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Original article

Campylobacter jejuni in *Musca domestica*: An examination of survival and transmission potential in light of the innate immune responses of the house flies

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Abstract

The house fly, *Musca domestica,* has been implicated as a vector of *Campylobacter* spp., a major cause of human disease. Little is known whether house flies serve as biological amplifying hosts or mechanical vectors for *Campylobacter jejuni*. We investigated the period after *C. jejuni* had been ingested by house flies in which viable *C. jejuni* colonies could be isolated from whole bodies, the vomitus and the excreta of adult *M. domestica* and evaluated the activation of innate immune responses of house flies to ingested *C. jejuni* over time. *C. jejuni* could be cultured from infected houseflies soon after ingestion but no countable *C. jejuni* colonies were observed > 24 hours post-ingestion. We detected viable *C. jejuni* in house fly vomitus and excreta up to 4 hours after ingestion, but no viable bacteria were detected ≥ 8 hours. Suppression subtractive hybridization identified pathogen-induced gene expression in the intestinal tracts of adult house flies 4-24 hours after ingesting *C. jejuni.* We measured the expression of immune regulatory (*thor*, *JNK,* and *spheroide*) and effector (*cecropin*, *diptericin*, *attacin, defensin* and *lysozyme*) genes in *C. jejuni*-infected and -

uninfected house flies using quantitative real time PCR. Some house fly factor, or combination of factors, eliminates *C. jejuni* within 24 hours post-ingestion. Because *C. jejuni* is not amplified within the body of the housefly, this insect likely serves as a mechanical vector rather than as a true biological, amplifying vector for *C. jejuni*, and adds to our understanding of insect-pathogen interactions.

Keywords   antimicrobial peptides , *Campylobacter* spp., Campylobacteriosis, house fly, innate immunity, *Musca domestica*, suppression subtractive hybridization (SSH), vector

Introduction

Campylobacteriosis is a severe gastroenteric human disease caused by bacteria of the genus *Campylobacter*, and is one of the most common and widespread food-borne infections globally and is an emerging or re-emerging pathogen that contributes to acute and chronic gastrointestinal disease (Kaakoush *et al*., 2015a,b). In the European Union > 220 000 cases were confirmed in 2011 (ECDC, 2013) but estimates suggest that only a small percentage of all cases are reported; the true incidence of campylobacteriosis is approximately nine million cases per year (EFSA, 2011) and an estimated $8.0 billion are spent annually in the USA on *Campylobacter* spp. infections and associated sequelae in humans (Buzby *et al*., 1997). In addition to gastrointestinal disease *Campylobacter* sp. has been linked to inflammatory bowel disease, colorectal cancer (Man, 2011) and autoimmune conditions such as Guillain-Barré syndrome and Miller Fisher syndrome (Kaakoush *et al*., 2015a).

The primary source of *Campylobacter* spp*.* in human infections is contaminated food sources, especially contaminated poultry products, which cause more than 50% of all cases (EFSA, 2014; WHO, 2013). The infection of poultry with *Campylobacter* spp*.* primarily occurs through environmental contamination of poultry facilities (van de Giessen *et al*., 1992; Jacobs-Reitsma *et al*., 1995; Nayduch *et al*., 2013). Numerous sources have been implicated in the introduction of *Campylobacter* spp*.* into, and subsequently throughout, poultry facilities including contaminated water, feed, rodents and insects (WHO, 2013) and among these, house flies (*Musca domestica*) are considered to be major vectors (Rosef & Kapperud, 1983; Shane *et al*., 1985; Hald *et al*., 2004; Hald *et al*., 2008). The prevalence of *Campylobacter* spp*.* in poultry correlates with high numbers of house flies during the summer months (Skovgård & Jespersen, 2000; Nichols, 2005) and house flies can disperse indiscriminately over large distances, transporting bacteria between distant farms (Kjærsgaard *et al*., 2015; Nazni *et al*., 2005). Physically preventing house flies from entering poultry facilities, by using fly screens, can reduce substantially the prevalence of *Campylobacter jejuni* in poultry facilities (Hald *et al*., 2007; Bahrndorff *et al*., 2013).

The association between house flies and bacteria, including *Campylobacter* spp*.,* is not surprising because house flies forage, breed, and develop in vast numbers in animal manure and human excrement where bacteria are abundant (Pell, 1997; Guan & Holley, 2003; Szalanski *et al*., 2004; Klein *et al*., 2010; Joyner *et al*., 2013; Fleming *et al*., 2014). Foraging flies, therefore, may have their bodies contaminated with *Campylobacter* spp. 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 *Campylobacter* spp*.* either from the mouth (vomitus) or anus (excreta) (Hewitt, 1914; Joyner *et al*., 2013). Although other microbial pathogens multiply and persist within the alimentary tract of adult flies (Kobayashi *et al*., 1999; Sasaki *et al*., 2000; Joyner *et al*., 2013; Fleming *et al*., 2014), *C. jejuni* is thought to survive for relatively short periods in house flies, usually < 24 hours (Bahrndorff *et al*., 2014c; Gill, 2014; Skovgård *et al*., 2011).

