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Immune defense mechanisms against a systemic bacterial infection in the cat flea (*Ctenocephalides felis*)

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ABSTRACT

A significant amount of work has been devoted towards understanding the cellular and humoral immune responses in arthropod vectors. Although fleas (Siphonaptera) are vectors of numerous bacterial pathogens, few studies have examined how these insects defend themselves from infection. In this study, we investigated the immune defense mechanisms in the hemocoel of cat fleas (*Ctenocephalides felis*), currently the most important flea pest of humans and many domestic animals. Using model species of bacteria (*Micrococcus luteus*, *Serratia marcescens*, and *Escherichia coli*), we delivered a systemic infection and measured the following: antimicrobial activity of hemolymph, levels of free radicals resulting from the induction of oxidase-based pathways, number of circulating hemocytes, phagocytosis activity of circulating hemocytes, and *in vivo* bacteria killing efficiency when phagocytosis activity is limited. Our results show that the antimicrobial activity of flea hemolymph increases in response to certain species of bacteria; yet, a systemic infection with the same bacterial species did not influence levels of hydrogen peroxide (H_2O_2), a reactive intermediate of oxygen, at the same time. Additionally, the number of circulating hemocytes increases in response to *E. coli* infection, and these cells display strong phagocytic activity against this bacterium. Moreover, limiting phagocytosis by injecting polystyrene beads subsequently increases flea susceptibility to *E. coli* infection when compared to injury controls; however, impairing the cellular immune response itself did not increase flea susceptibility to infection when compared to untreated fleas. Overall, this work yields significant insight into how fleas interact with bacterial pathogens in their hemocoel, and suggests that cellular and humoral immune responses cooperate to combat systemic bacterial infections.

1. Introduction

The immune system in the insect body cavity (hemocoel) is divided into humoral and cellular components that collectively provide defense against a range of microbes (Beckage, 2008; Hillyer, 2016). The humoral defenses include production of soluble effector molecules, such as antimicrobial peptides, complement-like proteins, reactive oxygen species (ROS) and reactive nitrogen species, and components of the phenoloxidase-based melanization cascade. Immune cells called hemocytes, whose primary immune mechanism is phagocytosis, but also produce many humoral immune molecules, mediate the cellular defenses. Hemocytes either circulate with the hemolymph (blood) – called circulating hemocytes – or remain attached to tissues – called sessile hemocytes (Beckage, 2008; Hillyer and Strand, 2014). Together, these two branches are crucial to the immune response of invertebrates, and are directly related to insect survivability against systemic infections.

In the case of arthropod vectors, the invertebrate immune system plays a key role in the spread and transmission of pathogens that cause human diseases. For example, in species of human lice, it is hypothesized that head lice (*Pediculus humanus capitis*) are not competent vectors for the deadly pathogens transmitted by body lice (*Pediculus humanus humanus*) because head lice have a stronger immune response against bacterial infections (Kim et al., 2011; Kim et al., 2017; Previte et al., 2014). Likewise, a comparative analysis of the tick immune response against *Borrelia* spirochetes in susceptible (*Ixodes scapularis*) and refractory (*Dermacentor variabilis*) tick species showed that *D. variabilis* has a much stronger immune response against spirochetes than *I. scapularis* (Johns et al., 2001; Johns et al., 2000). Additionally, numerous studies have shown that transmission of *Plasmodium* parasites ultimately depends on immune resistance and tolerance strategies in *Anopheles*

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mosquitoes (Dimopoulos et al., 1997; Dimopoulos et al., 1998; Dong et al., 2006; Habtewold et al., 2017; Luckhart et al., 1998; Richman et al., 1997). Finally, it is hypothesized that bed bugs do not transmit harmful pathogens because they possess immune-active substances that attenuate bacterial virulence factors and, thereby, decrease their competence as vectors of infectious human diseases (Lai et al., 2016).

