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The role of reactive oxygen species in the gut immune response of cat fleas (Ctenocephalides felis)

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The role of reactive oxygen species in the gut immune response of cat fleas (*Ctenocephalides felis*)

An Honors Thesis submitted in partial fulfillment of the requirements for Honors in Biology

By
Clark Hall

Under the mentorship of Dr. Lisa Brown

ABSTRACT

Fleas transmit numerous deadly and debilitating diseases, including the causative agents of murine typhus and plague. Because initial entry of these infectious agents occurs while blood feeding, the immune response in the flea gut is considered to be the first line of defense against invading microbes. However, relatively few studies have identified the flea immune molecules that effectively resist or limit infection in the gut. In other hematophagous insects, an immediate immune response to imbibed pathogens is the generation of reactive oxygen species (ROS). In this study, we utilized cat fleas (*Ctenocephalides felis*) to investigate whether natural infections with bacteria induce ROS synthesis in the flea gut, and whether production of ROS provides a defense mechanism against microbial colonization. Specifically, we assessed the generation of ROS via quantitative peroxide assays from fleas that were given uninfected and bacteria-infected blood meals. Additionally, we treated fleas with an antioxidant before infection with bacteria, and then measured the resultant bacterial load within each flea. Our data shows that ROS levels increase in response to infection in the flea gut, and that this increase helps to strengthen the flea immune response through the microbicidal activity of ROS. Overall, these data yield significant insight into how fleas interact with pathogens in their gut lumen, as well as the challenges faced by pathogens upon entering the flea host.

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1. INTRODUCTION

Fleas are parasitic insects that feed on mammalian or avian blood in order to survive. Among the 2,500 described flea species found throughout the world, the cat flea (*Ctenocephalides felis*) is considered to be the most important flea pest of humans and many domestic animals (Lemaitre and Hoffmann, 2007, Rust, 2017, Zhou et al., 2012). Fleas are responsible for the transmission of numerous pathogens that cause deadly and debilitating human diseases, including cat scratch disease, flea-borne spotted fever, murine typhus, and plague (Azad et al., 1989, Chomel et al., 2006, Eisen et al., 2006, Perry, 2003, Yazid Abdad et al., 2011). This makes their interactions with domesticated pets, and subsequently humans, of particular interest as fleas represent a potential threat to public health. Thus, it is crucial to understand the mechanisms that lead to the transmission of flea-borne pathogens, as well as the flea's ability as a host to combat these infections.

Because fleas are obligatory blood-feeders, they remain in close contact with their vertebrate host and are exposed to their hosts' microorganisms. Fleas acquire an infection while feeding on a diseased host and later transfer the infection to a new host during a subsequent bloodmeal. For example, *Yersinia pestis*, the causative agent of plague, creates a biofilm that blocks the entrance to the flea's digestive tract. This blockage, paired with the rapid consumption of large quantities of blood, results in regurgitation of plague bacilli into the vertebrate host (Beran, 1994, Jarrett et al., 2004). This feeding behavior results in dehydration and starvation, which causes the flea to increase its attempts to blood feed (Jarrett et al., 2004). In contrast, murine typhus (*Rickettsia typhi*) and cat scratch disease (*Bartonella henselae*) are excreted in the flea's feces and gain

access to the vertebrate host when the bite site is scratched (Brown et al. 2019). The natural transmission cycle of flea-borne spotted fever (*Rickettsia felis*) is unknown, but this bacterium eventually migrates to and invades the salivary glands of infected flea species (Legendre and Macaluso, 2017, Macaluso et al., 2008). Thus, although the final mode of transmission may differ, the flea gut is the first organ encountered by flea-borne pathogens.

In general, interactions with disease-causing microorganisms prompt an innate immune response in the host to limit and reduce the harmful effects of pathogens; however, the immune defense mechanisms utilized by fleas against their pathogens are largely unexplored. The generation of reactive oxygen species (ROS) is an immediate immune response that limits bacterial growth in the gut of most animals (Lemaitre and Hoffmann, 2007). Reactive oxygen species are natural by-products of aerobic respiration, but can also be produced by certain enzymes as an immune defense mechanism (Ha et al. 2005). The fruit fly (*Drosophila melanogaster*) has served as a model organism to elucidate the role of ROS against ingested microbes. In this insect, dual oxidase (DUOX), a member of the NADPH oxidase family, synthesizes H_2O_2, which is eventually converted to HOCl—an extremely microbicidal ROS (Fig. 1) (Ha et al., 2005).