Little is known of the impact of ingested microorganisms on house flies, but they, like all invertebrates, possess an effective innate immune system to eliminate potential pathogens. Pattern recognition receptors (PRRs) (Medzhitov & Janeway, 1997) recognize conserved pathogen-associated molecular patterns (PAMPs) on the outer surfaces of pathogens, and activate components of the humoral and cellular responses via multiple signalling cascades, including the Toll, Imd, RNAi, JNK and JAK-STAT pathways (Boutros et al., 2002, Leclerc & Reichhart, 2004, Tsakas & Marmaras, 2010). This response culminates in numerous effector mechanisms, including the expression of multiple antimicrobial peptides (AMPs) that target and kill microorganisms (Lowenberger *et al*., 1995; Bulet *et al*., 1999; Lowenberger *et al*., 1999; Lowenberger, 2001; Lopez *et al*., 2003; Boulanger *et al*., 2004, Boulanger *et al*., 2006; Ursic-Bedoya & Lowenberger, 2007; Ursic-Bedoya *et al*., 2011) . While the majority of immune responses are expressed in the hemocoel of insects (Lowenberger *et al*., 1995; Lowenberger, 2001), several AMPs also are expressed in the insect alimentary tract to prevent the overproliferation of non-desirable symbionts (Lowenberger *et al*., 1995; Ursic-Bedoya & Lowenberger, 2007; Ursic-Bedoya *et al*., 2011).

We demonstrated previously that *C. jejuni* ingested by house fly larvae survive in large numbers through moults into pupae, but not into the adult stage (Bahrndorff *et al*., 2014b) and 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. This is similar to the fate of *Staphylococcus aureus* in house flies (Nayduch *et al*., 2013) but differs from other bacteria that replicate within the house fly (Nayduch *et al*., 2002; McGaughey & Nayduch, 2009). *Pseudomonas aeruginosa*, for example, showed an initial decline and subsequently an increase in bacterial numbers (Joyner *et al*., 2013). The relationship between bacteria and house flies, therefore, may be species specific and warrants further study on the specificity of the molecular interactions between host and pathogen that eliminate or modulate pathogen numbers (Lopez *et al*., 2003; Ursic-Bedoya *et al.*, 2011). There are conflicting reports on the presence, absence, and expression levels of AMPs in house flies that have ingested different strains of bacteria (Liang *et al*., 2006; Wang *et al*., 2006; Dang *et al*., 2010;, Joyner

et al., 2013; Nayduch & Joyner, 2013; Fleming *et al*., 2014), and with the recently published genome of *M. domestica* available, no doubt other AMPs will be identified (Scott *et al*., 2014).

In this study, we determined the temporal fate of ingested *C. jejuni* in house flies by determining the period during which viable bacteria could be cultivated from the whole bodies, vomitus, and faeces of infected house flies. We used suppression subtractive hybridization (SSH) to identify and characterize differentially expressed genes in the GI tracts of house flies in response to ingested *C. jejuni*. The viability of *C. jejuni* was correlated with expression levels of immune-related genes, effectors and regulators, in the GI tracts of adult house flies.

Materials and methods

Insect colony maintenance

A colony of *M*. *domestica*, 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). The house flies were fed on milk powder, sugar, and tap water.

Cultivation of Campylobacter jejuni for infection of house flies

A green fluorescent protein (GFP) labeled *C. jejuni* strain RM1221 (pWM1007) (Miller *et al*., 2000) was used to infect house flies. Bacterial cultures were reconstituted from brain-heart-infusion glycerol stocks and grown overnight on blood-agar 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 resuspended in sterile 0.9% NaCl at an optical density (OD) of 0.6 at 620 nm (approximately 10^9 colony forming units (CFU)/mL). The presence of *C. jejuni* was confirmed using fluorescent microscopy and the suspensions were kept on wet ice throughout the experiments. The concentration of *C. jejuni* in the saline solution was determined before and after exposure by plating serial dilutions and counting bacterial colonies to ensure all flies were exposed to approximately the same number of bacteria.

Exposure of adult flies to C. jejuni

The protocol to expose adult house flies to *C. jejuni* was modified after Skovgard *et al*. (2011). Briefly, 5 day ± 24 h old male house 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. Flies that declined or stopped feeding were removed from the study. After ingestion, house flies were maintained individually in sterile 50 ml Falcon tubes (BD, Sparks, MD, USA) that were covered with a foam stopper. All house flies had access to an 8% sucrose solution *ad*

libitum. 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 house flies (5 replicates of two individual flies per replicate per time point) were collected at 4, 8, 12, and 24 h after exposure to *C. jejuni* or saline solution to estimate numbers of *C. jejuni*. Individual house flies were weighed, diluted 1:10 w/v in 0.9% saline and homogenized using a mortar and pestle. Bacterial numbers were determined by plating ten-fold serial dilutions of the sample in a 0.9% saline solution onto *Campylobacter* spp. selective modified charcoal-cefoperazone-deoxycholate agar (mCCDA; CM 739 plus SR155E, Oxoid Ltd., Basingstoke, Hampshire, UK) plates. 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 were estimated. These plates with bacteria were visualized using fluorescent microscopy (Zeiss Canada LTD., Toronto, ON, Canada) to confirm that the bacteria were GFP labeled *C. jejuni*.

Visualization of C. jejuni in the vomitus of infected flies

Four *C. jejuni*-exposed flies per time point from each of 2 replicates were sampled at < 1, 2, 4, 8, 12, and 24 h following ingestion and evaluated for the presence of GFP-labeled *C. jejuni* in the regurgitate. Houseflies can be induced to regurgitate by applying light pressure to the body while holding the flies. Individual flies were placed at 4°C for approximately 5 minutes to reduce activity. Chilled flies were grasped with a pair of sterile forceps, the abdomen was squeezed lightly and the mouth parts were placed on a microscope slide upon which they regurgitated. The vomitus spot was marked, covered with a cover slip, and examined immediately at 1000× under oil immersion using fluorescent microscopy (Zeiss Canada LTD., Toronto, ON, 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.

