Cathepsin E & L Expression in Response to Septic Injury in *Drosophila melanogaster*

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

in

Biology

by

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2013
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2013
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Acknowledgements

I would like to acknowledge a group of individuals at the University of California, San Diego who have assisted me towards the completion of this project.

First and foremost, I would like to thank my adviser, Dr. Willis Li, for his guidance throughout the course of this project. His direction and mentoring were indispensable in shaping my approach to the scientific questions addressed and also helped me grow a great deal as a researcher during my time as a student.

I would like to thank Dr. Pranab Dutta, who helped me learn a variety of experimental techniques. Thanks to Jingtong Wang who aided in developing the methods used. Additional thanks to all of the undergraduate students who worked on this project, including Taeisk You and Rachel Meza. I would also like to thank all members of the Li Lab for their support and input including: Areana Park, Andre Loyola, Alex Chavez, and Vahid Niki.

Thanks to the members of my thesis committee, Dr. Steven Wasserman and Dr. Ethan Bier, for their support and suggestions regarding my research.

Additionally, I would like to thank my family and friends for their support over the course of my research and throughout my life.
ABSTRACT OF THE THESIS

Cathepsin E & L Expression in Response to Septic Injury in Drosophila melanogaster

by

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Master of Science in Biology

University of California, San Diego, 2013

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Professor Steven Wasserman, Co-Chair

Chronic inflammation is a problem associated with many diseases such as: rheumatoid arthritis, asthma, and inflammatory bowel disease. [4] Understanding the long term effects of chronic inflammation on lifespan and immunity could inform our treatment of such diseases. JAK/STAT gain of function (hopTum-l) flies exhibit chronic inflammation due to overexpression of cytokines such as upd and antimicrobial peptides. Previous research has shown that altering the levels of cathepsin E & L in Drosophila melanogaster extends lifespan in Drosophila cancer models, additionally JAK/STAT targets cathepsins E & L have been implicated in immunity to microbial infection. [20] We show that cathepsin E & L expression levels in fat body are increased
in response to septic injury. We also investigated the bacterial counts in JAK/STAT gain of function (hop\textsuperscript{Tum-l}), JAK/STAT loss of function (hop\textsuperscript{3}), and wild type flies at time points after septic injury with \textit{E. coli}. We found that wild type flies are able to eliminate bacteria in an exponential fashion within 15 minutes. In both hop\textsuperscript{Tum-l} and hop\textsuperscript{3} flies, bacterial counts were best modeled by a parabolic equation, while bacterial counts eventually increased in both mutants, they showed a longer initial decrease in hop\textsuperscript{Tum-l} flies. These results suggest that cathepsin E and L proteases are expressed in fat body as part of the humoral response to bacterial infection and are possibly involved in the production of antimicrobial peptides. Additionally, the higher levels of cathepsin E & L as seen in hop\textsuperscript{Tum-l} mutants allow them to counteract bacterial infection more efficiently than loss of function mutants.
Introduction

*Drosophila melanogaster* are a particularly useful model in which to study concepts of human disease and immunity because there is a high level of conservation of the *Drosophila* innate immune system as compared to humans. Due to their lack of adaptive immunity, studies in *Drosophila* can focus on the components and effects of the innate immune system. [18]

There are three divisions of the *Drosophila* innate immune response: humoral response, cellular response, and melanization. During the humoral response, immune challenge stimulates the fat body to produce antimicrobial peptides. In the cellular response, plasmocytes phagocytose invading microorganisms. Lastly, melanization occurs at the site of a wound in order to facilitate healing. [19]

Cellular Response & Melanization

*Drosophila* have three types of blood cells: lamellocytes, crystal cells, and plasmocytes. Lamellocytes are involved in encapsulation and crystal cells are involved in melanization. The cellular response is enacted by blood cells and leads to phagocytosis of microbes by plasmocytes. [1] Melanization is involved in both wound healing and encapsulation, and generates toxic intermediates that kill invaders. [19]

Humoral Response

As mentioned previously, the humoral response leads to the production of antimicrobial peptides by the fat body.

Currently, there are seven known antimicrobial peptide families in *Drosophila*. Drosomycin and Cecropin are active against fungi, Defensin is active against gram-positive pathogens. Metchnikowin is active against both fungi and gram-positive pathogens. Additionally, Attacin, Cecropin, Diptericin, and Drosocin are active against gram-negative pathogens. Transcription of the genes for these peptides is induced in the fat body soon after immune challenge. [19]
The humoral response is controlled by three signaling pathways, two of which have been well characterized. These are the Imd(immune deficiency) and Toll pathways, both of which are activated in response to septic injury. [18]

The Imd pathway controls the systemic expression of antimicrobial peptides by fat body as well as the local expression by gut epithelium in response to challenge by Gram-negative bacterial infections. The Imd pathway is turned on in response to detection of DAP-type Gram-negative peptidoglycan. Subsequent recruitment of the Imd molecule starts a cascade which results in activation of Imd controlled genes. [18]

The Drosophila Toll pathway controls the expression of antimicrobial peptides by the fat body in response to Gram-positive and fungal infections. Toll signaling is turned on by the detection of lysine-type Gram-positive peptidoglycan as well as by fungal cell wall components. This is followed by a cascade that results in translocation of the Dif and Dorsal proteins into the nucleus where they turn on expression of Toll controlled genes. [18]

The Janus kinase-signal transducer and activator of transcription(JAK/STAT) pathway is the third signaling pathway which controls the humoral response and it is not as well characterized. [18] The Drosophila JAK/STAT pathway is homologous to its mammalian counterpart. Drosophila are a good model in which to study the JAK/STAT pathway because they have only one Janus kinase(Hopscotch) and one STAT protein(STAT92E) compared to mammals which have three types of JAK enzyme, and seven types of STAT protein. In Drosophila, the canonical JAK/STAT pathway is activated in response to binding of the cytokine upd to a JAK-coupled Domeless receptor which causes activation of JAK enzymes that phosphorylate cytoplasmic STAT. Subsequently, phosphorylated STAT proteins dimerize and translocate to the nucleus where they activate transcription of JAK/STAT target genes. [21]

Two cathepsin enzymes, cathepsins E & L, are of interest because previous research has determined that the genes for cathepsins E and L are targets of the JAK/STAT pathway. [20] It was determined that changing levels of cathepsin expression in Drosophila
resulted in altered lifespan. Decreased expression of cathepsin E was lethal in the pupa stage, while overexpression caused decreased lifespan in wild type models. Both increasing and decreasing the levels of cathepsin L in wild type Drosophila decreased lifespan. Lifespan studies were also performed in relevant mutants. hopc111 mutants have a loss of function hopscotch allele and hop^{Tum-l} mutants have a hyperactive hopscotch allele. The hop^{Tum-l} mutation is known to be oncogenic due to overproliferation of hemocyte stem cells which leads to blood tumors similar to those seen in leukemia. [10] Notably, the combination of cathepsin E deficiency mutation with the hop^{Tum-l} mutation resulted in an increased lifespan compared to hop^{Tum-l} mutants. Levels of cathepsin L expression were elevated in response to injury, and flies with cathepsin E(RNAi) were more susceptible to bacterial infection and had shorter lifespans, a phenotype that was rescued upon treatment with antibiotics. The study concluded that a delicate balance of cathepsin levels is necessary for optimum lifespan and immunity. [20] Because alteration of cathepsin expression levels caused differences in septic injury survival and the lethality of cathepsin E downregulation was rescued upon treatment with antibiotics, we hypothesize that cathepsins E & L are involved in microbial immunity in some capacity. It is unknown where cathepsins E& L are expressed in Drosophila. Therefore, this study aims to elucidate where cathepsins E& L are expressed in response to septic injury, as well as gain insight into how the immune response to septic injury in JAK/STAT mutants differs from that of wild type flies.

