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Staphylococcus aureus in the House Fly: Temporospatial Fate of Bacteria and Expression of the Antimicrobial Peptide *defensin*

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Abstract

House flies disseminate numerous species of bacteria acquired during feeding and breeding activities in microbe-rich habitats. Previous house fly surveys have detected the pathogen *Staphylococcus aureus* Rosenbach 1884, which causes cutaneous and septic infections in mammals, and enterotoxic food poisoning. We assessed the fate of GFP-expressing *S. aureus* (GFP-*S. aureus*) in the house fly alimentary canal with microscopy and by culture of whole flies and excreta. Furthermore, the concurrent expression of the antimicrobial peptide gene *defensin* was measured in the crop, proventriculus, midgut, and fat body. As soon as 4 h postingestion (PI), GFP-*S. aureus* were visualized as cocci or diplococci in the hindgut and rectum of flies fed $\approx 10^5$ colony forming units. Bacteria persisted up to 6 h PI but significantly decreased. Excretion of viable GFP-*S. aureus* peaked at 2 h PI and, although significantly less, continued up to 4 h PI. *defensin* was highly upregulated locally in the alimentary canal and systemically in fat body at 2, 4, and 6 h PI making this study the first to report, to our knowledge, an epithelial and systemic response to a bacterium with lysine-type peptidoglycan in flies exposed via feeding. While flies harbored *S. aureus* for up to 6 h PI, the highest probability of vectoring biologically relevant amounts of bacteria occurred 0–2 h PI. The combined effects of excretion, digestion and antimicrobial effectors likely contribute to loss of ingested bacteria. Nonetheless, house flies are relevant vectors for *S. aureus* up to 2 h PI and environmental reservoirs up to 6 h PI.

Keywords

bacteria; culture recovery; excreta; midgut; fat body

House flies (*Musca domestica* L.; Diptera: Muscidae) have been shown to harbor, transport, and vector numerous species of human and livestock pathogenic bacteria (Graczyk et al. 2001). Because larvae have a nutritional requirement for bacteria (Zurek et al. 2000), adult flies (particularly females) are attracted to septic substrates for oviposition (West 1951). Subsequently when adults search for sugar- or protein-rich foods in human or animal habitats, flies deposit live microbes via physical contact or excreta. In agricultural settings, bacteria transmitted by flies frequently are enteropathogens such as *Escherichia coli* O157:H7, *Salmonella* spp., and *Campylobacter* spp., which are picked up by flies during associations with animal waste or contaminated environments (Rosef and Kapperud 1983, Moriya et al. 1999, Szalanski et al. 2004, Kinde et al. 2005, Holt et al. 2007). However, in human habitats, house flies have been shown to harbor a wide range of pathogenic and nonpathogenic microbes from various taxa (Cohen et al. 1991; Fotedar et al. 1992a,b;

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Graczyk et al. 2001; Gupta et al. 2012). In this study, we chose to look at the fate of *Staphylococcus aureus* Rosenbach 1884 in flies, because this pathogen has been isolated in several of the surveys above and causes cutaneous, systemic, and enterotoxigenic diseases in humans and livestock (Ben Zakour et al. 2008, Fitzgerald 2012).

The fly's vector potential for particular pathogens is affected by location of bacteria either on or in the fly, fly antimicrobial defenses, and inherent species-specific properties of bacteria that interact with either of these factors. For example, bacteria harbored externally may dry during flight and lose viability (Yap et al. 2008), but bacteria harbored internally face digestion and defensive responses from gut epithelia. Ingested bacteria usually are initially stored in the fly crop, where food can be predigested via salivary carbohydrases and/or regurgitated to liquefy the next meal (Lehane and Billingsley 1996). Once food is partitioned past the proventriculus to the midgut, it cannot be regurgitated and passes posteriorly via peristalsis. Along the entire midgut, food and bacteria are physically separated from the delicate epithelium by a type-II peritrophic matrix (PM); however, digestive enzymes and defensive effectors secreted by the epithelium can traverse the PM and act on ingested substrates (Richards and Richards 1977).

