Fall 2022

Hemocyte-mediated immune response to a bacterial infection in the cat flea (Ctenocephalides Felis)

Melanie Muñoz

HEMOCYTE-MEDIATED IMMUNE RESPONSE TO A BACTERIAL INFECTION IN THE CAT FLEA (*CTENOCEPHALIDES FELIS*).

by

MELANIE MUNOZ

(Under the direction of Lisa D. Brown)

ABSTRACT

Despite their continued medical and veterinary importance, the vectorial capacity of fleas (Order Siphonaptera) is often underestimated. Fleas are best described as ectoparasites of a variety of mammalian and aviary hosts. In humans, fleas are infamous for the transmission of several detrimental agents, including those that cause plague, murine typhus, and cat-scratch disease. Although their significance in flea immune responses remains unknown, phagocytic immune cells, known as hemocytes, are important players in the cell mediated immune response of insects. Among invertebrates, hemocytes provide defense against foreign microbes via phagocytosis, cellular encapsulation, and the production of humoral immune factors. As mentioned above, current information on the types of hemocytes fleas produce, their relative abundance, and their functions is limited. This is a significant gap in knowledge, as improved understanding of how fleas defend themselves from infection could lead to the refinement of disease control strategies. To investigate the role of hemocytes in flea immunity, cat fleas (*Ctenocephalides felis*) were infected with the Gram-negative bacterium, *Escherichia coli*. Specifically, an *E.coli* infection was delivered via a septic pinprick, and the following parameters were measured: (1) *in vivo* bacteria killing efficiency; (2) quantification of circulating hemocytes; and (3) the relative capacity of hemocyte phagocytosis. The data show that hemocytes proliferate in response to a bacterial infection, and that these immune cells are highly phagocytic. Overall, this study provides important insight into how fleas interact with Gram-negative bacterial pathogens in their hemocoel, which can directly affect transmission of flea-borne diseases.

INDEX WORDS: Fleas, cat flea, *Ctenocephalides felis*, immunity, phagocytosis, hemocytes

HEMOCYTE-MEDIATED IMMUNE RESPONSE TO A BACTERIAL INFECTION IN THE CAT FLEA (*CTENOCEPHALIDES FELIS*).

by

MELANIE MUNOZ

B.S., Georgia Southern University, 2019

A Thesis Submitted to the Graduate Faculty of Georgia Southern University in Partial

Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

HEMOCYTE-MEDIATED IMMUNE RESPONSE TO A BACTERIAL INFECTION IN THE CAT FLEA (*CTENOCEPHALIDES FELIS*).

by

MELANIE MUNOZ

Major Professor:	Lisa D. Brown
Committee:	Lance A. Durden
	Joshua D. Gibson

DEDICATION

For my family, thank you for your love and patience. All of this is for you and because of you. Wherever I am, we are.

For Justin, I am humbled by your love and support. To know you is to love you – thank you for this chapter and every chapter that follows.

For my Father, I'm excited to see what you'll do next.

ACKNOWLEDGMENTS

I am eternally grateful for the unyielding support by several individuals during this process, as I could not have completed this alone.

Firstly, I would like to thank my advisor, Dr. Lisa D. Brown, for her constant support both throughout my undergraduate and graduate career at Georgia Southern University. Her mentorship has been a cornerstone for my development as a young professional. I wholeheartedly believe that I could not have had a better advisor.

Secondly, I would like to thank my thesis committee members, Dr. Lance Durden and Dr. Josh Gibson. Their expertise has propelled me and my project to new heights.

Finally, I would like to thank my family and friends for their unwavering support throughout my career. My achievements are not my own, but a result of the constant support I have been given by my loved ones.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Purpose of Study

The insect immune system is divided into humoral and cellular components that provide defense against a range of microbes (Beckage 200, Hillyer 2016, Bartholomay and Michel 2018). The humoral defenses include production of soluble effector molecules (*e.g.*, antimicrobial peptides, complement-like proteins, reactive oxygen species (ROS), reactive nitrogen species, and components of the phenoloxidase-based melanization cascade), while cellular defenses are mediated by immune cells called hemocytes. Hemocytes are located within the insect body cavity (hemocoel), and either circulate with the hemolymph (blood) – called circulating hemocytes – or remain attached to tissues – called sessile hemocytes (Strand 2008). The primary defense response of both populations of hemocytes is phagocytosis, whereby a foreign body is (1) recognized by pathogen recognition receptors (PRRs) on the cell surface, (2) internalized into a phagosome, which fuses with a lysosome, and (3) hydrolytically digested by toxic substances produced in the phagolysosome (Fig. 1) (Hillyer and Strand 2014). Additionally, in the event of larger foreign objects, such as eukaryotic parasitic organisms, multiple hemocytes can irreversibly bind to form a protective multicellular sheath (Satyavathi *et al.* 2014). Overall, these phagocytic cells are crucial to the immune response of invertebrates and are directly related to insect survivability against systemic infections (Hillyer *et al.* 2004, Pham *et al.* 2007, Kwon and Smith 2019).