Cathepsins are classified into categories based on the key residues involved in their catalytic mechanisms. Cathepsin E is an aspartic protease, and cathepsin L is a cysteine protease. Previous research has shown that cathepsins are involved in a variety of aspects of immunity and cancer.

**Cathepsins**

Cathepsins are a group of protease enzymes that were originally discovered in the lysosome. The first cathepsin, cathepsin C, was described by Gutman et. al. Sub-
sequently, many other cathepsins were discovered including cysteine cathepsins B, H, L, C, K, O, F, V, X and W as well as aspartic cathepsins D and E. Eventually, the structure of the cathepsin enzymes was determined. The general structure of a cysteine cathepsin is a monomer with a light and heavy chain connected by disulfide bonds. The cathepsin active site is V-shaped and contains conserved secondary structures including alpha helices and a beta barrel. Aspartic cathepsins require two aspartic amino acids in the active site. [15]

Although the cathepsins were originally discovered in the nucleus, various mechanisms have been discovered through which different cathepsin L variants can localize to other parts of the cell. Cathepsin L variants transcribed from downstream start sites do not contain the signal peptide that targets them to the ER. Cathepsin L variants that are targeted to the ER are glycosylated, if they are to be targeted to the lysosome, they are processed in the golgi apparatus where mannose residues are changed to mannose-6-phosphates. These cathepsins then bind a mannose-6-phosphate receptor that targets them to the lysosome. If the change to mannose-6-phosphate does not occur, these cathepsin L variants are exocytosed. There is also evidence that cathepsins can be recruited from late endosomes or lysosomes and then be secreted into the extracellular space when the organelles in which these enzymes are contained fuse to the cells plasma membrane. Aside from the lysosome, cathepsin L has been found to be active in the nucleus, secretory vesicle, mitochondrial matrix, and the extracellular space. [26] Meanwhile, cathepsin E has been found to be involved in antigen presentation and cathepsin E knockout mice have been found to have a reduced number of bacteria specific TLRs. [20]

**Cathepsin L**

Recent research shows a variety of roles for cathepsin L (cat L) in normal cell cycle progression, antigen presentation, and cancer. Cathepsin L is generally expressed and secreted at higher levels in tumor cells than in normal cells. Additionally, Cathepsin L has been shown to efficiently degrade components of the basement membrane includ-
ing collagen, laminin, and elastin. [6] One of the products of cathepsin L cleavage of collagen XVIII in murine hemangioendothelioma cancer cells is endostatin, an angiogenesis inhibitor which also slows tumor growth. Felbor et. al. propose that secretion of cathepsin L and the subsequent production of endostatin by cancer cells is part of feedback regulation of angiogenesis that prevents the growth of secondary cancers and far reaching metastases as seen in cancer patients. [9]

Additionally, shorter isoforms of cathepsin L generated through the use of downstream transcription start sites are localized to the nucleus during the G1-S transition where they process the CDP/Cux transcription factor to its active form. As seen from transgenic organisms, CDP/Cux plays an important roll in differentiation and growth. Growth retardation or multiorgan tissue hyperplasia is exhibited in cux-1 inactivated or over expressed mutants respectively. [13]

Cathepsin L is also involved in the processing of multiple peptide neurotransmitters including enkephalin, neuropeptide Y, proopiomelanocortin, dynorphins, cholecystokinin, and catestatin in the secretory vesicle. Additional research has shown that cathepsin L is involved in matrix degradation, cell signaling, and atherosclerosis. [17]

Cathepsin L has also been proven to be involved in DNA repair. It is known that cathepsin L levels are higher in cancer, but it was found that cathepsin L is involved in the degradation of 53BP1, a mediator/adaptor protein, recruited to double-stranded DNA breaks and involved in the non-homologous end joining (NHEJ) pathway of DNA Damage Response. [12] Further research implicated this pathway in BRCA-1 deficient breast cancer cells. High levels of 53BP1 resulted in use of NHEJ, a more error prone method of DNA damage repair. The resulting chromosomal instability caused growth arrest and death. On the other hand, in tumor cells without BRCA-1 that upregulate cathepsin L to decrease 53BP1 levels, the homologous recombination (HR) pathway of DNA Damage Repair was rescued allowing the tumor cells to bypass growth arrest. As a result, levels of cathepsin L and 53BP1 are now being considered as markers of BRCA-1 tumors. [14]
Cathepsin L also has a role in the MHC II pathway of antigen presentation. Cathepsin L was found to be important in the degradation pathway of the invariant chain(Ii) in cortical thymic epithelial cells, involved in the positive selection of CD4+ T cells, but not in bone marrow derived antigen presenting cells, which are involved in negative selection. [22]

When cat L knockout mice were crossed with congenic mice bred to develop pancreatic tumors, it was observed that tumors in the cathepsin L−/− mice had decreased tumor proliferation and growth, and decreased tumor invasiveness and malignancy. Additionally, it was determined that E-cadherin, a cell-adhesion protein that is characteristically absent in tumors that are invasive, was found to be a substrate of cathepsin L. [11] Therefore cysteine cathepsins such as cathepsin L are possible targets for anti-cancer drugs. [20]

Cathepsin E

Cathepsin E is expressed in a variety of lymphocytes. It is found in microglia, dendritic cells, and macrophages in its active form and in other antigen-presenting cells in its proform. Expression of cathepsin E in microglia was found to be increased upon treatment with IFN-γ which causes MHC class II expression and was found to be involved in the antigen processing of ovalbumin and other antigens. [24] Cathepsin E was also implicated in MHC class II antigen processing in dendritic cells. [5] Additionally, macrophages and B cells have been shown to use cathepsins including cathepsin E in the digestion of complex antigens as well as the presentation of cleavage products to T lymphocytes. [20]

Due to its involvement in antigen processing, cathepsin E was also found to play a part in the antigen sensitization that is central to allergic asthma. Proliferation and infiltration of lymphocytes, a defining characteristic of allergic asthma, was decreased through the use of cathepsin E deficient mice. [25]

In cancer, cathepsin E causes the release of tumor necrosis factor related apop-
tosis inducing ligand (TRAIL) and thereby inhibits tumor growth and metastasis. [20]

Cathepsin E null mice exhibit increased susceptibility to bacterial infection and decreased macrophage activity in response to infection. Additionally, they have been shown to have decreased expression of bacteria specific TLRs. They are also susceptible to chronic inflammatory diseases such as atopic dermatitis. [20]

**Septic Injury**

Significant research has been done into the relationship between *Drosophila* and various microbes. Ren et. al. found that bacterial load increases with aging, but that reducing bacterial load did not extend lifespan. [27]

Septic injury refers to the pricking or injecting of flies with a needle of *E. coli* culture or pelleted cells. In *Drosophila*, septic injury triggers the expression of *upd3*, the cytokine that activates the JAK/STAT pathway, as well as the secretion of a large amount of antimicrobial peptides by the fat body. Two pathways are already proven to be involved in this process, the Toll and Imd pathways, are activated by receptors in the peptidoglycan recognition protein family. Additionally, septic injury causes expression of *totA* a gene regulated by the JAK/STAT pathway. STAT is activated in the fat body in response to septic injury. The expression of JAK/STAT target genes in fat body is dependent on the *upd3* expressed by hemocytes. [2] Studies have been performed to observe how *Drosophila* respond to infections with microorganisms of interest, including *P. aeruginosa* and *S. marcescens*. [3] [23]
Materials & Methods

Detecting Cathepsin mRNA Expression

We determined the location of cathepsin mRNAs using the methods diagrammed in Figure 1.