Retention of viable C. jejuni in the vomitus and excreta of infected flies

In order to evaluate the presence of GFP-labeled *C. jejuni* in the vomitus and excreta of houseflies, houseflies were exposed to *C. jejuni* as described above and then were placed individually in 1.5 mL Eppendorf tubes with a foam stopper and access to 8% sucrose solution *ad libitum.* Two replicates of five infected and control flies per time point were removed from their individual microcentrifuge tubes at < 1, 2, 4, 8, 12, and 24 h after exposure. Tubes were rinsed with 100 μL of 0.9% NaCl, vortexed, and three aliquots (20 μL) of each were plated onto mCCDA plates, incubated microaerobically at 42°C for 48 hours, and evaluated for the growth of *C. jejuni* as described above

Tissue dissection, RNA extraction, and poly-A purificaton

House flies were exposed, as described above, to a suspension of GFP-labeled *C. jejuni* with a concentration of approximately 2.1×10^9 CFU/mL. The GI tracts were dissected from 15 infected and control adult flies per time point at 4, 8, 12, and 24 h following ingestion of *C. jejuni* for the first biological replicate and from 5 infected and 5 control flies per time point for the second and third biological replicates from 2, 4, 8, 12, and 24 h after ingestion of *C. jejuni*. Fewer flies were used in replicates 2 and 3 as the amount of RNA extracted was far more than required. Dissected tissues, including the intestine, Malpighian tubules, salivary glands and crop, were stored at -80°C. Total RNA extraction from the GI tracts was performed using 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. Subsequently 0.5 µg of mRNA from time points 4, 8, 12, and 24 h post ingestion were pooled (total of 2 µg) separately for positive (infected) and negative (non-infected) samples.

Subtractive library construction and efficiency analysis

A subtractive library was generated from the pooled mRNA samples using the PCR-select cDNA Subtraction Kit following the manufacture's recommendations (Clontech, Palo Alto, CA, USA) and as described previously (Ursic-Bedoya & Lowenberger, 2007; Barón *et al*., 2010). Briefly, the 2 µg of mRNA was used in first and second strand cDNA synthesis following the manufacturer's protocols. Subsequently the cDNAs were digested with RSA I enzyme and specific adaptors were ligated to both ends of the cDNAs, followed by subtractive hybridization, and PCR amplification with specific adaptor primers as described in the protocols. In SSH amplification of hybrids corresponding to common sequences is suppressed, yielding a library enriched for differentially expressed sequences in the *C. jejuni*-exposed house flies. The efficiency of the subtraction process was estimated by comparing the abundance of the constitutively expressed genes, *β*-actin and GAPDH, before and after subtraction as described previously (Ursic-Bedoya & Lowenberger, 2007; Barón *et al*., 2010). The primers for *β*-actin and GAPDH amplification, shown in Table 1, were used in standard PCR reactions under the following conditions: 94°C for 1 min, followed by 33 cycles of 94°C for 10 s, 60°C for 10 s and 72°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 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 X-gal, and 0.5 mmol/L IPTG, and incubated overnight at 37°C as described (Ursic-Bedoya & Lowenberger, 2007). Individual white colonies were screened using PCR to confirm the presence of an insert; colonies were resuspended individually in

10 μL ddH$_2$O and 1 μL of this solution was used in a standard PCR reaction using SP6 and T7 primers that flank the multiple cloning site in the vector and are used to estimate the size of cloned inserts. The conditions used were 96°C for 2 min, followed by 40 cycles of 96°C for 10 s, 50°C for 10 s, and 72°C for 30 s. PCR products were size fractionated on 1% agarose gels and stained with Gel-Red to confirm the presence of, and estimate the size of, any 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 each clone was 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. We used the official Gene Ontology browser and search engine, AmiGO (Ashburner *et al.*, 2000; Carbon *et al.*, 2009), for a functional prediction of our ESTs (expressed sequence tags) and 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 Centre for Biotechnology Information (NCBI) and assigned accession nos. 78910768-78911127 [GenBank JZ545987-JZ546346] described in more detail in Table S1.

cDNA synthesis and Real Time Quantitative PCR (qRT-PCR)

We used qRT-PCR to evaluate the temporal expression of the differentially expressed genes identified through SSH. We used the RNA extracted at the different time points from the intestinal tracts of control or *C. jejuni*-exposed insects described above (4–24 h post ingestion in replicate 1 and 2–24 h in replicates 2 and 3). Total RNA was quantified using a NanoDrop 2000C (Thermo Fisher Scientific, Wilmington, DE, USA). cDNAs from each time point were synthesized with 5 μg of total RNA using MMLV-RT (Promega, Madison, WI, USA) and a dT primer with a 5' extension (5'-CGGGCAGTGAGCGCAACG(T)$_{14}$-3') as previously described (Lopez *et al.*, 2003; Ursic-Bedoya *et al.*, 2011).

