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Temporospatial fate of bacteria and immune effector expression in house flies (*Musca domestica* L.) fed GFP-*E. coli* O157:H7

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Abstract

House flies (Diptera: Muscidae; *Musca domestica* L.) harbor and transmit a variety of human enteropathogens including *E. coli* O157:H7. Interactions between ingested bacteria and the fly gut directly impact bacterial persistence, survival and ultimately fly vector competence. We assessed the temporospatial fate of GFP-*E. coli* O157:H7 (GFP-ECO157) in house flies along with fly antimicrobial responses for 12 h post-ingestion. In flies fed GFP-ECO157, culture and microscopy revealed a steady decrease in bacterial load over 12 h, which was likely attributable to the combined effects of immobilization within the peritrophic matrix, lysis and peristaltic excretion. However, flies can putatively transmit this pathogen in excreta because intact bacteria were observed in the crop and rectum. qRT-PCR analysis of antimicrobial peptides (AMP) and *lysozyme* gene expression showed minimal upregulation in both the gut and carcass of house flies fed GFP-ECO157. However, these genes were upregulated in fly heads and salivary glands, and effector proteins were detected in the gut of some flies. Collectively, these data indicate that house flies can serve as reservoirs of *E. coli* O157:H7 for up to 12 h, and factors in addition to AMPs and lysozyme may contribute to bacteria destruction in the gut.

Keywords

Musca domestica; antimicrobial peptides; cecropin; defensin; diptericin; immunofluorescence; lysozyme; qRT-PCR; vector

Introduction

House flies (*Musca domestica* L.) feed and breed on septic substrates, putting them in direct contact with a multitude of pathogenic microorganisms. Since house flies are synanthropic organisms, they transport these pathogens from septic environments to domestic habitats. As a result, flies have long been implicated as agents in the spread of human disease pathogens (Hawley, 1951; West, 1951; Greenberg, 1959). Despite the recognized relevance of house flies in harboring and disseminating a wide variety of infectious agents affecting humans (Graczyk *et al.*, 2001), only recently has focus shifted to elucidating the role house flies serve beyond their well-known capacity as a mechanical vector.

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The potential of house flies to serve as competent vectors for bacteria has been investigated in studies that determined the fate of bacteria and transmission potential in experimentally-infected flies. Zurek *et al.* (2001) found that bacteria-fed flies harbored the zoonotic turkey pathogen *Yersinia pseudotuberculosis* for up to 36 h post-exposure. In 2002, Nayduch *et al.* demonstrated that house flies could harbor the enteropathogen *Aeromonas caviae* up to 8 d after feeding and that flies transmitted viable bacteria in excreta. Subsequent experiments revealed that a related species, *Aeromonas hydrophila*, survived in the house fly crop and was recoverable from vomit specks, which indicated that the primary mode of transmission of *Aeromonas* was via regurgitation (McGaughey and Nayduch, 2009). More recently, the fate of *Staphylococcus aureus* in house flies was investigated (Nayduch *et al.*, 2013), and although bacteria counts declined rapidly in the fly alimentary canal, substantial numbers of this pathogen were excreted within 2 h after ingestion. Collectively, these studies demonstrate that house flies harbor a number of pathogens in the alimentary canal and can serve as significant reservoirs and transmitters of some bacterial species.

Although house flies internally harbor human and animal pathogens, they rarely become diseased themselves, showing remarkable resilience and what must be a very efficient defense response in conjunction with physical barriers against bacteria in the alimentary canal. The midgut epithelium of the house fly is protected by a type II peritrophic matrix (PM), an acellular double layer secreted by a section of the proventriculus known as the cardia (Lehane, 1997). The PM protects the epithelium from directly contacting ingested material, including microbes (Lehane, 1997). Bacteria do not traverse the PM and are sequestered therein by size exclusion, and some bacterial species appear to be immobilized within the PM by an unknown mechanism (McGaughey and Nayduch, 2009). The PM is continuous throughout the midgut and terminates at the hindgut. The hindgut and rectum epithelia are lined in cuticle, which offers protection from microbial invasion in the absence of the PM.

In addition to physical barriers, physiological defenses such as digestive processes, pH/ionic fluxes, and innate immune responses help to protect the fly gut from microbes. The dipteran innate immune system combats microbes on both a systemic (i.e. fat body) and local epithelial (e.g., the alimentary canal) level. Flies such as *Drosophila melanogaster* detect microbes when microbe-associated molecular patterns (MAMPs) like bacteria peptidoglycan (PGN) bind pathogen recognition receptors (PRRs) that subsequently activate signaling pathways of the humoral response (Lemaitre and Hoffmann, 2007). In the gut, small dimers of PGN are able to traverse the PM; therefore, PRRs on epithelial cells detect pathogens without directly contacting bacteria (Charroux and Royet, 2010). Activation of these pathways results in the expression of effector molecules including antimicrobial peptides (AMPs) (Lemaitre and Hoffmann, 2007). AMPs show target specificity in induction and activity, enabling an efficient innate immune response to invading microbes. In fruit flies, diptericin, attacin, drosocin, and cecropin target Gram-negative bacteria and defensin targets Gram-positive bacteria (Lemaitre and Hoffmann, 2007). However, in filth flies such as house flies and blow flies, defensin has demonstrated more broad-spectrum activity (Lambert *et al.* 1989; Dang *et al.* 2010). In addition to the antimicrobial activity of AMPs, the peptidoglycan-digesting enzyme lysozyme has shown extensive bacteriolytic activity in

