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SPATIAL AND TEMPORAL IMMUNE RESPONSE IN HOUSE FLIES IN
RESPONSE TO INGESTION OF *BACILLUS CEREUS* AND *ESCHERICHIA COLI*
O157:H7

by

ADAM FLEMING

(Under the direction of DANA NAYDUCH)

ABSTRACT

House flies (*Musca domestica* L.) feed and breed on septic substrates, putting them in direct contact with a multitude of disease causing agents and can act as a bridge for those agents to humans. The house fly has previously been shown to carry many different species of bacteria that are pathogenic. *Escherichia coli* O157:H7 is a pathogenic enterohemorrhagic serotype of *E. coli* that can be vectored by the house fly. *Bacillus cereus* is a foodborne pathogen that has also been isolated from the house fly in previous studies. To examine vector potential for these pathogens, house flies were fed green fluorescent protein (GFP)-expressing *E. coli* O157:H7 or *B. cereus* and then bacterial fate and localization of fly defensive responses were analyzed at various hours post-ingestion (h PI). Bacterial fate was assessed qualitatively by localizing bacteria via microscopy and quantitatively by culturing whole fly homogenate. House fly defensive responses, including three antimicrobial peptides (AMPs; Defensin, Diptericin, and Cecropin) and the peptidoglycan cleaving enzyme Lysozyme, were analyzed using immunofluorescent localization. Localization of *B. cereus* and *E. coli* O157:H7 at various time points correlated with evidence of lysed bacteria in microscopy, a decrease in recovered bacteria, and observed expression of AMPs and Lysozyme. Bacterial recovery showed that *B. cereus* decreased steadily up to 24 h PI and *E. coli* O157:H7 decreased

steadily up to 12 h PI. Flies fed *B. cereus* induced Defensin, Diptericin, and Lysozyme expression that peaked in the midgut at 6 h PI. In contrast, flies fed *E. coli* O157:H7 showed noticeable expression of only Lysozyme and Diptericin at 2 and 6 h PI in the midgut and proventriculus. This study shows that *B. cereus* elicits a strong immune response from the house fly and can persist in the gut until 24 h PI, while *E. coli* O157:H7 elicits little immune response and can persist up to 12 h PI. These findings help to define whether or not pathogenic bacteria can survive at infectious levels within the fly, how the house fly responds to ingestion of these pathogens, and finally how long the bacteria can persist within the fly.

INDEX WORDS: *Musca domestica*, Bacteria, Pathogens, Immunity, Disease, Antimicrobial, Humoral Immunity, Alimentary Canal, Proliferation, Expression

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by

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ADAM FLEMING

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Electronic Version Approved: July 2012

DEDICATION

This work is dedicated to my father, William Fleming. He's always been a source of inspiration, motivation, and insight throughout my life. Hope it reads good (*sic*) dad.

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INTRODUCTION

House Flies as Vectors

House flies (*Musca domestica* L.) have long been implicated as agents in the spread of disease (Hawley, 1951; West, 1951; Greenberg, 1959). Adults feed and breed on septic substrates, putting them in direct contact with a multitude of pathogenic microorganisms. Being synanthropic, flies are constantly found in contact with humans in a variety of scenarios, both rural and metropolitan. House flies provide a bridge for pathogens between septic environments, such as waste reservoirs and refuse, and human hosts. The World Health Organization (WHO) has reported that as many as 1.5 million children die each year as a result of diarrheal illness (WHO, 2009), which can be caused by many of the different bacterial species that have been found associated with the house fly (Cohen *et al.*, 1991; Grübel and Cave, 1998; Fotedar, 2001; Nichols, 2005; Förster *et al.*, 2007).

Although house flies have been identified as a factor in the epidemiology of a wide variety of infectious diseases affecting humans (Graczyk *et al.*, 2001), relatively little research has been done to assess the role of the house fly as a vector beyond simple mechanical transmission. First Hawley (1951), then Greenberg (1964; 1970) examined the possibility of multiplication of various types of bacteria within the house fly by feeding them suspensions of known quantities of bacteria, immobilizing them using paraffin and collecting their feces. Among the bacteria examined were *Escherichia coli*, *Salmonella* spp., and *Shigella* spp. These studies showed that bacteria were not only being passed in the feces of the house fly, but were in some cases multiplying within the

fly. Greenberg *et al.* (1970) showed that gnotobiotic house flies fed as few as 20 cells of *S. typhimurium* were able to excrete the pathogen at a rate of about 1.4×10^7 cells per 4 hours 3 days after ingesting the initial dose.

In terms of the practical ability of the house fly to transmit bacteria, Moriya *et al.* (1997) showed that a 1996 outbreak of enterohemorrhagic *E. coli* in a nursery in Japan was likely caused by house fly transmission from a nearby cattle farm on to the food, plates, and utensils used in the nursery dining room. This outbreak led to a series of studies examining the ability of the house fly to transmit *E. coli* O157:H7, and further reinforced the need to more thoroughly examine its role in spreading human and animal pathogens. Grübel and Cave (1998) examined the potential of house flies to carry *H. pylori* both externally and internally, and this pathogen was able to persist at higher numbers within the house fly than it would in ambient air. Zurek *et al.* (2001) examined the potential of the house fly to transmit *Yersinia pseudotuberculosis*, an important animal pathogen in turkey farming. They found that the house fly was capable of harboring this pathogen for up to 36 hours after exposure, although they noticed a gradual decline in viable cells recovered from whole fly homogenate over time. In 2002, Nayduch *et al.* demonstrated the capacity of the house fly to harbor the enteropathogen *Aeromonas caviae* up to 8 days after feeding on an infectious dose, with the bacteria recovered increasing in numbers for up to 2 days after infection. McGaughey and Nayduch (2009) found that *Aeromonas hydrophila* was able to survive in the house fly crop and recovered viable bacteria from vomit specks. However, they found that no viable bacterial cells were excreted in feces.

***Escherichia coli* O157:H7**

Escherichia coli O157:H7 is a pathogenic strain of *E. coli* that produces verotoxin, a shiga-like toxin named for its similarity to a toxin produced by *Shigella dysenteriae* (Calderwood *et al.*, 1987). The O157:H7 strain of *E. coli* can cause bloody diarrhea and is therefore classified as being an enterohemorrhagic *E. coli* (EHEC). In addition, this strain can cause hemolytic uremic syndrome, which can lead to kidney failure in humans, with children and the elderly at a greater risk (Griffin *et al.*, 1988). This bacterium occurs naturally in the intestines of dairy cows and can be recovered from manure (Moriya *et al.*, 1999). Keen *et al.* (2003) reported that 11.4% of agricultural fair livestock tested positive for *E. coli* O157:H7 and cattle that shed *E. coli* O157:H7 can be expected to shed at least 1×10^6 CFU/g of feces (Callaway *et al.*, 2009).

A 1996 outbreak of *E. coli* O157:H7 in a Japanese nursery underscored the need for research the role of the house fly in spreading this bacteria. Analysis of the etiology of this outbreak implicated that house flies were brought the *E. coli* from the cattle farm to the nearby school, creating a bridge between the two sites (Moriya *et al.*, 1997). Iwasa *et al.* (1999) sampled 4 different farm locations in Japan and confirmed that the house fly was capable of harboring *E. coli* O157:H7 under natural conditions by isolating the pathogen from wild caught flies. Kobayashi *et al.* (1999) and Sasaki *et al.* (2000) specifically examined the potential of the house fly to be more than just a mechanical vector of *E. coli* O157:H7. Sasaki *et al.* (2000) showed that flies fed approximately 10^9 colony forming units (CFU) of bacteria harbored cells in their intestinal tract and also continued to excrete viable bacteria up to 3 days post ingestion. Kobayashi *et al.* (1999)

reported a decline of bacterial load in the house flies over four days, from 10^6 CFU/fly to 10^3 CFU/fly.