**Figure 1: Methods**- The location of cathepsin mRNAs was determined using the methods outlined in (A). Maps of the plasmids containing cathepsin E and L cDNA are shown in (B) and (C) respectively. Plasmids were transformed into *E. coli* DH5α and the bacteria were allowed to replicate. Subsequently, replicated plasmids were purified from the bacterial cells and linearized by digestion with *EcoRI*. This linearized plasmid was used as a template for an in vitro transcription reaction using T7 RNA Polymerase which incorporated DIG-labeled uracils to create an antisense RNA probe to the cathepsin of interest. These probes were incubated with sagittal sections of *Drosophila* and detected using an alkaline phosphatase conjugated anti-DIG antibody and alkaline phosphatase substrate solution.

**Cathepsin cDNA Plasmids**

pBluescript plasmids containing the cathepsin E and L cDNA between T3 and T7 promoters as well as an ampicillin resistance gene were obtained from lab stocks.
**Transformation**

Competent DH5α *E. coli* cells were removed from storage at -80°C and placed on ice to thaw for 10 minutes. After which, 1µL of plasmid DNA was added to the cells. Subsequently, cells were left on ice for 30 minutes and then subjected to heat shock at 42°C for 90 seconds. Cells were then allowed to cool on ice for 2 minutes. 1mL of SOC media or LB broth was added to the cells and then they were placed in a 37°C water bath to incubate for 1 hour after which they were plated on LB media with ampicillin. The resulting plates were incubated overnight at 37°C.

**Liquid Culture**

Discrete colonies were chosen for liquid culture. 3mL of LB and 3 µL 100mg/mL ampicillin were added to a culture tube. Each tube was inoculated with a colony from the plate. The resulting liquid culture was incubated in a shaker overnight at 37°C and 240 rpm.

**Plasmid Purification**

Replicated plasmids were purified from the *E. coli* liquid culture using the Qiagen QuickLyse Miniprep Kit as follows. 1.5mL of culture was transferred to a lysis tube and centrifuged at 12,000rpm for 1 minute at room temperature. The supernatant was removed and 400 µL of ice cold Lysis Solution was added to the resulting pellet. The solution was vortexed for 30 seconds at high speed and then incubated at room temperature for 3 minutes. The lysate was placed into a Quicklyse spin column and centrifuged at 12,000rpm for 1 minute at room temperature. Subsequently 400 µL of Buffer QLW was added to the column and it was again centrifuged at 12,000 rpm for 1 minute. The flow through was discarded and the tube was centrifuged again for 1 minute at 12,000rpm. Then, spin column was placed inside a collection tube and 50 µL of Buffer QLE was added. Finally, the tube was spun at 12,000rpm for 1 minute to elute the DNA. A nanodrop spectrophotometer was used to determine the concentration of
the purified DNA.

**Linearizing cDNA**

The purified plasmid was linearized by restriction digest with *EcoRI*. The reaction mixture consisted of 4 µL 10X *EcoRI* Buffer, 2 µL *EcoRI*, 28 µL purified plasmid DNA, and 6 µL sterile water. This mixture was incubated in a 37°C water bath for 1 hour.

**Purification of Digest Product**

The desired digest fragment was purified from the reaction mixture using the QIAquick PCR Purification Kit as follows. After adding five volumes of Buffer PB, the sample was applied to a spin column and centrifuged for 1 minute at 12,000rpm. The flow through was discarded. 0.75mL Buffer PE was added and the solution was then centrifuged again at 12,000rpm for 1 minute. To elute, the solution was placed into a collection tube to which 50 µL Buffer EB was added, and then centrifuged for 1 minute at 12,000rpm. A nanodrop spectrophotometer was used to determine the concentration of the purified digest product. If necessary, ethanol precipitation was used to concentrate DNA from different preparations.

Tables 1 and 2 show the concentrations and purities of cathepsin E &L plasmids and probes. The purity values are based on the absorbance of the sample at various wavelengths. Nucleic acids have maximum absorbance at 260nm and proteins at 280nm. Therefore the 260 value is indicative of the nucleic acid content, and the 280 of protein. A 260/280 value of 1.8 is pure. A common rule of thumb is that 260/280 values of 1.5-2.0 are acceptable. A lower 260/280 value implies that the absorbance at 280nm is higher and that the sample is contaminated with protein. Absorbance at 230nm comes from sample contamination by organic ions and compounds. 260/230 values of 2.0-2.2 are pure. [28] [29]

Analyzing the purity values seen in Table 1, tube 1 of the linearized cathepsin E plasmid has a 260/280 value of 1.8, which is pure. The other two tubes are not
Table 1: Cathepsin E Plasmid & Probe Concentrations—Concentrations of cathepsin E plasmid and probe.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>Purity: 260/280(DNA) or 260/230(RNA)</th>
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</thead>
<tbody>
<tr>
<td>Cat E Linearized Tube 1</td>
<td>4.30ng/µL</td>
<td>1.8</td>
</tr>
<tr>
<td>Cat E Linearized Tube 2</td>
<td>2.70ng/µL</td>
<td>1.67</td>
</tr>
<tr>
<td>Cat E Linearized Tube 3</td>
<td>4.73ng/µL</td>
<td>1.35</td>
</tr>
<tr>
<td>Cat E Antisense RNA Probe</td>
<td>99.57ng/µL</td>
<td>1.56</td>
</tr>
</tbody>
</table>

Table 2: Cathepsin L Plasmid & Probe Concentrations—Concentrations of cathepsin L plasmid and probe.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>Purity: 260/280(DNA) or 260/230(RNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat L Linearized Plasmid DNA</td>
<td>105.35ng/µL</td>
<td>1.85</td>
</tr>
<tr>
<td>Cat L Antisense RNA Probe</td>
<td>138.14ng/µL</td>
<td>2.20</td>
</tr>
</tbody>
</table>

completely pure, but tube 2 falls in the acceptable range. The 260/280 value for tube 3 at 1.35 is quite low, indicating protein contamination. The spectrophotometry values for the linearized plasmid come from the purified product of the restriction digest reaction with EcoRI, it is possible that protein components of the reaction such as the EcoRI restriction enzyme were not completely purified out and provide some absorbance at 280nm contaminating the sample. The 260/230 value for the antisense RNA probe is 1.56 which is lower than 2.0, indicating that there is contamination providing absorbance at 230nm from organic compounds.

The purity values for cathepsin L in Table 2 show that both the linearized plasmid DNA and the antisense RNA probe are pure. The 260/280 value of the linearized plasmid is 1.85 which is very close to the 1.8 pure value, and the 260/230 value of 2.20 of the antisense RNA probe falls into the 2.0-2.2 pure range.
Figure 2: 

**Cathepsin E Restriction Digest Gel**-An agarose gel comparing the plasmid used for transformation, the plasmid purified out of bacterial cells after replication, and the linearized plasmid after restriction digest. The linearized plasmid is of the correct size, approximately 4,000bp. 2µL of DNA were loaded in each lane.