We designed primers (Table 1) for use in qRT-PCR to compare the expression of select immune-related regulatory genes (*spheroide*, *JNK*, and *thor*), AMPs (*defensin, cecropin, attacin, diptericin),* a digestive enzyme *(lysozyme*) and housekeeping genes (*β-actin* and *GAPDH*) as previously described (Bahrndorff *et al.*, 2014b; Gill, 2014). Amplicons generated with these primers were size-fractionated on 1% agarose gels, bands were excised, purified (QIAquick Gel Extraction Kit (Qiagen, Netherlands), and sequenced using Big-Dye chemistry (Life Technologies, Carlsbad, CA, USA). The efficiencies of PCR amplification for each primer pair were determined as described (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008; Bahrndorff *et al*., 2014b; Gill, 2014,).

qRT-PCR was used to measure expression of the target mRNAs in cDNAs from infected and control fly GI tracts at different timepoints after ingesting saline or *C. jejuni*. All qRT-PCR 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.

qRT-PCR results were analyzed using described methodologies (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). 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-exposed) flies as the second calibrator. In the graphs the levels expressed in the control flies are arbitrarily set to 1 and the levels in the exposed flies represent fold changes with respect to the control flies. The results are presented as the means and standard errors of gene expression from cDNAs generated from 2–3 independent biological replicates, and with each sample run in duplicate. Fold changes > 2 are considered statistically significant.

Results

Temporal decline in C. jejuni following ingestion

The *C. jejuni* suspension used to infect the house flies was estimated to contain 2.1×10^{9} CFU/mL. All pools of 2 *C. jejuni*-exposed flies sampled at 4 and 8 h after exposure were positive for *C. jejuni*, with a mean CFU/g of 1.90×10^{6} and 5.08×10^{5}, respectively (Fig. 1). At 12 h post-exposure colonies were observed at dilutions down to 10^{-3} and at 24 h at 10^{-1}, but individual colonies were not abnormal and were not discernible. All flies fed on saline alone were negative for *C. jejuni*.

Visualization of Campylobacter jejuni in house fly vomitus

The saline solution of GFP-labeled *C. jejuni* showed individual and discernible bacteria, approximately 4 μm in length, under fluorescent microscopy (Fig. 2A). Similarly, intact GFP-labeled *C. jejuni* were evident in the house fly vomitus up to 4 hours after ingestion, but intact and individual bacteria became progressively more difficult to discern as they were degraded or digested over time (Fig. 2B–D). Although fluorescence was detected, this was associated with broken or digested and non-viable bacteria. No fluorescence of GFP-labeled *C. jejuni* was detected ≥ 8 hours (Fig. 2E) after

ingestion of *C. jejuni* and no GFP-labeled *C. jejuni* were found in control fly regurgitate at any time point.

Retention of viable C. jejuni in house fly vomitus and excreta

Fly vomitus and/or excreta on the inside of the microcentrifuge tube was found to contain viable *C. jejuni* up to 4 h after ingestion (Table 2). Forty percent of flies at < 1 h and 4 h were *C. jejuni*-positive. No viable *C. jejuni* was found 8–24 h following ingestion and all control flies were negative for bacterial growth.

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. Twenty-six clones (6.7%) that either had inserts <60 bp in length or which had poor quality sequence were excluded from the analysis. In total, 109 clones (28.2%) corresponded to unique EST sequences, which are described in more detail in Table S1. 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). Redundant clones were included in the functional analysis (Fig. 3) 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*.

All identified genes were clustered into functional groups according to their putative biological function; binding, cytoskeleton, defence (immunity), metabolism, mitochondrial, protease inhibitor, protease/proteolysis, ribosomal, transcriptional/translational control, transport and other (Fig. 3). 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 defence (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 has been reported in other SSH studies (Ursic-Bedoya & Lowenberger, 2007; Barón *et al*., 2010). The genes associated with regulators of immunity included a putative eukaryotic translation initiation factor 4E-binding protein (thor), a putative c-Jun N-terminal kinase (JNK), and a putative serine protease of the Toll pathway (spheroide).

Gene expression

The temporal expression of selected AMP and immune-related transcripts in infected and control adult house fly GI tracts was compared. When comparing the putative immune-regulation

genes, there was no significant change in the expression of *JNK* at any time point after ingestion (Fig. 4A) but *Spheroide* and *thor* demonstrated an early peak in expression at 2 hours of 2- and 5.5-fold, respectively, and their expression levels fell to control levels at all subsequent time points (Fig. 4B,C).

The expression levels of the different effector AMPs are shown in Figure 5. All AMPs show minimal changes in expression at 2 h post ingestion. *Cecropin*, *defensin*, and *attacin* all showed a large increase in expression by 4 h after ingestion, and a peak in expression at 8 h of 35-, 9-, and 24-fold, respectively (Fig. 5A,B,D). Whereas *attacin* expression remained up-regulated at 12 and 24 h, expression levels of *cecropin* and *defensin* fell to control levels by 12 h. *Diptericin* demonstrated a 19-fold peak in expression at 4 h and remained up-regulated at all subsequent time points (Fig. 5C). The digestive *Lysozyme* demonstrated no significant difference in mRNA expression at any timepoint after ingestion (Fig. 5E).

