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House Fly (*Musca Domestica* L.) Temporal and Spatial Immune Response to *Streptococcus Pyogenes* and *Salmonella Typhimurium*: Role of Pathogen Density in Bacterial Fate, Persistence and Transmission

Rabecca Chifanzwa

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HOUSE FLY (*MUSCA DOMESTICA* L.) TEMPORAL AND SPATIAL IMMUNE RESPONSE  
TO *STREPTOCOCCUS PYOGENES* AND *SALMONELLA TYPHIMURIUM*: ROLE OF  
PATHOGEN DENSITY IN BACTERIAL FATE, PERSISTENCE AND TRANSMISSION

by

RABECCA CHIFANZWA

(Under the direction of DANA NAYDUCH)

ABSTRACT

House flies (*Musca domestica* L), feed and breed in decomposing organic waste and therefore are constantly in contact with different species of microorganisms. Because house flies live in close proximity to human and animal habitats, they pose a danger of transmitting pathogenic microorganisms from diseased sources to new environments. To elucidate pathogen vector potential of house flies, this study investigated dose-dependent survivability of GFP-expressing *Streptococcus pyogenes* and *Salmonella typhimurium* SR11 within the fly alimentary canal both spatially via epifluorescence microscopy and quantitatively via culture-recovery. Adult house flies were fed known amounts of bacteria (high or low dose), and were dissected to remove the entire alimentary canal for microscopy or were homogenized and cultured at intervals within 24 h post-ingestion. Excreta also were cultured to determine transmission potential. Also investigated was the dose-dependent local intestinal epithelial immune response of house flies to *S. pyogenes* and *S. typhimurium*, where the upregulation of three antimicrobial peptides Defensin, Cecropin and Dipteracin were investigated. Both bacterial dose and species affected survivability of these pathogens in the house fly alimentary canal. High dose of both species survived throughout the 24 h period. The number of viable *S. typhimurium* increased in

numbers in both high and low dose, whereas *S. pyogenes* decreased in number with time progression. Viable *S. typhimurium* were recovered in large quantities from excreta as compared to *S. pyogenes*. Both bacterial species and dose also affected the temporal, spatial and class of AMP expression profiles in the gut. In *S. pyogenes*-fed flies, only Defensin was regionally produced in midgut tissue. In contrast, tissues from flies that were fed *S. typhimurium* expressed both Cecropin and Diptericin. In both species, the higher dose of bacterial challenge induced greater AMP expression than the low dose. The region of the gut showing AMP expression in both bacterial challenges was mainly the midgut, and peak expressions correlated with high numbers of bacteria as determined by culture-recovery. Dose-dependent effects on survival and transmission of bacteria from house flies has significant implications on vector potential.

INDEX WORDS: *Musca domestica*, Bacteria, Midgut, Proliferation, Excreta, Pathogens, Immune,

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## CHAPTER 1

COMPARATIVE DOSE-DEPENDENT SURVIVABILITY OF GFP-EXPRESSING  
*STREPTOCOCCUS PYOGENES* AND *SALMONELLA TYPHIMURIUM* IN HOUSE FLIES  
AND IMPLICATIONS ON VECTOR POTENTIAL

## ABSTRACT

When house flies feed on septic substrates, bacteria can be harbored internally and later transmitted via regurgitation and/or defecation. The ingested bacterial dose may affect survival within the gut and subsequently impact vector potential. This study investigated the dose-dependent fate of two human pathogens within the fly alimentary canal via epifluorescence microscopy and culture-recovery. Adult house flies were fed two doses of GFP-expressing *Streptococcus pyogenes* and *Salmonella typhimurium* SR-11 ( $10^4$  and  $10^5$  CFU for each species), and bacteria were visualized and enumerated at 2, 4, 6, 12 and 24 h post-ingestion. Bacteria also were cultured from excreta to assess transmission potential. Both bacterial dose and species affected survival within the house fly alimentary canal. Within each species, higher doses of bacteria showed significantly enhanced survivability. Although each species survived throughout the digestive tract and were cultured from excreta, over the 24 h period *S. typhimurium* proliferated up to ten times the initial dose inside the house fly and persisted in flies fed both bacterial doses, while *S. pyogenes* showed a steady decline over 24 h and eventually was eliminated from the lower dose-fed flies. In addition, flies transmitted greater numbers of *S. typhimurium* in excreta than *S. pyogenes*. We speculate that the ability of *S. typhimurium* to proliferate, survive and persist in house flies may have been bolstered by inherent resistance to bacteriolytic enzymes along with the ability to utilize motility to avoid unfavorable conditions in the gut. KEY WORDS: *Musca domestica*, bacteria, midgut, proliferation, excreta, pathogens

## INTRODUCTION

Due to their coprophagous habits, house flies (*Musca domestica* L.; Diptera: Muscidae) have long been considered vectors or transporters of pathogenic microorganisms (West 1951). House flies breed in animal wastes and decaying organic material (Greenberg and Klowden 1972), during which their external surfaces and alimentary canal become contaminated by numerous microbes. Flies move from these septic substrates to domestic environments where they alight and feed on human or animal food. Because of this indiscriminate and synanthropic feeding behavior, flies transfer contaminants from decayed and diseased sources to other environments (Butler 2010). This transfer may be solely due to dislodgement of microorganisms from their external body parts (Meerburg et al. 2007, Yap et al. 2008). However, microbes are also transferred when flies regurgitate septic crop contents to liquefy dry food substrates and facilitate ingestion, or during defecation, which sometimes occurs concurrently with feeding (West 1951). As a result of their potential to harbor and disseminate pathogenic bacteria, house flies serve as mechanical and biological vectors of microorganisms that pose a danger to human and animal health.

Numerous species of pathogenic bacteria have been isolated from house flies collected from different settings such as restaurants (Butler et al. 2010), hospitals (Fotedar et al. 1992), city streets (Rahuma et al. 2005), poultry farms (Fukushima et al. 1979) and slaughter houses (Vazirianzader et al. 2008). House flies can carry bacteria that cause diarrheal diseases including *Vibrio cholera* (Fotedar et al. 2001), *Shigella* spp. (Levine and Levine 1991) and *Salmonella enterica* (Olsen and Hammack 2000, Holt et al. 2007). An epidemic of typhoid fever outbreaks among military camps during the Spanish-American war was attributed to house flies that were transmitting *Salmonella typhi* (Cirillo 2006). House flies also were implicated as the cause of an

outbreak of colitis at a nursery school in rural Japan in 1999, where flies carried and transmitted the *Escherichia coli* O157:H7 from a nearby animal holding facility (Moriya et al. 1999). Further, experimental evidence has demonstrated that house flies can vector bacteria such as *E. coli* O157:H7 (Kobayashi et al. 1999, Talley et al. 2009), and *Yersinia enterocolitica* (Fukushima et al. 1979).

Some of the microbes that house flies carry within their bodies are pathogenic (Greenberg et al. 1970), but the house flies themselves apparently do not become diseased. Protection from microbial assault may be due to physical barriers such as the cuticle of the exoskeleton and, in the midgut, the double-layered Type II peritrophic matrix (PM), which lines and protects the epithelium (Lehane 1997). Previous studies have shown that bacteria do not escape entrapment within the house fly PM (McGaughey and Nayduch 2009). In addition, the midgut secretes digestive enzymes including amylases, lipases, proteinases, and lysozyme that function to breakdown food and hydrolyze ingested bacteria (Terra et al. 1988, Shanbhag and Tripathi 2009).

Detection of bacteria-associated molecules such as peptidoglycan by receptors on gut epithelia also can induce systemic (fat body) or local (epithelial) immune responses in insects (Kurata 2004, Cherry and Silverman 2006, Lemaitre and Hoffmann 2007, Kavanagh and Reeves 2007). Interestingly, insects show discriminatory detection of lysine-type (Lys-type) and diaminopimelic acid-type (Dap-type) peptidoglycan (PGN), stimulating either the Toll or Imd pathway of immune responses, respectively (Lemaitre and Hoffmann 2007). However, it has been proposed that only the Imd pathway operates in gut epithelial immunity, at least in the fruit fly *Drosophila melanogaster* (Zaidman-Remy et al. 2006). Despite these numerous defense mechanisms, ingested bacteria still may endure and exit the house fly alimentary canal, either via

regurgitation from the crop, or via surviving passage through the hostile midgut and exiting in feces.

Such survival and passage of bacteria from the fly directly impacts vector potential. Consequently, it is important to identify microbiological variables that differentially affect the survivability of bacteria during transit through the alimentary canal. Flies encounter variable amounts of bacteria in their environment (depending on the septic level of substrate), and the ingested dose of bacteria may influence overall survival. For instance, high doses of bacteria may induce immune responses in the gut (Zaldman-Remy et al. 2010), yet substantial numbers may still survive if microbes are resistant to these responses. On the other hand, while low doses of bacteria may not be immunostimulatory, bacteria still could be susceptible to other barriers like digestive enzymes and pH changes in the alimentary canal. In addition to dose, bacterial species may also affect survival, due to structural differences in immunostimulatory molecules (i.e., Lys-type or Dap-type PGN) and the resulting upregulation of different signaling pathways in epithelial cells. Thus, it follows that there may be both dose-dependent and species-specific effects on bacterial survival in the house fly gut. The main objective of this study was to compare the survivability and transmission of “low” and “high” doses of bacteria ( $10^4$  and  $10^5$  colony forming units, respectively) in house flies, while concurrently examining the role of species having different types of PGN. The species fed to flies were *Streptococcus pyogenes* (Lys-type PGN) which causes wound and systemic infections in humans (Bessen and Lizano 2010) and *Salmonella typhimurium* (Dap-type PGN) which is a major cause of gastroenteritis, and is also carried by domestic animals such as poultry and cattle (Oloya et al. 2009). Both species have been previously isolated from wild-caught house flies (Nmorsi et al. 2007, Wang et al. 2011). We assessed the effects of bacterial dose (within species) and species (within dose) on the

location and survival of these pathogens within in the alimentary canal, along with excretion from house flies.

## MATERIALS AND METHODS

### House Fly Rearing and Preparation

House fly pupae were collected from colonies originally established at Georgia Southern University in 2004 and were surface-sanitized by submerging them in 10% bleach solution for 5 min, followed by rinsing in sterile deionized (DI) water. Pupae were kept in a sterile closable container at 30°C until emergence. Flies were housed in individual jars and fed 5  $\mu$ l of 10% sucrose solution as a droplet on a piece of Parafilm® (Fisher Scientific, Atlanta, GA), then were fasted for 24 h after which they were fed the bacteria species and dose of interest. All house flies used in the experiments below were processed in this manner.

### Bacteria Preparation

Group A *Streptococcus pyogenes* SF370 transformed with eGFP-expressing plasmid pCM18 was donated by Dr. Vince Fischetti at Rockefeller University. Bacteria were cultured for 12 h in Brain Heart Infusion (BHI; Fisher Scientific, Atlanta, GA) broth containing 5  $\mu$ g/ml erythromycin. The culture was then diluted in sterile BHI broth with the above antibiotic concentration to an OD<sub>600</sub> of ~1.0 for the high dose or OD<sub>600</sub> of ~0.5 for low dose feeding experiments. The average amounts of bacteria fed to flies are mentioned in each of the corresponding experiments below.

*Salmonella typhimurium* SR-11 (provided by Dr. John Maurer at the University of Georgia) were transformed with 1  $\mu$ g of pGFPuv plasmid (Clontech Laboratories, Mountain

View, CA) carrying both kanamycin and ampicillin resistance genes as previously described (McGaughey and Nayduch 2009). Bacteria were cultured on Luria-Bertani (LB) agar (Fisher Scientific, Atlanta, GA) containing 50 µg/ml kanamycin and 50 µg/ml ampicillin for 48 h. Bacteria were suspended and diluted in BHI broth to an OD<sub>600</sub> of ~ 0.090 for high dose and ~ 0.011 for low dose, with actual colony forming units (CFU) fed described below.

### **House Fly Bacterial Feeding and Microscopic Examination**

To determine the spatial and temporal location of bacteria in the house fly, newly eclosed mixed sex house flies (n=30) were isolated and housed separately as described above for each experiment. Two different dosages (high and low) of each bacterial species were fed in separate experiments.

For the high dose *S. pyogenes* experiments, house flies (n=25) were fed a mean of  $3.2 \times 10^5$  CFU (SD= $1.3 \times 10^4$ ) of bacteria in 2 µl droplet placed on a piece of Parafilm®. For the low dose experiments, house flies were similarly fed a mean of  $1.4 \times 10^4$  CFU (SD= $8.0 \times 10^3$ ). Each of the experiments were replicated 3 times for a total of n=75 flies. In each experiment, 5 additional flies were fed sterile BHI broth with 5 µg/ml erythromycin for control purposes.

