathogens.

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Other structures such as the legs and body can also transport pathogens. Since adult

house flies and stable flies have different methods of feeding, their methods for acquiring

pathogens differ.

2.4.1. Morphology

Adult house flies have sponging mouthparts that trap dissolved food particles. The

labella are well developed and act as a sponge that filters and absorbs liquids. The part of

the labella in contact with the substrate bears rows of small parallel channels called

pseudotracheae. When the labella fill with liquid, these pseudotracheae act like gutters

and drain the liquid to the mouth opening and up to the food canal (Greenberg 1973). The

space between these channels is approximately five microns (Greenberg 1973). The

labella also serve to scrape the substrate with the prestomal teeth located before the

opening of the food canal. The teeth are delicate blade-like structures, and their number

and shape vary among species (Greenberg 1973, Broce and Elzinga 1984). Although

house fly prestomal teeth are smaller than those of other species, they can still cause

some tissue damage during feeding (Kovacs et al. 1990). The flies can also feed directly

from the opening of the food canal, which enables them to ingest small particles like

helminth eggs (Greenberg 1973). Since house flies are found in many different

environments and feed off a variety of substances, they are exposed to a wide range of

pathogens. Particles can adhere to the minute spaces of the sponging mouthparts and be

transferred to the next surface the fly feeds on. House flies fed *E. coli* O157:H7 had

bacterial cells attached to the inner and outer surfaces of the labella 24 h after feeding

(Kobayashi et al. 1999).

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Other structures of the fly's morphology facilitate pathogen transport. The body is hairy, and so are the legs. Hairs on the exoskeleton can easily trap droplets of liquids or small solid particles as adult flies walk on a substrate during feeding or oviposition (Tan et al. 1997). The feet also have sticky pads covered with tiny hair that enable them to walk on walls and ceilings; these also can trap micro-organisms. These morphologic factors are more important for coprophagous and saprophagous flies because more of their body is in contact with the contaminated medium.

Recent work on mechanical transport of the rotavirus by the legs and wings of the house fly indicates that flies picked up more viruses in a solution containing faeces than in a clear suspending medium (Tan et al. 1997). This was attributed to the hairs' and bristles' efficiency in catching suspended particles containing viruses. All tested flies transferred the largest amount of virus particles to the first surface visited, regardless of its type or of the time they were allowed to walk on that first surface. The proportion of virus particles transferred was influenced by the nature of the surface, where a soft surface was found to remove more particles from the flies' legs. When virus particles adhered to the underside of the wings, more than 95 percent were removed within the first 11 seconds of flight (Tan et al. 1997).

Blood feeding flies, such as the stable fly, pierce skin in order to feed. Adult stable flies are called pool feeders, or telmophages, and have short and sturdy mouthparts adapted for cutting the skin rather than penetrate it as in the case of vessel feeders. The maxillae and mandibles are blade-like, and the labella are large and surround the flowing blood to direct it to the food canal (Teskey 1990). The teeth inside the labella rasp the skin surface while the maxillae, mandibles and hypopharynx stab the skin to make the

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blood flow to the surface. Body contamination of blood feeding flies is less important in disease transmission since only the proboscis is in contact with the potentially infected blood or fluid during feeding (Glass and Gerhardt 1984).

This type of feeding is painful for the host, and defensive behaviours often dislodge the fly before a full blood meal is ingested. Dislodged flies will keep biting until they get the amount of blood they require. This repeated biting can occur on the same or a different animal and lead to pathogen transmission. Pathogens can also stay in the spaces between the different components of the mouthparts (Butler et al. 1977). *Trypanosoma brucei* and *T. vivax* were relatively easily transmitted by African Stomoxinae after an interrupted meal on infected blood: the success rate with *T. brucei* was of 11.5 percent, and with *T. vivax* it was 3.4 percent (Mihok et al. 1995). This was compared to transmission with a needle where the success rate with *T. brucei* was 80 percent, and 100 percent with *T. vivax*. However, the needle probably penetrates deeper than the slashing mouthparts of the Stomoxinae, placing the pathogens directly into the blood stream, and this may increase the chances of a successful infection.

2.4.2. Regurgitation

Regurgitation during feeding can occur in both species and can also be an important contribution to pathogen transmission. House flies regurgitate on the substrate during feeding and reabsorb the liquid in which some of the substrate has dissolved. Studies performed on face flies (*Musca autumnalis*) indicate the nature of the substances ingested by flies has an effect on regurgitation frequency. Face flies offered only water did not regurgitate after feeding, but face flies fed various concentrations of trypticase soy broth

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(TSB) regurgitated for over three hours after ingestion of the solution (Coleman and Gerhardt 1988a). Furthermore, regurgitation increased proportionally with the volume ingested and the concentration of the TSB solution (Coleman and Gerhardt 1988a). Environmental factors such as temperature and relative humidity may also play a role in regurgitation frequency (Coleman and Gerhardt 1988b). Interactions between flies can also affect regurgitation. In a group of flies, the amount of regurgitation drops per fly remains the same whether the group is large or small; however, the arrival of unfed flies increases the number of regurgitation drops per fly (Coleman and Gerhardt 1987). Regurgitation also occurs more often when face flies are fed on or near a host compared with when they are fed in a lab (Coleman and Gerhardt 1987).

The how's and why's of regurgitation are not well understood, but it is clear the process has a significant role to play in mechanical transmission. Enteric pathogens are often isolated from the faeces of infected humans or animals. In low sanitation areas or close to animal production facilities, coprophagous flies have relatively free access to contaminated stools where they readily ingest these pathogens. If fresh food is to be found nearby, these same flies may feed from it, and regurgitate part of their previous meal onto it. Selective regurgitation may occur. Protozoan cyst and helminth eggs are retained in the alimentary canal and may not be regurgitated as easily as are smaller organisms such as trypanosomes and bacteria (Greenberg 1973). House flies were fed eggs of hookworm and *Ascaris lumbricoides* in human faeces and the development of the regurgitated eggs was assessed. No difference was observed in the developmental time of regurgitated and control eggs (Dipeolu 1982). When the flies were fed larvae of both helminth species, regurgitation of infective larvae was observed up to 4-5 hours after

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intake for hookworm larvae, and longer in the case of *A. lumbricoides*. The digestive

process in the fly gut seemed to affect the larvae of hookworm, whereas the ascarid

larvae were protected by their characteristic shell (Dipeolu 1982). Regurgitation was also

held responsible for the persistent contamination of house fly mouthparts with bacteria

days after the initial contact (Kobayashi et al. 1999).

Blood feeding flies also regurgitate during feeding (Butler et al. 1977). Straif et al.

(1990) demonstrated regurgitation of both red blood cells and pathogens from

S. calcitrans after a meal of infected blood. Pathogens studied included *Trypanosoma*

brucei brucei, Borrelia burgdorferi, and *Plasmodium yoelii nigeriensis*. Simple

contamination of the mouthparts occurred almost regularly with *Trypanosoma*, and

occasionally with *Plasmodium*. Regurgitation of all pathogens was observed. These

results were confirmed by interrupted feeding experiments on mice where the proboscis

of the flies was cleaned before the transfer on the healthy mouse. Only infection trials

with *Trypanosoma* were conclusive. This could explain the transmission of *T. brucei* in

regions of the world where tsetse are absent, but where Stomoxinae are present (Straif et

al. 1990). The authors believe infection with *Plasmodium* failed because infected

erythrocytes could not reach the general circulation and travel to the liver, whereas

Trypanosoma is motile and could migrate from the surface of the wound where it was

regurgitated to the general blood circulation. In the case of *Borrelia*, they suspect the

number of cells transmitted was too low to cause an infection. The experiment by Mihok

et al. (1995) also confirms the *Trypanosoma* transmission results, although no

differentiation was made between mouthparts contamination and regurgitation. The

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interrupted feeding of stable flies can also transmit cutaneous leishmaniasis (Faust et al. 1968).

2.4.3. Digestion and excretion

The ingested pathogens that are not regurgitated proceed through the gut where they are either destroyed or excreted. House flies and stable flies secrete a peritrophic matrix that surrounds the food bolus. The peritrophic matrix is a membranous sac made of chitin, proteins and proteoglycans secreted by the gut, and acts as a substantial physical barrier for pathogens (Tellam 1996). The matrix is permeable to certain enzymes, ions, and small molecules, but normally retains larger particles such as bacteria and viruses. There are two types of peritrophic membranes: type I is formed during feeding and is synthesised by the midgut epithelium; type II is continually produced by specialised anterior gut cells and is usually present before food is ingested (Tellam 1996). House flies and stable flies produce a type II peritrophic matrix both as larvae and as adults (Tellam 1996, Lehane 1997). In adult house flies, digestion begins in the crop where starches are broken down to reduce the starch content in the anterior gut. This makes bacteria more vulnerable to the action of the low pH level of the midgut (approximately 3.1-3.3) and the lysozymes (Terra et al. 1996).

Large amounts of bacteria must be ingested to permit bacterial persistence in adult house fly guts. House flies fed 10^9 CFU/ml of *E. coli* O157:H7 excreted 10^4 CFU/ml up to three hours after feeding (Sasaki et al. 2000). Pathogenic bacteria are not always available in such high concentration in the environment. The prevalence of *E. coli* O157:H7 in cattle rarely reaches more than 40% (Zhao et al. 1995, Bach et al. 2002) and

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the bacterium is shed intermittently in bovine faeces at levels ranging from 10^2 to 10^5

CFU/g (Zhao et al. 1995, Shere et al. 1998). Flies may need to acquire large amounts of

bacteria in order to transmit an infectious dose, even if the infectious dose of a bacterium

is low (DuPont et al. 1989, Kobayashi et al. 1999).

Interspecific competition among bacteria in the fly gut may also inhibit pathogen

transmission. Flies fed on a bacterial suspension of *Yersinia pseudotuberculosis*

contaminated agar plates up to 18 hours after the initial feeding; further detection of

Y. pseudotuberculosis was made impossible by the growth of other enteric bacteria

(Zurek et al. 2001). The elimination of the bacterium as a result of competition is one

possibility, but it is also possible *Y. pseudotuberculosis* was more digestible than the

other bacterial species.

Most particles, pathogen or other, pass through the gut within a few hours after

ingestion, both for larvae and adults (Espinoza-Fuentes and Terra 1987, Sasaki et al.

2000, Mumcuoglu et al. 2001). However, adult house flies fed *E. coli* O157:H7 excreted

viable bacterial cells for at least three days (Kobayashi et al. 1999). House flies fed on

bovine faeces containing 2×10^5 oocysts/ml of *Cryptosporidium parvum* excreted an

average of seven oocysts per faecal spot over a period of 8 days (Graczyk et al. 1999).

Infection with this protozoan can be caused by as few as 30 oocysts in healthy human

beings.

2.4.4. Gonotrophic cycle

From an epidemiological point of view, the gonotrophic cycle of the females is also

important in pathogen transmission. Non-gravid females need more blood to develop

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eggs and are more persistent feeders than gravid females. Saprophagous female flies also have increased nutritional requirements during early gonotrophic development. House flies with developing eggs feed and excrete more often than males and more than females with fully developed eggs (Sasaki et al. 2000). Females with mature eggs feed and excrete less than males.

2.5. Alternate routes of acquisition

In the early 20th century, the United States government was involved in a large campaign to expose flies as a health threat, and even today, extension services try to educate the population about the health hazard flies represent for people and their family. House flies and stable flies undoubtedly acquire pathogens throughout their adult life, but so do larvae developing in manure and decomposing matter. Since flies undergo a complete metamorphosis, the fate of the pathogens may be of epidemiological importance if they are passed on to the adult fly. House fly and stable fly larvae harbour total bacterial populations of about 10^7 CFU per larva (Greenberg 1959d, 1962), but emerging adults usually harbour few bacteria or none at all (Radvan 1960b), regardless of the rearing medium of the larvae (Greenberg 1959a). Contamination of the adult was reported to be mostly detected on the surface of the fly and was therefore considered of low epidemiological significance (Radvan 1960b). Surface contamination of adult flies at emergence is probably greater in a natural environment than in a laboratory (Greenberg 1959c). There are two major declines in the bacterial population during fly metamorphosis (Greenberg 1959a). The first decline occurs at the prepupal stage and

averages a hundred-fold decrease. The second decline may be related to the shedding of the old digestive tract during pupal metamorphosis. A large number of bacteria can be recovered from the empty pupal case (Radvan 1960a, Greenberg 1962, 1964), and while some species of bacteria survive more successfully through the metamorphosis, there is no evidence the "normal flora" of saprophagous bacteria tolerate the changes better than pathogens (Greenberg 1959a, Radvan 1960a).

However, these data are incomplete as they do not consider specific host-pathogen relationships. Not all bacteria from the development habitat are digested and used by the larvae, and some remain in the digestive system until pupation. There may be a relationship between pathogen retention to the adult stage and digestion, or lack of digestion, of these pathogens in the larval gut. The association of *E. coli* and synanthropic flies is a good model to investigate this potential for pathogen transfer. *E. coli* is a common bacterium, and is known to be associated with cattle and other vertebrates. It can easily be sampled in feedlots and dairies where both species of flies are present. House flies and stable flies are pests of confined animals, and therefore also occur in feedlots and dairies. The importance of adult flies in disease transmission has been established, but the role of immature flies has been overlooked in the whole pathogen transmission cycle. It is important to determine if larvae can acquire specific pathogens such as *E. coli* and retain them into the adult stage.

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CHAPTER 3.   MATERIALS AND METHODS

3.1. Stock insect colonies

House flies and stable flies were obtained from stock colonies maintained using

procedures outlined in Lysyk (1998, 2001). The adults were maintained at 25°C with a

photoperiod of 16:8 (L:D) h. House flies were fed a diet of granulated sugar and dilute

evaporated milk (1:1) in small specimen dishes with crushed absorbent paper to prevent

flies from drowning. Eggs were laid on the milk-soaked paper. Stable flies were fed

defibrinated bovine blood and 10% sucrose in water in small dishes with a gauze pad that

also served as ovipositional substrate. Larvae were reared in a mixture of wheat bran,

dried brewer's grain, alfalfa meal, water, and brewer's yeast Stable fly larval rearing

medium also included sawdust. House fly pupae were removed from the rearing medium

by forced air circulation and stable fly pupae were scooped from the rearing medium.