Among insect vectors responsible for the spread of human diseases, the hemocyte-mediated immune response is best described in mosquitoes (Order Diptera). Mosquito hemocytes are highly phagocytic in response to a range of bacteria and can initiate this process within

minutes of pathogen exposure (Hillyer *et al.* 2003a, Hillyer *et al.* 2003b, King and Hillyer 2013). Additionally, mosquito hemocyte numbers increase in response to blood-feeding and infection by certain pathogens (Christensen *et al.* 1989, Baton *et al.* 2009, Castillo *et al.* 2011, Coggins *et al.* 2012, King and Hillyer 2013, Bryant and Michel 2014). On average, adult mosquitoes contain between 500 and 4000 circulating hemocytes, the majority of which will readily engage in phagocytosis (up to 95%) and are capable of internalizing hundreds of bacteria within 24 hours of infection (Hillyer *et al.* 2005, Hillyer *et al.* 2007). Thus, although mosquitoes are susceptible to a wide variety of infectious pathogens, they mount powerful cellular immune responses against these invading microorganisms to limit or resist infection. In fact, such findings have led the scientific community to hypothesize that insect immune responses can be harnessed to control the spread of vector-borne pathogens. Yet, few studies exist that have examined the cellular immune response in other insect vectors (*e.g.*, fleas, lice, kissing bugs, sandflies, tsetse flies) to the same extent as mosquitoes.

Fleas (Order Siphonaptera) are insect vectors for several harmful human bacterial diseases, including murine typhus (caused by *Rickettsia typhi*), plague (caused by *Yersinia pestis*), and cat scratch disease (caused by *Bartonella henselae*); however, little is known about the immune system of these vectors against bacterial pathogens. In particular, the hemocyte-mediated immune response of fleas is unknown. This is a significant gap in knowledge, as improved understanding of how fleas defend themselves from systemic bacterial infections could lead to the development or refinement of vector-borne disease control strategies. As stated above, phagocytosis is a key defense mechanism in the immune response of another group of blood-feeding insects (mosquitoes) against an array of harmful pathogens. In particular, hemocytes are responsible for the phagocytosis of bacterial pathogens, such as those transmitted

to humans by fleas (Strand 2008, League *et al.* 2017, Pham *et al.* 2007, Hillyer & Strand 2014, Coggins *et al.* 2012, Hillyer 2015, Sheehan *et al.* 2018). Thus, I hypothesize that hemocyte-mediated phagocytosis is a key defense mechanism against bacterial pathogens in the flea hemocoel. To test this hypothesis, cellular defense mechanisms were examined in the cat flea (*Ctenocephalides felis*), one of the most important arthropod pests of humans and many domestic animals (Durden and Hinkle 2019). Specifically, the hemocyte-mediated immune response was examined by measuring hemocyte numbers in response to a Gram-negative bacterial infection (*Escherichia coli*). Additionally, phagocytic trends were measured by quantifying the phagocytic capacity and phagocytic index of flea hemocytes following microbial challenge. Finally, phagocytic activity was impaired to understand the importance of phagocytosis against a systemic infection. Overall, improved understanding of the *C. felis* immune response against a bacterial pathogen may result in a more effective method of inhibiting transmission of flea-borne pathogens to human hosts.