Bacillus cereus

Bacillus cereus is a Gram-positive facultatively-anaerobic spore-forming rod, which is ubiquitous in the environment and able to survive in conditions ranging from 3 °C - 75 °C in its vegetative form (Drobniewski, 1993). This pathogen can cause abdominal cramps and diarrhea in humans. Symptoms are usually caused by ingestion of a secreted enterotoxin that can be preformed in food or produced in the small intestine (Drobniewski, 1993). An emetic syndrome due to *B. cereus* results in fever and vomiting (CDC, 1994), and is caused by a highly stable peptide produced by the bacterium during the late exponential to stationary growth phase (Drobniewski, 1993). Food contamination frequently occurs when *B. cereus* contaminates dishes that are cooled to room temperature and then reheated at a later time, such as fried rice. In addition to food borne illnesses, this organism has been associated with localized necrotic cutaneous infections, ocular infections, and in some cases even systemic infections (Drobniewski, 1993).

Early studies by Ledingham (1911) examined the potential persistence of *Bacillus* species in the house fly and through its life stages. His studies were inconclusive though they did point to the decline of bacterial load over time. There was persistence of a bacterium designated "Bacillus A", indicating the possibility of some *Bacillus* species to persist in the house fly. Banjo *et al.* (2005) demonstrated that *B. cereus* could be isolated from both the interior gut as well as the exterior of house fly maggots. Adult house flies have also been shown to harbor different species of *Bacillus*, including *B. cereus* and *B.*

megaterium (Hawley *et al.*, 1951). Unlike with *E. coli* O157:H7, the vector potential of the house fly in regards to the spread of *B. cereus* after ingestion has yet to be examined.

House Fly Alimentary Defenses

Although they are constantly exposed to any number of human and animal pathogens, house flies manage to rarely become diseased themselves, showing remarkable resilience and what must be a very efficient immune response in conjunction with multiple physical barriers against bacteria. To completely understand the role that house flies play in disease transmission, it is necessary to understand how the house fly responds to an ingested bacterial challenge, and the resulting impact of fly defenses on bacterial fate and persistence.

After ingestion, bacteria are either directed into the crop or to the midgut by the fly. If food is stored in the crop, it can be regurgitated and possibly re-consumed at a later time and directed through the proventriculus to the midgut. The midgut epithelium is protected by a type II peritrophic matrix, an acellular double layer secreted at the cardia (a section of the proventriculus), which consists of proteins, glycoproteins, and chitin microfibrils and runs the length of the midgut (Lehane, 1997). In addition to this physical barrier there also are drastic pH shifts at different areas of the midgut, from pH 3.5 to pH 8.5 which facilitate the function of various digestive enzymes (Terra, 1988). Peristalsis moves ingested materials through the midgut and food is compressed into fecal pellets or food boluses, which pass through the hindgut and out the rectum (Nayduch *et al.*, 2005). The peritrophic matrix pore size allows for the passage of molecules and digestive enzymes, but physically excludes bacteria thereby rendering them unable to contact the

midgut epithelium. This allows the house fly to excrete digestive and immune response proteins into the gut while separating bacteria from the epithelium.

All of the physical defenses described above contribute to the house fly's gut defenses, however, there are additional means by which the house fly can protect itself from ingested bacteria. Expression of antimicrobial peptides (AMPs) and their corresponding signal transduction pathways have been thoroughly studied in *Drosophila melanogaster* (Lemaitre and Hoffmann, 2007). The two major immune response pathways seen in *D. melanogaster* are the Toll pathway, which is activated in response to fungi and the Lysine-type peptidoglycan found in most Gram-positive bacteria, and the Imd pathway, activated by DAP-type peptidoglycan found mostly in Gram negative bacteria. Peptidoglycan molecules, referred to as microbe-associated molecular patterns or MAMPs, bind receptors and induce signaling pathways resulting in expression of effector molecules such as AMPs. In *D. melanogaster*, AMPs show target specificity in induction and activity: for Gram-negative bacteria, Diptericin, Attacin, Drosocin, and Cecropin; for Gram-positive bacteria, Defensin; for fungi, Drosomycin and Metchnikowin. Studies in *D. melanogaster* have provided us with a helpful basis for examining immune responses in the house fly since both species are higher dipterans.

Park *et al.* (2007) recently suggested that partial digestion of bacterial peptidoglycan with Lysozyme may be integral to triggering the Toll pathway in the hemolymph of *D. melanogaster*. Previous studies have suggested Lysozyme plays a role in defenses in the house fly gut (Terra *et al.*, 1988). Lysozyme is a broad spectrum digestive enzyme that cleaves the glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan of both Gram-positive and Gram-negative cell

walls (Ren *et al.*, 2008). Highly cross-linked Lys-type peptidoglycan may be resistant to digestion by Lysozyme, as well as Gram negative peptidoglycan as it is covered by a layer of lipopolysaccharide (LPS) (Park *et al.*, 2007). House fly Lysozyme is active at low pH (optimum pH 3.5), which makes it functional primarily in the mid-midgut region of the fly (Ren *et al.*, 2009). Lysozyme is found in both larval and adult midgut of *M. domestica* (Terra *et al.*, 1988). House fly larvae show high Lysozyme expression in midgut and low expression levels in the hemolymph as well as presence of mRNA transcripts for Lysozyme in both the midgut tissues and the fat body (Ren *et al.*, 2009).

Rationale and Significance

In order to examine the events that occur after a house fly ingests bacteria it is important to examine the expression of effector molecules proximal to the bacteria in the gut as well as the number and location of bacteria at various time points after ingestion. By knowing where the pathogens are, in what numbers they occur, and how the house fly responds to them after ingestion we will contribute to a model for how effectively the house fly can respond to ingestion of disease causing agents and provide insight into potential avenues and targets for fly control. With a better understanding of this process of pathogen ingestion, fly response, and bacterial fate, it may be possible to identify steps that can be modified by humans in order to reduce transmissibility of pathogens or cause mortality in the fly.

Hypotheses

Because of the resistance of *B. cereus* to many environmental and physiological stresses in both its vegetative form and through its ability to form endospores (Araki *et al.*, 1972) and the demonstrated ability of *E. coli* O157:H7 to pass through the house fly gut and be excreted (Koybayashi *et al.*, 1999). It is hypothesized that: 1) both *B. cereus* and *E. coli* O157:H7 will survive passage through the house fly gut and 2) the immune response to *B. cereus* and *E. coli* O157:H7 will be similar due to the fact that both species possess DAP-type peptidoglycan, even though *E. coli* O157:H7 peptidoglycan is protected by a layer of lipopolysaccharide.

MATERIALS AND METHODS

House fly rearing

House flies were reared at Georgia Southern University (Statesboro, Georgia) from a stable colony started in 2004. Flies were provided *ad libitum* water and fly food consisting of powdered egg yolk (20 %), powdered sugar (40 %), and powdered milk (40 %). The colony was maintained at 30 °C with a 12L:12D photoperiod. Larval media consisting of wheat bran and vermiculite (3:1, w/w) was saturated with tap water and placed in the breeding cage to allow for oviposition. Puparia were collected from the larval rearing dish and stored in a separate container until eclosion.

Bacterial infection of house flies

Newly emerged (2-3 days old), mixed-sex flies were used for all experiments. Eclosed flies were fed sterile 10 % fly food solution *ad libitum* on a sanitized rectangular piece of Parafilm® (40 mm × 40 mm; Fisher Scientific, Atlanta, GA, USA) for 24 h.

Then, flies were individually housed in sterile glass jars and fed 5 μ l of sterile 5 % sucrose solution on a sanitized Parafilm® square (15 mm \times 15 mm). Jars were covered with aluminum foil and flies were held at room temperature (22-25 °C) for 12 h, after which they were again provided with 5 μ l of 5 % sugar water. After 12 h, flies were immobilized by chilling them for 5 min at 0° C and transferred into new glass jars covered with foil. The flies were fasted for at least 12 h and then were placed in 30 °C incubator for ~ 2 h and afterwards the 37 °C incubator for ~1 hour to induce feeding. A 2 μ l droplet of culture of either bacterial species (described below) was fed to each individually housed fly. Time of feeding for each fly was recorded, and all flies used for experiments fed within 1 hour.