Discussion

Campylobacter jejuni has become one of the most common bacterial causes of gastroenteritis (WHO, 2013) and other sequelae such as acute and chronic gastrointestinal disease inflammatory bowel diseases (IBD), and colorectal cancer (Kaakoush *et al*., 2015a; Man, 2011). Bilions of dollars could be saved each year in the United States by eliminating this emerging disease (Buzby et al., 1997; Altekruse *et al*., 1999). *Musca domestica* is regarded as a principal insect vector of *C. jejuni*, yet we know relatively little of its role as an amplifying host. While data are available on house fly interactions with several bacteria, there are few available data on the molecular interactions between *C. jejuni* and the house fly and specifically how this relates to the epidemiology of campylobacteriosis. 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 to which house flies might be exposed as they forage and feed in animal manure (Bahrndorff *et al*., 2014a; Szalanski *et al*., 2004).

The bacterial load (CFU/mL) in the whole bodies of house flies was estimated at different time points post-ingestion. Because the house flies had been starved overnight, they all fed to repletion quickly. The effect of this food deprivation on other resident gut microbiota is unknown. The results show a large reduction in bacterial numbers within the first 8 h and no countable colonies were present 12–24 h after exposure (Fig. 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. Similar results have been reported for house flies infected with *S. aureus* (Nayduch *et al*., 2013) whereas some bacteria, *Aeromonas hydrophilia*, *Aeromonas caviae*, and *Enterococcus faecalis* have been reported to multiply within the house fly (Nayduch *et al*., 2002; McGaughey & Nayduch, 2009; Doud & Zurek, 2012). *Aeromonas hydrophilia*,

A. caviae, and *C. jejuni* are Gram-negative bacteria whereas *E. faecalis* and *S. aureus* are Gram-positive bacteria which indicates that the response, replication or not within the house fly, is not defined by class of bacteria. Indeed the response by the house fly may depend on the molecular patterns found on the surface of the bacteria. There may be significant differences in the lipopolysaccharides found on Gram-negative bacteria, and if this serves as the PAMP that activates the innate immune response of the house fly then we would expect to see differences in immune responses to these bacteria. Alternatively, the bacteria simply may have died due to an inhospitable environment in the GI tract of the flies rather than a directed response by the innate immune system.

Visualization of house fly regurgitate using fluorescent microscopy demonstrated that *C. jejuni* was detectable in the vomitus up to 4 hours after ingestion. The bacteria, however, appear to be progressively degraded and digested over time (Fig. 2B–D), with no *C. jejuni* detected ≥ 8 h (Fig. 2E). The fly vomitus and/or excreta collected from the holding tubes and cultured on CCDA plates indicated that material collected up to 4 hours after ingestion contained viable *C. jejuni*, but no viable bacteria were present on plates seeded with vomitus and/or excreta collected ≥ 8 h post exposure (Table 2). Although we did not distinguish between vomitus and faecal specks, it is most likely that vomitus was present prior to 4 hours and excreta later (Hewitt, 1914).

Several genes were up-regulated in house flies in response to the ingestion of *C. jejuni*, including several immune-responsive genes described in other SSH studies (Ursic-Bedoya & Lowenberger, 2007; Barón *et al.*, 2010). These include genes classified as immune regulators and effector genes. The putative immune regulatory genes included a putative eukaryotic translation initiation factor 4E-binding protein (thor), a putative c-Jun N-terminal kinase (JNK), and a putative serine protease of the Toll pathway (spheroide). There was an early increase in the expression of *thor* (Fig. 4C) similar to the response of *Drosophila melanogaster* to infection with Gram-negative bacteria (Rodriguez *et al.*, 1996) and whose role is related to the early translational regulation of immune factors (Bernal & Kimbrell, 2000). *Spheroide* also demonstrated an early pulse of expression (Fig. 4B) as might be expected in its putative role in the Toll signalling pathway that results in the expression of defensin (Hoffmann & Reichhart, 2002). Both *thor* and *spheroide* fell to normal levels of expression by 4 hours (Fig. 4B,C). *JNK* is normally activated in response to Gram-negative bacteria lipopolysaccharides (Sluss *et al.*, 1996; Wojda *et al.*, 2004) and results in the release of AMPs (Mizutani *et al.*, 2003), but in this study *JNK* expression did not change following ingestion. JNK up-regulation may be very fast, within 10 min of infection, and peak expression levels are reported in < 60 min(Wojda *et al.*, 2004), suggesting we may not have measured expression levels early enough. Even slight alterations in the expression of important signalling modulators could have major implications for host immunity with signalling cascades so intimately entwined (Tsakas & Marmaras, 2010).

We examined the expression of insect AMPs commonly studied in other insects (Tzou et al., 2000; Hoffmann & Reichhart, 2002; Liehl *et al.*, 2006; Lemaitre & Hoffmann, 2007). All AMPs tested (*cecropin*, *defensin*, *attacin*, and *diptericin*) other than *lysozyme* were up-regulated in response to *C. jejuni* ingestion (Fig. 5). Lysozymes can function as digestive or immune peptides, or as both (Lemos

& Terra, 1991; Ursic Bedoya *et al*., 2005; Ursic-Bedoya *et al*., 2008). The lysozyme studied here is a putative digestive enzyme (Nayduch *et al*., 2013; Bahrndorff *et al*., 2014b) and although not considered significant, there was an increase in *lysozyme* expression in *C. jejuni*-fed flies at 8 hours (1.8-fold; Fig. 5E). This fell below control fly *lysozyme* expression levels at 12 hours, and remained 3-fold lower at 24 hours, which may highlight the fact that most bacteria had been degraded by this point and digestion was no longer required. There may be a constitutive level of lysozyme expression in house flies that may be altered during infection, and midgut infections may activate the systemic expression of other lysozymes and potentially other AMPs (Nayduch & Joyner, 2013). It should be noted that many AMPs are expressed by hemocytes or the fat body and it is possible that some of these tissues may have adhered to the GI tract during dissection. If the genes we measured were expressed in adhering hemocytes then there must be a signalling mechanism to induce their expression in the body cavity while the bacteria remain in the GI tract.

