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Expression of Lysozyme in the Life History of the House Fly (*Musca domestica* L.)

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Abstract

From egg to adult, all life history stages of house flies associate with septic environments teeming with bacteria. House fly lysozyme was first identified in the larval midgut, where it is used for digestion of microbe-rich meals because of its broad-spectrum activity against gram-positive and gram-negative bacteria as well as fungi. This study aimed to determine the temporal expression of lysozyme in the life history of house flies (from egg through adults) on both the mRNA and protein level, and to determine the tissue-specific expression of lysozyme in adult flies induced by feeding *Staphylococcus aureus*. From 30-min postoviposition through adulthood, all life history stages of the house fly express *lysozyme* on the mRNA level. In adult flies, *lysozyme* is expressed both locally in the alimentary canal and systemically in the fat body. Interestingly, we found that during the normal life history of flies, lysozyme protein was only detected in larval stages and older adults, likely because of ingestion of immune-stimulating levels of bacteria, not experienced during egg, pupa, and teneral adult stages. Constitutive expression on the mRNA level implies that this effector is a primary defense molecule in all stages of the house fly life history, and that a mechanism for posttranscriptional control of mature lysozyme enzyme expression may be present. Lysozyme active enzyme primarily serves both a digestive and defensive function in larval and adult flies, and may be a key player in the ability of *Musca domestica* L. to thrive in microbe-rich environments.

Keywords

stage-specific expression; tissue-specific expression; *Staphylococcus aureus*

Because larval house flies (*Musca domestica* L.) have been shown to require the ingestion of live bacteria to undergo development and pupariation (Zurek et al. 2000), there is an underlying necessity for all life history stages of flies to occupy a septic trophic niche. Although adults will feed on various substrates, usually in liquefied form and not necessarily septic, both males and females spend a large majority of time associated with filth for breeding purposes. Thus, all stages of house fly development are constantly exposed to a diverse array of bacteria and fungi, both on their surfaces and in their food.

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Microbes have the opportunity to infect and infest house flies once internalized in the alimentary canal. Therefore, insects have in place both physical and secreted defenses in the gut. Bacteria ingested by adult flies usually are first stored in the sac-like crop lined with protective cuticle. Subsequently the meal is regurgitated anteriorly to a cuticle-lined area of the proximal foregut, adjacent to the proventriculus, and then transferred posteriorly where it enters the midgut. The delicate midgut epithelium is protected from bacteria by a double-layered type II peritrophic matrix (PM) via physical exclusion (Tellam 1996, Lehane 1997). The PM terminates at the midgut–hindgut junction, where the epithelium of the hindgut and rectum are again protected by cuticle. In addition to physical barriers, the house fly gut epithelium secretes lytic digestive enzymes and presents variable pH zones in the alimentary canal (Terra et al. 1988). In larvae, midgut digestion of bacteria likely results from the combined action of pepsin, low pH, and a digestive lysozyme (Espinoza–Fuentes and Terra 1987).

Lysozyme is an antibacterial enzyme found in higher metazoa, whose main function is to cleave the β 1,4-glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine found in the peptidoglycan (PGN) of bacterial cell walls (Phillips 1967). Studies in *M. domestica* larvae have shown that house fly lysozyme has both antibacterial (i.e., PGN-digesting) and chitinase functions, providing the added ability to digest fungal cell walls (Espinoza–Fuentes and Terra 1987, Lemos et al. 1993). In addition, this “digestive” lysozyme has optimal activity at pH 3.5–5 (Terra et al. 1988, Ito et al. 1995), consistent with the acidic midgut of house fly larvae and adults. Initial studies characterizing lysozyme activity in larvae showed active enzyme in the middle midgut (where pH averages 3.1) and not in other tissues (Lemos and Terra 1991, Terra and Ferreira 1994).