Culture of GFP-expressing *Bacillus cereus* and *Escherichia coli* O157:H7

A pathogenic GFP-expressing strain of *B. cereus* (GFP-BC) transformed with the plasmid pRS601 was obtained from Dr. Ray Schuch at Rockefeller University. Stock cultures of GFP-BC were maintained in Brain-Heart infusion (BHI) agar plates with spectinomycin and kanamycin (BHISK: 25 g/L (w/v) BHI agar powder, 250 μ g/ml (w/v) of spectinomycin sodium, 20 μ g/ml (w/v) kanamycin sulfate; Fisher Scientific, Atlanta, GA, USA). Bacteria were cultured in 50 ml of sterile BHISK broth (BHISK: 25 g/L (w/v) BHI broth powder, 250 μ g/ml (w/v) of spectinomycin sodium, 20 μ g/ml (w/v) kanamycin sulfate; Fisher Scientific, Atlanta, GA, USA) for 12 h while shaking at 30 °C after which 1 ml of the culture was inoculated into 9 ml of sterile BHISK broth and incubated at 30 °C while shaking until an OD₆₀₀ of 1.00-1.20 (\pm 0.05) was reached, which equated to a mean of $2.4 \pm 0.9 \times 10^4$ CFU/2 μ l.

Escherichia coli O157:H7 EDL 933 (EC-O157) was transformed with the plasmid pGFPuv (Clontech, Mountain View, CA, USA) which contained additional kanamycin resistance (gift from Dr. Brian Weiss, Yale University, CT, USA) to allow for dual antibiotic selection as previously described (McGaughey and Nayduch, 2009). Stock cultures of GFP-ECO157 were maintained in Luria-Bertani agar plates with ampicillin and kanamycin (LBAK: 25 g/l (w/v) LB agar powder, 100 µg/ml (w/v) of ampicillin sodium, 50 µg/ml (w/v) kanamycin sulfate; Fisher Scientific, Atlanta, GA, USA). Bacteria were cultured in 50 ml of sterile LBAK broth (LBAK: 25 g/l (w/v) LB powder, 100 µg/ml (w/v) of ampicillin sodium, 50 µg/ml (w/v) kanamycin sulfate; Fisher Scientific, Atlanta, GA, USA for 8-9 h while shaking at 37 °C after which 1 ml of the culture was inoculated into 25 ml sterile LBAK broth and incubated at 37 °C while shaking until an OD₆₀₀ of 1.00-1.20 (± 0.05) was reached, which equated to a mean of $1.8 \pm 2.6 \times 10^6$ CFU/2 µl.

Culture recovery and enumeration of bacteria from house flies

For bacterial enumeration, flies (n=20) that were fed bacteria were immobilized at 2, 4, 6, 12 and 24 h post-ingestion (h PI) by chilling at 0° C (n=5 per time point), and homogenized in 500 µl sterile 1 × PBS (per L: 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄). The homogenate was serially diluted in sterile 1 × PBS, vortexed and plated, in duplicate, on BHISK or LBAK agar for GFP-BC or GFP-ECO157 recovery, respectively. Culture plates were incubated at 30 °C for 24 h for GFP-BC and 37 °C for 24 h for GFP-ECO157, after which the CFU were enumerated. The above fly treatment procedure was replicated three times for GFP-BC and four times for GFP-ECO157. The

initial culture was serially diluted and plated on BHISK or LBAK plates to enumerate the CFU of bacteria fed to the flies. The plates were incubated at 30 °C for 24 h for GFP-BC and 37 °C for GFP-ECO157. A group of control flies (n=5 per replicate) were individually fed 5 µl of sterile 5% sucrose and plated as a negative control.

Microscopic localization of bacteria in the house fly alimentary canal

Flies (n=20 per replicate for GFP-BC; n=12 per replicate for GFP-ECO157) that fed on a 2 µl droplet of bacteria ($1.8 \pm 2.6 \times 10^6$ CFU/2 µl for GFP-ECO157 and $2.4 \pm 0.9 \times 10^4$ CFU/2 µl for GFP-BC) were immobilized by chilling them at 0 °C. Time post ingestion for dissection was 2, 6, 12 and 24 h PI for GFP-BC (n=5 per time point) and 2, 4 and 6 h PI for GFP-ECO157 (n=4 per time point). The entire alimentary canal (proventriculus, crop, midgut, hindgut and rectum) was removed by dissection and viewed with an epifluorescent microscope to localize GFP-expressing bacteria (Leitz Laborlux 12 epifluorescence microscope; Wetzlar, Germany). Images of bacteria in the alimentary canal were captured using a Leica DFC 420 digital camera (Leica Microsystems Ltd., Germany) mounted on the microscope. The location, motility and cellular integrity of bacteria were observed and recorded. This procedure was repeated three times for GFP-ECO157 and twice for GFP-BC. A group of control flies (n=5 per replicate) were individually fed 5 µl of sterile 5% sucrose, dissected, and examined via microscopy as a negative control.

Dissections and immunofluorescence microscopy

Flies were fed GFP-BC or GFP-ECO157 according to the above protocol ($8.85 \pm 6.4 \times 10^4$ for GFP-BC; $2.36 \pm 1.7 \times 10^5$ for GFP-ECO157; n=20 per replicate, 2 replicates per bacteria). At 2, 4, 6, 12, and 24 h PI, 5 flies were killed and dissected to remove the

entire alimentary canal as described above. Whole fly guts were fixed in 4% paraformaldehyde for two hours, taken through a graded series of alcohols (50%-100%), cleared in CitrisolvTM (Fisher Scientific, Atlanta, GA), were pooled by time point and embedded in Paraplast[®] plus Tissue Embedding Medium (Fisher Scientific; n=5 per time point). Serial sections 5 μ m thick were sliced from each block and affixed to slides (Superfrost[®], Fisher Scientific). Tissue sections were then rehydrated and blocked with StartingBlockTM T20 Blocking Buffer (Thermo Scientific) for one hour. Polyclonal antibodies (Genscript, Piscataway, New Jersey) were applied overnight at room temperature: rabbit anti-Cecropin (20 μ g/ml), rat anti-Lysozyme (43.2 μ g/ml), chicken anti-Defensin (5 μ g/ml), or mouse anti-Diptericin (6.69 μ g/ml). Slides were washed in 1 \times PBS (pH 7.4) for five minutes at room temperature and incubated with Alexa Fluor[®] (Invitrogen, Grand Island, New York) fluorescent secondary antibodies (2 μ g/ml) overnight at room temperature: Alexa Fluor[®] 568 goat anti-rabbit, Alexa Fluor[®] 568 goat anti-rat, Alexa Fluor[®] 488 goat anti-chicken, or Alexa Fluor[®] 488 goat anti-mouse. Slides were visualized and images captured using a Leica DFC420 microscope camera. Of specific interest were the regions of the proventriculus, crop, midgut, hindgut, and rectum. Two biological replicates were done for each species of bacteria.

As negative technical controls each group of treated slides also included two slides that were treated without primary antibody. This was in order to ensure that there was no non-specific binding of the secondary antibody when applied to the slides. As a negative biological control, a group (n=10) of adult flies were individually fed 2 μ l of sterile BHISK broth and dissected at 5 h PI. Guts were then fixed, sectioned and treated with antibodies as described above.