Although insects do not possess an adaptive immune system, they are able to discriminate among various classes of microorganisms, resulting in the induction of specific effector AMPs (Tzou *et al*., 2000). Our data demonstrate that the 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 (Fig. 5A–D). The IMD pathway is activated rapidly against Gram-negative bacteria, such as *C. jejuni* (Hoffmann & Reichhart, 2002*)*, and studies have demonstrated a specificity in the midgut response of *M. domestica* to *Streptococcus pyogenes* and *Salmonella typhimurium* (Chifanzwa, 2011). *Attacin*, *diptericin*, and *cecropin*, are strongly induced in the gut and Malpighian tubules of *D. melanogaster* 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 (Fig. 5A–D). A similar pattern of midgut expression levels of *cecropin* and *defensin* were seen in house flies infected with *E. coli*; but the expression levels and pattern for *lysozyme* differed from the current data (Fleming *et al*., 2014), although it is unclear if the same lysozyme gene was evaluated in the two studies. There were similarities in the pattern of expression of *cecropin* in house flies infected with *P. aeruginosa* (Joyner *et al*., 2013) and *C. jejuni* (this study) but differences in the pattern of expression of *defensin* and *diptericin* in these same studies. Diptericin is known to reach high concentration levels in the 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 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*.

The moderate up-regulation of some genes in the *C. jejuni*-exposed house flies may be due to the means by which we exposed flies to the bacteria. We exposed flies orally to ecologically-relevant doses, which differs from other studies in which bacteria were injected into the insect (Ursic-Bedoya

& Lowenberger, 2007, Wang et al., 2006). Injection into the hemocoel does not reflect natural conditions, but often is used to activate immune genes to the highest level for gene identification (Liehl et al., 2006). The expression levels of immune genes in the midgut of most insects are usually lower than levels seen 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).

Our data suggest that *C. jejuni* remains viable in the vomitus and faecal specks of adult house flies for up to 4 hours. Subsequently, house fly excreta appear to be *C. jejuni*-free, possibly due to the activation of the immune and digestive responses in the alimentary tract of the flies, corroborating previous studies that found relatively short retention times of viable *C. jejuni* within the bodies of house flies (Skovgård *et al*., 2011; Bahrndorff *et al*., 2014b). There is no doubt that there are many other bacteria in the midguts of house flies, but we isolated *C. jejuni* on selective growth media and visualized the presence of GFP labeled *C. jejuni* using fluorescent microscopy to follow its development or elimination by the insect.

While these data highlight the role of house flies as mechanical, rather than biological amplifying vectors (Bahrndorff *et al*., 2014b; Gill, 2014), house flies continue to play a major role in the transmission of *C. jejuni* into and throughout poultry facilities. The data suggest that regurgitation and defecation of viable *C. jejuni* likely plays a significant part in disseminating the bacteria but that this window of opportunity for successful transmission is limited. Physical barriers that prevent the entry of house flies may ultimately provide the best means to reduce transmission of *C. jejuni* to poultry (Hald *et al*., 2007; Bahrndorff *et al*., 2013) and ultimately the incidence of campylobacteriosis in humans.

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Disclosure

The authors report no financial, scientific or intellectual conflicts of interest.

Supplementary Table S1. Novel ESTs.

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Figure Legends

Figure 1. Mean number (± SD) (CFU/g) of *Campylobacter jejuni* enumerated from the whole bodies of house flies at different time points after ingestion. The stock solution used for infection was estimated to contain 2.10×10^9 CFU/ml of *C. jejuni*. All data points represent five replicates of two individual flies per replicate.
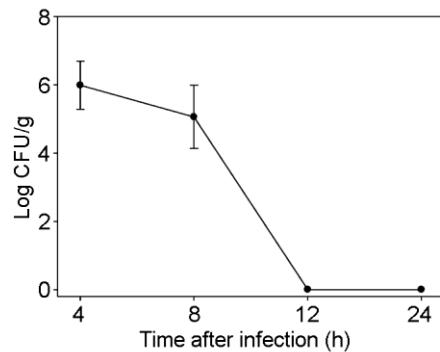