**Gel Eletrophoresis**

A 0.8% agarose gel was run comparing digested and non-digested plasmid in order to confirm that the restriction digest was successful. Figure 2 shows cathepsin E while Figure 3 shows cathepsin L.

**In vitro transcription**

In vitro transcription was carried out in a thermocycler using the Roche DNA Labeling Kit(SP6/T7). Approximately 1.0-1.2 µg of DNA in 13µL of sterile water was added to a PCR tube which was heated to 95°C for 15 minutes and then cooled to 4°C in order to dehybridize the DNA. Subsequently the vial was removed and the following components were added: 2µL 10X NTP Labeling Mixture, 2µL 10X Transcription Buffer,
Figure 3: Cathepsin L Restriction Digest Gel-An agarose gel comparing the plasmid purified out of bacterial cells after replication, and the linearized plasmid after restriction digest. The linearized plasmid is of the correct size, approximately 4,000bp. 2µL of DNA were loaded in each lane.

1 µL Protector RNase Inhibitor, and 2 µL RNA Polymerase T7. After mixing, the tube was returned to the cycler. The temperature was raised to 37°C for 20 hours and then to 75°C for 10 minutes to denature the polymerase. The solution was then kept at 4°C till retrieval upon which it was stored at -20°C until use. Figure 4 shows an agarose gel of the probes. The fact that there is no visible band for the cathepsin L probe can be attributed to the fact that the gel was run for 45 minutes and not immediately imaged. RNA probes being run on a DNA gel are extremely susceptible to degradation, and at least 100ng of RNA needs to be present in the 2µL of probe loaded in order to be visible. The presence and concentration of RNA in the cathepsin L probe solution were verified using nanodrop spectrophotometry as seen in Table 2.

Preparation of Drosophila Tissue Sections

Two day old w1118 flies were used. Nothing was done to flies in the non-injured category. PBS injured flies were pricked in the right thorax with a 21G1 needle dipped in
Figure 4: Probe Gel—An agarose gel showing the cathepsin E and L RNA probes. 2µL of probe were loaded into each lane.

PBS. For septic injury, *E. coli* DH5α cells transformed with a pUAST plasmid were liquid cultured overnight. 500µL of the culture was pelleted by centrifugation at 5,000rpm for five minutes. The 21G1 needle was dipped into the pellet and then used to prick w1118 flies in the right thorax. 24 hours later, flies were fixed in 4% paraformaldehyde for 48 hours. Subsequently, the flies were placed in a softening solution of 30mL distilled water, 80mL 95% ethanol and 4mL melted phenol for 24 hours in order to soften the chitin of the cuticle. These flies were then paraffin embedded and sectioned using a microtome.

In situ hybridization

The following procedure was used to hybridize the probe to paraffin-embedded sections of w1118 *Drosophila melanogaster*. Sections were washed with the following: twice with xylene for 3 minutes, xylene:100% ethanol (1:1) for 3 minutes, twice with 100% ethanol for 3 minutes, 95% ethanol for 3 minutes, 70% ethanol for 3 minutes, and 50% ethanol for 3 minutes. The paraffin was then rinsed off of the slides with sterile
Subsequently the following washes were performed: 70% ethanol for 1 minute, 95% ethanol for 1 minute, and 100% ethanol for 1 minute. After the slides were allowed to air dry, hybridization solution was added to each section and the slides were incubated in hybridization chambers at 55°C for one hour. Subsequently, probe diluted 1:10 with hybridization buffer was heated to 95°C in a PCR machine to dehybridize, and then a drop was added to each section. The sections were covered with a coverslip, sealed with rubber cement, and placed back in the hybridization chamber at 55°C to incubate overnight.

The following day, the coverslip was removed and the sections were washed three times for 30 minutes in formamide wash buffer, once in 0.2X SSC for 30 minutes and once in PBT for 20 minutes. Tissues were then blocked with 10% NGS in TBST for 30 minutes after which alkaline phosphatase conjugated anti-DIG antibody diluted 1:1000 in TBST 5% NGS was added. The sections were covered with a coverslip sealed with rubber cement and allowed to incubate with the antibody overnight.

The next day, slides were washed four times for 15 minutes in TBST. Then NBT/BCIP alkaline phosphatase substrate solution was added. After two hours if color was not visible, the slides were allowed to incubate with substrate solution overnight at 4°C. Finally, tissues were mounted with DAPI Vectashield and imaged.

**Infection Study**

Flies were chosen for infection, for wild type, both male and female w1118 flies were chosen. For JAK gain of function, heterozygous hop<sup>Tum-l</sup> aG/FM7 females with the Barr marker mutation were chosen. For JAK loss of function, heterozygous hop<sup>3</sup> GFP/FM7 females with the Barr marker mutation were chosen. A 21G1 needle was dipped in a 1mL aliquot of overnight culture of DH5α pUAST E. coli and then used to prick anesthetized flies after which they were returned to food vials. At the corresponding timepoint after infection 50µL of LB was pipetted onto a glass coverslip and the fly was placed in the liquid. A second coverslip was used to crush the fly and release the
hemolymph. 800µL LB was used to wash the resulting tissue and liquid into a culture flask. LB was added to the 3mL mark and 3µL of carbenicillin were also added to the flask. Flasks were incubated at 37°C and shaken at 240rpm for 10-12 hours at which point cultures were diluted and plated onto LB+carbenicillin plates. Plates were incubated overnight at 37°C.

**Positive Control**

As a positive control, 50µL of LB broth was pipetted onto a coverslip. A 21G1 needle dipped in DH5α pUAST E. coli liquid culture was then dipped into the LB. Subsequently, 800µL of LB broth was used to wash the solution into a culture tube. LB broth was added to the tube to the 3mL mark and 3µL of carbenicillin was added. The culture was then incubated at 37°C and shaken at 240rpm for the same amount of time as the fly samples, after which it was diluted and plated.

**Negative Control**

As a negative control, uninfected flies were sacrificed, incubated and plated. Although, the results are not shown, these plates did not grow colonies.

**Calculations**

Upon removal from the incubator, colonies were counted, and the original number of colony forming units in the fly at the time of death was calculated using the equation:

\[ N_f = N_0 2^n \]

\[ N_0 = \frac{N_f}{2^n} \]

\( N_f \) is the number of colonies on the plate incorporating the dilution factor, \( n \) is the number of generations calculated from the length of time the liquid culture was incubated for using a generation time of 30 minutes, and \( N_0 \) is the initial number of colony forming units at the time of death.
\( N_0 \) from the positive control was considered as the maximum possible amount of bacteria that the fly was inoculated with at time 0. The bacterial counts were also expressed as a percentage of this inoculum as such:

\[
\text{Percent of Inoculum} = \left( \frac{\text{CFU}_{\text{sample}}}{\text{CFU}_{\text{control}}} \right) \times 100
\]

**Statistical Tests**

**Dixon’s Q-test:** We used a Dixon’s Q-test to identify outliers in our infection study data. The Q-test is a statistical test specifically designed for data sets with small numbers of observations. The principle behind the test is that it estimates a central value for all the data in a group and implements confidence limits while minimizing the effect of the possible outlier on the estimated central value. [7] [30]

In a Q-test the Q value is calculated as follows:

\[
Q = \frac{\text{gap}}{\text{range}}
\]

where

\[
\text{gap} = (\text{possible outlier}) - (\text{closest value in data set to the outlier})
\]

Q is then compared to \( Q_{\text{table}} \). \( Q_{\text{table}} \) values can be seen in Table 3. If \( Q > Q_{\text{table}} \) then the value can be rejected as an outlier with the associated level of confidence.