Epithelial immunity in the house fly gut has been understudied, but insight can be gleaned from studies on the model dipteran *Drosophila melanogaster* (Meigen), because fruit flies also commonly associate with bacteria during feeding and breeding activities. Numerous studies on the local epithelial response in the gut of naturally infected *D. melanogaster* show recognition of gram-negative bacteria via binding of diaminopimelic acid-type peptidoglycan (DAP-PGN) fragments to transmembrane peptidoglycan recognition receptors (PGRPs) (Stenbak et al. 2004, Vodovar et al. 2005, Liehl et al. 2006, Ryu et al. 2006, Buchon et al. 2009, Charroux and Royet 2010). These small subunits of DAP-PGN are able to cross the peritrophic matrix and stimulate immune cascades, which eventually produce antimicrobial peptides (AMPs), secreted bactericidal effector molecules (Lemaitre and Hoffmann 2007). In this study, the expression of *defensin* (*def*) was examined because this broad-spectrum AMP is particularly effective against gram-positive cocci with lysine-type PGN (LYS-PGN) (Lambert et al. 1989, Hoffmann and Hetru 1992, Yamada and Natori 1993). Defensin from the blow fly *Lucilia sericata* Meigen (= "lucifensin") has shown bactericidal activity against pathogens such as methicillin-resistant *S. aureus* (MRSA) and, thus, may be the key effector for successful maggot therapy treatment of refractory wounds (Andersen et al. 2010, Cerovsky et al. 2010). Additionally, the spectrum activity of purified house fly Defensin was recently analyzed and revealed activity against bacteria with DAP- and LYS-PGN as well as fungi (Dang et al. 2010). However, both of these studies involved larvae and pupae, respectively, and did not determine local expression in the alimentary canal, proximal to bacteria. Thus, in our study we examined the spatial and temporal expression of *def* both locally in the gut and systemically in the fat body of adult house flies.

The objective of this study was to determine the fate of GFP-expressing *S. aureus* in house flies by microscopical examination of the location of bacteria in the gut over time, and by culture of viable bacteria from whole fly homogenate as well as excreta. Further, the concurrent expression of the AMP gene *def* was quantified to assess the house fly response to ingested bacteria. Examining the interaction of house flies and bacteria from the perspective of both organisms provided an opportunity to uniquely observe the dynamics of bacteria fate and ultimately vector potential for this pathogen.

Materials and Methods

House Fly Rearing

Adult house flies from a colony established in 2004 at Georgia Southern University were maintained on a diet of 2:2:1 powdered sugar: milk:egg at 30°C with a photoperiod of 12:12 (L:D) h. Flies were provided moistened wheat bran media for oviposition and larva development. Pupae collected from this colony were allowed to emerge in sterile petri dishes and separated in individual, sterile jars for bacterial feeding. House flies were determined to be free of *S. aureus* but did contain low numbers (<1,000 CFU [colony forming unit]) of bacteria carried over through pupariation including *Bacillus* spp.

S. aureus Culture

FP-expressing *S. aureus* RN6390 strain ALC 1743 with plasmid psk236 (GFP-*S. aureus*) was provided by A. Cheung (Dartmouth College) and maintained at 37°C in tryptic soy broth plus chloramphenicol (10 μg/ml) for all experiments (Fisher, Atlanta, GA). Liquid culture was inoculated with a single colony of GFP-*S. aureus* and shaken (120 rpm) at 37°C for 2–6 h, until the OD_{600} reached ≈0.60, which corresponded to ≈10^8 CFU/ml.

Fly Feeding

Within 24 h posteclosion, adult flies were segregated into individual jars and fed 5 μl 10% sterile sucrose to ensure proper nutrition to produce AMPs and reduce the amount of indigenous microbiota in the house fly digestive tract. Flies then were fasted overnight (8–12 h) and each fed a single 2 μl droplet of *S. aureus,* which were later quantified by serial dilution and plating in duplicate on chloramphenicol (10 μg/ml) tryptic soy agar (TSA). Mean counts (i.e., CFU in the 2 μl droplet) of bacteria fed to each fly in the following experiments were as follows: for epifluorescence microscopy, 2.0 ± 10^5 CFU; for culture-recovery, 6.75 ± 1.44 ×10^5 CFU; for excreta recovery, 3.2 ± 1.4 × 10^5 CFU; for qRTPCR, 4 × 10^5 CFU.