Fleas (Order Siphonaptera) are insect vectors of numerous human pathogens, including *Bartonella henselae* (cat scratch disease), *Rickettsia felis* (flea-borne spotted fever), *Rickettsia typhi* (murine typhus), and *Yersinia pestis* (plague) (Brown, 2019; Durden and Hinkle, 2019). Despite their medical and veterinary importance, relatively little is known about the immune defense mechanisms in the flea hemocoel. To gain a better mechanistic understanding of flea immune responses against systemic infections, we used model bacterial species to measure humoral and cellular responses in the hemocoel of cat fleas (*Ctenocephalides felis*). We found that humoral antimicrobial activity in the flea hemolymph increases in response to select bacterial species, but this enhanced immunity did not correlate with the generation of hydrogen peroxide (H_2O_2), a reactive intermediate of oxygen and component of the melanization cascade. Additionally, the number of circulating hemocytes increases in response to bacterial infection, and these cells display strong phagocytic activity. Moreover, impairing phagocytosis by injecting polystyrene beads subsequently increases flea susceptibility to bacterial infection when compared to injury controls. However, limiting the cellular immune response itself did not increase flea susceptibility to infection when compared to untreated fleas, which suggests that cellular and humoral immune responses cooperate to combat systemic bacterial infections.

2. Methods

2.1. Flea rearing and maintenance

Cat fleas (*C. felis* Bouché) were collected from colonies maintained at Georgia Southern University as previously described (Brown et al., 2021). Briefly, immature stages were reared in an incubator at 25 °C and ≥ 85 % relative humidity (RH). Larvae were fed the dried feces from blood-feeding adults. Adults were fed 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 and 3 days post-emergence.

2.2. Flea injection and bacterial infection

Adult fleas were cold anesthetized and injected at the intersegmental suture between the mesonotum and metanotum plates of the thorax. For experiments involving bacterial infections, a stainless-steel insect pin (size 000) was dipped into a concentrated solution of the following: (a) live, ampicillin resistant, GFP-expressing *Escherichia coli* (ATCC® 25922GFP; *E. coli*) in tryptic soy broth (TSB) with 100 μg/mL ampicillin; (b) heat-killed *E. coli* in TSB with 100 μg/mL ampicillin; (c) live *Serratia marcescens* (D1, Carolina Biological Supply Company) in nutrient broth (NB); (d) heat-killed *S. marcescens* in NB; (e) live *Micrococcus luteus* (Carolina Biological Supply Company) in NB, or (f) heat-killed *M. luteus* in NB. For phagocytosis blocking assays (see below), approximately 0.2 μl of a polystyrene bead solution was injected using an aspirator (Drummond™ Aspirator Tube) attached to a finely pulled glass capillary tube.

In a shaking incubator, *E. coli* was grown overnight at 37 °C, and *S. marcescens* and *M. luteus* were grown overnight at 25 °C. 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), and was standardized across all trials for each bacterium (*E. coli*: $OD_{600} = 100$; *S. marcescens*: $OD_{600} = 1$; *M. luteus*: $OD_{600} = 1$). For phagocytosis blocking assays (see below), the absolute dose of *E. coli* delivered was 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. Plates were grown overnight at 37 °C, then the resulting colony forming units (CFUs) were counted and the dose was calculated. To confirm that all colonies originated from the *E. coli* inoculums, plates were also screened for GFP fluorescence. For experiments using heat-killed bacteria, 50 μl of bacterial culture was incubated at 95 °C for 10 min on a Fisherbrand™ Isotemp™ heating block, and injected after cooling to room temperature. Plating of the heat-killed cultures on either TSA with ampicillin (*E. coli*) or nutrient agar (*S. marcescens*, *M. luteus*) resulted in no CFUs, which confirmed that all bacteria were dead prior to injection.

2.3. Quantification of antimicrobial hemolymph activity

Hemolymph was extracted at 24 h after treatment from fleas that were naïve, injured (sterile pinprick), or injected with heat-killed bacteria (*E. coli, S. marcescens, M. luteus*). For each flea, an incision was made across the lateral and ventral portions of the suture that joins the 6th and 7th abdominal segment. Freshly wounded individuals were placed together inside a single unfiltered 200 μl pipette tip. This tip was then inserted into a 1.5 mL microcentrifuge tube and was centrifuged at 2150 RCF for 10 min at 4 °C. This resulted in the collection of 2 – 3 μl of hemolymph from each treatment group of pooled individuals. Antimicrobial activity of flea hemolymph was then measured using a zone of inhibition assay as described by others (Haine et al., 2008; League et al., 2017; Moret and Schmid-Hempel, 2000). Briefly, a 3 mL *M. luteus* culture was grown overnight in NB at 25 °C in a shaking incubator until it reached approximately $OD_{600} = 10$. One mL of the *M. luteus* culture was then added to 10 mL of sterile, liquid LB agar that was maintained at 55 °C in a water bath. The *M. luteus*-LB agar solution was mixed, and then poured in duplicate volumes (approximately 5 mL) into two standard size Petri plates (technical replicates). After solidifying, equidistant holes were created in the agar of the Petri dish using a 1 mm tip sterile glass Pasteur pipette. Each hole was then loaded with 1 μl of hemolymph, and the plates were incubated overnight at room temperature. For each sample, the diameters of the individual zones of bacterial growth inhibited were measured using a fine precision ruler, and the average of the two technical replicates was used to calculate the areas of the zones of inhibition. Three independent trials were conducted, and each trial consisted of approximately 18 – 20 fleas per treatment group. 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).