For the high dose *S. typhimurium* experiments, house flies (n=25) were fed a mean of  $3.3 \times 10^5$  CFU (SD= $1.7 \times 10^4$ ) as a droplet on a piece of Parafilm® for a total of 3 replicates (n=75). For low dose experiments, house flies were similarly fed bacteria a mean of  $1.5 \times 10^4$  CFU (SD= $6.1 \times 10^3$ ). Each of the experiments was replicated 3 times for a total of n=75 flies. In addition, 5 flies were fed sterile BHI broth containing 50 µg/ml each of ampicillin and kanamycin, and served as controls for each experiment.



At 2, 4, 6, 12 and 24 h post-ingestion (PI), the entire intact guts (crop, proventriculus, midgut, hindgut and rectum) were aseptically removed from bacteria fed flies (n=5) and from broth fed flies (n=1). Guts were placed on glass slides for observation of the spatial location of GFP-expressing bacteria via epifluorescence and bright field microscopy. Images were captured using Leica DFC420 digital camera system for microscopy fitted on a Laborlux 12 microscope (Leitz, Germany).

### **Enumeration of *S. pyogenes* Recovered from House Flies**

Two doses of bacteria were used for the determination of *S. pyogenes* survivability in the house flies. For the “high dose” culture recovery experiment, individual house flies (n=15) were fed a mean of  $3.5 \times 10^5$  CFU (SD= $5.9 \times 10^3$ ) per fly. The experiment was replicated 5 times, for a total n=75 bacteria-fed flies. In each replicate, at 2, 4, 6 12 and 24 h PI, 3 bacteria-fed flies were immobilized by chilling them at  $-20^\circ$  C for five minutes and processed to enumerate bacteria (described below). For the “low dose” experiment, individual flies (n=25) were fed an average of  $1.5 \times 10^4$  CFU (SD=  $2.0 \times 10^3$ ) *S. pyogenes*. The experiment was replicated three times for a total of n=75 bacteria-fed flies. In each replicate, at the same time points above, 5 bacteria-fed flies were immobilized by chilling in preparation for bacterial enumeration. In each experiment and replicate, 5 flies were fed sterile broth with appropriate antibiotics for negative control purposes. To enumerate bacteria, immobilized flies first were surface sanitized by submerging them in 10% bleach for 5 min followed by 1 min in 70% ethanol. Individual flies were homogenized in 0.5 ml sterile phosphate buffered saline (PBS) using an electric grinder, and homogenate was serially diluted in PBS and cultured in duplicate on Tryptic Soy Agar (TSA; Fisher Scientific, Atlanta, GA) containing 10% sheep blood and 5  $\mu$ g/ml erythromycin.

Cultures were incubated at 37° C for 24 h, after which the number of GFP-expressing viable cells recovered from each fly was enumerated.

### **Enumeration of *S. typhimurium* Recovered from House Flies**

Like the *S. pyogenes* experiments above, two doses of bacteria were used for the determination of *S. typhimurium* survivability in house flies. For *S. typhimurium* “high dose” culture recovery experiments, individual house flies (n=25) were fed an average of  $4.1 \times 10^5$  CFU (SD= $9.0 \times 10^4$ ) bacteria; the experiment was replicated 3 times, for a total n=75 bacteria-fed flies. For the “low dose” experiment, individual house flies (n=25) were fed an average of  $1.5 \times 10^4$  CFU (SD= $2.9 \times 10^3$ ) *S. typhimurium*. The experiment was repeated 3 times, for a total of n=75 bacteria-fed flies. Five flies were fed sterile broth (per dose/replicate) and served as negative controls. In each replicate, bacteria-fed flies (n=5) were collected at 2, 4, 6, 12, and 24 h PI and processed as described above. Homogenate was serially diluted in and cultured in duplicate on LB agar containing 50 µg/ml kanamycin and 50 µg/ml ampicillin. Cultures were incubated at 37° C for 24 h. Colony forming units were enumerated for estimation of viable *S. typhimurium* recovered from each fly.

### **Assessment of Viable Bacteria from House Fly Excreta**

To obtain quantitative data on the transmission of bacteria by house flies, three experiments were conducted for each bacterial species. For all experiments, house flies were individually housed in sterile glass jars and kept at room temperature (~22° C). For each replicate of each species, individual house flies (n=10) were fed either an average of  $1.7 \times 10^5$  CFU (SD= $1.2 \times 10^3$ ) *S. pyogenes* or  $2.9 \times 10^5$  (SD= $1.0 \times 10^5$ ) CFU *S. typhimurium* in BHI broth containing appropriate antibiotics. All flies were fed 2 µl sterile 10% sucrose solution at 2 and 4

h after bacteria ingestion to induce peristalsis and excretion. In each experiment, continuous visual observation of the flies was made to check for excretion, for a total period of 6 h. When excretion was observed (either vomit or feces) each freshly deposited excreta speck was collected from the jars using an inoculation loop. The loop of excrement was suspended in 100  $\mu$ L PBS which was then cultured on appropriate selective media. Cultures were incubated at 37<sup>o</sup> C for 24 h, and viable CFU were enumerated.

### **Statistical Analyses**

The variables used in our statistical analyses were change in survival and percent survival. Change in survival was determined by subtracting the amount bacteria fed from the amount of bacteria recovered at each time point and percent survival was calculated as the number of CFU recovered divided by initial dose fed, multiplied by 100. Although these values clearly are related to each other, we were interested in using both measurements of survival since they give different insights into vector potential and account for the ingested dose in separate ways. Since the data were not normally distributed, all analyses were performed using Mann-Whitney U tests. We determined the effects of dose within species and of species within dose by pairwise comparisons. To determine the difference in number of viable bacteria over the collection period, percent survival was compared between time points within each species and dose. Analyses were performed using JMP statistical software, (SAS Institute Inc., 2001).

## **RESULTS**

### ***Streptococcus pyogenes* Microscopical Observation**

At 2 h PI, viable cells were microscopically observed in the crop and midgut of house flies that were fed *S. pyogenes*. In all 15 flies that were observed, bacterial cells were in chains of

at least eight to twelve cells in flies fed both low and high doses of bacteria (Fig. 1, a and b). At 4 h PI, bacteria continued to persist in chains greater than four cells in the crop and midgut in all of the 15 flies observed (Fig. 1c) in each of the experiment (high and or low dose feeding), and were confined within the PM in the midgut. At 6 h PI, *S. pyogenes* cells were seen as individual cocci or diplococci, and were present in the crop, midgut and posterior midgut near the hindgut junction in 14 of the 15 flies observed in high dose fed flies (Fig. 1d). Similar observations were made in 7 of the 15 flies in low dose experiment. Cells also appeared to be adhered to PM in the midgut at this time. Qualitative observations throughout the experiment indicated that the number of cells had visibly decreased as time progressed. At 12 and 24 h PI, viable cells only were observed in flies that had been fed the high dose of bacteria. At 12 h PI, few individual cells were observed in the midgut and hindgut of 9 of the 15 bacteria-fed flies that were dissected. At 24 h PI, a few individual cells were observed in the hindgut of 7 of the 15 flies, but in lower density as compared to high dose experiment. However, high levels of auto-fluorescence of the hindgut tissue coupled with low density of *S. pyogenes* cells made it difficult to conclusively assess the presence of viable cells in this region at these late time points. Progression of cells was remarkably similar during early time points (2-6 h) in flies fed both high and low doses of bacteria. No GFP-expressing *S. pyogenes* cells were observed in control flies fed sterile broth.

### ***Salmonella typhimurium* Microscopical Observation**

In the high dose *S. typhimurium* experiment, viable bacteria were consistently observed in the crop (Fig. 2a) and in the midgut at 2 h PI in all 15 bacteria-fed flies. At 4 h PI, viable bacteria were observed in the entire gut which included crop, anterior midgut (Fig. 2b), hindgut and rectum (Fig. 2c) in all 15 flies that were dissected. The number of visible cells appeared to

increase (Fig. 2b) as compared to observations at 2 h PI (not shown). Similar observations were noted in flies observed at 6 h PI, with actively motile aggregates of bacteria being seen in the midgut (Fig. 2d) in all 15 flies observed. At 12 h PI, bacteria were observed in all regions of the gut in all 15 flies; however, the number of bacteria visually appeared to be less than those in earlier observations. At all time points, bacteria were observed to be trapped within the inner PM of the midgut (Fig. 2e), yet the majority of cells were highly motile within the lumen. In all 15 flies observed at 24 h PI, viable and highly-motile *S. typhimurium* were seen throughout the gut. The number of cells visually appeared to have increased as compared to the 12 h observation.

We also observed a similar pattern in the low dose bacteria-fed flies during early time points (2-6 h PI). At 2 h PI, *S. typhimurium* cells were observed in the crop and midgut in all 15 flies observed. At 4 h PI, bacteria were observed in the entire gut (crop, midgut, hindgut and rectum) and were actively motile in all of 15 flies observed. At 6 h PI, bacteria were seen in all regions of the gut and visually appeared to have increased in number as compared to previous observations in all 15 flies. At 12 h PI, bacteria were present in all regions of the gut of all 15 flies. At 24 h PI, bacteria were also present in the entire gut in all 15 flies observed, but cells visually appeared to be fewer than in earlier observations (midgut is shown in Fig. 2f). No GFP-expressing *S. typhimurium* were observed in guts of control flies that had been fed sterile broth.

### ***Streptococcus pyogenes* Culture Recovery**

Culture recovery experiments supported the microscopical observations made above. Statistical analyses included pairwise comparisons of each time point change in survival with the initial dose fed. Throughout the 24 h period, the number of viable cells continued to significantly decline (Fig. 3). In the high dose experiment, flies that were fed an average of  $3.5 \times$

$10^5$  CFU showed an initial decline in bacterial load, with an average of  $2.4 \times 10^5$  CFU (SD= $4.2 \times 10^4$ ) being recovered 2 h PI, (~32% reduction,  $U=135$ ,  $P < 0.0001$ ). At 4 h PI, an average of  $1.2 \times 10^5$  CFU (SD= $4.5 \times 10^4$ ) were recovered, which represented ~66% of the initial dose. At 6 h PI, the average CFU recovered was  $6.3 \times 10^4$  (SD= $3.9 \times 10^4$ ) a number that is approximately one order of magnitude less than the initial dose (~82% reduction,  $U=120$ ,  $P < 0.0001$ ). The steady decline continued, with an average of  $2.4 \times 10^4$  CFU (SD= $5.8 \times 10^3$ ) recovered at 12 h PI (~6% of the initial dose,  $U=120$ ,  $P < 0.0001$ ) and at 24 h an average of  $7.4 \times 10^3$  CFU (SD= $4.4 \times 10^3$ ) which represented ~ 2% of initial dose ( $U=120$ ,  $P < 0.0001$ ).

For the low dose experiment, house flies ingested an average of  $1.5 \times 10^4$  CFU (SD= $2.0 \times 10^3$ ) *S. pyogenes*. At 2 h PI, an average of  $8.8 \times 10^3$  CFU (SD= $6.0 \times 10^3$ ) were recovered representing ~42% reduction of the initial dose ( $U=135$ ,  $P < 0.0001$ ). At 4 h PI an average of  $1.9 \times 10^3$  CFU (SD= $1.4 \times 10^3$ ) were recovered representing ~88% reduction of the initial dose, ( $U=120$ ,  $P = 0.0001$ ). The average *S. pyogenes* recovery at 6 h PI was  $4.8 \times 10^2$  CFU (SD= $8.9 \times 10^2$ ) representing ~3% of the initial dose remaining ( $U=120$ ,  $P < 0.0001$ ). At 12 h PI, in all replicates combined, we recovered viable cells from only 3 flies and those were too few to count for statistical analysis. There were no viable *S. pyogenes* cells recovered from any flies fed the low dose of bacteria at 24 h PI (Fig. 3).