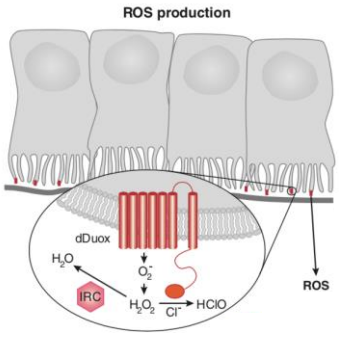

Figure 1. Gut immune response in fruit flies. ROS are produced by the Duox protein in the barrier epithelia of *D. melanogaster*, and the extracellular peroxidase domain of *Drosophila* DUOX can transform H_2O_2 into the microbicidal HOCl. (Lemaitre & Hoffmann 2007).

In this study, we utilized cat fleas to investigate whether natural infections with bacteria induce ROS synthesis in the flea gut, and whether production of ROS provides a defense mechanism against microbial colonization. Specifically, we assessed the generation of ROS via quantitative peroxide assays from fleas that were given uninfected and bacteria-infected blood meals. Additionally, we treated fleas with an antioxidant before infection with bacteria, and then measured the resultant bacterial load within each flea. Our results suggest that ROS levels increase in response to infection in the flea gut, and that this increase helps to strengthen the flea immune response through the microbicidal activity of ROS. Overall, these data yield significant insight into how fleas interact with pathogens in their gut lumen, as well as the challenges faced by pathogens upon entering the flea host.

2. MATERIALS AND METHODS

2.1. Flea maintenance and bacterial infection

Newly emerged, unfed cat fleas were purchased from Elward II (EL) Laboratory (Soquel, CA, USA). Adult fleas were maintained on defibrinated bovine blood (HemoStat Laboratories) within an artificial feeding system (Fig. 2) (Wade & Georgi 1988). This system was kept in a walk-in environmental chamber at 25°C, 55% relative humidity (RH), and a 12:12 h photoperiod. Eggs were collected onto sand in a Petri dish to complete development to adulthood, and hatched larvae were fed the dried feces from blood-feeding adults. Immature stages were reared at 25°C and ≥ 85% RH in an incubator with no light source.

To infect fleas, the Gram-negative bacterium *Serratia marcescens* (Carolina Biological Supply Company) was grown overnight in a shaking incubator at 25°C in

nutrient broth. Infection dose was estimated prior to beginning an experiment by measuring the OD_{600} of the bacterial culture in a BioPhotometer D30 (Eppendorf AG, Hamburg, Germany). Once an optical density of approximately $OD_{600} = 5$ was reached, bacterial cultures were pelleted by centrifugation at 13,000 g for 5 min and resuspended in 600 µL of heat-inactivated (HI) blood. Fleas were allowed to feed on the infected bloodmeal for 24 h. Absolute doses were determined by plating serial dilutions of the treatment culture on nutrient agar, growing them for 48 h at room temperature, and then counting the resultant colony forming units (CFUs).