2.4. Quantification of ROS in hemolymph

Using the method described for the zone of inhibition assay, hemolymph was extracted from naïve, injured, *E. coli*-, *S. marcescens*- and *M. luteus*-infected fleas at 24 h post-treatment. The concentration of hydrogen peroxide (ROS) was then measured for each group using the Pierce™ Quantitative Peroxide Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions for the aqueous-compatible procedure. Briefly, immediately following extractions, 2 μl of hemolymph was mixed with 40 μl of PBS containing 2 mg/mL of 3-amino triazole (catalase inhibitor). Each sample was transferred in duplicate to a microwell plate (20 μl per well), and 200 μl of the working reagent was then added to each well. Following a 20 min incubation at room temperature, the absorbance was then measured at 595 nm using a plate reader (Synergy H1 plate reader, BioTek, Winooski, VT, USA). Seven independent trials were conducted per treatment group, each consisting of hemolymph pooled from 18 to 25 fleas. 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).

2.5. Quantification of circulating hemocytes

Using a perfusion method adapted from mosquitoes (Brown et al., 2018; League et al., 2017), circulating hemocytes were collected from naïve, injured, and *E. coli*-infected fleas at 24 h post-treatment. Briefly, an incision was made across the second to last abdominal segment, a microinjection needle was inserted into the thorax, and approximately 200 µl of PBS was injected. The diluted hemolymph that exited through the abdominal incision was collected within a 1-cm-diameter etched ring on a Rite-On glass slide (Gold Seal). After allowing the hemocytes to adhere to the slide for 20 min at room temperature, cells were fixed and stained using Hema 3 (Fisher Scientific), dried, and mounted under a coverslip using Poly-Mount (Polysciences). The total number of hemocytes was then counted under bright-field illumination at 40X magnification using a Nikon Eclipse E200 compound microscope (Nikon, Tokyo, Japan). Three independent trials were conducted, and each trial consisted of approximately 10 fleas per treatment group. 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).

2.6. Quantification of phagocytosis by circulating hemocytes

Fleas were injected with *E. coli*, and 1 h later, the hemocytes were collected by perfusion. Hemocytes were allowed to adhere to the slide for 20 min at room temperature, fixed for 5 min by adding 100 µl of 4 % formaldehyde, and coverslips were mounted using VECTASHIELD® Antifade Mounting Medium with DAPI. Hemocytes were examined under 40X magnification using fluorescence illumination on a Nikon Eclipse E200 compound microscope. For each flea, the number of bacteria that had been phagocytosed by each of the first 100 hemocytes viewed was recorded. These values were used to calculate two parameters: (a) the phagocytic index, which is defined as the percentage of cells that engage in phagocytosis; and (b) the phagocytic capacity, which is defined as the average number of phagocytosed bacteria per hemocyte. Three independent trials were conducted, and each trial consisted of approximately 5 fleas. Data were combined and analyzed by descriptive statistics in GraphPad Prism version 8 (GraphPad software, San Diego, CA, USA). To better visualize phagocytic hemocytes under fluorescence 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.

2.7. Phagocytosis limitation assays

Using an assay adapted from 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™ FluoroMax Fluorescent Beads) in PBS was injected using an aspirator to limit phagocytosis. A solution of PBS alone was also injected using an aspirator as an injury control. Fleas were placed 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; (3) PBS injection, and 24 h later, an *E. coli* injection. A subset of fleas from group one was processed immediately as described above in section 2.2. to determine the infectious dose delivered. Twenty-four hours post-infection, surviving fleas from all three groups were homogenized in PBS and plated on TSA with ampicillin. Plates were incubated overnight at 37 °C, and the number of CFUs was counted and used to calculate infection intensity (the mean number of *E. coli* per flea). Again, to confirm that all colonies originated from the *E. coli* inoculums, plates were also screened for GFP fluorescence. Three independent trials consisting of ≥ 15 fleas per treatment 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).