Due to the proposed broad-spectrum activity of house fly lysozyme, this study aimed to investigate the role of this enzyme throughout the life history of the fly by 1) determining expression of lysozyme protein and mRNA in life history stages from egg through adult and 2) quantitative analysis of the spatial and temporal expression of *lysozyme* in adult house flies both locally in the alimentary canal (proximal to bacteria) and systemically in the fat body. We hypothesized that lysozyme would be expressed as a constitutive first line of defense throughout the life stages of this insect. Further, we predicted that lysozyme would be constitutively expressed primarily in the gut, where it would be used for bacterial digestion at low pH.

Materials and Methods

House Fly Rearing

House fly colonies 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 cycle. Small containers of 75% wheat bran and 25% vermiculite saturated with tap water were placed in colony cages as oviposition (larva) medium. Larva medium incubated for 24 h at 30°C became readily contaminated with ubiquitous bacteria (“ripened”), and this microbial floral growth served as a food source for larval development.

Life Stage Collections

Five larvae were removed from larva medium at 1, 3, and 5 d after oviposition (keyed to first, second, and third instars, respectively) and processed (see below). Third instar larvae or early puparia were collected from larva medium, placed in sterile petri dishes, maintained at 30°C and removed at 1, 2, and 3 d postpupariation, which corresponded with white, amber, and dark brown colored puparia, respectively ($n = 5$, each). Adult flies were eclosed from puparia in sterilized 50-ml jars, and either remained unfed ($n = 5$) or were fed sterile

10% sucrose for experiments below. Oviposited egg samples were obtained by preparing ripened larval medium contaminated with ubiquitous bacteria as above. Approximately 5 ml of ripened medium was added to a petri dish, which was placed in the fly colony cage to observe oviposition. Using this method, we obtained three clusters each of 15–20 eggs $\approx$ 0–10 min old and three clusters of 15–20 eggs $\approx$ 30–35 min old. To test unfertilized and fertilized house fly eggs, newly eclosed females ($n = 9$ per treatment) were either kept isolated from males (unmated) or were placed with nine males in a 500 ml container. Flies were provided ad libitum sterile 10% liquid fly food and maintained for 120 h at 30°C. After this time, flies were chilled and dissected to collect three pools of three pairs of ovaries from each treatment group for processing (below). “Old” flies used in expression analyses below were five flies (mixed sex, >1 wk old) removed from our colony, which were normally exposed to ubiquitous bacteria on feeding sponges and fly food in mating cages where they were housed.

Adult Fly Bacteria Feeding

For bacterial feeding, newly eclosed mixed-sex adults were fed sterile sucrose for 36 h, and were then fasted for 12 h to clear out gut bacteria and induce selective feeding. Bacteria were cultured in Luria-Bertani (LB) broth (Fisher, Atlanta, GA) and included *E. coli* K12 (OD₆₀₀ = 2.329) and *Staphylococcus aureus* RN6390 (OD₆₀₀ = 2.695). Five hundred microliters of each culture were combined (1 ml total) and serially diluted on LB agar for enumeration. Two microliters of the mixed culture (1.2×10^5 CFU/ μ l) were fed to 12 individual flies, and at 2, 6, 12, and 24 h postingestion, three flies were pooled and processed for RNA extraction (below).

RNA Extraction and Standard Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from homogenized specimens using the Ribopure kit (Ambion, Austin, TX) according to manufacturer instructions. Reverse transcription (cDNA synthesis; RT) was performed by using the Quantitect (Qiagen, Valencia, CA) kit according to manufacturer instructions with 1 μ g DNase-treated RNA serving as template. Polymerase Chain Reaction (PCR) was performed subsequently in 20 μ l reactions as follows: 0.2 μ l GoTaq polymerase, 4 μ l GoTaq buffer, 0.4 μ l of each 20 μ M primer, 1 μ l template cDNA, 2 μ l dNTP mix, 1.25 μ l 25 mM MgCl₂, and 10.75 μ l of nuclease-free water (Fisher). Each sample cDNA was run in two reactions, one with *lysozyme* primers as a target gene and one with *rps18* primers as a reference gene (Table 1). Cycling parameters for PCR were: 2 min at 94°C, 35 cycles of 15 s at 94°C, 15 s at 55°C, and 15 s at 72°C, and a 5 min terminal extension at 72°C. Products were analyzed by electrophoresis (0.9% agarose TBE gel) for 45 min at 150 v.