### ***Salmonella typhimurium* Culture Recovery**

Culture recovery experiments supported the microscopical observations made above, with the pattern of *S. typhimurium* survivability being markedly different to *S. pyogenes* (Fig. 3). Statistical analyses included pairwise comparisons of change in survival at each time point to the initial dose fed. For high dose experiments, flies were fed an average dose of  $4.1 \times 10^5$  CFU

(SD= $9.0 \times 10^4$ ). At 2 h PI, a mean of  $5.6 \times 10^5$  CFU (SD= $1.4 \times 10^5$ ) was recovered, which accounts for ~36% increase as compared to the initial dose of bacteria that were fed ( $U=270$ ,  $P < 0.0089$ ). By 4 h PI,  $1.5 \times 10^6$  CFU (SD= $2.1 \times 10^6$ ) were recovered, which is an increase of greater than three and a half times the initial dose, ( $U=345$ ,  $P < 0.0001$ ). At 6 h PI, an average of  $2.6 \times 10^6$  CFU (SD= $2.4 \times 10^6$ ) were recovered, which represents a greater than 6-fold increase from the initial dose of bacteria that were fed, ( $U=330$ ,  $P < 0.0001$ ). This was followed by a decrease in the average number of viable cells at 12 h PI, where  $4.7 \times 10^5$  CFU (SD= $2.4 \times 10^5$ ) were recovered, which represents ~14% more CFU than the initial dose ( $U=240$ ,  $P < 0.7701$ ) but an 82% decrease as compared to enumerations from the previous time point. However, this was followed by an increase in number of recovered bacteria at 24 h PI, where an average of  $2.0 \times 10^6$  CFU, (SD= $2.1 \times 10^6$ ), which represents almost 5-fold increase from the initial dose ( $U=310$ ,  $P < 0.0013$ ), and also ~4-fold increase compared to the previous collection period.

For low dose experiments, flies were fed a mean of  $1.5 \times 10^4$  CFU (SD= $2.9 \times 10^3$ ). The average viable cells recovered at 2 h PI was  $6.2 \times 10^4$  CFU (SD= $9.0 \times 10^4$ ) representing a 4-fold increase in bacteria compared to the initial dose ( $U=345$ ,  $P < 0.0001$ ). At 4 h PI, the average number of viable cells recovered was  $1.6 \times 10^5$  CFU (SD= $2.1 \times 10^5$ ), which represents a ~10 fold increase compared to the initial dose ( $U=345$ ,  $P < 0.0001$ ). At 6 h PI, recoveries were similar to those at 4 h PI, where the number of bacteria recovered averaged  $1.5 \times 10^5$  CFU (SD= $1.1 \times 10^5$ ;  $U=345$ ,  $P < 0.0001$ ). However, at 12 h PI, the number of bacteria recovered decreased to a mean of  $9.9 \times 10^4$  CFU (SD= $1.6 \times 10^5$ ), representing 6-fold increase from the initial dose, ( $U=290$ ,  $P < 0.0173$ ), but a 34% decrease from the previous count. At 24 h PI, a further decrease in number of viable cells occurred, with a mean of  $1.8 \times 10^4$  CFU (SD= $1.2 \times 10^4$ ) being recovered, representing a 15% increase from the initial dose but was not statistically

different to the initial dose fed, ( $U=255$ ,  $P < 0.3583$ ). This recovery represented ~81% decrease from the previous count (12 h PI; Fig. 3).

### **Statistical Analyses of Effect of Dose and Species on Bacterial Survival**

For *S. pyogenes*, Mann-Whitney U test pairwise comparisons using change in survival data showed that dose significantly affected survival of bacteria at all time points (Table 1). Similarly, when tested using percent survival, dose significantly affected survival of bacteria at all time points except 2 h PI ( $P = 0.5335$ ). For, *S. typhimurium*, Mann-Whitney U test pairwise comparisons showed that dose significantly affected survival of bacteria at all time points (Table 1). Using the same analysis of percent survival, dose also significantly affected the survival of bacteria at 2 h PI ( $P = 0.0130$ ) and 4 h PI ( $P = 0.0045$ ) but not at other time points.

We also performed Mann-Whitney U test pairwise comparisons of change in survival across species and within dose at each time point. Analyses showed that bacterial species significantly affected the change in survival within similar doses (Table 1). These results show that the two bacteria species differ significantly in their temporal survivability patterns inside the house fly alimentary canal. For example, in high dose fed flies at 6 h PI, about a fifth of the initial dose of *S. pyogenes* was recovered, while in house flies that were fed *S. typhimurium*, bacteria increased in number to almost 6 times the initial dose ( $P < 0.0001$ ). At 24 h PI, in low dose experiments, there were no bacteria recovered in *S. pyogenes* fed flies, but in *S. typhimurium* fed flies, bacteria continued to persist in all flies.

### **Enumeration of Viable Bacteria from Excreta**

Since very few *S. pyogenes* survived in flies fed the “low” dose of bacteria (presented above), bacteria were enumerated from excreta only from house flies fed “high” doses of either



bacterial species. House flies (n=10, replicated three times) ingested a mean of  $1.7 \times 10^5$  (SD= $7.0 \times 10^4$ ) CFU *S. pyogenes* and were continuously observed for excretion for a 6 h period. Viable *S. pyogenes* cells only were recovered from excreta in 7/30 flies. In the first experiment, viable bacteria were cultured from 3 different flies during the entire 6 h collection period, amounting to 3, 5 and 10 CFU from each fly, respectively. These were obtained 5 h PI from single defecations from each fly. In the second experiment, bacteria were recovered from single defecations from two different flies: 30 CFU were obtained 3 h PI, and 10 CFU were obtained 5 h PI. In the third experiment, two flies excreted viable *S. pyogenes* at 4 h PI; one fly shed 19 CFU and the other fly shed 12 CFU.

We also collected and cultured *S. typhimurium* from house fly excreta in 3 different experiments for a total of 30 flies (n=10/replicate). House flies were fed an average of  $2.9 \times 10^5$  CFU (SD= $1.1 \times 10^5$ ). Viable *S. typhimurium* was recovered from house fly excreta at various time points in the 6 h collection period (Table 2). The number of CFUs cultured from single defecation or vomitus specks varied widely among flies (range = 2-180 CFU). Viable *S. typhimurium* was cultured from 18/30 flies, with 10/18 flies excreting bacteria more than once during the 6 h observation period. Although flies excreted at various times throughout the collection period, we noted that the largest amount of *S. typhimurium* was excreted at approximately 3 h PI. These results support our microscopical data above, where viable *S. typhimurium* was observed in the rectum at 4 h PI (Fig. 2c).

## DISCUSSION

The primary objective of this study was to assess the species-specific and dose-dependent effects on bacterial survivability in the alimentary canal of house flies. Our results showed that *S.*

*pyogenes* and *S. typhimurium* exhibited different survivability patterns when in the gut of *M. domestica*. A number of factors may contribute to the disparate survivability we observed for these two human pathogens in flies. Factors that would bolster survivability in the gut include the ability of bacteria to resist unfavorable conditions of the host digestive tract including digestive processes, peristalsis and hosts defense mechanisms such as the PM and innate immune response. For instance, bacteria entering the house fly gut face an onslaught of different types of digestive enzymes including amylase, proteinases and lipases secreted by midgut epithelia (Terra et al. 1988). These digestive enzymes have different optimum pH at which they efficiently work, and therefore they are variably activated along the length of the gut depending on the degree of acidity (Blahovec et al. 2006). In addition, pH varies spatially along the length of the gut, as low as 3.5 in the anterior midgut and as high as 8.0 in the hindgut (Terra et al. 1988, Chapman 1998). In the house fly midgut, ingested materials are sequentially digested by the enzymes while remaining within the PM and moved towards defecation via peristalsis (Lehane 1997). Thus, for bacteria to survive they must be either resistant to these physiological changes or employ motility to avoid unfavorable conditions.

For *S. pyogenes*, both microscopical and culture-recovery experiments showed a temporal decrease in the number of cells in both high and low dose experiments (Fig. 3). Since *S. pyogenes* are Gram-positive bacteria, their peptidoglycan (PGN) is not protected by an outer membrane, and therefore, they may be more vulnerable to lysis due to pH changes, ensuing ionic or osmotic fluctuations, and digestive enzymes as compared to Gram-negative organisms that are shielded by an outer membrane and a dense coat of lipopolysaccharide (LPS). Additionally, the peptidoglycan-digesting enzyme lysozyme, a product of the alimentary canal epithelium which serves to digest bacteria (Lemos and Terra 1991, Cançado et al. 2007), is expressed in the adult

midgut after bacteria ingestion (Joyner and Nayduch unpublished). Evidence of possible lysis included the observed single cocci or diplococci forms of bacteria in both high and low dose *S. pyogenes* feedings, where either digestive enzymes and/or lysozyme could cleave streptococci chains or even lyse individual cells within the chains. In addition, since streptococci are non-motile, passage through the alimentary canal is susceptible to the host's peristaltic movements, resulting in (1) continuous posterior movement towards the rectum and excretion and (2) bacterial cells being unable to induce negative chemotaxis to avoid digestive enzymes, pH changes or other adverse conditions in the gut. Since we could not account for our initial dose being entirely lost by excretion (i.e. recoveries of *S. pyogenes* from excreta were extremely low), it is inferred that lysis, by unknown mechanism(s) occurs and few cells survive and are moved peristaltically to the rectum. Interestingly, although *S. pyogenes* are facultative anaerobes, they are not well adapted to living in places of hypoxia such as the insect midgut, which may have thwarted their ability to proliferate and recover from lytic or peristaltic effects. Group A streptococci naturally thrive in areas of high and continuous oxygen supply such as the skin, flesh wounds, throat, blood and heart (Gibson et al. 2000).

Dose-dependent effects on bacterial fate in the alimentary canal were observed in *S. pyogenes*-fed flies. In high dose-fed flies, we observed the sharpest decline in CFU recovery between 6 and 12 h PI, while in low dose-fed flies, this decline occurred between 4 and 6 h PI. In addition, few bacterial cells persisted at 24 h PI in high dose-fed flies, and there was complete elimination of *S. pyogenes* at 12 and 24 h PI in the low dose experiment. We infer that lysis of bacteria, by either digestive enzymes, osmotic/pH changes, or possibly the epithelial immune response (discussed below for *S. typhimurium*) resulted in these observed differences, where the higher initial dose was able to persist for an extended period of time due to sheer numbers of

bacteria. This difference can be attributed to the rate at which digestive enzymes or other effectors act upon substrates (in this case, bacteria PGN). Irrespective of dose, we observed (1) a decline in recoverable bacteria with time progression and (2) the conversion of chains to diplococci or single cocci forms. This indicates that dose of *S. pyogenes* had an effect on the dynamic persistence of bacteria within flies, but not on the ultimate fate of this species.

In contrast to *S. pyogenes*, *S. typhimurium* proliferated in flies, survived throughout the alimentary canal in high numbers, and more viable cells were cultured from excreta in *S. typhimurium* fed flies than in flies that were fed *S. pyogenes*. Enhanced survival of *S. typhimurium* in house flies can be attributed to several factors. For instance, since *S. typhimurium* are Gram-negative bacteria, their cell wall is protected by an outer membrane densely covered with LPS, which has been shown to act as a physical barrier to digestive enzymes and lysozyme (Nakimbugwe et al. 2005) and to antimicrobial peptides (Papo and Shai 2005). Further, after sensing changes in the physiological environment (such as pH, osmotic or other chemical changes), *S. typhimurium* are highly motile in the fly gut and could chemotactically avoid conditions that would otherwise promote lysis (Bren and Elsenbach 2000). Motility also allows *S. typhimurium* to move counter to host peristalsis and avoid expulsion in feces. Notably, *S. typhimurium* are facultatively anaerobic enteropathogens (Schiemann and Shoper 1984) that are well adapted to living in the gastrointestinal tract of diverse organisms including mammals, reptiles, and birds (Roy and Malo 2002, van der Woude and Baumler 2004). Since digestive enzymes along with variation in intestinal pH and osmolality also exists in these vertebrate hosts, *S. typhimurium* could be naturally adapted to the relatively less complex, yet similar, dynamic conditions present in the house fly gut.

We infer that since *S. typhimurium* could putatively escape these primary defenses (digestive enzymes, lysozyme, and pH variations) they were able to initially proliferate in the house fly gut. However, we interestingly observed stabilization of the number of bacteria in the gut in both our high and low dose feeding experiments. Although the initial proliferation occurred in both dose feedings, the fold-increase and time of this proliferation differed between doses. For example, the greatest amount of proliferation in the high dose experiment occurred 6 h PI and was 6 times the initial dose, while in the low dose experiment this occurred 4 h PI and was 10 times the initial dose. Further, after these peaks in proliferation, we noted a marked decrease in each dose experiment when bacteria were recovered at the next time interval. We speculate that the epithelial immune response was activated at the peak proliferation of each species, and that the observed decrease (though notably not complete elimination) was a result of antimicrobial effector molecules.