Pupae were placed in small salad dishes and held in clean cages for eclosion.

3.2. Bacteria preparation

Bacterial strains used in the experiments were reconstituted from glycerol stock

cultures of bacteria. *Empedobacter brevis* (Holmes & Owen) and *Flavobacterium*

odoratum Stutzer were previously isolated from stable fly eggs obtained from a

laboratory colony (Lysyk et al. 1999). Bacteria were grown overnight at room

temperature on nutrient agar (Difco, Becton Dickinson, MD). The *E. coli* strain used was

previously isolated from horn fly larval gut (Perotti et al. 2001). The bacterium was grown overnight at 37°C on Luria-Bertani (LB) plates and broth. Bacteria collected from the plates were re-suspended in sterile distilled water unless otherwise stated and bacterial density was adjusted by optical density at 600 nm. *E. coli* populations from larvae, pupae and adults were enumerated by spreading serial dilutions onto MacConkey agar and counting red colonies (Clark 1971).

3.3. Gnotobiotic larval rearing procedure

Larvae used in all experiments were reared on agar medium. House fly and stable fly eggs obtained from the stock colonies were rinsed in distilled water, washed in three changes of freshly made 0.26% sodium hypochlorite solution for a total of 15 minutes (Perotti et al. 2001) and rinsed in three changes of sterile distilled water. Plates containing egg yolk medium four (Watson et al. 1993) were inoculated with 100 µl of a mixed bacterial suspension of *E. brevis* and *F. odoratum* as these were found to be most favourable for the development of stable flies (Lysyk et al. 1999). Two pieces of sterile #1 Whatman filter paper (Whatman Ltd, UK) were also placed on the plates. The surface-sterilised eggs were aseptically transferred onto the filter paper with a sterile brush at a density of about 40 eggs per plate. Plates were incubated at 25°C throughout the life cycle.

3.4. Persistence of *E. coli* in larval guts of house flies and stable flies

3.4.1. Short term persistence

This experiment was conducted to evaluate the persistence of *E. coli* in the larval gut of house flies and stable flies over a period of five hours. The general procedure was to feed larvae of both species an *E. coli* suspension, rear larvae on water agar, and sample larvae hourly to determine temporal changes in *E. coli* load.

E. coli cultures were grown overnight in LB broth at 37°C with constant agitation (200 rpm). Cultures were transferred to cuvettes and diluted to an optical density of 0.800 (± 0.005) at 600 nm with sterile LB broth. Viable counts of the bacterial suspensions were performed to determine the concentration of bacteria fed to the larvae. Larvae were reared from egg to third instar on agar medium using the gnotobiotic procedure. Third instar larvae were aseptically removed from rearing plates and starved on water agar for a day before each trial. Larvae were assigned to one of three treatment groups: *E. coli*-fed, broth-fed or unfed. The *E. coli*-fed larvae were used to determine *E. coli* persistence in the larval guts. Six larvae were placed in each of two sterile 1.5 ml microfuge tubes with 35 µl of coloured *E. coli* suspension and allowed to feed for 20 minutes. One drop of green food colouring (Club House, McCormick Canada Inc.) was added to the solutions to ensure the larvae had ingested the solution. The coloured solution was visible in the guts of feeding larvae. Broth-fed larvae were used as controls to ensure that contamination did not result from rearing. Six larvae were placed in a sterile 1.5 ml microfuge tube with 35 µl of coloured sterile LB broth and allowed to feed for 20 minutes. After feeding, larvae were removed from the tubes with sterile insect handling

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forceps, surface-sterilised in three changes of 0.26% sodium hypochlorite for a total of 15

minutes and rinsed in three changes of sterile distilled water. Unfed larvae were used to

determine the efficiency of surface-sterilisation. Six larvae were dipped in a coloured *E.*

coli solution for less than two seconds each and surface-sterilised following the same

method. Larvae in all treatments were handled similarly following treatment. Larvae

were placed on sterile water agar and incubated at 25°C for 0, 1, 2, 3, 4 and 5 h after

surface-sterilisation. Incubation was completed on water agar to reduce contamination

from excreted bacteria. Each hour, two *E. coli*-fed larvae, one broth-fed larva and one

unfed larva were collected, individually homogenised in 1 ml phosphate buffered saline

(PBS) (pH 7.2: NaH_2PO_4 0.2M, 14.0 ml/l; Na_2HPO_4 0.2M, 36.0 ml/l; NaCl, 8.0 g/l) with

a sterile plastic pestle and centrifuged at $800 \times g$ for two minutes to produce a clear

supernatant. Serial dilutions of the supernatant were plated in duplicate. A total of 50

E. coli-fed larvae, 25 broth-fed larvae and 25 unfed larvae were examined for each time

point and species. For each larva, the amount of *E. coli* in the larval gut was recorded as

the average count from the two plates. Logistic regression was used to determine if

change in the proportion of infected larvae over time was consistent among species

(SPSS Inc. 1989-1999). The number of bacteria per larva was transformed to $\log(y + 1)$.

A two-way analysis of variance (ANOVA) was used to determine if the change in *E. coli*

populations in the larval guts varied over time and between species. A linear regression

model was used to estimate temporal changes in the *E. coli* load in larval guts for each

species.

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3.4.2. Long term persistence

The same general procedure was used to examine the persistence of *E. coli* in the

larval gut of house flies and stable flies over a period of 48 h. Larvae of both species

were fed, reared on water agar and sampled daily to determine temporal changes in

E. coli.

E. coli suspensions and larvae of both species were prepared as previously

described. Larvae were reared from egg to third instar on agar medium using the

gnotobiotic procedure. Larvae were assigned to the same three groups: *E. coli*-fed, broth-

fed and unfed. *E. coli*-fed larvae were produced by placing 40 larvae in four sterile 1.5 ml

microfuge tubes (10 per tube), each with 35 µl of coloured *E. coli* suspension. Broth-fed

larvae were placed in two sterile 1.5 ml microfuge tube (10 larvae per tube) with 35 µl of

coloured sterile LB broth. Larvae were allowed to feed for 20 minutes, removed from the

tubes with sterile insect handling forceps and surface-sterilised as previously described.

Unfed larvae were produced by placing 20 larvae in a coloured *E. coli* suspension for less

than two seconds each and surface-sterilised to serve as controls for sterilisation. After

treatment, larvae were placed on sterile water agar at 25°C. Larvae were initially sampled

within five minutes of surface-sterilisation (zero hour) and again at 24 and 48 h. At each

time point, 10 *E. coli*-fed larvae, five broth-fed larvae and five unfed larvae were

collected and individually homogenised as previously described. Larvae sacrificed at 24

and 48 h post-feeding were surface-sterilised again prior to homogenisation. Enumeration

of *E. coli* populations was done as previously described and the experiment was

replicated five times. A total of 50 *E. coli*-fed larvae, 25 broth-fed larvae and 25 unfed

larvae were examined for each time interval and species. The number of bacteria per

larva was transformed to log(y + 1). Logistic regression with time as a categorical variable was used to determine the change in the proportion of infected larvae over time for each species separately. Two-way ANOVA and linear regression were used to estimate temporal changes in *E. coli* load in larval gut for each species.

3.5. Dose-dependent persistence of *E. coli* in house fly and stable fly larval guts

Changes in the *E. coli* gut population of house fly and stable fly larvae fed a low or a high concentration of bacteria was examined. Larvae of both species were fed on either a low or a high dose of *E. coli* in suspension and sampled immediately after ingestion and again after 48 h to determine the temporal changes in *E. coli* gut population.

E. coli cultures were grown overnight in LB broth at 37°C with constant agitation (200 rpm). Cultures were transferred to cuvettes and adjusted to an optical density of 0.800 (± 0.005) at 600 nm with sterile LB broth. This high dose *E. coli* suspension was serially diluted to 1:10 000 to produce the low dose *E. coli* suspension. The concentration of bacteria fed to the larvae was determined by viable counts of the high dose bacterial suspension. Larvae were reared from egg to third instar on agar medium using the gnotobiotic procedure. Third instar larvae of both species were removed from the rearing plates and starved on water agar for 24 h prior to the experiment. Larvae were assigned to one of four groups: *E. coli*-fed with a high dose suspension, *E. coli*-fed with a low dose suspension, broth-fed or unfed. *E. coli*-fed larvae were produced by placing 40 larvae in four sterile 1.5 ml microfuge tubes (10 per tube) with either 35 µl of a coloured high dose

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E. coli suspension or 35 µl of a coloured low dose *E. coli* suspension. Larvae were

allowed to feed for 20 minutes. Broth-fed larvae were produced by placing 40 larvae in

four sterile 1.5 ml microfuge tube (10 per tube) with 35 µl of coloured sterile LB broth

and were used as controls to ensure *E. coli* did not come from contamination of the

rearing medium. Broth-fed larvae were left to feed for 20 minutes and were then surface-

sterilised as previously described. Unfed larvae were dipped in a coloured *E. coli* solution

for less than two seconds each and served as controls for sterilisation. Once treated,

larvae were placed on water agar at 25°C. Larvae were initially sampled within 5 minutes

of surface-sterilisation (zero hour) and again at 48 h after surface-sterilisation. At each

time point, 10 *E. coli*-fed larvae fed a high dose, 10 *E. coli*-fed larvae fed a low dose, five

broth-fed larvae and five unfed larvae were individually homogenised in 1 ml PBS with a

sterile pestle. Homogenates were centrifuged at 800 × g for two minutes and dilutions of

the supernatant were plated in duplicate. Because of low bacterial density, larvae fed a

low dose *E. coli* suspension and sacrificed immediately after ingestion were homogenised

in 100 µl of PBS and the whole volume was plated on a single MacConkey plate as it

represented the total *E. coli* in the gut. The experiment was replicated five times and a

total of 50 *E. coli*-fed larvae, 25 broth-fed and 25 unfed larvae were treated for each time,

dose and species. The number of *E. coli* in each larva was transformed to $\log(y + 1)$ and

data were analysed using a three-way analysis of variance (SPSS Inc. 1989-1999) with

species, dose and time as main effects. All two-way interactions were examined.

3.6. Survival and development of house fly and stable fly larvae reared on three different bacterial cultures

This experiment was designed to evaluate the survival of house fly and stable fly larvae reared on *E. coli* and other bacterial cultures. Rearing plates were inoculated with bacterial mixtures and surface-sterilised fly eggs, and survival to pupation and to adult emergence were assessed.

E. coli was grown on LB agar plates incubated at 37°C while *E. brevis* and *F. odoratum* were grown on nutrient agar plates incubated at 25°C. Bacterial cells of all species were suspended in sterile distilled water and diluted to an optical density of 0.25 (± 0.02) at 600 nm (Lysyk et al. 1999). Egg yolk medium plates were inoculated with the following bacterial suspensions: (1) 50 µl of *E. brevis* and 50 µl of *F. odoratum*, (2) 100 µl of *E. coli*, and (3) 33 µl of *E. brevis*, 33 µl of *F. odoratum* and 33 µl of *E. coli*. Three plates were inoculated for each bacterial combination and for each fly species, for a total of 18 plates. Initial cell densities were determined by viable counts of the bacterial suspensions. For each species, 20 surface-sterilised eggs were placed on a moist sterile filter paper and added to each plate. Egg hatch was determined using an aliquot of 100 surface-sterilised eggs placed on water agar for 72 h. All plates were incubated at 25°C and a photoperiod of 16:8 (L:D) h. Plates were examined daily and pupae removed with sterile forceps, rinsed, weighed, and placed on water agar until adult emergence. The entire experiment was replicated four times. The number of pupae per plate per day was used to determine the larval development time and survival. The number of flies emerged per plate per day was used to calculate pupal development time and survival. Pupal

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weight was also recorded. Data were analysed using analysis of variance to determine if

survival, developmental time of larvae and pupae, and pupal weight varied among

treatments. Means were compared using least significant difference (LSD) (SPSS Inc.

1989-1999).

3.7. Persistence of *E. coli* during the development of house fly and stable fly pupae

This experiment was undertaken to determine if *E. coli* cells acquired during larval

feeding survive through pupation. Larvae were left to feed on *E. coli* lawns until

pupation. Reared pupae were sampled every two days to determine temporal changes in

E. coli load.

E. coli cells were collected from LB plates and suspended in sterile distilled water.

Egg yolk medium plates were inoculated with 100 µl of this *E. coli* suspension and

incubated at 37°C overnight. Larvae were reared from egg to third instar on agar medium

using the gnotobiotic procedure. Third instar larvae were aseptically transferred from the

rearing plates to the *E. coli* lawns at a density of about 40 maggots per plate. Plates were

incubated at 25°C with a photoperiod of 16:8 (L:D) h until pupation. Pupae were

removed daily with sterile forceps, rinsed in distilled water, washed in 3 changes of

0.26% sodium hypochlorite for a total of 15 minutes and rinsed in 3 changes of sterile

distilled water. The surface-sterilised pupae were placed on water agar and incubated at

25°C for 1, 3, 5 or 6 days for house flies and 7 days for stable flies. Individual pupae

were homogenised with a sterile plastic pestle in 1 ml PBS and centrifuged at 800 × g for

two minutes. *E. coli* populations were quantified as previously described. A total of 200

pupae were processed for each species, 50 per time interval. The number of *E. coli* CFU

per pupa was transformed to $\log(y + 1)$. Logistic regression was used to estimate the

change in proportion of infected pupae. Relationship between *E. coli* load and time was

described using quadratic regressions for each species.

3.8. *E. coli* contamination of teneral adult house flies and stable flies

This experiment was conducted to determine if *E. coli* cells present in the pupa

contaminated the emerging adult, and if the contamination was internal, external or both.