3. Results

3.1. Hemolymph antimicrobial activity

Inside the insect hemocoel, multiple tissues produce humoral immune factors that lyse pathogens. To test whether a bacterial infection in the flea hemocoel alters the overall antimicrobial activity of hemolymph, we conducted a zone of inhibition assay at 24 h post infection. We found that flea hemolymph antimicrobial activity was dramatically altered by the presence of specific bacterial species (Fig. 1A; ANOVA: $p < 0.0001$). In particular, the antimicrobial activity of Gram (-) *E. coli*- and Gram (+) *M. luteus*-infected fleas was significantly higher when compared to naïve (Tukey's: $p < 0.0001$ for both bacteria) and injury controls (Tukey's: $p < 0.0001$ for both bacteria). Furthermore, this difference was due to an induced response, rather than a constitutive response, as the antibacterial activity in the hemolymph of naïve and injured fleas did not differ (Tukey's: $p > 0.9999$). Interestingly, although there was an induced response with Gram (-) *E. coli*, the antimicrobial activity of Gram (-) *S. marcescens*-infected fleas did not differ when compared to naïve and injured fleas (Tukey's: $p = 0.3521$ for both comparisons). Taken together, although the doses – and subsequent levels of pathogenicity – were not equivalent across the different species of bacteria, these experiments highlight the relative strength of hemolymph antimicrobial activity in fleas against a range of microbes.

3.2. Hemolymph peroxide levels

In addition to their function in cellular immunity, ROS are also involved in humoral immune responses in the insect hemocoel. For example, hydrogen peroxide, which functions as both a messenger molecule and an immune effector, is produced by multiple tissues in insects and is an important component of the antibacterial response. To test whether a bacterial infection in the flea hemocoel alters synthesis of ROS, we measured hydrogen peroxide levels in hemolymph at 24 h post infection. We found that a bacterial infection in the flea hemocoel did not have a meaningful effect on the production of hydrogen peroxide (Fig. 1B; ANOVA: $p = 0.1803$). Although peroxide levels from *E. coli*-infected fleas increased by roughly 18 % and 20 % when compared to naïve and injured fleas, respectively, this was not a significant difference. Moreover, with the exception of *E. coli*, all other treatments actually resulted in slightly lower peroxide levels when compared to naïve individuals (1 %, 11 %, and 9 % for injury, *M. luteus*, and *S. marcescens*, respectively); however, again, this decrease did not reach statistical significance. Taken together, these data indicate that although humoral antimicrobial activity in the flea hemolymph increases in response to bacterial infection at 24 h post infection, this enhanced immunity does not correlate with increased levels of hydrogen peroxide at the same time.

3.3. Hemocyte numbers and hemocyte-mediated phagocytosis

The number of hemocytes in circulation is a key component to the differences in immune proficiency between insects, as well as the frequency or degree to which those hemocytes engage in phagocytosis. To test whether flea hemocyte numbers increase in response to a bacterial infection, individuals were injected with *E. coli* and the number of circulating immune cells was counted 24 h later. Additionally, to test the phagocytic activity of individual hemocytes, another group of fleas was injected with *E. coli* and the phagocytic burden was quantified 1 h later. We found that the number of circulating hemocytes changed significantly as a consequence of *E. coli* infection in the flea hemocoel (Fig. 2C; ANOVA: $p < 0.0001$). In particular, *E. coli*-infected fleas had 85 % (Tukey's: $p < 0.0001$) and 46 % (Tukey's: $p = 0.0039$) more circulating hemocytes than naïve and injury controls, respectively. Furthermore, this difference was due to the bacterial infection as the number of circulating hemocytes in naïve and injured fleas did not differ (Tukey's:

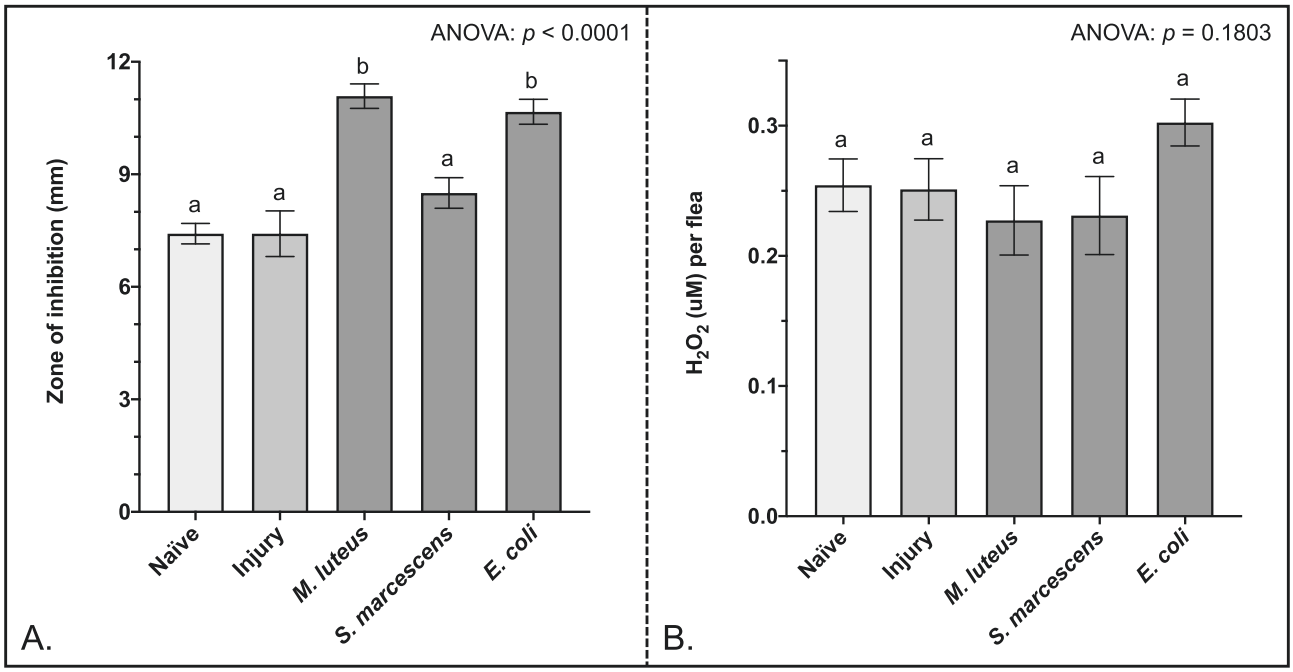

Fig. 1. Measurement of humoral immune responses. Column heights represent the mean diameter of lytic activity **(A)** or the average amount of hydrogen peroxide **(B)** in hemolymph from fleas that were naïve, injured, or injected with heat-killed **(A)** or live **(B)** bacteria (*E. coli, S. marcescens, M. luteus*). Bars represent the standard error of the mean (SEM), and data were analyzed by a one-way ANOVA followed by a Tukey's multiple comparison test. Different lowercase letters denote significant differences between treatments.

$p = 0.2766$). Also, after *in vivo* inoculation of fleas with *E. coli*, hemocytes displayed strong phagocytic activity. The percentage of hemocytes actively engaged in the phagocytosis of *E. coli* (defined as phagocytic index) was 80 % (±2% SEM) at 1 h post-infection. When only hemocytes that phagocytosed bacteria were included in the analysis, 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. Taken together, these data show that the number of circulating hemocytes in the flea hemocoel increases in response to a bacterial infection, and that these immune cells mount a strong phagocytic response.