Interestingly, bacteria-feeding flies such as house flies and *Drosophila* spp. do not have constitutively expressed immune responses in the gut, despite the fact that bacteria are normally present in their meals. Recent studies have revealed that in the gut of *Drosophila melanogaster*, circulating (i.e. not membrane-bound) peptidoglycan recognition proteins (PGRPs) modulate immune reactivity of the gut epithelium via a PGN-scavenging feedback mechanism. Immune signaling pathways such as Imd are normally stimulated by dimeric moieties of PGN, which are released either when bacteria are lysed by other mechanisms, or even when bacteria are dividing (Lemaitre and Hoffman 2007). However, circulating PGRPs with amidase function cleave the immunostimulatory tetrapeptide from Dap-PGN and therefore block constitutive immune stimulation on a dose-dependent basis. PGRP-SC1 and PGRP-SC2 have been shown to degrade peptidoglycan in *Drosophila* larvae that were infected with Gram-negative bacteria. An over

activation of the Imd pathway was observed in *Drosophila* PGRP-SC mutants after Gram-negative bacterial challenge (Bischoff et al. 2006). Similarly, PGRP-LB functions to degrade DAP-type PGN and minimize immune stimulation. *In-vitro* injection of recombinant PGRP-LB to knockdown *Drosophila melanogaster* resulted in reduced AMP expression in response to bacteria challenge (Zaldman-Remy et al. 2006). A feedback loop is established whereby (1) amidase PGRPs deactivate a limited amount of free dimeric PGN, keeping the epithelial response unstimulated then (2) bacteria multiply and the amount of PGN overwhelms the amidase PGRPs and thus is able to bind the membrane PGRPs, which activate the signaling cascade and (3) the effector molecules (such as AMPs) are expressed and released which kill bacteria presumably until populations fall below some unknown immunostimulatory level. Thus, we speculate that *S. typhimurium* was able to proliferate in the gut and initially was non-immunostimulatory; however, release of increasing levels of free peptidoglycan (which occurs during cell division) led to amidase PGRPs being saturated which subsequently allowed for epithelial immune activation. Studies of AMP gene expression in response to bacterial challenge in *Drosophila melanogaster* show that the peak immune activation was ~ 6 h post infection (Chermysh et al. 1997, Shia et al. 2009), which is consistent with the subsequent decreases in bacterial load observed in our study at 12 h PI. Interestingly, *S. typhimurium* has been shown to be resistant to AMPs secreted from their vertebrate hosts (Guina et al. 2000), so it would be intriguing to investigate if house fly AMPs are indeed bactericidal against this species.

We also noticed an increase in *S. typhimurium* numbers in high dose-fed flies at 24 h PI. As an alternative explanation to the feedback mechanism described above, the resurgence of bacterial proliferation may also be due to depletion of house fly nutritional resources. Since flies were not allowed to feed since ingestion of bacteria, it is possible that flies did not have essential

nutrients required to mount a potent immune response at these later time points. Nutritional status plays an important role to immune functioning and combating infection, as energy is needed to sustain an immune response (Harbige 1996). In contrast, in the low dose feeding experiment, bacteria decreased in number at 24 h PI. The question arises as to why *S. typhimurium* did not proliferate at late time points as was shown in high dose experiments, and whether the high dose experiments would show a subsequent decrease at later time points after 24 h PI.

In our experiments, lower numbers of surviving *S. typhimurium* at late time points may also be attributed to suppressive overgrowth by the indigenous flora. Since our flies were raised gnotobiotically, yet not aposymbiotic, transient Gram-positive and Gram-negative species of bacteria were present in the gut, including those that survive pupariation such as *Bacillus* spp. and *Pseudomonas* spp. (data not shown). The number of transient species ranged between  $10^2$  to  $10^5$  CFU in control flies, and we are uncertain if these bacteria remain present in the flies fed our test species (data not shown). Nonetheless, in our low dose experiments, the number of *S. typhimurium* may have been outnumbered by indigenous gut bacteria. Zurek et al. (2001) suggested that house fly intestinal conditions sometimes promote only certain types of microbes, and newly introduced ones are out-competed and unable to establish. Greenberg (1959) also demonstrated the effect of competing microflora in the house fly alimentary canal. In house flies that were orally co-infected with *S. typhimurium* and *Proteus mirabilis* ( $\sim 10^3$  CFU of each species), numbers of excreted *S. typhimurium* continued to decrease over time, and this species was totally eliminated from house flies within 2 d after initial exposure. In a subsequent experiment, when flies were fed a similar amount of *S. typhimurium* alone, excretion of the bacterium was noted to continue up to 8 d (Greenberg et al. 1970).

In regards to vector potential of house flies for pathogens, it is important to assess both the infectious dose of bacteria and the resulting number of organisms shed. Previous studies on wound management showed that, in acute infection,  $\sim 10^3$  CFU *S. pyogenes* can be obtained from a single swabbing of a wound (Bowler et al. 2001), which is notably below the dose fed to flies in our study. The infectious dose of *S. pyogenes* (i.e., resulting in disease) is approximately 100 CFU (Greig 2010), although this number may vary depending on the host immune condition. Although, we did not recover a considerable number of *S. pyogenes* from excreta, it is likely that in environments such as hospitals in underdeveloped countries, where sanitation conditions may be poor and flies can feed on patients' wound discharge, transmission of bacteria may occur by multiple flies alighting and feeding on open wounds.

In regards to vector potential for *Salmonella enterica* spp. (the species group to which *S. typhimurium* belongs), infected animals can shed as many as  $10^5$  CFU/g of feces (Sanchez et al. 2002). In addition, Holt et al. (2007) reported that house flies carry as many as  $10^6$  CFU *Salmonella* spp. on their bodies when they feed on *Salmonella*-contaminated substrates such as poultry feces. These studies imply that house flies may naturally ingest or encounter amounts of bacteria similar to the dose ranges fed in our study. Further, since the infectious dose of *S. typhimurium* is only 15-22 cells (Greig 2010), and flies in our study consistently shed numbers exceeding this range, house flies are likely efficient vectors and disseminators of this species of bacteria.

We demonstrated a dose-dependent survivability of the two human pathogens, *S. pyogenes* and *S. typhimurium*, inside the house fly alimentary canal. While both bacterial species faced the onslaught of defenses including lysozyme, digestive enzymes, pH changes, and possibly epithelial immunity, each species and dose (within species) exhibited significant



differences in survival. Previous studies have shown that bacterial survival in house flies differs among species. For instance, house flies that were experimentally fed *Aeromonas caviae*, a Gram-negative human pathogen, showed that bacteria were harbored in alimentary canal 24 h PI and bacteria proliferated within flies (Nayduch et al. 2002). Kobayashi et al. (1999) demonstrated survival and harboring of *E. coli*-O157:H7 by house flies for several days and viable bacteria were defecated 3 d after initial exposure. We speculate *S. typhimurium* may persist in the gut lumen for several days. Preliminary experiments have shown that  $>10^4$  viable *S. typhimurium* can be recovered by culture 54 h PI from flies that were fed a high dose of bacteria (data not shown). Further investigations will look into the length of time that *S. typhimurium* persists in the fly gut and is shed in feces. While we showed that *S. pyogenes* and *S. typhimurium* differ in their survivability in house fly gut, there is still very limited information regarding survival of other Gram-positive bacteria species in flies. Future experiments are aimed at understanding the fate of several species bacteria in house flies.

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Table 1. Effects of dose and species on bacterial survival in house flies. Mann Whitney U test was used for pairwise comparisons of the effect of dose (within species) and the effect of species (within dose) at each time point after bacteria ingestion. Analyses included change in survival ( $\Delta$  survival) and percent survival (% survival); calculations are described in the text.

		Time post ingestion (h)				
		2	4	6	12	24
<i>Effect of dose on survival within species</i>						
<i>S. pyogenes</i>	U-value	135	120	120	120	120
	$\Delta$ survival	<b><i>P</i> &lt; 0.0001</b>	<b><i>P</i> &lt; 0.0001</b>	<b><i>P</i> &lt; 0.0001</b>	<b><i>P</i> &lt; 0.0001<sup>a</sup></b>	<b><i>P</i> &lt; 0.0001<sup>a</sup></b>
	U-value	217	330	332	345	345
	% survival	<i>P</i> = 0.5335	<b><i>P</i> &lt; 0.0001</b>	<b><i>P</i> &lt; 0.0001</b>	<b><i>P</i> &lt; 0.0001<sup>a</sup></b>	<b><i>P</i> &lt; 0.0001<sup>a</sup></b>
<i>S. typhimurium</i>	U-value	321	312	298	134	335
	$\Delta$ survival	<b><i>P</i> = 0.0003</b>	<b><i>P</i> = 0.0011</b>	<b><i>P</i> &lt; 0.0070</b>	<b><i>P</i> &lt; 0.0001</b>	<b><i>P</i> &lt; 0.0001</b>
	U-value	196	164	220	208	183
	% survival	<b><i>P</i> = 0.0130</b>	<b><i>P</i> = 0.0045</b>	<i>P</i> = 0.6187	<i>P</i> = 0.3095	<i>P</i> = 0.726
<i>Effect of species on survival within dose<sup>b</sup></i>						
Sp vs. St	U-value	120	120	120	130	145
	High dose	<b><i>P</i> &lt; 0.0001</b>	<b><i>P</i> &lt; 0.0001</b>	<b><i>P</i> &lt; 0.0001</b>	<b><i>P</i> &lt; 0.0001</b>	<b><i>P</i> &lt; 0.0001</b>
Sp vs. St	U-value	120	120	120	130	145
	Low dose	<b><i>P</i> &lt; 0.0001</b>	<b><i>P</i> &lt; 0.0001</b>	<b><i>P</i> &lt; 0.0001</b>	<b><i>P</i> &lt; 0.0001<sup>a</sup></b>	<b><i>P</i> &lt; 0.0001<sup>a</sup></b>

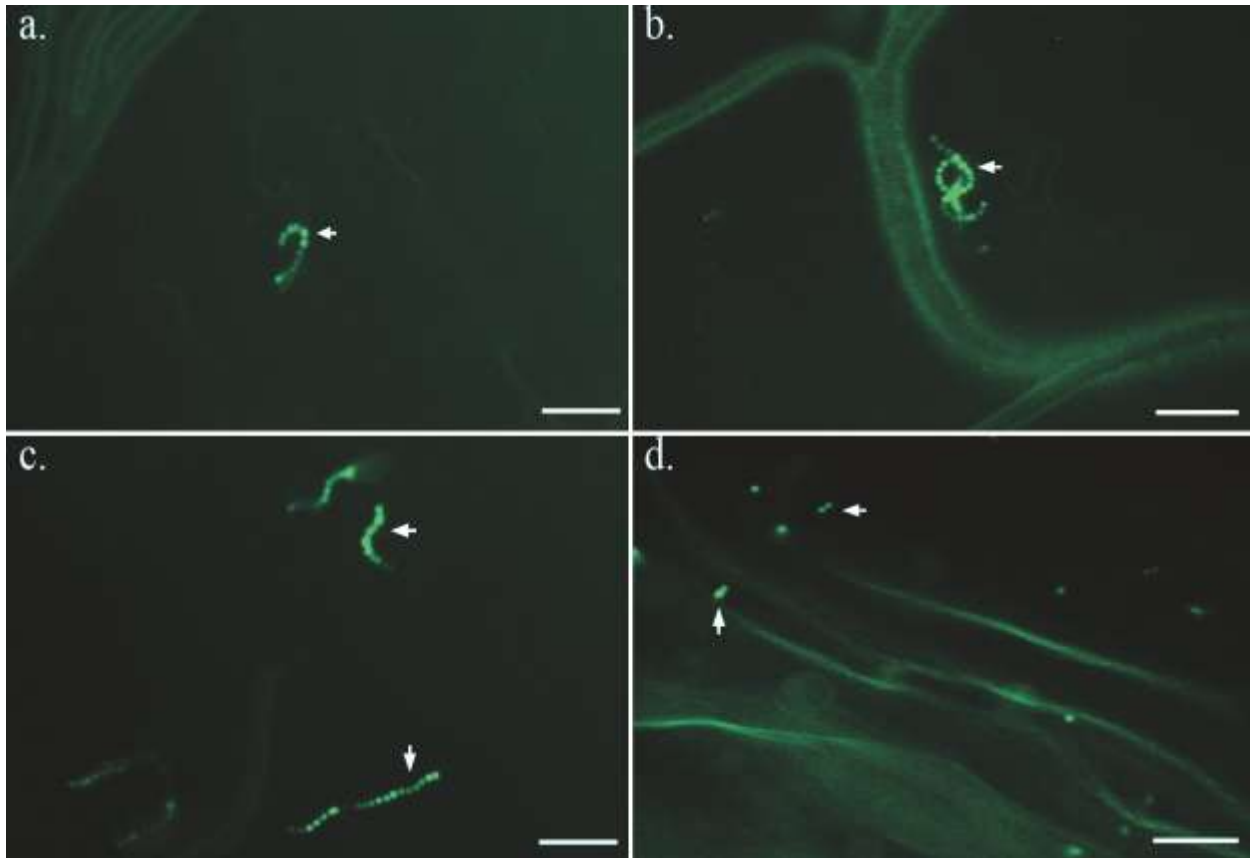
All bolded entries indicate statistical significance ( $P < 0.05$ ). <sup>a</sup>, no *S. pyogenes* were recovered in low dose experiments at those time points. <sup>b</sup>,  $\Delta$  survival was used to determine the effect of species on bacterial survival within similar doses. *Sp* = *S. pyogenes*, *St* = *S. typhimurium*.