Epifluorescence Microscopy

Adult house flies (n = 15) were fed 2 μl-droplets of GFP-*S. aureus,* and at 2, 4, and 6 h postingestion (PI) five flies were randomly chosen for dissection and aseptic removal of the entire alimentary canal including proventriculus, crop, midgut, hindgut, and rectum. Guts were placed on microscope slides, and the location of GFP-*S. aureus* was examined using epifluorescent microscopy as previously described (McGaughey and Nayduch 2009).

Culture Recovery

Adult flies (n = 15 per n = 3 biological replicates) were fed a single 2 μl droplet of GFP-*S. aureus,* and at 2, 4, and 6 h PI, five flies were immobilized by chilling and surface-sanitized by submersion in 10% bleach followed by 70% alcohol for at least 10 min each. Flies were rinsed in sterile water, air-dried, and homogenized in 500 ml of sterilized 1× phosphate buffered saline (Fisher). Homogenate was serially diluted and cultured on chloramphenicol (10 μg/ml) TSA agar, which was incubated at 37°C overnight for enumeration of CFU/fly. Cultured dilutions not within 30–300 CFU were not considered to be in the acceptable countable range and were excluded. Mean CFU recoveries from three combined replicates had normal distributions and were analyzed by one-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) posttest for log transformed CFU counts (GraphPad Prism five for Mac OSX, GraphPad Software, LA Jolla, CA, www.graphpad.com).

Excreta Recovery

Following the same feeding protocol, house flies (n = 5 per replicate, for three replicates) were put in sterile jars and fed 2 μl droplets of *S. aureus.* After consumption of bacteria, flies were transferred to sterile 35 mm petri dishes (Fisher). At 2, 3, and 4 h PI, flies were chilled at 4°C for temporary removal, and the dishes were washed with 500 μl sterile 1× PBS to collect fecal and vomit specks. The same flies were transferred to new petri dishes for the next 1 h interval, after which time the washes were repeated. Each wash was serially diluted, plated on TSA with chloramphenicol (10 μg/ml), and incubated overnight at 37°C for CFU enumeration. At each time point, only flies with recoverable CFU were included in the analysis. CFU recoveries from excreta did not fit Gaussian distribution, so nonparametric Kruskal–Wal-lis test was performed with Dunn's multiple comparison posttest being used to compare mean excreta CFU recoveries between time intervals.

Tissue-Specific Analyses of *def* Temporal Expression

Flies (n = 30 per replicate, with two parallel biological replicates) were individually housed in glass jars and fed 2 μl GFP-*S. aureus.* Tissues (i.e., proventriculus, midgut, crop, and fat body) were aseptically dissected from 10 flies at 2, 4, and 6 h PI and pooled by tissue for RNA extraction (Ribopure kit; Ambion, Austin, TX) and cDNA synthesis (Quantitect kit; Qiagen, Valencia, CA) following manufacturers' protocols. Quantitative polymerase chain reaction (PCR) was performed with three technical replicates for each primer set on cDNA (quantitative reverse-transcriptase PCR, qRTPCR) using primers for two reference genes, *tubulin* and *rps18,* and *def* (Table 1), with the 5-Prime SYBR-ROX kit (Fisher) following manufacturer's instructions with 250 nM of each primer and 1:40 diluted cDNA template (1 μl). PCR cycling was performed on an Eppendorf Realplex thermal cycler with the following parameters: 2 min at 95°C, 40 cycles of 59°C for 20 s, 68°C for 15 s, 95°C for 15 s, followed by 2 min at 68°C. Threshold cycles (C_T) for each reaction were collected and analyzed using the Relative Expression Software Tool (REST; Pfaffl et al. 2002), which allows for group wise comparative statistical analyses of relative expression while accounting for primer-efficiency differences.

