Investigating the Vector Competence of the House Fly (*Musca domestica*) for *Campylobacter jejuni*

by

Carson Gill

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Name: **Carson Gill**

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Examining Committee: **Chair:** Michael Silverman, Associate Professor

Carl Lowenberger
Senior Supervisor
Professor

Norbert Haunerland
Supervisor
Professor

Margo Moore
Internal Examiner
Professor

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Abstract

Campylobacteriosis is a severe gastroenteric disease in humans caused by the bacterium, *Campylobacter jejuni*, typically obtained through the ingestion of contaminated poultry products. Poultry facilities become contaminated through the introduction of pathogens, including *C. jejuni*, by the house fly, *Musca domestica*. This thesis investigates the vector competence of *M. domestica* for *C. jejuni* to determine if the bacteria survive house fly metamorphosis from larva to adult, and can multiply within adult flies to enhance transmission, and whether innate immune factors of the house fly can clear *C. jejuni* infections. We demonstrate that *M. domestica* mounts an effective innate immune response that prevents transmission of *C. jejuni* from larva to adult, and eliminates *C. jejuni* from adult house fly gastrointestinal tracts within hours. We propose that *M. domestica* serves as a mechanical vector, rather than as a true, amplifying, biological vector. These findings will help elucidate the elusive epidemiology of campylobacteriosis.

Keywords: campylobacteriosis; *Campylobacter jejuni*; house fly; vector; innate immunity; antimicrobial peptides

To my family,

for your endless love and support.

And to my dear nephew Finley,

may you grow to inspire our next generation.

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Table of Contents

List of Tables

List of Figures

List of Acronyms

AMP	Antimicrobial Peptide
ANOVA	Analysis of Variance
CDT	Cytolethal Distending Toxin
CTL	C-Type Lectin
DDT	Dichlorodiphenyltrichloroethane
EID	Emerging Infectious Disease
EST	Expressed Sequence Tag
GBS	Guillain-Barré Syndrome
GFP	Green Fluorescent Protein
GI	Gastrointestinal
Imd	Immune Deficiency
JAK	Janus Kinase
JNK	c-Jun N-terminal Kinase
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
LTA	Lipotechoic Acid
MAPK	Mitogen-Activated Protein Kinase
mCCDA	Modified Charcoal-Cefoperazone-Deoxycholate Agar
MMLV-RT	Moloney Murine Leukemia Virus Reverse Transcriptase
NCBI	National Center for Biotechnology Information
PAMP	Pathogen Associated Molecular Pattern
PGN	Peptidoglycan
PRR	Pattern Recognition Receptor
qPCR	Real Time Quantitative Polymerase Chain Reaction
RISC	RNA-Induced Signalling Complex
ROS	Reactive Oxygen Species
SARS	Severe Acute Respiratory Syndrome
SSH	Suppression Subtractive Hybridization
STAT	Signal Transducer and Activator of Transcription
VBNC	Viable But Non-Culturable
WHO	World Health Organization

Glossary

Bacteriocins	Ribosomally synthesized antimicrobial peptides produced by bacteria and archaea, many of which are considered probiotics.
Campylobacteriosis	Human gastroenteric disease resulting from infection with *Campylobacter* spp., most commonly, *C. jejuni*.
Cytolethal Distending Toxins	A recently discovered family of toxins in Gram-negative bacteria that cause cell cycle arrest.
Emerging Infectious Disease	Infections that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range.
Expressed Sequence Tag	Small pieces of DNA sequence (usually 200 to 500 nucleotides long) that are generated by sequencing one or both ends of an expressed gene.
Guillain-Barré Syndrome	Human autoimmune disease that results in acute neuromuscular paralysis.
Lipooligosaccharide	Major glycolipids expressed on some mucosal Gram-negative bacteria. Analogous to lipopolysaccharides (LPSs) also found in Gram-negative bacteria, but lack O-antigen repeating units.
Lipotechoic acid	Major constituent of the cell wall of Gram-positive bacteria.
RNA interference	The silencing of gene expression by the introduction of double-stranded RNAs that trigger the specific degradation of a homologous target mRNA and often subsequently decrease production of the encoded protein.
Transcriptome	The complete collection of mRNAs in a particular cell or population of cells.
Zoonosis	Disease in humans that results from a pathogen being passed from an animal (in some instances, by a vector).

Chapter 1. Introduction

GILL Carson
Simon Fraser University
Department of Biological Sciences
Burnaby, British Columbia, V5A 1S6, Canada

Emerging Infectious Diseases

Emerging infectious diseases (EIDs) are historically among the leading causes of death and disability worldwide. Defined as "infections that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range" (Morse 1995), EIDs have been familiar threats since ancient times (Morens et al. 2004). Historically, these diseases include those that caused the great plagues, or Black Death in Europe during the 14th century (Achtman et al. 2004), the epidemics of yellow fever that plagued development of the New World (Acuna-Soto et al. 2000, Gubler 2009), and more recently, the HIV/AIDS pandemic (Jones et al. 2008).

EID events have risen significantly since 1940 (Jones et al. 2008) and infectious diseases are estimated to cause 15 million deaths annually worldwide (Morens et al. 2004). This dramatic disease emergence is thought to be driven primarily by the socioeconomic, demographic, and environmental transformations that have occurred globally since World War II (Wilcox and Gubler 2005). However, the causal linkages remain to be fully elucidated and despite advances in technology and preventative medicine, infectious diseases continue to emerge and re-emerge, leading to unpredictable epidemics and difficult challenges for public health: not unlike the severe acute respiratory syndrome (SARS) coronavirus outbreak of 2003 (Morens et al. 2004).

Vector-borne Diseases

Vectors are organisms that transmit pathogens from one infected person or animal to another, causing serious diseases in human populations. Vector-borne diseases account for approximately 17% of the estimated global burden of infectious diseases (WHO 2004, Townson et al. 2005), with the majority transmitted by arthropods. Arthropod-borne diseases, including malaria, dengue, yellow fever, trypanosomiasis, chagas, encephalitis, leishmaniasis, and filariasis, are considered the most important vector-borne diseases in terms of their impact on human health and account for more than 1.5 million deaths per year (WHO 2004, Hill et al. 2005).

Although the discovery of dichlorodiphenyltrichloroethane (DDT) as an insecticide in the 1930s significantly reduced the number of agricultural pests and disease transmitting arthropods, increased emergence of insecticide resistance coupled with the ban of DDT in the 1970s due to environmental concerns have resulted in the re-emergence of vector-borne diseases worldwide (WHO 1979, Gubler 1998). Malaria caused more than 600,000 deaths in 2012 and remains the most deadly vector-borne disease (WHO 2014b), while dengue is the world's fastest growing vector-borne disease. An estimated 2.5 billion people are currently at risk of dengue virus infection, which has seen a 30-fold increase in incidence since the ban of DDT (WHO 2009, Guzman et al. 2010). No vaccines or medical treatments currently exist for numerous vector-borne diseases, including dengue, thus vector control is the only means of protecting populations from infection (Townson et al. 2005).

Foodborne Zoonoses

A zoonotic disease, or zoonosis, is any infectious disease that manifests following the transmission of a pathogen (in some instances, by a vector) from animals to humans. Most pathogenic microorganisms (61%) are considered zoonotic, and the majority of EIDs (75%) are considered zoonoses (Taylor et al. 2001, Jones et al. 2008). Among these, foodborne diseases are an important cause of morbidity and mortality. Although the global burden of foodborne disease is unknown, an estimated 48-76 million illnesses result from the consumption of contaminated food in the US annually (Mead et al. 1999, Scallan et al. 2011a). The World Health Organization (WHO) is currently developing a comprehensive strategy for foodborne disease surveillance to provide more accurate estimates (WHO 2014a).

Agricultural intensification to meet the increasing global demand for food and the concomitant increase in interactions between humans, livestock, and wildlife, is linked to the emergence of zoonoses (Jones et al. 2013). More than 200 foodborne diseases have been identified (Bryan 1982), however, the industrialization and intensification of food production has led to numerous new or re-emerging foodborne infections (Frenzen 2004, Scallan et al. 2011a, Koluman and Dikici 2013). Indeed, the epidemiology of foodborne disease is changing as new pathogens emerge and well-recognized

pathogens increase in prevalence or become associated with new food vehicles (Altekruse et al. 1997, Tauxe 1997).

Major Causes of Human Foodborne Diseases

Foodborne diseases are often colloquially referred to as 'food poisoning' and typically result in inflammation of the gastrointestinal tract, or gastroenteritis, which leads to nausea, diarrhoea and vomiting (Bennett 1995). Many bacteria, along with some viruses and parasites, are among the main causative agents of gastroenteritis, including *Campylobacter* spp., *Salmonella* spp., and *Escherichia coli*, which together account for more than 90% of all reported bacterial cases. Poultry and poultry products are considered the primary source of foodborne disease caused by these bacteria, which are found naturally in the intestines of poultry, livestock, and numerous other animals, and contaminate food during processing (Thorns 2000, CFIA 2013). Although *Salmonella* spp. command the majority of media attention, *Campylobacter* spp. have emerged relatively recently and have become one of the most common causes of bacterial gastroenteritis in the world (EFSA 2013, WHO 2013). In 2011, there were approximately 220,000 and 7,000 confirmed human cases of *Campylobacter* spp. infection in the European Union (EU) (EFSA 2013) and US (CDC 2012), respectively. However, estimates suggest that the true incidence is actually upwards of 9 million cases in the EU (EFSA 2011) and 0.8-2.5 million cases in the US per annum (Mead et al. 1999, Scallan et al. 2011b).

Campylobacteriosis

Human disease resulting from infection with *Campylobacter* spp. is termed campylobacteriosis and typically manifests as acute gastroenteritis. Anyone can become infected with *Campylobacter* spp.; however, the immunocompromised, children younger than five years, and young adults are more likely to get sick (PHAC 2013). Campylobacteriosis typically presents as watery or bloody diarrhoea with abdominal pain, cramping and fever, and may be accompanied by nausea and vomiting (Moore et al. 2005). Disease develops two or three days after ingestion of contaminated food and although symptoms typically resolve themselves within a week, severe post-infection

complications can arise, including Guillain-Barré syndrome (GBS), reactive arthritis, and irritable bowel syndrome (WHO 2013). GBS, the most common cause of acute neuromuscular paralysis in humans, is an autoimmune disease that targets the peripheral nervous system through molecular mimicry and often manifests following *Campylobacter* spp. infection (Poropatich et al. 2010). Antibiotics are available for the clinical therapy of campylobacteriosis, however, they should only be administered to immunocompromised individuals as there is controversy regarding their efficacy and increasing evidence for the emergence of antibiotic resistant *Campylobacter* spp. (Luangtongkum et al. 2009). In the US alone, up to $8.0 billion in human illness costs are spent annually on campylobacteriosis and associated sequelae, placing an enormous burden on public health and the economy (Buzby et al. 1997).

Campylobacter

A Historical Perspective

Campylobacter spp. were first described in 1886 in the stools of children by Theodor Escherich, the German paediatrician who discovered *E. coli*. However, attempts to culture the bacteria were unsuccessful and Dr. Escherich's data, published in German, remained unrecognized for many decades (Butzler 2004). Throughout the early-mid 20th century the bacteria were described as *Vibrio* spp., and it was not until 1963, that the genus *Campylobacter* was established following its isolation from animal fetuses (Debruyne et al. 2008). Despite *Campylobacter* spp. likely causing illness in humans for centuries, it was not until 1968 that techniques were developed to isolate the bacteria from faeces and *Campylobacter* spp. were finally recognized as human pathogens (Dekeyser et al. 1972).

Taxonomy

The taxonomy of the genus *Campylobacter* has changed dramatically since its inception in 1963. It belongs to Campylobacterales, an order of Epsilonproteobacteria, which also consists of the human gastric pathogen, *Helicobacter pylori*, formerly known as *Campylobacter pylori* (Perez-Perez and Blaser 1996). At present the genus

Campylobacter is comprised of 25 species, however, this number continues to grow as new (or 'emerging') species and subspecies are identified (Man 2011). The most abundant and well-known members are *C. jejuni* and *C. coli*, which cause over 80% and approximately 10% of human campylobacteriosis cases, respectively (EFSA 2011).

Structure and Physiology

The name *Campylobacter*, meaning 'twisted bacteria,' describes the appearance of the organisms. In young cultures, *Campylobacter* spp. are comma, spiral, or S-shaped, however, as cultures age or become stressed, the bacteria may appear round or coccoid (Perez-Perez and Blaser 1996). As a Gram-negative bacteria, *Campylobacter* spp. possess a thin peptidoglycan cell wall located between an inner and outer cell membrane, and often possess an external capsule in addition to highly variable lipooligosaccharides (LOSs) on their outer membranes. The bacteria are highly motile with one or two polar flagella, and are capable of directed movements via chemotaxis (Young et al. 2007). *Campylobacter* spp. are able to modify their surface structures, which are required for numerous biological processes and likely to evade host defenses, using two protein glycosylation systems: O-linked glycosylation modifies serine or threonine residues on flagellin and N-linked glycosylation modifies asparagine residues on many proteins. These bacteria are extremely unique in that they are the only known non-eukaryotes to possess an N-linked glycosylation system (Szymanski and Wren 2005).

Campylobacter spp. are non-spore forming and are unable to grow at temperatures below 30^{o}C. These organisms require temperatures of 37-42^{o}C and oxygen at much lower concentrations (~5%) than what is present in the atmosphere for optimal growth. In addition, *Campylobacter* spp. are nutritionally fastidious and sensitive to many external physical conditions, including water activity, pH, heat, UV light and salt, and unlike most foodborne pathogens are considered somewhat fragile (EFSA 2011, Bronowski et al. 2014). Given these characteristics, warm-blooded hosts are required for *Campylobacter* spp. multiplication, and the principal reservoir is the alimentary tract of domesticated and wild birds and mammals, primarily poultry. However, *Campylobacter* spp. are found ubiquitously in the environment, capable of surviving for

up to three months in soil, manure, and water (Nicholson et al. 2005) in a viable but non-culturable (VBNC) state (Rollins and Colwell 1986, Murphy et al. 2006). In the VBNC state, the bacteria reduce their metabolic activity and lose the ability to form colonies, but retain viability and the potential to recover and cause infections (Barer and Harwood 1999, Bronowski et al. 2014).

Campylobacter spp. Colonization: Pathogenic Versus Commensal

In contrast to the severe intestinal inflammation and disease caused in humans, *Campylobacter* spp. infection in chickens is benign, but the basis for the differential host response is unknown. Very rarely do these bacteria cause disease in animals and *Campylobacter* spp. are actually considered as commensal organisms in poultry (de Zoete et al. 2010). Fewer than 10^2 cells are sufficient to colonize chickens and the bacteria reach large numbers (10^9 CFU/g) within 24 hours in the caeca of chickens, the predominant site for colonization, where *Campylobacter* spp. can persist for long periods and are continually shed with the faeces (Beery et al. 1988, Hermans et al. 2011a). Although chickens can carry a high load of *Campylobacter* spp. without clinical signs, a recent study suggests that infection in chickens may result in changes to the intestinal barriers and an associated decrease in growth (Awad et al. 2014). Therefore, despite chickens not developing disease, there may be some associated costs to *Campylobacter* spp. infection.

The lack of an adequate or suitable animal model for campylobacteriosis has hindered our understanding of human pathogenesis and contributed to the paucity of effective intervention strategies. The completion of the *C. jejuni* genome in the year 2000, however, was a remarkable step forward and has aided in identifying and understanding the mechanism of many pathogenicity-associated factors (Parkhill et al. 2000). It is certainly evident that the highly variable surface structures (capsule, LOSs, flagella, glycosylated proteins) have vital roles for host-bacterium interactions (Young et al. 2007), and the inflammatory pathology in humans suggests strong induction of innate immune responses to these bacterial structures (de Zoete et al. 2010). One interesting finding, is that a cytolethal distending toxin (CDT) produced by *C. jejuni* is only required for human infection and is responsible, at least in part, for the severe inflammatory

response characteristic of campylobacteriosis. This recently discovered family of toxins in Gram-negative bacteria that cause cell cycle arrest, however, do not appear to play a role in poultry colonization (Biswas et al. 2006, Young et al. 2007).

Transmission Dynamics of *Campylobacter* spp.

Chickens as the Primary Source

Poultry meat is considered as the primary source of *Campylobacter* spp. for human infection, with up to 80% of campylobacteriosis cases attributed to chickens. The vast numbers of *Campylobacter* spp. colonizing the intestinal tract of chickens contaminates the meat during processing, and the handling, preparation and consumption of contaminated chicken meat may result in disease (EFSA 2011). Indeed, the incidence of campylobacteriosis cases among humans correlates with the prevalence of *Campylobacter* spp. among broiler chickens (Jore et al. 2010), and upwards of 80% of boiler flocks in the EU may be *Campylobacter* spp.-positive at any given time (Figure 1-1; Mølbak 2001, EFSA 2013). Furthermore, because chickens are asymptomatic carriers of *Campylobacter* spp. (Awad et al. 2014), the presence of the bacteria is not obvious. Given that *Campylobacter* spp. are the most common cause of bacterial gastroenteritis in the world, on-farm control of *Campylobacter* spp. in chickens would reduce the risk of human exposure and have a significant impact on food safety and public health (Lin 2009).

On-Farm Control Measures

Theoretically, on-farm control could be achieved in numerous ways, including hygienic and biosecurity measures, poultry vaccinations, antibiotics, or probiotic and bacteriocin application. However, no interventions have succeeded in controlling chicken infections to date (Hermans et al. 2011b). The application of probiotics in chickens have recently demonstrated some promising results, especially via their production of bacteriocins, ribosomally synthesized antimicrobial peptides produced by bacteria and archaea (Dobson et al. 2012); however, despite potentially reducing the *Campylobacter* spp. load in the gut of colonized chickens, further research concerning

long-term efficacy and the conductance of large-scale field trials are required before they can become commercially available (Hermans et al. 2011b). Antibiotics have also been shown to reduce *Campylobacter* spp. counts in chickens (Farnell et al. 2005), but their use is controversial given the valid concerns of antibiotic resistance developing in the bacteria and compromising the treatment of human campylobacteriosis. Chicken vaccinations have reported variable results and currently an effective vaccine to combat caecal *Campylobacter* spp. colonization in chickens is unavailable (Hermans et al. 2011b). Furthermore, while hygienic and biosecurity measures have demonstrated reduced *Campylobacter* spp. colonization in chickens, even strict compliance with these has failed to control infections (Bahrndorff et al. 2013). Therefore, major efforts must be made to understand the transmission dynamics of *Campylobacter* spp. in order to develop effective intervention strategies and reduce its prevalence in chickens.

Transmission of *Campylobacter* spp. to Chickens

Although there is considerable variation between countries in poultry production facilities, contamination of poultry barns and the infection of chickens with *Campylobacter* spp. primarily occurs through horizontal transmission from the environment (Figure 1-2; Jacobs-Reitsma et al. 1995, Sahin et al. 2002). Potential sources and vectors for contamination are infected livestock (van de Giessen et al. 1992), rodents (Zweifel et al. 2008), insects (Hald et al. 2008, Hazeleger et al. 2008), and contaminated surface water, feed (WHO 2013), personnel and farm equipment (Ramabu et al. 2004). Among these, studies have repeatedly suggested that flies play an important and central role in transmitting *Campylobacter* spp. into poultry facilities from environmental sources (Rosef and Kapperud 1983, Berndtson et al. 1996). Hundreds of flies per day pass through ventilation inlets into chicken facilities (Hald et al. 2004, Hald et al. 2008) and recent demonstrations suggest that physical interventions (screens) can prevent flies from entering these facilities and substantially reduce the prevalence of *Campylobacter* spp. in chickens (Hald et al. 2007, Bahrndorff et al. 2013). The house fly (*Musca domestica*) is the fly species most commonly found carrying *Campylobacter* spp. (Hald et al. 2008) and studies have demonstrated these flies readily transmit the bacteria to non-infected chickens (Shane et al. 1985). Thus, it is likely that

M. domestica plays an extremely important role in the epidemiology of campylobacteriosis.

The House Fly (*Musca domestica*)

Musca domestica, first described in 1758 by Linnaeus, is an arthropod belonging to the order Diptera. It is one of, if not the, most widely distributed insects in the world and the most common fly, following humans over the entire globe (Hewitt 1914). House flies are always found in association with humans and have historically been a nuisance, as well as an important player in the epidemiology of numerous human diseases.

Life History

The house fly undergoes complete metamorphosis with distinct egg, larval, pupal and adult stages (Figure 1-3). The entire life cycle from the deposition of the eggs till the emergence of the adults varies widely due to temperature, food and other factors, but typically requires approximately 14 days (Hewitt 1914). Mating takes place 2-12 days after the adults emerge and the female can deposit up to 150 eggs four or more times during her lifetime. These eggs hatch in 12-24 hours and the feeding larvae pass through three larval stages within 8 days. When the larvae are mature they cease feeding and pupate (Matheson 1950). The pupal stage lasts 4-5 days, after which the adult fly emerges and typically lives for 15-25 days, but can survive for up to two months (Greenberg 1973).