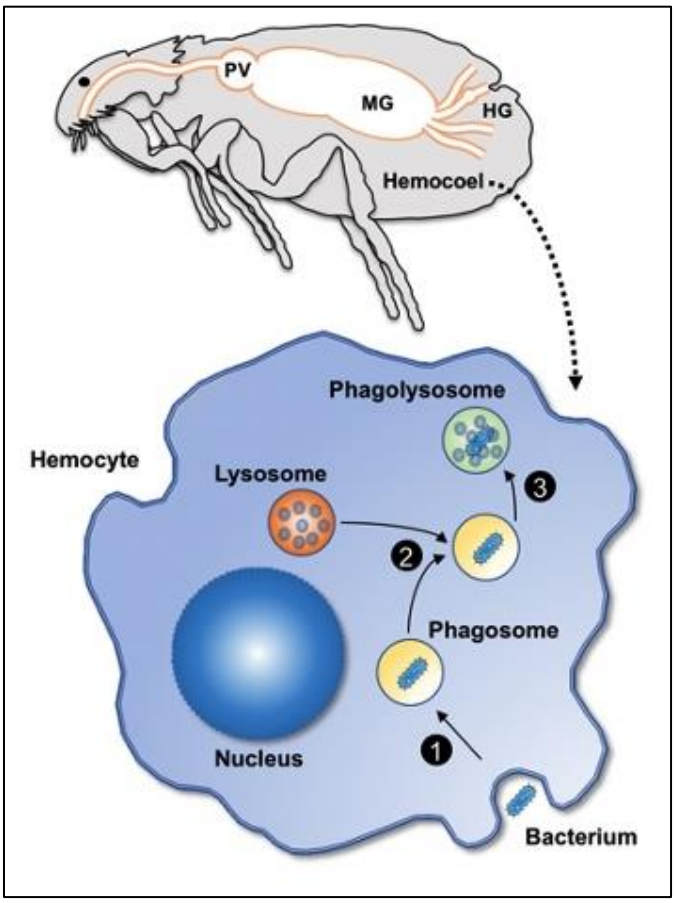

Figure 1. Representative diagram of phagocytosis by insect hemocytes. Circulating hemocytes are immune cells that circulate throughout the insect body cavity (hemocoel) with hemolymph (blood). The primary defense mechanism of hemocytes is phagocytosis, whereby a foreign body is **(1)** recognized by pathogen recognition receptors (PRRs) on the cell surface, **(2)** internalized into a phagosome, which fuses with a lysosome, and **(3)** hydrolytically digested by toxic substances produced in the phagolysosome. HG = Hindgut; MG = Midgut; PV = Proventriculus.

CHAPTER 2

METHODS

Flea rearing and maintenance

Cat fleas (*Ctenocephalides felis* Bouché) were collected from a laboratory colony at Georgia Southern University, which was initiated in 2019 with newly emerged, unfed, adults purchased from Elward II Laboratory (Soquel, CA, USA) (Brown *et al*. 2021). Immature stages were reared in an incubator at 25°C and ≥ 85% relative humidity (RH). Larvae were fed dried feces from blood-feeding adults. Adult fleas were maintained on defibrinated bovine blood (Hemostat Laboratories, Dixon, CA, USA) within an artificial feeding system (Wade and Georgi 1988). All experiments were performed on adult females between 2-3 days post-emergence.

Flea injection and bacterial infection

For experiments involving bacterial infections, a stainless-steel insect pin (size 000) was dipped into a concentrated solution of ampicillin resistant, green fluorescent protein (GFP)-expressing *Escherichia coli* (ATCC® 25922GFP), and then the insect pin was used to prick a previously cold anesthetized flea in the dorsal portion of their thorax. In a shaking incubator, bacteria were grown overnight at 37°C in tryptic soy broth (TSB) with 100 µg/mL ampicillin. Prior to beginning an experiment, the infectious dose was estimated by measuring the OD_{600} of the bacterial culture in a BioPhotometer D30 (Eppendorf AG, Hamburg, Germany). The absolute doses of bacterial inoculums were determined by infecting a set of fleas as described above, immediately placing each flea into an individual microcentrifuge tube on ice, homogenizing the flea in 200 µl of phosphate-buffered saline (PBS), and plating 27 µl of the homogenate on tryptic soy agar (TSA) with 100 µg/mL ampicillin. These plates were grown overnight at 37°C, then the resulting colony forming units (CFUs) were counted and the doses calculated.

Quantification of circulating hemocytes

Fleas were divided into three treatment groups: (1) naïve (no injection), (2) injured (sterile pinprick), and (3) injected with *E. coli* via pinprick. At 24 h post-treatment, circulating hemocytes were collected from all three groups by volume displacement, also known as perfusion, as described by others (League *et al.* 2017, Brown *et al.* 2019). Briefly, an incision was made across the lateral and ventral portions of the last suture that joins the flea abdominal segments. A finely pulled glass capillary needle was inserted into the flea thorax, approximately 200 µL of PBS was perfused through the flea hemocoel, and the diluted hemolymph was collected within 1 cm diameter etched rings on a Rite-On glass slide (Portsmouth, NH, USA). Following this procedure, hemocytes were allowed to adhere to the slide for 20 min at room temperature, and then were fixed and stained using Hema 3 (Fisher Scientific, Pittsburgh, PA, USA). After drying, slides were mounted with coverslips using Poly-Mount (Polysciences, Warrington, PA, USA), and the total number of hemocytes were counted at 40X magnification using a Nikon 90i compound microscope (Nikon, Tokyo, Japan). Three independent trials consisting of approximately 10 individuals per treatment group were conducted. Data were combined and analyzed by ANOVA, followed by Tukey's Multiple Comparison Test in GraphPad Prism version 8 (GraphPad software, San Diego, CA, USA).

Quantification of phagocytosis by circulating hemocytes

Fleas were injected with *E. coli*, and after 1 h, perfusions were conducted as described above. Hemocytes were allowed to adhere to the slide for 20 min at room temperature before they were fixed for 5 minutes by adding 100 µL of 4% formaldehyde. Next, coverslips were mounted using VECTASHIELD® Antifade Mounting Medium with DAPI. Hemocytes were then visualized under 40X magnification using fluorescence illumination on a Nikon Eclipse

E200 compound microscope. For each flea, the number of bacteria phagocytosed by the first 100 hemocytes was recorded. Two parameters were derived from these values: phagocytic index and phagocytic capacity. The phagocytic index is defined as the percentage of cells that engage in phagocytosis; whereas, the phagocytic capacity is defined as the average number of bacteria phagocytosed by each hemocyte. Three independent trials of approximately 5 fleas per trial were conducted. Data were combined and analyzed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). In order to more efficiently visualize hemocytes under fluorescent illumination, these experimental fleas were injected with a larger volume of *E. coli* (approximately 0.2 µl) using an aspirator as opposed to the septic pinprick method.