**T-test:** We used a one-tailed unpaired t-test to test whether the bacterial counts in mutant flies were significantly higher than those in wild type flies since t-tests are used to compare two groups of data and our data are not paired. We chose a one-tailed t-test because we were interested in positive differences between the groups. [31]

The t-value is calculated using the following formula for two sample groups with unequal variances:

\[
t = \frac{x_1 - x_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}
\]
Table 3: $Q_{table}$ Values with Confidence Limits—$Q_{table}$ values used to determine whether or not to reject the possible outlier.

<table>
<thead>
<tr>
<th>Number of Values</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Q_{90%}$</td>
<td>0.941</td>
<td>0.765</td>
<td>0.642</td>
<td>0.560</td>
<td>0.507</td>
</tr>
<tr>
<td>$Q_{95%}$</td>
<td>0.970</td>
<td>0.829</td>
<td>0.710</td>
<td>0.625</td>
<td>0.568</td>
</tr>
<tr>
<td>$Q_{99%}$</td>
<td>0.994</td>
<td>0.926</td>
<td>0.821</td>
<td>0.740</td>
<td>0.680</td>
</tr>
</tbody>
</table>

Where $x_1$ = mean of group 1, $x_2$ = mean of group 2, $s_1$ = unbiased estimated variance of group 1, and $s_2$ = unbiased estimated variance of group 2.

This $t$-value can then be correlated with a $p$-value using a $t$-table. The $p$-value represents the probability that the observed difference between the values could be observed due to chance rather than an actual difference between the populations.

Regression Models & Transformation Values

Regression models were fit to the average bacterial counts, expressed as percent of inoculum, after septic injury for each fly type. Wild type results were best modeled by an exponential regression model as determined by $R^2$ value, hop$^{Tum-l}$ by a second degree polynomial decrease, and hop$^3$ by a second degree polynomial increase.

After the regression equations were determined, transformation equations and values were calculated.

The first transformation value is seen in Figure 26. The transformation value is some value $m$ by which the regression curve for one sample type can be multiplied to get the regression curve of another sample. In Figure 26 the transformation value is the
value by which the bacterial counts, expressed as percent of inoculum, in wild type flies must be multiplied to get the counts seen in LB broth, the optimum growth condition for *E. coli*.

\[ m = \frac{\text{Regression Equation}_{LB}(x)}{\text{Regression Equation}_{WildType}(x)} \]

The transformation values for the hop\(^{Tum-l}\) and hop\(^{3}\) seen in Figure 27 were calculated in a similar fashion:

\[ m = \frac{\text{Regression Equation}_{Mutant}(x)}{\text{Regression Equation}_{WildType}(x)} \]

The first of these transformation values represents the factor by which the fly environment negatively impacts bacterial growth. The second represents the factor by which the mutant environment differs from the wild type.
Cathepsin E is Expressed in Gut and Fat Body in Response to Injury and Microbial Infection

Areas of cathepsin E mRNA expression were detected using an antisense RNA DIG-labeled probe made from a pBluescript plasmid containing the cathepsin E cDNA between T3 and T7 promoters. The probe was hybridized to endogenous cathepsin E mRNA in sagittal sections of *Drosophila* exposed to no injury, PBS injury, and septic injury. The probe was detected by using an alkaline phosphatase conjugated anti-DIG antibody and subsequently adding alkaline phosphatase substrate solution (NBT/BCIP).

As seen in Figure 7, the staining seen in non-injured flies was non-specific. Figure 7D shows the gut lumen, as evidenced by the columnar gut epithelium. There is dark staining in the lumen of the gut, but it is difficult to determine from a sagittal section whether this is fly tissue or something the fly has ingested.

As seen in Figure 8 and 9, there is a higher level of cat E expression in the fat
Figure 6: Drosophila Digestive System Anatomy—This Figure is taken from *Atlas of Drosophila Development* [16] and shows components of the *Drosophila* digestive system abbreviated as follows: ph-pharynx, sd-salivary ducts, es-esophagus, pv-proventriculus, sg-salivary glands, cr-crop, mg-midgut, hg-hindgut, mp-malpighian tubules, amp-rectal ampulla.

body and gut of PBS injured flies as compared to non-injured flies.

As seen in Figure 10, increased cathepsin E expression is seen in the fat body of flies exposed to septic injury.

**Cathepsin L is Expressed in the Fat Body in Response to Septic Injury**

As seen in Figure 11, there is no detectable expression of cathepsin L in non-injured w1118 flies.

As seen in Figure 12, cathepsin L expression is induced in the fat body in response to PBS injury as compared to non-injured flies.

As seen in Figure 13, there is increased expression of cathepsin L in the fat body in response to septic injury as compared to PBS injury alone. Additionally, there are high levels of expression of cathepsin L expression in the gut of flies exposed to septic injury as compared to non-injured and PBS injured flies.
Figure 7: Cathepsin E Expression in Non-injured Flies-Staining is visible in the lumen of the gut. In situ hybridization with antisense DIG-labeled probes to cathepsin E was performed to determine areas of cathepsin E mRNA expression in non-injured flies. (A) w1118 non-injured fly section 2X. (B) w1118 non-injured fly abdomen 10X. (C) w1118 non-injured fly abdomen 20X. (D) w1118 non-injured fly abdomen 40X. (E) Control w1118 non-injured fly abdomen 40X no probe added

Infection Study

In order to gain further insight into the effects of cathepsin E and L levels on immunity to microbial infection, experiments were carried out to determine the length of time DH5 E. coli with pUAST were able to survive in wild type(w1118), JAK/STAT gain of function and tumor model(hop\textsuperscript{Tum-1}), and JAK/STAT loss of function(hop\textsuperscript{3}) flies before being acted upon or eliminated by the immune system.