Pupae were reared and prepared as described earlier, and incubated on water agar at 25°C

until the day of emergence. Adult flies less than 15 minutes old were assigned to one of

two groups: newly emerged flies and rinsed flies. Newly emerged flies were

homogenised without further treatment and were used to determine the total

contamination of the fly, both internal and external. Rinsed flies were treated prior to

homogenisation to remove external bacteria and were used to differentiate between

external and internal contamination. Newly emerged flies were transferred with sterile

insect handling forceps to a sterile 1.5 ml microfuge tube and homogenised in 100 µl of

PBS with a sterile plastic pestle, centrifuged at 800 × g for two minutes, and the

supernatant plated onto MacConkey agar. Flies rinsed before homogenisation were

transferred to a sterile 1.5 ml microfuge tube with 100 µl PBS, vortexed for 30 seconds,

aseptically transferred to another tube containing 100 µl PBS and homogenised as

previously described. The rinse solution and the homogenised rinsed flies were plated on

separate MacConkey agar plates. The empty pupal case of each fly was homogenised in 1 ml of PBS and serial dilutions were plated onto MacConkey agar. A total of 50 newly emerged flies and 50 rinsed flies were processed for each species. Plates labelled "too numerous to count" were assigned the maximum number of colonies counted on plates of the same group: 2 000 CFU per newly emerged fly, 500 CFU/100 µl of rinse solution, and 700 CFU per rinsed fly. Student's t-tests were used to determine differences between minimal mean *E. coli* populations between species. The correlation between the contamination of flies and pupal cases was determined using Spearman's rank correlation.

CHAPTER 4.   RESULTS

4.1. Persistence of *E. coli* in larval guts of house flies and stable flies

House fly and stable fly larvae were homogenised following ingestion of an *E. coli* suspension to assess the persistence of the bacterium in the larval gut. The experiment was conducted over a short term period (zero to five hours post-ingestion) and a long term period (zero to 48 h post-ingestion).

4.1.1.   Short term persistence

Larvae of both species were fed an *E. coli* suspension, but not all larval guts were infected with the bacterium after 20 minutes of feeding. House fly and stable fly larvae were fed bacterial suspensions containing an average of $3.2 \pm 2.7 \times 10^{10}$ ($n = 5$) and $2.6 \pm 1.8 \times 10^{10}$ ($n = 5$) *E. coli* CFU/ml, respectively. The amount of bacteria fed to the larvae did not vary significantly between species ($t = 0.18$; df = 8; $P = 0.863$).

E. coli was detected in $41.5 \pm 6.2\%$ of house fly ($n = 65$) larvae and $95.2 \pm 2.8\%$ of stable fly ($n = 62$) larvae fed bacteria. The incidence of infected house fly larvae over five hours ranged from 14.3% to 61.5%, and that of stable fly larvae ranged from 81.8% to 100% (Figure 4.1.1). Logistic regression indicated the proportion of infected larvae was relatively constant over time, but varied between species and the change across time was consistent between species (Table 4.1.1).

The *E. coli* load of infected house fly ($n = 27$) larvae was $6.3 \pm 1.3 \times 10^4$ CFU

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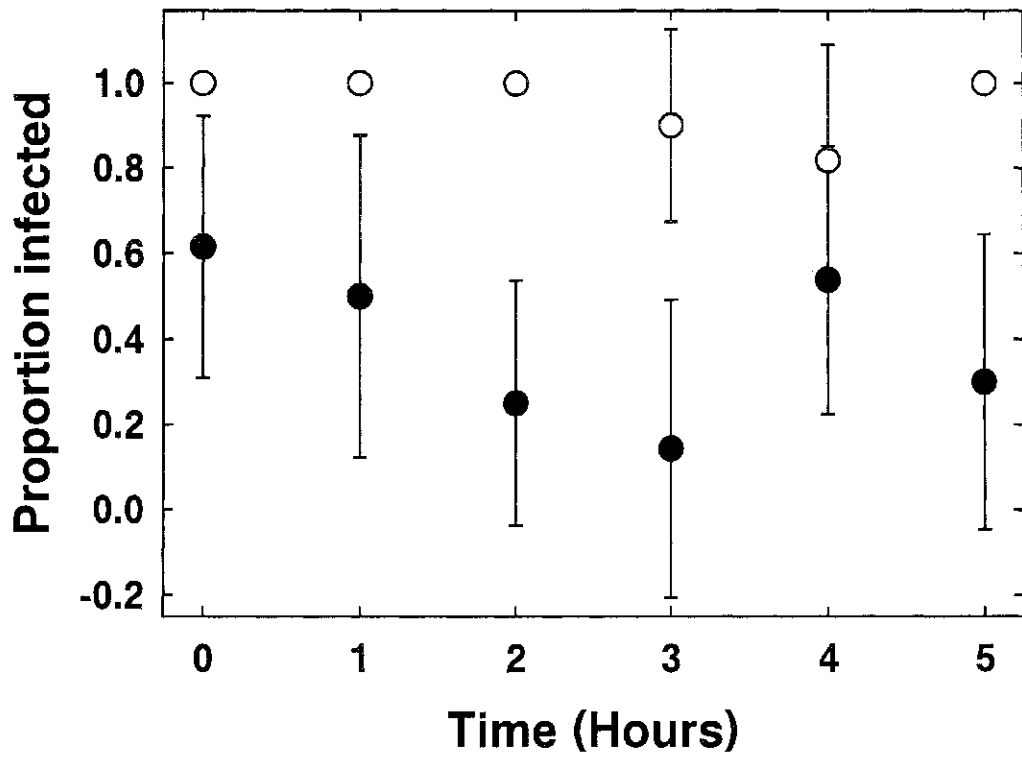
Figure 4.1.1. Proportion of house fly (●) and stable fly (○) larvae infected

with *E. coli* over a period from zero to five hours. Error bars represent 95%

confidence intervals, n = 65 for house fly larvae, n = 62 for stable fly larvae.

Table 4.1.1. Relationship between the proportion of house fly and stable fly larvae infected with *E. coli* and time from zero to five hours.

	Estimate ± SE	Wald	P (Wald > 0)
Constant	4.768 ± 1.704	7.83	-
Time	-0.596 ± 0.443	1.81	0.178
Species	-4.703 ± 1.756	7.17	0.007
Species*time	0.424 ± 0.466	0.83	0.363

Relationship is $P (Y = 1) = 1/(1 + exp(-(a + bT + cS + dTS)))$ where $P (Y = 1)$ is the proportion of infected larvae, T is hours since infection, $S = 1$ for house fly ($n = 65$) and $S = 0$ for stable fly ($n = 62$) and a, b, c and d are estimated parameters using logistic regression (SPSS Inc. 1989-1999).

per larva lower than infected stable fly (n = 59) larvae. Larvae randomly selected from the rearing plates and used as negative controls never tested positive for *E. coli* (Table 4.1.2). The surface sterilisation of house fly larvae was efficient, as indicated by the absence of *E. coli* CFU in the sterilisation controls (Table 4.1.2). However, a small number of *E. coli* CFU were detected on the sterilisation controls of the stable fly larvae (Table 4.1.2). This surface contamination of the larvae was negligible, representing 0.3% or less of the *E. coli* population of tested larvae.

Analysis of variance indicated that *E. coli* load varied between species and time as evidenced by the significant species and time effects (Table 4.1.3). The rate of change in bacterial load over time was similar between species as evidenced by the non-significant species*time effect (Table 4.1.3). The regression model was therefore established as

$$\log(\text{CFU/larva} + 1) = 4.852 - 0.097 \times T - 1.260 \times S$$

where T is hours post infection, S = 1 for house fly and S = 0 for stable fly (F = 51.63; df = 2, 83; P < 0.0001). The final model indicated that *E. coli* load was 1.260 ± 0.128 log(CFU/larva + 1) lower in house fly larvae, but that it declined by 0.097 ± 0.035 log(CFU/larva + 1) per hour for both species (Figure 4.1.2). The model accounted for 55.4% of the variation in bacterial load.

4.1.2. Long term persistence

House fly and stable fly larvae were fed an *E. coli* solution but not all larval guts were infected with the bacterium after ingestion. The concentration of *E. coli* fed to larvae averaged $5.8 \pm 0.7 \times 10^8$ (n = 4) CFU/ml for house fly larvae and $5.5 \pm 5.4 \times 10^{10}$

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Table 4.1.2. *E. coli* population (mean ± SE) in the guts of house fly and stable fly larvae over a period of five hours.

Time	Control larvae n	Control larvae Mean ± SE	Sterilisation control larvae n	Sterilisation control larvae Mean ± SE	Infected test larvae n	Infected test larvae Mean ± SE
			House flies			
0	7	0.0 ± 0.0	7	0.0 ± 0.0	8	$20 \pm 15 \times 10^3$
1	7	0.0 ± 0.0	7	0.0 ± 0.0	5	$5.3 \pm 2.4 \times 10^3$
2	7	0.0 ± 0.0	7	0.0 ± 0.0	3	$4.1 \pm 2.5 \times 10^3$
3	7	0.0 ± 0.0	7	0.0 ± 0.0	1	23×10^3
4	7	0.0 ± 0.0	7	0.0 ± 0.0	7	$1.7 \pm 0.7 \times 10^3$
5	7	0.0 ± 0.0	7	0.0 ± 0.0	3	$7.7 \pm 6.5 \times 10^3$
Mean	42	0.0 ± 0.0	42	0.0 ± 0.0	27	$9.5 \pm 4.7 \times 10^3$
			Stable flies			
0	7	0.0 ± 0.0	7	132.1 ± 132.1	11	$71 \pm 16 \times 10^3$
1	7	0.0 ± 0.0	7	205.0 ± 144.2	11	$82 \pm 17 \times 10^3$
2	7	0.0 ± 0.0	6	122.5 ± 118.5	12	$110 \pm 20 \times 10^3$
3	7	0.0 ± 0.0	7	246.4 ± 222.3	9	$91 \pm 35 \times 10^3$
4	7	0.0 ± 0.0	7	6.4 ± 6.4	9	$29 \pm 9 \times 10^3$
5	7	0.0 ± 0.0	7	7.1 ± 3.1	7	$32 \pm 10 \times 10^3$
Mean	42	0.0 ± 0.0	41	119.9 ± 52.1	59	$72 \pm 9 \times 10^3$

Table 4.1.3. Variation in *E. coli* load between the larval guts of house flies and stable flies over a period of five hours.

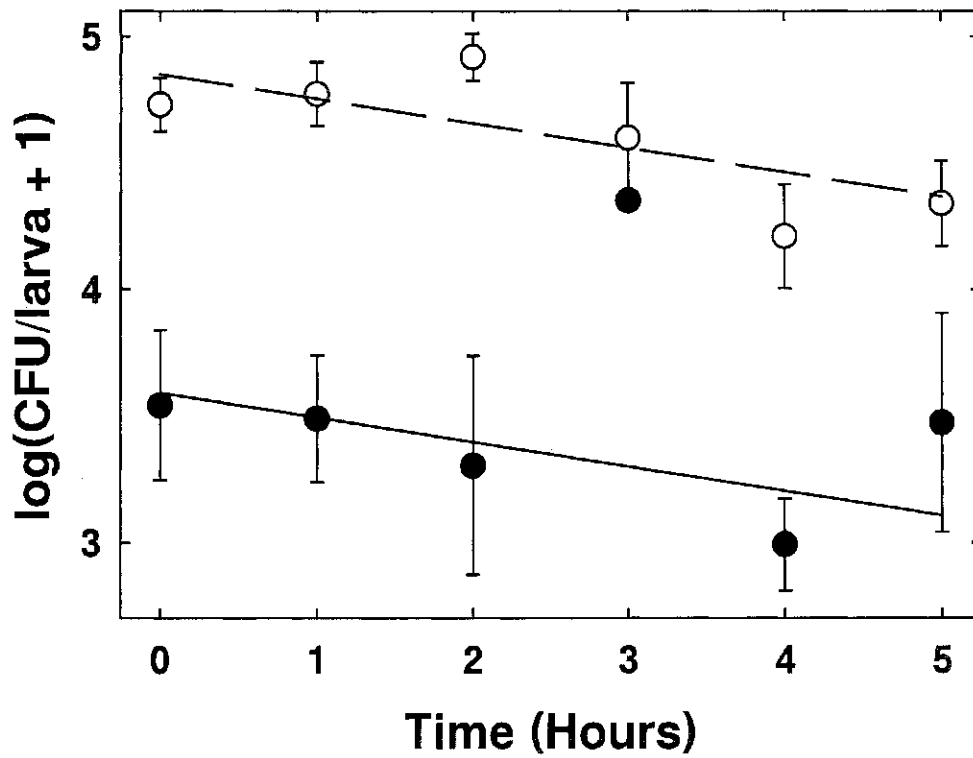| Source	df	SS	MS	*F*	*P (F)*
Time	5	3.532	0.706	2.42	0.043
Species	1	6.556	6.556	22.48	< 0.0001
Species*time	5	1.484	0.297	1.02	0.414
Error	74	21.585	0.292		

Figure 4.1.2. Mean *E. coli* population in house fly (●) and stable fly (○) larval

guts over a period of five hours. Error bars represent SE of the mean, n = 27 for

house fly larvae, n = 59 for stable fly larvae. Lines represent the fitted model for

house fly larvae (——) and stable fly larvae (— —). Point for house fly at 3 h

represents 1 larva, rest represent 3-12 larvae.

(n = 5) CFU/ml for stable fly larvae. On a log scale, the means were 8.757 ± 0.050 and 9.413 ± 0.543 CFU/ml for house fly larvae and stable fly larvae respectively. There was no significant difference in the amount of bacteria fed to both species (t = -1.064; df = 7; P = 0.323).