Phagocytosis limiting assay

To limit phagocytosis, a method was derived from an assay previously used in fruit flies (Elrod-Erickson *et al.* 2000, Pham *et al.* 2007). A solution of 0.2% solids 1- µm-diameter red fluorescent (542/612) polystyrene particles (Thermo Scientific™ Fluoro-Max Fluorescent Beads) in PBS was injected using an aspirator. An injury control group was created by injecting a solution of PBS alone into selected fleas. Fleas were separated into three groups, and received one of the following treatments: (1) *E. coli* injection, (2) polystyrene bead injection, and 24 h later, an *E. coli* injection, and (3) PBS injection, and 24 h later, an *E. coli* injection. At 24 h post-infection, surviving fleas from all groups were homogenized in PBS and plated on TSA with ampicillin. Plates were incubated overnight at 37°C, and the number of CFUs was used to calculate the amount of *E. coli* per flea (infection intensity). To confirm that plates contained colonies originating from *E. coli* inoculums, each plate was screened using GFP fluorescence. Three independent trials consisting of ≥15 fleas per treatment group were conducted. Data were

combined and analyzed by ANOVA, followed by Tukey's Multiple Comparison Test in GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA).

CHAPTER 3

RESULTS

Hemocyte numbers and hemocyte-mediated phagocytosis

To determine whether the number of circulating hemocytes increases in response to infection, immune cells were collected via perfusion from naïve, injured, and *E. coli*-infected fleas at 24-h post-treatment (Fig. 2A & 2B). There was a significant increase in the number of hemocytes as a consequence of *E. coli* infection in the flea hemocoel (Fig. 3; ANOVA: $p < 0.0001$). Specifically, *E. coli*-infected fleas had 85% (Tukey's: $p < 0.0001$) and 46% (Tukey's: $p = 0.0039$) more circulating hemocytes compared to naïve and injury controls, respectively. This difference was due to *E. coli* as the number of circulating hemocytes in naïve and injured fleas did not differ (Tukey's: $p = 0.2766$). Additionally, hemocytes displayed strong phagocytic activity against *E. coli* (Fig. 2C). The percentage of hemocytes actively engaged in the phagocytosis (defined as phagocytic index) was 80% (± 2% SEM) at 1 h post-infection. When only hemocytes that phagocytosed bacteria were considered, the number of *E. coli* phagocytosed by individual hemocytes (defined as phagocytic capacity) averaged 4 (± 0.2 SEM) bacteria per cell, with as many as 25 bacteria observed inside a single hemocyte. In summary, circulating hemocyte numbers increased in adult female fleas following exposure to a systemic bacterial infection, and these immune cells mounted a strong phagocytic response.

Phagocytosis limiting assays

To determine the importance of phagocytosis in the flea immune response, phagocytosis was limited by injecting polystyrene beads into the hemocoel 24 h prior to an *E. coli* infection (Fig. 2D). Fleas were placed into the following four groups: (1) *E. coli* injection, then processed immediately to determine the infectious dose delivered (infectious dose); (2) *E. coli* injection,

then processed 24 h later (24 h post-infection); (3) polystyrene bead injection, and 24 h later, an *E. coli* injection (bead injection); and (4) PBS injection, and 24 h later, an *E. coli* injection (PBS injection). After 24 h, infection intensity (the mean number of *E. coli* per flea) in the hemocoel differed significantly between treatment groups (Fig. 4; ANOVA: $p < 0.0001$). When phagocytosis was uninhibited, infection intensity in the flea hemocoel decreased by 55% compared to the infectious dose originally administered (group 1 vs. 2; Tukey's: $p < 0.0001$). Additionally, when phagocytosis was limited (bead injection), infection intensity decreased by 65% compared to uninhibited fleas (group 2 vs. 3; Tukey's: $p < 0.0001$). However, examination of the two groups that received an injection (beads or PBS injection) prior to an *E. coli* infection revealed that infection intensity increased by 2,445% in fleas with compromised hemocytes compared to injury controls (group 3 vs. 4; Tukey's: $p = 0.0440$). Taken together, these data show that a prior tissue injury (beads or PBS injection) increases bacteria killing efficiency in the flea hemocoel, but this heightened immune activity is considerably reduced when phagocytosis is limited.