Breeding and Feeding Habits

House flies are commonly referred to as 'filth flies,' as they breed in animal wastes, human excrement and decaying organic material. House fly larvae require bacteria as one of their main sources of nutrition, which are highly abundant in fermenting organic matter (Greenberg and Klowden 1972). Livestock and poultry manure are favourite breeding grounds of the house fly and vast numbers are produced; one pound of manure can yield more than 1,200 flies. House flies are also capable of

overwintering in either the larval or pupal stage under manure piles and in other protected locations, persisting through unfavourable conditions (Matheson 1950).

The proboscis and mouth parts of the house fly are adapted for sucking and absorbing liquid or liquefied food. They are incapable of biting and in order to feed on dry substances, such as sugar or manure, the fly must liquefy them. To do so, house flies secrete saliva on to their food, and following the ingestion of a meal, continually regurgitate drops of 'vomit' to further mix and digest their food through extracorporeal digestion (Hewitt 1914). The rate of digestion depends on the temperature and the nature of the food, with faeces usually deposited numerous times several hours after a meal. Following a meal, more than 30 spots of faeces and vomitus may be deposited within 24 hours by a single house fly (Hewitt 1914, Greenberg 1973).

Pathogen Transmission

The transmission of pathogens by insects may be biological or mechanical. Biological vectors carry pathogens that can multiply within their bodies and also may undergo developmental changes prior to transmission to a new host (Rochon 1998). Conversely, mechanical vectors transport pathogens externally on their body surface or mouthparts through simple body contact, or internally in their gastrointestinal tract through regurgitation or defaecation (Hewitt 1914, Greenberg 1973, Rochon 1998).

Given their filth and coprophagous habits, house flies have long been considered vectors of pathogenic microorganisms. During breeding, feeding and foraging, the bodies and alimentary tracts of house flies become contaminated by numerous microbes and these may be disseminated to domestic environments (Chifanzwa 2011). Indeed, the synanthropic house fly is a natural carrier of numerous pathogenic microorganisms, including viruses, fungi, parasites and bacteria (Greenberg 1971, Greenberg 1973). Among these are many bacteria that can cause human gastroenteritis, including *Salmonella* spp. (Greenberg et al. 1970), *E. coli*, and *Campylobacter* spp. (Szalanski et al. 2004). While house flies have demonstrated the potential to act as biological vectors for some bacteria, including *Salmonella* spp. (Greenberg et al. 1970, Chifanzwa 2011), typically they are considered as mechanical vectors (Meerberg et al. 2007). The

association between flies and enteric bacteria has been studied extensively (Rochon 1998), however, few studies have evaluated the vector competence of house flies for *Campylobacter* spp.

Vector competence is a complex characteristic governed by intrinsic factors that influence the ability of a vector to transmit a pathogen (Hardy et al. 1983, Beerntsen et al. 2000, Osei-Poku 2012). Vector-pathogen interactions are often very specific and many biochemical and physiological factors can influence vector competence (Hardy et al. 1983). These include the nutritional state of the vector, digestive enzymes, bacterial symbionts and the immune system (Ursic Bedoya 2008). Arthropod vector immune responses are paramount in limiting pathogen infection and transmission (Beerntsen et al. 2000, Cirimotich et al. 2010, Chifanzwa 2011); however, very few studies have evaluated the immune response of house flies. Doing so could elucidate the specific interactions between the house fly and *Campylobacter* spp. and its potential as a mechanical or biological vector.

Insect Immunity

Insects lack an adaptive immune system to confer specific and long lasting immunity. Nevertheless, the innate immune system of insects exhibits striking similarities with those of vertebrates and is extremely effective in combating pathogens (Nappi and Ottaviani 2000). Insects are the most diverse and prolific animal group to inhabit land and much of their success is due to a potent innate immune response (Cooper and Mitchell-Foster 2011).

The first line of defense in insects consists of structural barriers, including the exoskeleton or cuticle, the peritrophic matrix and the gastrointestinal epithelium (Royet 2004). A diverse repertoire of cellular and humoral responses is activated if a pathogen breaches these barriers. These include the production of reactive oxygen species (ROS), antimicrobial peptides (AMPs), and hemocyte-mediated phagocytosis, melanization and encapsulation (Cooper and Mitchell-Foster 2011). These responses are initiated directly or indirectly through an array of signalling pathways that are triggered by the recognition of pathogens as non-self (Tsakas and Marmaras 2010).

This process of recognition is mediated by pattern recognition receptors (PRRs) that bind to conserved structures on the surface of pathogens referred to as pathogen-associated molecular patterns (PAMPs; Medzhitov and Janeway Jr 2002).

PRRs recognize a limited but conserved set of PAMPs. These include Gram-negative bacteria lipopolysaccharides (LPSs), Gram-positive bacteria peptidoglycan (PGN) and lipotechoic acids (LTAs), or fungal β1,3-glucans (Royet 2004). PRR-PAMP binding triggers defense reactions that mediate and signal pathogen killing directly through phagocytosis and melanization, or indirectly through the activation of proteolytic cascades and signaling pathways that control the expression of immune effector genes (Tsakas and Marmaras 2010, Cooper and Mitchell-Foster 2011). Among these immune effectors are AMPs, which are ubiquitous and multipotent components of insect immune systems with a broad range of antimicrobial activity (Bulet et al. 1999). AMPs are synthesized by the fatbody, the midgut epithelium, and hemocytes, and their production is regulated largely by the Toll, Immune Deficiency (Imd), JAK-STAT, and apoptosis immune signalling pathways (Hoffmann and Reichhart 2002, Lemaitre and Hoffmann 2007, Cooper and Mitchell-Foster 2011).

The insect immune response exhibits a certain degree of specificity. Traditionally we have considered that infections by Gram-positive bacteria and fungi stimulate the Toll pathway and induce the expression of AMPs such as, defensin, drosomycin, and metchnikowin whereas infections with Gram-negative bacteria stimulate the Imd pathway and induce expression of AMPs such as, diptericin, attacin, cecropin, and drosocin (Hoffmann and Reichhart 2002, Hoffmann 2003). There is increasing evidence that there is significant crosstalk between many immune-related pathways as some immune-induced genes can be induced by multiple cascades and some molecules play key roles in multiple pathways, providing a certain level of redundancy (Tsakas and Marmaras 2010, Cooper and Mitchell-Foster 2011).

Thesis Objectives

The overall goal of this thesis was to characterize the vector competence of house flies for the most prevalent species of *Campylobacter*, *C. jejuni*. Despite the well-

known correlation between high numbers of house flies and the presence of *Campylobacter* spp. in poultry, we know little of the specific molecular interactions between pathogen and vector. The mechanisms of *Campylobacter* spp. transmission by house flies remain untested, especially if house flies function as a biological or a mechanical vector, and thus, their potential to amplify the inoculum or simply disseminate the pathogen.

Preliminary studies have demonstrated that *C. jejuni* can be recovered from both the external surfaces and the viscera of adult house flies exposed to contaminated faeces (Shane et al. 1985), but the retention of *C. jejuni* in adult flies has been found to be relatively short (< 24 hours) (Skovgård et al. 2011). Limited work has been done in recent years to examine the persistence of other bacteria throughout the life cycle of house flies, likely because early studies concluded these bacteria were destroyed by the acidic midgut of larvae or by other events during metamorphosis (Ledingham 1911, Greenberg 1973, Rochon 1998). However, it is important to determine if house fly larvae that feed on *Campylobacter* spp.-infected substrates can maintain infection throughout fly metamorphosis and amplify the bacteria. Furthermore, it is important to characterize the immune response of house flies to *C. jejuni* and determine its effects on the vector competence of house flies.

This thesis, therefore, aims to determine whether *C. jejuni* survives house fly metamorphosis and multiplies within adult house flies to enhance transmission, and whether host innate immune factors of the house fly can kill *C. jejuni*. Three main objectives guide this dissertation:

1. Characterize one component of the innate immune response (AMP expression) in the intestinal tract of *M. domestica* after exposure to *C. jejuni*, using suppression subtractive hybridization (SSH) and confirming the temporal patterns of these differentially expressed genes using real time quantitative PCR.

2. Determine if *C. jejuni* ingested by larvae can survive the metamorphosis to adults, and relate this time course with the expression of AMPs.

3. Determine how long after ingestion *C. jejuni* remains viable in the adult house fly, including in the vomitus, gastrointestinal tract, and excreta, and estimate the number of bacteria that can be harboured over what time periods.

Achieving these goals will allow us to determine with more clarity the specific interactions between vector and pathogen and especially elucidate the biological or mechanical potential of these insects in the transmission of *C. jejuni*.

Throughout this thesis we use the terms ‘infected’ and ‘exposed’ interchangeably. We exposed house flies to *C. jejuni* and evaluated gene expression and the presence of damaged and viable bacteria in the insects at different times after exposure. Given that bacteria are a common source of food for these insects, there is some debate regarding the validity of the term ‘infected,’ however, we believe that all house flies exposed to *C. jejuni* became infected, albeit for a brief period. Any confusion caused by these terms is the fault of the author.

Figures

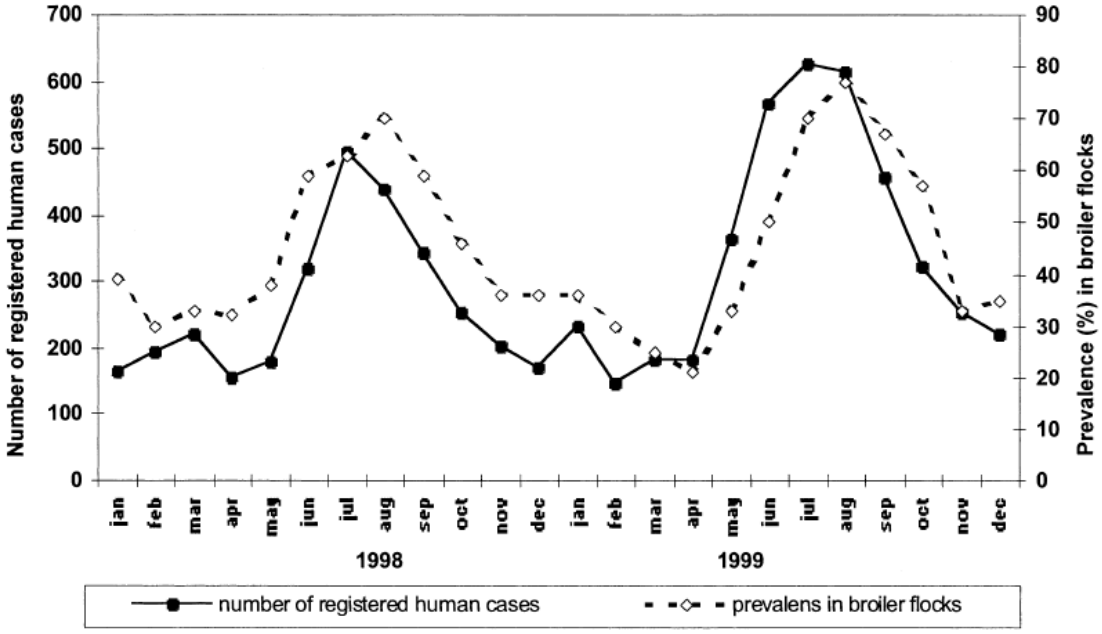

Figure 1-1 Correlation of chicken and human *Campylobacter* spp. infections.
The number of human *Campylobacter* spp. infections and the prevalence of *Campylobacter* spp. among broiler chicken flocks have a similar seasonal pattern. Upwards of 80% of boiler flocks may be *Campylobacter* spp.-positive during the summer months, which correlates strongly with the peak in incidence of campylobacteriosis cases among humans. This data was collected in Denmark from broiler flocks at slaughter by month between 1998 and 1999 (Mølbak 2001).

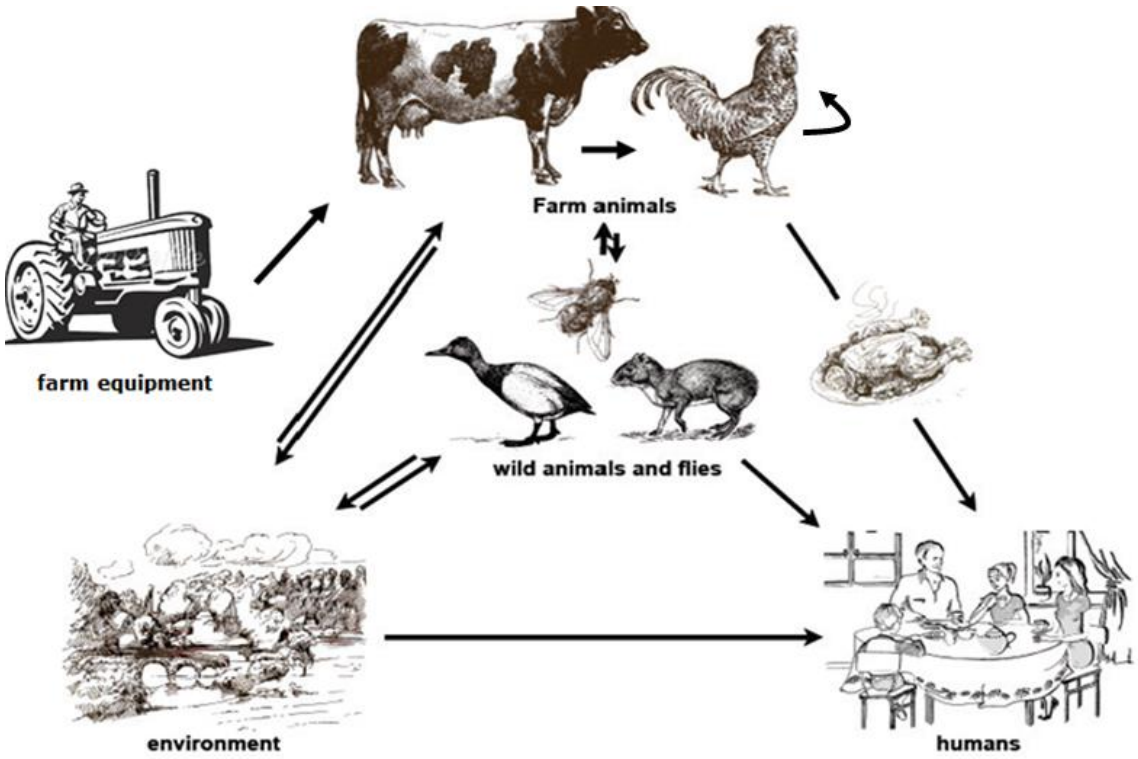

Figure 1-2 Proposed routes of transmission for *Campylobacter* spp.
Numerous routes of *Campylobacter* spp. transmission to humans have been proposed. Poultry products, primarily chicken meat, are considered the primary source for human infection. Chickens become infected from the horizontal transmission of *Campylobacter* spp. from environmental sources including, infected livestock, wild animals and insects, and contaminated personnel and farm equipment. Flies play a central role in the proposed transmission routes of *Campylobacter* spp. and thus, an important role in the epidemiology of campylobacteriosis. This figure is modified from the original version available from the Federation of European Microbiological Societies (FEMS) Microbiology Letters, Volume 356, published by Jonh Wiley and Sons Ltd. (Bronowski et al. 2014).

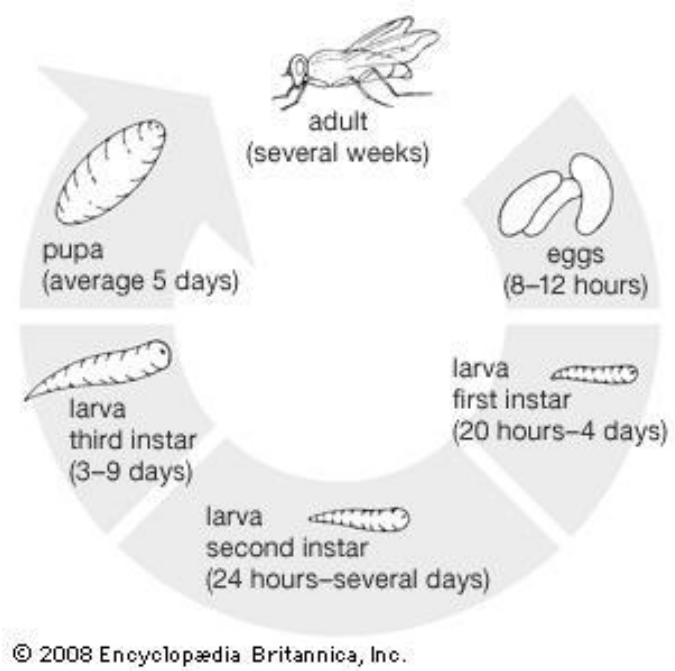

Figure 1-3 *Musca domestica* life cycle.
The house fly, *Musca domestica*, undergoes complete metamorphosis with distinct egg, larval, pupal and adult stages. Larvae feed and pass through three larval stages. The entire life cycle from the deposition of the eggs till the emergence of the adults varies widely due to temperature, food and other factors (Encyclopaedia Britannica, Inc. 2008).

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Connecting Statement 1

In the Introduction, I presented background information on *Campylobacter* spp. and the putative role of house flies (*Musca domestica*) in transmitting *Campylobacter* spp. into and throughout poultry facilities. One of the roles of innate immune responses in insects is to recognize and eliminate pathogens. This has been best described in the hemocoel of insects, but also occurs in the gastrointestinal (GI) tract of invertebrates. Because of the potential role of antimicrobial peptides (AMPs) to eliminate pathogens in the GI tract of many insects we investigate the global expression of immune-related genes in the GI tracts of *M. domestica* in response to *C. jejuni* exposure using suppression subtractive hybridization (SSH) in the next chapter. In addition, we estimate the number of viable *C. jejuni* that can be retained over time in house flies following exposure and determine if it correlates with the expression of the identified immune-related genes.

Chapter 2. Identification of Immune-Related Genes in the House Fly (*Musca domestica*) in Response to Ingestion of *Campylobacter jejuni* Using Suppression Subtractive Hybridization

GILL Carson, BAHRNDORFF Simon, LOWENBERGER Carl
Simon Fraser University
Department of Biological Sciences
Burnaby, British Columbia, V5A 1S6, Canada

Contributions:
I assisted with conception and design of the study, as well with all experimental protocols, including: infection experiments, dissections, CFU determinations, subtraction library generation, cloning and transformations. I performed all RNA extractions, cDNA syntheses and qPCR reactions, and analyzed all sequencing and qPCR data, in addition to writing the first draft of the manuscript.

Abstract

The house fly, *Musca domestica*, is regarded as the principal insect vector of *Campylobacter* spp., one of the most prominent causes of bacterial-induced diarrheal disease worldwide. Little is known, however, regarding the immune response of *M. domestica* to ingested *Campylobacter* spp. We report here, a global immune response in the gastrointestinal tract of the house fly following the ingestion of *C. jejuni*. RNA isolated from adult houseflies 4, 8, 12 and 24 hours after ingestion of *C. jejuni* was used in suppression subtractive hybridization to identify pathogen-induced gene expression in these flies. In addition, we determined the number of viable *C. jejuni* in the flies at the different time points. Three hundred and eighty-six clones were sequenced from the subtracted library and a functional annotation identified a broad array of genes, which included genes involved in immunity, proteolysis, transcription and translation. Because no viable bacteria were observed > 24 hours post-ingestion we propose that *M. domestica* serves as a mechanical vector rather than as a true biological amplifying vector.

Introduction

Campylobacteriosis is a severe gastroenteric human disease caused by bacteria of the genus *Campylobacter*, and is one of the most common foodborne infections. In the European Union (EU), > 220,000 cases were confirmed in 2011 (EFSA 2013), but estimates indicate that only 2.1% of all cases are reported; the true incidence of campylobacteriosis is approximately nine million cases per year (EFSA 2011). In the United States alone, up to $8.0 billion in human illness costs are spent annually on *Campylobacter* spp. infections and associated sequelae (Buzby et al. 1997).

The primary source of *Campylobacter jejuni* in human infections is poultry meat contaminated by the contents of the alimentary tract during processing (EFSA 2011, WHO 2013). Contamination of poultry facilities primarily occurs through horizontal transmission from external sources (van de Giessen et al. 1992, Jacobs-Reitsma et al. 1995) and numerous studies have implicated the house fly, *Musca domestica,* in the transmission of *C. jejuni* into, and within, poultry facilities (Szalanski et al. 2004, Hald et al. 2008). Physical interventions using screens to prevent flies from entering poultry facilities can substantially reduce the prevalence of *Campylobacter* spp. in broiler chickens (Hald et al. 2007, Bahrndorff et al. 2013).

Recent studies investigating the vector competence of *M. domestica* for *C. jejuni* demonstrate that *C. jejuni* in house fly larvae survive in large numbers through moults into pupae, suggesting that infected larvae may play a role in transmission via ingestion by poultry (Bahrndorff et al. 2014). *Campylobacter jejuni,* however, is not detectable in adult house flies following eclosion and other reports indicate that *C. jejuni* ingested by adult house flies does not survive for periods > 24 hours (Skovgård et al. 2011). Whether this is due to the bacteria being digested or whether ingested *C. jejuni* elicit an immune response by adult house flies is unknown.