On average, 90.2 ± 3.1% of house fly (n = 92) larvae and 92.0 ± 2.4% of stable fly (n = 125) larvae were positive for $E.$ $coli$. The proportion of infected house fly larvae varied significantly over time (χ^2 = 7.94; df = 2; P = 0.019) but not in a linear trend (Figure 4.1.3). The proportion of infected house fly larvae decreased slightly from 90.9 ± 5.1% to 81.8 ± 7.0% after 24 h, then increased to 100% infection at 48 h. Stable fly larvae demonstrated a constant linear increase in the proportion infected from 85.4 ± 5.6% after ingestion to 100% at 48 h, as indicated by the significant logistic regression (Table 4.1.4).

$E.$ $coli$ was not detected in the control larvae for either species (Table 4.1.5), however, it was found on the surface of larvae used to determine the efficiency of surface sterilisation. This amount was negligible for both species, representing on average less than 0.5% of the $E.$ $coli$ load found in fed larvae of either species (Table 4.1.5). $E.$ $coli$ infected house fly larvae (n = 83) harboured an average of 4.8 ± 0.8 × 10^4 CFU per larva, ranging from 390 to 3.3 × 10^5 CFU per larva. The $E.$ $coli$ load of infected stable fly larvae (n = 115) averaged 7.8 ± 0.8 × 10^4 CFU per larva, ranging between 3.0 × 10^3 and 3.0 × 10^5 CFU per larva. $E.$ $coli$ cells were detected on the surface of the water agar where larvae were reared before sampling, but no quantification was performed.

Analysis of variance indicated the changes in $E.$ $coli$ over time were not consistent between species as indicated by the significant species*time interaction (Table 4.1.6).

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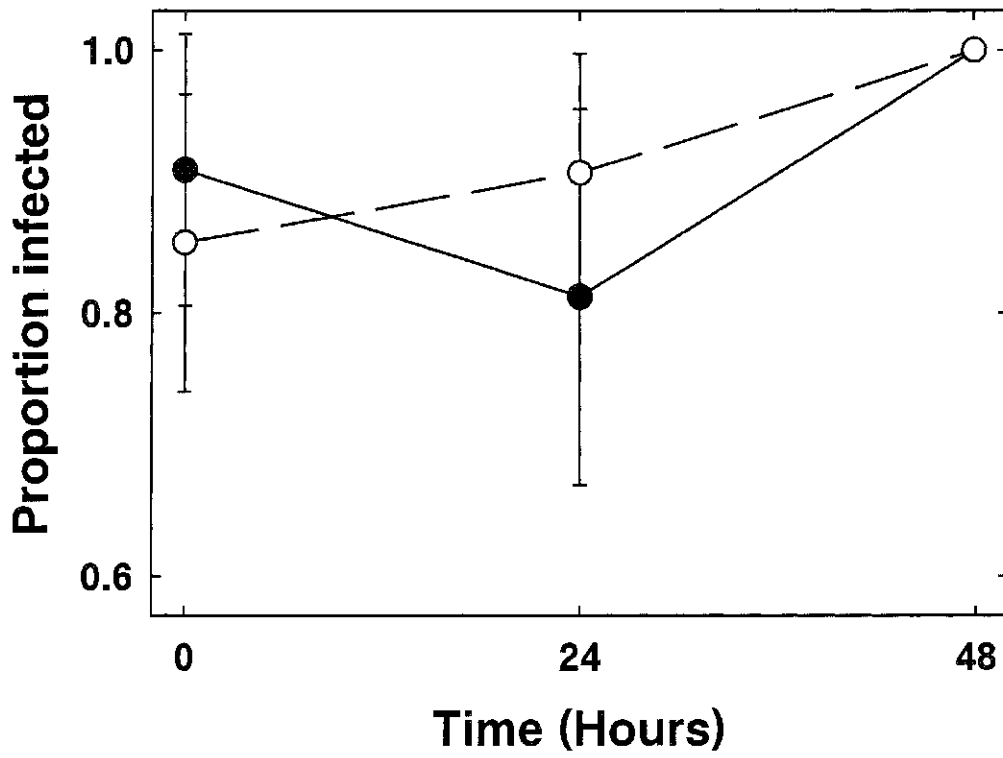
Figure 4.1.3. Proportion of house fly (●) and stable fly (○) larvae infected

with *E. coli* over a period of 48 hours. Error bars represent 95% confidence

intervals, *n* = 92 for house fly larvae, *n* = 125 for stable fly larvae.

Table 4.1.4. Relationship between the proportion of house fly and stable fly larvae infected with _E. coli_ and time from zero to 48 hours.

Species	n	$a \pm$ SE	$b \pm$ SE	χ^2	$P(\chi^2)$
House fly	92	1.831 ± 0.478	0.020 ± 0.019	1.14	0.286
Stable fly	125	1.622 ± 0.409	0.049 ± 0.022	6.45	0.011

Relationship is $P(Y = 1) = 1/(1 + exp(-(a + bT)))$ where $P(Y = 1)$ is the proportion of infected larvae, T is hours since infection and a and b are estimated parameters using logistic regression (SPSS Inc. 1989-1999).

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Table 4.1.5. *E. coli* population (mean ± SE) in the guts of house fly and stable fly larvae over a period 48 hours.

	Control larvae		Sterilisation control larvae		Infected test larvae	
Time	n	Mean ± SE	n	Mean ± SE	n	Mean ± SE
House flies						
0	25	0.0 ± 0.0	24	29 ± 18	30	$11 \pm 2 \times 10^4$
24	25	0.0 ± 0.0	22	89 ± 40	26	$2.4 \pm 1.1 \times 10^4$
48	25	0.0 ± 0.0	14	370 ± 130	27	$0.6 \pm 0.2 \times 10^4$
Mean	75	0.0 ± 0.0	60	130 ± 40	83	$4.8 \pm 0.8 \times 10^4$
Stable flies						
0	25	0.0 ± 0.0	24	80 ± 40	35	$10 \pm 2 \times 10^4$
24	19	0.0 ± 0.0	24	210 ± 140	39	$5.1 \pm 1.0 \times 10^4$
48	18	0.0 ± 0.0	18	140 ± 90	41	$8.2 \pm 1.2 \times 10^4$
Mean	62	0.0 ± 0.0	66	140 ± 60	115	$7.8 \pm 0.8 \times 10^4$

Table 4.1.6. Variation of *E. coli* population between house fly and stable fly larvae over a period of 48 hours.

Source	df	SS	MS	*F*	*P (F)*
Time	2	1.733	0.866	2.42	0.091
Species	1	0.561	0.561	1.57	0.212
Species*time	2	15.719	7.859	21.99	< 0.0001
Error	192	68.616	0.357		

The regression model was

$$\log(\text{CFU/larva} + 1) = 4.598 - 0.025 * T * S$$

where T is hours post infection, $S = 1$ for house fly and $S = 0$ for stable fly ($F = 99.73$; df $= 1, 196$; $P < 0.0001$). This indicates a reduction in $E.$ $coli$ population of 0.025 ± 0.003 $\log(\text{CFU/larva} + 1)$ per hour for house fly larvae and no reduction in $E.$ $coli$ population for stable fly larvae (Figure 4.1.4). The model accounted for 33.7% of the variation in bacterial load.

4.2. Dose-dependent persistence of *E. coli* in house fly and stable fly larval guts

The effect of the amount of $E.$ $coli$ fed to larvae on the persistence of the bacterium in the larval gut was evaluated. Larvae of both species were fed a low or a high dose suspension of $E.$ $coli$ prior to homogenisation.

The high dose suspension fed to house fly and stable fly larvae averaged $5.4 \pm 0.8 \times 10^8$ and $8.2 \pm 0.7 \times 10^8$ $E.$ $coli$ CFU/ml, respectively. The low dose suspension was a 1:10 000 dilution of the high dose suspension. The amount of $E.$ $coli$ fed to house fly larvae averaged 0.197 ± 0.077 $\log(\text{CFU/larva} + 1)$ lower than the amount fed to stable fly larvae ($t = -2.56$; df $= 8$; $P = 0.034$).

On average, $71.9 \pm 4.8\%$ of house fly ($n = 89$) larvae fed a low dose suspension were infected with $E.$ $coli$, the proportion ranging from $57.1 \pm 7.1\%$ ($n = 49$) after ingestion to $90.0 \pm 4.8\%$ ($n = 40$) after 48 h. All house fly ($n = 72$) larvae fed a high dose suspension were infected at zero ($n = 32$) and 48 ($n = 40$) h. The proportion of infected
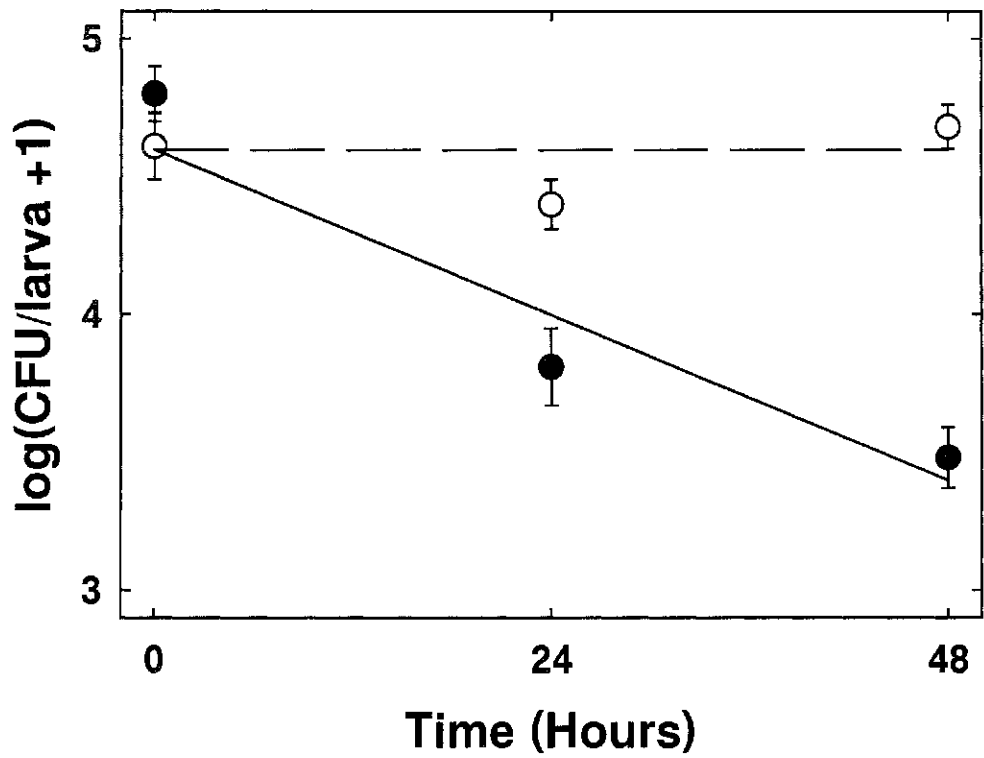
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Figure 4.1.4. Mean *E. coli* population in house fly (●) and stable fly (○) larval

guts over a period of 48 hours. Error bars represent SE of the mean, n = 83 for

house fly larvae, n = 115 for stable fly larvae. Lines represent the fitted model for

house fly larvae (——) and stable fly larvae (— —).

stable fly (n = 79) larvae fed a low dose suspension averaged 93.7 ± 2.8%, ranging from

89.8 ± 4.4% (n = 49) after ingestion to 100% (n = 30) after 48 h. All stable fly (n = 67)

larvae fed a high dose were positive for $E.$ $coli$ at zero (n = 29) and 48 (n = 38) h.

The control larvae were negative for $E.$ $coli$ throughout the entire experiment

(Table 4.2.1). The amount of $E.$ $coli$ CFU found on the surface of the larvae used to

determine the efficiency of the surface sterilisation was negligible, representing less than

0.05% of the amount found in the larvae fed $E.$ $coli$ (Table 4.2.1). A three-way analysis

of variance indicated species, dose and time all had significant main effects (Table 4.2.2).

The two-way interactions for species*time and dose*time were also significant (Table

4.2.2), indicating that changes in $E.$ $coli$ population in larval guts over time were not

consistent between species or doses.

The $E.$ $coli$ population in house fly larval guts averaged 73.5 ± 14.0 (n = 28) CFU

per larva after infection with a low dose suspension. After 48 h, the $E.$ $coli$ load had

reached 2.0 ± 0.5 × 10^4 (n = 36) CFU per larva. The $E.$ $coli$ population therefore

increased by 2.277 ± 0.114 log(CFU/larva + 1) after 48 h. The confidence intervals for

this change did not include zero, which indicated the change in $E.$ $coli$ population was

significantly different from zero. House fly larvae fed a high dose suspension had an

average of 9.9 ± 1.8 × 10^4 (n = 32) CFU per larva. After 48 h, this decreased to 3.6 ±

0.7 × 10^4 (n = 40) CFU per larva (Figure 4.2.1). The bacterial population decreased by

0.422 ± 0.092 log(CFU/larva + 1) CFU over 48 h. The confidence intervals for the

change indicated this change was significantly different from zero.

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Table 4.2.1. *E. coli* population (mean ± SE) in the guts of house fly and stable fly larvae over a period of 48 hours after ingestion of a low or a high dose suspension of bacteria.

Dose	Time (h)	n	Control larvae Mean ± SE	n	Sterilisation control larvae Mean ± SE	n	Infected test larvae Mean ± SE
					House flies		
Low	0	5	0.0 ± 0.0	5	0.0 ± 0.0	28	73.5 ± 14.0
	48	5	0.0 ± 0.0	5	0.0 ± 0.0	36	$2.0 \pm 0.5 \times 10^4$
High	0	5	0.0 ± 0.0	5	3.8 ± 2.5	32	$9.9 \pm 1.8 \times 10^4$
	48	5	0.0 ± 0.0	5	15.4 ± 10.8	40	$3.6 \pm 0.8 \times 10^4$
					Stable flies		
Low	0	5	0.0 ± 0.0	5	0.0 ± 0.0	44	94.1 ± 9.7
	48	5	0.0 ± 0.0	5	0.0 ± 0.0	30	$2.7 \pm 0.7 \times 10^5$
High	0	5	0.0 ± 0.0	5	0.2 ± 0.2	29	$1.2 \pm 0.3 \times 10^5$
	48	5	0.0 ± 0.0	5	36.9 ± 20.9	38	$5.5 \pm 1.1 \times 10^5$

Table 4.2.2. Variation of *E. coli* population in the gut of house fly and stable fly larvae over time after ingestion of a low or a high dose suspension of bacteria.