3.4. Phagocytosis limitation assays

Hemocytes are often considered the first responders to a systemic infection in the insect hemocoel. To determine the importance of these phagocytic cells in fleas, we limited phagocytosis by injecting polystyrene beads into the hemocoel 24 h prior to bacterial challenge. Specifically, fleas were placed into four groups, and received one of the following treatments: (1) *E. coli* injection, then processed immediately to determine the infectious dose delivered (0 h post-infection); (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 (processed 24 h post-infection); and (4) PBS injection, and 24 h later, an *E. coli* injection (processed 24 h post-infection). First, we found that flea hemocytes readily phagocytose polystyrene beads (Fig. 2D). After 24 h, the percentage of circulating hemocytes actively engaged in the phagocytosis of beads was 71 % (±4% SEM), and averaged 4 (±0.2 SEM) beads per cell. Second, we found that infection intensity (the mean number of *E. coli* per flea) in the hemocoel differed significantly between treatment groups (Fig. 2F; ANOVA: $p < 0.0001$). In particular, when phagocytosis was unlimited, infection intensity in the flea hemocoel decreased by 55 % compared to the infectious dose originally administered (group 1 vs 2; Tukey's: $p < 0.0001$). Next, contrary to our expectation, when phagocytosis was limited (polystyrene bead injection), infection intensity decreased by 65 % compared to unlimited fleas (group 2 vs 3; Tukey's: $p < 0.0001$). However, when we examined the two groups that received a treatment (polystyrene beads or PBS injection) prior to an *E. coli* infection, we found that infection intensity increased by 2,445 % in fleas with compromised hemocytes compared to injury controls (group 3 vs 4; Tukey's: $p = 0.0440$). Thus, these data show that a prior tissue injury (polystyrene beads or PBS injection) actually increases bacteria killing efficiency in the flea hemocoel, but this heightened immune activity is considerably reduced when phagocytosis is limited.

4. Discussion

The importance of immunity in arthropod vectors is well understood; yet, relatively little is known about the immune defense mechanisms in fleas. This is a major oversight, as the flea immune system likely, in part, determines subsequent transmission of disease-causing pathogens to humans and other animals. Here, we present an empirical study of the immune response mounted by cat fleas (*C. felis*) against systemic bacterial infection.

Hemolymph is the primary extracellular fluid in insects, consisting of the liquid plasma and the cellular hemocytes. It is also the major transport medium for immune effector molecules and signaling proteins secreted by hemocytes and other tissues. In the rat flea (*Xenopsylla cheopis*), injection of the plague bacterium (*Y. pestis*) directly into the hemocoel resulted in the identification of several infection-induced transcripts with sequence similarities to known arthropod immune genes, including a β-1,3 glucan recognition protein, a matrix metalloproteinase with a putative peptidoglycan binding domain, a putative small lysozyme protein, and a serine protease inhibitor (Zhou et al., 2012). Additionally, experiments with *X. cheopis* and a laboratory strain of *E. coli* revealed an infection-induced response, rather than a constitutive response, in the antimicrobial activity of flea hemolymph (Driver, 2002). Moreover, this systemic *E. coli* infection also led to the identification of transcripts in *X. cheopis* with homology to arthropod immune genes, such as antimicrobial peptides (defensin, cecropin, attacin, coleoptericin, apidaecin, and diptericin B), a peptidoglycan recognition protein, and a serine protease (Driver, 2002). In the present study, we