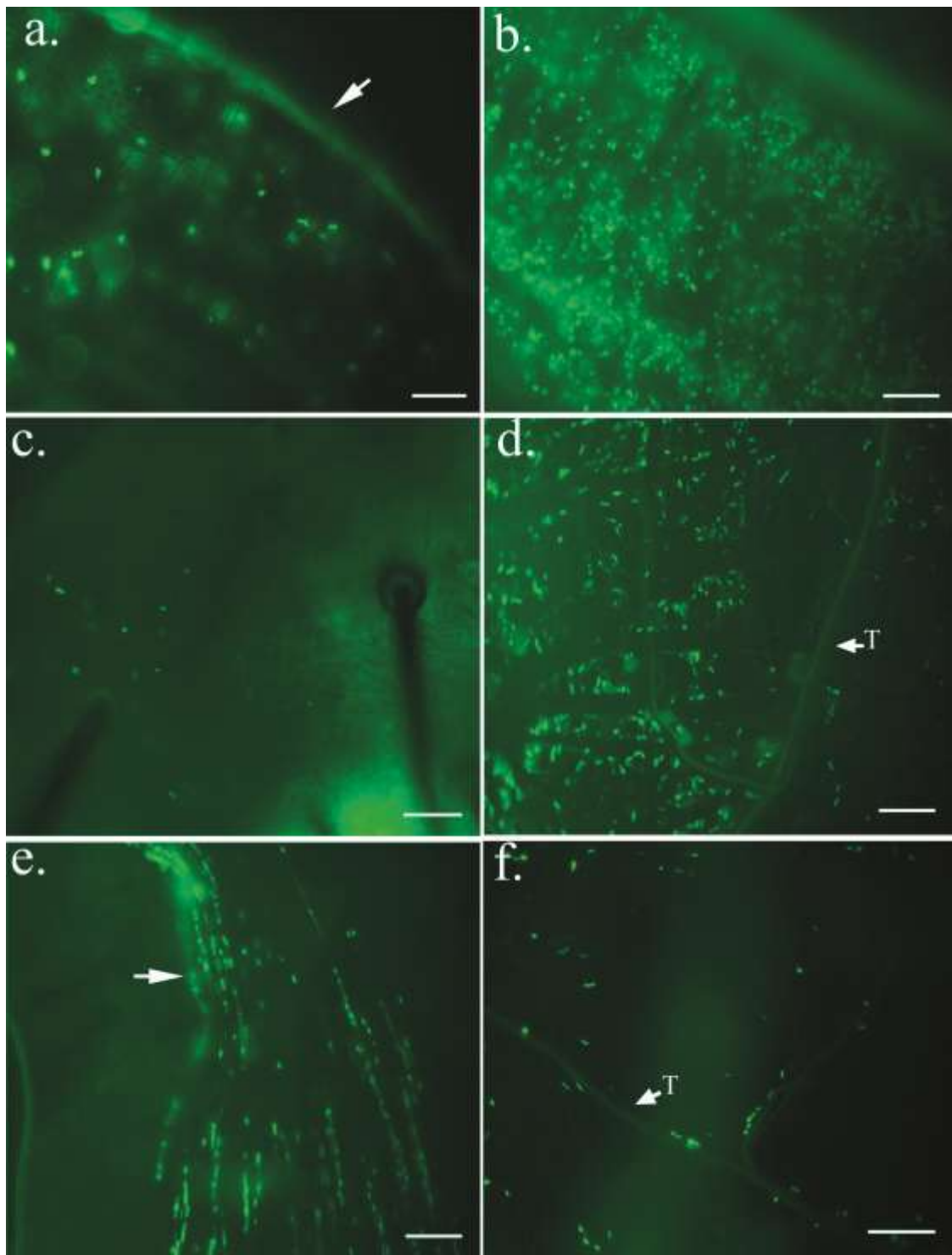
Table 2. *Salmonella typhimurium* recovery from house fly excreta. House flies (n=10) were fed an average of  $2.9 \times 10^5$  CFU (SD=  $1.0 \times 10^5$ ) in 3 experiments. Observations began right after feeding, and the first excreta were seen and collected 1 h PI. Each entry represents the average number of viable CFU cultured at each time point (with standard deviation in parentheses), and below the entry is the number of flies that excreted bacteria within that time interval. Also shown is the total CFU recovered for the entire 6 h period in each replicate and the total number of flies whose excreta contained viable *S. typhimurium*.

Replicate	Time interval of excreta recovery (h)					Total CFU
	1-2	2-3	3-4	4-5	5-6	
1	160 (99)	210	220 (14)	180	21	791
	n=2	n=1	n=2	n=1	n=1	n=5
2	0	35 (13)	27	165 (40)	228 (76)	495
	n=0	n=3	n=1	n=3	n=3	n=7
3	0	304 (31)	338 (28)	355 (106)	140	1137
	n=0	n=3	n=4	n=2	n=1	n=6

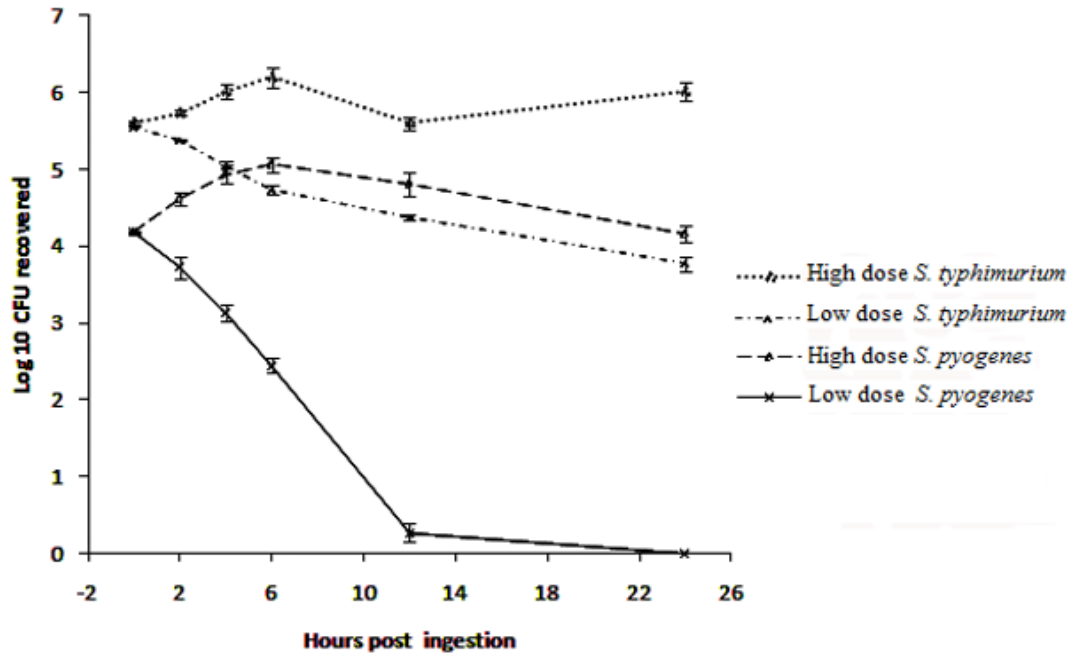
## FIGURES



**Fig. 1.** *Streptococcus pyogenes* in the alimentary canal of the house fly. At 2 h PI, *S. pyogenes* were seen in chains (arrows) in the midguts of flies fed either low (a.) or high (b.) doses of bacteria. (c.) At 4 h PI, chains of bacteria (arrows) were seen in the midgut in flies fed either dose of bacteria (high dose-fed fly shown here). At 6 h PI, individual cocci or diplococci were seen in the crop (d.; representative image from high dose-fed fly). Details on amounts of bacteria fed can be found in the text. Scale bar = 10  $\mu$ m.



**Fig. 2. *Salmonella typhimurium* in the alimentary canal of the house fly.** (a.) At 2 h PI, bacteria were seen in the crop (image from low dose-fed fly; arrow indicates edge of crop). At 4 h PI, numerous cells were seen in the midgut (b.) and bacteria also were in the rectum (c.) (images from high dose-fed fly). (d.) At 6 h PI, bacteria were seen the midgut and were highly motile (image from low dose-fed fly). (e.) At 12 h PI, bacteria were enclosed in the PM and some were adhered luminal surface of the inner PM (arrows; image from high dose-fed fly). At 24 h PI, bacteria were in the midgut (f.) and were motile (image from low dose-fed fly; T=trachioles). Details on amounts of bacteria fed can be found in the text. Scale bars =20  $\mu\text{m}$  except in (b.) where scale bar = 25  $\mu\text{m}$ .



**Fig. 3: Comparative survival of *Streptococcus pyogenes* and *Salmonella typhimurium* in the house fly.** House flies were fed an average of  $3.5 \times 10^5$  and  $1.5 \times 10^4$  CFU *S. pyogenes* and  $4.1 \times 10^5$  and  $1.5 \times 10^4$  CFU *S. typhimurium*, for high dose and low dose experiments within each species, respectively. CFU recoveries of *S. pyogenes* showed a steady temporal progression, and no bacteria were recovered at 24 h PI in flies fed the low dose of bacteria. *S. typhimurium* proliferated in flies fed either dose of bacteria. Statistical analysis of bacteria recoveries are discussed in the text, and effects of dose (within species) and species (within dose) are shown in Table 1. Mean Log<sub>10</sub> CFU are shown. Error bars = standard error.

## CHAPTER 2

DOSE-DEPENDENT LOCAL IMMUNE RESPONSE IN THE ALIMENTARY CANAL OF  
*MUSCA DOMESTICA* AFTER INGESTION OF *STREPTOCOCCUS PYOGENES* AND  
*SALMONELLA TYPHIMURIUM*

## ABSTRACT

House flies are exposed to diverse species and numbers of microorganisms when they utilize microbe-rich environments to oviposit or feed. *Studies in Drosophila melanogaster show that flies* defend themselves from pathogens in the alimentary canal by secreting antimicrobial peptides (AMPs), and the number of bacteria in the gut modulates activation of immune-signaling pathways. To investigate this phenomenon in the alimentary canal of house flies, different doses of *Streptococcus pyogenes* and *Salmonella typhimurium* were fed, and the upregulation of three AMPs (Cecropin, Defensin and Dipteracin) was examined temporally and spatially over 24 h using immunofluorescence microscopy. Both bacterial species and dose affected the temporal, spatial and class of AMP expression profiles in the gut. In *S. pyogenes*-fed flies, only Defensin was regionally produced in midgut tissue. In contrast, tissues from flies that were fed *S. typhimurium* expressed both Cecropin and Dipteracin within the first 6 h after ingestion. Cecropin mainly was localized in the midgut and hindgut, while Dipteracin was detected in the proventriculus and midgut. In both species, the higher dose of bacterial challenge induced greater AMP expression than the low dose. To our knowledge, this is the first demonstration of a local immune response to Gram positive bacteria (i.e., *S. pyogenes*) in Diptera.

**KEY WORDS:** bacteria, infection, immune, antimicrobial peptides, expression

## INTRODUCTION

House flies (*Musca domestica* L.) utilize decomposing organic matter as both food substrates and as developmental media for their larvae. Life in this septic niche results in constant contact with diverse microorganisms, including bacterial pathogens (Greenberg et al. 1970, Graczyk et al. 2005). Interestingly, house flies themselves apparently are very resistant to infection, disease and permanent colonization by these microbes (McGaughey and Nayduch 2009). This resistance may result from physical barriers such as the peritrophic matrix, or physiological defenses such as digestive enzymes or local immune responses, all of which are present in the alimentary canal.