Insects possess an effective innate immune response that recognizes conserved pathogen-associated molecular patterns (PAMPs) located on the surface of microbes with pattern recognition receptors (PRRs; Cooper and Mitchell-Foster 2011). This

recognition activates components of the humoral and cellular immune responses (Leclerc and Reichhart 2004) initiating phagocytosis and the expression of reactive oxygen intermediates (Carton and Nappi 2001, Christensen et al. 2005, Kocks et al. 2005), encapsulation (Lemaitre and Hoffmann 2007), melanisation (Bidla et al. 2005, Christensen et al. 2005), or the expression of several antimicrobial peptides (AMPs) that target and kill bacteria, fungi, and parasites (Bulet et al. 1999, Lowenberger et al. 1999a, Lowenberger et al. 1999b, Lowenberger 2001). Some AMPs are strictly immune-related while others may serve as both immune molecules and digestive enzymes (Lopez et al. 2003, Boulanger et al. 2004, Boulanger et al. 2006, Ursic-Bedoya and Lowenberger 2007, Ursic-Bedoya et al. 2011). Whereas many immune-related AMPs are expressed in the hemocoel of insects (Lowenberger et al. 1995, Lowenberger 2001), several isoforms of these AMPs also are expressed in the insect digestive tract to prevent the over proliferation of non-desirable symbionts (Lowenberger et al. 1995, Ursic-Bedoya and Lowenberger 2007, Ursic-Bedoya et al. 2011).

Studies on the molecular interactions between bacteria and house flies are scarce. There are reports that cecropin is absent in naïve larvae and adults (Liang et al. 2006), that attacin is absent in larvae (Dang et al. 2010) and that defensin expression is not detectable in larvae or adults (Wang et al. 2006). Our data, using real time quantitative PCR, demonstrate that cecropin, defensin, attacin, diptericin, and lysozyme are detectable in all developmental stages of *M. domestica*, but differed in the level and timing of expression of each gene (Bahrndorff et al. 2014). No studies to date have investigated whether ingested *C. jejuni* activates the immune response of the house fly. We used suppression subtractive hybridization (SSH) to identify and compare a set of differentially expressed genes in the transcriptome of the gastrointestinal tract of adult *M. domestica* in response to ingestion of *C. jejuni*. In addition, we determined the duration and viability of *C. jejuni* after ingestion by adult *M. domestica*.

Material and Methods

Insect Colony Maintenance

A *Musca domestica* colony, originally purchased from Beneficial Insectary Inc. (Redding, CA, USA), has been maintained in the insectary at Simon Fraser University since 2012. Adult individuals were reared in the laboratory at 25ºC with a photoperiod of 16:8 (Light:Dark) hours. The flies fed on milk powder, sugar, and tap water.

Experimental Protocol

Preparation of Campylobacter jejuni strain

We used a green fluorescent protein (GFP) labelled *C. jejuni* strain (Miller et al. 2000) obtained from the National Food Institute DTU (Technical University of Denmark, Mørkøj, Denmark) to infect house flies. Bacterial cultures were reconstituted from brain-hearth-infusion 20% glycerol stocks and grown overnight on blood-agar plates incubated at 42ºC in a micro-aerobic atmosphere generated using GasPak^{TM} EZ Campy Container System Sachets (BD, Sparks, MD, USA) in a Brewer's jar. Subsequently, bacteria were collected from the plates and re-suspended in sterile 0.9% NaCl at an optical density (OD) of 0.8 at 620 nm (approximately 10^9 colony forming units; CFU/ml) and the presence of *C. jejuni* was confirmed using fluorescent microscopy. Suspensions were kept on wet ice throughout the experiments.

Exposure of adult flies to C. jejuni

The protocol to expose adult house flies to *C. jejuni* was modified after Skovgård et al. (2011). Briefly, 5 day ± 24 h old male flies that had been starved overnight were anesthetized with CO_2 and subsequently fixed individually inside a sterile pipette tip that allowed the head and proboscis to protrude. A pipette tip containing 1 µl of the *C. jejuni* suspension was presented as a drop to each fly, and the solution was ingested entirely. Flies that declined or stopped feeding were removed from the study. After ingestion, each individual fly was released into a sterile 50 ml Falcon tube with access *ad libitum* to an 8% sugar solution for the remainder of the study. The control flies were subject to the

same exposure protocols, but ingested 1 µl of sterile 0.9% saline solution containing no bacteria.

Campylobacter jejuni CFU determinations

Ten adult flies (5 replicates of two individual flies per replicate) were collected at 4, 8, 12, and 24 hours following *C. jejuni* or saline exposure to estimate numbers of bacteria. Individual flies were weighed, 0.9% saline solution (1:10 w/v) was added, and flies were homogenized using a mortar and pestle. The supernatant was further diluted in a 10-fold dilution series in 0.9% saline solution. Aliquots (20 µl) of each dilution were plated onto *Campylobacter* spp. selective modified charcoal-cefoperazone-deoxycholate agar (mCCDA) plates. The plates were incubated microaerobically at 42°C for 48 hours before the *C. jejuni* colonies on the plates were counted and the number of bacteria per sample estimated.

Dissections

The gastrointestinal (GI) tracts including the intestine, malpighian tubules, salivary glands and crop were dissected from 15 infected and control adult flies at 4, 8, 12, and 24 hours following ingestion of bacteria and stored at -80^{o}C.

Total RNA and mRNA Isolation

Total RNA extraction from the GI tracts was performed using the TRI Reagent® RNA Isolation Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's specifications. Total RNA was quantified using a NanoDrop 2000C (Thermo Fisher Scientific, Wilmington, DE, USA). mRNA was isolated using Purist poly-A micro-spin columns (Ambion, Austin, TX, USA) according to the manufacturer's protocols: 0.5 µg of poly-A RNA from each time point was pooled (total of 2 µg) separately for positive (infected) and negative (non-infected) samples and used to construct the subtracted library.

Subtractive Library Construction

We generated a subtractive library from pooled mRNA samples using the PCR-select cDNA Subtraction Kit according to the manufacture's recommendations (Clontech, Palo Alto, CA, USA) and as described previously (Baron et al. 2010). Briefly, specific adaptors were ligated to both ends of the cDNAs, followed by subtractive hybridization, and lastly by PCR amplification with specific adaptor primers. Amplification of hybrids corresponding to common sequences is suppressed, yielding a library enriched for differentially expressed sequences in the *C. jejuni*-exposed house flies.

Subtractive Efficiency Analysis

The efficiency of the subtraction process was estimated using PCR by comparing the abundance of the constitutively expressed gene, GAPDH, before and after subtraction. The primers for this gene are described in Table 2-1 and were used in standard PCR reactions under the following conditions: 94^{o}C for 1 min, followed by 33 cycles of 94^{o}C for 10 s, 60^{o}C for 10 s and 72^{o}C for 30 s. Five microliter aliquots were removed from each reaction after 18, 23, 28 and 33 cycles, examined by electrophoresis on a 2% agarose gel, and stained with Gel-Red to evaluate subtraction success.

Cloning, Plasmid Isolation, DNA Sequencing and Database Search

An aliquot (4 µl) of the secondary PCR product from the subtracted library was ligated overnight at 4ºC into pGEM-T Easy plasmid vector (Promega, Madison, WI, USA), transformed by heat shock into *Escherichia coli* JM109 ultra competent cells (Promega, Madison, WI, USA), plated onto LB plates supplemented with 100 µg/ml carbenicillin, 80 µg/ml Xgal, and 0.5 mM IPTG, and incubated overnight at 37ºC as described (Ursic-Bedoya and Lowenberger 2007). Individual white colonies were screened using PCR to confirm the presence of an insert; colonies were resuspended individually in 10 µl ddH_2O and 1 µl of this solution was used in a standard PCR reaction containing SP6 and T7 primers that flank the multiple cloning site in the vector. The conditions used were 96^{o}C for 2 min, followed by 40 cycles of 96^{o}C for 10 s, 50^{o}C for 10 s, and 72^{o}C for 30 s. PCR products were size fractionated on 1% agarose gels and

stained with Gel-Red to confirm the presence of an insert. Positive colonies were grown overnight in 5 ml of LB medium with 5 µl carbenicillin (100 µg/µl). Glycerol stocks of the overnight cultures (100 µl) were aliquoted into 96-well plates and stored at -80ºC. Plates were sent to the Genome Sciences Centre, BC Cancer Agency (Vancouver, Canada), where colonies were grown, processed and sequenced.

Analysis of the sequence data, detection of open reading frames and sequence alignment, were performed using DNAstar modules Seqman, MegAlign, Editseq (DNAstar, Madison, WI, USA), and Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Database search was performed using BLAST-X and BLAST-N against non-redundant databases at NCBI with default parameters. The best annotated BLAST-X match from the similarity search was retained and BLAST-N matches were only used when a BLAST-X search resulted in no sequence similarity with an Expect (E) value less than 0.1. For the functional prediction of ESTs (expressed sequence tags) found in the database we used the official Gene Ontology browser and search engine, AmiGO (Ashburner et al. 2000, Carbon et al. 2009). We used the BLAST Search annotation tool (BLASTX and/or BLASTP) of AmiGO to cluster the ESTs based on the biological process annotation when available. Novel ESTs were submitted to dbEST at the National Center for Biotechnology Information (NCBI) and assigned accession nos. 78910768-78911127 (Gene-Bank accession JZ545987-JZ546346).

Validation of Differential Expression by Real Time Quantitative PCR (qPCR)

We used qPCR to confirm differential expression of selected genes identified through the SSH process in the GI tracts of *M. domestica* at different times post-ingestion of *C. jejuni*. We designed specific primers based on the EST sequences (Table 2-1). We amplified the target sequences in standard PCR conditions, electrophoresed the samples on 1% agarose gels, excised the bands, purified the DNA (QIAquick Gel Extraction Kit, Qiagen, Netherlands), and sequenced each amplicon using Big-Dye chemistry (Life Technologies, Carlsbad, CA, USA) to confirm we had amplified the correct gene, and subsequently, the efficiency of amplification of each primer pair was determined as described (Livak and Schmittgen 2001, Schmittgen and Livak 2008).

We used these primers in standard qPCR conditions to confirm differential expression. All qPCR reactions were performed with a Rotor-Gene 3000 (Corbett Research, Australia) using the PerfeCTa SYBR Green SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). We used 1 µl cDNA with 6.25 µl of SYBR Green SuperMix, 0.5 µl (25 ng) of each primer in 13 µl reactions under the following conditions: 95^{o}C for 2 min, followed by 35 cycles of 95^{o}C for 10 s, 55^{o}C or 60^{o}C for 15 s, and 72^{o}C for 30 s. Quantity values were calculated as $2^{(-\Delta\Delta C_T)}$ based on C_T values (Livak and Schmittgen 2001) and using GAPDH and β-actin as reference genes for normalization. All data represent duplicate runs of independently generated cDNAs.

Results

Temporal Decline in *Campylobacter jejuni* Following Ingestion

The *C. jejuni* suspension used to infect the house flies was estimated to contain $2.1x10^9$ CFU/ml. All flies sampled at 4 and 8 hours after exposure were positive for *C. jejuni*, with a mean CFU/ml of $1.90x10^6$ and $5.08x10^5$, respectively (Figure 2-1). At 12 hours post-exposure colonies were observed at dilutions up to 10^{-3} and at 24 hours at 10^{-1}, but individual colonies were not countable. All flies used as negative controls were negative for *C. jejuni*.

GI-Tract Subtracted Library in Response to Bacterial Ingestion

We sequenced 386 independent clones from the *M. domestica* GI-tract subtracted library in response to *C. jejuni* ingestion**.** After sequencing, we excluded from our analysis 26 clones (6.7%) that either had inserts < 60 bp in length or which had poor quality sequence. In total, 109 clones (28.2%) corresponded to unique EST sequences (Table 2-2). NCBI database searches using BLAST-X and BLAST-N resulted in 16 clones with no significant match, and 7 to hypothetical, uncharacterized, proteins deduced *in silico* from genome sequencing and annotation projects. Forty six (42.2%) of the putative genes had more than one copy, and 27 (24.8%) of these were highly redundant (more than 3 copies). We included these redundant clones in our functional

analysis (Figure 2-2) as this may reflect the importance of these genes in the physiology and immunity in the GI tract of *M. domestica* in response to *C. jejuni* ingestion.

All identified genes were clustered into functional groups according to their putative biological function as binding, cytoskeleton, defense (immunity), metabolism, mitochondrial, protease inhibitor, protease/proteolysis, ribosomal, transcriptional/ translational control, transport and other (Figure 2-2). The majority of genes were found in the protease/proteolysis (15.0%), transport (14.4%), and metabolism (10.0%) groups, with 6.9% of genes found in the defense (immunity) group. Some housekeeping genes (ribosomal, mitochondrial: 10.3% and 1.9%, respectively), whose amplification is normally repressed, were also found in the library as seen in other SSH studies (Ursic-Bedoya and Lowenberger 2007, Baron et al. 2010).

Several ESTs mapped to genes known to participate in different innate immune responses. These included a translation initiation factor 4E (eIF4E)-binding protein, JNK-like protein kinase, lectin, argonaute-2-like protein, lysosomal aspartic protease, and ovochymase-2-like protein. Due to their putative role in response to *C. jejuni* and immunity in flies these ESTs were chosen for further exploration and quantification via qPCR.

Real Time Quantitative PCR (qPCR)

We used qPCR to confirm the differential expression of selected transcripts at the different time points in our *C. jejuni*-exposed house flies. All six selected ESTs were up-regulated at one or more of the four time points in the *C. jejuni*-exposed group compared to the control (Figure 2-3). The *ovochymase-2*-like protein and the *JNK*-like protein kinase were up-regulated at all time points, the *eIF4E*-like and *argonaute-2*-like protein at three time points, whereas the *lectin* subunit was up-regulated at two time points. The *lysosomal aspartic protease* was the only EST up-regulated at a single time point. These data suggest that the temporal expression of these immune-related proteins is different.

Discussion

To date, no studies have investigated the effect of *Campylobacter* spp. ingestion on the immune system of its vectors. We used SSH to identify novel ESTs up-regulated in response to the ingestion of *C. jejuni*. Our data demonstrate the up-regulation of several immune-responsive genes using EST classifications described in other SSH studies (Figure 2-2; Ursic-Bedoya and Lowenberger 2007, Baron et al. 2010). qPCR confirmed a moderate up-regulation of these genes in the *C. jejuni*-exposed house flies (Figure 2-3), possibly due to the means by which we exposed flies to the bacteria. We exposed flies orally to ecologically-relevant doses, which is different from other similar studies where bacteria were injected into the insect (Wang et al. 2006, Ursic-Bedoya and Lowenberger 2007). Injection into the hemocoel does not reflect natural conditions, but often is used to activate immune genes to the highest level for identification (Liehl et al. 2006). The activation of immune genes in the midgut of most insects is much less than in the hemocoel (fat body), and in many insects is down-regulated to ensure that essential microbial symbionts are not eliminated (Lopez et al. 2003, Ursic-Bedoya et al. 2011).

We estimated the bacterial load (CFU/ml) in the house flies at different time points post-exposure. The results clearly show a significant reduction in bacterial numbers within the first 8 hours and no countable colonies were present 12-24 hours after exposure (Figure 2-1) as seen in previous studies (Skovgård et al. 2011). *Campylobacter* spp. may change morphology (become coccoidal) and exhibit poor growth when stressed (Mihaljevic et al. 2007, Cameron et al. 2012). We used serial dilutions of whole fly homogenates and it is possible that some component from the flies affected bacterial growth, although this has not been reported previously.

We describe here, in more detail, the identification of six ESTs that map to genes with immune-related functions and which could play a role in the immune response of *M. domestica* in clearing *C. jejuni*. Further studies are underway to evaluate these molecules in more detail.

eIF4E-binding protein (thor): We identified an EST that matched a predicted eukaryotic translation initiation factor 4E (eIF4E)-binding protein of *M. domestica* with

high homology to Thor of *Glossina morsitans* (Tsetse fly) and *Drosophila melanogaster* (common fruit fly). Thor is a member of the 4E-binding protein (4E-BP) family. Mammalian 4E-BPs have been defined as critical regulators in the pathway that controls initiation of translation, binding and sequestering the eukaryotic initiation factor 4E (Preiss and Hentze 1999). When 4E-BP is bound to eIF4E, eIF4E cannot bind appropriately to form the translation initiation complex. In *D. melanogaster*, Thor participates in host immune defense and is up-regulated in response to wounding and infection with bacteria or fungi (Bernal and Kimbrell 2000, Levitin et al. 2007). In addition, Thor-deficient strains of *D. melanogaster* are severely immunocompromised. The suggested role of Thor is either for translational regulation in humoral immunity or a new non-translational function (Bernal and Kimbrell 2000).

JNK: The c-Jun N-terminal kinase (JNK) is a group of signal transducing, mitogen-activated, protein kinases (MAPKs), which have been implicated as components of the insect innate immune system that is activated in response to bacterial infection (Sluss et al. 1996, Mizutani et al. 2003a, Mizutani et al. 2003b, Wojda et al. 2004). Specifically, JNK activation occurs in response to lipopolysaccharide (LPS) stimulation (Sluss et al. 1996), which is the principal cell wall component on Gram-negative bacteria (Boutros et al. 2002), such as *C. jejuni*. JNK activation by bacteria binding to the insect cell surface is followed by the release of antibacterial peptides (Mizutani et al. 2003a, Mizutani et al. 2003b) and detectable antibacterial activity (Wojda et al. 2004).

Cathepsin D-like (lysosomal aspartic protease): House flies possess an acidic midgut (pH 3.0-3.2) (Vonk and Western 1984) and have Cathepsin D-like proteinases active only in this region (Lemos and Terra 1991, Padilha et al. 2009). Cathepsin D is the major aspartic protease of the lysosomal compartment and functions primarily in the intracellular degradation of proteins (Tang and Wong 1987), cleaving peptide bonds flanked by bulky hydrophobic amino acids under acidic conditions (Marchler-Bauer et al. 2013). We identified an EST that mapped to a preprocathepsin D-like protease previously identified in *M. domestica* (Padilha et al. 2009). These proteases have been suggested to play an extracellular role in an acidic midgut to deal with bacteria-rich food (Padilha et al. 2009). Cathepsin D has also been described as an important regulator of

innate immunity in humans (Conus et al. 2008), catfish (Feng et al. 2011), and fruit flies (Loseva and Engstrom 2004) after bacterial infection. In addition, Cathepsin D expression in the midgut of the kissing bug, *Rhodnius prolixus*, is up-regulated upon ingestion of a blood meal containing the parasite, *Trypanosoma cruzi*, compared with expression levels in uninfected insects (Borges et al. 2006).

Serine proteases (ovochymase-2-like protein): Serine proteases regulate several invertebrate defense responses, including hemolymph coagulation, antimicrobial peptide synthesis, and melanisation of pathogen surfaces (Gorman and Paskewitz 2001). We identified an EST, belonging to the trypsin-like serine protease super family, that matched a predicted ovochymase-2-like protein of *M. domestica* with high homology to a predicted alpha-like trypsin in *Ceratitis capitata* (Mediterranean fruit fly) and Spheroide in *D. melanogaster.* This gene was up-regulated at all time points after infection. Spheroide is involved in the Toll immune signalling pathway and is proposed to be one of the serine proteases that activates Spaetzle (Kambris et al. 2006). Inactivation of Spheroide produces an immune-deficient phenotype similar to Toll pathway mutants (Kambris et al. 2006).

Lectin C-type: Lectins, together with other pattern recognition receptors, play an important role in the insect innate immune system (Yu and Kanost 2000). C-type lectins (CTLs) share a carbohydrate recognition domain and may act as receptors in pathogen recognition. In mosquitoes, some CTLs are required for the clearance of Gram-negative bacteria, but not Gram-positive bacteria, and RNA interference (RNAi) silencing of CTLs reduces mosquito survival only following infection with Gram-negative bacteria (Schnitger et al. 2009). Several CTLs that bind to different bacteria have been discovered in *D. melanogaster* (Tanji et al. 2006). We identified an EST that matched a putative lectin alpha-subunit from *M. domestica* with high homology to a lectin subunit from the salivary glands of *Lucilia sericata* (Green bottle fly) (Andersen et al. 2010). Whether this EST acts as a receptor for pathogen recognition remains to be fully tested.

Argonaute: The RNAi pathway is one of the major pathways that invertebrates utilize for antiviral defense (van Rij and Berezikov 2009). A key step in this pathway is the interplay between viral small interfering RNAs and Argonaute-2, a core catalytic

component of the RNA-induced silencing complex (RISC; van Mierlo et al. 2012). We identified an EST that matched a predicted Argonaute-2-like isoform of *M. domestica* with high homology to Argonaute-2 of *D. melanogaster*. Although this is commonly described as an antiviral response, bacteria have been shown to activate the RNAi pathway in *D. melanogaster* (Teixeira et al. 2008, Osborne et al. 2009). Whether this is due to direct involvement in antibacterial responses or due to the crosstalk among different immune pathways is unknown (Boutros et al. 2002, Cooper 2008).