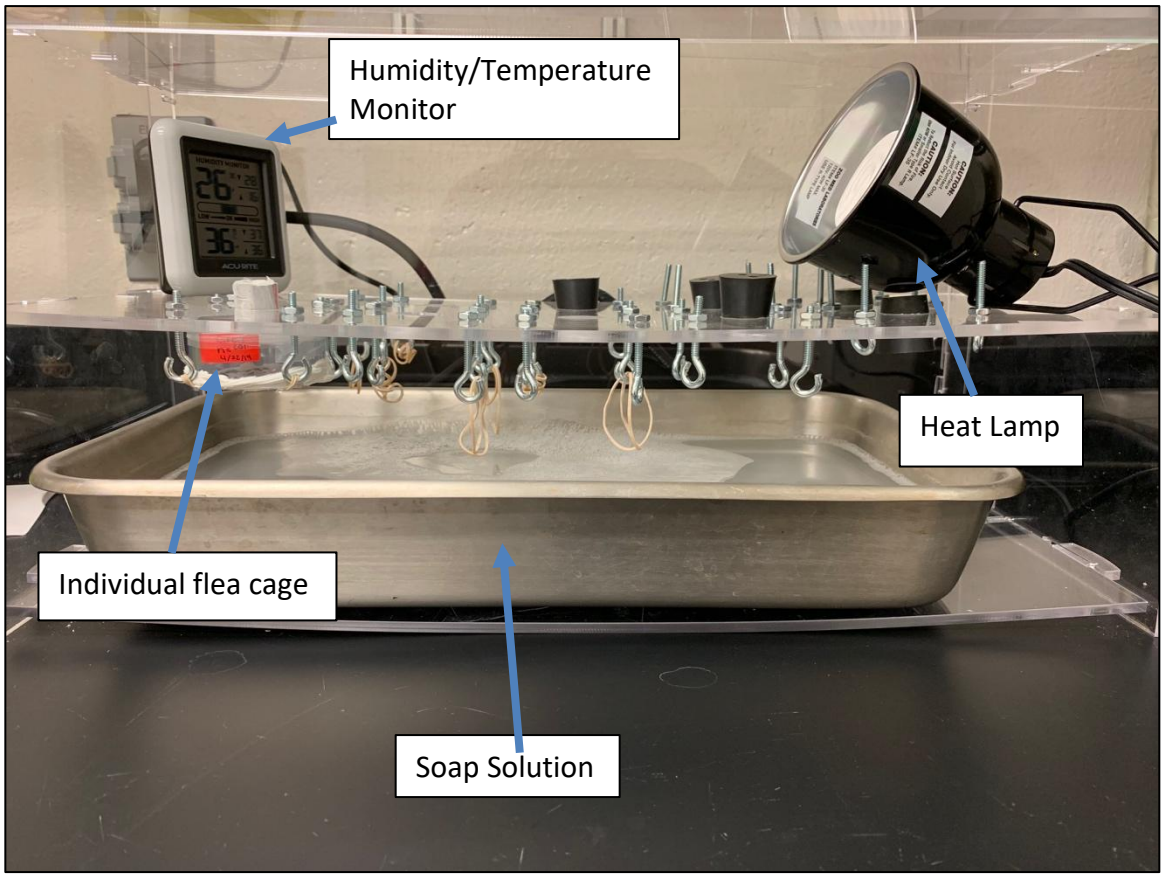

Figure 2. Individual cages within the "artificial dog" unit. The soap solution is a mechanism used to prevent fleas from escaping and infesting the room. The soap breaks the surface tension of the water causing fleas to sink to the bottom. The heat lamp allows for similar temperatures to that of a warm-blooded host. The metal piping maintains the blood's warm temperature while the parafilm provides a thin membrane through which the flea can feed in a controlled manner.

2.2. Hydrogen peroxide assays

To compare the levels of ROS synthesis, fleas were placed into four groups and exposed to one of the following treatments: (1) untreated blood; (2) blood infected with bacteria; (3) blood mixed with an antioxidant (see below); or (4) blood mixed with an antioxidant, and then blood infected with bacteria (Table 1; Fig. 3). After each treatment, the concentration of hydrogen peroxide (ROS of interest) was measured for each group using the Pierce™ Quantitative Peroxide Assay Kit according to the manufacturer's instructions for the lipid-compatible procedure. Briefly, whole guts from 20 female fleas were hand-dissected, pooled, and homogenized in 200 μL of PBS containing 2 mg/mL 3-amino triazole (catalase inhibitor). Because fleas had blood in their gut, both "test" (methanol-only) replicates and "TCEP Reference" replicates (Bond-Breaker™ TCEP Solution, Thermo Scientific) were created as suggested for metal-containing samples. Additionally, a blank was generated that omitted Reagent A. Each sample was transferred in duplicate to a microwell plate, and absorbance was measured at 595 nm using a plate reader (Synergy H1 plate reader, BioTek). Following subtraction of absorbance for the blank, the "TCEP Reference" absorbance was then subtracted from the "test" absorbance. Finally, a hydrogen peroxide standard curve was generated to calculate the concentration of the samples for each group. Three independent trials were conducted per treatment group, and the data were combined and analyzed by ANOVA, followed by Tukey's Multiple Comparison Test using GraphPad Prism.