Flies were infected with carbenicillin resistant bacteria. At defined time points, flies were sacrificed, their tissue incubated in liquid culture, and subsequently plated on LB + carbenicillin plates.
Figure 8: Cathepsin E Expression in PBS Injured Flies Section 1-In flies subjected to PBS injury, there is a higher level of cathepsin E expression as compared to non-injured flies and notably high levels in fat body. In situ hybridization with antisense DIG-labeled probes to cathepsin E was performed to determine areas of cathepsin E expression in PBS-injured flies (A) w1118 PBS injury fly section 2X (B) w1118 PBS injury fly head and thorax 10X (C) w1118 PBS injury fly abdomen 10X (D) w1118 PBS injury fly abdomen 10X (E) w1118 PBS injury fly thorax 20X (F) w1118 PBS injury fly abdomen 20X (G) w1118 PBS injury fly abdomen 20X (H) Control w1118 non-injured fly abdomen 40X no probe added
Figure 9: Cathespin E Expression in PBS Injured Flies Section 2-In PBS injured flies there are high levels of cathepsin E expression gut. In situ hybridization with antisense DIG-labeled probes to cathepsin E was performed to determine areas of cathepsin E expression in PBS-injured flies. (A) w1118 PBS injury fly section 2X (B) w1118 PBS injury fly abdomen 10X (C) w1118 PBS injury fly abdomen 20X (D) w1118 PBS injury fly abdomen 40X (E) w1118 PBS injury fly abdomen 40X (F) w1118 PBS injury fly abdomen 40X (G) Control w1118 non-injured fly abdomen 40X no probe added

Figure 10: Cathepsin E Expression in Septic Injury Flies-Septic injury induces cathepsin E expression in the fat body. In situ hybridization with antisense DIG-labeled probes to cathepsin E was performed to determine areas of cathepsin E expression in septically-injured flies. (A) w1118 Septic Injury Fly Section 2X (B) w1118 Septic Injury Fly Abdomen 10X (C) w1118 Septic Injury Fly Abdomen 20X (D) w1118 Septic Injury Fly Abdomen 10X (E) Control w1118 Fly Abdomen 40X no probe added
Figure 11: Cathepsin L Expression in Non-injured Flies—There is no notable expression of cathepsin L in non-injured flies. In situ hybridization with antisense DIG-labeled probes to cathepsin L was performed to determine areas of cathepsin L expression in non-injured flies. (A) w1118 non-injured fly section 2X (B) w1118 non-injured fly abdomen 10X (C) w1118 non-injured fly abdomen 20X (D) Control w1118 non-injured fly abdomen 20X with no probe added.

Bacterial Counts in Wild Type (w1118) Flies Decrease Steadily After Septic Injury

We counted the bacteria remaining in flies at certain time points after infection in order to characterize the ability of the immune system to fight off microbial infection in JAK/STAT mutants and correlate the immune response with levels of cathepsins E & L.

After infection, flies were crushed to release hemolymph. Then the fly tissue and resulting fluid were incubated in a culture flask for 10-12 hours. Subsequently the culture was plated allowing for back calculation of the initial number of colony forming units in the culture.

As seen in Figure 14, bacterial counts consistently decrease in wild type flies after time of infection.

As seen in Figure 15, which shows average bacterial counts after septic injury expressed as percent of inoculum, in wild type flies bacteria introduced via septic injury
Figure 12: Cathepsin L Expression in PBS Injured Flies-PBS injury induces cathepsin L expression in the fat body. In situ hybridization with antisense DIG-labeled probes to cathepsin L was performed to determine areas of cathepsin L expression in non-injured flies. (A) w1118 PBS-injured fly section 2X (B) w1118 PBS-injured fly abdomen 20X (C) w1118 PBS-injured fly abdomen 40X (D) Control w1118 non-injured fly abdomen 20X no probe added
Figure 13: Cathepsin L Expression in Septic Injury Flies—Septic injury induces high levels of cathepsin L expression in the fat body and gut. In situ hybridization with antisense DIG-labeled probes to cathepsin L was performed to determine areas of cathepsin L expression in non-injured flies. (A) w1118 septic injury fly section 2X (B) w1118 septic injury fly abdomen 10X (C) w1118 septic injury fly abdomen 20X (D) Control w1118 non-injured fly abdomen 20X no probe added

are generally eliminated by 15 minutes after infection.

In order to better understand the immune response of wild type flies to septic injury, we investigated which regression model best fit the average bacterial counts following septic injury. As seen in Figure 16, average bacterial counts in wild type flies were best modeled using an exponential regression with an $R^2$ value of 0.9935.

$$w1118 \text{ Regression Equation} = 47.367e^{-0.3589x}$$

Bacterial Counts in hop$^{Tam-l}$ Flies Show a Lengthened Initial Decrease and then Increase after Septic Injury

Using the same techniques explained above, we analyzed the bacterial counts in JAK/STAT gain of function (hop$^{Tam-l}$) flies. Figure 17 shows the bacterial counts recovered in each trial.

Figure 18, shows the average bacterial counts at different time points after septic}
Figure 14: Bacterial Counts in Wild Type Flies after Septic Injury—Wild type (w1118) flies were infected with cultures of DH5 *E. coli* pUAST and sacrificed into liquid cultures at certain time points after infection. Incubation and subsequent plating of these cultures allowed for back calculation of the number of colonies present in the fly at the time of death. *E. coli* counts in wild type flies steadily decrease after infection.
Figure 15: w1118 Average Bacterial Counts after Septic Injury-Wild type(w1118) flies were infected with cultures of DH5 E. coli pUAST and sacrificed into liquid cultures at certain time points after infection. Incubation and subsequent plating of these cultures allowed for back calculation of the number of colonies present in the fly at the time of death. Average bacterial counts show that in the majority of cases, all E. coli are eliminated within 15 minutes of infection.

Figure 16: Regression Model of w1118 Average Bacterial Counts-Different regression models were compared for their fit to the average bacterial counts in w1118 flies after septic injury. An exponential regression presented the best fit with an $R^2$ value of 0.9935.
Figure 17: Bacterial Counts in hop\textsuperscript{Tum-1} Flies after Septic Injury- hop\textsuperscript{Tum-1} flies were infected with cultures of DH5 \textit{E. coli} pUAST and sacrificed into liquid cultures at certain time points after infection. Incubation and subsequent plating of these cultures allowed for back calculation of the number of colonies present in the fly at the time of death.
Average bacterial counts after septic injury indicate that bacterial counts decrease after infection for 15 minutes and then increase. As seen in Figure 19, we fit a regression model to the hop<sup>Tum-l</sup> data in order to better characterize and compare the immune responses between the different types of flies being studied. In hop<sup>Tum-l</sup> flies, the change in bacterial counts over time was best modeled by a second degree polynomial regression with an R<sup>2</sup> value of 0.8492.

\[
\text{hop}^{\text{Tum-l}} \text{ Regression Equation} = 0.2007x^2 - 3.1136x + 24.423
\]

**Bacterial Counts in hop<sup>3</sup> Flies Show a Short Initial Decrease and then Increase after Septic Injury**

Bacterial counts after septic injury were determined in JAK/STAT loss of function(hop<sup>3</sup>) flies. Figure 20 shows the original bacterial counts from each septic injury trial.

As seen in Figure 20 the positive control value for Culture 4 is significantly higher than those of all other trials. Using a Q test, the culture 4 positive control was removed as an outlier with 99% confidence. All positive control values for all fly types were Q-tested and this was the only outlier. Therefore, culture 4 was removed because there was
Figure 19: Regression Model of hop\textsuperscript{Tum-1} Average Bacterial Counts—After septic injury, the bacterial counts in hop\textsuperscript{Tum-1} flies change in a manner that is best modeled by a second degree polynomial regression model.

no way to calculate % of inoculum for the other culture 4 values without the positive control. Figure 21 shows the final hop\textsuperscript{3} data with culture 4 removed.

Figure 22 shows the average bacterial counts, expressed as percent of inoculum, in hop\textsuperscript{3} flies after septic injury. Similar to hop\textsuperscript{Tum-1} flies, bacterial counts initially decrease and then increase in hop\textsuperscript{3} flies.

As seen in 23, average bacterial counts in hop\textsuperscript{3} flies were best modeled by a second order polynomial regression with an R\textsuperscript{2} value of 1.

hop\textsuperscript{3} Regression Equation = 0.0437x\textsuperscript{2} – 0.2694x + 0.8625

Due to the small size of the dataset, we will present the average counts and the regression model calculated with the outlier included as well. Figure 24 shows the average bacterial counts and Figure 25 shows the regression model.