Our results indicate the activation of several immune-related genes in adult house flies in response to *C. jejuni* ingestion. The proteins and molecules these genes encode, as well as other digestive enzymes, likely play a significant role in clearing the bacteria, as no viable *C. jejuni* were observed > 24 hours post-ingestion. Thus, we propose that *M. domestica* serves as a mechanical vector rather than as a true biological amplifying vector of *C. jejuni*, and future studies should aim to elucidate and characterize this vector-pathogen relationship.

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Figures

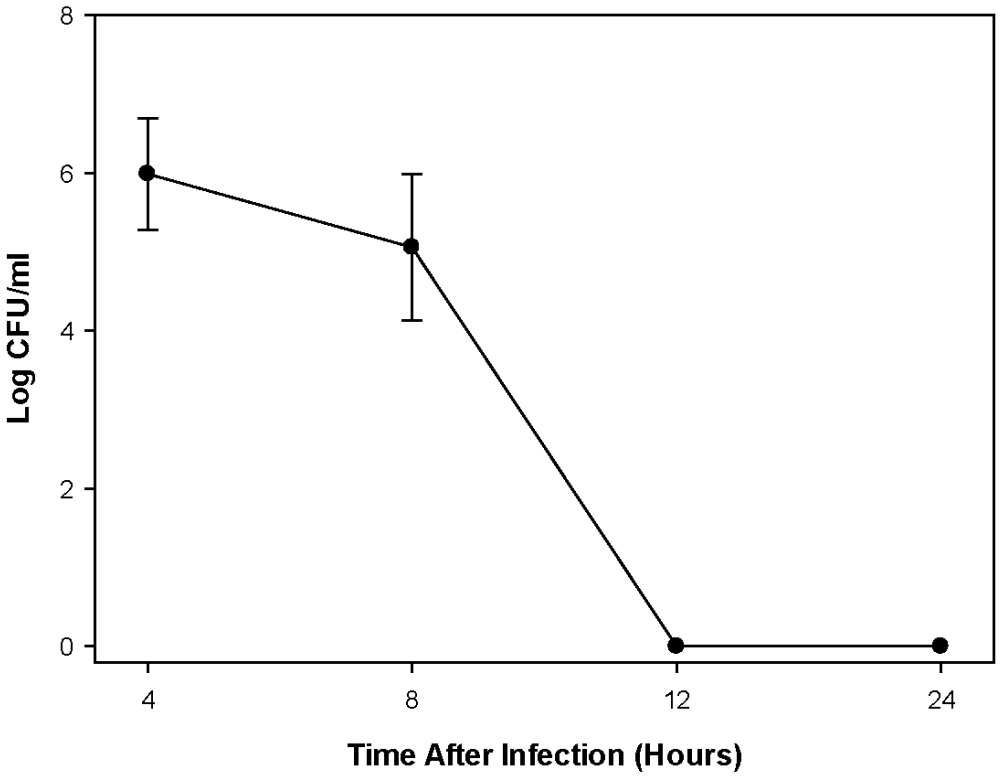

Figure 2-1 Retention of *Campylobacter jejuni* in adult house flies.
Number of *Campylobacter jejuni* enumerated from the whole bodies of *Musca domestica* at different time points following oral ingestion. All insects were raised and maintained at 25^{o}C. The stock solution used for infection was estimated to contain 2.10x10^{9} CFU/ml *C. jejuni*. All data points represent five replicates of two individual flies per replicate. Errors bars represent SD.

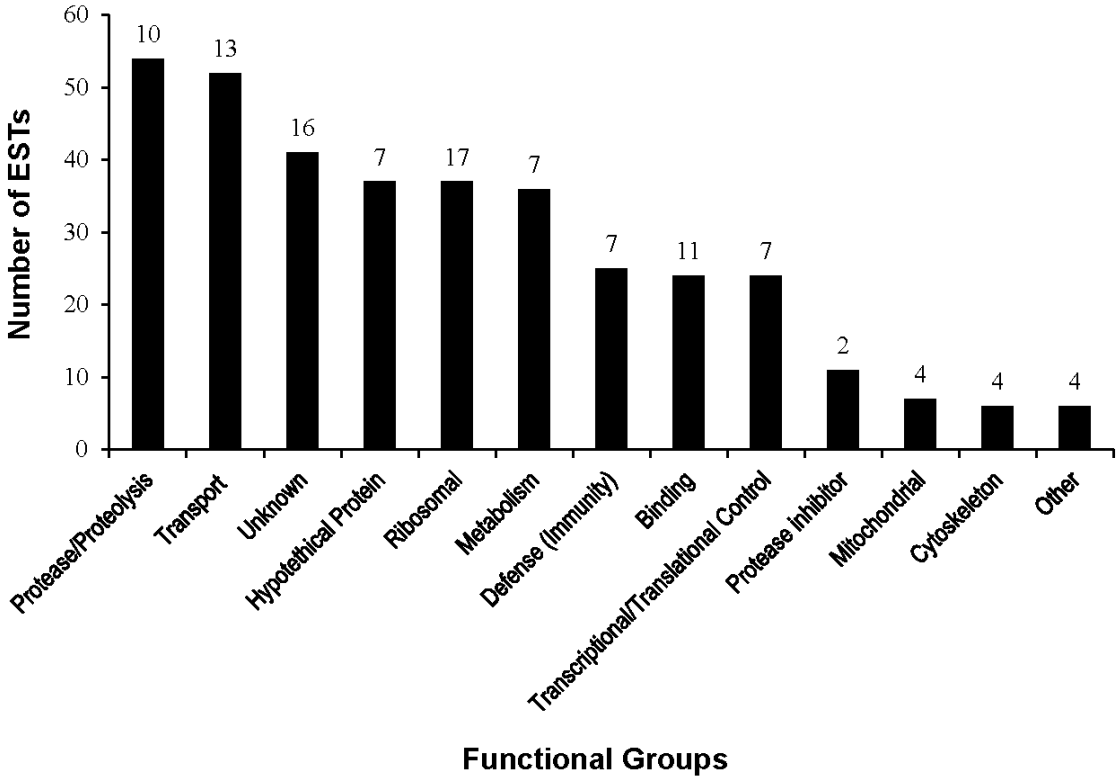

Figure 2-2 Functional prediction and classification of generated ESTs of *Musca domestica* in response to ingestion of *Campylobacter jejuni*.

Functional prediction and classification of the generated ESTs based on gene ontology using AmiGO. Number of ESTs includes redundant clones. Numeric superscripts represent the number of independent, unique, ESTs (109 total) excluding redundant clones. Novel ESTs were submitted to dbEST at the National Center for Biotechnology Information (NCBI) and assigned accession nos. 78910768-78911127 (Gene-Bank accession JZ545987-JZ546346).

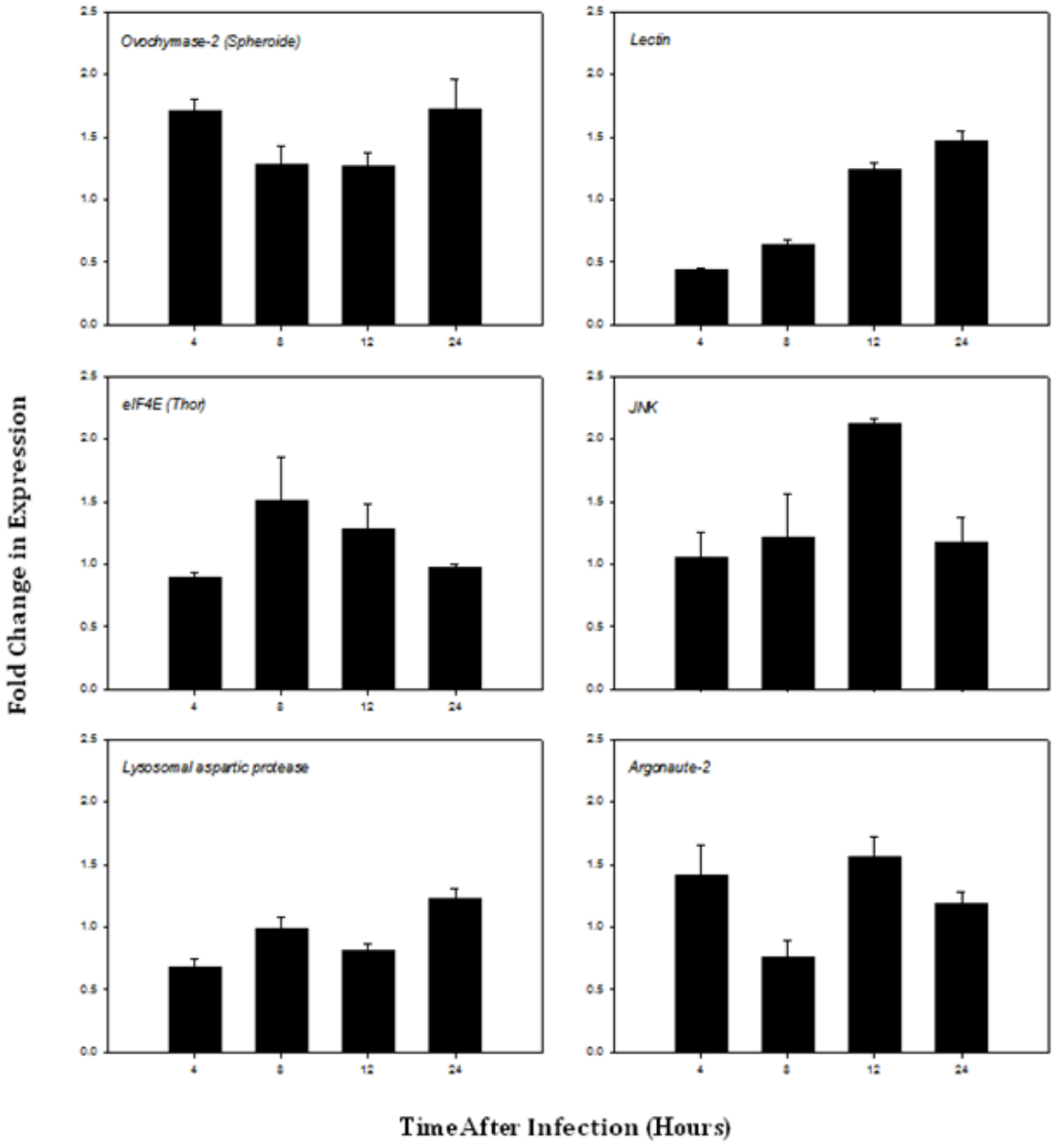

Figure 2-3 Temporal expression of select immune-related ESTs in adult *Musca domestica* in response to ingestion of *Campylobacter jejuni*

Expression patterns of six differentially expressed ESTs obtained from the GI-tracts of *Campylobacter jejuni*–exposed *Musca domestica*. The expression levels of each gene were measured by qPCR at four time points following infection. Expression levels were normalized with GAPDH. The expression levels in control (naïve) flies were selected as the calibrator and arbitrarily given a value of 1 for each time point. The vertical axis represents the fold change in expression in infected flies compared with control flies. The bars represent duplicate runs of independently generated cDNAs. Error bars indicate SD.

Tables

Table 2-1 Primers of select ESTs and housekeeping genes used for real time quantitative PCR.

Gene	Primer sequence (5' → 3')	Temp* (°C)	Amplicon size (bp)
GAPDH			
F	ACA ACG AAT TCG GTT ACT CC	52.6	219
R	CCT GTC TGA TGA TGT GCG	53.3	
β-actin			
F	GGT GTC ATG GGT TGG TAT GGG AC	59.8	225
R	ACG ATT AGC CTT GGG ATT CAA TGG G	59.2	
lectin			
F	GTG TGG CAA CGA ATT ATC G	51.8	168
R	TCA AAC AAT CTC GAT CAT TCC	50.5	
eIF4E (Thor)			
F	ATT CCG CAA GTG TGT GCC	56.3	134
R	GGA GGA AGC ACG GTG TAT AG	54.8	
ovochymase-2 (Spheroide)			
F	GCA AGA ATG CTT ACA GTG CC	54.6	214
R	ACT ACA ACA GAT TGA ATC CAG G	52.4	
JNK			
F	AGC TAC ATT CAT GTT TGG TAT GAC	53.2	163
R	CAT TGG TAT TAT TAC TGG TAT GGG	51.1	
lysosomal aspartic protease			
F	CTC TTC GAT AGT GGA TCT TCC	51.9	193
R	CAA CGG TTA CGG TAT CTT GC	53.7	
argonaute-2			
F	AAG ACG CTG CTC GTA TTC AC	55.1	280
R	CTA TAA CGT TAT CAT GGA CGA GTC	52.7	

*Melting temperature

Table 2-2 ESTs identified in the gastrointestinal tract of *Musca domestica* that had ingested *Campylobacter jejuni.*

ESTs classified based on BLAST-X or BLAST-N analysis against non-redundant database at NCBI.
NSM: No significant match.

Clone	NCBI gi	Amplicon size (bp)	No. Redundant clones	Blast-X Match (*Indicates Blast-N Match only)	Accession	*E*	Putative gene function
2A07	557778212	489	1	*M. domestica* lectin subunit, alpha-like	JZ546075	2.00E-88	Binding
2F09	557774616	318	3	*M. domestica* troponin C, isoform 3	JZ546135	1.00E-60	Binding
2G09	557763088	255	1	*M. domestica* E3 ubiquitin-protein	JZ546146	1.00E-39	Binding
3H08	557764361	295	2	*M. domestica* coactosin-like protein	JZ546244	1.00E-63	Binding
4F05	557784788	515	1	*M. domestica* mucin-2-like	JZ546313	3.00E-13	Binding
4H10	557766774	322	1	*M. domestica* mucin-2-like	JZ546341	2.00E-28	Binding
4G10	557771500	457	1	*M. domestica* metallothionein-1-like mRNA*	JZ546330	2.00E-119	Binding
4F11	557771498	437	2	*M. domestica* metallothionein-1-like mRNA*	JZ546319	2.00E-163	Binding
1E07	557781898	180	10	*M. domestica* RNA-binding protein 42 mRNA*	JZ546030	4.00E-52	Binding
3B02	442626157	135	1	*D. melanogaster* Msp-300, transcript variant H, mRNA*	JZ546173	6.00E-05	Binding
3E08	557769495	258	1	*M. domestica* protein GDAP2 transcript variant X5, mRNA*	JZ546214	4.00E-114	Binding
4C07	557769697	147	1	*M. domestica* senecionine N-oxygenase-like, mRNA*	JZ546279	2.00E-49	Catalysis
1F03	557756399	421	1	*M. domestica* tubulin-specific chaperone cofactor E-like protein	JZ546038	7.00E-92	Cytoskeleton
1F06	557763696	153	1	*M. domestica* Ras-related protein, Rab-1A-like	JZ546040	3.00E-26	Cytoskeleton
2A05	557784471	475	3	*M. domestica* dystonin-like isoform X14	JZ546073	3.00E-57	Cytoskeleton

2B04	557752681	577	1	*M. domestica* elongation of fatty acids protein 7-like isoform X1	JZ546084	8.00E-23	Cytoskeleton
4C08	557768127	246	1	*M. domestica* ornithine decarboxylase antizyme-like	JZ546280	8.00E-24	Decarboxylase inhibitor
1A03	557765093	330	1	*M. domestica* uncharacterized protein; Edin [*Drosophila*]	JZ545988	1.00E-24	Defense (Immunity)
1F05	557784371	685	4	*M. domestica* ovochymase-2-like; Spheroide [*Drosophila*]	JZ546039	1.00E-113	Defense (Immunity)
4B07	557783583	293	1	*M. domestica* stress-activated protein kinase, JNK-like	JZ546267	2.00E-29	Defense (Immunity)
4A08	557754873	305	1	*M. domestica* protein argonaute-2-like isoform X2	JZ546256	2.00E-56	Defense (Immunity)
1A11	557782126	274	1	*M. domestica* carboxypeptidase Y-like isoform X1	JZ545993	2.00E-16	Defense (Immunity)
1B02	557782128	272	16	*M. domestica* carboxypeptidase Y-like isoform X2	JZ545996	3.00E-29	Defense (Immunity)
1G01	557776381	226	1	*M. domestica* eukaryotic translation initiation factor 4E-binding protein 2; Thor [*Drosophila*]	JZ546046	9.00E-15	Defense (Immunity)
1G07	557752144	391	2	*M. domestica* adult cuticle protein 1-like	JZ546052	3.00E-09	Development
1A12	557769933	172	19	*M. domestica* uncharacterized protein	JZ545994	8.00E-32	Hypothetical protein
2B09	557760330	198	5	*M. domestica* uncharacterized protein	JZ546089	4.00E-17	Hypothetical protein
1E03	557765191	92	6	*M. domestica* uncharacterized protein	JZ546027	6.00E-11	Hypothetical protein

1G10	557767662	235	3	*M. domestica* uncharacterized protein	JZ546055	1.00E-38	Hypothetical protein
1H05	557754929	114	1	*M. domestica* uncharacterized protein	JZ546062	2.00E-18	Hypothetical protein
4A07	557767979	167	2	*M. domestica* uncharacterized protein	JZ546255	6.00E-10	Hypothetical protein
4C04	430802840	208	1	*U. yaschenkoi* hypothetical protein 11	JZ546276	8.00E-06	Hypothetical protein
1B03	557751638	200	24	*M. domestica* aldehyde dehydrogenase	JZ545997	9.00E-27	Metabolism
1H02	557756861	248	1	*M. domestica* dehydrogenase/ reductase SDR family 11-like	JZ546059	1.00E-50	Metabolism
2A10	557779158	290	1	*M. domestica* uncharacterized oxidoreductase like isoform X1	JZ546078	4.00E-51	Metabolism
2C08	557778609	614	1	*M. domestica* aldose 1-epimerase	JZ546100	5.00E-100	Metabolism
2C11	557759992	355	4	*M. domestica* Acyl-CoA desaturase-like	JZ546102	7.00E-79	Metabolism
2D03	557753208	382	4	*M. domestica* cytochrome P450 4g1-like	JZ546106	2.00E-44	Metabolism
2G02	557768372	302	1	*M. domestica* molybdenum cofactor sulfurase 3-like	JZ546140	3.00E-60	Metabolism
2H07	557763125	228	4	*M. domestica* cytochrome b-c1 complex subunit 7-like, mRNA*	JZ546155	9.00E-100	Mitochondrial
1C12	557758892	570	1	*M. domestica* dynamin-1-like protein-like	JZ546014	1.00E-37	Mitochondrial
2H02	557750146	166	1	*M. domestica* cytochrome b-c1 complex subunit 9-like	JZ546151	1.00E-24	Mitochondrial

3G11	557770554	476	1	*M. domestica* cytochrome c oxidase subunit 6B2-like	JZ546235	3.00E-18	Mitochondrial
4C11	557775536	356	2	*M. domestica* multiple inositol polyphosphate phosphatase 1	JZ546283	1.00E-78	Phosphatase
1B04	557765971	183	9	*M. domestica* male accessory gland serine protease inhibitor	JZ545998	1.00E-09	Protease inhibitor
1G06	557760227	235	2	*M. domestica* leukocyte elastase inhibitor-like isoform X2	JZ546051	2.00E-12	Protease inhibitor
1A01	557767471	323	5	*M. domestica* serine proteases 1/2-like	JZ545987	6.00E-70	Protease/ Proteolysis
4G02	557767471	349	15	*M. domestica* serine proteases 1/2-like	JZ546322	4.00E-47	Protease/ Proteolysis
1D02	557777994	177	17	*M. domestica* uncharacterized protein	JZ546016	2.00E-32	Protease/ Proteolysis
1H01	557776111	192	5	*M. domestica* trypsin alpha-like	JZ546058	9.00E-36	Protease/ Proteolysis
4E05	557774956	601	5	*M. domestica* lysosomal aspartic protease-like	JZ546301	3.00E-141	Protease/ Proteolysis
2C07	557784742	311	3	*M. domestica* proteasome subunit beta type-4-like	JZ546099	7.00E-69	Protease/ Proteolysis
2H10	557765201	211	1	*M. domestica* zinc metalloproteinase nas-13-like	JZ546158	1.00E-32	Protease/ Proteolysis
3A07	557776113	401	1	*M. domestica* trypsin alpha-4-like	JZ546166	2.00E-42	Protease/ Proteolysis
4H06	557764066	674	1	*M. domestica* lectizyme-like	JZ546337	2.00E-142	Protease/ Proteolysis
3E10	557780306	280	1	*M. domestica* signal peptidase complex subunit 3-like	JZ546216	2.00E-54	Protease/ Proteolysis
1E02	557783118	263	1	*M. domestica* 60S ribosomal protein L10-like	JZ546026	9.00E-54	Ribosomal