Results

Temporal and Spatial Fate of GFP-*S. aureus* in the Gut

Epifluorescence microscopy was used to detect viable GFP-*S. aureus* in distinct regions of the digestive tract including the crop, midgut, hindgut, and rectum at 2 h intervals for 6 h PI. At 2 h PI, viable bacteria were visualized in the crop and midgut, and in the midgut, bacteria were restricted to the inner PM (Fig. 1A, B). At 2 h PI and throughout the observation period, cells appeared not as typical staphylococcal clusters but as single cocci or diplococci (Fig. 1B). At 4 h PI, noticeably fewer viable bacteria were observed expressing GFP, and clumps of remaining bacteria moved distally, presumably by peristalsis, toward the rectum. Clumps of bacteria cells were observed in the distal midgut (Fig. 1C, D) and hindgut/rectum (data not shown). Although bacteria were observed at 6 h PI, few viable cells were found in the crop. At 6 h PI, clumps of GFP-*S. aureus* mostly were seen in the hindgut (Fig. 1E, F) and rectum (not shown). Similar patterns of progression of bacteria in the gut of five flies examined at each time point were noted, and the only variability observed was a slight difference in the number of viable bacteria observed in the crop of flies at 2 or 4 h PI. All flies examined had viable GFP-*S. aureus* in the hindgut and rectum area as soon as 4 h PI.

Culture Recovery of GFP-*S. aureus* From Whole House Flies and Excreta

GFP-*S. aureus* was cultured from whole fly homogenate at all time points (Fig. 2), and a significant effect of time on bacterial recovery was observed ($F_{(3,29)}$ = 9.262; P = 0.0002).

Between 0 and 2 h PI, bacteria recovery from whole flies did not change significantly, but interestingly, we recovered a mean of 7,908 CFU from fly excreta during the same time interval (12/15 flies; Fig. 3). Throughout the 6 h collection period, recoverable numbers of bacteria from homogenized flies decreased, but significant differences occurred between both 2 and 4 h PI and 6 h PI (mean CFU at 6 h = 8.0×10^4; Tukey's HSD, $P < 0.05$; Fig. 2). Although 10/15 and 12/15 flies continued excreting bacteria at three and 4 h PI, respectively, CFU counts were nearly two orders of magnitude less than those at 2 h PI. Kruskal–Wallis analysis revealed a significant effect of time on mean CFU in excreta (df = 2; H = 10.02; $P = 0.0067$), and recoverable CFU of GFP-*S. aureus* in excreta at 4 h PI differed significantly from 2 h PI (Dunn's test; $P < 0.05$). Of note, while all 15 flies excreted recoverable bacteria during at least one of the time intervals, the amount of bacteria excreted varied greatly. The fewest CFUs recovered were 10 cells at 2 h PI, which also was the only time this fly excreted bacteria. The largest total number of CFU recovered was 30,470 CFU from one fly over the entire collection period; this fly also had the largest single excreta recovery, which was 30,000 CFU at 2 h PI. Overall, the average CFU per fly excreta (±SD) was 6,646 ± 9,302, which further demonstrates the great fly-to-fly variability in recovery of bacteria from excreta.

Temporal and Spatial Expression of *def* in Flies Fed GFP-*S. aureus*

Tissue-specific qRTPCR analyses of flies fed GFP-*S. aureus* were analyzed separately, as replicates could not be combined because of inter-replicate variability. *def* was upregulated in all tissues examined at 2, 4, and 6 h postingestion, in comparison to teneral flies that served as calibrators (Fig. 4). REST analysis revealed that expression differed significantly from that of teneral flies in proventriculus, crop, and fat body at all time points in both replicates ($P < 0.05$). However, upregulation in the midgut differed from baseline only at 2 h PI in the first biological replicate while upregulation was observed at all time points in the second biological replicate (Fig. 4; $P < 0.05$). The range of *def* upregulation in tissues of flies fed GFP-*S. aureus* was between ≈180 to >16,000 times the teneral level (Fig. 4).