the house fly gut (Terra *et al.*, 1988; Ren *et al.*, 2009) and is constitutively expressed when flies are in contact with large amounts in bacteria (Nayduch and Joyner, 2013).

E. coli O157:H7 is an important human pathogen commonly isolated from wild-caught house flies (Forster *et al.*, 2007). Previous studies (Kobayashi *et al*., 1999; Sasaki *et al*., 2000) have demonstrated *E. coli* O157:H7 colonization of fly mouthparts and persistence in the alimentary canal as well as transmission, but did not observe the role of fly-microbe interactions in these phenomena. Therefore, the aims of this study were to (1) determine the temporospatial fate of *E. coli* O157:H7 within house flies over 12 h and to (2) assess the concurrent temporospatial expression of immune effectors (AMPs and lysozyme) mounted by the fly after ingestion of *E. coli* O157:H7.

Materials and Methods

Bacteria culture

Escherichia coli O157:H7 EDL 933 (ECO157) was transformed with the plasmid pGFPuv (Clontech, Mountain View, CA, USA) with an additional kanamycin resistance cassette as previously described (McGaughey and Nayduch, 2009). Stock cultures of GFP-expressing *E. coli* O157:H7 (GFP-ECO157) were maintained on Luria-Bertani media (Fisher Scientific, Atlanta, GA, USA) with 100 μg/ml (w/v) of ampicillin sodium and 50 μg/ml (w/v) kanamycin sulfate (LBAK agar or broth). Prior to fly feeding, bacteria were cultured in 50 ml LBAK broth for 8–9 h while shaking at 37°C, and 1 ml was sub-cultured in 25 ml LBAK broth until an OD_{600} of 1.00–1.20 (± 0.05) was reached.

House fly rearing and bacteria feeding

House flies were reared as described, and puparia were kept in sterile glass jars until eclosion (McGaughey and Nayduch 2009). Newly emerged (2–3 day-old), mixed-sex flies were used for all experiments. Eclosed flies were fed sterile 10% (w/v) fly food solution (40% powdered sugar, 40% powdered milk, 20% powdered egg) *ad libitum* for 24 h, then transferred to individual sterile glass jars where they were maintained on sterile 5% sucrose at room temperature (22–25°C) overnight. To induce feeding, flies were fasted for at least 12 h, placed in 30°C incubator for approximately 2 h, and finally placed in the 37°C incubator for 1 h before each experiment. A 2 μl droplet of GFP-ECO157 liquid culture (described above) was placed on a small (15mm × 15mm) Parafilm® M square (Fisher Scientific) in each jar, and the flies were monitored until the entire droplet was consumed. House flies that did not feed within 30 min were discarded, and the remaining flies were maintained at room temperature for the rest of the experiment.

Enumeration of GFP-ECO157 from house flies

Flies (total $n = 76$ in four biological replicates) were individually-fed a 2 μl droplet of GFP-ECO157 (mean CFU ± SE in droplet: $2.6 \pm 0.9 \times 10^6$) and at 2, 4, 6, and 12 h post-ingestion (h PI), flies ($n = 3$ to 6 per replicate per time point) were immobilized by chilling at 0° C. A group of control flies fed sterile 5% sucrose ($n = 5$ per replicate) served as negative controls. Individual flies were homogenized in 500 μl sterile PBS (per 1 L: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, pH 7.4) and homogenate was serially diluted and plated in

duplicate on LBAK agar for GFP-ECO157 recovery. Culture plates were incubated at 37°C for 24 h. The total number of CFU recovered from each fly were $\log_{10}$ transformed and data were analyzed using Kruskal-Wallis/Dunn's Test (JMP ® 9; SAS Institute Inc., Cary, NC, USA). Bacterial plate counts above 300 CFU and below 30 CFU were excluded from statistical analyses, as is common convention (Madigan, 2009). Counts below 30 CFU are subject to estimation error, and above 300 CFU cannot reliably be counted on culture plates since there is an increased chance of merged colonies due to crowding.