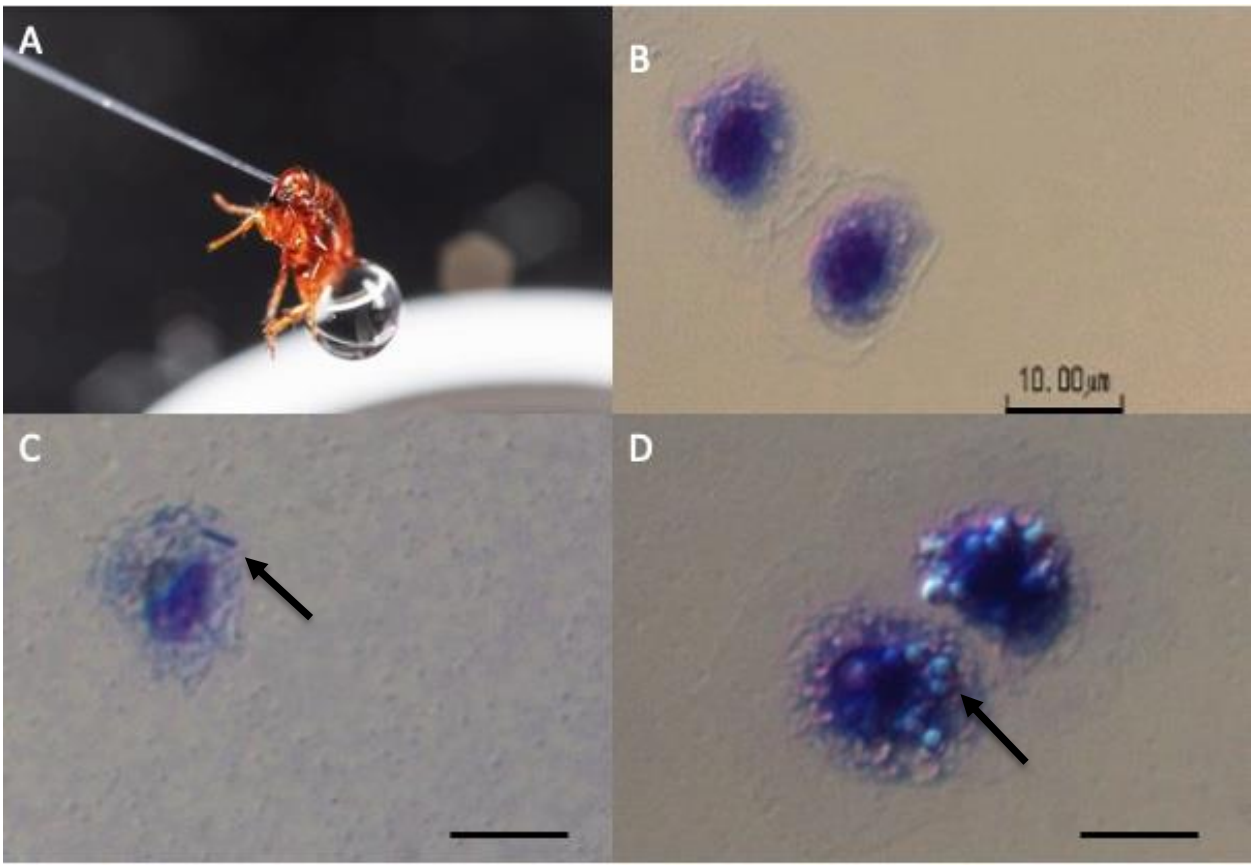

Figure 2. Images of flea perfusion method and stained hemocytes. **A.** Adult female flea undergoing hemolymph perfusion; **B.** Adult female flea circulating hemocytes; **C.** Phagocytosis of *E. coli* (black arrow) by flea hemocyte; **D.** Microscopic beads (black arrow) phagocytosed by flea hemocytes. Hemocytes images were examined under both bright-field and DIC conditions at 40-100 X magnification (black line = 10 µm).

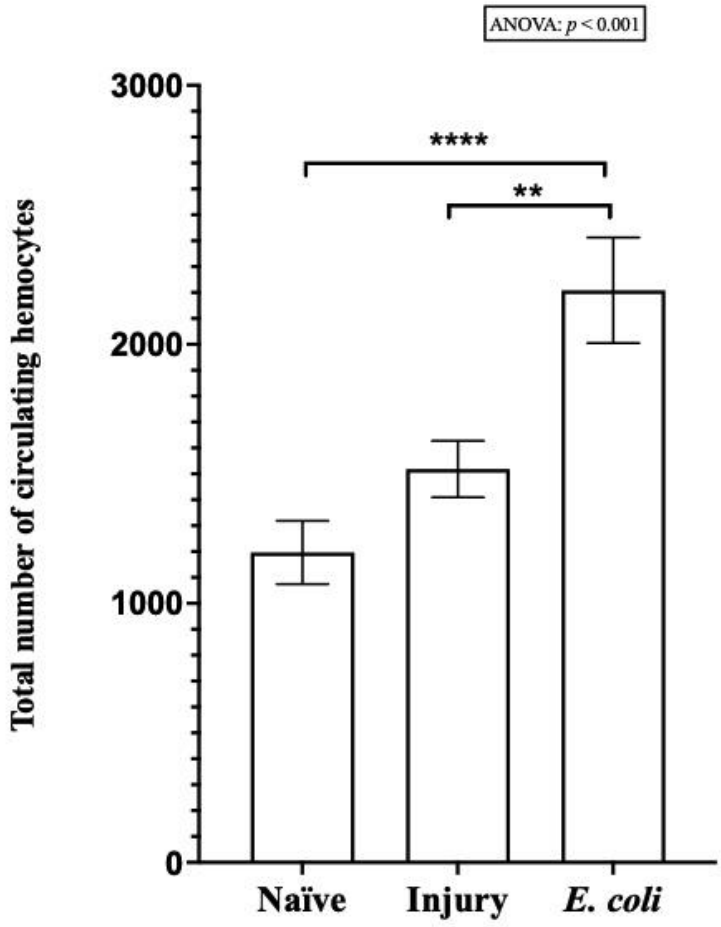

Figure 3. Total number of circulating hemocytes in naïve, injury, and *E. coli*-infected fleas. Column heights represent the average number of circulating hemocytes and error bars denote the standard error of the mean. Asterisks denote significant differences as determined by One-Way ANOVA, followed by Tukey's Multiple Comparison Test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$) GraphPad Prism V8.