Table 1. Bloodmeal treatment schedule. Fleas were placed into four groups and exposed to one of the following treatments: (1) untreated blood (control); (2) blood infected with bacteria; (3) blood mixed with an antioxidant; or (4) blood mixed with an antioxidant, and then blood infected with bacteria.

Group	24 h	72 h	120 h	144 h
1	Untreated	Untreated	Untreated	End treatment
2	Untreated	Untreated	Infected	
3	Antioxidant	Antioxidant	Untreated	
4	Antioxidant	Antioxidant	Infected	

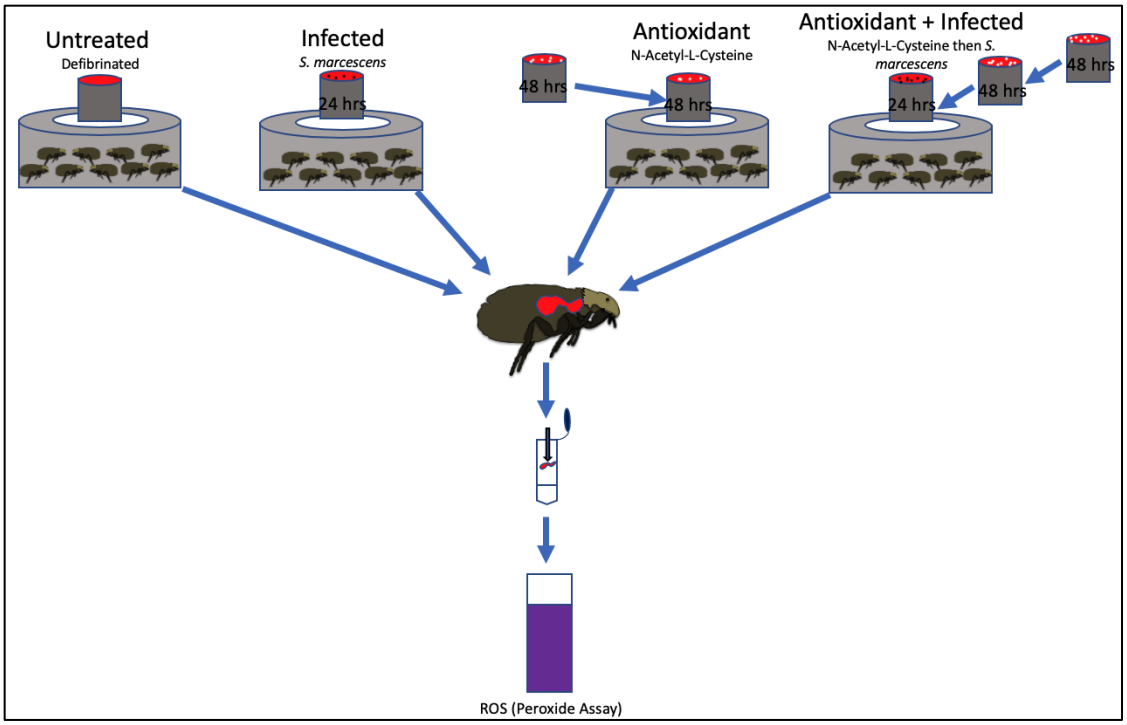

Figure 3. Diagram of hydrogen peroxide assays. Guts were dissected from four groups of 20 female fleas that were untreated, fed an infected bloodmeal, fed an antioxidant, or were fed an antioxidant before an infected bloodmeal. Peroxide levels in the pooled guts were determined by Pierce™ Quantitative Peroxide Assay Kit according to the lipid compatible procedure.