Comparison of Bacterial Counts in Different Fly Types

Figure 26 shows a comparison of bacterial growth in LB broth to that in wild type flies as well as the transformation value by which wild type counts must be multiplied to return to the LB broth values. This transformation value represents the factor by which
Figure 20: Bacterial Counts in hop<sup>3</sup> Flies after Septic Injury: Original Data.

hop<sup>3</sup> flies were infected with cultures of DH5 E. coli pUAST and sacrificed into liquid cultures at certain time points after infection. Incubation and subsequent plating of these cultures allowed for back calculation of the number of colonies present in the fly at the time of death.
Figure 21: Bacterial Counts in hop\(^3\) Flies after Septic Injury Final Data-hop\(^3\) bacterial count data with Culture 4 outlier removed.
Figure 22: hop$^3$ Average Bacterial Counts after Septic Injury - hop$^3$ flies were infected with cultures of DH5 E. coli pUAST and sacrificed into liquid cultures at certain time points after infection. Incubation and subsequent plating of these cultures allowed for back calculation of the number of colonies present in the fly at the time of death. The number of bacterial colonies in infected hop$^3$ flies decreases for the first 5 minutes after infection and then increases between 5 and 10 minutes after infection.

Figure 23: Regression Model of hop$^3$ Average Bacterial Counts - Average bacterial counts in hop$^3$ flies after infection are best modeled by a second order polynomial regression with an $R^2$ value of 1.
Figure 24: hop$^3$ Average Bacterial Counts: Original Data-Average bacterial counts in hop$^3$ flies with outlying culture included.

Figure 25: Regression Model of hop$^3$ Average Bacterial Counts: Original Data-Average bacterial counts in hop$^3$ flies with the outlying culture showed a general trend of increase modeled by a second degree polynomial.
the change from an optimum growth environment to that of the fly affects bacterial growth.

The transformation value was calculated as follows:

\[ E. \ coli \ Regression \ Equation: \ y_1 = 99.998e^{0.0231x} \]

\[ \text{w1118 Regression Equation: } y_2 = 47.367e^{-0.3589x} \]

Transformation Value: \( m(x) = \frac{y_1(x)}{y_2(x)} \)

Figure 27 shows the regression curves for bacterial counts in wild type, hop\textsubscript{Tum-l}, and hop\textsuperscript{3} as well as the transformation values to return each mutant to the growth levels seen in wild type flies. This transformation value represents the effects of the mutant fly environment on bacterial growth as compared to wild type flies. The graph shows that bacterial counts were highest at time zero in wild type flies, then hop\textsuperscript{Tum-l}, and least in hop\textsuperscript{3}. This was not what we expected, based on our hypothesis, we expected that bacterial counts would be the highest in hop\textsuperscript{3} at time zero, then hop\textsuperscript{Tum-l}, and least in wild type. Looking back at the data though, this phenomenon can be explained by the fact that the average bacterial concentration, represented by the positive control values were highest for wild type then hop\textsuperscript{Tum-l} then hop\textsuperscript{3}.

The transformation values were calculated as follows:

\[ \text{w1118 Regression Equation: } y_2 = 47.367e^{-0.3589x} \]

\[ \text{hop}\textsuperscript{Tum-l} \ Regression \ Equation: \ y_3 = 0.2007x^2 - 3.1136x + 24.423 \]

\[ \text{hop}\textsuperscript{3} \ Regression \ Equation: \ y_4 = 0.0437x^2 - 0.2694x + 0.8625 \]

\[ \text{hop}\textsuperscript{Tum-l} \ Transformation \ Value: \ m(x)_{\text{hop}\textsuperscript{Tum-l}} = \frac{y_3}{y_2} \]
Figure 26: Comparison of Bacterial Counts in LB Broth and Wild Type Flies-Bacteria display exponential growth when cultured in LB broth and exponential decline when grown in wild type *Drosophila*. The transformation factor represents the effect that the environment of the fly has on bacterial growth as compared to optimum conditions, which increases over time and is manifested in a decrease in bacterial counts in wild type due to the immune system.

\[ \text{hop}^3 \text{ Transformation Value: } m(x)_{\text{hop}^3} = \frac{y_1}{y_2} \]
Figure 27: Comparison of Bacterial Counts in wild type, hop^{Tum-1} and hop^{3}

Flies-Wild type flies are exhibit the most efficient response to microbial infection and are able to eliminate bacteria in an exponential manner. Bacterial counts in hop^{Tum-1} and hop^{3} flies showed an initial decrease and then increase best modeled by parabolas. Transformation values show that bacterial counts in hop^{3} flies were closer to wild type than those in hop^{Tum-1}. 
Discussion

Detection of Cathepsin E & L Expression in Response to Septic Injury

In this study, we detected areas of cathepsin E & L mRNA expression in response to injury and septic injury in Drosophila melanogaster. Antisense DIG-labeled RNA probes created from cathepsin cDNAs were hybridized to Drosophila sagittal sections and detected using an alkaline phosphatase conjugated anti-DIG antibody detection system as well as NBT/BCIP substrate which stains dark blue or purple upon cleavage by alkaline phosphatase.

We observed no significant cathepsin E expression in non-injured flies aside from some staining in the lumen of the gut which has been disregarded because of the fact that it is impossible to tell whether this is fly tissue or something that was ingested.

We subjected flies to PBS injury with a 21G1 needle in order to determine the level of cathepsin E expression in response to injury alone for subsequent comparison to expression in response to septic injury. PBS injury was found to cause notable levels of cathepsin E expression in the fat body and gut.

In flies subjected to septic injury, with a 21G1 needle dipped in E. coli DH5α pUAST, higher levels of cathepsin E expression were observed in the fat body.

The location of cathepsin E expression in the fat body and gut in response to septic injury is corroborated by Chintapalli et. al. [10] who found cathepsin E expression in the gut and fat body of adult flies. Overall, these results are supported by those of Larson, who found that cathepsin E expression levels are high at the time of infection and remain high up to six hours afterwards. [20] Our results show that this high level of cathepsin E expression in septic injury flies extends to 24 hours post-infection, the time at which these flies were sacrificed.

Levels of cathepsin L mRNA expression were also tested in non-injured, PBS injured, and septic injury flies thorough hybridization of antisense DIG-labeled RNA probes from cathepsin L cDNAs which were hybridized to paraffin embedded tissue sections.
In non-injured flies, there was no significant expression of cathepsin L mRNAs. PBS injured flies were tested to elucidate the effect of injury alone on cathepsin L expression, we observed that PBS injury resulted in low levels of cathepsin L expression in the fat body 24 hours after infection. The expression was specifically localized to the fat body.

In septic injury flies infected with *E. coli* we observed a marked increase in cathepsin L expression, much higher than the low levels observed in response to PBS injury. In septic injury flies a drastic increase in cathepsin L expression is observed in the fat body, there is also previously absent expression in the gut. Additionally, we once again observed staining of objects within the gut lumen but disregarded them because we were unable to determine whether it was fly tissue or an ingested object.

In total, these results suggest that both cathepsin E and L expression are upregulated in the fat body in response to injury and even more so in septic injury. Additionally, PBS injury causes an increase in expression of cathepsin E in a wide variety of tissues, an increase which is not observed with cathepsin L expression in response to septic injury.