Microscopic localization of bacteria in the house fly alimentary canal

Flies (total $n = 28$ in two biological replicates) were fed a 2 μl droplet of GFP-ECO157 (mean CFU ± SE in droplet: $1.8 \pm 0.9 \times 10^6$) and were immobilized by chilling at 0°C at 2, 4, and 6 h PI with one replicate extending to 12 h PI ($n = 4$ flies per time point in each replicate). The entire alimentary canal (i.e. proventriculus, crop, midgut, hindgut and rectum) was removed by aseptic dissection and viewed with an epifluorescence microscope to visualize GFP-ECO157 (Leitz Laborlux 12 epiflourescence microscope; Wetzlar, Germany). Images of bacteria in the alimentary canal were captured using a Leica DFC 420 digital camera (Leica Microsystems Ltd., Buffalo Grove, IL, U.S.A.), while also noting bacteria location, motility and cellular integrity. A group of negative control flies ($n = 5$ per replicate) were individually fed 5 μl of sterile 5% sucrose, dissected, and examined via microscopy as above to confirm no contamination with the test strain.

Temporospatial qRTPCR analysis of AMP and lysozyme expression in GFP-ECO157-fed flies

Flies (total $n = 95$ in three biological replicates) were fed 2 μl GFP-ECO157 (mean CFU ± SE in droplet: $1.40 \pm 0.30 \times 10^6$) as described above, and in each replicate 7 or 8 flies were aseptically dissected at 2, 4, 6, and 12 h PI to remove (1) the head including attached salivary glands, (2) the alimentary canal (i.e. crop, from proventriculus to rectum), and (3) the remaining carcass with fat body. LBAK broth-fed flies (n = 4) served as controls and were dissected as above. The dissected tissues were pooled for each replicate and homogenized in 500 μl TRIzol® reagent (Invitrogen, Grand Island, NY, U.S.A.). RNA was extracted using the Ambion® Ribopure™ kit (Ambion Life Technologies, Grand Island, NY, U.S.A.) following the manufacturer's protocol. One microgram of RNA was then reverse transcribed using a Quantitect® Reverse Transcription kit (Qiagen Inc., Valencia, CA, U.S.A.) following the manufacturer's instructions. After synthesis, cDNA was diluted 1:10 and used in qRT-PCR (quantitative reverse-transcriptase PCR) analysis. Each reaction was performed using the RealMasterMix SyBR ROX kit (5-Prime Inc., Gaithersburg, MD, USA) with 500 nM of sense and antisense primers of target (AMPs, *lysozyme*) and reference (*rps18*) genes (Table 1). The reactions were performed on an Mastercycler Realplex^2 (Eppendorf, Happuage, NY, USA) with the following program: 2 min at 95°C, 32 cycles of 20 s at 61°C, 15 s at 68°C, 15 s at 95°C, and a final extension for 2 min at 68°C. Negative controls were included during each run and contained all components of the reaction except cDNA template, which was replaced with DEPC treated nuclease-free water (Ambion Life Technologies).

For relative expression analysis, C_T values for calibrator conditions (i.e. flies fed LBAK broth) and treatment groups (i.e. flies fed bacteria) were analyzed using REST-MCS® software and Pairwise Fixed Reallocation Randomization Tests® (Pfaffl, 2002). Log_2 fold-change in expression ratios of the target genes *cecropin*, *defensin*, *diptericin* and *lysozyme* (*cec*, *def*, *dpt*, and *lyz*) were compared to the calibrator condition using the reference gene *rps18*. In addition, the effect of time or tissue (head, gut, carcass) on effector expression was analyzed. A Shapiro-Wilk test was performed and determined that expression ratios for *cec* and *dpt* were normally distributed ($P>0.05$) while *def* and *lyz* were not normally distributed ($P<0.05$). Therefore, ANOVA with Tukey-Kramer post hoc tests were used for *cec* and *dpt* analyses, and Kruskal-Wallis with Dunn's test was used for *def* and *lyz* analyses. All statistical tests were performed using JMP9® (SAS Institute Inc.).

Immunofluorescent detection of AMPs and lysozyme in GFP-ECO157-fed flies

Flies (total $n = 40$ in two biological replicates) were fed GFP-ECO157 (mean CFU ± SE: $2.36 \pm 0.3 \times 10^5$) as described above. At 2, 4, 6, and 12 h PI, the alimentary canal was removed ($n = 5$ flies per time point) and fixed in 4% paraformaldehyde for 2 h. Tissues were dehydrated through a graded series of alcohols (50%–100%), cleared in Citrisolv™ (Fisher Scientific), pooled by time point and embedded in Paraplast® Plus (Fisher Scientific). Serial sections (5 μm) were affixed to slides (Superfrost®, Fisher Scientific) and rehydrated and blocked with StartingBlock™ T20 Blocking Buffer (Thermo Scientific) for one hour. Slides were incubated individually in one of four polyclonal antibodies overnight at room temperature (RT) (Genscript, Piscataway, NJ, U.S.A.) at the following concentrations in 0.1% bovine serum albumin (BSA): rabbit anti-cecropin (20 μg/ml), rat anti-lysozyme (43.2 μg/ml), chicken anti-defensin (5 μg/ml), mouse anti-diptericin (6.69 μg/ml). Slides were washed in PBS (pH 7.4) for 5 min at RT and incubated with appropriate Alexa Fluor® (Invitrogen) fluorescent secondary antibodies (2 μg/ml) overnight at RT: 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. Technical controls consisted of at least two slides per time point that were incubated with secondary antibodies only. Biological controls were adult flies (n=10) that were individually-fed 2 μl of sterile LBAK broth and dissected at 5 h PI and processed as above. Images were captured using a Leica DFC420 microscope camera (Leica Microsystems Ltd.).