Data analysis

Bacterial plate counts above 300 CFU and below 30 CFU were not included in these analyses because samples above 300 CFU may not be distinguishable from one another on a plate count, and samples below 30 CFU may not be representative of the sample (Madigan, 2009). The total number of GFP-BC recovered from each fly at each h PI was log transformed, tested for normality and homogeneity of variance, and analyzed using an ANOVA test ($P < 0.05$). To further understand the differences in change in survival of GFP-BC between time points the means of each time point were compared using the Tukey-Kramer test ($P < 0.05$). The total number of GFP-ECO157 recovered from each fly at each h PI was log transformed and found to not be normally distributed. As a result, the GFP-ECO157 data was analyzed using the Kruskal-Wallis test ($P < 0.05$). To further understand the differences in survival of EC-O157 between time points the means of each time point were compared using the Dunn's test ($P < 0.05$). All analyses were performed using JMP ® 9 (SAS Institute Inc., Cary, NC, USA, 2009).

RESULTS

Bacterial Survivability in House Flies

GFP-BC culture recovery showed that viable bacteria within the fly decreased significantly from a mean of $2.47 \pm 0.98 \times 10^4$ CFU fed to $1.27 \pm 0.6 \times 10^3$ CFU, a decrease of 95% over 24 h (Fig. 1). At 2 h PI, bacterial load had decreased to a mean of $8.58 \pm 5.4 \times 10^3$, representing 34% of the dose fed, although this did not represent a significant change from the initial dose ($P=0.17$), possibly due to high variability across individual flies. By 6 h PI, mean bacterial load in the fly had decreased significantly to $3.91 \pm 2.37 \times 10^3$, representing only 15% of the initial dose ($P=0.0054$). No significant difference was seen in the average amount of bacteria recovered between 6 and 12 h PI ($P=0.90$). The bacteria present at 12 h PI represented 12% of the original fed dose, a decrease of 3% from what was found at 6 h PI. Only 5% of the fed amount of bacteria persisted at 24 h PI, but there was no significant difference between the amount of bacteria recovered between 12 and 24 h PI ($P=0.53$). No CFU of the GFP-BC were recovered from control flies fed sterile 5% sucrose.

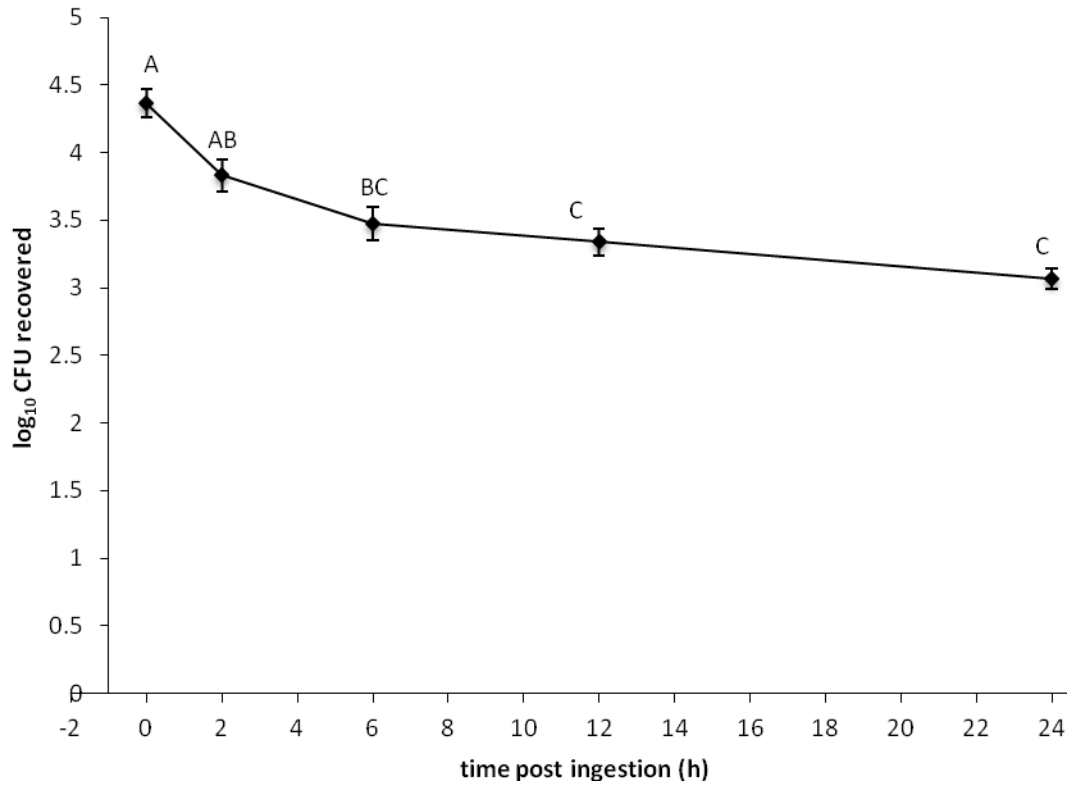


Figure 1: Recovery of GFP-expressing *Bacillus cereus* from the house fly. Flies were fed an average of $2.47 \pm 0.98 \times 10^4$ CFU. Mean recoveries are shown for 3 replicate experiments (n=15 per time point). Different letters denote significant difference between pairs ($P < 0.05$). Error bars represent standard error.

Culture recovery of GFP-ECO157 revealed a continuous decrease in bacterial load over 12 h from $2.65 \pm 1.8 \times 10^6$ to $9.83 \pm 10.9 \times 10^4$, an overall decrease of 96.3% with the final CFU recovered representing 3.7% of the initial dose fed (Fig. 2). At 2 h PI mean CFUs recovered were $1.38 \pm 1.2 \times 10^6$ (56% of initial dose), at 4 h PI $9.53 \pm 9.8 \times 10^5$ (35% of initial dose), and at 6 h PI $4.9 \pm 4.17 \times 10^5$ (18% of initial dose). The mean CFUs recovered at these time points did not differ significantly from one another ($P > 0.05$). Finally, at 12 h PI, mean CFU recovered was $9.8 \pm 10.9 \times 10^4$, which differed significantly from the amount fed, and recoveries at 2 and 4 h PI ($P \leq 0.0097$). No CFU of the GFP-ECO157 were recovered from control flies fed sterile 5% sucrose.

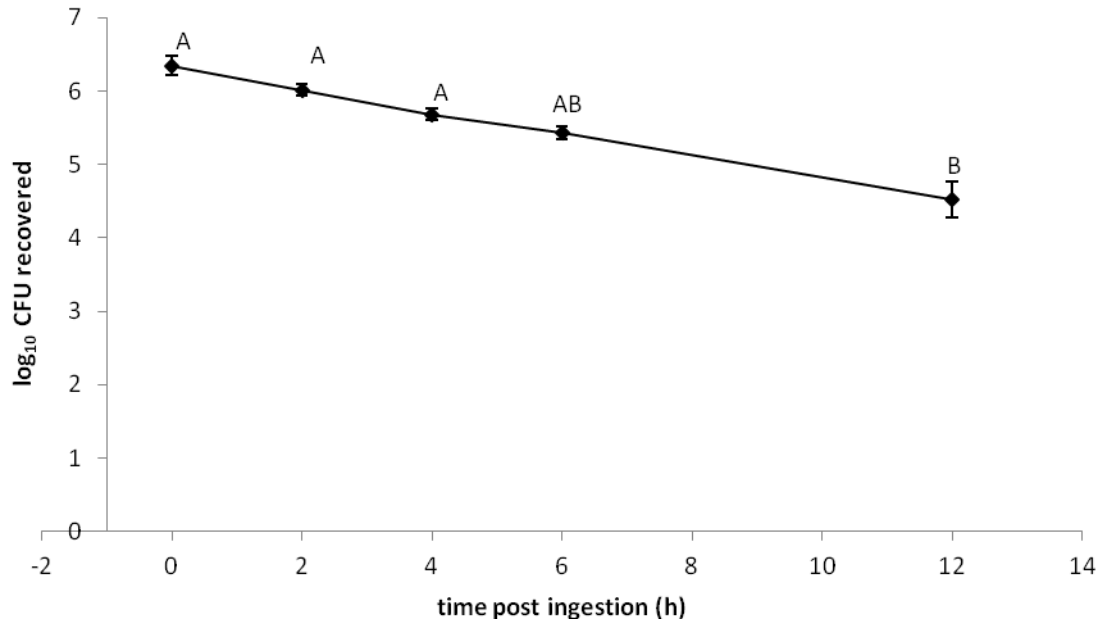


Figure 2: Recovery of GFP-expressing *Escherichia coli* O157:H7 from the house fly.