2.3. Antioxidant treatment

To assess the relative strength of ROS production as a flea defense mechanism, a group of fleas was fed on two consecutive occasions, 48 h apart, on blood containing 20 mM *N*-acetyl-L-cysteine (antioxidant supplement) (Fig. 4). An additional group was fed an untreated bloodmeal. Two days after the second treatment, both groups were given an infected bloodmeal as described above. After feeding on infected blood for 24 h, fleas were collected for enumeration of *S. marcescens* (see below). The same protocol for antioxidant treatment was followed for the hydrogen peroxide assays (Table 1, groups 3 & 4).

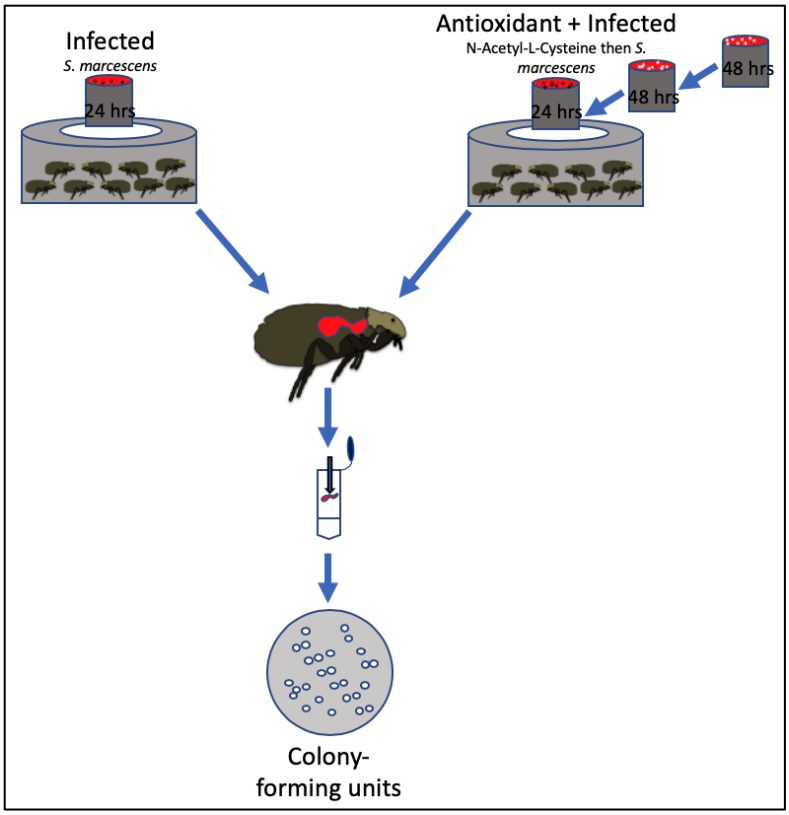

Figure 4. Diagram of infection intensity assays. Guts were dissected from two groups of 20 female fleas there were either treated with an antioxidant before an infected bloodmeal or received an infected bloodmeal only (no antioxidant treatment). Infection intensity per individual flea was determined by plating each homogenized gut on nutrient agar and counting the resultant CFU.

2.4. Measurement of bacterial infection in fleas

Twenty-four hours post infection, whole guts from fleas were hand-dissected, homogenized in PBS, and a diluted sample for each individual was spread on nutrient agar plates. Plates were incubated at room temperature for 48 h, the number of CFUs was recorded, and the CFU number was then used to calculate *S. marcescens* infection intensity in each flea. In order to confirm that all colonies originated from the original inoculum, a group of untreated fleas was also examined for the presence of gut microbes that may form red colonies similar to *S. marcescens*. Five independent trials were conducted, and each trial consisted of approximately 20 fleas per treatment group. Data were combined and analyzed using an unpaired t-test in GraphPad Prism.