Results

Enumeration of GFP-ECO157 from house flies

Culture recovery of GFP-ECO157 from whole house flies revealed a 96.3% reduction in fly bacterial load from 0 to 12 h PI, decreasing from mean CFU ± SE fed: $2.65 \pm 0.9 \times 10^6$ to $9.83 \pm 3.1 \times 10^4$ ($n = 12$), respectively (Fig. 1). The percentage of the fed CFU remaining in flies steadily declined over this time interval: at 2 h PI, 56% of the initial dose was recovered (mean CFU ± SE: $1.38 \pm 0.3 \times 10^6$, $n = 16$); at 4 h PI, 35% of initial dose (mean CFU ± SE: $9.53 \pm 2.5 \times 10^5$, $n = 15$); at 6 h PI, 18% of initial dose (mean CFU ± SE: $4.9 \pm 1.1 \times 10^5$, $n = 14$); at 12 h PI, 3.7% of the initial dose ($9.83 \pm 3.1 \times 10^4$, $n = 12$). The mean CFUs recovered from flies at 12 h PI differed from the amount fed as well as the mean CFUs recovered at 2 and 4 h PI ($P \leq 0.0097$), but other time points did not differ

significantly from one another or the amount fed in the droplet ($P > 0.05$). GFP-ECO157 was not recovered from control flies fed sterile 5% sucrose. A total of 17 flies across all time points and replicates had colony counts outside the countable range (i.e., 30–300 CFU). Of those, 3 were above the countable range and 14 below the countable range. These flies were not included in the enumerations above. Additionally, across all replicates 2 flies died during the course of the experiment, and were not included in the analyses.

GFP-ECO157 visualization in the house fly gut

Although intact GFP-ECO157 persisted in the alimentary canal up to 12 h PI, as soon as 2 h PI, aggregates of immobilized, yet intact, bacteria were visible in the crop and midgut (Fig. 2A; Table 2). In the midgut, bacteria were confined within the PM (Fig. 2). Immotile, intact cells also were observed in the rectum as soon as 2 h PI (Table 2). At 4 h PI, both free GFP (likely due to lysis of GFP-ECO157) and intact, immotile bacteria were observed in food boluses in the midgut of all flies (Fig. 2B; Table 2). Large numbers of intact cells were observed in the crop in over 50% of flies, but all bacteria were immotile.

At 6 h PI, GFP-ECO157 were visible throughout the midgut of all flies and in the crop, hindgut and/or rectum of approximately 50% of flies (Fig. 2C; Table 2). When compared to earlier observations, fewer bacteria were visible in the gut at 6 h PI, and all bacteria were nonmotile, irrespective of location (Table 2). To determine the fate of GFP-ECO157 in house flies past 6 h PI, one biological replicate was extended to 12 h. At this time point, no GFP-ECO157 were observed in the midgut of any flies (n=4), and only small numbers of immotile bacteria were visible in the hindgut and rectum of two flies, as well as the crop (Fig. 2D; Table 2).

Analysis of temporospatial expression of AMPs and lysozyme by qRT-PCR

qRTPCR analysis of AMP and *lysozyme* expression revealed that the most immune-responsive tissues in flies fed GFP-ECO157 were the head and salivary glands. In these tissues, *cec* and *lyz* were significantly upregulated from baseline expression levels at all time points ($P \leq 0.05$; Fig. 3), and likewise *def* and *dpt* were significantly upregulated at 2, 6 and 12 h PI ($P < 0.05$) but not at 4 h PI ($P > 0.05$). Interestingly, AMP and *lyz* expression in the gut and carcass was not significantly different from baseline levels except for *dpt* in the gut at 6 h PI (Fig. 3, Panel C; $P < 0.05$).

The change in effector gene expression over time was also evaluated. Unexpectedly, there was no significant change in the temporal expression of *cec*, *dpt*, *def*, or *lyz* after ingestion of GFP-ECO157 within any of the tissues examined ($P > 0.05$) despite the observed increases above baseline expression. Further, comparative analyses of gene expression across tissues, but within time points, only showed a significant difference in *lyz* expression between head/salivary glands and gut tissues at 12 h PI ($P < 0.04$; Fig. 3, Panel D).