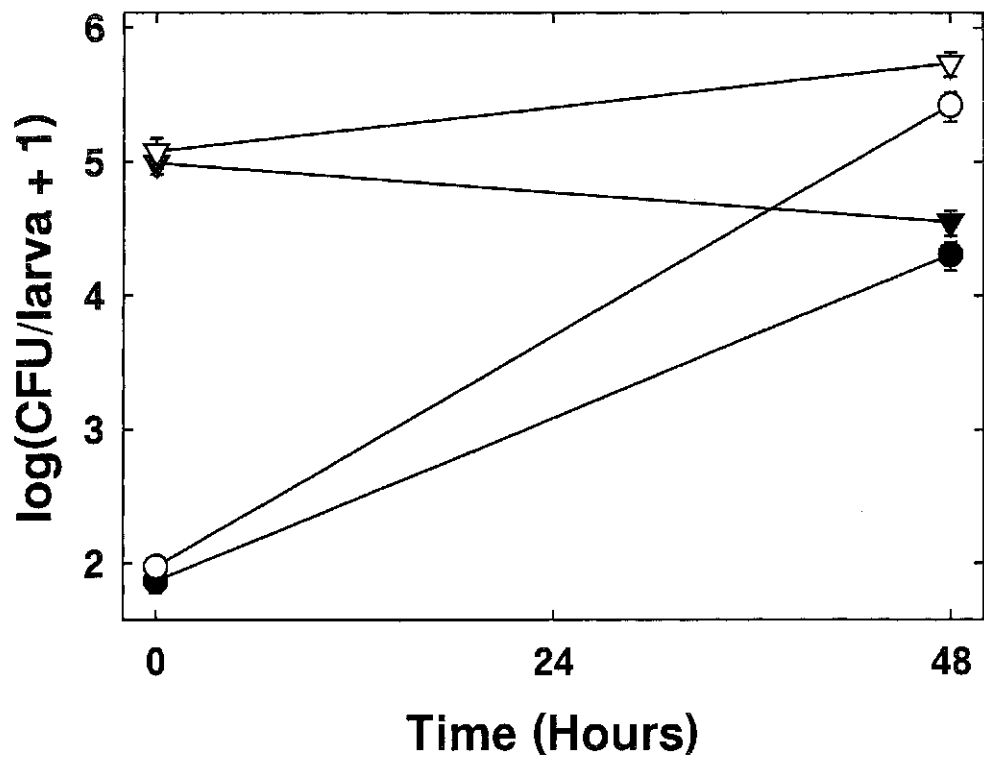| Source	df	SS	MS	F	P (F)
Replicate	4	1.377	0.344	0.94	0.444
Species	1	26.207	26.207	71.18	< 0.0001
Dose	1	185.048	185.048	502.57	< 0.0001
Time	1	136.553	136.553	370.86	< 0.0001
Species*dose	1	0.417	0.417	1.13	0.288
Species*time	1	17.697	17.697	48.06	< 0.0001
Dose*time	1	116.455	116.455	316.28	< 0.0001
Species*dose*time	1	0.094	0.094	0.26	0.614
Error	265	97.574	0.368		

Figure 4.2.1. Mean *E. coli* population in house fly and stable fly larvae over a period of 48 hours after ingestion of a low or a high dose suspension of bacteria. Error bars represent SE of the mean; ●, house fly larvae fed low dose suspension; ▼, house fly larvae fed high dose suspension; ○, stable fly larvae fed low dose suspension; ▽, stable fly larvae fed high dose suspension.

Stable fly larvae *E. coli* load following infection with a low dose suspension averaged 94.1 ± 9.7 ($n = 44$) CFU per larva, and increased to $2.7 \pm 0.7 \times 10^5$ ($n = 30$) *E. coli* CFU per larva after 48 h. This increase of 3.248 ± 0.095 log(CFU/larva + 1) CFU per larva over 48 h was significantly different from zero as indicated by the confidence intervals not overlapping zero. When a high dose suspension of *E. coli* was fed to stable fly larvae, the gut population averaged $1.2 \pm 0.3 \times 10^5$ ($n = 29$) and $5.5 \pm 1.1 \times 10^5$ ($n = 38$) *E. coli* CFU per larva immediately after infection and 48 h later, respectively (Figure 4.2.1). The increase was 0.659 ± 0.115 log(CFU/larva + 1). The confidence intervals did not include zero, which indicates this change was significant.

4.3. Survival and development of house fly and stable fly larvae reared on three different bacterial cultures

Rearing plates were inoculated with an initial average dose of $2.8 \pm 0.4 \times 10^8$, $8.0 \pm 2.2 \times 10^7$ and $1.6 \pm 1.2 \times 10^8$ CFU/ml for *E. coli*, *E. brevis* and *F. odoratum*, respectively. Egg hatch for house fly eggs averaged $74.5 \pm 4.2\%$ and $62.8 \pm 7.2\%$ for stable fly eggs.

4.3.1. Larval survival

The proportion of larvae to reach pupation when reared on different cultures was recorded. House fly larval survival varied significantly among treatments ($F = 4.06$; df = 2, 30; $P = 0.027$). Survival on *E. coli* plates averaged $62.3 \pm 7.1\%$ (Figure 4.3.1) and was
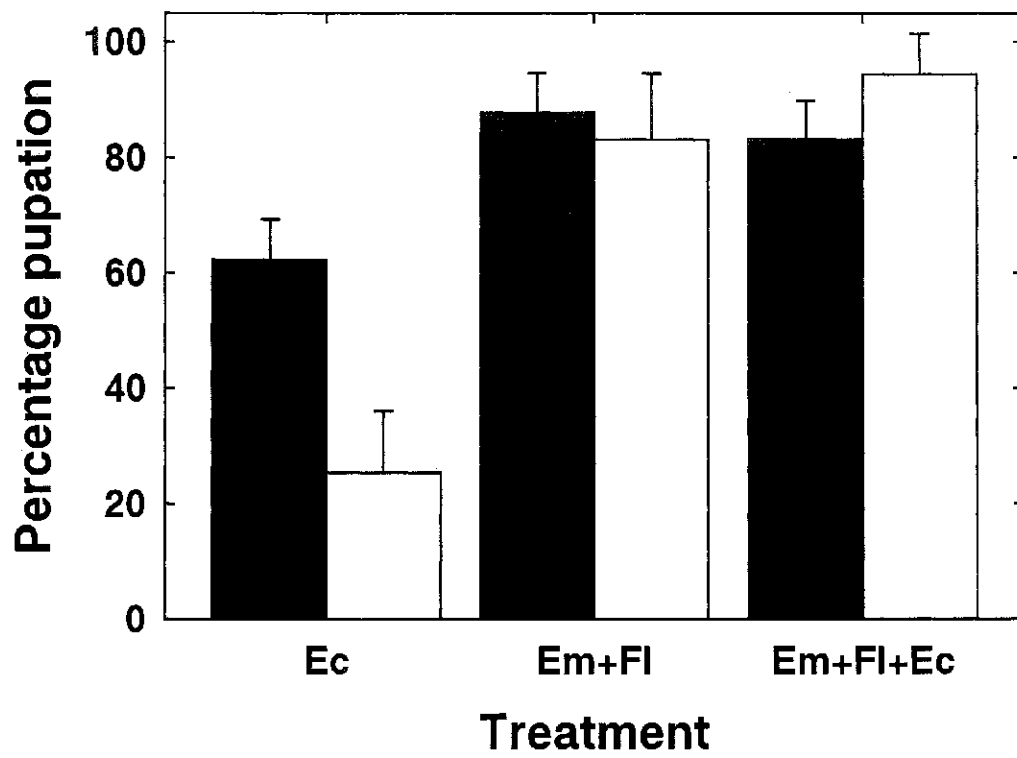
49

Figure 4.3.1. Survival to pupation of house fly (■) and stable fly (□) larvae reared on three different bacterial cultures. Bars represent percentage ± SE.

lower than the other treatments. House fly larval survival was highest on the *E. brevis* + *F. odoratum* combination with 87.8 ± 6.8% (Figure 4.3.1) and this was similar to the 83.2 ± 6.7% survival on the *E. brevis* + *F. odoratum* + *E. coli* plates.

The bacterial inocula also had a significant effect on stable fly survival (F = 20.81; df = 2, 30; P < 0.0001). The survival of stable fly larvae was highest on plates containing *E. brevis* + *F. odoratum* + *E. coli*, averaging 94.4 ± 7.0% (Figure 4.3.1). This was not significantly different from survival on *E. brevis* + *F. odoratum* combination. Survival of stable fly larvae was lowest on *E. coli* plates, averaging 25.4 ± 10.7% (Figure 4.3.1).

4.3.2. Larval and pupal development

The time required by larvae to reach pupation and the time required by pupae to reach emergence was recorded. Analysis of variance indicated that development time of house fly larvae varied among treatments (F = 59.41; df = 2, 407; P < 0.0001). House fly larvae developed fastest on *E. brevis* + *F. odoratum* + *E. coli* plates, reaching pupation in 6.8 ± 0.1 days (Figure 4.3.2). Larvae reared on the *E. brevis* + *F. odoratum* bacterial mixture had an average larval development time of 7.0 ± 0.1 days (Figure 4.3.2). Larval development time was significantly greater on *E. coli* plates, requiring 7.9 ± 0.1 days to reach pupation. There was no effect of treatment on the development time of house fly pupae (Figure 4.3.3).

The bacterial food source had an effect on the development time of stable fly larvae, as indicated by the significant term for treatment (F = 37.91; df = 2, 286; P < 0.0001). On average, stable fly larvae developed 1.8 ± 0.2 days faster on *E. brevis* + *F. odoratum* + *E. coli* plates compared with the *E. brevis* + *F. odoratum* plates (Figure
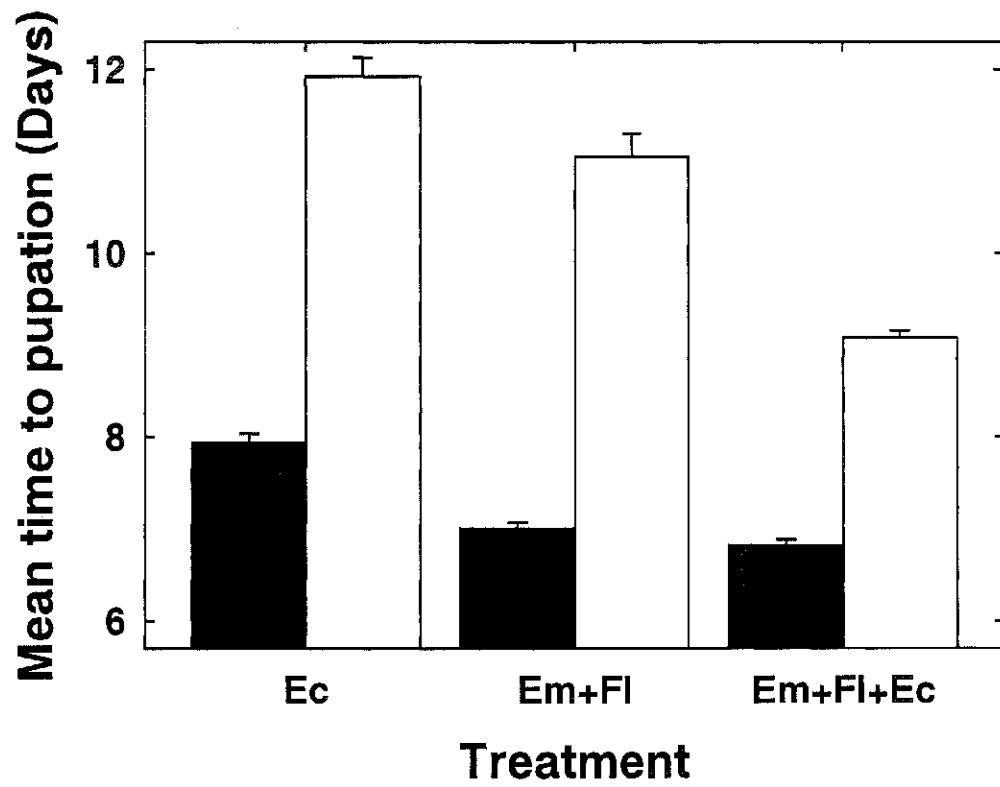
Figure 4.3.2. Time (in days) required by house fly (■) and stable fly (□) larvae to reach pupation when reared on three different bacterial cultures. Bars represent mean ± SE.
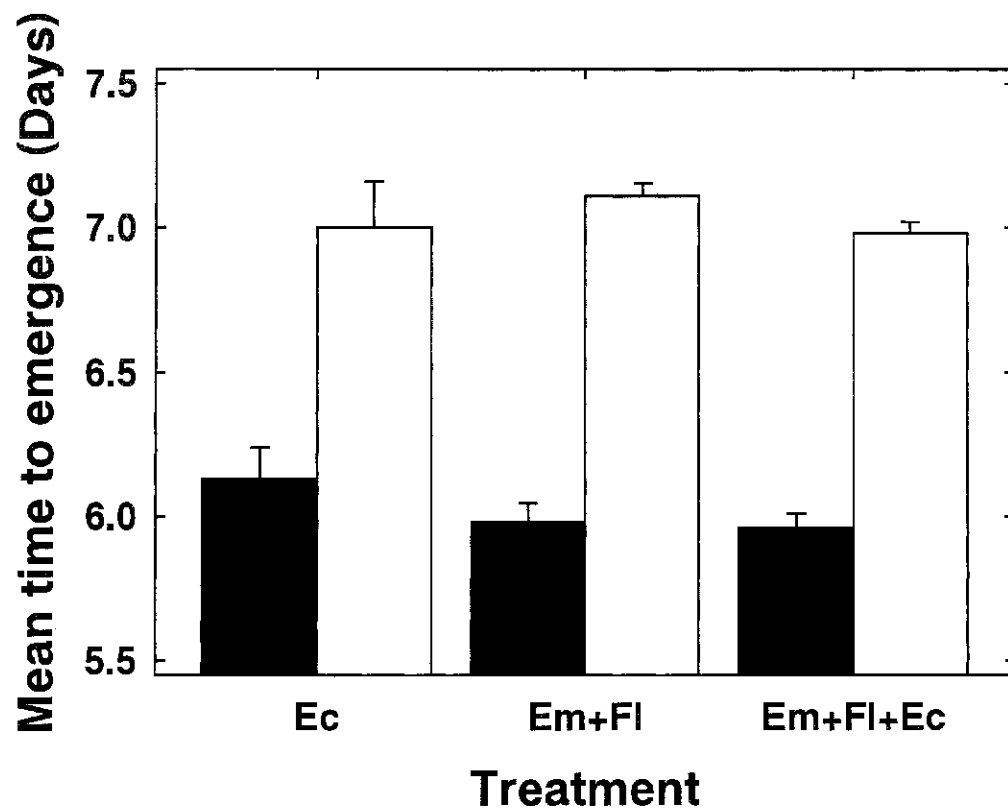
Figure 4.3.3. Time (in days) required by house fly (■) and stable fly (□) pupae reared on three different bacterial cultures to reach the adult stage. Bars represent mean SE.