Flies were fed an average of $2.65 \pm 1.8 \times 10^6$ CFU. Mean recoveries are shown for 4 replicate experiments (n=12-16 per time point). Different letters denote significant difference between pairs ($P < 0.05$). Error bars represent standard error.

Localization of GFP-expressing bacteria

GFP-BC cells were visible in the alimentary canal throughout all time points analyzed, and in all tissues analyzed. At 2 h PI, large groups of free viable cells were visible in the crop of 3/10 flies (Fig. 3B) and in the midgut of 6/10 flies (Fig. 3A). Cells were also visible in the rectum of 1/10 flies at this time point (data not shown). At 6 h PI viable cells were visible in the crop and midgut in 6/10 flies (Fig. 3C, 3D); a large amount of free GFP was present in the midgut and rectum of three of the remaining flies, with one fly showing no presence of bacteria or free GFP. Interestingly, 5/10 flies observed at 6 h PI showed viable GFP-BC in the rectum. At 12 h PI cells were visible in fewer numbers as compared to earlier time points, cells were present in the crop and rectum of 4/10 flies with 6/10 flies showing free GFP in the midgut and rectum (not shown). At 24 h PI, 4/10 flies showed cells present in the crop at comparable levels as observed at 12 h PI, with free GFP in the rectum of 5/10 flies. At this time point 2/10 flies showed no signs of bacteria or free GFP in their alimentary canal. Localization of GFP-BC showed that viable cells were able to persist in the crop up to 24 h PI, although only in small numbers. Control slides of flies fed sterile 5% sucrose showed no presence of GFP-expressing bacteria.

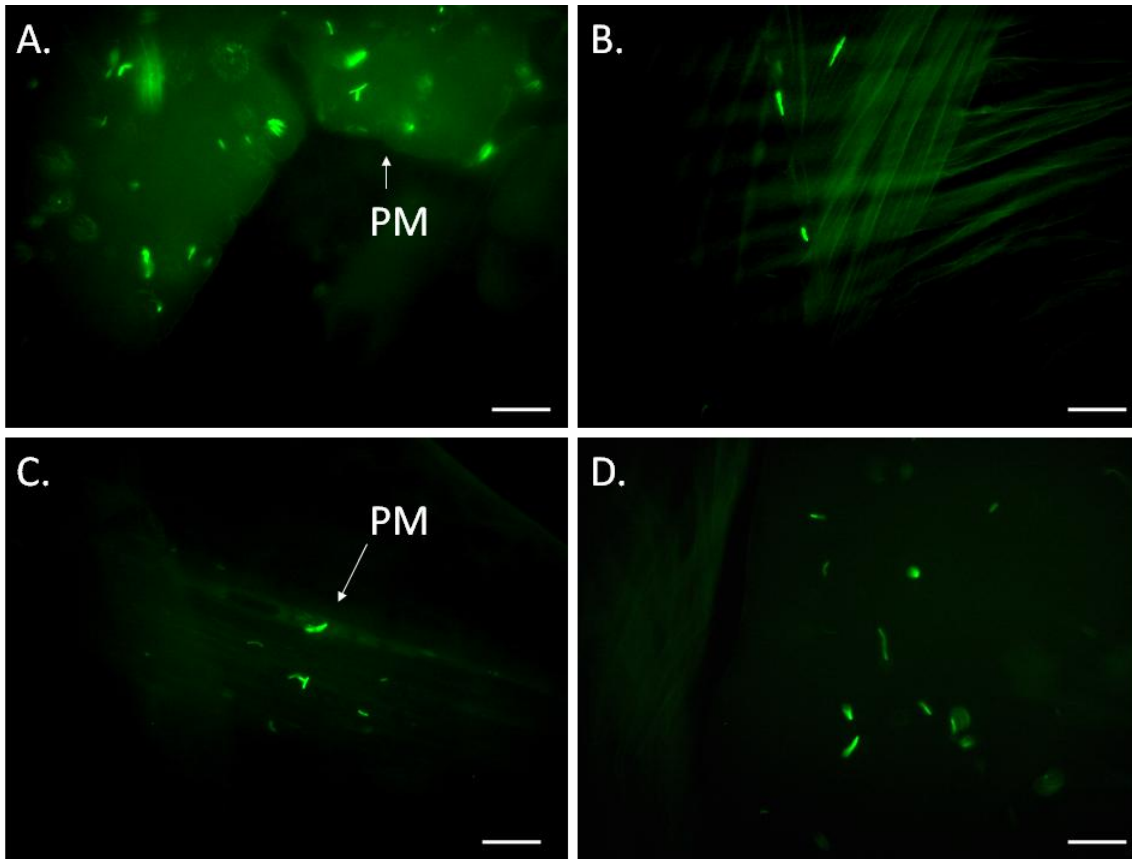


Figure 3. Viable GFP-*Bacillus cereus* cells observed in the house fly alimentary canal. GFP-expressing *B. cereus* cells (green) were visualized at 2 h PI (A, midgut; B, crop) and 6 hours post-ingestion (C, midgut; D, crop). Arrows indicate viable cells. PM refers to the peritrophic matrix. Scale bar is 10 μ m.

Viable GFP-ECO157 cells persisted in the alimentary canal up to 12 h PI, although at early time points the cells were clumped and immobilized in the PM. At 2 h PI, clumps of cells were visible in the crop of 5/8 flies and immobilized in the midgut of 7/8 flies (Fig. 4A). Viable cells were present in the rectum of 1/8 flies. At 4 h PI, food boluses containing a mixture of free GFP and immotile cells were observed in all flies, and 3/8 flies still had large numbers of cells in the crop, although they were also not motile. Immobilized cells were present in the midgut of 8/8 flies, although there was also a large amount of free GFP present (Fig. 4B). At 6 h PI, less cells were visible as compared to earlier time points, and 4/8 flies showed a range of a few cells to large numbers of cells in the crop. Bacteria were still visible throughout the midgut in all flies, and in the hindgut area of 4/8 flies (Fig. 4C). One replicate was extended to 12 h PI with no flies having cells present in the midgut and 2/4 flies showed individual cells in the hindgut at this time point. Immotile cells were visible in the crop of 2/4 flies (Fig. 4D). No GFP-ECO157 were seen in control flies fed sterile 5% sucrose.