Discussion

One goal of this study was to determine the fate of the pathogen *S. aureus* in the house fly using microscopy and culture recovery (from whole flies and excreta). These methods, along with the use of a GFP-expressing bacterial strain, allowed us to closely monitor the location and viability of bacteria over time. In addition, we concurrently assessed the expression of the antimicrobial peptide gene *def* both locally in the gut, proximal to bacteria, and systemically in the fat body. The combination of these experiments gave us a distinct spatial and temporal view of house fly-microbe interactions from the perspective of both organisms.

Spatial observation of bacteria in the house fly alimentary canal helped in determining critical time points to be included in subsequent culture-recovery experiments and provided insight into the mode by which flies could putatively excrete bacteria. Up to 2 h PI, viable cells of GFP-*S. aureus* were observed in the crop and midgut, primarily as diplococci and cocci. Thus, we suspect at early time points post ingestion, vomitus is the likely means of excretion of the large amounts of viable bacteria, similar to what has been demonstrated with other bacteria such as *Aeromonas hydrophila* (Chester) Stainer (McGaughey and Nayduch 2009) and *Enterococcus faecalis* (Andrewes and Horder) Schleifer (Doud and Zurek 2012). At later time points, bacteria more likely were dispersed in feces, because as soon as 4 h PI we observed bacteria in the hindgut and rectum. However, we observed lysis, clumping, and free GFP in several regions of the gut, which indicates that fly feces should contain fewer numbers of bacteria compared with vomitus at 2 h PI. While excreta were not collected at 6 h PI, our microscopical examinations indicated that the number of bacteria

shed at this time point would continue to temporally decrease, because clumps of bacteria and GFP were observed in the hindgut/rectum (Fig. 1F).

Culture recovery of bacteria from whole flies and excreta over time provided information not only on the vectoring and dissemination of *S. aureus* by flies, but also the extent to which flies may serve as environmental reservoirs for this pathogen. At 2 h PI, bacteria recovery from whole flies was not significantly different than the number of CFU fed (Fig. 2), yet a mean of ≈8,000 CFU (with up to 30,000 CFU) bacteria were cultured from fly excreta during that same time interval (Fig. 3), which equates to 1–10% of the amount of bacteria fed. It is important to note that recovery of an apparently large number of CFU from excreta may be artifactual interpretation, because of the discrepancy in what comprises a "CFU" between fed cultures and recovered in vivo *S. aureus.* In liquid culture, *S. aureus* were in clusters of 2 to >12 cells (data not shown), but in vivo we observed only cocci and diplococci (Fig. 1). Thus, because this morphological inconsistency exists, all recoveries from whole fly homogenate and excreta could reflect an apparent, but not actual, increase in CFU. We can further infer that the differences between fed and recovered amounts (whole fly or excreta) is actually greater than what was measured. Future experiments could be designed to sonicate or vortex liquid cultures before feeding to flies, with the alternate caveat being that this type of pretreatment would not represent the morphology of *S. aureus* that flies encounter in natural conditions.