Local Versus Systemic *lysozyme* Expression via Quantitative Reverse-Transcriptase PCR (qRT-PCR)

Flies ($n = 30$ per replicate, two biological replicates) were individually housed in glass jars and fed 2 μ l *S. aureus* RN6390 (OD₆₀₀ = 0.97; $\approx 4 \times 10^5$ CFU/fly) as above. Tissues (proventriculus, midgut, hindgut, and fat body) were aseptically dissected from 10 flies at 2, 4, and 6 h postingestion and pooled by tissue for RNA extraction and cDNA synthesis as above. qRT-PCR was performed on an Eppendorf Realplex instrument (Eppendorf NA, Inc., Hauppauge, NY) using primers for two reference genes, *tubulin* and *rps18*, and *lysozyme* (Table 1). PCR master mix contained 14.85 μ l 5-Prime SYBR-ROX (Fisher), 3.3 μ l each of 2.5 μ M primer, 1.55 μ l nuclease-free water; 6.7 μ l of this mixture was added to each of three separate wells in a PCR plate for three technical replicates. To each well, 3.3 μ l of 1:40 diluted cDNA template was added. PCR cycling parameters were: 2 min at 95°C, followed

by 40 cycles of 20 s at 59°C, 15 s at 68°C, 15 s at 95°C, and a final extension for 2 min at 68°C. Melting curve analysis was performed on all samples to ensure single product amplification. Threshold cycles (C_T) for each reaction (e.g., tissue, time point, and gene) were collected and analyzed using the Relative Expression Software Tool (REST; Pfaffl et al. 2002), which allows for group wise comparisons and statistical analyses of relative expression, and corrects for differences in primer efficiency.

Western Analysis of *lysozyme* Expression Across Life History Stages

Unfertilized and fertilized ovaries (dissected from $n = 3$ flies), 15 and 30 min egg clutches (as described above for RNA analysis), larval and pupal stages as above ($n = 4$ each), and teneral (newly emerged, unfed) or old adult flies (mixed sex, $n = 4$ each) were homogenized in protein lysis buffer as previously described by Edery et al. (1994), boiled at 100°C for 10 min, and centrifuged for 1 min at 11,000 rpm. Protein concentration was determined by Bradford Coomassie protein assay (Thermoscientific, Rockford, IL) following the manufacturer's protocol. Equivalent amounts of protein (30 µg/lane) were separated on 4–20% Glycine–SDS–polyacrylamide gels (Pierce, Rockford, IL) by electrophoresis at 60 V for 2 h in running buffer (100 mM Tris, 100 mM HEPES, 3 mM SDS, pH = 8.0). The gel was transferred at 152 mA for 70 min in transfer buffer (25 mM Tris, 192 mM Glycine, pH = 8.0) via semidry dry blotter (Fisher) to a PVDF membrane (Bio-Rad, Hercules, CA). The blot was blocked in 5% nonfat dry milk dissolved in TBS (25 mM Tris, 0.15 M NaCl, pH = 7.2) for 1 h at RT. After blocking, the blot was washed in TBST (25 mM Tris, 0.15 M NaCl, pH = 7.2, 0.2% Tween 20 [vol:vol]) for 5 min. Rat anti-lysozyme primary polyclonal antibodies (Genscript, Piscataway, NJ) were generated to the peptide CQPSNGRFSYN-ECHL, a sequence specific to *M. domestica* lysozyme 1 (MdLys1; Cançado et al. 2008), which is the major lysozyme expressed in house fly larvae and adults. The blot was incubated with polyclonal rat anti-lysozyme antisera (17.28 µg/ml) for 2 h at RT, then washed twice in TBST for 10 min. Following washes, the blot was incubated for 1 h with horseradish peroxidase-conjugated anti-rat antibody (1 µg/ml in TBST; Genscript), 0.01% SDS, and 5% nonfat dry milk at room temperature. After two 10-min TBST washes, and one 5-min TBS wash, the blot was visualized by a Pierce ECL chemiluminescence system (Thermoscientific, Rockford, IL) following manufacturer's instructions, and exposed for 15 min (Kodak, Rochester, NY).