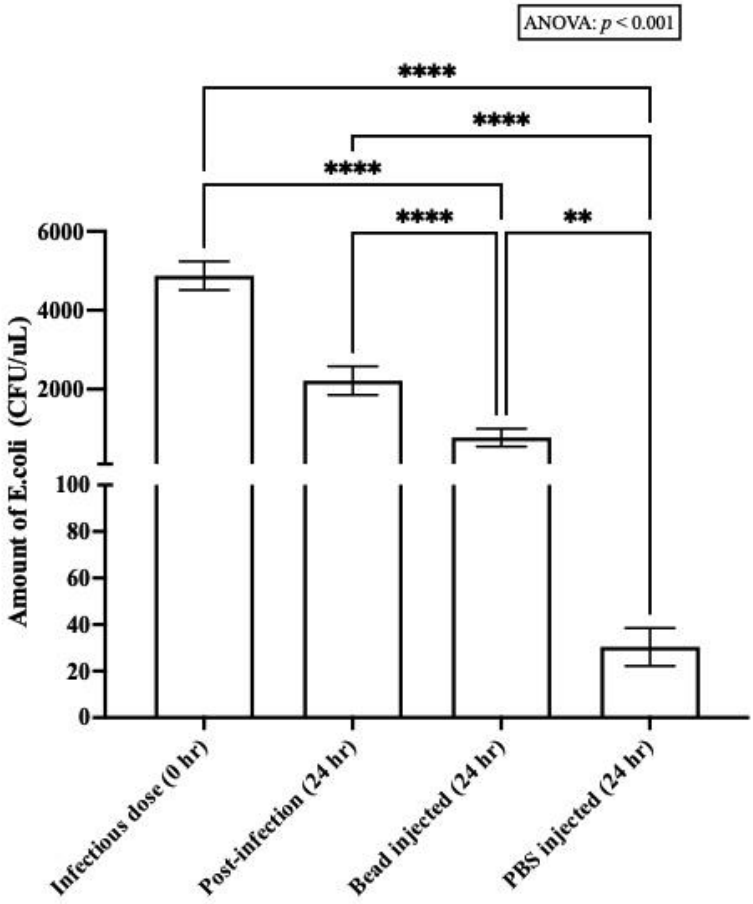

Figure 4. Number of *E. coli* per infected flea (infection intensity). Column heights mark the average CFUs, and error bars denote the standard error of the mean. Asterisks denote significant differences as determined by One-Way ANOVA, followed by Tukey's Multiple Comparison Test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$) in GraphPad Prism V8.

CHAPTER 4

DISCUSSION

Very little is known regarding the flea immune system, despite their continued veterinary and medical importance as vectors of harmful human pathogens (Brown 2019). In fact, fleas are often underestimated as vectors of agents of disease compared to other arthropods (*e.g.*, mosquitoes, sandflies, ticks and tsetse flies). Insects possess an innate immune system, which is less specific than the adaptive immune system in vertebrate animals (Hillyer 2015, Gasteiger *et al.* 2017). Innate immunity depends on the recognition of pathogen molecular moieties, and is divided into cellular and humoral components (Hillyer & Strand 2014). Cell-mediated immune responses protect against pathogens via phagocytosis by hemocytes (Gasteiger *et al.* 2017, Strand 2008, Hillyer 2015).

Phagocytosis is known as an evolutionarily conserved process in invertebrates, and is initiated when immune cells recognize foreign objects (Hillyer 2015). In mosquitoes, hemocytes are able to initiate this process within seconds of pathogen recognition, at which point pathogens are internalized and degraded (Hillyer 2015). Over the last 35 years, several labs have identified the range of circulating hemocytes in mosquitoes to be approximately 500 to 4000 cells (Christensen *et al.* 1989, Castillo *et al.* 2006, Baton *et al.* 2009, Castillo *et al.* 2011, Telang *et al*. 2012, King & Hillyer 2013, Bryant & Michael 2014, Hillyer & Strand 2014). Additionally, the average range of hemocytes in the well-studied fruit fly (*Drosophila melanogaster)* falls between 1000 – 2000 circulating hemocytes (Lanot *et al*. 2001). As outlined in Table 1, the range of circulating hemocytes differs for various insects (Castro *et al*. 2009, Coggins *et al*. 2012, Figuerido *et al*. 2006, Horn *et al*. 2014, Oliver *et al*. 2011, Weiss *et al.* 2014). These data are similar to the amount of circulating hemocytes found in the current study. In *C. felis*, the total

amount of circulating hemocytes in naïve fleas range from 650 – 2800. Moreover, there was an infection-induced increase in the number of these cells in the flea hemocoel. Additionally, the data show that flea hemocytes in circulation readily phagocytose bacteria. Taken together, flea hemocytes actively respond to bacterial infection by increasing in numbers and engaging in phagocytosis.