Immunofluorescent detection of effector molecules in the house fly gut

Immunofluorescence microscopy of alimentary tissues showed the presence of effector molecules at 2 and 6 h PI in only one of the two replicates examined. Diptericin and lysozyme were both expressed on the apical portion of midgut cells in flies from one

replicate at 2 h PI (Fig. S1A, S1C). Lysozyme was detected in the apical and basal portion of the midgut and in the caudal (midgut proximal) region of the proventriculus of flies in one replicate at 6 h PI. Control house flies did not show AMP expression (Fig. S1B), however lysozyme expression was observed in the midgut (Fig. S1D), but not proventriculus of LBAK broth-fed flies.

Discussion

The current study determined that house flies can serve as short-term reservoirs for *E. coli* O157:H7 by simultaneously monitoring the location and number of GFP-ECO157 within the fly after ingestion. It was found that *E. coli* O157:H7 formed clumps, adhered to the inner PM, and that many bacteria were lysed in the gut of flies that were fed bacteria. Similar observations of immobilization, clumping and apparent adherence to the PM have been previously observed in flies fed *A. hydrophila* (McGaughey and Nayduch, 2009). However despite these effects, the current study detected intact GFP-ECO157 persisting in the crop, hindgut, and rectum of some flies up to 12 h post-ingestion, which implies that house flies could feasibly excrete by vomit and defecation.

Consistent with microscopy observations, GFP-ECO157 abundance steadily decreased within flies over the same time interval. These findings are similar to those of Kobayashi *et al*. (1999) that showed a gradual decrease of bacterial load over 4 days in flies that had been fed a comparable dose *E. coli* O157:H7. However, in Kobayashi et al. (1999), approximately 10^4 cells/fly were recovered at 24 h PI, and in our study, a similar amount of bacteria was recovered at 12 h PI. The more rapid clearing of bacteria in the current study may be attributable to the use of teneral flies in our study as opposed to fully mature adult flies in Kobayashi *et al.* (1999), as well the method of feeding a single droplet as opposed to allowing the flies to feed *ad libitum.* Since teneral flies tend to have low bacteria counts in the gut, their gut defense, digestive and immune status can likely differ from fully-developed adults. Further, feeding bacteria *ad libitum* increases the possibility of multiple small ingestion events as opposed to one large ingestion event of a single bolus, and this difference can potentially affect the temporospatial location of bacteria. It is important to point out that mean recoveries in the current study are likely an overestimate, since culture plates that were outside of the countable range (30–300 CFU) were excluded. Also, two flies died during the course of the experiment (probably due to fasting), and this resulted in 19/76 flies being excluded from the data set. Of these 19 flies, 73% had too few colonies to count (<30 CFU).

For the duration of the current study, the mean bacterial load within flies remained well above the potential infectious dose of 10–100 cells (Todar, 2008). Taken in conjunction with previous data (Kobayashi *et al.*, 1999; Sasaki *et al.*, 2000), this indicates that house flies potentially serve as significant reservoirs for this pathogen for at least 12 h. Interestingly, recent work by Schuster *et al.* (2013) has shown that house fly larvae that fed on *E. coli*-contaminated substrate carried bacteria trans-stadially, and newly-eclosed flies harbored as many as 50 CFU. The possibility of trans-stadial survival of *E. coli* O157:H7 deserves further study, as this would complicate the epidemiological picture. A more thorough

examination of how larval exposure to bacteria impacts bacterial load, persistence and dissemination in adult house flies is a focus for future studies.

Despite the steady decrease of GFP-ECO157 within the fly after exposure, the current study did not detect a robust effector response against the ingested bacteria. Although effectors were upregulated in some fly tissues, there were no significant changes of *cec*, *dpt*, *def*, or *lyz* gene expression over time. Similarly there was little observable local immune expression and the gut only showed significant upregulation of *dpt* at 6 h PI. This was reflected at the protein level with the exception of diptericin being observed at 2 h PI instead of 6 h PI, and the presence of lysozyme at levels equal to that of broth fed controls. This low level of lysozyme expression in adult flies exposed to ubiquitous bacteria has been reported previously (Nayduch and Joyner, 2013). qRT-PCR and immunofluorescence analyses indicate a lack of utilization of the effectors observed here against *E. coli* O157:H7. This is in contrast to previous experiments with *S. aureus*-fed flies (Nayduch *et al.*, 2013), which showed significant upregulation of *def* in response to bacterial ingestion. Similarly, flies fed *P. aeruginosa* significantly upregulated AMPs on both the mRNA and protein level in the gut (Joyner et al., in press). In this study, the steady decrease of ingested GFP-ECO157 can be a result of numerous other house fly gut defenses, including: lectin-mediated immobilization and/or adherence to the PM, peristalsis and excretion, shifting pH and osmolarity, production of reactive oxygen species (ROS), and/or lysis by other AMPs or digestive enzymes (Peters *et al.*, 1983; Terra *et al.*, 1988; Ryu *et al.*, 2006; Lemaitre and Hoffmann, 2007).