Results

lysozyme mRNA Expression in House Flies

mRNA of *lysozyme* was not detected in either fertilized or unfertilized eggs dissected from gravid females (Fig. 1). However, *lysozyme* expression was detected in house fly eggs as soon as 30 min postoviposition onto bacteria-rich medium, and this expression continued throughout life history, irrespective of obvious exposure to bacteria (Fig. 1). Tissue-specific qRT-PCR analyses of flies fed *S. aureus* were analyzed separately, as replicates could not be combined because of variability. *lysozyme* was upregulated in the hindgut and fat body, and downregulated in the proventriculus and midgut at 2, 4, and 6 h postingestion, in comparison with teneral flies, which served as calibrator (Fig. 2). REST analysis revealed that expression differed significantly in hindgut ($P = 0.001$) and fatbody ($P = 0.048$) of flies fed bacteria at all time points in both replicates when compared with baseline levels in teneral flies. However, downregulation in the midgut differed from baseline only at 4 and 6 h postingestion in the first biological replicate ($P = 0.001$).

Lysozyme Protein Detection in Life History of the House Fly

Incongruent with mRNA expression, lysozyme protein only was detected in feeding larval stages (all three instars) and adults >1 wk old (Fig. 3). The approximate molecular weight of

secreted peptide is between 13 and 14 kDa, and larger signal detected (≈ 15 kDa) may represent full unprocessed peptide still retaining signal sequence (Ren et al. 2009), as protein was extracted from whole tissue. Neither fertilized nor unfertilized eggs produced lysozyme protein, which correlated with RT-PCR results (Fig. 1); however, lysozyme protein was not detected in pupae or teneral flies (Fig. 3), unlike *lysozyme* mRNA (Fig. 1). We also were unable to detect lysozyme protein in clutches of eggs 15 or 30 min postoviposition (data not shown).

Discussion

House flies began expressing mRNA of *lysozyme* within the interval of 10–30 min postoviposition and continued expressing this gene at the mRNA level trans-stadially throughout the life history. The molecular trigger and signaling pathway that may induce transcription of *lysozyme* in the egg remains undiscovered, but expression may commence upon detection of microbe-associated molecular patterns (MAMPs) present on bacteria in larva medium. Because the vitelline membrane of the fly egg is surrounded by perforated chorion (Smith 1968), MAMPs such as small peptidoglycan dimers feasibly can traverse and bind MAMP receptors such as Peptidoglycan-Recognition Proteins (PGRPs; Charroux and Royet 2010), if present, on the cell membrane of the developing zygote. An alternative mechanism of *lysozyme* transcription induction could be restoration of the diploid state after fertilization and syngamy, events that occur during ovulation and after oviposition, respectively (Schwalm 1988). For example, although fertilization by sperm occurs during travel of the egg through the oviduct, fusion of gametic pronuclei in *Drosophila melanogaster* is delayed 10–15 min postoviposition (Foe et al. 1993). To our knowledge, the timing of syngamy in house flies has not been determined, but feasibly could occur within the time interval where *lysozyme* is first detected. Investigation of the actual trigger for *lysozyme* induction, and constitutive expression throughout life of the fly, merits further study as this gene product may serve a critical role in the house fly's successful occupation of a septic niche.