4.3.2). Development time was longer when larvae were reared on *E. coli* (11.9 ± 0.2

days) but this was similar to the 11.1 ± 0.3 days required on *E. brevis + F. odoratum*

plates (Figure 4.3.2). Development time of stable fly pupae did not vary between

bacterial treatments (Figure 4.3.3).

4.3.3. Pupal weight

The weight of each pupa formed on the different bacterial cultures was recorded.

House fly pupal weight varied among treatments, as indicated by the significant term for

treatment in the analysis of variance ($F = 14.17$; df = 2, 406; $P < 0.0001$). House fly

pupae reared on *E. coli* plates were smaller by an average of 1.4 ± 0.4 and 2.1 ± 0.4 mg

compared to pupae reared on *E. brevis + F. odoratum* and *E. brevis + F. odoratum +*

E. coli plates, respectively (Figure 4.3.4). The largest house fly pupae were reared on the

E. brevis + F. odoratum + E. coli plates and weighed an average of 18.7 ± 0.3 mg.

Stable fly pupal weight varied significantly among treatments ($F = 15.03$; df = 2,

286; $P < 0.0001$). The smallest stable fly pupae were reared on *E. brevis + F. odoratum*

plates and had an average weight of 10.6 ± 0.2 mg (Figure 4.3.4). The average weight of

stable fly pupae reared on *E. brevis + F. odoratum + E. coli* plates and on *E. coli* plates

was 11.8 ± 0.1 and 11.0 ± 0.3 mg, respectively, and the difference was not significant.

4.3.4. Pupal survival

The proportion of adult flies emerged from the pupae was recorded. House fly

pupae survival was not consistent between treatments ($F = 8.52$; df = 2, 30; $P = 0.001$).

House fly pupae survival was highest when pupae were reared on *E. brevis +*
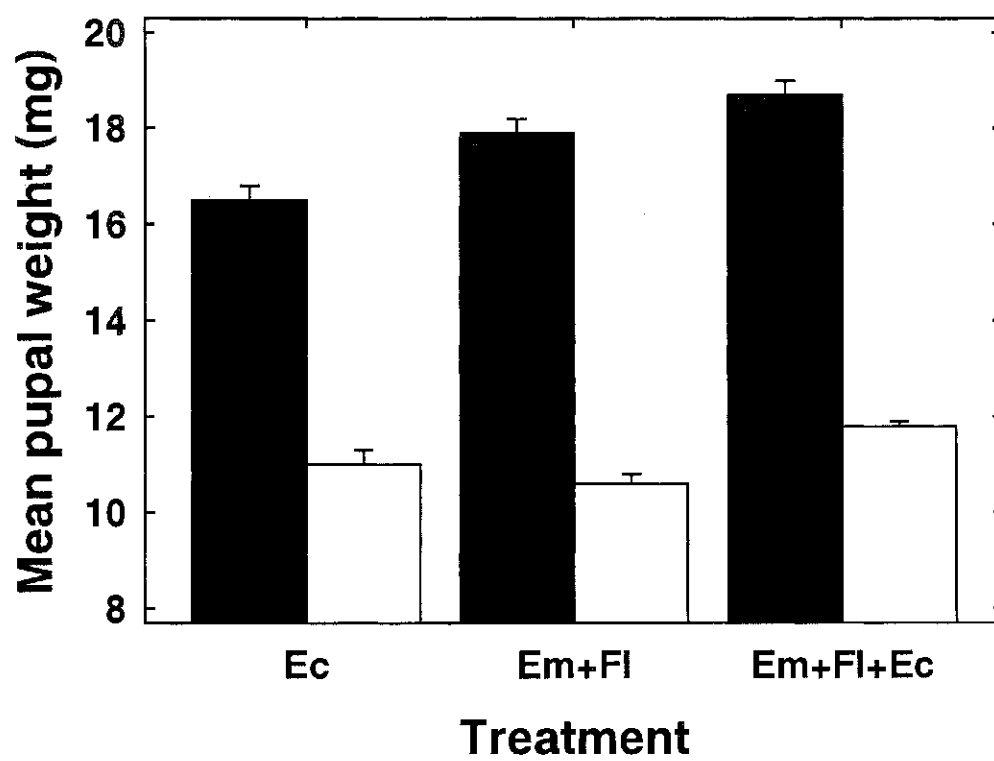
Figure 4.3.4. Weight (in mg) of house fly (■) and stable fly (□) pupae reared on three different bacterial cultures. Bars represent mean ± SE.

F. odoratum plates, averaging 72.9 ± 6.7% and was not significantly different from

71.8 ± 5.6% survival on *E. brevis* + *F. odoratum* + *E. coli* plates (Figure 4.3.5). Average

survival of house fly pupae reared on *E. coli* plates was 39.5 ± 9.6%, a significant

difference compared to the other two treatments.

Survival of stable fly pupae was different between bacterial treatments as indicated

by the significant term for treatment (F = 17.05; df = 2, 30; P < 0.0001). The survival rate

of stable fly pupae reared on *E. brevis* + *F. odoratum* + *E. coli* plates was highest with

95.0 ± 2.5%, and was not significantly different from the 82.4 ± 8.3% survival of pupae

reared on *E. brevis* + *F. odoratum* plates (Figure 4.3.5). Pupae reared on *E. coli* plates

had a significantly lower survival rate of 32.2 ± 1.2% (Figure 4.3.5).

4.4. Persistence of *E. coli* during the development of house fly and stable fly pupae

Pupae formed on *E. coli* plates were homogenised at different time intervals to

evaluate the persistence of the bacterium in the developing pupae. The proportion of

pupae infected with *E. coli* was 98.0 ± 1.0 % and 99.5 ± 0.5% for house fly (n = 200) and

stable fly (n = 200) pupae, respectively. The logistic regression between the proportion of

infected pupae and time was not significant (Table 4.4.1), indicating the proportion of

infected pupae remained constant throughout the entire pupal development.

The bacterial load in pupae averaged 1.7 ± 0.2 × 10^5 and 2.4 ± 0.3 × 10^5 *E. coli*

CFU per pupa for house fly and stable fly pupae, respectively. *E. coli* populations in
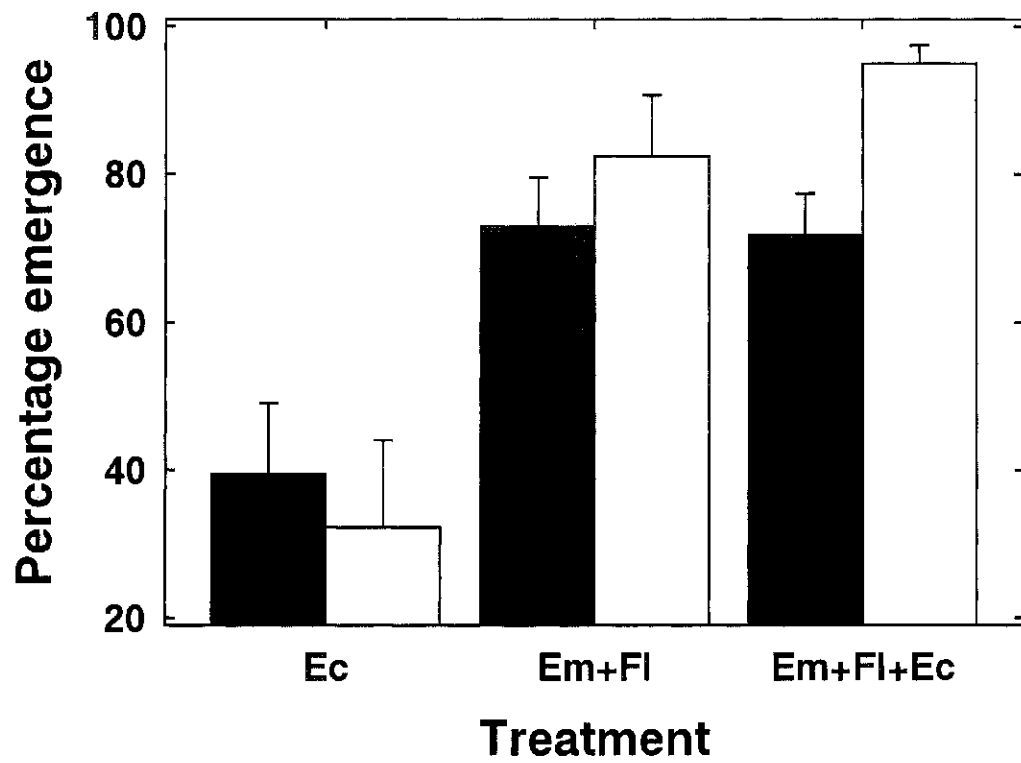
Figure 4.3.5. Survival to adult emergence of house fly (■) and stable fly (□)

pupae reared on three different bacterial cultures. Bars represent percentage ± SE.

Table 4.4.1. Relationship between the proportion of house fly and stable fly pupae infected with _E. coli_ and time since pupation.

Species	n	$a \pm$ SE	$b \pm$ SE	χ^2	$P (\chi^2)$
House fly	200	4.477 ± 1.293	-0.146 ± 0.281	0.286	0.593
Stable fly	200	-0.665 ± 47.158	4.556 ± 47.136	2.787	0.095

Relationship is $P (Y = 1) = 1/(1 + exp(a + bT))$ where $P (Y = 1)$ is the proportion of infected pupae, T is days since pupation and a and b are estimated parameters using logistic regression (SPSS Inc. 1989-1999).

pupae ranged from 600 to 2.0×10^6 CFU per pupa for house flies and from 2.9×10^3 to 2.8×10^6 *E. coli* CFU per stable fly pupa.

The relationship between bacterial population and time after pupation varied significantly between fly species ($F = 14.20$; df = 3, 389; $P < 0.0001$). *E. coli* load in house fly pupae increased between the first and the third day of development, and decreased by average of 0.648 log(CFU/pupa + 1) between the fifth and sixth day of the pupal stage (Figure 4.4.1). The relationship between bacterial load and time since pupation for house fly larvae was described by the quadratic regression

$$\log(\text{CFU/pupa} + 1) = 3.636 + 0.854*T - 0.119*T^2$$

where T is days since pupation ($F = 20.47$; df = 2, 193; $P < 0.0001$). The model accounted for 17.5% of the variation of *E. coli* population in house fly pupae.

In stable fly pupae, there was also an increase in the *E. coli* population between days one and three, but populations did not decrease before the emergence of the adult fly from the pupa (Figure 4.4.1). The relationship between bacterial load and time since pupation for stable flies was described by the quadratic regression

$$\log(\text{CFU/pupa} + 1) = 4.265 + 0.310*T - 0.028*T^2$$

where T is days since pupation ($F = 13.58$; df = 2, 196; $P < 0.0001$). The model accounted for 12.2% of the variation in bacterial load of stable fly pupae.

4.5. *E. coli* contamination of teneral adult house flies and stable flies

Teneral adult flies were tested to determine infection with *E. coli* of the adult following emergence from the pupal case. The proportion of newly emerged house flies
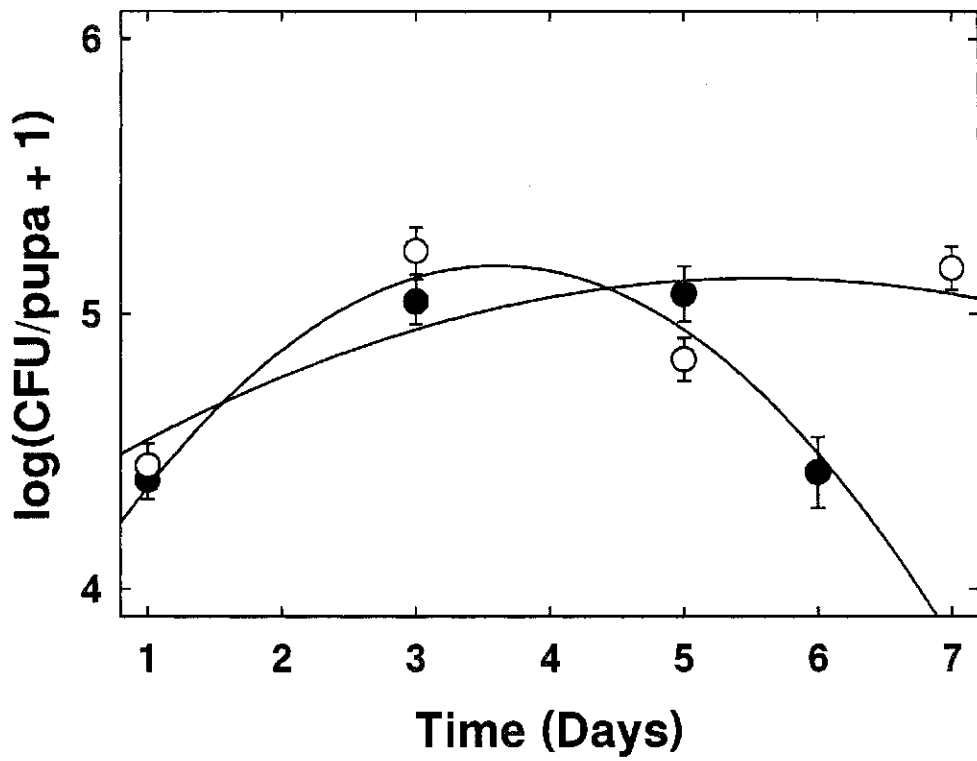
Figure 4.4.1. *E. coli* populations in house fly (●) and stable fly (○) pupae since pupation. Error bars represent SE of the mean.