In contrast to the gut, the house fly head and salivary gland tissues showed significant upregulation of AMPs and *lyz* at almost all time points post-ingestion. Production of AMPs in the salivary glands has previously been observed in other species such as *D. melanogaster* (Tzou *et al.*, 2000), *Aedes aegypti* (Valenzuela *et al.*, 2002), *Anopheles gambiae* (Dimopoulos *et al.*, 1998) and *Stomoxys calcitrans* (Wang *et al.*, 2009). Though the current study could not distinguish between expression in the salivary glands and expression in the head (including mouthparts) in this study, it is possible that AMP production from these locations can contribute to the lysis of EC-O157 by being swallowed and passing into the gut and crop. The continued upregulation of AMPs in the salivary glands and head may indirectly indicate persistence of bacteria in the mouthparts of the fly after feeding, as has been previously observed up to at least 24 h post-ingestion (Kobayashi *et al.* 1999). Further protein-level analyses are needed to detect AMP production from the salivary glands, and to distinguish expression in the salivary glands from expression in the rest of the head or mouth parts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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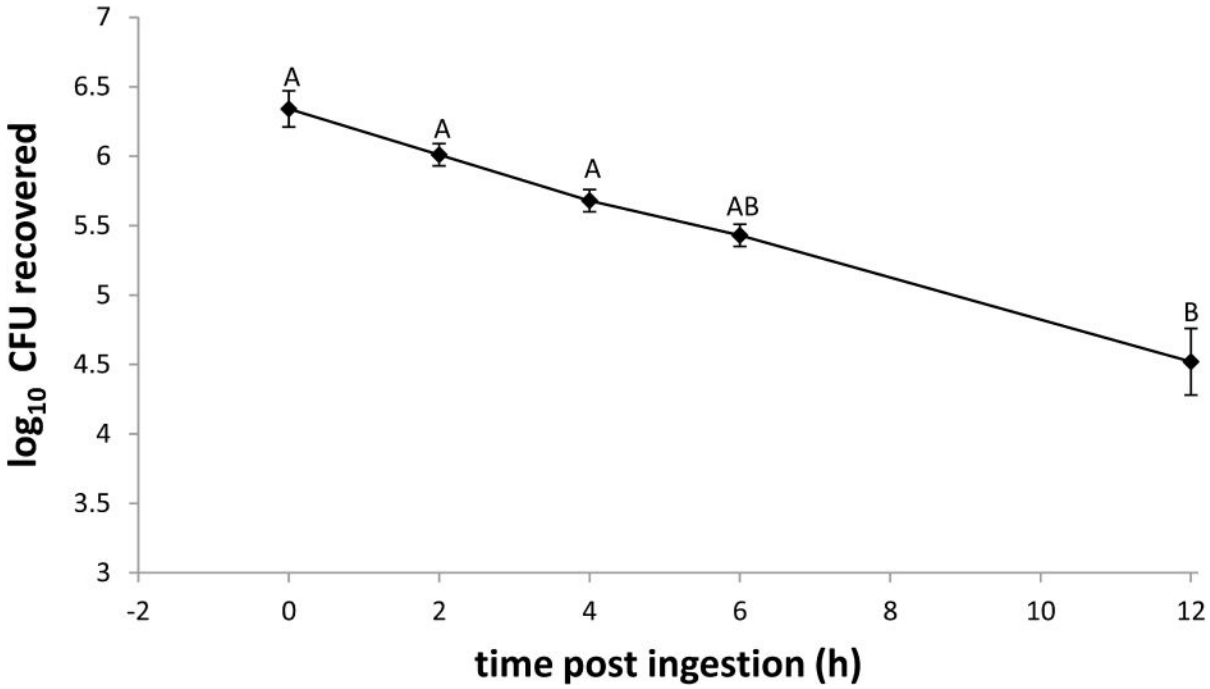

Figure 1. Recovery of GFP-*Escherichia coli* O157:H7 from the house fly

Flies (total $n = 76$ in four biological replicates) were fed an average of $2.65 \pm 0.9 \times 10^6$ CFU (mean ± SE) GFP-expressing *E. coli* O157:H7. Mean recoveries from whole house flies are shown. Different letters denote statistical significance difference ($P < 0.05$). Error bars are standard error.

