4-29-2024

Antimicrobial activity of cat flea (Ctenocephalides felis) gut proteins on different days after blood feeding

Dhruva K. Karnik

Antimicrobial activity of cat flea (*Ctenocephalides felis*) gut proteins on different days after blood feeding

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

By
Dhruva Karnik

Under the mentorship of Dr. Lisa D. Brown

Abstract

Cat fleas (*Ctenocephalides felis*) are a blood-feeding ectoparasitic insect and a common domestic pest found throughout the world. Because of their reliance on host blood, fleas are exposed to blood-borne pathogens; however, the flea gut lumen is a hostile environment for microbial colonization. For example, the gut epithelia differentially express immune genes in response to feeding. In the present study, we measured the antimicrobial activity of gut proteins from cat fleas at different days after feeding (2, 5, 7, and 14). Dissected flea guts were homogenized, passed through a syringe filter, and measured in a protein assay kit. Antimicrobial activity was assessed by incubating flea gut samples with the bacterial species *Micrococcus luteus* overnight in a microtiter plate, and then plating the samples on nutrient agar to compare bacterial growth. Our results shows that protein concentrations increase with feeding days, and this increase generally corresponds to a higher antimicrobial activity.

Thesis Mentor: Dr. Lisa D. Brown

Honors Dean: Dr. Steven Engel

April 2024
Biology
Honors College
Georgia Southern University

Acknowledgements

Firstly, I would like to sincerely thank Dr. Brown for reaching out to me and offering the opportunity to work with her. In a time where I was feeling burnt out and down from the repeated assignments, working in a lab allowed me to understand the practical application of what I was learning, and more importantly, how to apply it. Her patience, ability to accommodate, help design a research project and troubleshoot the various issues which pop up every step of the way have allowed me to learn countless lessons which I otherwise would never have been able to learn.

I would like to thank my parents for the sacrifices they have made and continue to make for me and my sister everyday. Despite the chaos that is acquiring American Citizenship, their tireless efforts have made each endeavor fall in place perfectly, shielding me from the harshness of a lot of things.

I would also like to thank my friends Adam, Sean, and Garett for being synonymous to brothers for me throughout my time at the University. Without their friendship, jokes and support I would never have been able to enjoy my days at Georgia Southern.

Introduction

Cat fleas *(Ctenocephalides felis)* are the most common domestic pest found in North America (CDC 2020). Unlike other ectoparasites *(e.g.*, mosquitoes, sandflies, triatomine bugs), adult cat fleas remain in constant contact with their host and feed on blood almost continuously. Cat fleas are carriers of various ailments, such as the human bacterial diseases murine typhus, cat scratch disease, flea-borne spotted fever, and plague (Yu Zhang et al. 2021). Strategies to control flea infestations have made strides in recent decades, with the overwhelming majority of pet owners opting to use products such as fluralaner, afoxolaner or spinosad (Rust MK. 2020). The flea gut lumen is a hostile environment for bacterial colonization, as blood feeding induces the expression of immune genes (Brown LD. 2019). However, bacterial pathogens continue to propagate through the flea vector, serving as excellent carriers due to their ectoparasitic nature.

Mechanisms used by bacteria to overcome immune defense mechanisms and establish replicative reservoirs within fleas are relatively unknown. In arthropods, two pathways govern the system of innate immune defense: the Immune Deficiency Pathway (IMD) and the Toll pathway. Typically, the IMD pathway responds to Gram-negative bacteria, while the Toll pathway acts on Gram-positive bacteria (Bland et al. 2020). In insects, these two pathways produce antimicrobial peptides and serve as a first line of defense against potential invaders. Fleas are known to have microbicidal proteins in the form of defensins, lysozymes and serpins, which differ in their effectiveness against Gram-positive and Gram-negative bacteria (Dreher-Lesnick et al. 2010). These antimicrobial proteins are effective against a wide range of microorganisms due to their

broad range of immunological activities. Studies with the fruit fly (*Drosophila melanogaster*) have indicated that the cat flea shares a level of the immune-system architecture in the form of the IMD and Relish pathways (Renoll et al. 2018), in conjunction with the Dual Oxidase (DUOX) pathway that produces hypochlorous acid (Brown et al. 2021).

Additionally, although the lifespan of adult fleas is relatively short (about one month), age-dependent variations in the physiology of fleas have not been examined. In the present study, we aimed to determine whether the antimicrobial activity of flea gut contents decreases with age. Specifically, we determined the protein concentration from cat fleas at different days post blood feeding (2, 5, 7 and 14) using a protein assay kit and homogenized flea gut samples. Next, flea gut samples were incubated with the Gram-positive bacterium *Micrococcus luteus* overnight, after which bacterial growth was measured on nutrient agar plates. We found that protein concentrations in the flea gut increase with feeding days, but the strength of antimicrobial activity is dependent on flea age.

Methods

Flea maintenance:

Newly emerged, unfed, adult cat fleas (*C. felis* Bouché) were purchased from EctoServices, Inc. (Henderson, NC, USA). Adult fleas were fed defibrinated bovine blood (Hemostat Laboratories, Dixon, CA, USA) using an artificial feeding system as previously described (Wade and Georgi, 1988; Brown et al. 2021). Fleas were collected from cages at 2-, 5-, 7- and 14-days post-blood feeding. Three independent trials were conducted, and each trial consisted of 40 female fleas per time point.

Bacterial culture:

Micrococcus luteus (Carolina Biological Supply Company) was grown overnight in a shaking incubator at 25°C in nutrient broth. After 24 hours, the optical density of the bacterial culture was measured in a BioPhotometer D30 (Eppendorf AG, Hamburg, Germany). Once an optical density of approximately $OD_{600} = 5$ was reached, *M. luteus* was diluted 1:100 in phosphate buffered saline (PBS) for use in antimicrobial activity assays.