Def was upregulated both locally, proximal to bacteria (i.e., crop, proventriculus, and midgut), and systemically (e.g., fat body) in flies. Systemic *def* expression may be stimulated by diffusing dimers of lysine-type peptidoglycan (LYS-PGN, comprising cell wall of gram-positive cocci) or by primary recognition via receptors on gut epithelia followed by secondary communication with the fat body, mediated by signaling molecules (Hao et al. 2003, Gendrin et al. 2009). In *D. melanogaster* gut AMP responses are entirely mediated through DAP-PGN recognition by trans-membrane PGRPs and the Imd pathway (Lhocine et al. 2008, Leulier and Royet 2009). How the house fly epithelial cells recognize LYS-PGN remains unknown. Because Defensin is classically associated with the Toll pathway systemically, where LYS-PGN would act upstream of Toll and not directly bind transmembrane PGRPs on the fat body (Wang et al. 2006), it would be interesting to determine if gut epithelial PGRPs have different affinity for PGN, or perhaps if cross activation of Toll and Imd occurs in the house fly gut as seen systemically in *D. melanogaster* (Tanji and Ip 2005, Tanji et al. 2007). Nonetheless, this is the first demonstration, to our knowledge, of epithelial *def* expression in the alimentary canal of cyclorrhaphous Diptera in response to feeding LYS-PGN bacteria. *Def* transcripts have been detected locally in the alimentary canal of other flies. For example, blood feeding alone stimulates *def* upregulation in the stable fly *Stomoxys calcitrans* L. (Munks et al. 2001), and DAP-PGN gram-negative bacteria like *E. coli* or even protozoan parasites induce *def* expression in the gut, proventriculus, and fat body of *Glossina morsitans* Westwood (Hao et al. 2001, 2003). Ongoing studies in our laboratory have determined that Defensin is upregulated in the gut of the house fly on both mRNA and protein levels in response to ingestion of both DAP-PGN and LYS-PGN expressing bacteria and protozoan parasites, but not in teneral flies that contain low levels of bacteria (<1,000 CFU) carried over from pupariation (D.N. et al., unpublished data). We have not yet determined whether all paralogs of *def* or, alternatively, transcript variants, were detected in our qRTPCR assays, but future work is aimed at a house fly transcriptome that should help elucidate the regulation of *def* transcription.

House fly Defensin may be one of the primary effectors contributing to observed GFP-*S. aureus* lysis and loss in the house fly gut because MRSA lysis by Defensin from other filth flies has been demonstrated (Andersen et al. 2010). Bacteria lysis in vivo likely results from

the joint action of several effectors (e.g., other AMPs), digestive enzymes, and pH changes in concert with mechanical removal via peristalsis (Lehane and Billingsley 1996). Nonetheless, survival of bacteria in the crop or rectum, for oral or fecal excretion, substantiates house fly vector potential for this organism. The mean CFU excreted from flies in our study was >6,000, which is within the clinically significant range. For example, 10^3 CFU of *S. aureus* can establish infection in 100% of guinea pig animal models (Vaudaux et al. 2002) and 3×10^2 CFU was found to be the minimal infectious dose for mice to establish long-term infection (John et al. 2011). Further, this is a sufficient inoculum for human or animal foods that could lead to enterotoxic food borne illness. The Food and Drug Administration (FDA 2012) has determined that only 10^5 CFU *S. aureus* per gram of food produces enough toxin for gastrointestinal illness (Adams and Moss 2008). Considering the generation time can be as short as ≈30 min in optimal conditions (Okuma et al. 2002) and the average inoculum deposited by flies in our study, generation of sufficient endotoxin could occur within 2 h after fly excreta contamination of food items. In summary, although flies only harbor *S. aureus* for a short period of time, and lysis of bacteria may occur via AMPs like Defensin and other processes in the gut, vector and dissemination potential for this pathogen is possible, albeit transiently.

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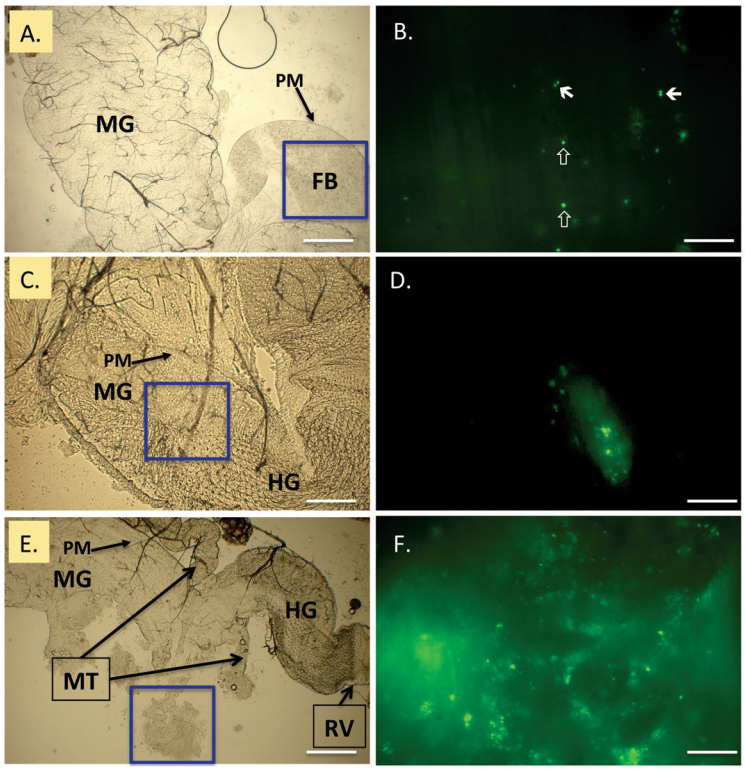