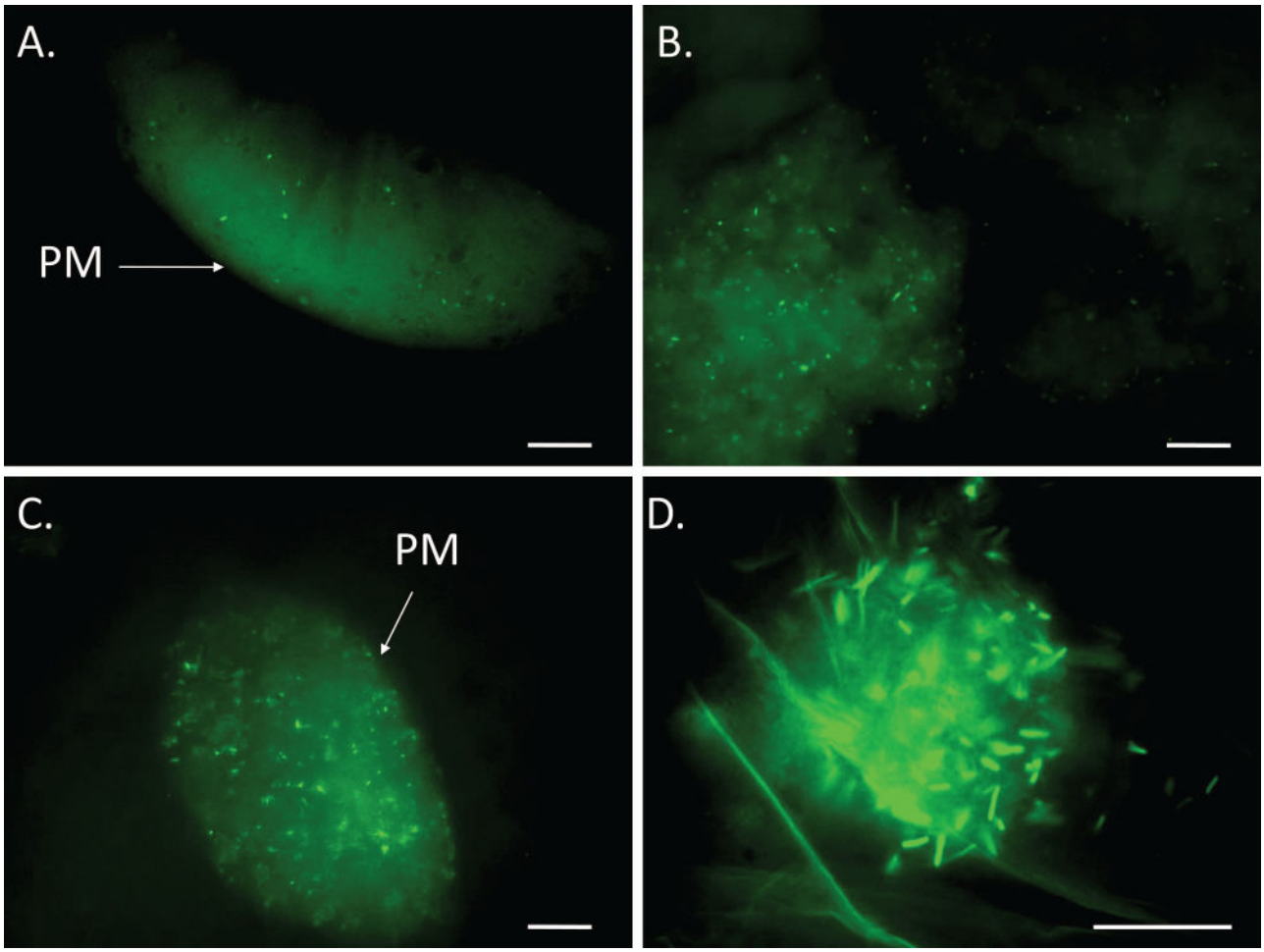

Figure 2. GFP-*Escherichia coli* O157:H7 in the alimentary canal of the house fly

House flies (total $n = 28$ in three biological replicates) were fed $1.8 \pm 0.9 \times 10^6$ CFU (mean ± SE) GFP-expressing *E. coli* O157:H7. Bacterial cells (green rods) were visible in the midgut at all time points including 2 h PI (A), 4 h PI (B), and 6 h PI (C). Clumps of intact cells were visible in the crop at all time points including 12 h PI (D) in one replicate. PM, peritrophic matrix. Scale bar is 10 μm.