3C05	557783118	400	1	*M. domestica* 60S ribosomal protein L10-like	JZ546188	3.00E-43	Ribosomal
2G04	557767072	348	2	*M. domestica* 40S ribosomal protein S2-like	JZ546142	4.00E-43	Ribosomal
2H12	557767072	238	3	*M. domestica* 40S ribosomal protein S2-like	JZ546159	2.00E-40	Ribosomal
1F08	557777590	348	5	*M. domestica* 60S ribosomal protein L28-like	JZ546042	3.00E-39	Ribosomal
1G04	557751433	169	4	*M. domestica* 40S ribosomal protein S6-like	JZ546049	2.00E-28	Ribosomal
2G05	557762644	406	6	*M. domestica* 60S ribosomal protein L7a-like	JZ546143	5.00E-48	Ribosomal
2A01	557754865	414	6	*M. domestica* 60S ribosomal protein L26-like isoform X1	JZ546069	1.00E-30	Ribosomal
2F12	557765239	226	1	*M. domestica* 60S ribosomal protein L23a-like	JZ546138	6.00E-46	Ribosomal
2G10	557784489	491	1	*M. domestica* 60S ribosomal protein L8-like	JZ546147	4.00E-68	Ribosomal
3D12	557763890	539	1	*M. domestica* 60S ribosomal protein L3-like	JZ546206	2.00E-66	Ribosomal
3E05	557777195	400	1	*M. domestica* 60S ribosomal protein L13a-like	JZ546211	2.00E-50	Ribosomal
4D11	557769548	191	1	*M. domestica* 60S ribosomal protein L37a-like	JZ546295	7.00E-37	Ribosomal
4E10	557776960	324	1	*M. domestica* 60S ribosomal protein L5-like	JZ546306	9.00E-69	Ribosomal
4F07	557755280	627	1	*M. domestica* 60S acidic ribosomal protein P2-like	JZ546315	3.00E-37	Ribosomal

4G09	334349312	351	1	*Monodelphis domestica* 40S ribosomal protein S5-like	JZ546329	8.00E-43	Ribosomal
4B05	557773489	65	1	*M. domestica* 60S acidic ribosomal protein P1-like, mRNA*	JZ546265	5.00E-16	Ribosomal
3A03	557756345	307	1	*M. domestica* histone H4-like, mRNA*	JZ546162	2.00E-153	Transcriptional/ Translational Control
1F01	557756739	396	2	*M. domestica* elongation factor 1-gamma-like	JZ546036	2.00E-56	Transcriptional/ Translational control
2A03	557777337	367	1	*M. domestica* mediator of RNA polymerase II transcription subunit	JZ546071	3.00E-28	Transcriptional/ Translational control
2A04	557755992	428	3	*M. domestica* heterogeneous nuclear ribonucleoprotein R-like isoform X10	JZ546072	6.00E-96	Transcriptional/ Translational control
2H06	557753139	244	1	*M. domestica* ATP-dependent RNA helicase WM6-like	JZ546154	4.00E-19	Transcriptional/ Translational control
3B09	557784529	667	1	*M. domestica* RNA polymerase-associated protein Rtf1-like	JZ546180	9.00E-83	Transcriptional/ Translational control
3F07	557765758	251	15	*M. domestica* elongation factor 2-like, mRNA*	JZ546223	6.00E-97	Transcriptional/ Translational control
3C11	557769904	177	1	*M. domestica* AQPcic-like, mRNA*	JZ546194	7.00E-30	Transport
1D11	513027246	135	6	*H. glaber* ADP-ribosylation factor 1 isoform X1	JZ546024	2.00E-15	Transport
1A09	557782888	404	1	*M. domestica* alpha-tocopherol transfer protein-like	JZ545991	3.00E-09	Transport
1C04	557766522	438	2	*M. domestica* AP-1 complex subunit sigma-2-like	JZ546009	1.00E-94	Transport
1D01	557750241	177	19	*M. domestica* V-type proton ATPase subunit C-like	JZ546015	1.00E-32	Transport

1D03	307695436	313	1	*H. armigera* V-ATPase B	JZ546017	3.00E-69	Transport
1G05	557780138	177	1	*M. domestica* synaptic vesicle glycoprotein 2B-like	JZ546050	2.00E-31	Transport
4G07	557780138	487	1	*M. domestica* synaptic vesicle glycoprotein 2B-like	JZ546327	4.00E-30	Transport
2A12	557752592	451	1	*M. domestica* sodium-independent sulfate anion transporter-like	JZ546080	2.00E-87	Transport
2C04	557756725	321	1	*M. domestica* V-type proton ATPase subunit E-like	JZ546096	4.00E-70	Transport
2C06	557765707	343	14	*M. domestica* V-type proton ATPase 116 kDa subunit a isoform 1-like	JZ546098	3.00E-46	Transport
2E07	557765707	418	3	*M. domestica* V-type proton ATPase 116 kDa subunit a isoform 1-like	JZ546121	1.00E-15	Transport
3D10	557771605	536	1	*M. domestica* facilitated trehalose transporter Tret1-like isoform X1	JZ546204	3.00E-09	Transport
1F12		426	3	NSM	JZ546045		Unknown
2B08		270	12	NSM	JZ546088		Unknown
1A08		403	2	NSM	JZ545990		Unknown
3F10		60	1	NSM	JZ546225		Unknown
4D06		80	1	NSM	JZ546290		Unknown
2G12		154	7	NSM	JZ546149		Unknown
4H07		232	1	NSM	JZ546338		Unknown
1C02		239	1	NSM	JZ546007		Unknown
1A10		246	5	NSM	JZ545992		Unknown
2F04		259	1	NSM	JZ546130		Unknown
4D02		268	1	NSM	JZ546286		Unknown
3D04		291	1	NSM	JZ546198		Unknown
3E06		275	1	NSM	JZ546212		Unknown
4G04		290	1	NSM	JZ546324		Unknown
3B01		551	1	NSM	JZ546172		Unknown
4F10		580	2	NSM	JZ546318		Unknown

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Connecting Statement 2

In Chapter Two, we used a functional genomics approach termed suppression subtractive hybridization (SSH) to identify expressed sequence tags (ESTs) up-regulated in response to immune challenge with *Campylobacter jejuni* from house fly (*Musca domestica*) intestinal tissues. Putative functional annotation of these ESTs based on similarity searches, revealed that several of the molecules these encode are immune-related. Because of short retention times (< 24 hours) of viable *C. jejuni* in exposed house flies we proposed that *M. domestica* functions as a mechanical vector of the bacterium, rather than a biological vector. In the next chapter, we investigate whether *C. jejuni* ingested by *M. domestica* larvae can survive house fly metamorphosis to adults and relate this to the expression of antimicrobial peptides (AMPs) during each life stage. If so, *M. domestica* larvae may amplify the number of *C. jejuni* and play a major role in the transmission dynamics of the bacterium.

Chapter 3. The Effects of Temperature and Innate Immunity on Transmission of *Campylobacter jejuni* between Life Stages of *Musca domestica*

*BAHRNDORFF Simon, *GILL Carson, LOWENBERGER Carl, SKOVGÅRD Henrik, HALD Birthe
Simon Fraser University
Department of Biological Sciences
Burnaby, British Columbia, V5A 1S6, Canada
*Considered Co-First Authors

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Contributions:
Infection experiments and CFU determinations were performed by Dr. Bahrndorff and his colleagues in Denmark. I assisted with conception and design of the study and performed all RNA extractions, cDNA syntheses and qPCR reactions, as well as analyzed all qPCR data. The first draft of the manuscript was written by Dr. Bahrndorff and I.

Abstract

The house fly (*Musca domestica*) is a well-established vector of human pathogens, including *Campylobacter* spp., that can cause infection of broiler chicken flocks, and through infected chicken meat can cause outbreaks of campylobacteriosis in humans. We investigated whether *Campylobacter jejuni* could be transferred between life stages of *M. domestica* (larvae-pupae-adults) and determined bacterial counts of *C. jejuni* at different time points after bacterial exposure. *Campylobacter jejuni* was transmitted from infected larvae to pupae, but not to the adult stage. Infected larvae maintained at 25ºC showed mean bacterial numbers of 6.5 ± 0.2 SE $\log_{10}$ (colony forming units [CFU]/g) that subsequently dropped to 3.6 ± 0.3 SE $\log_{10}$ (CFU/g) only 8 hours after infection. Pupae originating from infected larvae showed mean bacterial numbers of 5.3 ± 0.1 SE $\log_{10}$ (CFU/g) but these numbers dropped to 4.8 ± 0.1 SE $\log_{10}$ (CFU/g) 24 hours after pupation. The decline in *C. jejuni* CFUs during pupation coincided with increased expression of antimicrobial peptides (AMPs), including *cecropin*, *diptericin*, *attacin* and *defensin*, in the larva-pupa transition stage and a later second peak in older pupae (4 or 48 h). Conversely, there was a reduced expression of the digestive enzyme, *lysozyme*, in pupae and adults compared with larvae.

Introduction

Campylobacter jejuni is a Gram-negative, microaerophilic, non-spore forming bacteria. It is recognized as one of the leading bacterial causes of gastroenteritis in the world with more than 220,000 reported cases in 2011 in the European Union (EU) (EFSA 2013). It has been estimated, however, that only 2.1% of all cases are currently reported (EFSA 2011). Campylobacteriosis is largely perceived to be a foodborne disease and contaminated poultry is considered as the primary source (EFSA 2011). Many Diptera play a significant role in the transmission of viruses, fungi, bacteria and parasites and can harbour up to 100 different species of pathogenic microorganisms (Greenberg 1971, Greenberg 1973, Forster et al. 2007) including *Campylobacter* spp. (Rosef and Kapperud 1983, Szalanski et al. 2004, Hald et al. 2008). Recent studies suggest that *Musca domestica* plays a significant role in transmitting *Campylobacter* spp. under natural conditions (Shane et al. 1985, Hald et al. 2004, Hald et al. 2007). The incidence of campylobacteriosis in humans correlates with the prevalence of *Campylobacter* spp. in broilers (Patrick et al. 2004), which itself correlates with high numbers of house flies (Skovgård and Jespersen 2000, Nichols 2005). Infected flies can transmit the bacteria to broiler chickens (Shane et al. 1985) and the use of fly screens to physically prevent flies from entering poultry houses can significantly reduce the prevalence of chicken infections (Hald et al. 2007, Bahrndorff et al. 2013).

Despite the role of house flies in the transmission of *Campylobacter* spp., very little is known about the vector competence of *M. domestica* for *Campylobacter* spp*.,* whether the bacteria multiply within *M. domestica*, and if transstadial transmission of *Campylobacter* spp. can occur. Transstadial transmission may depend on the location of pathogens within vectors, environmental conditions, host immune responses and interaction between these (Murdock et al. 2012). Insects use pattern recognition receptors (PRRs) to recognize conserved pathogen-associated molecular patterns (PAMPs) located on the surface of microbes. PRR-PAMP binding activates downstream signalling cascades (Imd and Toll pathways) that induce the expression of antimicrobial peptides (AMPs) to eliminate potential pathogens (Medzhitov and Janeway Jr 1997, Welchman et al. 2009, Tsakas and Marmaras 2010). Gram-negative bacteria

predominantly activate the Imd pathway, which induces the expression of AMPs such as diptericin, cecropin, and attacin, whereas the Toll pathway is primarily activated by Gram-positive bacteria and fungi, inducing the expression of AMPs such as defensin (Michel et al. 2001, Hoffmann and Reichhart 2002), although there is growing information that there is considerable crosstalk among and between immune pathways (Cooper et al. 2009, Tsakas and Marmaras 2010).

Insects may use AMPs as immune-related peptides (Lopez et al. 2003, Boulanger et al. 2004, Boulanger et al. 2006), as digestive enzymes (Regel et al. 1998), or for both functions (Ursic-Bedoya and Lowenberger 2007, Ursic-Bedoya et al. 2011). Whereas cecropin, defensin, attacin and diptericin are generally considered to be components of the innate immune response in the hemocoel of insects (Lowenberger 2001), several isoforms of these AMPs also are expressed in the insect digestive tract to prevent the overproliferation of non-desirable symbionts.

We report here that the survival of *C. jejuni*-exposed *M. domestica* larvae is temperature dependent and that *C. jejuni* can survive the transformation from larvae to pupae, but not to adults. The number of bacteria drops significantly in pupae, which corresponds with an increased expression of AMPs.

Materials and Methods

Experimental Protocol

The experiments were run in two series. The first was designed to evaluate whether *C. jejuni* could be transferred between life stages of *M. domestica* (larvae-pupae-adults) and how bacterial numbers changed over time in larvae and pupae. The second experiment was designed to determine the expression of AMPs in the different life stages of *M. domestica*.

Insect Rearing

Two colonies of *M. domestica* were used in this study. The first colony originated from a Danish dairy cattle farm (DK) in 1989 and has been maintained in the laboratory at population sizes of 2-3000 at the Flakkebjerg Research Station (Slagelse, Denmark). The DK population was used for infection of *M. domestica* larvae with *C. jejuni.* The second colony was originally purchased from Beneficial Insectary Inc. (Redding, CA, USA) and has been maintained for two years at Simon Fraser University (SFU). The SFU population was used to study life-stage-specific expression of AMPs. Both colonies were maintained under laboratory conditions at 25°C and 80% relative humidity, and with a photoperiod of 16:8 (Light:Dark) hours. Flies were fed on water, sugar and milk powder. Newly laid eggs were transferred to fresh larval medium consisting of wheat bran (24.6%), alfalfa (12.3%), yeast (0.6%), malt sugar (0.9%) and tap water (61.6%).

Preparation of *Campylobacter jejuni* Strains

The *C. jejuni* strain used in the present study was a strain (DVI-SC181) belonging to the most common serotype (Penner serotype 2) and flaA type (1/1) and was obtained originally from a Danish broiler farm (Bang et al. 2003). Bacterial cultures were reconstituted from brain-heart-infusion 20% glycerol stock and incubated overnight at 42ºC on blood-agar plates in a microaerobic atmosphere (6% O_2, 6% CO_2, 4% H_2 in N_2). Subsequently, bacteria were collected from the plates and re-suspended in sterile 0.9% NaCl to an optical density (OD) of 0.6 at 620 nm (approximately 10^9 colony forming units [CFU]/ml). To ensure that larvae were infected with the same number of *C. jejuni*, the number of bacteria in the bacterial solution was determined before and after exposure by plating serial dilutions and counting bacterial colonies. The solution was kept on wet ice throughout the experiments.

Exposure of Larvae to *C. jejuni*

Egg yolk agar plates (Watson et al. 1993) were inoculated with 500 µl of the *C. jejuni* suspension (approximately 10^9 CFU/ml). Third-instar *M. domestica* larvae were placed on the plates for 4 hours, at which point they were transferred to sterile egg yolk plates and maintained there for different time periods and at different temperatures. The

temperature and time exposures were based on a pilot study that demonstrated that larvae kept on control plates (no bacteria) for up to 24 hours showed < 2% mortality. To infect pupae, late third-instar larvae were exposed to a *C. jejuni* suspension as described above and the resulting pupae were transferred to new sterile plates. The number of *C. jejuni* were measured in larvae kept at 25 and 35°C at 0, 2, 4, 8 and 20 hours post-infection, and in pupae kept at 25°C at 0, 2, 4, 8 and 24 hours post-exposure as larvae. A subset of the insects kept at 25°C was allowed to pupate and eclose as adults and these were used to determine the presence or absence of *C. jejuni* in adults 24 hours post-emergence. Flies from the DK population were used for this work.

Campylobacter jejuni CFU determinations

Insects that were used to determine *C. jejuni* numbers were surface sterilized with 0.26% sodium hypochlorite for 4 min and washed twice with sterile distilled water for 2 min to avoid contamination from the surface of the insect. This procedure was repeated three times. Emerging adults were not surface sterilized and the pupal cases were excluded from *C. jejuni* determination. Larvae, pupae, and adults were subsequently weighed, diluted 1:10 wt:vol in 0.9% saline, and homogenized using a mortar and pestle. Bacterial numbers were determined by plating 10-fold serial dilutions of the sample in a 0.9% saline solution onto *Campylobacter* spp. selective Abeyta-Hunt-Bark agar plates with 1% triphenyltetrazoliumchloride. The plates were incubated microaerobically at 42°C for 48 hours before the *C. jejuni* colonies on the plates were counted and bacterial numbers for each replicate estimated.

RNA Extraction and cDNA Generation

RNA was extracted from four individuals each of third-instar larvae, larvae that had initiated pupation, pupae (0, 2, 4, 8, 24 and 48 hour old) and adult flies (24 hour post-emergence) using TRI Reagent RNA Isolation Reagent (Sigma-Aldrich, St. Louis, MO, USA) following manufacturer's instructions. Total RNA was quantified using a NanoDrop 2000C (Thermo Fisher Scientific, Wilmington, DE, USA). cDNAs were synthesized with 5 µg of total RNA using Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Promega, Madison, WI, USA) and a dT primer with a unique 5'

extension (5'-CGGGCAGTGAGCGCAACG$(T)_{14}$-3') as previously described (Ursic-Bedoya et al. 2011). Flies from the SFU population were used for this work.

Real Time Quantitative PCR (qPCR)

Primers for use in qPCR were designed for *M. domestica* defensin, cecropin, attacin, diptericin, lysozyme, β-actin and GAPDH based on published sequences (EF17879.1, AF416602.1, DQ062744.1, FJ748596.1, HQ897688.1, JN969088, and AY675185.1, respectively; Table 3-1). The efficiencies of PCR amplification were done with each purified amplicon using 10-fold dilution series to confirm similar amplification efficiencies (within ± 10% of 1) for comparative analysis (Livak and Schmittgen 2001, Schmittgen and Livak 2008). qPCR was used to measure expression of the target mRNAs in each life stage. All qPCR reactions were performed on a Rotor-Gene 3000 (Corbett Research, Mortlake, NSW, Australia) using the PerfeCTa SYBR Green SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). We used 1 µl cDNA with 12.5 µl of SYBR Green SuperMix, 1 µl (50 ng) forward primer, 1 µl (50 ng) reverse primer in 25 µl reactions under the following conditions: 95^{o}C for 2 min, followed by 35 cycles of 95^{o}C for 10 s, 55^{o}C or 60^{o}C for 15 s, 72^{o}C for 30 s. Quantity values were calculated as the $2^{(-\Delta\Delta CT)}$ based on the C_T values (Livak and Schmittgen 2001) and using β-actin and GAPDH as reference genes for normalization. All data represent duplicate runs of independently generated cDNAs.

Statistical Analysis

Each temperature treatment and time point was replicated five times. At each time point insects were collected and analyzed in pools of five larvae or pupae. For adult house flies, we used 25 replicates, each containing a pool of two flies. Analysis of variance (ANOVA) of bacterial counts for each replicate across time intervals was performed. Time series at different temperatures and life stages were analyzed separately as data were not balanced. Bacterial numbers were log (x) transformed to meet assumptions of equal variance. If ANOVA revealed significantly different (P < 0.05) counts between time points, pairwise comparisons were conducted using the

Bonferroni post hoc test. Analyses were performed using the statistical package IBM SPSS 20.0 (IBM Corp. 2011).

Results

Campylobacter jejuni Dynamics in Larvae

Larvae kept at 35ºC showed lower mean, but not statistically significant bacterial counts of *C. jejuni* compared with larvae kept at 25ºC (Figure 3-1). Bacterial numbers changed over time in a similar way at both temperatures. At 25ºC the bacterial numbers sharply dropped from 6.5 ± 0.2 (mean ± SE) $\log_{10}$ (CFU/g) to 3.6 ± 0.3 $\log_{10}$ (CFU/g) 8 hours after infection. At 20 hours after infection, only larvae maintained at 25ºC were alive; all larvae kept at 35ºC were dead. There was a significant effect of time on bacterial numbers both at 25ºC (F = 15.7; df = 4; P < 0.001) and 35ºC (F = 37.2; df = 3; P < 0.001). Individual comparisons revealed that bacterial numbers at 25ºC at time point 0 hours were significantly higher than at 4, 8 and 20 hours post-infection (P < 0.001). At 35ºC bacterial numbers at time point 0 hours were significantly higher compared to 2, 4 and 8 hours post-infection (P < 0.001).

Campylobacter jejuni Dynamics in Pupae

All pools of pupae originating from exposed larvae were found to be *C. jejuni* positive. Newly pupated individuals showed mean bacterial numbers of 5.3 ± 0.1 $\log_{10}$ (CFU/g; Figure 3-2). Bacterial numbers stayed at this level over the next 8 hours and dropped to 4.8 ± 0.1 $\log_{10}$ (CFU/g) after 24 hours. There was a significant effect of time on bacterial numbers (F = 4.243; df = 4; P < 0.012) and individual comparisons revealed that numbers at 0 and 4 hours post-pupation were significantly higher compared to 24 hours post-pupation (P = 0.036 and P = 0.029, respectively).

Transstadial Transmission of *C. jejuni*

Campylobacter jejuni was transmitted from larvae to pupae. However, none of the adult house flies exposed to *C. jejuni* as larvae were *C. jejuni*-positive. Whether the

bacteria were killed before metamorphosis to adults or after the adult was fully formed could not be determined.