When microbes are ingested by house flies they can be stored in consumed liquids in the crop, from which they can be regurgitated. However, microbes eventually are redirected through the proventriculus which sits at the proximal junction to the midgut. The primary function of this organ in some insects is to begin food processing, but interestingly, in other dipterans such as *Drosophila melanogaster* and tsetse flies, the proventriculus has an immune function as well (Hao et al. 2003, Gely et al. 2008). Once in the midgut, the food bolus is sequestered in a Type-II double-layered peritrophic matrix (PM) which lines the majority of the alimentary canal (Zhuzhikov 1964). The PM originates from the cardia, extends posteriorly through the hindgut and opens into the rectum (Richards and Richards 1997). The PM functions to encase the food bolus and, therefore, protects the gut epithelial tissue from damage from ingested matter and microbial invasion (Lehane 1997). Bacteria enclosed within the PM are unable to escape into the ectoperitrophic space or hemocoel (Kobayashi et al. 1999, Nayduch et al. 2005).

Besides physical barriers, bacteria face an onslaught of physiological changes and effectors present in the house fly digestive tract. pH levels vary tremendously along the length of the alimentary canal, from less than pH 4.0 in the anterior midgut to 8.0 in the rectum (Chapman 1998). Such variation aims to facilitate sequential digestion of ingested materials, including bacteria, by activating enzymes such as peptidases, lipases and amylases (Espinoza-Fuentes and Terra 1987). Additionally, the house fly midgut secretes a digestive lysozyme (Ito et al. 1995), which has been shown to cleave bacterial peptidoglycan and also has putative chitinase functions. Lysozyme has been shown to be highly expressed in house flies that were bacteria challenged (Ren et al. 2009, Joyner and Nayduch unpublished). This enzyme is considered of paramount importance as a primary defense molecule in the midgut environment, especially for house fly larvae (Terra et al. 1988, Markart et al. 2004).

Upon bacterial challenge, insects also mount immunological responses to components of bacterial cell walls such as the DAP-type or the Lys-type peptidoglycan (PGN), or  $\beta$ -1,3-glucan of fungi. These pathogen-associated molecular patterns (PAMPs) are recognized by the host immune system (Lemaitre and Hoffmann 2007). Insects recognize these PAMPs via peptidoglycan recognition proteins (PGRPs; Werner et al. 2000). Following microbial challenge, two signal transduction pathways are activated based on the PAMP present: (1) Toll is activated by Gram-positive bacteria (Lys-PGN) or fungi (glucans) and results in activation of NF- $\kappa$ B transactivators Dif and Dorsal or (2) the immune deficient (Imd) pathway is activated in response to Gram-negative bacteria (DAP-PGN) and results in activation of NF- $\kappa$ B transactivator Relish (Hoffman and Reichhart 2002, Rutschmann et al. 2002). The transactivators migrate to the nucleus and induce transcription of antimicrobial peptide (AMP) genes, which code for effector molecules that bind and/or lyse bacteria (Lemaitre et al. 1997, De Gregorio et al. 2002). AMPs



are secreted systemically by the fat body and locally from barrier epithelia such as the alimentary canal and tracheae (Tzou et al. 2000, Kurata 2004, Lemaitre and Hoffmann 2007, Gendrin et al. 2009).

AMPs have different spectra of activity, and their microbial targets have been well demonstrated in *D. melanogaster*. For example, Attacins, Drosocins and Diptericin show efficacy in killing Gram-negative bacteria (Bulet et al. 1996, Dushay et al. 2000, Schmidt et al. 2008 ). Drosomycin and Metchnikowin exhibit antifungal activity, although Metchnikowin also has anti-Gram-positive properties (Levashina et al. 1995). In *D. melanogaster*, Defensins have antibacterial activity against many Gram-positive bacteria (Bulet et al. 2005), although recently it has been shown that house flies upregulate expression of this AMP during Gram-negative and Gram-positive bacterial infections (Dang 2010). Cecropins are primarily induced with Gram-negative bacterial infections, but overlapping bacteriocidal effects towards Gram-positive organisms have been demonstrated (Hoffman et al. 1997, Ganz 2003). AMPs are believed to function via electrostatic interactions with the bacterial cytoplasmic membrane, which causes changes in membrane potential resulting in bacterial death (Bulet et al. 1999, Ganz 2003). The mechanisms by which Defensin kills bacteria is not well known but, it is assumed that Defensins interfere with cell wall biosynthesis, which is particularly effective against Gram-positive bacterial infections (Lehrer and Ganz 1999). Studies in *D. melanogaster* have shown that the above AMP classes are highly expressed in the alimentary canal upon challenge with Gram-negative bacteria (Reviewed in Charroux and Royet 2010). Although both the Toll and Imd signaling pathways control systemic AMP expression, local expression from epithelia is solely dependent on the Imd cascade in *D. melanogaster*. Further, Imd activation by DAP-PGN results in different AMP classes being expressed in a tissue-specific manner. Interestingly, flies

do not mount a constitutive antimicrobial immune response in the gut, despite being consistently exposed to microbes in the environment. It has recently been shown that the circulating PGRPs with amidase function are responsible for cleaving otherwise immunostimulatory PGN thereby downregulating immune activation in a dose-dependent manner (Mellroth et al. 2003, Zaldman-Gely 2010).

House flies encounter various species and quantities of bacteria in their environment and have been shown to harbor as many as 100 species of microbes ranging between  $10^7$  to  $10^{10}$  CFU per fly (Greenberg 1971; Greenberg 1973). This study aimed to elucidate the dose-dependent epithelial antimicrobial response to two human pathogens with different types of peptidoglycan, *Salmonella typhimurium* (Gram-negative; DAP-PGN) and *Streptococcus pyogenes* (Gram-positive; Lys-PGN), as these bacteria have been isolated from house flies in previous surveys (Greenberg 1973, Nmorsi et al. 2007, Wang et al. 2011). We investigated the spatial and temporal tissue-specific expression of the AMPs Cecropin, Defensin and Diptericin, in the alimentary canal, including the crop, proventriculus, midgut, hindgut and rectum in response to two different doses of each bacterial species.

## MATERIALS AND METHODS

### **House Fly Rearing**

House flies (*Musca domestica* L.) from colonies established at Georgia Southern University in 2004 were isolated and maintained as previously described (See chapter 1). Individual house flies were fed a known amount of either *S. typhimurium* or *S. pyogenes* suspension.

### **Bacteria Preparation**

Group A *Streptococcus pyogenes* transformed with eGFP-expressing plasmid pCM18, obtained from Dr. Vince Fischetti at Rockefeller University, were cultured in 5 ml Brain Heart Infusion (BHI) broth (Fisher Scientific, Atlanta, GA) with 5 µg/ml erythromycin for ~12 h at 37°C without shaking. The culture was diluted in BHI with the above antibiotic concentration to an optical density (OD<sub>600</sub>) of ~1.0 which corresponded to a mean of  $2.6 \times 10^8$  CFU/ml (SD=  $6.3 \times 10^6$ ) for high dose feeding, and to an OD<sub>600</sub> of 0.5 which corresponded to a mean of  $1.9 \times 10^7$  CFU/ml (SD=  $7.9 \times 10^5$ ) for low dose feeding.

*Salmonella typhimurium* SR11 was obtained from John Maurer at University of Georgia and was transformed with 1 µg of pGFPuv plasmid (Clontech Laboratories, Mountain View, CA) ligated with both kanamycin and ampicillin resistance genes as previously described (McGaughey and Nayduch 2009). Bacteria were cultured for 48 h at 37°C on Luria-Bertani (LB) agar (Fisher Scientific, Atlanta, GA) containing 50 µg/ml kanamycin and 50µg/ml ampicillin. Bacteria were suspended in BHI broth with the above antibiotic concentrations and diluted to an OD<sub>600</sub> of 0.090 or 0.011 which corresponded to  $2.9 \times 10^8$  CFU/ml (SD= $1.8 \times 10^6$ ) for high dose feeding and  $1.6 \times 10^7$  CFU/ml (SD= $3.5 \times 10^3$ ) for low dose feeding.

### **House Fly Infection, Dissection and Tissue Preservation**

To determine the house fly alimentary canal immune response individual house flies (n=30) for each experiment were isolated and maintained as previously described (See Chapter 1). Two different dosages (high and low) of each bacterial species were fed in separate experiments.

For *S. pyogenes* experiments, individually-housed flies (n=25) were fed 2 µl of bacterial suspension corresponding to  $5.2 \times 10^5$  and  $3.8 \times 10^4$  CFU for high and low dose experiments respectively on a piece of Parafilm®. Per each experiment, 5 additional flies were fed sterile BHI with 5 µg/ml erythromycin for control purposes. At each time point, 2, 4, 6, 12 and 24 h post ingestion (PI), bacteria-fed flies (n=5) were dissected to remove the whole intact gut, including the crop, proventriculus, midgut, hindgut and the rectum. Guts were fixed in 4% paraformaldehyde (Fisher, Scientific, Atlanta, GA) for 2 h and dehydrated using an increasing gradient alcohol series. The tissues were cleared with CitriSolv® (Fisher Scientific, Atlanta, GA) and embedded in paraffin wax (Fisher Scientific, Atlanta, GA). Sterile broth-fed flies were similarly dissected at 6 h PI, fixed and embedded as above. Each experiment was repeated three times.

For *S. typhimurium* experiments, house flies were similarly fed bacteria in 2 µl of bacterial suspension and corresponding to  $5.8 \times 10^5$  and  $3.2 \times 10^4$  CFU for high and low dose experiments respectively. Five additional flies were fed sterile BHI with 50 µg/ml kanamycin and 50 µg/ml ampicilin. Flies were similarly dissected for the whole intact gut and processed as in *S. pyogenes* above.

### **Local Expression of Antimicrobial Peptides in the Alimentary Canal**

To examine antimicrobial peptide (AMP) expression following ingestion of bacteria, immunofluorescence assays were performed on the embedded gut tissues. Tissues were sectioned to 5 µm using a microtome and were mounted on Superfrost® amino-silane positively charged slides (Fisher Scientific, Atlanta, GA). A decreasing gradient alcohol series was used to rehydrate tissue samples. To avoid non-specific binding of antibodies, tissue samples were

incubated at room temperature (RT; ~21°C) for 1 h in Starting Blocking buffer (Thermo Scientific, Rockford, IL). For the detection of AMPs *in-situ*, tissues were incubated for 24 h at RT in custom-made primary polyclonal IgG antibodies (rabbit anti-cecropin, chicken anti-defensin, and mouse anti-diptericin; Genscript, Piscataway, NJ) diluted to 1:100 (0.06ng/ml) in 0.1% Bovine Serum Albumin (BSA; Fisher Scientific, Atlanta, GA). This was followed by incubation of tissues in conjugated Alexa Fluor® secondary antibodies (goat anti-cecropin, goat anti-mouse, goat anti-defensin; Invitrogen, Carlsbad, CA) diluted to 1:1000 in 0.1% BSA for 24 h in the dark at RT. The tissue samples were mounted with ProLong Gold® antifade reagent (Invitrogen, Carlsbad, CA) containing DAPI nuclear stain. Immunoreactivity was viewed using a Laborlux 12 microscope (Leitz, Germany) equipped with appropriate bandpass filters. Images were captured using a Leica DFC420 digital camera system (Leitz, Germany) for microscopy fitted on the microscope. Tissue sections from flies that were fed sterile broth were mounted and incubated with antibodies as above and viewed for control purposes.

## RESULTS

To determine the sites of AMP expression in the house fly alimentary canal, tissues were processed for visual observation. Because there was so much consistency within trials, only representative figures for each AMP (Cecropin, Defensin and Diptericin) expression localization are shown.