Fig. 1.

GFP-expressing *Staphylococcus aureus* in the house fly alimentary canal. Flies were fed 2.0 × 10^5 CFU, and bacteria were examined in dissected alimentary canals at 2, 4, and 6 h postingestion (PI). Epifluorescent microscopy (right, B, D, F) of blue boxed regions in bright field panels (left, A, C, E) revealed bacteria throughout the alimentary canal. Bacteria mainly appeared as single coccic and diplococcic forms as soon as 2 h (B., closed and open arrows, respectively), and were contained within food boluses (FB) in the peritrophic matrix (PM) of the midgut. Viable bacteria were visualized in the distal midgut (MG) and hindgut (HG) at 4 h PI. At 6 h PI, clumps of lysed and viable bacteria were released from the terminal open end of the PM, near the midgut/hindgut junction (E, boxed, freed during preparation and magnified for detail in F). MT = Malpighian tubules; RV = rectal valve. Scale bars: A, C, E, 200 μm; B, D, F, 20 μm. (Online figure in color.)

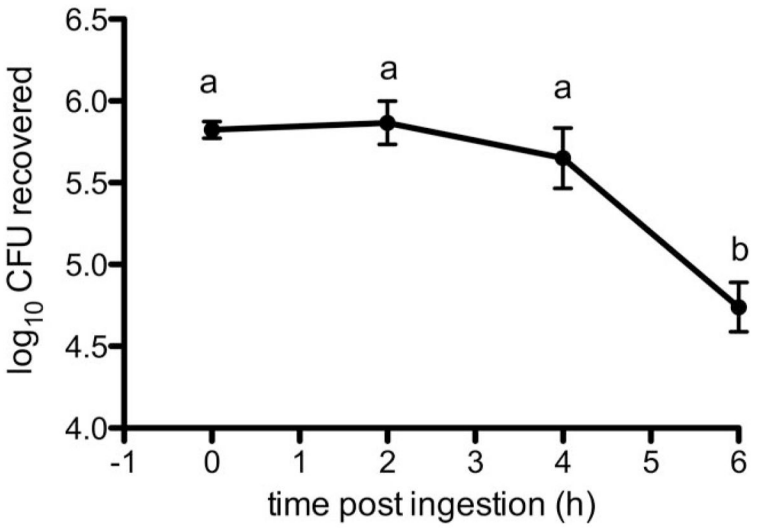

Fig. 2.

Recovery of *Staphylococcus aureus* from whole fly homogenate. Flies were fed 6.75 ± 1.44 × 10^5 CFU bacteria, and mean CFUs recovered for three replicate experiments are shown (n = 15 flies per replicate). Mean recoveries of bacteria from whole fly homogenate are shown ± SE. Different letters represent statistical significance (Tukey's; $P < 0.05$).