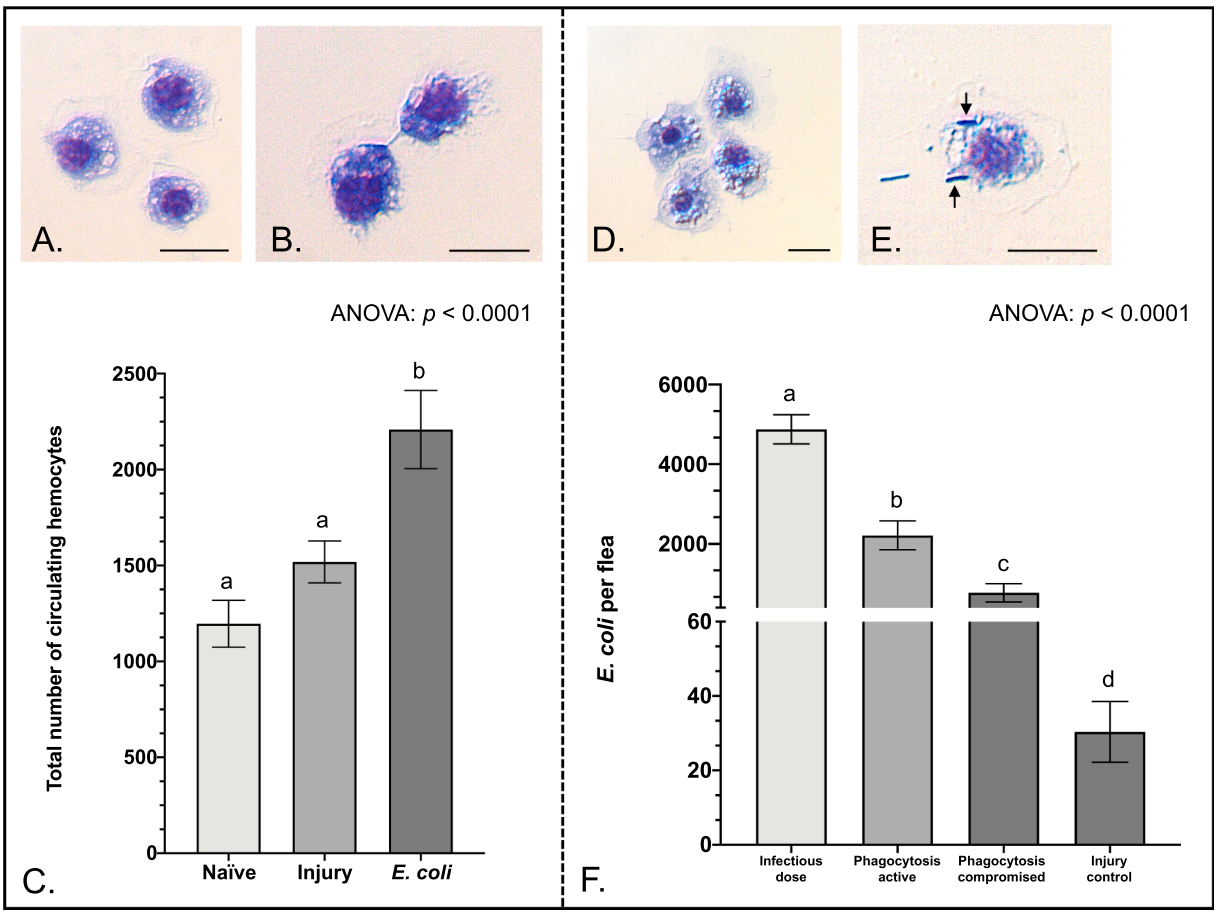

Fig. 2. Measurement of cellular immune responses. (A) Circulating hemocytes collected by hemolymph perfusion, fixed and stained using Hema 3, and examined under both bright-field and DIC conditions at 40 – 100X magnification (black line = 10 μm); **(B)** Hemocytes that appear to have finished dividing (nuclei at opposite poles of cell, midbody channel formed at the end of cytokinesis), suggesting that they are undergoing mitosis; **(C)** Colum heights represent the average number of circulating hemocytes from fleas that were naïve, injured, or injected with *E. coli*; **(D)** Hemocytes that have phagocytosed polystyrene beads**; (E)** Hemocyte that has phagocytosed *E. coli* (black arrows)**; (F)** Column heights represent the mean number of *E. coli* in the hemocoel of fleas from naïve, injured, or injected with polystyrene beads. Bars represent the standard error of the mean (SEM), and data were analyzed by a one-way ANOVA followed by a Tukey's multiple comparison test. Different lowercase letters denote significant differences between treatments. Images were captured on a Nikon Eclipse Ni upright compound microscope (Nikon Corp., Tokyo, Japan) equipped with a Nikon DS-Fi2 CCD camera.

showed that *C. felis* exhibit strong antimicrobial activity in their hemolymph. In contrast to *X. cheopis*, the hemolymph of cat fleas showed antimicrobial activity in both naïve and injured individuals, in addition to increasing in strength after an infection with Gram (-) *E. coli* and Gram (+) *M. luteus*. Interestingly, this increase in antimicrobial activity was not observed using another Gram (-) bacterium, *S. marcescens*. At first glance, these data suggest that the flea immune system can discern between different species of bacteria; however, this assumption lacks support because the doses were not equivalent across the two G (-) bacterial treatments. Pilot experiments revealed that higher doses of *S. marcescens* (OD_{600} = >1) resulted in significant mortality of fleas; thus, the antimicrobial activity of flea hemolymph against G (-) bacteria may be induced in a dose dependent manner. Overall, these data show constitutive antimicrobial activity in the hemolymph of cat fleas, and, depending on the flea-pathogen combination, this activity may increase or remain the same following infection.