To determine the importance of phagocytosis as an immune defense mechanism in the flea hemocoel, polystyrene beads were injected to limit the phagocytic activity of hemocytes against a future bacterial infection. The data show that an injection of beads prior to an *E. coli* infection resulted in a higher number of bacteria compared to individuals that did not receive the bead treatment. Interestingly, fleas in the injury control group (PBS injected) had the lowest number of bacteria compared to all other groups. This phenomenon has been documented in the well-studied fruit fly (*D. melanogaster*), and is referred to as immune priming (Pham *et al.* 2007). This offers insight into the ability of an injury to initiate an immune response to a future bacterial infection in insects (Pham *et al.* 2007). Taken together, these data suggest that defects in the cellular immune response may be overcome by other immune defense molecules in the flea hemocoel.

Conclusion

In conclusion, studying flea immunity is crucial to our general understanding of how these insect vectors fight against the pathogens that they transmit to humans and other vertebrates. In the cat flea, a systemic bacterial infection initiates the proliferation of hemocytes and subsequent phagocytosis of foreign invaders. The use a phagocytic limiting assay showed that the flea immune system relies heavily on cellular mechanisms to protect itself against bacterial pathogens. Hence, studies on the immune system of fleas, which are the major vectors

of plague, murine typhus, and cat-scratch disease, could aid in the development of novel pest and disease control methods tailored to these organisms.

Table 1. Average number of circulating hemocytes, phagocytic index, and phagocytic capacity of different invertebrate species.

	Ctenocephalides felis	***Drosophila melanogaster***	***Aedes aegypti***	***Rhodinus prolixus***
# of circulating hemocytes	1,000-2,500	1,000-2,000	1,000-2,000	10-1,000
Phagocytic index	80%	24%	42%	70%
Phagocytic capacity	4/ cell	4.5/ cell	7.7 / cell	15 / cell

REFERENCES

Bartholomay LC, Michel K. 2018. Mosquito immunobiology: the intersection of vector health and vector competence. *Annu Rev Entomol,* 63:145-167.

Baton LA, Robertson A, Warr E, Strand MR, Dimopoulos G. 2009. Genome-wide transcriptomic profiling of *Anopheles gambiae* hemocytes reveals pathogen-specific signatures upon bacterial challenge and *Plasmodium berghei* infection. *BMC Genom*, 10:257.

Beckage NE. 2007. Insect immunology. Academic Press. 360 p.

Brown LD, Shapiro LLM, Thompson GA, Estévez-Lao TY, Hillyer JF. 2019. Transstadial immune activation in a mosquito: adults that emerge from infected larvae have stronger antibacterial activity in their hemocoel yet increased susceptibility to malaria infection. *Ecol Evol,* 9:6082-6095.

Brown LD, Maness R, Hall C, Gibson JD. 2021. Reactive oxygen species-mediated immunity against bacterial infection in the gut of cat fleas (*Ctenocephalides felis*). *Insect Biochem Mol Biol*, 136:103620.

Bryant WB, Michel K. 2014. Blood feeding induces hemocyte proliferation and activation in the African malaria mosquito, *Anopheles gambiae* Giles. *J Exp Biol,* 217:1238–1245.

Castillo JC, Robertson AE, Strand MR. 2006. Characterization of hemocytes from the mosquitoes *Anopheles gambiae* and *Aedes aegypti*. *Insect Biochem Mol Biol,* 36:891-903.

Castillo JC, Brown MR, Strand MR. 2011. Blood feeding and insulin-like peptide 3 stimulate proliferation of hemocytes in the mosquito *Aedes aegypti*. *PLoS Pathog,* 7:e1002274.

Castro DP, Figueiredo MB, Genta FA, Ribeiro IM, Tomassini TC, Azambuja P, Garcia ES. 2009. Physalin B inhibits *Rhodnius prolixus* hemocyte phagocytosis and microaggregation by the activation of endogenous PAF-acetyl hydrolase activities. *J Insect Physiol*, 55:532-537.

Christensen BM, Huff BM, Miranpuri GS, Harris KL, Christensen LA. 1989. Hemocyte population changes during the immune response of *Aedes aegypti* to inoculated microfilariae of *Dirofilaria immitis*. *J Parasitol,* 75:119–123.

Coggins SA, Estevez-Lao TY, Hillyer JF. 2012. Increased survivorship following bacterial infection by the mosquito *Aedes aegypti* as compared to *Anopheles gambiae* correlates with increased transcriptional induction of antimicrobial peptides. *Dev Comp Immunol,* 37:390-401.