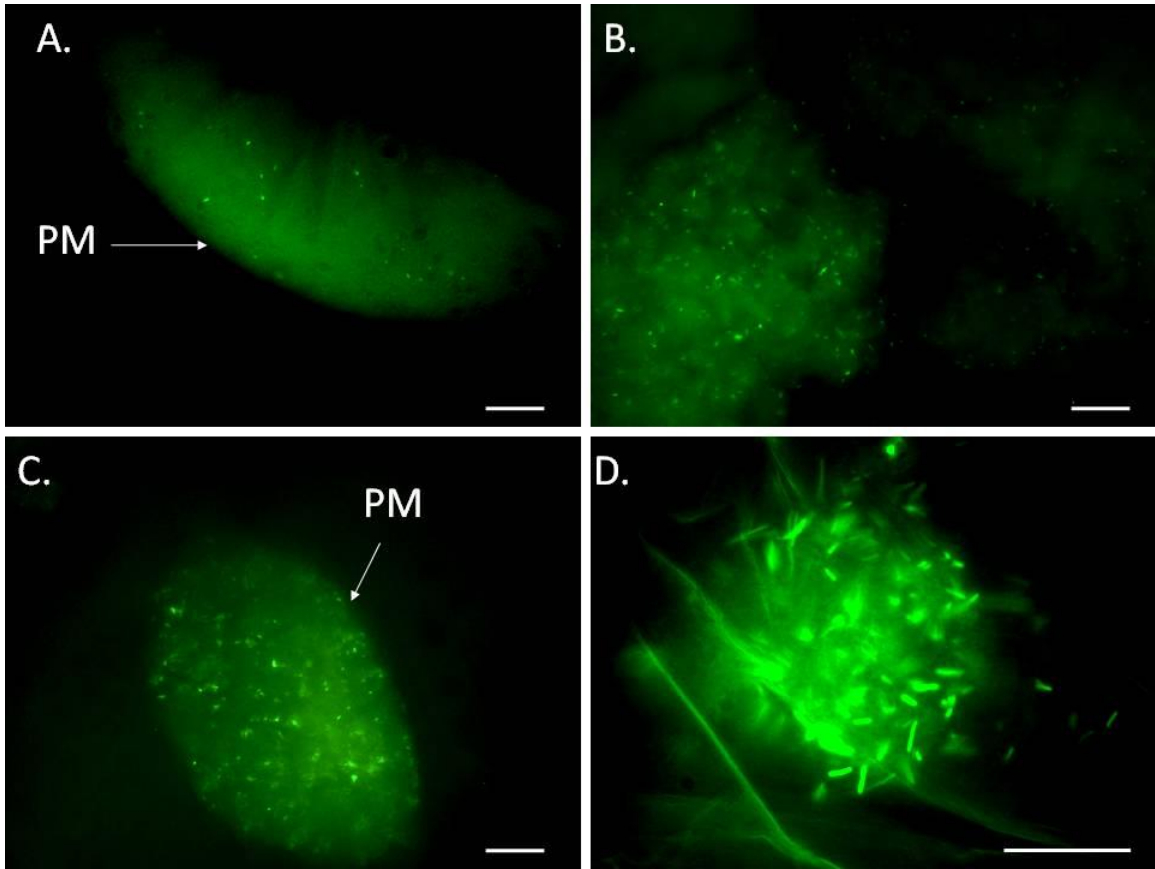


Figure 4. Viable GFP-*Escherichia coli* O157:H7 cells observed in the midgut and crop of the house fly. GFP-expressing *E. coli* O157:H7 cells (green rods) were visible in the midgut at (A) 2 h PI, (B) 4 h PI, and (C) 6 h PI. Clumps of viable cells are visible in the crop at 12 h PI (D). PM indicates the peritrophic matrix. Scale bar is 10 μ m.

Local Immune Response

Immunofluorescent staining of alimentary tissues from two replicates of flies fed GFP-BC (n=20 per replicate) was conducted and analyzed at 2, 6, 12, and 24 h PI. Five fly guts were pooled in a single block for each time point within each replicate. Analysis showed no upregulation of Cecropin and varied presence of Lysozyme, Diptericin, and Defensin across 24 h. Lysozyme was detected in the apical portion of the cells of the proventriculus as at 2 h PI in one pooled replicate. At 6 h PI, defensin and lysozyme were highly expressed in both the midgut and foregut regions of the proventriculus. Diptericin also was detected in the proventriculus at 6 h PI and was mainly expressed in the apical portion of the foregut cells in one pooled replicate. The tissue that showed the most AMP expression in both replicates across all time points was the midgut. At 6 h PI, in both replicates, Defensin was observed in basal vesicles in the midgut (Fig. 5A), and Lysozyme and Diptericin were expressed in apical vesicles (Fig. 5C, 5D). No upregulation of Cecropin was observed (Fig. 5B). At 12 h PI Defensin and Diptericin were present at lower levels than previously observed in midgut cells. Finally, at 24 h PI, Lysozyme and Defensin were observed in the midgut of both replicates with expression levels lower than had been previously observed. Only once were AMPs or Lysozyme observed in the crop, with one replicate showing Lysozyme at 6 h PI.

Immunofluorescence analysis of alimentary tissues of flies fed GFP-ECO157 was conducted in two replicates, each analyzed at 2, 4, 6, and 12 h PI. Five fly guts were pooled in a single block for each time point within each replicate. There was little detection of AMPs or Lysozyme in either replicate with exceptions at 2 and 4 h PI. Diptericin and Lysozyme were both expressed in small amounts on the apical portion of

midgut cells at 2 h PI in one replicate (Fig. 6A, 6B). Lysozyme was present in the midgut and proventriculus at 6 h PI in both the apical and basal portion of cells in one replicate (Fig. 6C, 6D). The Lysozyme observed in the proventriculus at 6 h PI appeared mostly in the midgut cells of the proventriculus.

No AMP or Lysozyme expression was detected in technical or biological controls, which indicates absence of secondary antibody non-specific binding as well as absence of protein level expression of these effectors in broth-fed control flies.

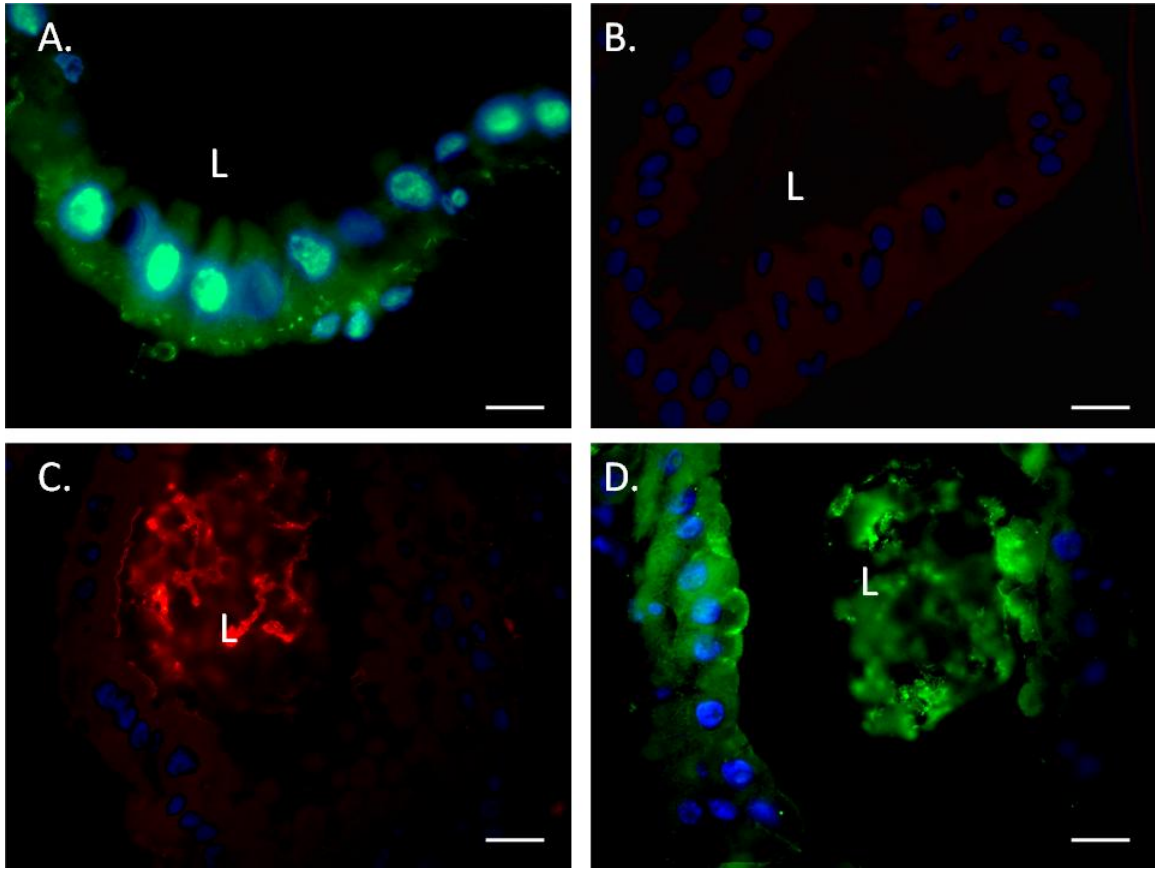


Figure 5. AMP and Lysozyme expression in the midgut of house flies after ingestion of GFP-expressing *Bacillus cereus*. House fly immune effectors Defensin (A; Alexa Fluor® 488 goat anti-chicken; green), Cecropin (B; Alexa Fluor® 568 goat anti-rabbit; red), Lysozyme (C; Alexa Fluor® 568 goat anti-Rat; red), and Dipteracin (D; Alexa Fluor® 488 goat anti-chicken; green) were detected at 6 h PI using immunofluorescence (arrows). Nuclei are stained with DAPI (blue). L indicates gut lumen. Scale bars = 10 μm .