Figure 2. Visualization of GFP-labeled *Campylobacter jejuni* in the vomitus of adult house flies at different time points after ingestion using fluorescent microscopy at 1000x magnification and oil immersion. Panel a: Suspension of *C. jejuni* used for infection depicted here as a positive control; Panels b–e: Regurgitate visualized at < 1, 2, 4, and 8 h after ingestion. No fluorescence was evident ≥ 8 h or in any of the control flies.
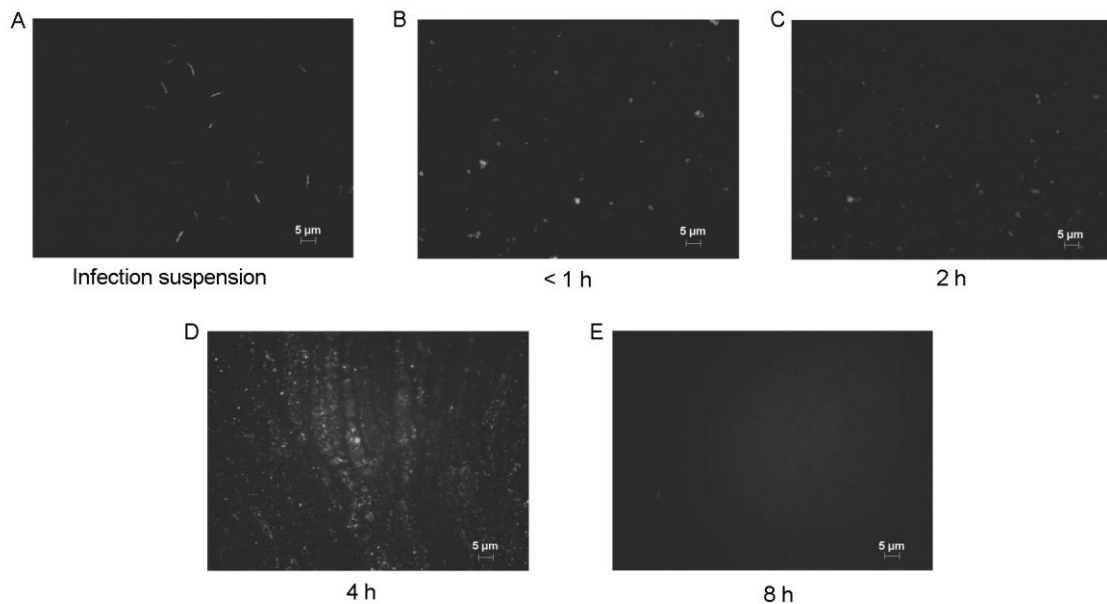
Figure 3. Functional prediction and classification of differentially expressed ESTs in the GI tracts of adult house flies after ingesting *Campylobacter jejuni.* EST numbers include redundant clones and numerical superscripts represent the number of independent, unique, ESTs (109 total) excluding redundant clones. Novel ESTs were submitted to dbEST at the National Center for Biotechnology Information (NCBI) and assigned accession nos. 78910768-78911127 [GenBank: JZ545987-JZ546346].
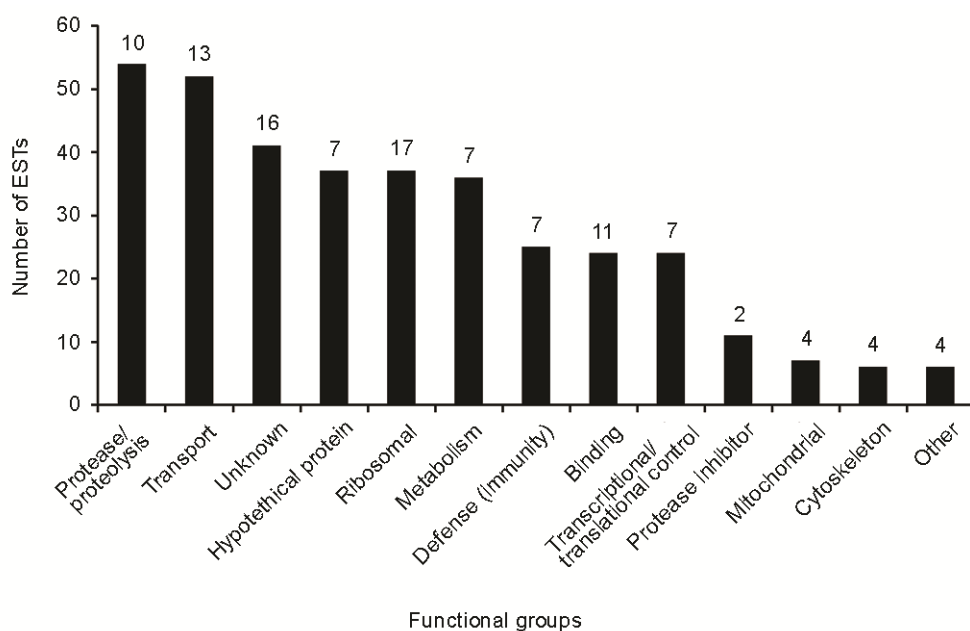