Expression of AMPs

We compared the expression of AMP transcripts in larvae, pupae and adults and evaluated their expression using two different reference genes, β-actin and GAPDH (Figure 3-3). The pattern of expression was similar with both reference genes. We used the expression in the larvae (L-III) as the calibrator and present the expression levels as fold changes related to the levels in larvae. The expression levels of the individual AMPs differed substantially between life stages. With *lysozyme*, there was a decrease in expression in all pupal and adult stages compared with the larvae. There was a spike (4-45 fold) in the expression of *diptericin*, *defensin*, *attacin* and *cecropin* in the pre-pupae (L-pup). These levels fell as they entered the unsclerotized white pupal stage. *Attacin* and *cecropin* expression increased significantly 2-8 hours post-pupation, peaking at 4 hours with increases of 150- and 90-fold, respectively. *Cecropin* had another peak of expression at 48 hours, which coincided with the peak in expression of *diptericin* (140-fold). Increases in *defensin* expression were less than 5-fold in all stages with maximum expression at 4 hours.

Discussion

Enteritis in humans due to *Campylobacter* spp. infections is an important health problem with high economical costs (Altekruse et al. 1999). Poultry meat is considered the primary source of *Campylobacter* spp. and flies play an important role in transmitting *C. jejuni* to broiler chicken flocks (Hald et al. 2007, Hald et al. 2008, Bahrndorff et al. 2013). While *M. domestica* is the fly most often found carrying *Campylobacter* spp., the stable fly (*Stomoxys calcitrans*), the false stable fly (*Muscina stabulans*), the noon fly (*Mesembrina meridian*), the black dump fly (*Hydrotaea* sp.), and the green bottle fly (*Lucilia caesar*) have also been found to carry *Campylobacter* spp. (Skovgård et al. 2011).

For epidemiological reasons it is important to understand how flies carry and transmit *Campylobacter* spp. in order to reduce pathogen transmission and ultimately the incidence of campylobacteriosis cases. In the current study, the mean bacterial numbers of *C. jejuni*-exposed larvae decreased from 6.5 ± 0.2 $\log_{10}$ CFU/g a time 0 hours to 2.7 ± 0.7 $\log_{10}$ CFU/g at 20 hours after infection when kept at 25ºC. Similarly, larvae kept at 35ºC showed an initial decrease in bacterial numbers, but mortality occurred between 8 and 20 hours post-infection, suggesting that the negative impacts of *C. jejuni* on the fly hosts are temperature dependent. Similar temperature-dependent mortality was reported in the wax moth, *Galleria mellonella*, infected with *C. jejuni* at 37ºC (Champion et al. 2010). Larvae of *M. domestica* have a wide temperature tolerance ranging from 17 to 35 ºC (Stafford and Bay 1987). When kept on agar plates at 35ºC larvae show mean mortality rates of less than 2%.

The number of *C. jejuni* counted in the larvae could have been affected by recontamination after the initial exposure as larvae were not surface sterilized before the transfer to sterile plates. Larvae were, however, surface sterilized before being homogenized and the number of *C. jejuni* determined. Bacterial numbers in pupae remained high and stable up to 24 hours after pupation began. The numbers of *C. jejuni* in the pupae may reflect both bacteria ingested by the larvae and possibly some bacteria attached to the integument of the larvae.

Several studies have investigated the change in expression of AMPs in different insect life stages including the house fly (Ito et al. 1995, Liang et al. 2006, Wang et al. 2006, Dang et al. 2010). Lysozymes can either function as digestive or immune peptides (Lowenberger 2001, Ursic-Bedoya et al. 2008). The lysozyme studied here is a putative digestive c-type enzyme based on BLAST analysis, and likely is used by larvae to digest bacteria. The expression of lysozyme should be constitutive in an actively feeding insect, and levels may vary depending on the feeding rate. Lysozyme expression, therefore, was highest in the actively-feeding larvae where it was required to digest bacteria. The decline in *lysozyme* expression after pupation is likely due to the fact that the pupa is in a non-feeding stage and there are no ingested bacteria to digest.

Studies on *M. domestica* AMPs using northern blots and qPCR indicated that transcripts of *cecropin* were absent in naïve larvae and adults (Dang et al. 2010), *attacin* was absent in larvae (Wang et al. 2006), and that *defensin* expression was not detectable in larvae and adults (Ren et al. 2009). High expression of AMPs in pupae has been reported in several insects (Lowenberger et al. 1999, Wang et al. 2006, Dang et al. 2010). Our data, using qPCR, show that the expression of *cecropin*, *defensin*, *attacin*, *diptericin*, and *lysozyme* was detectable in all developmental stages, but differed in the level and timing of expression of each gene. In naïve individuals, the expression levels for *defensin*, *attacin*, *cecropin* and *diptericin* increased as pupation began and peaked 4-48 h after pupation. Innate immune responses, including AMP expression, are increased during metamorphosis, may be tissue specific, and developmentally regulated as reported in several studies (Lowenberger et al. 1999, Altincicek and Vilcinskas 2006, Cooper et al. 2009, Verma and Tapadia 2012). In the transformation from larva to pupa to adult, there is a histolysis of the larval gut that is replaced by an adult gut. The expression of high pulses of different AMPs in pupae eliminates larval gut bacteria and prevents a septicaemia of the hemolymph and pathogen-induced death of the insects during metamorphosis. This process in holometabolous insects such as the house flies differs from what happens in hemimetabolous insects such as *Rhodnius prolixus*, in which no destruction of the GI tract occurs as nymphs moult to larger nymphs, and therefore no bacteria, or obligate symbionts, ever enter the hemocoel. Our data indicate that transstadial transmission of *C. jejuni* occurred between larvae and pupae, but that no *C. jejuni* were found in adults 24 hours after emergence. It is possible that some bacteria may have remained on empty pupal cases, but this was not determined in this study. Other studies have demonstrated partial or full transstadial transmission of bacteria in insects (Greenberg 1959, Rochon et al. 2005), which suggests that results may depend on experimental conditions and specific pathogen-host interactions (Parola and Raoult 2001).

The results of this study indicate that *C. jejuni* does not multiply within larvae or pupae of the house fly, suggesting that *C. jejuni* is transmitted mechanically by adult flies that become infected after eclosion. Reducing transmission from flies to broiler poultry may, therefore, be done best by physically preventing the entry of flies into broiler houses using screens as described previously (Hald et al. 2007, Bahrndorff et al. 2013).

Acknowledgements

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Figures

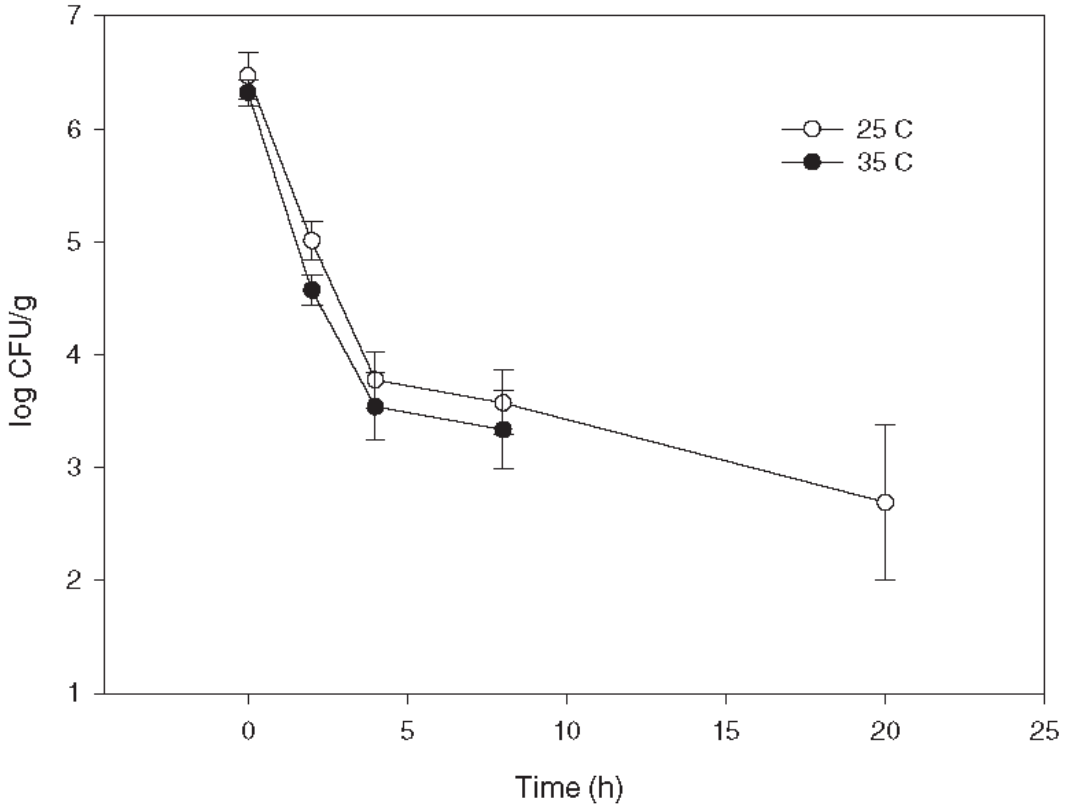

Figure 3-1 *Campylobacter jejuni* dynamics in *Musca domestica* larvae.
Mean Colony Forming Units (CFU) (± SE) of *C. jejuni* at different time points post-infection in third-instar larvae of *M. domestica* infected and maintained for 4 hours at either 25ºC or 35ºC. Larvae kept at 35ºC showed lower mean, but not statistically significant bacterial counts of *C. jejuni* compared with larvae kept at 25ºC. All larvae kept at 35ºC were dead at 20 hours. There was a significant effect of time on bacterial numbers both at 25ºC ($F = 15.7$; df = 4; $P < 0.001$) and 35ºC ($F = 37.2$; df = 3; $P < 0.001$). Individual comparisons revealed that bacterial numbers at 25ºC at time point 0 hours were significantly higher than at 4, 8 and 20 hours post-infection ($P < 0.001$). At 35ºC bacterial numbers at time point 0 hours were significantly higher compared to 2, 4 and 8 hours post-infection ($P < 0.001$).

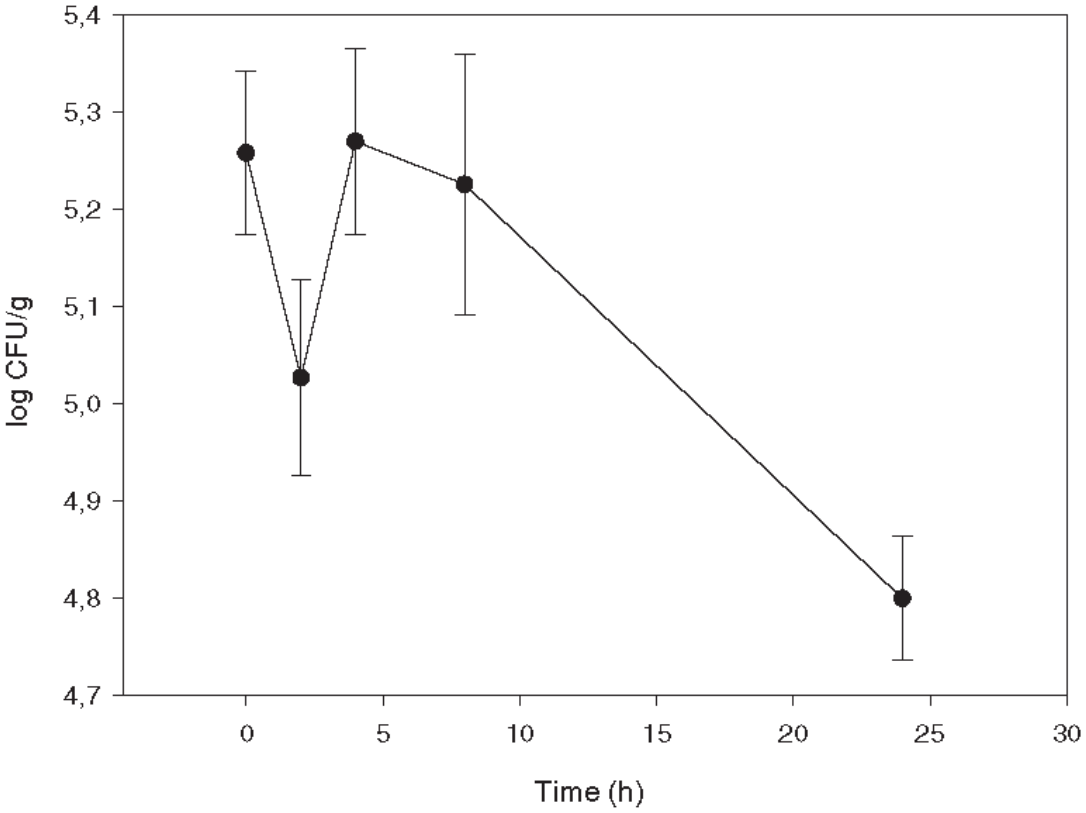

Figure 3-2 *Campylobacter jejuni* dynamics in *Musca domestica* pupae.
Mean Colony Forming Units (CFU) (± SE) of *C. jejuni* at different time points post-infection in pupae of *M. domestica* kept at 25ºC. Pupae originated from late third-instar larvae that had been infected for a 4 hour period at 25ºC. All pools of pupae originating from infected larvae were found to be *C. jejuni* positive. There was a significant effect of time on bacterial numbers (F = 4.243; df = 4; $P < 0.012$) and individual comparisons revealed that numbers at 0 and 4 hours post-pupation were significantly higher compared to 24 hours post-pupation ($P = 0.036$ and P = 0.029, respectively).

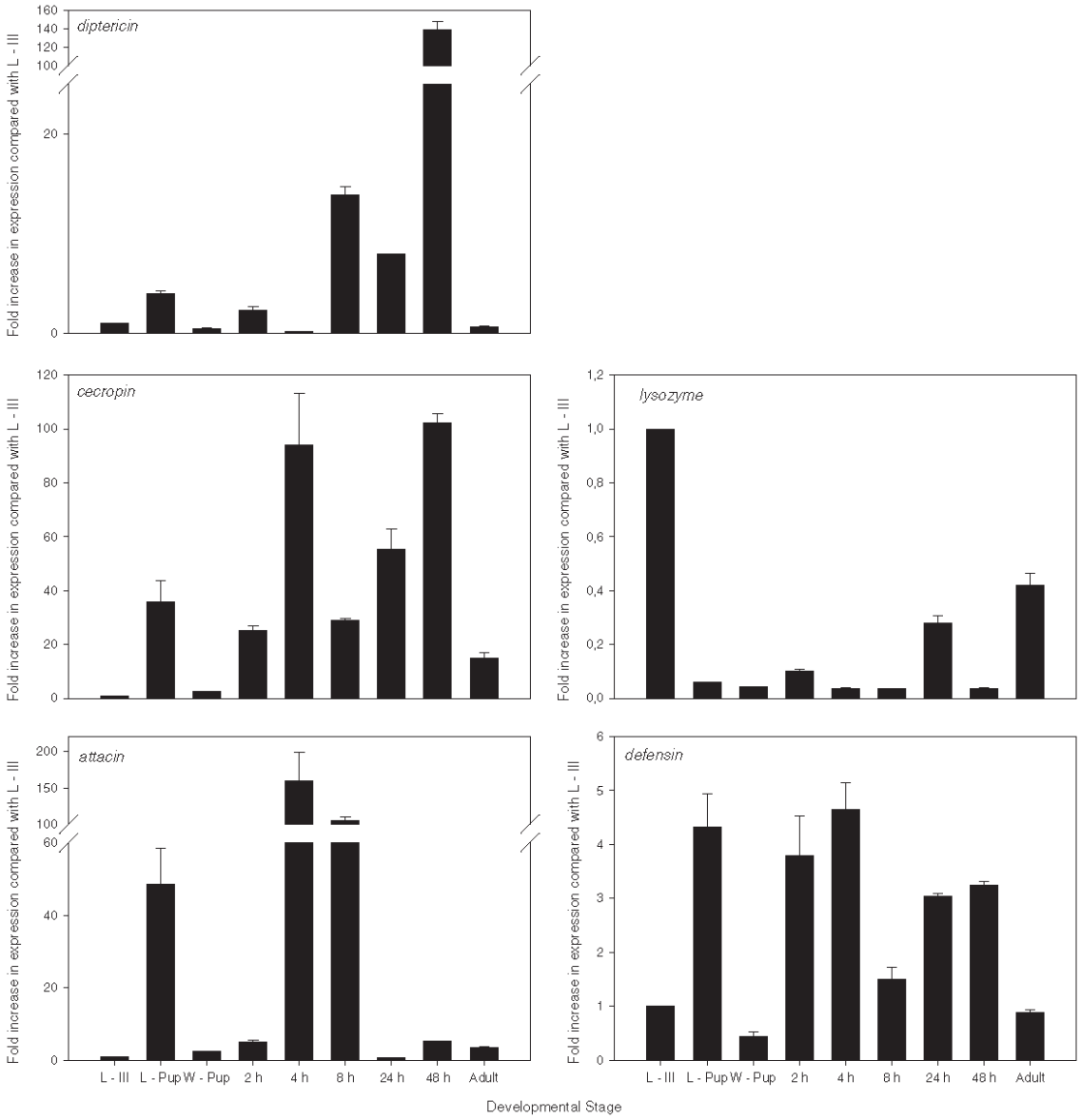

Figure 3-3 Expression of antimicrobial peptides in different developmental stages of *Musca domestica*.

The expression levels of each gene, as measured by qPCR, were normalized with *β-actin*. The expression levels in third-instar larvae (L-III) were selected as the calibrator and arbitrarily given a value of 1 for each panel. The expression in all other developmental stages represents the fold differences in expression compared with L-III. The scales on the y-axes are different for each AMP due to significant variation in expression levels of the different genes. Bars represent means ± SD of duplicate runs of independently generated cDNAs. The stages compared were: L-III: third instar larvae, L-Pup: larva-pupa transition stage, W-Pup: initial unsclerotized white pupal stage, 2-48 h: pupa collected at different time points after the W-pup stage, and adult: 24 h old adults.

Tables

Table 3-1 Primers used in real time quantitative PCR of selected AMPs and housekeeping genes in *Musca domestica*.

Gene	Primer sequence (5' → 3')	Temp* (°C)	Amplicon size (bp)
GAPDH			
F	ACA ACG AAT TCG GTT ACT CC	52.6	218
R	CCT GTC TGA TGA TGT GCG	53.3	
β-actin			
F	GGT GTC ATG GGT TGG TAT GGG AC	59.8	225
R	ACG ATT AGC CTT GGG ATT CAA TGG G	59.2	
defensin			
F	TGT CGC TGT TTT CTT GGC	53.6	254
R	CAA ACA CAA ACA CCT TTG CC	53.2	
cecropin			
F	CTT GGC TGT TTG CAT TGG	52.5	99
R	TTG AAT TGT AGC ATC GCG	50.7	
attacin			
F	TGG TCC TGT AAC CAG AGG AG	55.7	226
R	GAT TGT AAT CAA GAC CAC CAC C	53.6	
diptericin			
F	GCT CTA AGT GCC GCT CTT GTG G	60.3	240
R	CGC CAC GGT AAT CAG GAC GAC	60.2	
lysozyme			
F	CAA CGG CCG TTT CTC CTA C	56.0	137
R	ACT TCC AGG TGG ACC AGG C	59.8	

*Melting Temperature

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Connecting Statement 3

In Chapter Three, we reported that *Campylobacter jejuni* could be partially transferred between life stages of *Musca domestica*. House fly larvae and pupae maintain viable *C. jejuni*, however, the bacteria do not multiply, and within 24 hours of eclosion, adult *M. domestica* emerge *C. jejuni*-negative. The decline in *C. jejuni* numbers during house fly pupal development coincides with increased expression of antimicrobial peptides (AMPs). In the next chapter, we investigate the potential of adult house flies to pick up and then disseminate *C. jejuni* through their vomitus and excreta given their coprophagous habits. We characterize the temporal expression of AMPs and putative immune-regulation genes identified in Chapter Two in the gastrointestinal tracts of adult house flies, and discuss the role they may play in clearing *C. jejuni* infection. Determining the retention period of viable *C. jejuni* in adult house fly vomitus and excreta will help elucidate the potential of *M. domestica* to transmit the bacterium and further establishes the insect as a mechanical vector.

Chapter 4. Investigating the Immune Response of Adult House Flies (*Musca domestica*) on the Viability of Ingested *Campylobacter jejuni* in the Fly Vomitus and Excreta

GILL Carson, LOWENBERGER Carl
Simon Fraser University
Department of Biological Sciences
Burnaby, British Columbia, V5A 1S6, Canada

Abstract

Campylobacteriosis is a severe enteric disease in humans caused by the bacterium, *Campylobacter jejuni*. It is primarily regarded as a foodborne disease as infection arises from contaminated chicken meat. Along with other potential sources of contamination, house flies (*Musca domestica*) are known to play a major role in transmitting *C. jejuni* into and throughout poultry facilities. We investigated the retention period of viable *C. jejuni* after ingestion in adult *M. domestica* vomitus and excreta to elucidate *C. jejuni* dissemination patterns. *Campylobacter jejuni* was detected using fluorescent microscopy, and was found to be viable in house fly vomitus up to 4 hours following infection, however, no bacteria were detected ≥ 8 hours in the vomitus or in the excreta. We suggest that the house fly mounts an effective innate immune response in its alimentary tract against *C. jejuni*, as the retention period of the bacterium coincided with increased expression of multiple antimicrobial peptides (AMPs), including *cecropin*, *diptericin*, *attacin* and *defensin*, as well as a digestive enzyme, *lysozyme*. In addition, putative immune-pathway regulators including *Md-thor*, *Md-spheroide*, and *Md-JNK*, were detected in exposed house flies; these are proposed to play an important role in the regulation and initiation of immune signalling cascades in response to *C. jejuni* exposure. Our results indicate that *M. domestica* serves as a mechanical, rather than a biological vector of *C. jejuni*, through its feeding habits and extracorporeal digestion.