**Bacterial Counts after Septic Injury in wild type, hop^{Tum-l}, and hop^{3} Flies**

We quantified the number of bacteria present at certain time points after septic injury by crushing infected flies and culturing the resulting fluid and tissue in nutrient broth. Subsequently, the culture was diluted and plated in order to determine the number of colony forming units in the original culture at the time of death.

Of the types of flies tested, wild type flies eliminated bacteria the most efficiently, decreasing bacterial counts in an exponential fashion and eliminating the majority of bacteria before the 15 minute time point. In hop^{Tum-l} flies, bacterial counts decreased for the first 15 minutes after infection and then increased by the 20 minute time point, this change was best modeled by a parabolic equation. In hop^{3} flies bacterial counts decreased for 5 minutes after infection and then increased by the 10 minute timepoint
and were also best modeled by a second degree polynomial.

It is expected that the wild type flies are the most adept at countering microbial infection, and that any mutation in endogenous immune components would decrease the efficiency with which bacteria were eliminated. In comparison, both hop$^{Tum-l}$ and hop$^{3}$ were immuno-deficient with hop$^{Tum-l}$ flies being able to decrease bacterial counts for the first 15 minutes after infection and hop$^{3}$ flies being able to decrease bacterial counts for 5 minutes after infection, before bacterial proliferation was observed.

To assess the statistical significance of our data, we used a one-tailed T-test to see whether hop$^{3}$ and hop$^{Tum-l}$ bacterial counts were significantly higher than those in wild type. We compared the values at the latest shared time point between the two fly types.

We compared hop$^{Tum-l}$ and wild type bacterial counts at 15 minutes after infection and found that they differed with a p-value of 0.1506.

$$p-value_{hop^{Tum-l}15m} = 0.1506$$

We also compared bacterial counts in hop$^{3}$ and wild type at 5 minutes after infection in order to see if the hop$^{3}$ counts were significantly higher and found that they differed with a p-value of 0.1268.

$$p-value_{hop^{3}5m} = 0.1268$$

While low, these p-values are higher than the generally used cutoff of $p = 0.05$ for statistical significance. The high p-values can be explained by the small sample size. We tested five cultures per type of fly and for each culture, only one fly was analyzed at each time point. Performing further tests with larger sample sizes will allow for a decreased standard deviation which corresponds to lower p-values. Nonetheless, these preliminary results can still inform our understanding of the immune response to septic injury in Drosophila.
Regression Model Analysis

Analyzing the regression curves for all fly types, bacterial counts decrease in wild type and hop\textsuperscript{Tum-l} flies. hop\textsuperscript{3} flies were the only ones in which bacterial counts increased. The equations for hop\textsuperscript{Tum-l} and hop\textsuperscript{3} are as follows:

hop\textsuperscript{Tum-l} Regression Equation: \( y_3 = 0.2007x^2 - 3.1136x + 24.423 \)

hop\textsuperscript{3} Regression Equation: \( y_4 = 0.0437x^2 - 0.2694x + 0.8625 \)

Therefore, we can see that bacterial counts are higher at time 0 in hop\textsuperscript{Tum-l} flies than in hop\textsuperscript{3} because:

\[
hop\textsuperscript{Tum-l}: y_3(0) = 24.423 \\
hop\textsuperscript{3}: y_4(0) = 0.8625
\]

\( y_3(0) > y_4(0) \)

This is most likely because the maximum amount of bacteria injected into the hop\textsuperscript{Tum-l} flies, represented by the positive control values, were on average higher than in hop\textsuperscript{3} as seen in Figures 17 and 21.

In order to address this, further experiments could be carried out using bacterial cultures with similar optical density on the different fly types.

These data suggest that as expected, wild type flies are the most efficient at eliminating bacteria after infection. Comparing the hop\textsuperscript{Tum-l} and hop\textsuperscript{3} data, hop\textsuperscript{Tum-l} flies were able to decrease bacterial counts for a longer period of time than hop\textsuperscript{3} flies.

While both mutants have immune deficiencies, the major difference between these two types of flies is that hop\textsuperscript{Tum-l} flies have an overactive JAK/STAT pathway. They therefore have higher concentrations of JAK/STAT target proteins, including those for cathepsins E & L, which our data suggest are involved in the humoral response to septic injury and provide microbial immunity. Therefore, we propose that the higher levels of
cathepsins E & L in hop^{Tum-l} flies give them an advantage in countering infection and allows them to eliminate bacteria for a longer period after infection than hop^3 flies. hop^3 flies which have decreased levels of cathepsins E & L as compared to hop^{Tum-l} and wild type.

The fact that the bacterial counts in mutant flies after septic injury are best modeled by polynomial regressions indicates that these phenomena are not simply dependent on time. There are other factors involved. This is expected since the immune response is a very complex system with multiple components.

Optimally, these regression equations could be divided into two terms, one that reflects the overall state of the immune system which would be higher in wild type flies than hop^3 and hop^{Tum-l} flies, and the other that corresponds to the cathepsin levels in the flies for which hop^{Tum-l} > wild type > hop^3. Developing such an equation would be helpful and requires further study.

**Implications of Cathepsin Expression Locations for Function in Innate Immunity**

As mentioned before, the three divisions of Drosophila innate immunity are the cellular response, melanization, and the humoral response. The humoral response is characterized by production of antimicrobial peptides in the fat body and is controlled by three pathways: Toll, Imd, and JAK/STAT, which are activated in response to septic injury. Additionally, it has been shown that septic injury results in the production of antimicrobial peptides as well as STAT expression in the fat body. [8] Since previous research has shown that both cathepsins E and L are targets of the JAK/STAT pathway, and our results show that they are expressed in the fat body, it is possible that these proteases are expressed in the fat body as part of the humoral response to septic injury in order to process and cleave peptides to their active antimicrobial forms.

There are a few considerations within our experimental protocol that could be improved upon or lead to alternate interpretations of the data. In the infection study,
there is a need for multiple trials, not only for each time point but also for the positive control, to ensure that the effect of sampling error is minimized. It is also necessary to ensure that all injected bacteria are in the log phase of growth in order to consider them as similar tests. Use of bacterial cultures not in log phase could result in a lag phase before bacterial proliferation that might incorrectly be attributed to the fly immune response. In further experiments, spectrophotometry or plating can be used to ensure that cultures used for infection are in the log phase of growth. Also, since all tested mutant flies were females, all tested wild type flies should be female as well in order to avoid discrepancies due to the differing bacterial dosage in males which are smaller. Additionally, it is worth noting that the use of a culture period before assessing bacterial counts introduces additional experimental factors that could lead to error as compared to direct plating after infection. In setting up our experiments we originally tried the direct plating method, but were unsuccessful. Other groups have concentrated their bacterial cultures before infection [23] and used injection of bacterial culture rather than pricking. Implementation of one of these protocols could be used to introduce enough bacteria into the fly at the time of infection that colonies could be grown from direct plating of the fly after infection even at these early time points.

Further experiments are necessary to determine which substrates are cleaved by cathepsins E and L in response to septic injury, possibly through comparison of protein profiles in wild type, JAK gain of function, and JAK loss of function mutants. Subsequently, the exact impact of cathepsin E and L expression on the immune response to microbial infection could be further elucidated.
References