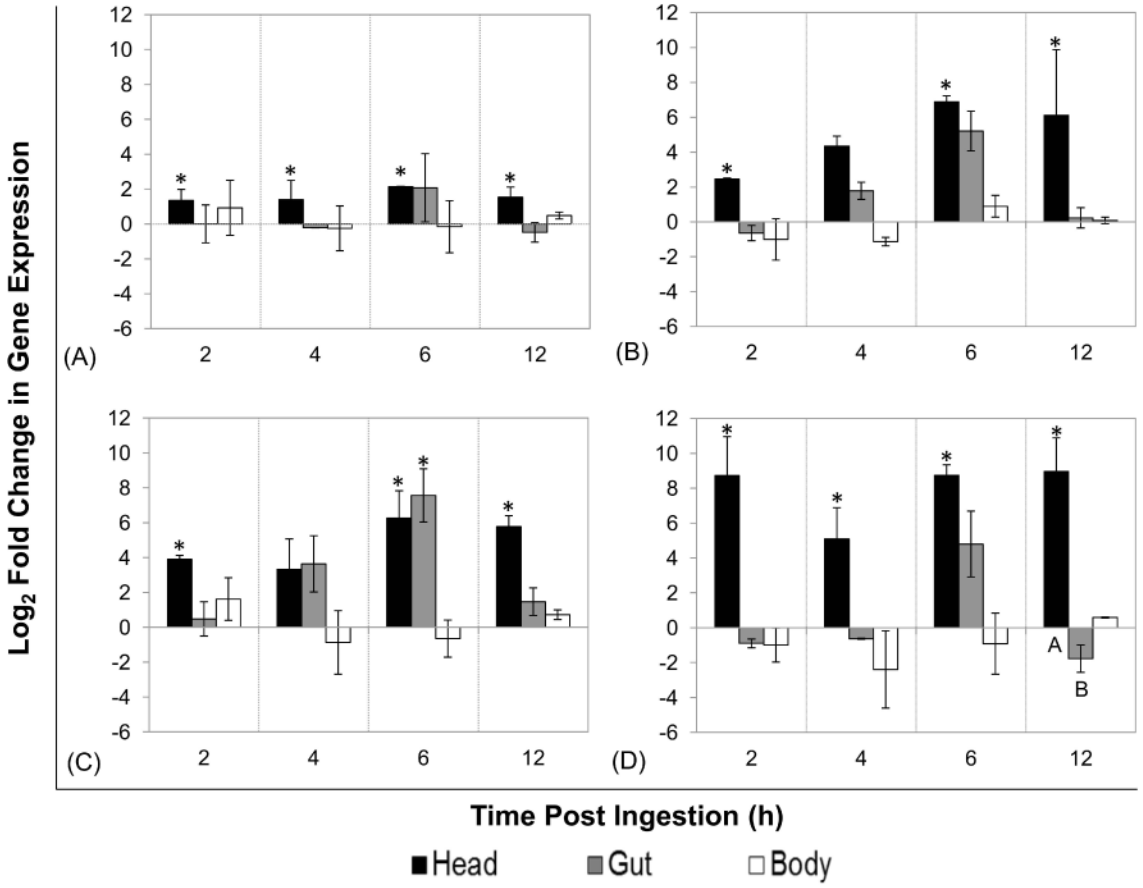

Figure 3. Expression of antimicrobial peptides and *lysozyme* in house flies after ingestion of GFP-*Escherichia coli* O157:H7

Flies (total n = 95 in three biological replicates) were fed GFP-expressing *E. coli* O157:H7 (mean CFU ± SE: 1.40 ± 0.30 × 10^6) and aseptically dissected at 2, 4, 6, and 12 h post-ingestion (n=7–8 per time point) to separate heads (with salivary glands), guts (i.e. proventriculus to rectum), and carcasses. qRT-PCR analysis was used to determine relative tissue-specific expression of *cecropin* (A), *defensin* (B), *diptericin* (C), and *lysozyme* (D) in house fly tissues (pooled per tissue within replicate). Log_2 fold change in expression was determined using the REST-MCS® software, with calibration to matched tissues from broth-fed control flies and the *rps 18* reference gene. The mean expression of three biological replicates along with standard error is shown. Different letters denote statistical significance ($P < 0.04$) between tissues within time point. Asterisks denote statistical significance from broth-fed control flies ($P < 0.05$)

Table 1

GenBank accession number, sense sequence, and antisense sequence of primers used for qRT-PCR analysis.

Gene	Accession Number	Sense Sequence	Antisense Sequence
rps18	ES608249.1	5′-GCGTGACGATTTGGAACGCTTGAA-3′	5′-TTCTTGGATACACCGACAGTGCGA-3′
cecropin	ES608437	5′-GGACAAAGTGAAGCTGGATGGTTG-3′	5′-GCTGGGCCACACCAATAGTTTGAA-3′
defensin	ES608345	5′-AAATTTCGTCCATGGAGCTGACGC-3′	5′-ACCGCTCAACAAATCGCAAGTAGC-3′
diptericin	ES608652	5′-AGTGCAACATTTGTGGTTGCCGAC-3′	5′-GCCATAACCTGCTGTGGCATCAAA-3′
lysozyme	DQ384635	5′-TCGAATGGCTCCAACGATTACGGT-3′	5′-TCCAGCCTTGTTGGGACTTGATCT-3′

Table 2

Temporal location of GFP-*E. coli* O157:H7 in the house fly alimentary canal. Time point is hours after ingesting bacteria (fed amount was $1.8 \pm 0.9 \times 10^6$ CFU (mean ± SE)), *n* is the sample size at that time point, location of bacteria indicates the number of flies with bacteria in the described region at that time point as observed using epifluorescence microscopy (Fig. 2).

		Location of Bacteria			
Time point (h PI)	***n***	**Crop**	**Midgut**	**Hindgut**	**Rectum**
2	8	5	7	0	1
4	8	3	8	0	0
6	8	4	8	4	4
12	4	2	0	2	2