The tissue-specific quantitative analysis of *lysozyme* expression in response to *S. aureus* oral ingestion revealed both local (alimentary canal) and systemic (fat body) upregulation. Lysozyme enzyme activity was first described in the gut of *M. domestica* larvae (Lemos and Terra 1991), and Ren et al. (2009) recently described the presence of *lysozyme* mRNA and protein in the fat body of larvae as well. To our knowledge, our study is the first report of *lysozyme* expression in the hindgut and fat body of adult house flies. Because house fly lysozyme has been previously shown to have an acidic optimal pH (Lemos and Terra, 1991, Ito et al. 1995), it remains unclear whether hindgut or fat body lysozyme protein would have effective glycolytic and chitinolytic activities in these anatomical locations, which are near neutral or basic pH. We did not determine whether adult fat body and hindgut produced lysozyme protein in this study, but recent experiments in our laboratory have demonstrated immunolocalization of lysozyme protein in hind gut tissue of flies fed high doses of bacteria (unpublished data). Further, it remains to be determined how the presence of gut bacteria would be detected by the fat body, which would be responsible for circulating lysozyme in the hemolymph, but such communication between these two compartments may be mediated by nitric oxide, as suggested in tsetse (Hao et al. 2003), or even diffusion of muropeptide dimers of peptidoglycan, as inferred in fruit flies (Stenbak et al. 2004, Zaidman-Remy et al. 2006, Gendrin et al. 2009). We do not know whether the fat body and hindgut *lysozyme* detected in this study and Ren et al. (2009) is an identical or a paralog of the predominant *lysozyme* in house flies, *Musca domestica lysozyme-1* (*MdLys1*) (Cançado et al. 2008), and it would be interesting to determine whether several paralogs of *lysozyme* exist, as is the case for *Drosophila melanogaster* (Meigen), especially since they often show tissue- and stage-specific regulation of expression (Daffre et al. 1994). The *lysozyme* primers

used in this study should preferentially amplify only MdLys1, as each primer differs in sequence from *lysozyme-2* (*MdLys2*) in the 3' region, thereby terminating polymerase translocation and strand extension if mispriming occurred. Therefore, it is unlikely that our primers identified both paralogs in the RT-PCR or qRT-PCR experiments, but we will investigate this possibility in future studies.

We separately analyzed the tissue-specific qRT-PCR analyses in flies fed *S. aureus* because of variability across biological replicates. This variability was not surprising, as flies can show differences in compartmentalization of the ingested meal (i.e., to crop or to midgut) as well as varying peristaltic rates, as a consequence of differences in the physiological state of each experimental cohort (e.g., larval nutrition, hydration status, and behavior). Nonetheless, *lysozyme* was upregulated in the hindgut and fat body, and downregulated in the proventriculus and midgut at 2, 4, and 6 h postingestion, in comparison with teneral flies, which served as calibrator. Interestingly, *lysozyme* expression was downregulated in the proventriculus and midgut of flies fed *S. aureus* (Fig. 2). Because the calibrator state used in REST analysis of qRT-PCR data were teneral flies, this implies that the teneral proventriculus and midgut *lysozyme* mRNA level exceeds that of flies fed bacteria. Indeed, standard RT-PCR analysis did detect teneral *lysozyme* expression in whole flies (Fig. 1), although the actual level was not quantified. Teneral expression of mRNA coding for effector molecules in the gut in the absence of microbial MAMP induction has been previously reported in other Diptera, including *Glossina* spp. (Hao et al. 2001, Nayduch and Aksoy 2007) and *D. melanogaster* (Tzou et al. 2000), which have shown tissue-specific constitutive expression of antimicrobial peptides (AMPs). As in the current study, it is thought that such uninduced expression serves as a primed first line of defense; should the insect encounter microbes in their environment or meal, mRNAs can rapidly be translated into effector molecules that target microbial threats.

A significant finding in this study was the incongruence between mRNA and protein level expression of lysozyme across the house fly life history. Lysozyme protein only was detected in stages of life directly exposed to high levels of bacteria, including feeding or wandering larval instars and breeding adults. We postulate that the house fly maintains a primed first line of defense by having mRNA available for rapid, imminent, and effective production in the event of bacterial encounter, yet tightly regulates translation to protein by an unknown posttranscriptional mechanism. The antibodies used for western analysis in this study were designed only to detect the “major” lysozyme, as described in Cançado et al. 2008 (MdL1). It would be interesting to determine the spatial and temporal expression of the second lysozyme (MdL2) across life history and in concert with MdL1 at both protein and RNA level.