(n = 50) and stable flies (n = 50) infected with *E. coli* was 78.0 ± 5.9% and 28.0 ± 6.4%, respectively. The proportion of infected house flies and stable flies was significantly different (z = 5.00; P < 0.0001). All pupal cases were infected with *E. coli*.

Some teneral adult house flies and stable flies were rinsed prior to homogenisation to evaluate the surface contamination with *E. coli*. Flies rinsed prior to homogenisation contaminated the rinse solution in 72.0 ± 6.4% of house flies (n = 50) and 28.0 ± 6.4% of stable flies (n = 50). The difference in proportion of rinse solutions infected was significant (z = 4.40; P < 0.0001). After rinsing, 66.0 ± 6.8% of house flies and 26.0 ± 6.3% of stable flies remained contaminated. The proportion of rinsed flies infected with *E. coli* was different between species (z = 4.01; P < 0.0001). There was no significant difference between the proportion of infected rinse solutions and the proportion of infected flies for either house flies (z = 0.65; P = 0.258) or stable flies (z = 0.23; P = 0.411).

The minimal *E. coli* population of infected newly emerged flies averaged 900.3 ± 151.5 and 1.2 ± 0.3 × 10^3 CFU per fly for house flies (n = 39) and stable flies (n = 14), respectively. There was no significant difference in the *E. coli* load of infected flies of both species (t = 0.54; df = 51; P = 0.592). House fly and stable fly pupal cases harboured an average of 2.9 ± 0.7 × 10^5 and 8.0 ± 1.4 × 10^5 *E. coli* CFU per empty case, respectively. House fly pupal cases contained, on average, 0.714 ± 0.157 log(CFU/case + 1) less *E. coli* CFU than stable fly cases (t = -4.55; df = 91.286; P < 0.0001). Pupal cases of infected house flies harboured an average of 0.884 ± 0.231 log(CFU/case + 1) more CFU per case than pupal cases from uninfected house flies (t = 3.83; df = 22.24; P = 0.001). Pupal cases from infected and uninfected stable flies harboured an average of

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$5.9 \pm 2.3 \times 10^5$ ($n = 13$) and $4.2 \pm 0.9 \times 10^5$ ($n = 36$) CFU per case, respectively, and the *E. coli* contamination of the cases was not significantly different ($t = 0.85$; df = 48; $P = 0.398$).

When the bacterial load from the empty pupal cases of newly emerged house flies was compared to the *E. coli* population in house fly pupae before emergence, the cases contained an average of 0.472 ± 0.199 log (CFU/case + 1) more *E. coli* CFU than the pupae ($t = 2.38$; df = 98; $P = 0.019$). Empty stable fly cases contained an average of 0.357 ± 0.123 log (CFU/case + 1) more *E. coli* than stable fly pupae before emergence ($t = 2.89$; df = 98; $P = 0.005$).

Emerged flies were rinsed to determine if the adult contamination with *E. coli* was mostly internal or external. The minimal contamination of the rinse solutions ranged from 1 to 500 *E. coli* CFU/ml for both species, and averaged 250.3 ± 37.2 and 367.0 ± 51.1 *E. coli* CFU/ml for house fly ($n = 50$) and stable fly ($n = 50$) rinse solutions, respectively. There was no difference in the amount *E. coli* found in the rinse solutions between species ($t = -1.57$; df = 48; $P = 0.122$). Infected stable flies ($n = 13$) contaminated the rinse solution by an average of 0.480 ± 0.176 log (CFU/ml +1) more CFU/ml than infected house flies ($n = 33$).

The minimal *E. coli* population of infected rinsed flies averaged 208.3 ± 45.8 for house flies ($n = 33$) and ranged from 1 to 700 CFU per fly. Rinsed infected stable flies ($n = 13$) had a minimal average of 336.7 ± 80.9 CFU per fly, ranging from 7 to 700 CFU per fly. There was no difference in the minimal *E. coli* load of infected rinsed flies of both species ($t = -1.83$; df = 44; $P = 0.075$). The *E. coli* contamination of pupal cases from rinsed house flies ($n = 50$) and stable flies ($n = 49$) averaged $3.1 \pm 0.7 \times 10^5$ and

$4.7 \pm 0.9 \times 10^5$ E. coli CFU per case, respectively. Pupal cases from rinsed stable flies

contained an average of 0.431 ± 0.172 log(CFU/case + 1) more CFU per case than pupal

cases from rinsed house flies ($t = -2.50$; df $= 94.34$; $P = 0.014$). Infected house flies

emerged from cases containing an average of 1.151 ± 0.206 log (CFU/case + 1) more

E. coli CFU per case than uninfected house flies ($t = 5.60$; df $= 42.37$; $P < 0.0001$). Cases

from rinsed infected and uninfected stable flies had a similar E. coli load ($t = 1.31$; df $=$

48; $P = 0.196$).

Empty cases from rinsed house flies contained, on average, 0.456 ± 0.203

log(CFU/case + 1) more E. coli CFU per case than six-day old pupae ($t = 2.24$; df $= 98$;

$P = 0.027$). There was no difference in the E. coli load of seven-day old stable fly pupae

and the empty cases from rinsed stable flies ($t = -0.28$; df $= 74.09$; $P = 0.778$).

Spearman's rank correlation was used to determine if the E. coli contamination of

the house fly and stable fly adults was related to the amount of bacteria found in the

pupal case. In house flies, the contamination of the newly emerged fly, the rinse solution

and the remaining contamination on the rinsed fly were all correlated with the amount of

E. coli in the case (Table 4.5.1). There was no correlation between the contamination of

the adult stable fly and the E. coli load in the pupal case (Table 4.5.1).

Table 4.5.1. Spearman's rank correlation between house fly and stable fly
E. coli **contamination and pupal case** _E. coli_ **contamination.**

	Case contamination		
	n	ϱ	$P(\varrho)$
	House flies		
Fly contamination	50	0.724	< 0.0001
Rinse contamination	50	0.817	< 0.0001
Rinsed fly contamination	50	0.711	< 0.0001
	Stable flies		
Fly contamination	50	0.067	0.643
Rinse contamination	50	0.142	0.327
Rinsed fly contamination	50	0.191	0.185

CHAPTER 5.   DISCUSSION

A non-pathogenic strain of *E. coli* was used during experimentation for safety

reasons, and likely had little effect on experimental outcome. This non-pathogenic strain

was less hazardous to work with and its detection was easier and less expensive than

detection of pathogenic strains such as *E. coli* O157:H7. Survival of *E. coli* O157:H7 in

the environment follows a similar pattern to that of non-pathogenic strains (Mubiru et al.

2000, Ogden et al. 2001). Also, the suspected acid tolerance of *E. coli* O157:H7 varies

from strain to strain (Miller and Kaspar 1994, Arnold and Kaspar 1995) and is

comparable to acid tolerance of some non-pathogenic strains (Duffy et al. 2000). The

E. coli strain in this study served as an indicator bacterium, and was used as a model for

the survival of possible pathogenic strains.

The persistence of *E. coli* in house fly and stable fly immatures was assessed during

short and long time periods in the larval and pupal stages through to adult emergence. For

both fly species, the bacterium was present in larvae, pupae and adults, but the overall

pattern of contamination varied between house flies and stable flies. The *E. coli*

population in house fly larvae was generally lower compared with stable fly larvae. Also,

the *E. coli* population tended to decrease over time in house fly larvae, while it remained

unchanged in stable fly larvae. During the pupal stage, the amount of *E. coli* in the house

fly pupa dropped just before adult emergence. This reduction in *E. coli* was not observed

in stable fly pupae. Resulting contamination of the adults also varied between both fly

species. Although adult flies of both species were contaminated externally and internally,

contamination occurred in a large proportion of house flies and in a smaller proportion of stable flies. These differences may be related to the use of *E. coli* by each species.

Greenberg (1959d) added strains of *Salmonella* or *Shigella* to the rearing medium of house fly larvae and found that most larvae sampled were free of the bacterium, indicating these bacterial species did not persist in maggots reared in their presence, and were perhaps destroyed in the larval gut. In the present study, the proportion of larvae free of the test bacterium ranged from about 5% to nearly 60%, depending on the experiment and the fly species. Two reasons could account for failure to detect *E. coli* in larvae. Either larvae did not ingest *E. coli* after the 20 minute feeding period, or *E. coli* was digested quickly. It was unlikely due to rapid digestion since the food bolus of house fly maggots has a passage time of about 10 and 25 minutes in the fore-midgut and the mid-midgut, respectively (Espinoza-Fuentes and Terra 1987). The mid-midgut is the region where bacteria are thought to be destroyed through the combined action of low pH, gut-specific lysozymes and digestive enzymes (Greenberg 1965, Espinoza-Fuentes and Terra 1987, Terra et al. 1988). Also, complete gut transit in third instar house flies is slightly over 100 minutes (Espinoza-Fuentes and Terra 1987), so bacteria consumed within the 20 minutes feeding period and not destroyed by digestion should not have been excreted before the first sampling of the maggots following surface-sterilisation. Furthermore, when larvae were fed different doses of bacteria, the ingestion of small amounts of *E. coli* by the larvae resulted in an increase of the bacterial population over time in both species, not a swift destruction through digestion.

The proportion of infected larvae did not change during the first five hours after infection, but changed over 48 h. The proportion of infected house fly maggots decreased

during the first 24 h, probably from digestion and from excretion of live bacteria, and then increased to reach 100% after 48 h of incubation. This was probably due to re-infection of the remaining larvae feeding on the excreted bacteria, and perhaps also because of a reduced digestion rate caused by a decrease in the amount of food in the gut. This trend was not seen in stable fly larvae, where there was a constant increase in the proportion of larvae infected. Live *E. coli* was also excreted by immature stable flies, but with apparently less effective *E. coli* digestion, maggots simply accumulated the bacteria, and remaining larvae contaminated themselves by feeding on the excreted cells. The survival of both species of larvae reared on *E. coli* may help provide an explanation for the persistence of the bacterium through development and will be discussed later.

Bacterial populations average 10^7 bacteria (total gut flora) in actively feeding house fly maggots and $10^5 - 10^6$ bacteria in the prepupa (Greenberg 1959a). Radvan (1960a) reported similar amounts of *E. coli* in house fly larvae prior to pupation. The normal flora of stable flies is similar in abundance to that of immature house flies (Greenberg 1962). The amount of *E. coli* detected in third instar larvae in this study was lower than those reported earlier (Greenberg 1959a, Radvan 1960a, Greenberg 1962). Contrary to the fore-mentioned experiments, the larvae used here were not reared on the detected bacteria, but only put in contact with *E. coli* for a period of 20 minutes. In this short period, it appears stable fly larvae were able to acquire equal or more bacterial cells than house fly larvae from cultures of similar bacterial densities. Over five hours, the initial *E. coli* gut population was different for both species, but the rate of change was comparable. This being only a very short portion of the global pattern over 48 h, the reduction of *E. coli* load could be linked to excretion of the bacteria.

The long term persistence probably represents a more accurate picture of *E. coli* persistence in the larvae. For house fly larvae, there was a decrease in the amount of *E. coli* in the larval gut over time. Some live bacteria were excreted onto the water agar and then re-ingested, but a portion of *E. coli* was probably digested at each passage through the gut, reducing the amount of bacteria excreted onto the water agar and available for re-consumption over time. Stable fly larvae may be less efficient at digesting *E. coli*, and the amount of bacteria in their gut remained fairly constant over time because the amount of bacteria excreted and available for re-ingestion was comparable.

This pattern of *E. coli* persistence appears when a high concentration of *E. coli* is fed to the larvae. The change in the *E. coli* gut population of larvae varies between species when fed a low concentration of bacteria. *E. coli* multiplies in both house fly and stable fly larval guts when acquired at a low dose. Over 48 h, the *E. coli* population increased by an average of 2.277 log(CFU/larva + 1) in immature house flies and 3.248 log(CFU/larva +1) in immature stable flies. This increase brought the *E. coli* larval load of both species to levels similar with the load of larvae 48 h after ingestion of a high dose of *E. coli* cells. This could be indicative of a carrying capacity for *E. coli* in this experimental system, where stable fly larvae can support a larger *E. coli* population in their gut compared with house fly larvae. The rate of change over time in the bacterial population is the difference between the rate of growth (multiplication and re-ingestion) and the rate of death (digestion and excretion) of the population. When growth is greater than death, the change is positive and the population increases; when death is greater than growth, the change is negative and population decreases. At low density, *E. coli* in the

gut of immature house flies and stable flies grows faster than it is destroyed or evacuated, probably because there are enough nutrients in the gut to sustain a certain bacterial population. *E. coli* is generally not known as a fastidious organism, and can probably grow on material found in the larval gut. As the larvae have been starved for a period of about 24 h before feeding, it is also possible that digestive processes slowed. The peritrophic matrix of starved dipterous larvae is secreted more slowly than the peritrophic matrix of actively feeding larvae (Jacobs-Lorena and Oo 1996). When only a small quantity of bacteria was ingested, digestion may not have been fully stimulated, giving an opportunity for *E. coli* population growth. Also, third instar house fly larvae starved for 24 h lose over 90% of the bacteria in their gut (Greenberg 1959c). This reduction of the competing flora may also have helped in the growth of the initially low *E. coli* population. When a large quantity of *E. coli* was ingested, the resources for growth were probably insufficient and the destruction of the bacteria through digestion more efficient, leading to a slower increase of the *E. coli* population in stable fly guts and a decrease in house fly guts.