### **Detection of Antimicrobial Peptides in *Streptococcus pyogenes*-Fed Flies**

Oral ingestion of high-dose *S. pyogenes* ( $5.2 \times 10^5$  CFU, SD= $1.3 \times 10^4$  in 2  $\mu$ l suspension) induced Defensin expression in the house fly alimentary canal epithelium (Fig. 1). Defensin expression was not observed in the entire gut of sterile broth-fed flies (Fig. 1a is

showing the proventriculus). Defensin expression was detected in midgut tissues of house flies that were fed *S. pyogenes* at 2 h PI, and was localized mostly on the basal side of the epithelia (Fig. 1b). This pattern of expression was consistent in all of the tissue sections that were observed. Defensin expression was not detected in the crop, proventriculus, hindgut or rectum of the high dose *S. pyogenes* fed flies. At 4 h PI, we did not detect Defensin in most of the gut tissue sections, except for low expression in a few samples from the midgut (Fig. 1c). Expression was observed on both the basal side and apical side of the epithelia. At 6 h PI, no Defensin expression was observed in any of the tissues (Fig. 1d shows the midgut). Similarly at 12 and 24 h PI, Defensin expression was not detected in tissues examined from flies that ingested high dose of *S. pyogenes* (not shown).

For flies that were fed low dose of *S. pyogenes* ( $3.8 \times 10^4$  CFU,  $SD=1.6 \times 10^3$  in 2  $\mu$ l suspension), Defensin expression was not detected in any tissues observed over the experimental time course within all flies from all replicates (data not shown). Also, Cecropin and Dipterocin expression were not observed in tissues from either high or low dose bacteria-fed flies or in control flies (data not shown).

### **Detection of Antimicrobial Peptides in *Salmonella typhimurium*-Fed Flies.**

Upregulation of Cecropin expression was observed in the alimentary canal of house flies fed both high and low doses of *S. typhimurium*. Representative images are from tissues of flies that were fed high dose of *S. typhimurium* ( $5.8 \times 10^5$  CFU;  $SD=3.6 \times 10^3$ ) are shown in Fig. 2. Cecropin expression was not observed in sterile broth-fed flies (Fig. 2a). At 2 h PI, Cecropin was detected on both the basal and apical side of the midgut epithelium (Fig. 2b). At 4 h PI, high levels of Cecropin were observed in the midgut (Fig. 3c) and hindgut tissue (not shown) of high

dose-fed flies. In the midgut, expression was concentrated on the basal side of the gut epithelium. In the hindgut tissue, Cecropin expression was detected on both the apical and basal edges of epithelia (data not shown). In contrast to earlier observations (2 and 4 h PI), Cecropin expression was observed mostly on the apical (lumen) side of the gut epithelium, at 6 h PI, with less expression being in the basal regions of the cells (Fig. 2d). At 12 h PI, expression of Cecropin was observed on the basal side of the midgut epithelia with low expression in the apical regions (Fig. 2e). Low levels of Cecropin expression were seen at 24 h PI at both the basal and apical regions of the midgut tissue (Fig. 2f). Interestingly, Cecropin was not detected in the proventriculus or crop at any time point in the high dose-fed flies.

In low dose *S. typhimurium*-fed flies ( $3.2 \times 10^4$  CFU, SD =  $3.5 \times 10^3$  in 2  $\mu$ l suspension), Cecropin expression was only detected in the midgut at 4 and 6 h PI (data not shown), but was not detected at 2, 12 and 24 h PI in all tissues observed. Similar to the observations in the high dose-fed flies, Cecropin was localized on the basal side of the midgut epithelium, although few tissue sections showed low expression on the apical side. Also, Cecropin was not detected in the proventriculus and crop at any time points in the study period.

Diptericin expression was neither observed in flies that were fed sterile broth (Fig. 3a; shown is proventriculus image) nor in high dose-challenged flies that were dissected at 2 h PI. However, at 4 h PI, Diptericin expression was detected in various regions of the proventriculus (Fig. 3b) in high dose-fed flies. At 6 h PI, expression was observed both in the proventriculus (not shown) and apical and basal side of the midgut (Fig. 3d) as well. No Diptericin was detected in any tissues of high dose-fed flies that were dissected at 12 and 24 h PI. In flies fed the low dose of bacteria, Diptericin expression was only observed at 6 h PI in the proventriculus (Fig. 3c). Diptericin expression was not observed at any other tissue sites or at other time points.

Defensin expression also was examined in *S. typhimurium* fed-flies, and no detectable levels of Defensin were seen in any tissue sections from flies fed either dose at all time points in the study period.

## DISCUSSION

Tissue-specific immune responses have been demonstrated in several insects such as *D. melanogaster* (Tryselius et al. 1992, Tzou et al. 2000, Senger et al. 2006), *Aedes aegypti* (Shin et al. 2006) and *Bombyx mori* (Brey et al. 1993). Barrier epithelia such as the cuticle, tracheae, reproductive tracts and the gut have been shown to actively upregulate AMPs in response to localized microbe infection (Gendrin et al. 2009). The purpose of this study was to understand the house fly's alimentary epithelium local immune response to *S. pyogenes* and *S. typhimurium* oral infection. Using immunofluorescence staining assays, we provided evidence that house flies synthesize the AMPs Cecropin, Defensin and Diptericin within hours following ingestion of bacteria. We also found that the specific type of AMP was expressed at different times after ingestion of bacteria in a dose-dependent and species-specific manner. As has been demonstrated previously for *D. melanogaster* (reviewed in Lemaitre and Hoffmann 2007), the house fly immune response differentiates Gram-positive and Gram-negative bacterial infections and mounts appropriate and distinct AMP expression.

Our results showed that flies fed the high dose of *S. pyogenes* induced local expression of Defensin in the midgut during early time points post-ingestion, a time when numbers of bacteria were still high (See chapter 1). At later time points, Defensin was not detected, although bacteria still persisted in the gut (data not shown). We speculate that Defensin is integral in reducing the number of viable *S. pyogenes* in the midgut to levels that did not cause further immune



stimulation. As noted in our previous study, the number of viable *S. pyogenes* decreased with time progression (see Chapter 1). We surmise that the decreased number of viable cells is no longer immunostimulatory. Defensin expression ceased with time progression in high dose-fed flies and was never observed in low dose-fed flies, suggesting that there is a dose dependent effect on the local expression of Defensin in the house fly gut. Dose-dependent immune activation in the gut has been demonstrated in *D. melanogaster* using Gram-negative bacteria (Zaidman-Remy 2009), and is modulated by circulating PGRPs with amidase function. PGRP-LB was shown to operate as a PGN scavenger, cleaving the immunostimulatory tetrapeptide chain (L-Ala-f-D-Glu-meso-DAP-D-Ala) from DAP-PGN and thereby down-regulating the local immune response. When PGRP amidase activity is overwhelmed by the presence of large numbers of bacteria and/or PGN, the Imd pathway becomes activated resulting in AMP upregulation (Steiner 2004). Thus amidase PGRPs act as molecular switches in the regulation of the immune response. Notably, this activity in local epithelia has only been shown in response to Gram negative bacterial infections in *D. melanogaster*. To our knowledge, no local immune response to Gram positive bacteria has been demonstrated. Our study is first to show a local and dose-dependent immune response to a Gram-positive bacteria species in Diptera. Since *S. pyogenes* have Lys-PGN, the mechanism of our observed dose-dependent activation remains unclear. However, PGRP-SC has been shown to have overlapping effects on the digestion of DAP- and Lys-type PGN in *D. melanogaster* during systemic challenge (Mellroth et al. 2003).

Defensin expression was not observed in the gut of flies that ingested *S. typhimurium*. Defensin has been characterized from other filth flies such as *Sarcophaga peregrina* where larvae that were injured and hemolymph withdrawn showed the induction of a peptide (Sapecin) which is now known to be Defensin (Matsuyama and Natori 1988), and *Phormia terranova* that

were systemically challenged with Gram positive bacteria such as *Micrococcus luteus* and *Bacillus megaterium* (notably Gram-positive bacteria, but with DAP-PGN) revealed upregulation of the proteins (Lambert et al. 1989). In house flies, Defensin is upregulated in pupae after immune stimulation by septic injury with bacterial species such as *Staphylococcus aureus* and *Escherichia coli* (Dang et al. 2010). Wang et al. (2006) similarly demonstrated the upregulation of Defensin in house fly larvae and adults that were infected with *S. aureus*, and larvae that were infected with *E.coli* through septic injury using Northern blot analysis. However, *in-situ* hybridization assays revealed no local expression of Defensin in the midgut after *E. coli* infection. In our study, we similarly failed to detect Defensin local expression in the midgut after *S. typhimurium* oral ingestion, we therefore conclude that the local expression of Defensin in the gut due to Gram-negative infection in house flies is either dose-dependent or does not occur.

Flies fed *S. typhimurium* upregulated Cecropin and Dipteracin in the alimentary canal. Cecropin expression was observed in high dose-fed flies in the midgut and in the hindgut tissues throughout the observational period, but flies fed the lower dose of bacteria, expression was observed in the midgut only at 4 and 6 h PI. Interestingly, this dose-dependent difference in the temporal and spatial expression of Cecropin may be related to the dynamics of bacterial populations in the gut. In Chapter 1, we demonstrated that *S. typhimurium* increased in number within 2 h although the number remained within the same order of magnitude as the amount initially fed ( $10^5$  CFU). With low dose fed flies, bacteria also increased in number at early time points from  $10^4$  CFU fed to  $10^5$  CFU at 4 and 6 h PI. Here we demonstrate that induction of the local expression of Cecropin in the gut of house flies may be dose-dependent. When *S. typhimurium* numbers were in the range of  $10^4$  CFU or less, Cecropin was not detected.

Cecropin may be effective in combating *S. typhimurium*, since we previously observed a decline in number of bacteria at 12 h PI in both high and low dose infection and at 24 h PI only with low dose, which is subsequent to the peak immune activation seen in this study (6 h PI). These results support the findings of Ling et al. (2005), where *cecropin* mRNA was detected via Northern blot, RT-PCR and *in-situ* hybridization in the fat body, epidermis and midgut of flies that were infected with *E. coli*. Also, in studies of *D. melanogaster*, a model system for studying insect defenses, Cecropin was shown to be strongly expressed *in-situ* in the hindgut of flies that were immune challenged with *Enterobacter cloacae*, another Gram-negative species of bacteria (Tryselius et al. 1992).

Ingestion of *S. typhimurium* also induced expression of Diptericin in the house fly proventriculus and midgut epithelia. The proventriculus has been shown to be an important immune organ in other dipteran insects such as the *D. melanogaster* and *Glossina* spp. (Hao et al. 2003, Nayduch and Aksov 2007, Gely et al. 2008, Nayduch and Aksov 2007). Diptericin expression is associated with Gram-negative bacterial infections in higher Diptera such as *D. melanogaster* (Wicker et al. 1990) and *P. terranova* (Dimarcq et al. 1988). We demonstrated that the expression of Diptericin in the midgut coincided with the expression of Cecropin. This demonstrates a putative synergistic role of these two AMPs in order to restrict the multiplication of pathogenic bacteria in the gut lumen. Synergistic interactions of antimicrobial peptides have been demonstrated to occur in some other higher eukaryotes (Westerhoff et al. 1995).

The upregulation of Cecropin and Diptericin corresponded with previously-observed decrease in the number of bacteria between 6-12 h PI (see Chapter 1), therefore, we can infer that the synergistic effect of the AMPs may have some effect on lysis of *S. typhimurium* at this time point. However, bacteria were not completely cleared in the gut, showing that some resistance to

lysis may exist in *S. typhimurium*. The mechanisms of bacterial resistance to AMPs are still a subject of investigation (Li et. al. 2007). Tenover (2006) reported that some bacterial species secrete proteases that deactivate AMPs and prevent them from acting on their microbial target. Kawasaki (2009) reported that *S. typhimurium* has the capability of changing the charge on the cell surface (LPS) through modification of the 4-amino-4-deoxy-L-arabinose of lipid A, which promotes bacterial survival and resistance to AMPs. Because *S. typhimurium* are motile, survival could also be linked to their ability to move away from the areas where AMPs are being secreted, thereby evading lysis.

In response to *S. pyogenes* or *S. typhimurium* infection, the house fly gut epithelium synthesizes antimicrobial peptides. The proventriculus and the midgut were the sites of AMP expression following microbial challenge. Expression of AMPs in the gut may be dependent on the number of microbes ingested. Overall, we demonstrated that a high infective dose of either species of bacteria induced greater expression of AMPs in house flies as compared to ingestion of lower doses of bacteria. The findings represent a novel contribution to the understanding of molecular strategies used by house flies to survive microbial encounters. Future studies will focus on looking at the house fly local epithelial responses to other species of pathogenic bacteria. Since house flies naturally feed on different septic substrates and encounter various bacterial species that they can harbor within their bodies, (Fotedar et al. 1992, Rahuma et al. 2005) understanding species-specific responses to bacteria will ultimately aid in assessing vector potential. From a practical application viewpoint, studies on the efficacy of house fly AMPs may reveal alternative methods in treatment of human and animal microbial infections.