Constitutive expression of lysozyme, even at the mRNA level, could be energetically costly to house flies; however, the digestive and defensive dual role of lysozyme allows house flies to exploit life in a septic niche where relatively few other metazoa thrive. Constitutive expression of mRNA for *lysozyme* has also been reported in *D. melanogaster*, whose larvae live in microbe-rich environments on rotting vegetation (Daffre et al. 1994). It would be interesting to determine whether other filth-dwelling invertebrates, especially coprophagous insects, constitutively express broad-spectrum defensive bacteriolytic or bacteriocidal genes in their life history as a similar life strategy. Notably, research in our laboratory has shown that house flies do not constitutively express AMPs such as *dipteracin*, *attacin*, and *cecropin* (D. Nayduch, C. Saski, and C. Joyner, unpublished). However, recent data in our lab suggest that lysozyme likely works in concert with enzymes, pH changes, and effectors such as AMPs to degrade microbial cell envelopes when the number of bacteria encountered are above a yet to be determined threshold level. Synergy of AMPs and digestive enzymes may further broaden spectrum of activity and facilitate lysozyme access to the cell wall (Chalk et

al., 1994, Mumcuoglu et al. 2001). Likewise, lysozyme processing of PGN has been shown to facilitate binding to PGRPs and induction of consequent immune response pathways (Park et al. 2007), further illuminating the complex and concerted response insects use in their antibacterial defense strategies.

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Fig. 1. RT-PCR analysis of *lysozyme* and *rps18* expression in life history stages of the house fly. Standard RT-PCR for *lysozyme* (*lys*) and the reference gene *rps18* was performed on developmental stages of flies and bacteria fed adults. For ovaries (eggs dissected from mated or unmated female house flies) and oviposited eggs, three biological replicates are shown for each. For larvae and pupae, pooled instars or stages, respectively, are described in the text. SA/EC fed were adult flies fed a mixture of *S. aureus* and *E. coli* and collected 2, 6, 12, and 24 h postingestion, and pooled for RNA extraction. Teneral (ten) and >1-wk-old adults from the breeding colony (old) also were pooled.

Fig. 2.

Tissue-specific qRT-PCR analysis of *lysozyme* expression in house flies after ingestion of *Staphylococcus aureus*. Fold changes in mRNA expression levels were calculated using REST-MCS software and calibrated to expression levels in teneral adults. Log₂ fold change in expression levels is shown for each tissue, time, and biological replicate, and error bars represent standard error of technical replicates. Asterisks denote significant upregulation or downregulation of *lysozyme* from baseline expression levels ($P < 0.05$). Biological replicates could not be combined, and are shown separately.

Fig. 3.

Lysozyme protein expression in developmental stages of the house fly. Protein (30 μ g/lane) from life stages was electrophoresed, blotted, and lysozyme was detected via western analysis as described in the text. Samples were eggs from mated (M) or unmated virgin (V) females, three larval instars (L1–L3), pupal stages (P1–P3; white, amber, dark brown, respectively) and teneral (ten) or >1-wk-old colony (old) adults. Approximate size of the major lysozyme detected was around 13–14 kDa, and a second larger signal detected ($\approx$ 15 kDa) may represent full unprocessed peptide.

Table 1
Sequences of primers used for PCR

Gene	Accession no.	Primer sequence
<i>rps18</i>	ES608249.1	Fwd: 5' -GTTGGTATTGCCATGACCGCCATT-3' Rev: 5'-ATGGGTGGAGATGATGGTGACGA-3'
<i>tubulin</i>	ES608434.1	Fwd: 5'-GCTTGCTGCATGTTGTATCGTGGT-3' Rev: 5'-CGAATTGAATGGTGCGCTTGGTCT-3'
<i>lysozyme</i>	KC685581	Fwd: 5' -TCGAATGGCTCCAACGATTACGGT-3' Rev: 5'-TCCAGCCTTGTTGGGACTTGATCT-3'