3. RESULTS

3.1. Peroxide levels increase after bacterial infection in fleas

To determine whether a bacterial infection alters ROS synthesis, fleas were fed one of four different bloodmeal treatments, and the concentration of hydrogen peroxide (ROS of interest) was measured from pooled gut samples. The level of hydrogen peroxide was altered by bloodmeal treatment (Fig. 5; ANOVA: $p < 0.0001$). Specifically, peroxide levels were almost twice as high in fleas that were fed bacteria than those that were fed an untreated bloodmeal (Fig. 5; Tukey's: $p < 0.005$). Additionally, incorporation of an antioxidant into a bloodmeal reduced the amount of ROS produced by roughly 75% and 85% when compared to untreated (Tukey's: $p = 0.0001$) and infected (Tukey's: $p < 0.0001$) bloodmeals, respectively. Moreover, fleas fed an antioxidant before an infected bloodmeal increased ROS synthesis by 50% when compared to the antioxidant treatment alone (Tukey's: $p = 0.1451$); however, peroxide levels were 50% and 70% lower than

that of untreated (Tukey's: p = 0.0030) and infected groups (Tukey's: $p < 0.0001$), respectively. Thus, these data show that the presence of bacteria increases the generation of ROS in the gut of fleas more than blood feeding alone.

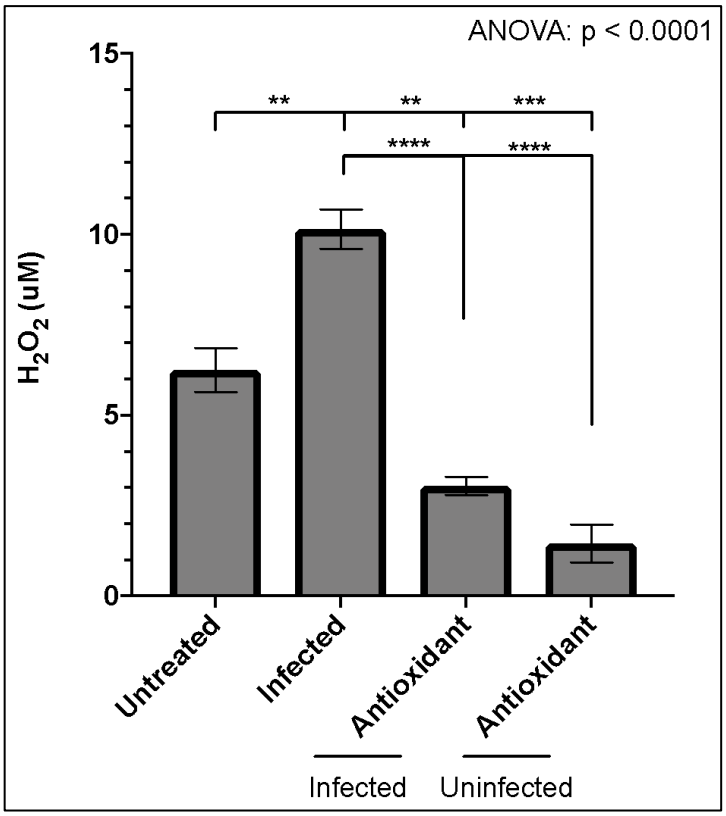

Figure 5. Data are shown as the mean (± S.E.M.) concentration of 3 independent trials combined. The data were analyzed by ANOVA followed by Tukey's Multiple Comparison Test. ** p < 0.005; *** p < 0.001; **** p < 0.0001

3.2. ROS synthesis limits survival of *S. marcescens* in fleas

To determine the effect of ROS generation on bacterial growth in the gut of fleas, two groups were fed either untreated or antioxidant-treated blood prior to an infected bloodmeal. The bacterial load was measured 24 h later by plating each individual gut on nutrient agar and counting the resultant CFUs. Across all experimental trials, the absolute dose of *S. marcescens* per infected bloodmeal was approximately 4.56×10^8 ($\pm 7.3 \times 10^7$ S.E.M.), while the average amount recovered from untreated flea guts was 4,583 (± 721

S.E.M.). Although fleas process their blood meals rapidly and digest little before defecation, infection prevalence (percentage of individuals infected) after 24 h was greater than 80% for the untreated groups. Among fleas that remained infected with *S. marcescens* (excluding individuals that cleared the infection), infection intensity increased by 70% when peroxide levels in the gut were lowered with the antioxidant treatment (Fig. 6; unpaired t test: $p = 0.0016$). Overall, these data show that ROS is an immune response that limits pathogen survival in the gut of fleas.