Very high proportions of pupae of both species were infected with *E. coli*. Since third instars were transferred to EYM plates with *E. coli* lawns and left to graze until pupation, larvae were in constant contact with the bacterium for a long time period. The average amount of *E. coli* detected in house fly pupae was similar to the pupal bacterial population of 10^4-10^5 stated in literature (Greenberg 1959a, Radvan 1960a). In the case of stable fly pupae, the average amount detected in this study was lower than the 10^5-10^6 previously reported (Greenberg 1962). The overall pattern of bacterial survival through metamorphosis was comparable between species with the bacterial load remaining

unchanged through pupation (Greenberg 1962). However, in this study, the pattern of change in the *E. coli* population of pupae varied between fly species. Although the *E. coli* population increased until day 3 in both species, there was a sudden decrease of the bacterial load in house fly pupae between day 5 and 6, the last day before adult emergence. This decrease was not observed in stable fly pupae where the *E. coli* population stabilised after day 3. This pattern differs from the increase in bacterial load (Radvan 1960a) or the lack of change in the bacterial population (Greenberg 1959a) reported in other studies.

The destruction of *E. coli* in the house fly pupa cannot be directly related to digestion, as there is no active feeding taking place during this stage. The process of metamorphosis involves the destruction and synthesis of tissues, and these histolytic processes may affect the *E. coli* population. However, these drastic changes occur within the first 48 h of pupation, where the puparium content is liquefied as the old larval tissue is being destroyed and new adult tissue is being synthesised (Greenberg 1959c, Radvan 1960b, Thomas 1985). Since the *E. coli* population grows in both species during this 48 h period, it is possible the bacteria present in the larval digestive system use some of the nutrients in the liquefied puparium content to grow. As the pupa takes form, the midgut lining of the old larva is shed to the internal surface of the puparium, while a new midgut is synthesized (West 1951, Greenberg 1959c, Radvan 1960b). It appears these major changes do not affect the size of the pupal *E. coli* population. Studies involving the dissection of pupae indicate the new pupal midgut to be largely free of bacteria while the exuvial membrane is highly contaminated (Greenberg 1959c, Radvan 1960b). The pupa then becomes more solid and the nutrients may be less accessible for the bacteria and the

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E. coli population enters the stationary phase. Because of the localisation of the bacteria outside of the forming adult, the contamination of the emerging fly was assumed to be mostly external (Radvan 1960a, b).

The contamination of newly emerged house flies and stable flies with *E. coli* also varied between species. While house fly pupae harboured less *E. coli* than stable fly pupae, the bacterium was detected on a significantly higher proportion of house fly adults. The proportion of infected house flies in this study is similar to what has been stated in the literature (Greenberg 1959a, Radvan 1960a). The proportion of infected stable flies (46%) reported by Greenberg (1962) is larger than what was found in this study, where *E. coli* was detected on only 28% of adult stable flies.

Detection of bacteria on the newly emerged fly indicated 2 to 10% of the total bacterial load of the pupa could be recovered from the adult (Radvan 1960a), which supported the hypothesis that most of the contamination was external to the fly. In experiments similar to the ones conducted in this study, the amount of bacteria on the surface of house flies was reported to be generally less than 100 CFU (Greenberg 1959b). Although most adult midguts were sterile, external contamination accounted for only part of the total bacterial load of teneral flies (Greenberg 1959b). The results of the present study indicate contamination with *E. coli* is both internal and external for house fly and stable fly adults. There was a high correlation between the amount of bacteria in the rinse solution and the amount of bacteria in rinsed flies for both species, which would indicate that flies externally contaminated are usually internally contaminated as well. Since the rinsed flies were not surface sterilised prior to homogenisation, it is possible some bacterial cells adhered to the cuticle. Therefore, the minimal *E. coli* load recorded for

rinsed flies may not represent only internal contamination. The goal of the experiment

was not to quantify *E. coli* contamination precisely, but to determine the relative

importance of external and internal contamination with bacteria carried from the larval

stage. The amount of bacteria in infected stable flies was reported to be higher than in

house flies (Greenberg 1962). Similarly, this study indicated infected stable flies

contaminated the rinse solutions more than did infected house flies. However, because

only minimal *E. coli* loads were recorded, it can only be said that there was no significant

difference in the *minimal* amount of bacteria between contaminated adult house flies and

stable flies. The average minimal amounts of *E. coli* reported in this study are higher than

amounts reported for both house flies (Greenberg 1959b) and stable flies (Greenberg

1962). The pupal cases of emerged stable flies were also significantly more contaminated

with *E. coli* than cases from emerged house flies. Yet, the proportion of contaminated

stable flies was much lower than the proportion of contaminated house flies. Greenberg

(Greenberg 1959c) reported there was no correlation between the amount of bacteria on

emerged adult house flies and the amount in their puparium. While this is true for stable

flies in this study, it is not for house flies. A strong correlation was found between the

contamination of house flies and the contamination of their respective puparia.

Ultimately, the amount of *E. coli* in the immature house fly has an influence on the

bacterial load of the adult, while the contamination of immature stable flies does not

influence adult contamination.

Results of the survival experiment indicate differences in the suitability of *E. coli*

for sustaining growth and development of house fly and stable fly larvae, and may help

explain some patterns of persistence. Both fly species completed development on all

treatments, and had higher survival rates when reared on other treatments compared to *E. coli* alone. House fly larvae developed reasonably well on *E. coli* with a mean pupation rate of 62.3%. This was lower than the 72% survival to pupation obtained by Watson et al (1993) on the same agar-based medium. Watson et al (1993) also recorded greater pupal weights and pupal survival. This variation in survival may be attributable to differences in the bacterial strain used to sustain house fly development. Schmidtmann and Martin (1992) demonstrated that house fly larvae reared on blood agar plates supplemented with *E. coli* had a survival rate of 40% on one *E. coli* strain, but reached almost 60% on a second *E. coli* strain. Survival differences may also be due to differences between house fly strains. Developmental time of house fly larvae was 7.9 days when reared on *E. coli* alone, significantly longer than the 7 days required on *E. brevis* + *F. odoratum*. Watson et al (1993) and Schmidtmann and Martin (1992) using agar medium did not report developmental time. However, house fly development in poultry manure requires an average of 8.1 days at 25°C (Lysyk and Axtell 1987), comparable to the 7.9 days required by house fly larvae reared on *E. coli* alone. Overall, the performance of house fly larvae and pupae was reduced on *E. coli* alone compared to the other treatments, and addition of *E. coli* to the *E. brevis* + *F. odoratum* inoculum did not have any significant impact, positive or negative, on survival compared with the other treatments.

Stable fly larvae and pupae had low survival when reared on *E. coli* alone. The mean survival rate of 25.4% was lower than reported by Lysyk et al (1999) under similar conditions, where the larval survival rate on *E. coli* varied between 36.8% and 49.2%. Larvae reared on *E. coli* reached pupation in an average of 11.9 days. Survival of stable

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fly larvae in traditional rearing medium at 25°C is ≈ 80% and requires about 8 days

(Lysyk 1998). Larval survival of stable flies on a mixture *E. brevis* + *F. odoratum* +

E. coli was 94.4%, indicating a positive interaction among the three bacterial species for

the development of immature stable flies, or the production of a favourable by-product.

Development time on the mixture of all three bacteria was also significantly shorter than

on the other treatments, averaging 9.1 days, a value comparable to development in

traditional rearing medium. Although pupal weight of stable flies reared on *E. coli* was

greater than pupae reared on *E. brevis* + *F. odoratum*, adult emergence on *E. coli* was

very low (32%) compared to other treatments (>80%). Lysyk et al (1999) reported stable

fly emergence ranging from 7.4 to 95.1% when reared on *E. coli*, while emergence from

stable fly pupae in traditional rearing medium at 25°C is ≈ 86% (Lysyk 1998). Although

pupal weight was high, pupal survival was low, indicating growth factors provided by

E. coli alone do not guarantee survival. The addition of *E. coli* to the control treatment

E. brevis + *F. odoratum* increased pupal survival to 95%, again suggesting a possible

bacterial interaction favourable to stable fly development.

Reduced survival of stable fly larvae on *E. coli* plates suggests *E. coli* may not be

effectively digested by immature stable flies, but may produce some by-products that are

favourable to the larvae, as the addition of *E. coli* to other bacteria had a noticeable

positive effect on immature stable fly growth and survival. In the case of house fly larvae,

E. coli was an adequate food source, but not an optimal one.

Persistence of bacteria in the gut can be indicative that the bacterial species are a

poor food source for larvae, as was the case with stable fly larvae. Larval survival of horn

flies on different bacterial isolates is inversely proportional to the prevalence of the

isolate in the gut (Perotti et al. 2001). Bacteria remaining in large amounts in the gut of larvae are not digested, are not used as a source of nutrients by the maggots, and consequently survival of the immature fly is reduced on this isolate. Also, survival on a particular bacterial isolate was positively correlated to its relative abundance in the rearing environment versus its abundance in the gut. Similarly, Zurek (2000) reared immature house flies on gut isolates of house fly larvae and found that some were unsuitable for house fly development, although all were isolated from the guts of growing larvae. There was no indication of the relative abundance of these bacterial isolates in the gut or in the original breeding environment.

Dairy and beef cattle are asymptomatic carriers of *E. coli* O157:H7. They are also the primary reservoir of the bacterium, and shed it in their faeces with higher prevalence in the summer months (Bach et al. 2002). *E. coli* O157:H7 can survive for extended periods of time in manure (Wang et al. 1996) and can also replicate in poorly ensiled forage (Bach et al. 2002). House flies and stable flies are also present in Alberta in the summer months. House fly populations show peaks in late June, July and August, while stable flies peak in August and September (Lysyk 1993a). Both house flies and stable flies are associated with cattle and can breed in manure. In Alberta dairy farms, house fly larvae can be found mostly in silage mounds and in residual manure and bedding inside farm buildings (Lysyk 1993b). Stable fly larvae can be found mostly in manure mounds, near fence lines and in silage mounds (Lysyk 1993b)]. In the United States, stable fly larvae were also observed in feed aprons (Meyer and Shultz 1990, Skoda et al. 1991).

House fly larvae will use *E. coli* as a food source. Since the house fly gut is moderately favourable for bacterial development, *E. coli* populations may increase within

the larval gut, interspecific bacteria competition permitting. If *E. coli* remains in the larval gut until pupation, there is a high probability the adult will harbour it, albeit in relatively low numbers. Although the teneral house fly adult is infective at emergence and has a preference for resting indoors, the contaminants acquired later in adult life are probably of equal epidemiological importance.

Stable flies, on the other hand, are more likely vectors of *E. coli* during the larval stage. By sequestering *E. coli* from the environment in their gut, stable fly larvae may actually concentrate the *E. coli* population in particular locations such as fly pupation sites. As the larvae seek a dryer area to pupate, they may contaminate feed by leaving behind highly infected pupal cases. This would lead to a higher incidence of infection in animals, and a greater quantity of infected faeces in the environment, increasing the odds of eventual human contact.

CHAPTER 6. CONCLUSION

E. coli can be acquired by house flies and stable flies in the larval stage and be carried through metamorphosis to the emerging adult. House fly and stable fly larvae feed on bacteria in their habitats. *E. coli* cells fed to house fly and stable fly larvae persists in the gut for over 48 h in both species. The *E. coli* population in the larval guts increased over time when house fly and stable fly larvae ingested a low dose of bacterial cells. However, growth of *E. coli* varied between species when larvae ingested a high concentration suspension. The *E. coli* population decreased over time in house fly larvae and increased in immature stable flies. Changes in the *E. coli* population over 48 h suggest each fly species has a different carrying capacity for the bacterium, where stable fly larval guts can sustain larger *E. coli* populations compared with house fly larval guts. The capacity to digest *E. coli* varied among fly species and affected the persistence of the bacteria in the larval gut. House fly larvae reared on *E. coli* alone survived well, suggesting they were able to digest and utilise the bacterium. Larval stable flies did not survive well on *E. coli* alone, suggesting they did not digest and utilise the bacterium. The undigested *E. coli* cells were unaffected by the digestive processes, could not be used as a food source, and remained in the digestive tract of third instars until pupation. During metamorphosis, *E. coli* populations increased until day 3 of the pupal stage in both species, but there was a decrease in bacterial populations in the last day of house fly metamorphosis while populations remained constant in stable fly pupae. At emergence, a large proportion of house flies were contaminated with *E. coli*, while only a small

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proportion of stable flies were contaminated. In both species, *E. coli* was found internally and externally. All empty pupal cases retained large amounts of *E. coli*.

The persistence of *E. coli* from the larva to the adult house fly is significant. Undigested bacteria from the larval gut can be found on most teneral adults, making house flies infective at emergence. The amount of bacteria and how long they persist in and on the adult are key factors to determining the epidemiological importance of the contaminants acquired as immatures. Contamination with *E. coli* from the larva to the adult is not very significant in stable flies. However, the interaction between this fly species and *E. coli* is still epidemiologically important. Because they cannot digest *E. coli*, stable fly larvae actually sequester and accumulate the bacteria in their gut. Although most teneral adults are free of *E. coli*, the pupal cases harbour large amounts of bacteria. Third instars and pupae in feed may contribute to the dissemination of the bacteria in animals.

The experiments conducted in this study determined the pattern of persistence of *E. coli* in house fly and stable fly larvae, and throughout most of the life cycle of these insects. It would be interesting to see if the patterns of persistence are similar when *E. coli* is fed in a mixture of digestible and indigestible bacterial species. This would take bacterial competition in the larval gut into account and give a more accurate portrait of the fate of *E. coli* in flies.

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