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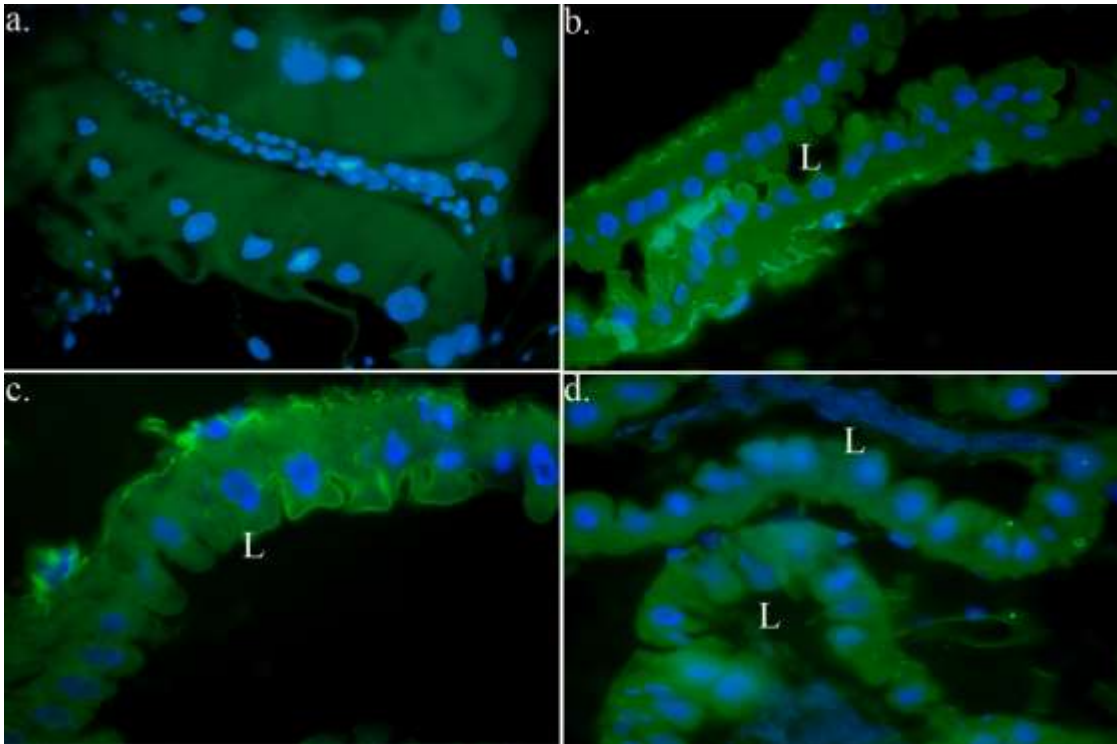
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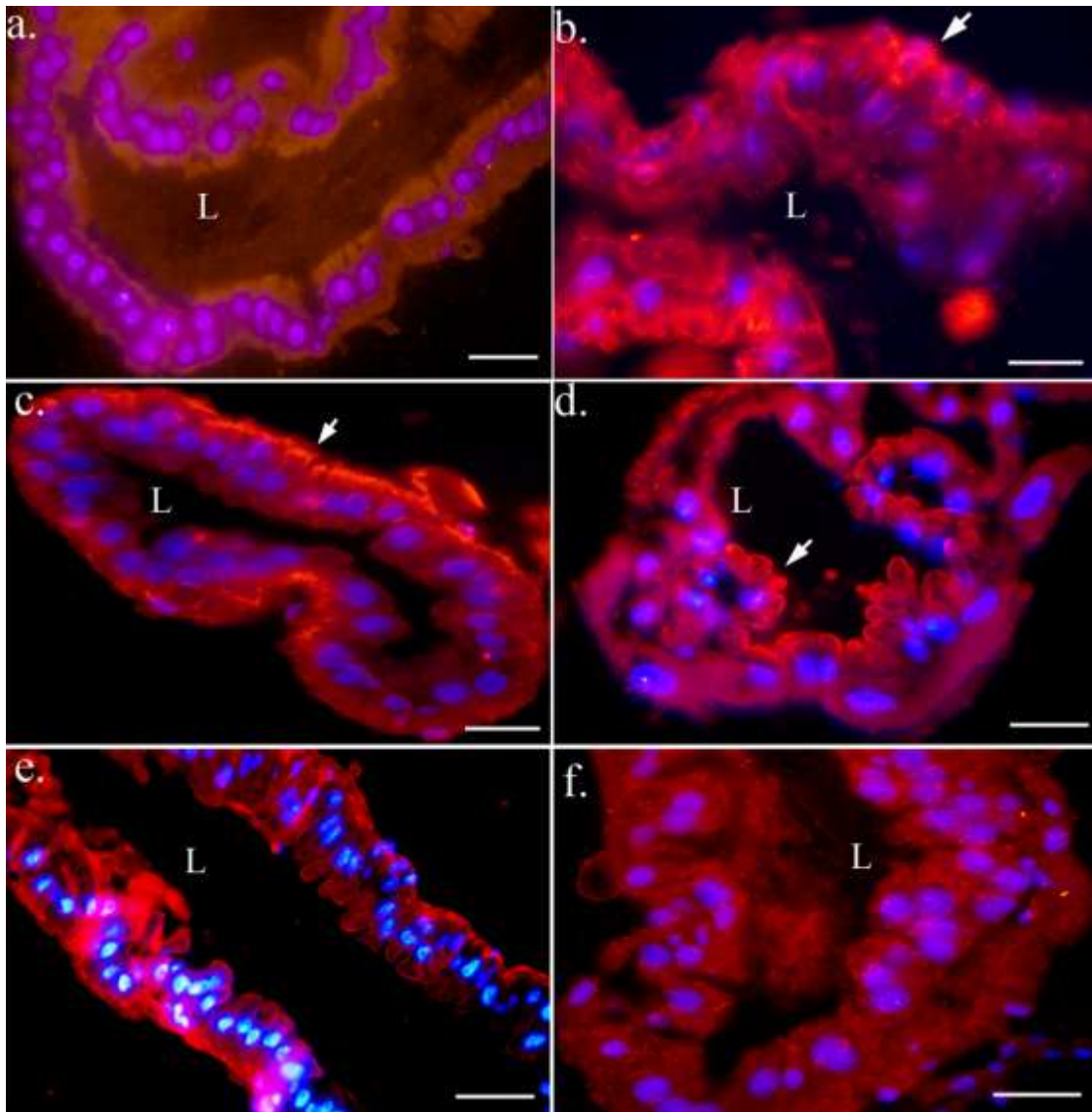
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## FIGURES

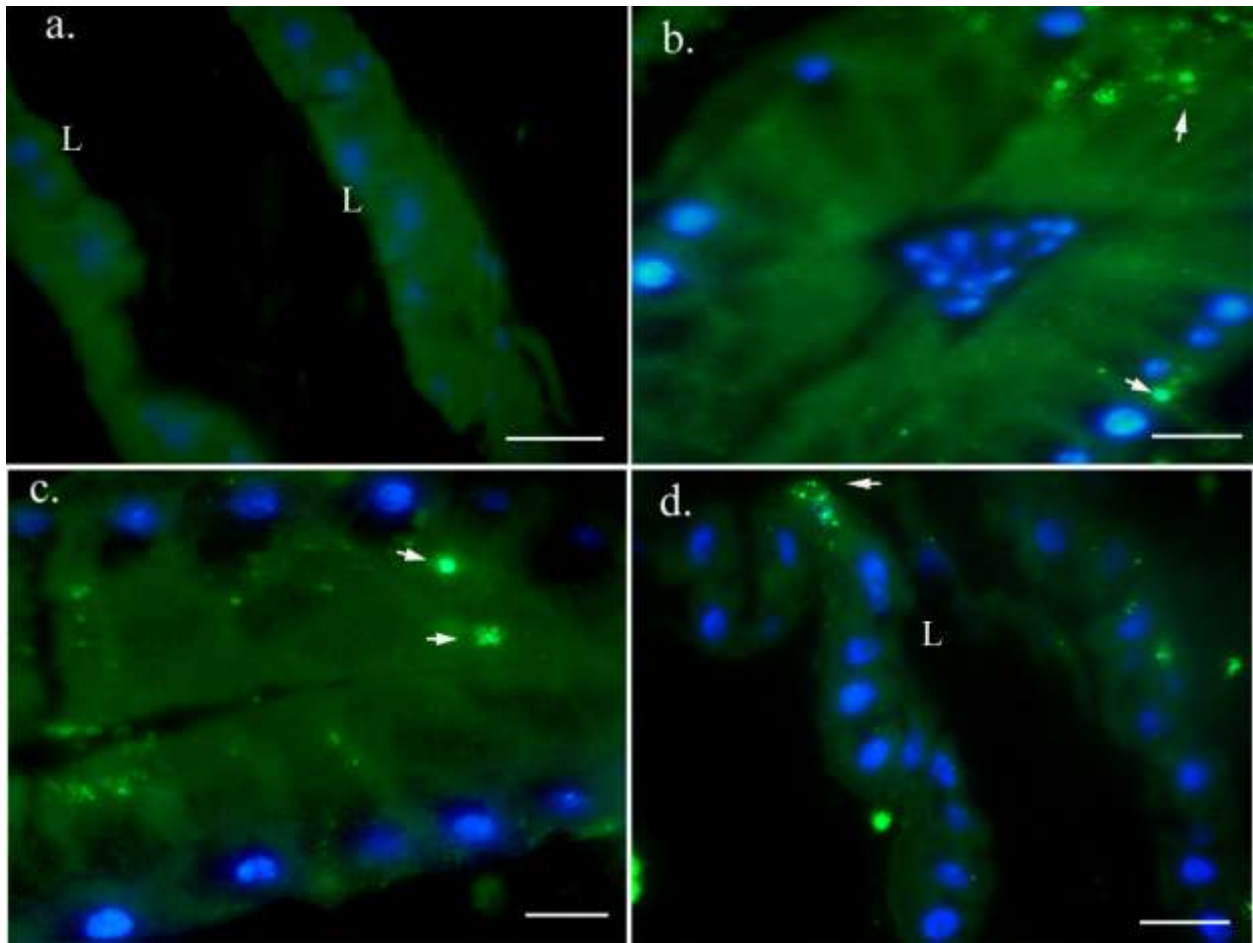


**Figure 1. Defensin local expression in *Streptococcus pyogenes*-fed flies.** Defensin expression was not observed in alimentary tissues of control flies (a., proventriculus shown). (b.) Defensin expression was observed (bright green) at 2 h PI in midgut epithelia of flies fed the high dose of bacteria (average  $5.2 \times 10^5$  CFU, SD= $1.3 \times 10^4$ ). (c.) At 4 h PI Defensin expression was also observed in the midgut. At 6 h PI Defensin expression was not observed in all tissues (d.; midgut shown). Blue = DAPI stained nuclei. L = lumen Scale bars are identical and represent 10  $\mu\text{m}$ .



**Figure 2. Cecropin local expression in *Salmonella typhimurium*-fed flies.** Representative pictures were taken from high dose fed flies (average  $5.8 \times 10^5$  CFU). Cecropin expression was not observed tissues of control flies (a., midgut shown). (b.) At 2 h PI, Cecropin expression (bright red) was observed in the midgut. Cecropin expression was observed in the midgut tissues at 4, 6, 12 and 24 (b-f, respectively) and hindgut (not shown). At these time points, Cecropin was mostly observed on the basal side of the epithelia except for 6 h PI, where expression was on the apical (lumen) side of the epithelia. Blue = DAPI stained nuclei. Scale bars = 10  $\mu$ m.





**Figure 3. Dipteracin expression in *Salmonella typhimurium*-fed flies.**

House flies were fed *S. typhimurium* (average CFU =  $5.8 \times 10^5$  and  $3.2 \times 10^4$ , for high and low doses, respectively). Dipteracin expression was not observed in all tissues from control flies (a., midgut). At 2 h Pi Dipteracin was not observed with either high or low dose experiment (not shown). At 4h PI Dipteracin expression (bright green vesicles; arrows) was only observed in the proventriculus (b.) in high dose-fed flies. At 6 h PI, Dipteracin expression (arrows) was observed in both high and low dose fed flies in the proventriculus (c., low dose-fed fly shown), and in the midgut of only high dose-fed flies (d.). Blue = DAPI stained nuclei. L = lumen. Scale bars = 10  $\mu\text{m}$ .