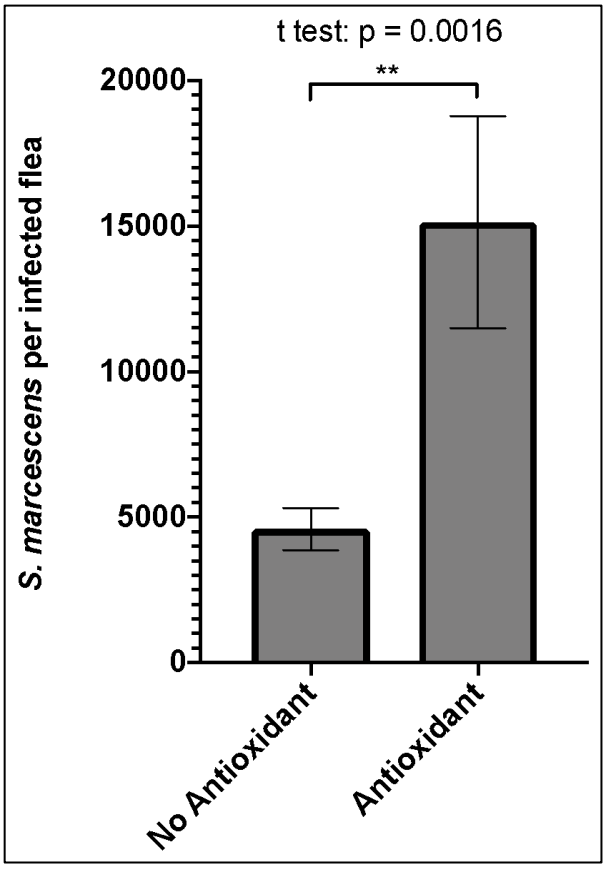

Figure 6. Data are shown as the mean (± S.E.M.) infection intensity of 5 independent trials combined. The data were analyzed using an unpaired t test.

4. DISCUSSION

Although fleas are efficient vectors of disease-causing pathogens, they are not willing hosts. Fleas, like all insects, possess an innate immune system that provides defense against invading microbes. However, the immune mechanisms that determine whether an infected flea eliminates or succumbs to a given pathogen are largely unexplored. Here, we present direct evidence that local production of microbicidal ROS provides defense against pathogens in the gut of cat fleas.

Our first experiments showed that, in the presence of bacteria, peroxide levels were significantly higher in the guts from infected fleas compared to those from untreated individuals. These data support our initial hypothesis that an immediate response to infection in the flea gut epithelia is the generation of ROS. This prompted further investigation to determine the impact of ROS synthesis on the subsequent survival of ingested pathogens. First, we demonstrated that incorporation of an antioxidant into the bloodmeal limits ROS production in the flea gut, and that ROS levels remained low when fleas were treated with the antioxidant before oral infection with *S. marcescens*. Next, we showed that pathogen survival was significantly higher in the guts from antioxidant treated fleas compared to untreated groups. Thus, these data further support our hypothesis that ROS production is an inducible immune defense mechanism in the gut of cat fleas. These findings are consistent with previous studies that focused on the immune strategies utilized by the gut epithelial cells of other insects, such as fruit flies, mosquitoes, and rat fleas (*Xenopsylla cheopis*) (Ha et al. 2005, Ha et al. 2009, Kumar et al. 2010, Zhou et al. 2012). Taken together, these results suggest that ROS production is a significant component of early flea immune response to bacterial infection.

Although we may not be the cat flea's primary host, they often find residence and nutritious blood meals on some of our beloved domesticated pets. Fleas are consistent parasites of canine and feline hosts. Our mutualistic relationship with domesticated dogs and cats leads cat fleas to interact with humans regularly. Increased risk of parasitic interactions with cat fleas leave humans susceptible to increased levels of exposure to deadly pathogens. The project yielded significant insight into how fleas interact with pathogens in their gut lumen and exposed some of the challenges faced by pathogens upon entering the flea host. Further exploration is needed to fully understand the entire repertoire of innate defense mechanisms available to fight off pathogens in these insect vectors.

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