Durden LA, Hinkle NC. 2019. Fleas (Siphonaptera), Medical and Veterinary Entomology. Elsevier, pp. 145-169.

Elrod-Erickson M, Mishra S, Schneider D. 2000. Interactions between the cellular and humoral immune responses in *Drosophila*. *Curr Biol,* 10:781-784.

Figueiredo MB, Castro DP, Nogueira NF, Garcia ES, Azambuja P. 2006. Cellular immune response in *Rhodnius prolixus*: role of ecdysone in hemocyte phagocytosis. *J Insect Physiol*, 52:711-716.

Gasteiger G, D'Osualdo A, Schubert DA, Weber A, Bruscia EM, Hartl D. 2017. Cellular innate immunity: an old game with new players. *J Innate Immun*, 9:111-125.

Hillyer JF, Schmidt SL, Christensen BM. 2003a. Hemocyte-mediated phagocytosis and melanization in the mosquito *Armigeres subalbatus* following immune challenge by bacteria. *Cell Tissue Res*, 313:117-127.

Hillyer JF, Schmidt SL, Christensen BM. 2003b. Rapid phagocytosis and melanization of bacteria and *Plasmodium* sporozoites by hemocytes of the mosquito *Aedes aegypti*. *J Parasitol*, 89:62-69.

Hillyer JF, Schmidt SL, Christensen BM. 2004. The antibacterial innate immune response by the mosquito *Aedes aegypti* is mediated by hemocytes and independent of Gram type and pathogenicity. *Microbes Infect*, 6:448-459.

Hillyer JF, Schmidt SL, Fuchs JF, Boyle JP, Christensen BM. 2005. Age-associated mortality in immune challenged mosquitoes (*Aedes aegypti*) correlates with a decrease in haemocyte numbers. *Cell Microbiol*, 7:39-51.

Hillyer JF, Barreau C, Vernick KD. 2007. Efficiency of salivary gland invasion by malaria sporozoites is controlled by rapid sporozoite destruction in the mosquito haemocoel. *Int J Parasitol*, 37:673–681.

Hillyer JF, Strand MR. 2014. Mosquito hemocyte-mediated immune responses. *Curr Opin Insect Sci*, 3:14–21.

Hillyer JF. 2015. Integrated immune and cardiovascular function in pancrustacea: Lessons from the Insects. *Integr Comp Biol*, 55:843–855.

Hillyer JF. 2016. Insect immunology and hematopoiesis. *Dev Comp Immunol*, 58:102-118.

Horn L, Leips J, Starz-Gaiano M. 2014. Phagocytic ability declines with age in adult *Drosophila* hemocytes. *Aging Cell*, 13(4):719-28.

King JG, Hillyer JF. 2013. Spatial and temporal *in vivo* analysis of circulating and sessile immune cells in mosquitoes: hemocyte mitosis following infection. *BMC Biol*, 11:55.

Kwon H, Smith RC. 2019. Chemical depletion of phagocytic immune cells in *Anopheles gambiae* reveals dual roles of mosquito hemocytes in anti-*Plasmodium* immunity. *Proc Natl Acad Sci U S A*, 116:14119-14128.

Lanot R, Zachary D, Holder F, Meister M. 2001. Postembryonic hematopoiesis in *Drosophila*. *Dev Biol*, 230: 243-257.

League GP, Estévez-Lao TY, Yan Y, Garcia-Lopez VA, Hillyer JF. 2017. *Anopheles gambiae* larvae mount stronger immune responses against bacterial infection than adults: evidence of adaptive decoupling in mosquitoes. *Parasit Vectors,* 10:367.

Oliver JD, Dusty Loy J, Parikh G, Bartholomay L. 2011. Comparative analysis of hemocyte phagocytosis between six species of arthropods as measured by flow cytometry. *J Invertebr Pathol*, 108:126-30.

Pham LN, Dionne MS, Shirasu-Hiza M, Schneider DS. 2007. A specific primed immune response in *Drosophila* is dependent on phagocytes. *PLoS Pathog,* 3:e26.

Satyavathi VV, Minz A, Nagaraju J. 2014. Nodulation: an unexplored cellular defense mechanism in insects. *Cell Signal*, 26:1753-63.

Sheehan G, Garvey A, Croke M, Kavanagh K. 2018. Innate humoral immune defences in mammals and insects: The same, with differences? *Virulence*, 9:1625-1639.

Strand MR. 2008. The insect cellular immune response. *Insect Science,* 15:1-14.

Telang A, Qayum AA, Parker A, Sacchetta BR, Byrnes GR. 2012. Larval nutritional stress affects vector immune traits in adult yellow fever mosquito *Aedes aegypti* (*Stegomyia aegypti*). *Med Vet Entomol,* 26: 271-281.

Wade SE, Georgi JR. 1988. Survival and reproduction of artificially fed cat fleas, *Ctenocephalides felis* Bouché (Siphonaptera: Pulicidae). *J Med Entomol*, 25: 186-190.

Weiss BL, Maltz M, Aksoy S. 2012. Obligate symbionts activate immune system development in the tsetse fly. *J Immunol,*188:3395–403.