Introduction

Campylobacteriosis is a severe gastrointestinal disease in humans caused by infections with *Campylobacter* spp., a genus of microaerophilic, Gram-negative bacteria. Of the currently described species, *Campylobacter jejuni* has emerged as the most common bacterial cause of foodborne disease in many industrialized countries (Dasti et al. 2010, WHO 2011) with an estimated 9 million cases in the European Union (EFSA 2011) and 2.5 million cases annually in the United States (Mead et al. 1999). *Campylobacter jejuni* is considered to be a commensal organism of poultry alimentary tracts, and most commonly, human campylobacteriosis arises after the consumption of contaminated chicken products (Young et al. 2007). Despite strict physical barrier regulations to prevent infection of poultry flocks (Hald et al. 2004), the incidence of disease among humans is increasing and correlates strongly with the prevalence of *C. jejuni* among chickens (Patrick et al. 2004).

While there are considerable differences in poultry production facilities around the world, the infection of poultry with *C. jejuni* primarily occurs through environmental contamination (van de Giessen et al. 1992, Jacobs-Reitsma et al. 1995). Numerous sources have been implicated in the introduction of *C. jejuni* into, and subsequently throughout, poultry facilities including contaminated water, feed, rodents and insects (WHO 2013), and among these, house flies (*Musca domestica*) are continually implicated as major vectors (Rosef and Kapperud 1983, Shane et al. 1985, Berndtson et al. 1996). Indeed, the prevalence of *C. jejuni* in chickens correlates directly with high numbers of house flies during the summer months (Skovgård and Jespersen 2000, Nichols 2005). House flies naturally carry *C. jejuni* (Rosef and Kapperud 1983, Hald et al. 2004, Förster et al. 2007, Hald et al. 2008) and transmit the bacteria to non-infected chickens (Shane et al. 1985). Moreover, physically preventing house flies from entering poultry facilities can reduce substantially the prevalence of *C. jejuni* in poultry (Hald et al. 2007, Bahrndorff et al. 2013).

The synanthropic house fly is a natural vector of many pathogenic microorganisms, including viruses, fungi, parasites and bacteria, and is known to play a role in the epidemiology of many diseases (Greenberg 1971, Greenberg 1973). The

association of house flies with *C. jejuni* is not surprising because they forage, breed, and develop in vast numbers in animal manure and human excrement (Matheson 1950) where the bacteria are often abundant (Pell 1997, Guan and Holley 2003, Szalanski et al. 2004, Klein et al. 2010). Foraging flies may have their bodies contaminated with *C. jejuni* and they also ingest large numbers of bacteria (Shane et al. 1985, Petridis et al. 2006). House flies continually regurgitate to liquefy, mix, and digest their food through extracorporeal digestion, and defaecate remains of previous meals, potentially disseminating *C. jejuni* either from the mouth (vomitus) or anus (excreta) (Hewitt 1914). To date, however, no studies have evaluated the retention of viable *C. jejuni* in house flies and whether *C. jejuni* can multiply within vomitus or excreta and the roles these play in the epidemiology of campylobacteriosis, to define whether these flies serve purely as mechanical vectors or whether they serve as a true, biological, amplifying host for this bacterium.

Despite other related microbial pathogens multiplying and persisting within the alimentary tract of adult house flies (Greenberg 1973, Kobayashi et al. 1999, Sasaki et al. 2000), *C. jejuni* only survives for a relatively short period of time (< 24 hours) (Gill et al. Unpublished). House flies possess an effective innate immune system that, upon recognizing conserved pathogen-associated molecular patterns (PAMPs) on pathogen surfaces via pattern recognition receptors (PRRs; Medzhitov and Janeway Jr 1997), activates components of the humoral and cellular responses (Leclerc and Reichhart 2004) via multiple signalling cascades, including the Toll, Imd, RNAi, JNK and JAK-STAT pathways (Boutros et al. 2002, Tsakas and Marmaras 2010). Together, these signalling pathways culminate in numerous effector mechanisms, such as phagocytosis (Kocks et al. 2005), encapsulation (Lemaitre and Hoffmann 2007), melanisation (Christensen et al. 2005), or the expression of multiple antimicrobial peptides (AMPs) that target and kill microorganisms (Lowenberger et al. 1995, Bulet et al. 1999, Lowenberger et al. 1999a, Lowenberger et al. 1999b, Lowenberger 2001). While the majority of immune responses are expressed in the hemocoel of insects (Lowenberger et al. 1995, Lowenberger 2001), several AMPS are also expressed in the insect alimentary tract to prevent overproliferation of non-desirable symbionts (Lowenberger et al. 1995, Ursic-Bedoya and Lowenberger 2007, Ursic-Bedoya et al. 2011).

Recent studies investigating the immune repertoire and response of house flies exposed to *C. jejuni* demonstrate that numerous AMPs and immune-related genes are expressed, but the levels and timing of expression vary considerably. AMPs are proposed to play a significant role in clearing *C. jejuni* from adult house flies and preventing full transstadial transmission of the bacteria from larvae to adults (Bahrndorff et al. 2014, Gill et al. Unpublished). In the current study, we characterized the vector competence of *M. domestica* for *C*. *jejuni*, evaluating immune gene expression in the gastrointestinal tracts of adult *M. domestica* and the viability of *C. jejuni* in different tissues at different times after ingestion by house flies, to elucidate the role of *M. domestica* in the epidemiology of campylobacteriosis. We first determined the temporal viability of *C. jejuni* in the vomitus and excreta of adult *M. domestica* following ingestion, and then correlated viability with the expression of AMPs and other immune-related genes in the intestinal tract of adult *M. domestica* after ingestion of *C. jejuni*. We propose that house flies serve as a mechanical vector rather than a biological vector of *C. jejuni*.

Materials and Methods

Insect Colony Maintenance

A *Musca domestica* colony, originally purchased from Beneficial Insectary Inc. (Redding, CA, USA), has been maintained in the insectary at Simon Fraser University since 2012. Adult individuals were reared in the laboratory at 25ºC and 80% RH with a photoperiod of 16:8 (Light:Dark) hours. The flies fed on milk powder, sugar, and tap water.

Preparation of *Campylobacter jejuni*

We used a green fluorescent protein (GFP) labelled *C. jejuni* strain (Miller et al. 2000) obtained from the National Food Institute DTU (Technical University of Denmark, Mørkøj, Denmark) to infect house flies. Bacterial cultures were reconstituted from brain-heart-infusion 20% glycerol stocks and grown overnight on *Campylobacter* spp. selective modified charcoal-cefoperazone-deoxycholate agar (mCCDA) plates incubated at 42ºC

in a microaerobic atmosphere generated using GasPak^{TM} EZ Campy Container System Sachets (BD, Sparks, MD, USA) in a Brewer's jar. Subsequently, bacteria were collected from the plates and re-suspended in sterile 0.9% NaCl at an optical density (OD) of 0.8 at 620 nm (approximately 10^9 colony forming units; CFU/ml) and the presence of *C. jejuni* was confirmed using fluorescent microscopy. To ensure that adult house flies were exposed to approximately the same number of *C. jejuni*, the number of bacteria in the inoculation solution was established before exposure by plating serial dilutions. Suspensions were kept on wet ice throughout the experiments.

Exposure of Adult House Flies to *C. jejuni*

The protocol to expose adult house flies to *C. jejuni* was modified after Skovgård et al. (2011). Briefly, 5 day ± 24 h old flies that had been starved overnight were anesthetized with CO_2 and subsequently placed individually inside sterile pipette tips in a manner that allowed the head and proboscis to protrude. A pipette tip containing 1 µl of the *C. jejuni* suspension was presented as a drop to each fly, and the solution was ingested entirely. Control flies were exposed similarly to 1 µl of sterile 0.9% saline solution containing no bacteria. Flies that declined or stopped feeding were removed from the study.

To investigate the presence of *C. jejuni* in house fly vomitus and excreta, *C. jejuni*- and saline-exposed flies were placed individually in a 1.5 ml microcentrifuge tube with access *ad libitum* to an 8% sugar solution for the remainder of the study. To test the expression of selected immune-related genes in the GI tracts of *C. jejuni*- and saline-exposed male flies, we maintained house flies individually in sterile 50 ml Falcon tubes following exposure, with access *ad libitum* to an 8% sugar solution for the remainder of the study. The exposure protocol was repeated for three biological replicates.

Visualization of *C. jejuni* in the Vomitus of Infected Flies

Four infected flies were taken at < 1, 2, 4, 8, 12, and 24 hours following exposure, to test for the presence of GFP-labelled *C. jejuni* in fly regurgitate. Flies were placed at 4^{o}C to reduce activity. Chilled flies were grasped with a pair of sterile forceps

and the mouth parts were placed on a microscope slide on which we could collect the regurgitated vomitus. The vomitus spot was covered with a cover slip and visualized immediately at 1000x under oil immersion using fluorescent microscopy (Zeiss Canada LTD., Toronto, ONT, Canada). The vomitus of control flies was visualized at each time point and the original solution used for infection of the flies was visualized as a positive control (Figure 4-1a).

Retention of Viable *C. jejuni* in the Vomitus and Excreta of Infected Flies

Five infected and control flies were removed from their individual microcentrifuge tubes at < 1, 2, 4, 8, 12, and 24 hours following exposure. Tubes were rinsed with 100 µl of 0.9% NaCl, vortexed, and three aliquots (20 µl) of each were plated onto mCCDA plates. The plates were incubated microaerobically at 42°C for 48 hours before they were checked for the growth of *C. jejuni*.

Tissue Dissection, RNA Extraction, and cDNA Synthesis

The GI tracts were dissected from 15 infected and control adult flies at 4, 8, 12, and 24 hours following exposure to *C. jejuni* for the first biological replicate and from 5 infected and control flies for the second and third biological replicates from 2-24 hours after exposure to the bacteria. Dissected GI tracts, including the intestine, malpighian tubules, salivary glands and crop, were stored at -80^{o}C.

Total RNA was extracted from pools of 15 (replicate 1) or 5 (replicates 2 and 3) GI tracts from infected and control flies at different time points (2, 4, 8, 12, 24 hours) using TRI Reagent® RNA Isolation Reagent (Sigma-Aldrich, St. Louis, MO, USA) following manufacturer's instructions. Total RNA was quantified using a NanoDrop 2000C (Thermo Fisher Scientific, Wilmington, DE, USA). cDNAs were synthesized with 5 µg of total RNA using MMLV-RT (Promega, Madison, WI, USA) and a dT primer with a unique 5' extension (5'-CGGGCAGTGAGCGCAACG$(T)_{14}$-3') as previously described (Ursic-Bedoya et al. 2011).

Real Time Quantitative PCR (qPCR) Assays and Analyses

We used qPCR to compare the expression of select AMPs and immune-related genes in the GI tracts of infected and control flies. We designed specific primers for *M. domestica* defensin, cecropin, attacin, diptericin, lysozyme, *Md*-spheroide, *Md*-JNK, *Md*-thor, β-actin and GAPDH (Table 4-1) as previously described (Bahrndorff et al. 2014, Gill et al. Unpublished). Amplicons generated with these primers were purified and sequenced to confirm their identity (Gill et al., Unpublished). Similar PCR amplification efficiencies for each purified amplicon were confirmed as described (Bahrndorff et al. 2014, Gill et al. Unpublished), and qPCR was used to measure expression of the target mRNAs in each pool of infected and control fly GI tracts. All qPCR reactions were performed on a Rotor-Gene 3000 (Corbett Research, Mortlake, NSW, Australia) using the PerfeCTa SYBR Green SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). We used 1 µl of cDNA with 12.5 µl of SYBR Green SuperMix, 1 µl (50 ng) of forward primer, and 1 µl (50 ng) of reverse primer in 25 µl reactions under the following conditions: 95^{o}C for 2 min, followed by 35 cycles of 95^{o}C for 10 s, 55^{o}C or 60^{o}C for 15 s, and 72^{o}C for 30 s. To ensure only a single product was amplified, a melt curve analysis was performed, and a non-template negative control was included for each primer set to check for primer-dimers and contamination in the reactions.

The qPCR results were analyzed using described methodologies (Livak and Schmittgen 2001, Schmittgen and Livak 2008) and modified after Ocampo et al. (2013). We normalized expression levels using the geometric mean (Vandesompele et al. 2002) of two internal controls (β-actin and GAPDH) to generate ΔCt values (Vandesompele et al. 2002, Zhong et al. 2013). We compared gene expression between infected and control flies using $2^{-\Delta Ct}_{Inf}/2^{-\Delta Ct}_{Con}$, with the results presented as fold changes using the control (non-infected) flies as the second calibrator, arbitrarily set to 1 (Figures 4-2 and 4-3). The results are presented as the means and standard errors of two-three independently generated cDNAs (biological replicates) with each sample run in duplicate.

Results

Infections

The majority of house flies exposed to the *Campylobacter jejuni*-positive and -negative solutions during the infection experiments entirely ingested the solution, with less than ten flies on average being removed per biological replicate for having declined or stopped feeding. The GI tracts were dissected from 230 flies in total for the gene expression analyses, while approximately 110 flies were sacrificed for the vomitus and excreta bacterial viability analyses.

Visualization of *Campylobacter jejuni* in House Fly Vomitus

The GFP-labelled *C. jejuni* suspension used to infect flies was estimated to contain 10^9 CFU/ml and when visualized under fluorescent microscopy, clearly showed individual and discernible bacteria, approximately 4 µm in length (Figure 4-1a). The fluorescence of the GFP-labelled *C. jejuni* suspension was evident in the vomitus up to 4 hours after ingestion, but became progressively more degraded and digested over time (Figure 4-1b-d). No fluorescence of GFP-labelled *C. jejuni* was detected ≥ 8 hours (Figure 4-1e) and control fly regurgitate at all time points showed no sign of the bacteria.

Retention of Viable *C. jejuni* in House Fly Vomitus and Excreta

The *C. jejuni* suspension used to infect flies for the viability study was estimated to contain 10^6 CFU/ml. Fly vomitus and/or excreta on the inside of the microcentrifuge tube was found to contain viable *C. jejuni* up to 4 hours after infection (Table 4-2). Forty percent of flies at < 1 hour and 4 hours were *C. jejuni*-positive, with 20% of flies *C. jejuni*-positive at 2 hours following infection. No viable *C. jejuni* was found 8-24 hours following infection and all control flies were negative for bacterial growth.

Gene Expression

We compared the temporal expression of select AMP and immune-related transcripts in infected and control adult house fly GI tracts and evaluated their

expression using two housekeeping genes, β-actin and GAPDH (Figures 4-2 and 4-3). We used expression of control (non-infected) flies as the calibrator and present the gene expression levels of infected flies as fold changes related to the levels in control flies. Fold changes > 2 are generally considered significant.

The expression levels of the different AMPs are shown in Figure 4-2. All AMPs show minimal changes in expression before 4 hours, with *lysozyme* demonstrating no changes at any point other than a 3-fold decrease at 24 hours following infection (Figure 4-2e). *Cecropin, defensin,* and *attacin* all show a large increase in expression at 4 hours after infection, with a peak in expression at 8 hours of 35-, 9-, and 24-fold, respectively (Figure 4-2a,b,d). *Attacin* remains up-regulated at 12 and 24 hours; however, *cecropin* and *defensin* fall to uninduced levels by 12 hours, with *defensin* slightly up-regulated again at 24 hours. *Diptericin* demonstrates a 19-fold peak in expression at 4 hours and remains up-regulated at all subsequent time points (Figure 4-2c). All AMPs show the most variability at 8 hours.

The expression levels of different putative immune-regulation genes are shown in Figure 4-3, including: c-Jun N-terminal kinase (JNK) of the JNK pathway, Spheroide of the Toll pathway, and Thor, a eukaryotic translation initiation transcription factor 4E-binding protein. *Md-JNK* shows no changes in expression at any time point following infection (Figure 4-3a). *Md-spheroide* and *Md-thor* demonstrate an early peak in expression at 2 hours of 2- and 5.5-fold, respectively, with their expression levels falling to control levels at all remaining time points (Figure 4-3b,c).

Discussion

Microbial pathogens in food are estimated to cause up to 76 million illnesses and more than 5,000 deaths in the United States each year (Mead et al. 1999). Among these, *Campylobacter jejuni* has emerged as the most common bacterial cause of gastroenteritis (WHO 2013) and an important health problem with high economical costs (Altekruse et al. 1999). Up to $5.6 billion in human illness costs could be saved each year in the United States by reducing *Campylobacter* spp. in food (Buzby et al. 1997), with contaminated poultry meat considered as the primary source of human

campylobacteriosis (EFSA 2011). To reduce *C. jejuni*-infected poultry requires an understanding of the potential sources of infection as even strict compliance with biosecurity measures has failed to control *C. jejuni* contamination of poultry facilities (Hald et al. 2004). *Musca domestica* is regarded as the principal insect vector of *C. jejuni*, but more studies are required to understand the potential of the house fly to disseminate this bacterium. Elucidating the vector competence of house flies for *C. jejuni* could aid in efforts to reduce transmission and ultimately the incidence of campylobacteriosis cases among humans.

We investigated the period during which *C. jejuni* remains viable in house fly vomitus and excreta following the ingestion of ecologically-relevant doses of the bacterium. This reflects natural conditions as flies typically forage and feed in animal manure where *C. jejuni* may be abundant (Matheson 1950, Szalanski et al. 2004). Visualization of house fly regurgitate using fluorescent microscopy demonstrated that *C. jejuni* is detectable in the vomitus up to 4 hours after ingestion; however, the bacteria appear to be progressively more degraded and digested over time (Figure 4-1b-d), with no *C. jejuni* detected ≥ 8 hours (Figure 4-1e). The fly vomitus and/or excreta were found to contain viable *C. jejuni* up to 4 hours after infection, but no viable bacteria were present ≥ 8 hours (Table 4-2). While we did not distinguish between vomit and faecal specks, it is likely that only vomitus was present prior to 8 hours as excreta is typically not deposited until several hours after a meal (Hewitt 1914); house flies can deposit up to 30 specks within 24 hours depending on environmental temperature and the nature of the food, the majority of which are vomitus (Hewitt 1914, Greenberg 1973).

Although further studies should be conducted to confirm that house fly excreta do not contain viable *C. jejuni*, we examined the expression of immune-related genes in the house fly that have been reported in other insect systems (Tzou et al. 2000, Hoffmann and Reichhart 2002, Liehl et al. 2006, Lemaitre and Hoffmann 2007). Our data demonstrate that all AMPs tested (*cecropin, defensin, attacin,* and *diptericin*) other than *lysozyme* are up-regulated in response to *C. jejuni* infection (Figure 4-2). Lysozymes can function as digestive or immune peptides and are characterized by their ability to break down bacterial cell walls (Lemos et al. 1993, Ursic Bedoya et al. 2005, Ursic-Bedoya et al. 2008). The lysozyme studied here is a putative digestive enzyme that

likely is used to digest bacteria (Nayduch and Joyner 2013, Bahrndorff et al. 2014), and although not considered significant, there was an increase in *lysozyme* expression in *C. jejuni*-fed flies that peaked at 8 hours (1.8-fold; Figure 4-2e). This fell below control fly *lysozyme* expression levels at 12 hours, and remained 3-fold lower at 24 hours, which may highlight that most bacteria had been degraded by this point and expression was no longer required.

Although insects do not possess an adaptive immune system, they are still capable of discriminating among various classes of microorganisms and the tissue-specific induction of effector AMPs via their innate immune systems (Tzou et al. 2000). Our data demonstrate that Immune Deficiency (Imd) pathway-associated AMPs (*attacin, cecropin*, *diptericin*) show a higher fold increase than the Toll pathway-associated *defensin* in *C. jejuni*-infected house flies (Figure 4-2a-d). The Imd pathway is often activated rapidly against Gram-negative bacteria, such as *C. jejuni* (Hoffmann and Reichhart 2002), and studies have demonstrated previously this response specificity in the midgut of *M. domestica* (Chifanzwa 2011). Other AMPs, *attacin*, *diptericin*, and *cecropin*, are strongly induced in the gut and malpighian tubules of *Drosophila* following ingestion of infectious Gram-negative bacteria (Tzou et al. 2000, Liehl et al. 2006). In addition, our results demonstrate that the expression levels for *defensin, attacin*, and *cecropin* peak at 8 hours after infection, while *diptericin* expression peaks at 4 hours (Figure 4-2a-d). Diptericin is known to reach high concentration levels in the fly midgut, as well as in the proventriculus, an organ that acts as a valve between the oesophagus and the anterior midgut, and may provide an early barrier allowing house flies to eliminate ingested bacteria efficiently and rapidly (Liehl et al. 2006). In contrast, *attacin* and *cecropin* are primarily expressed in the midgut or malpighian tubules (Tzou et al. 2000, Chifanzwa 2011), which may explain why the peak expression in these AMPs follows that of *diptericin*.