The function of ROS in immune responses is best known for the oxidative burst that leads to the destruction of engulfed bacteria in phagocytic cells (Bedard et al., 2007; Kawahara et al., 2007). However, in addition to their function in this cellular response, ROS also affects the lytic activity in the extracellular environment of hemolymph. For example, hydrogen peroxide, which functions as both a messenger molecule and an immune effector, is produced by the enzyme dual oxidase (DUOX) at the plasma membrane of hemocytes and epithelial cells in response to invasion of the hemocoel (Arbi et al., 2011; Chakrabarti and Visweswariah, 2020; Kumar et al., 2003; Razzell et al., 2013). Additionally, the enzymatic process that leads to melanization of hemolymph – the prophenoloxidase cascade – also produces ROS, such as hydrogen peroxide, superoxide anion (O_2^-), and hydroxyl radical (OH) (Beckage, 2008; Komarov et al., 2006). Although transcripts have been identified in *X. cheopis* with homology to genes involved in these biochemical pathways (Driver, 2002; Zhou et al., 2012), we found relatively constant levels of hydrogen peroxide in the hemolymph of *C. felis* across treatment groups (naïve, injured, and injected with heat-killed bacteria). Additionally, we did not visually observe a rapid darkening (*i.e.*, dark melanin deposits) of the flea hemolymph after extraction, which occurs in other insects with notable phenoloxidase activity (Insua et al., 2013; League et al., 2017; Li et al., 2019). Several possibilities may explain these findings in cat fleas. First, because free radicals also damage host cells, their production and removal can be rapid, from a few hours to a few days (Komarov et al., 2006; Kumar et al., 2003; Myers et al., 2018); thus, although the antimicrobial activity in *C. felis* hemolymph increased at 24 h post infection with the same pathogen, this may be too late of a time point to detect differences in ROS synthesis. Similarly, because peroxide levels were assayed at 24 h, a time when *C. felis* had significantly fewer bacteria in the hemocoel, it is possible that the peroxide levels returned to baseline given the observed lower infection intensity. Second, in addition to production of

intracellular and extracellular ROS, insect hemocytes may also uptake extracellular H_2O_2 as part of their response to tissue injury (Chakrabarti and Visweswariah, 2020; Myers et al., 2018); thus, because we did not lyse the hemocytes collected with the hemolymph, perhaps increased levels of extracellular H_2O_2 accumulated inside the flea hemocytes, which was beyond our method of detection. Third, similar to ticks, fleas probably possess a mechanism for hemolymph clotting, but activation of the prophenoloxidase cascade, which leads to reactive intermediates due to the production of melanin, may not occur (Hajdušek et al., 2013). Overall, additional studies are needed to determine the importance of extracellular ROS against bacterial infections in the hemolymph of fleas. These should include quantitative peroxide assays at earlier time points, quantification of phenoloxidase activity, gene expression analysis of transcripts with sequence similarities to components of oxidase-based pathways, and knockdown of pathway-related genes via RNAi to confirm gene function.

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, b; King and Hillyer, 2013). Additionally, mosquito hemocyte numbers increase in response to blood feeding and infection by certain pathogens (Baton et al., 2009; Bryant and Michel, 2014; Castillo et al., 2011; Christensen et al., 1989; Coggins et al., 2012; King and Hillyer, 2013). 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 h of infection (Hillyer et al., 2007; Hillyer et al., 2005). In the present study, we found similar densities of circulating hemocytes in *C. felis*, including an infection-induced increase in the number of these cells. Additionally, our data shows that flea hemocytes in circulation readily phagocytose bacteria and other small foreign entities, such as polystyrene beads. Moreover, when we limited phagocytosis by injecting polystyrene beads prior to bacterial challenge, we found that an inferior ability to engage in this process increased infection intensity in the flea hemocoel. Interestingly, we also demonstrated that a sterile injury provides protection to fleas against subsequent infection, which has been documented in other insects (Chakrabarti and Visweswariah, 2020; Moret and Siva-Jothy, 2003; Roth et al., 2009; Vargas et al., 2016). Taken together, our data suggests that phagocytosis by circulating hemocytes is an important component of the flea immune response against systemic bacterial infection, but that defects in the cellular immune response may be overcome by other immune defense molecules in the flea hemocoel.

In summary, the present study provides valuable insights into the immune responses in the hemocoel of cat fleas through the use of model bacterial species. We have shown that fleas mount powerful humoral and cellular immune responses to resist or limit systemic bacterial infection. Moreover, we have collected baseline data on flea immunity through the use of laboratory infection models, which will serve as a guide for later studies on natural flea-pathogen interactions. Overall, studies on the immune system of fleas are crucial to our understanding of flea-borne disease transmission to humans and other animals.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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