Figure 4. Expression levels (mean + SE) of putative regulatory genes, *JNK,* s*pheroide* and *thor* in the GI tracts of adult *M*usca *domestica* after ingesting *Campylobacter jejuni*. Expression levels in control, non-infected, flies were arbitrarily set at 1 (grey bars) and the expression levels in infected flies (black bars) represent fold-differences from these controls. The graphs represent data collected from 2–3 independently generated cDNAs from three biological replicates with each sample run in duplicate. Expression levels were normalized with *β-actin* and *GAPDH*.
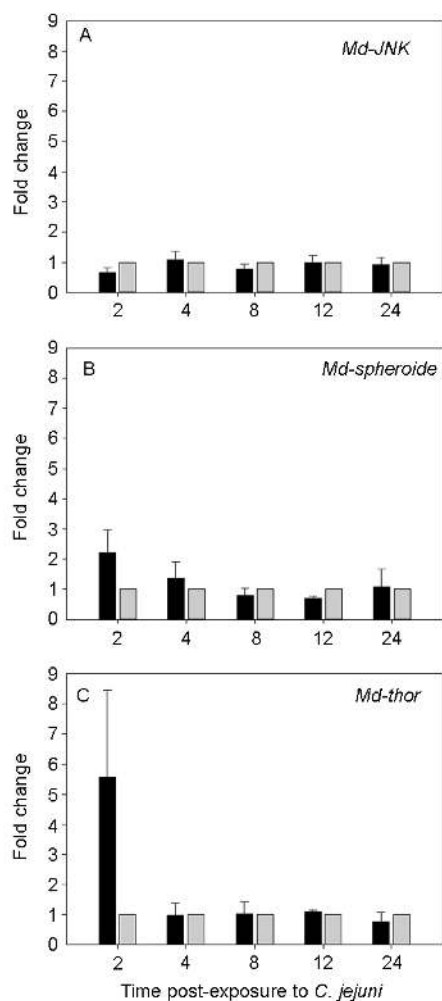
Figure 5. Expression levels (mean + SE) of selected effector antimicrobial peptides (*cecropin*, *defensin*, *diptericin*, *attacin* and *lysozyme)* in the GI tracts of adult *Musca domestica* after ingesting *Campylobacter jejuni*. Expression levels in control, non-infected, flies were arbitrarily set at 1 (grey bars) and the expression levels in infected flies (black bars) represent fold-differences from these controls. The results represent data collected from 2–3 independently generated cDNAs from three biological replicates with each sample run in duplicate. Expression level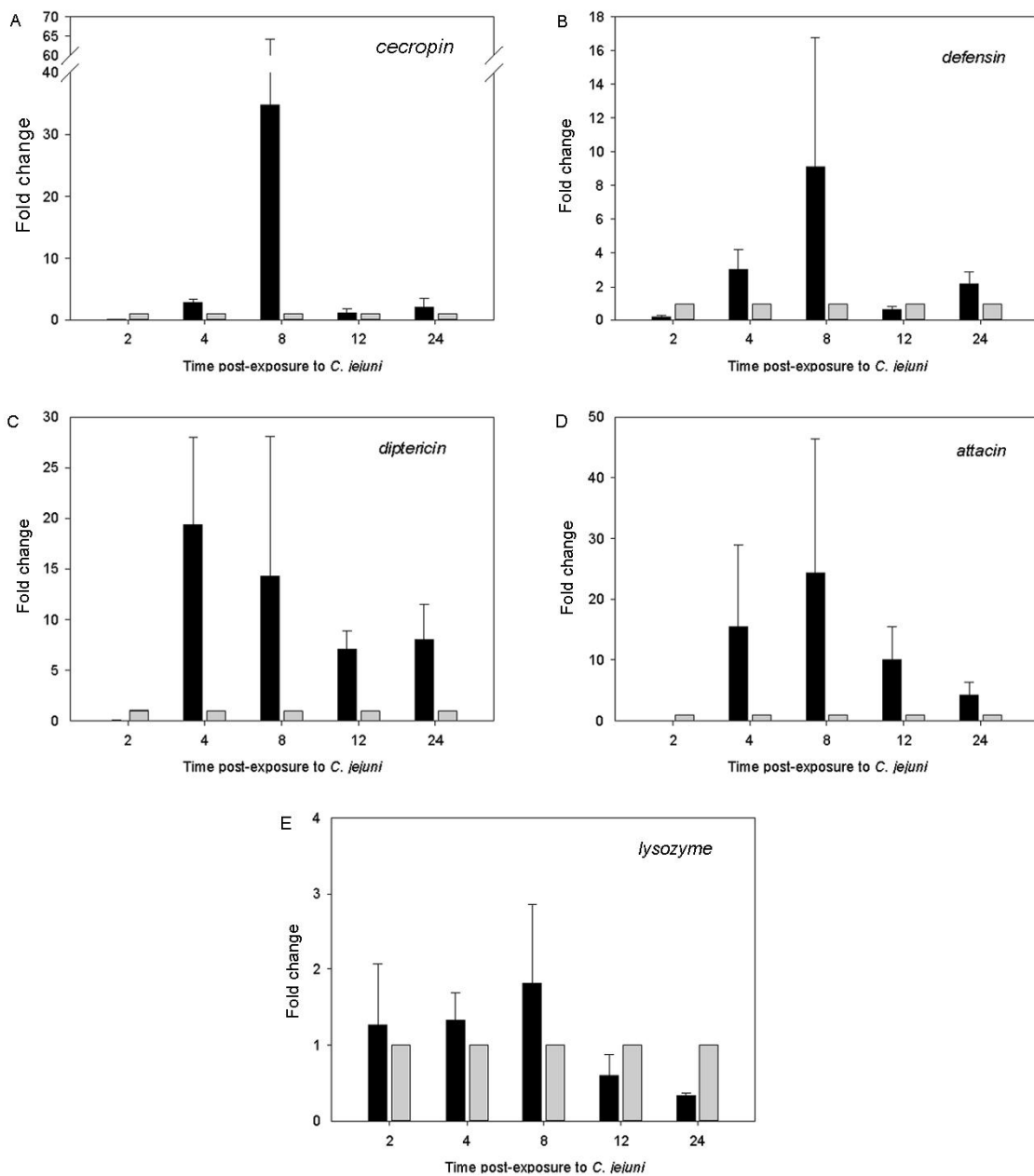s were normalized with *β-actin* and *GAPDH*. The scales on the y-axes are different due to significant variation in expression levels of the different genes.

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

Table 2. The retention of viable *Campylobacter jejuni* in the excreta and vomitus of *Musca domestica* following ingestion

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%)