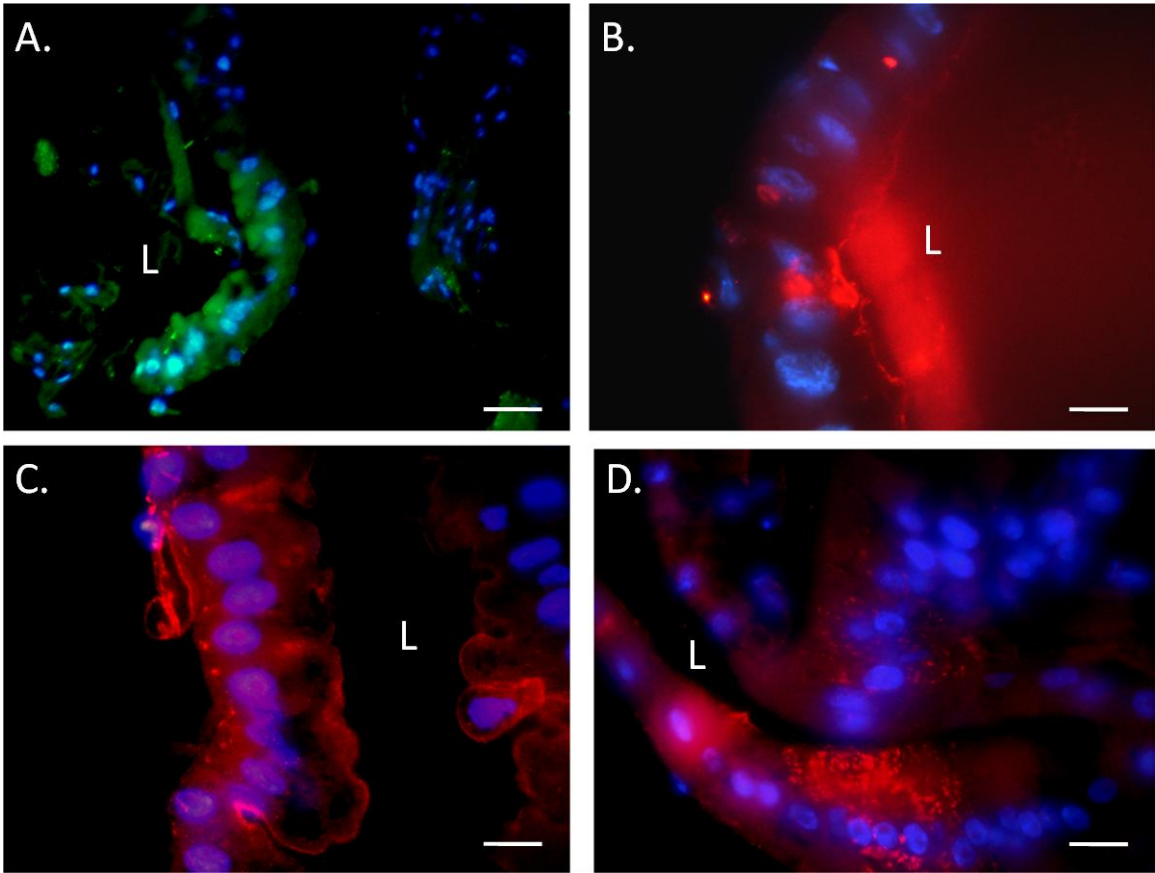


Figure 6. Dipteracin and Lysozyme expression in the midgut and proventriculus of house flies after ingestion of GFP-expressing *Escherichia coli* O157:H7. Immune effectors (AMPs and Lysozyme) were detected in the alimentary canal. Dipteracin (A; Alexa Fluor® 488 goat anti-chicken; green) and Lysozyme (B; Alexa Fluor® 568 goat anti-Rat; red) were detected in the midgut at 2 h PI. At 6 h PI, Lysozyme was also expressed in the midgut (C) and proventriculus (D). Cell nuclei stained with DAPI (blue). L indicates gut lumen. Scale bar = 10 μm .

DISCUSSION

The goals of this study were to examine the fate of either GFP-*E. coli* O157:H7 or GFP-*B. cereus* after ingestion by the house fly, and concurrently examine the house fly immune response. Fate was examined by analyzing the location of bacteria within the fly alimentary canal microscopically and assessing viability using culture-recovery methods. Immunofluorescent microscopy was used to localize temporal expression of three antimicrobial peptides (Defensin, Dipteracin, and Cecropin) and the digestive enzyme Lysozyme in the alimentary canal after bacterial ingestion. Examining where and when bacteria are located in the gut after ingestion, in what numbers, and how the fly responds to the bacteria provides insight into what happens within the fly after ingestion of a pathogenic bacteria.

The fate of GFP-BC and GFP-ECO157 shared similar aspects after ingestion by the house fly, with some notable exceptions. No bacterial propagation was observed, and both species were immobilized within the PM of the midgut and eliminated from the gut during the observation period. Overall, both species persisted in the gut for the entire time period assayed: 24 h for GFP-BC and 12 h for GFP-ECO157. However, GFP-BC showed a significant decrease from CFU fed at 6 h PI, while GFP-ECO157 did not show a significant decrease until 12 h PI. One possible reason for this observed difference in survival pattern between the two different species may be attributable to the different natural environments of *Bacillus cereus* and *E. coli* O157:H7. *Bacillus cereus* is a more ubiquitous bacterium, found widely in the environment (Drobniewski, 1993) and not specific to any area or animal. Despite its many resilient characteristics, such as

resistance to Lysozyme (Araki *et al.*, 1972) and the capacity to form endospores (Drobniewski, 1993), free GFP was visible in GFP-BC microscopy as early as 2 h PI. *Bacillus cereus* is not naturally found in animal alimentary canals, so it is likely not as resistant to the combined actions of immune secretion, drastic pH shifts, and digestive enzymes that occur in the house fly gut (Drobniewski, 1993; Terra, 1988). In contrast *E. coli* O157:H7 originates from the intestine of the cow (Moriya *et al.*, 1999), and therefore it may be well adapted to the types of physio-chemical stresses that are encountered in the fly gut. This adaptability is reflected in the observed high survivability of GFP-ECO157 in culture recovery experiments over 12 h.

Localization observations for both bacteria were similar across all time points observed. Both species of bacteria were seen in the fly rectum as soon as 2 h PI in at least one fly from each experiment, which suggests that fecal excretion of viable bacteria is possible at this time. Free GFP was visible in the midgut of two flies fed GFP-BC at 2 h PI, indicating the cell lysis was occurring even at that early time point. By 6 h PI flies fed GFP-BC and GFP-ECO157 had free GFP in the midgut, hindgut, and rectum. This widespread evidence of cell lysis in the alimentary canal at 6 h PI corresponds to the decline in numbers seen in the recovery data, as well as previously observed decline in recoverable numbers of other pathogens in the house fly after ingestion (McGaughey and Nayduch, 2009). This cell lysis could be attributed to an effective immune response, which indicates it may take up to 6 h for the fly to mount an immune response to ingested bacteria at the numbers fed here. Loss of bacteria before 6 h PI (as indicated by culture recovery) may be due to excretion of the bacteria from the alimentary canal in feces or

regurgitation from the crop. Experiments that collect excreta with concurrent culture recovery could be designed to determine loss by this mechanism.