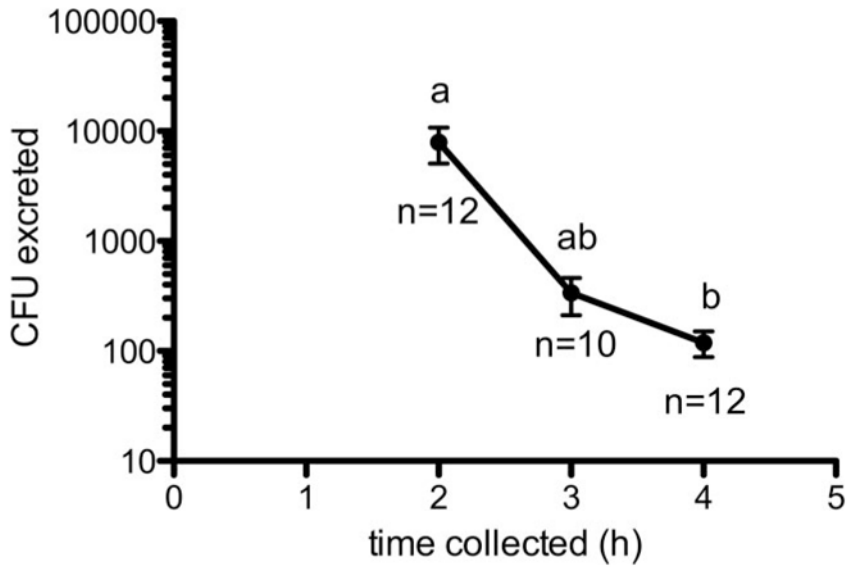

Fig. 3.

Recovery of *Staphylococcus aureus* from house fly excreta. Flies were fed a mean 3.2 ± 1.4 × 10^5 CFU bacteria in three separate experiments. Replicates were combined (n = 15 total flies). Mean recoveries of bacteria from excreta droplets (vomit and feces) are shown ± SE. Different letters above means represent statistical significance (Dunn's test; $P < 0.05$). Numbers of flies that excreted at that time point are indicated below means.

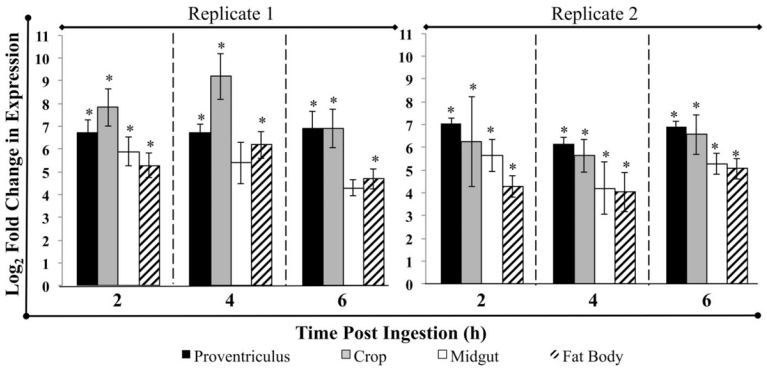

Fig. 4.

qRT-PCR analysis of tissue-specific expression of *defensin* in house flies fed GFP-*Staphylococcus aureus*. Fold changes in mRNA expression of *defensin* (*def*) were calculated using REST-MCS software and calibrated to expression levels in teneral adults and the reference genes *rps18* and *tubulin*. Log_2 fold change in expression levels is shown for each tissue, time and biological replicate, and error bars represent SE of technical replicates. Asterisks denote significant upregulation of *def* from baseline expression levels ($P < 0.05$).

Table 1

Primer sequences for qRTPCR

Gene	Accession no.	Primer sequence
rps18	ES608249.1	Fwd: 5′-GTTGGTATTGCCATGACCGCCATT-3′ Rev: 5′-ATGGGTTGGAGATGATGGTGACGA-3′
tubulin	ES608434.1	Fwd: 5′-GCTTGCTGCATGTTGTATCGTGGT-3′ Rev: 5′-CGAATTGAATGGTGCGCTTGGTCT-3′
defensin	DQ384634.1	Fwd: 5′-CAATTTCGTCCATGGAGCTGATGC-3′ Rev: 5′-ACCGCTCAACAAATCGCAAGTAGC-3′