There is increasing evidence for crosstalk between immune signalling pathways and that the multifaceted response of insects to specific microorganisms may be mediated by other immune factors and pathways (Hoffmann 2003, Cooper et al. 2009, Tsakas and Marmaras 2010). We used qPCR to evaluate the expression of select immune-related, but not effector, genes in house fly GI tracts following oral exposure to

C. jejuni. These include, 1) a putative eukaryotic translation initiation factor 4E-binding protein, *Md*-thor 2) a putative c-Jun N-terminal kinase (JNK), *Md*-JNK and 3) a putative serine protease of the Toll pathway, *Md*-spheroide. Our data indicate that there is an early up-regulation of *Md-thor* and *Md-spheroide*, but *Md-JNK* expression does not change following infection (Figure 4-3). Both *Md-thor* and *Md-spheroide* fall to normal levels of expression by 4 hours (Figure 4-3b,c).

Md-thor is a member of the 4E-binding protein (4E-BP) family that participates in *Drosophila* immunity against fungi, Gram-positive and Gram-negative bacteria (Rodriguez et al. 1996, Bernal and Kimbrell 2000, Levitin et al. 2007). In *Drosophila*, Thor is up-regulated following infection with Gram-negative bacteria at 2 hours (Rodriguez et al. 1996), as was the case with *Md-thor* in house flies (Figure 4-3c), and is suggested to play a role in the early translational regulation of immune factors (Bernal and Kimbrell 2000).

Md-spheroide is involved in the Toll immune signalling pathway and is proposed to be one of the serine proteases that cleaves and activates Spaetzle (Kambris et al. 2006), a key player in the activation of Toll and the initiation of the signalling cascade that culminates in the production of AMPs, such as defensin (Hoffmann and Reichhart 2002). However, defensin is not the major AMP expressed against Gram-negative bacteria (Hoffmann 2003). This could explain the early and small increase in expression of *Md-spheroide* in house flies in response to infection with *C. jejuni* (Figure 4-3b).

Md-JNK is a signal inducing, mitogen-activated, protein kinase (MAPK) that is activated in response to Gram-negative bacteria lipopolysaccharides (LPSs; Sluss et al. 1996, Wojda et al. 2004); activation of JNK results in the release of antibacterial peptides (Mizutani et al. 2003) and antibacterial activity (Wojda et al. 2004). Although we did not detect an increase in expression of *Md-JNK* (Figure 4-3a), it is very probable that any up-regulation occurs prior to 2 hours, as previous studies with *Galleria mellonella* indicate that JNK is activated within 10 min of infection and reaches peak expression levels in < 60 min (Wojda et al. 2004). Furthermore, JNK signalling is required in addition to Toll and Imd pathway signalling for the appropriate expression of antimicrobial genes, with deficiencies often implicated in abnormal control of apoptosis

(Boutros et al. 2002). As such, even slight alterations in the expression of important signalling modulators, such as Spheroide and JNK, could have major implications for host immunity with signalling cascades so intimately entwined (Tsakas and Marmaras 2010).

Our results indicate that *C. jejuni* ingested by *M. domestica* remains viable in the vomitus regurgitated by adult house flies for up to 4 hours. In contrast, house fly excreta appear to be *C. jejuni*-free because an extremely effective immune and digestive response is mounted in the alimentary tract of the flies. Through a number of intricately related signalling cascades, multiple effector AMPs are produced and may aid in clearing *C. jejuni* infection from the house fly gut. Therefore, it is not surprising that previous studies have found the retention of viable *C. jejuni* within the bodies of house flies to be relatively short (< 24 hours), and highlights the role of house flies as mechanical, rather than biological, vectors (Gill et al. Unpublished). Nonetheless, house flies continue to play a major role in the transmission of *C. jejuni* into poultry facilities, and regurgitation of viable *C. jejuni* likely plays a significant part in disseminating the bacteria among poultry. Future studies should aim to confirm the absence of viable *C. jejuni* in the excreta of house flies and continue to characterize the vector competence of *M. domestica* for *C. jejuni*. Doing so could aid in developing effective biosecurity interventions to reduce transmission of *C. jejuni* to poultry and ultimately the incidence of campylobacteriosis cases among humans.

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Figures

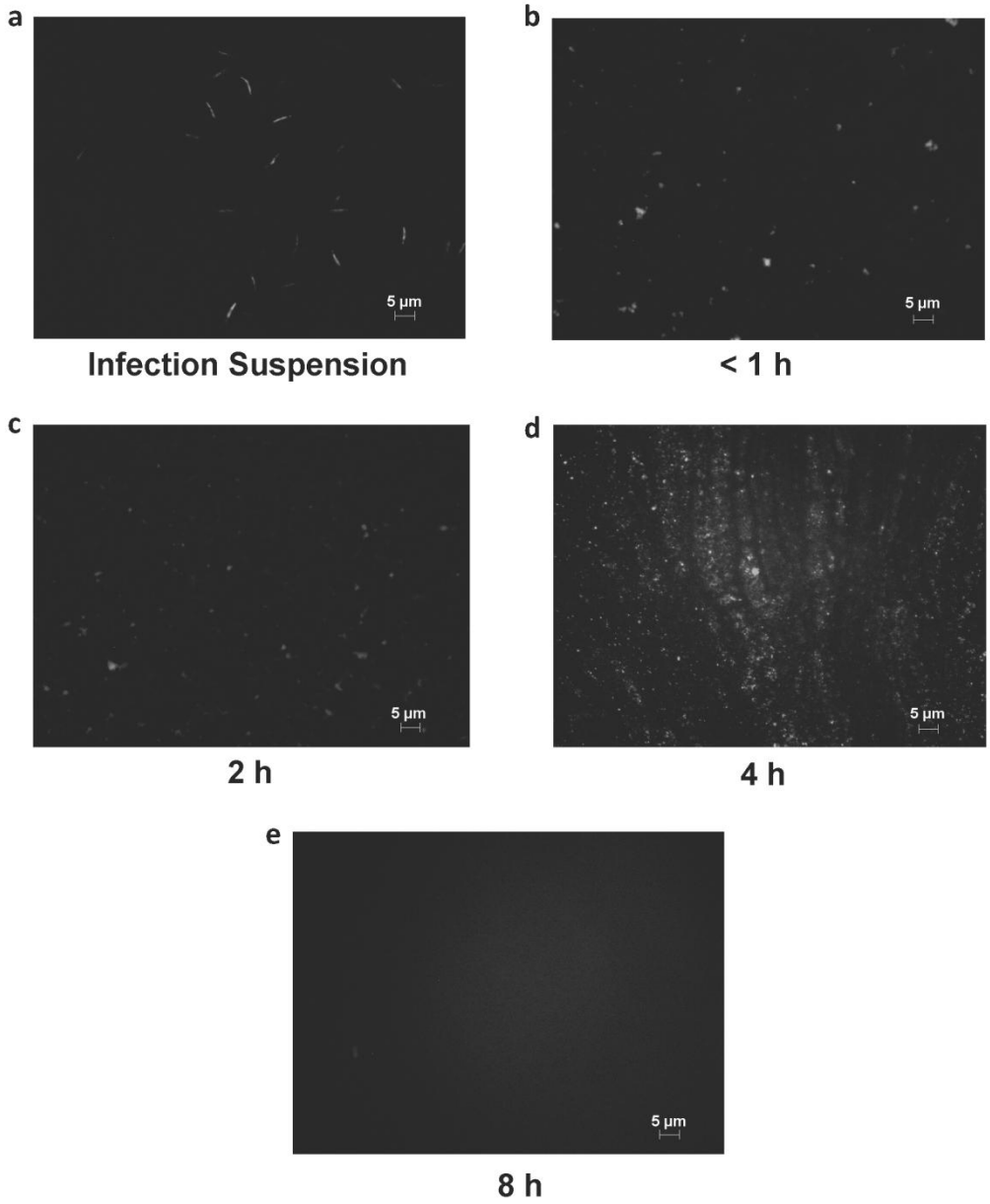

Figure 4-1 Visualization of GFP-labelled *Campylobacter jejuni* in *Musca domestica* vomitus.

Visualization using fluorescent microscopy of GFP-labelled *C*. *jejuni* at different times after infection in the vomitus of adult *M*. *domestica.* All images are magnified 1000x under oil immersion. a) Suspension of *C. jejuni* used for infection depicted here as a positive control. b-e) Regurgitate-*C. jejuni* visualized at < 1, 2, 4, and 8 hours. No fluorescence was evident ≥ 8 h or in any of the control flies.

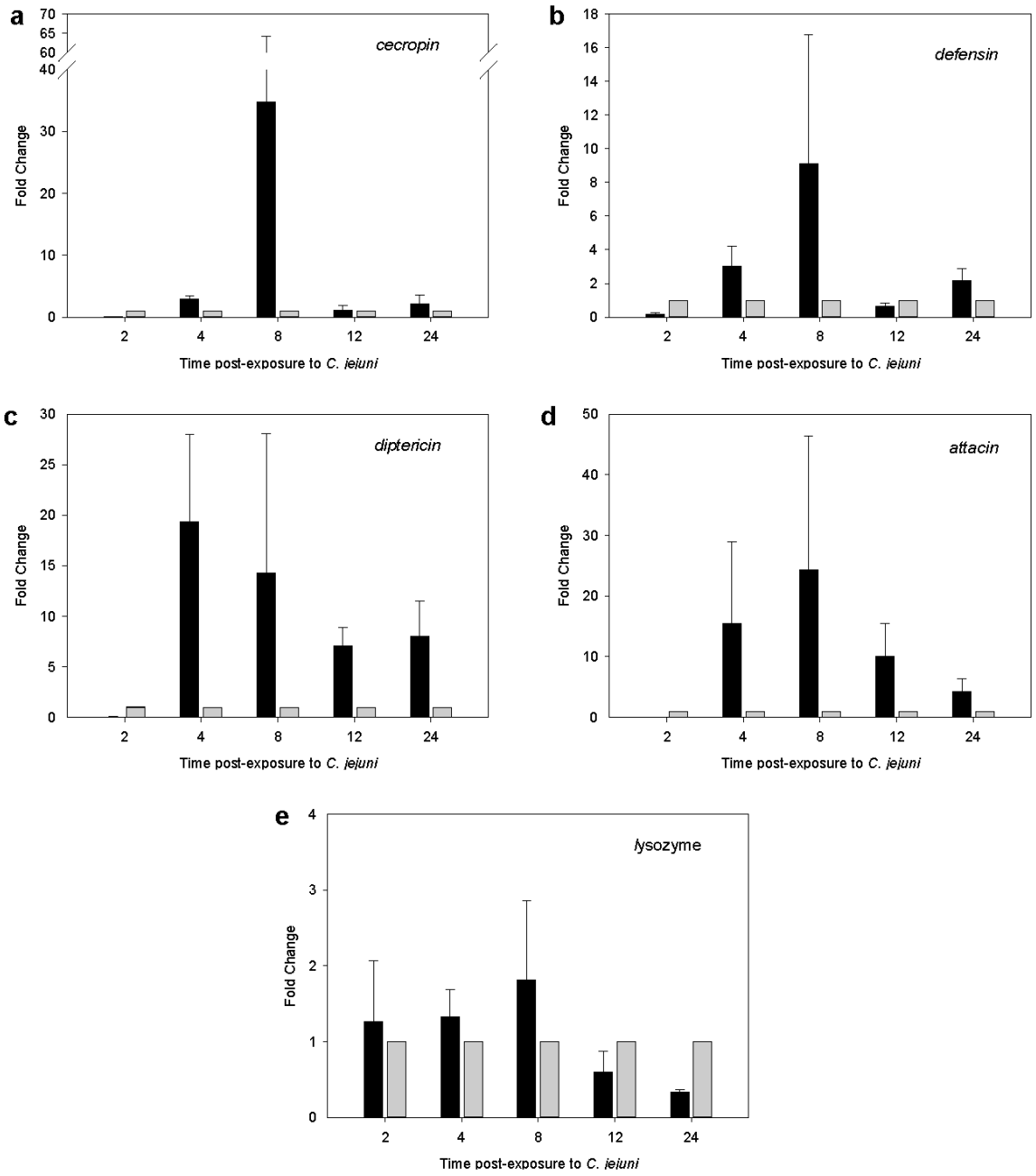

Figure 4-2 Temporal expression of select antimicrobial peptides in adult *Musca domestica* gastrointestinal tract.

Expression levels of selected effector AMPs in the GI tracts of adult *M*. *domestica* after oral exposure to *Campylobacter jejuni*. The expression levels in control, non-infected, flies were arbitrarily set at 1 (white bars) and the expression levels in infected flies (black bars) represent fold-differences from these controls. The results are presented as the mean and SE of 2-3 independently generated cDNAs from three biological replicates with each sample run in duplicate. Expression levels were normalized with *β-actin* and *GAPDH*. The scales on the y-axes are different due to significant variation in expression levels of the different genes. The genes used in this study were: a) *cecropin* b) *defensin* c) *diptericin* d) *attacin* e) *lysozyme*.

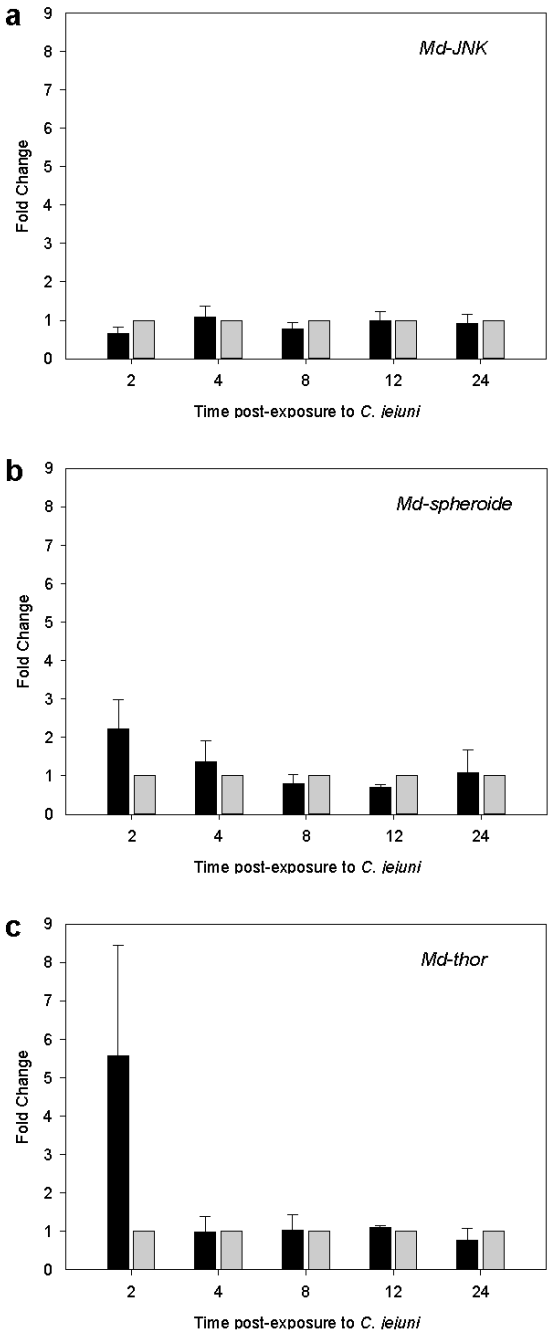

Figure 4-3 Expression of putative immune regulatory genes in *Musca domestica* gastrointestinal tract.

Expression levels of putative regulatory genes of immune function in the GI tracts of adult *M. domestica* after exposure to *Campylobacter jejuni*. The expression levels in control, non-infected, flies were arbitrarily set at 1 (white bars) and the expression levels in infected flies (black bars) represent fold-differences from these controls. The results are presented as the mean and SE of 2-3 independently generated cDNAs from three biological replicates with each sample run in duplicate. Expression levels were normalized with *β-actin* and *GAPDH*. The genes used in this study were: a) *Md-JNK* b) *Md-spheroide* c) *Md-thor*.

Tables

Table 4-1 **Primers used in real time quantitative PCR of selected housekeeping, AMPs, and immune-regulatory genes in *Musca domestica.***

Gene	Primer sequence (5' → 3')	Temp* (°C)	Amplicon size (bp)
GAPDH			
F	ACA ACG AAT TCG GTT ACT CC	52.6	219
R	CCT GTC TGA TGA TGT GCG	53.3	
β-actin			
F	GGT GTC ATG GGT TGG TAT GGG AC	59.8	225
R	ACG ATT AGC CTT GGG ATT CAA TGG G	59.2	
defensin			
F	TGT CGC TGT TTT CTT GGC	53.6	254
R	CAA ACA CAA ACA CCT TTG CC	53.2	
cecropin			
F	CTT GGC TGT TTG CAT TGG	52.5	99
R	TTG AAT TGT AGC ATC GCG	50.7	
attacin			
F	TGG TCC TGT AAC CAG AGG AG	55.7	226
R	GAT TGT AAT CAA GAC CAC CAC C	53.6	
diptericin			
F	GCT CTA AGT GCC GCT CTT GTG G	60.3	240
R	CGC CAC GGT AAT CAG GAC GAC	60.2	
lysozyme			
F	CAA CGG CCG TTT CTC CTA C	56	137
R	ACT TCC AGG TGG ACC AGG C	59.8	
Md-JNK			
F	AGC TAC ATT CAT GTT TGG TAT GAC	53.2	163
R	CAT TGG TAT TAT TAC TGG TAT GGG	51.1	
Md-spheroide			
F	GCA AGA ATG CTT ACA GTG CC	54.6	214
R	ACT ACA ACA GAT TGA ATC CAG G	52.4	
Md-thor			
F	ATT CCG CAA GTG TGT GCC	56.3	134
R	GGA GGA AGC ACG GTG TAT AG	54.8	

*Melting temperature

Table 4-2 **Retention of viable *Campylobacter jejuni* in the vomitus and excreta of *Musca domestica*.**

Time After Infection (h)	Number of *C. jejuni*-Positive Flies
< 1	2/5 (40%)
2	1/5 (20%)
4	2/5 (40%)
8	0/5 (0%)
12	0/5 (0%)
24	0/5 (0%)

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Chapter 5. General Conclusions

GILL Carson
Simon Fraser University
Department of Biological Sciences
Burnaby, British Columbia, V5A 1S6, Canada

Campylobacteriosis results from infections with *Campylobacter* spp. and has recently emerged as one of the most common foodborne human diseases caused by bacteria in the world (EFSA 2013, WHO 2013). Current control measures to limit poultry colonization, the primary source of *Campylobacter* spp. for human infection, have failed, and new strategies aimed at on-farm control are required to control this disease (Hermans et al. 2011b). However, this requires a better understanding of the transmission dynamics of *Campylobacter* spp. House flies (*Musca domestica*) have continually been suggested to play an important role in transmitting *Campylobacter* spp. from environmental sources into and throughout poultry facilities (Rosef and Kapperud 1983, Shane et al. 1985, Hald et al. 2004, Hald et al. 2008). However, few studies have addressed the specific interactions of *M. domestica* and *Campylobacter* spp. to elucidate their potential as a vector.

In this thesis I determined that:

1. Antimicrobial peptides (AMPs) and immune-related genes are up-regulated in adult house flies following *C. jejuni* exposure.

2. House fly larvae may ingest *C. jejuni*, and viable bacteria may survive metamorphosis from larvae to pupae, but do not survive to the adult stage.

3. Adults that ingest *C. jejuni* retain viable bacteria for very short periods and may play a minimal role in amplifying the bacteria.

In summary, the results presented in this dissertation suggest that *M. domestica* acts as a mechanical vector of *C. jejuni*, rather than a true, amplifying biological vector. *Campylobacter jejuni* is unable to multiply within any life stage of the house fly and its reduction and elimination from pupae and adult house flies, respectively, coincides with the up-regulation of numerous immune-related genes. Despite the relatively short retention period of *C. jejuni* in house flies, the potential of these insects to disseminate the pathogen cannot be understated. The vomitus of house flies maintains viable *C. jejuni* for up to 4 hours after a meal and can be continually spread to different sources, including poultry barns, during this time. House flies often breed and develop in livestock and poultry manure (Matheson 1950, BCMAFF 2003), and thus, once inside these poultry facilities, house flies have the potential to spread and maintain infection amongst an entire flock. Furthermore, because poultry are asymptomatic carriers of *Campylobacter* spp. (Awad et al. 2014), the presence of the bacterium is not obvious

and with immense numbers (10^9 CFU/g) of the bacteria present in the caeca of poultry (Newell and Fearnley 2003, Hermans et al. 2011a), contamination of food products is inevitable during processing regardless of the biosecurity interventions in place. Thus, given the role of house flies, and other insects, as mechanical vectors of *C. jejuni*, on-farm control measures should aim at preventing insects from entering poultry facilities using physical barriers such as screens to cover ventilation inlets and doors (Hald et al. 2007, Bahrndorff et al. 2013). This could aid in reducing the prevalence of *Campylobacter* spp. in poultry and ultimately reduce the prevalence of campylobacteriosis among humans.

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