Whether or not the flies stored consumed bacteria in the crop for later consumption or immediately directed the meal to the midgut varied from fly to fly. However, for both species at least one fly harbored bacteria within the crop at all time points. Variation in the nutritional state of the flies as they were fed the bacteria could be the cause of this, those flies that had consumed a meal immediately before isolation may have stored the bacteria in the crop. In all experiments, bacteria in the midgut were contained within the peritrophic matrix across all time points, which corresponds to observations from previous studies with other bacteria (Nayduch *et al.*, 2005), showing the effective activity of this aspect of the physical immune response on both species by preventing bacterial colonization.

Loss of bacteria via lysis, as evidenced by free GFP and declining recovery numbers, may have been at least partially mediated by the epithelial immune response. Antimicrobial peptides were expressed from the midgut epithelium in flies fed either bacterial species, and peak expression seemed to correlate with a subsequent decrease in recoverable CFUs and observed lysis. For example, immunofluorescence in flies fed GFP-BC showed the presence of Lysozyme, Diptericin, and Defensin in the midgut, with these three AMPs observed at 6 h PI (Fig. 3), the same time point with the first significant decrease in recovered CFUs (Fig. 1). Flies fed GFP-ECO157 elicited an immune response at 2 and 6 h PI, in only two tissues, the midgut and proventriculus (Fig. 4). GFP-ECO157 appears to either partially evade the immune response or the localized immune response is not sensitive to the presence of *E. coli*, as evidenced by the lack of

detected AMPs and the gradual decline of recovered bacteria from the fly over 12 h. Lysozyme is expressed after ingestion of a meal for digestion of bacteria (Terra and Ferreira, 1994) and may be responsible, along with aspects of the physical immune response, for the lysis of bacteria observed at early time points. Defensin and Diptericin are products of different known systemic immune pathways in *D. melanogaster*, Toll and Imd, respectively (Lemaitre and Hoffmann, 2007). The Toll pathway is dependent upon circulating PGRPs for activation, which would be swept away or denatured in the gut environment. The Imd pathway relies on PGRPs bound to the cell membrane, and thus is activated by MAMPs released in the gut, by cell lysis or cell division, that cross the PM. Buchon *et al.* (2009) showed that immune responses in the gut of *D. melanogaster* are regulated by the Imd and JAK-STAT pathway, but not Toll. Midgut Defensin, a Toll product, has been observed in another Muscid, *Stomoxys calcitrans* (Hamilton *et al.*, 2002) after a blood meal. Similar upregulation of Defensin has been observed in the midgut of *Glossina morsitans morsitans*, the tsetse fly, after ingestion of *E. coli* (Hao *et al.*, 2003). In *D. melanogaster*, epithelial responses are entirely mediated by IMD pathway and membrane-bound PGRPs, and therefore no Toll-associated AMPs are upregulated in the midgut (Charroux and Royet, 2010). Thus, in higher Diptera such as flesh flies, tsetse flies, stable flies and now house flies (Hamilton *et al.*, 2002; Hao *et al.*, 2003) the particular signal transduction pathway that leads to Defensin upregulation remains to be determined. Perhaps there may be cross regulation of Defensin expression via typical IMD transactivators. Alternatively another pathway activated molecules other than PGN may result in upregulation of AMPs in the gut of the higher Diptera (El Chamy *et al.*, 2008).

The lack of expression of all AMPs and Lysozyme at later time points corresponds to the loss of bacteria seen in culture recovery and microscopy. This indicates that there is likely a critical amount of bacteria, and thus a threshold amount of PGN, required to induce AMP and Lysozyme expression. As the bacterial PGN present is degraded by amidase PGRPs in the gut, the amount of PGN present declines below the threshold for induction of AMP expression (Lemaitre and Hoffman, 2007). We have recently identified regulatory PGRPs in an immune induced house fly transcriptome (Nayduch, unpublished) which may play a putative role in this type of feedback inhibition. The immune response to both species of pathogen is efficient, brief, and spatially regulated. House flies live and thrive in septic environments, so it is necessary to have such an efficient immune response to potential threats but also have a selective response in order to avoid constant immune stimulation. It seems that digestion, peristalsis, and the protection of the midgut by the PM is more than enough to ensure the elimination of the bacteria without the sustained activation of a variety of immune response genes, which may be activated as a 'backup' for the physical immune response in the event of high levels of bacteria or potential infection to the fly. The ability of the house fly to potentially 'ignore' a virulent pathogen after ingestion and simply excrete it via peristalsis through the alimentary canal with little or no immune upregulation or bacterial lysis can potentially lead to the fly transmitting that pathogen to humans via excretion. As the fly has differing responses to these two pathogens, which resulted in different fates for each, then each species of pathogen and the number of bacteria ingested of each pathogen may have a variable effect on fate of bacteria after ingestion, leading to different dissemination outcomes.

One of the goals in examining the fate of pathogens after ingestion was to assess whether or not the pathogens could be maintained over time at infectious levels within the fly after ingestion. The initial dose fed for both pathogens ($2.47 \pm 0.98 \times 10^4$ for GFP-BC; $2.65 \pm 1.8 \times 10^6$ for GFP-ECO157) was above the infectious dose to humans for each species (10^4 CFU/g for *B. cereus*; Gaulin *et al.*, 2002; 10 cells for *E. coli* O157:H7; FDA, 2009). Microscopy indicated that viable cells were present in the rectum as early as 2 h PI for both pathogens, with culture recovery from flies fed GFP-EC still showing well above the infectious dose ($1.38 \pm 1.2 \times 10^6$ CFU/fly) and flies fed GFP-BC already below the infectious dose ($8.58 \pm 5.4 \times 10^3$) at this time point. Previous studies have demonstrated the capacity of the house fly to excrete viable bacteria via vomitus within hours of ingestion (McGaughey and Nayduch, 2009) and the ability of the fly to excrete bacteria across the course of days (Greenberg *et al.*, 1970; Kobayashi *et al.* 1999; Sasaki *et al.* 2000). At 6 h PI for GFP-BC the mean CFU carried ($3.91 \pm 2.37 \times 10^3$ CFU) was below the infectious dose for this bacteria and significantly different from the fed dose (Fig. 1) indicating that the house fly may not be an important reservoir or vector in the epidemiology of this pathogen. Other studies have observed the capacity of the house fly to excrete viable *E. coli* O157:H7 in vomitus and feces (Kobayashi *et al.*, 1999; Sasaki *et al.*, 2000). Here, flies fed GFP-ECO157 maintained a level of $9.8 \pm 10.9 \times 10^4$ CFU (Fig. 2) recovered, which is still well above the infectious dose for this bacterium, so it seems very likely that a house fly ingesting this level of bacteria would be able to then excrete an infectious dose up the last time point observed, 12 h PI. Future studies should focus on the ability of the fly to excrete infectious doses of cells over time, as well as the effect of different fed doses on the fly immune response and fate of the pathogens.

This study provides insight into how the house fly responds to the types of bacterial challenges it encounters as a result of a life strategy that involves close association with septic substrates. The induction of the house fly immune response to lyse bacteria after ingestion relates directly to its potential to vector those pathogens to humans. The species specific responses by the fly and the fate of *B. cereus* and *E. coli* O157:H7 indicate that the fly is unlikely to be a potential vector for *B. cereus* and likely able to vector *E. coli* O157:H7, as they may encounter up to 1×10^6 CFU *E. coli* O157:H7/g of feces in the wild (Callaway *et al.*, 2009). Sasaki *et al.* (2000) have demonstrated the capacity for the house fly to excrete viable *E. coli* O157:H7 cells, although the dose used (10^9 CFU/ml) was much higher than the concentration of cells the fly would likely encounter in the wild, as is the dose fed here ($2.65 \pm 1.8 \times 10^6$ CFU/fly). It is unlikely for a house fly to consume large amounts of just one bacteria in the wild, so multiple species infection should be analyzed to examine its effect on the persistence of pathogens to assess vector potential under more natural conditions.

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