Preparation of flea gut samples:

Whole guts from individual fleas were collected as previously described (Brown et al. 2021). Briefly, fleas were cold anesthetized on ice, and guts were removed using two 27½ G needles to open the flea body cavity. Flea dissections were conducted under a dissecting microscope to ensure no tearing of the gut tissue. The collected guts from 40 female fleas were pooled into a single 1.5 mL microcentrifuge tube filled with 200 µL of PBS and 20 µL of a protease inhibitor (Sigma-Aldrich, Catalog No. P2714-1BTL). Samples were maintained on ice throughout the dissections. The contents of each tube were homogenized using a sterile pestle, and then centrifuged at 14,000 g at 4°C for 5 minutes. The supernatant was collected and passed through two syringe filters: a 0.45 µM PVDF membrane filter followed by a 0.22 µM PVDF membrane filter. Filtered supernatant was collected into a new microcentrifuge tube, and samples were stored at -20 °C until use.

Antimicrobial activity assays

Antimicrobial activity of flea gut contents was assessed *in vitro* using an assay adapted from Viera et al. (2014). First, protein concentrations were measured using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific) with bovine serum albumin standards. Next, 10 µL of flea gut sample were dispensed in triplicate into the wells of a 96-well half-area microtiter plate with 5 µL of the diluted *M. luteus*, 2.5 µL of nutrient broth, and 22 µL of PBS. Control wells without gut samples contained 5 µL of the diluted *M. luteus*, 2.5 µL of nutrient broth, and 32 µL of PBS. The microtiter plate was covered and incubated for 24 hours in a shaking incubator at 25°C. Following the 24-hour incubation period, the remaining volume of each well was plated on nutrient agar. Plates were incubated for 6 days at room temperature, or until observable bacterial growth had occurred on each plate. Individual *M. luteus* colonies on each plate were counted, and the average number of colony forming units (CFUs) between each group was calculated.

Statistical Analysis

The data were analyzed by ANOVA followed by Tukey's Multiple Comparison Test in GraphPad Prism V8. Data are reported as the mean ± standard error of the mean (SEM). Differences were considered significant at $p \leq 0.05$.

Results

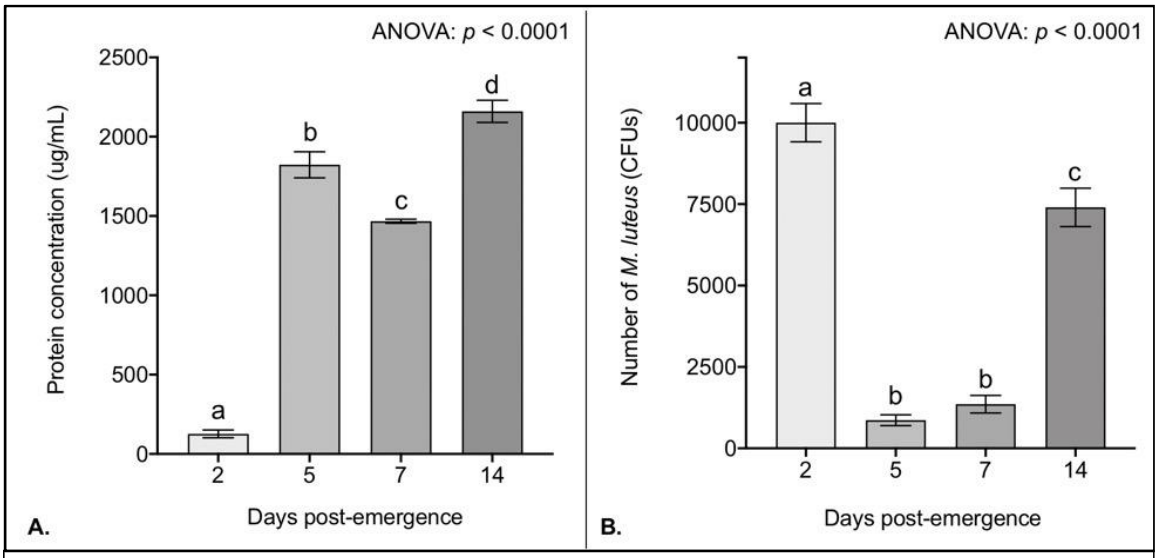

Figure 1. Antibacterial assay of flea gut contents on different days post-emergence. Data are shown as the mean (± S.E.M.) protein concentration (**A**) or number of *M. luteus* (**B**) from 3 independent trials combined. The data were analyzed by ANOVA followed by Tukey's Multiple Comparison Test in GraphPad Prism V8. Different lowercase letters denote significant differences ($p < 0.05$) between days.

To determine whether the antimicrobial activity of flea gut contents decreases with age, we conducted an antibacterial assay with uninfected, blood-fed, female fleas at 2-, 5-, 7-, and 14-days post-emergence. We found that protein concentration in the flea digestive tract differs significantly with age (Fig. 1A, ANOVA: $p < 0.0001$). Protein levels increased by 1823% between 2- and 5-days post-emergence (Fig. 1A, Tukey's: $p < 0.0001$), decreased by 19% between 5- and 7-days post-emergence (Fig. 1A, Tukey's: $p = 0.0085$), and increased by 47% between 7- and 14-days post-emergence (Fig. 1A, Tukey's: $p = 0.0001$).

Similarly, the antibacterial activity of flea gut contents also differed significantly with age (Fig. 1B, ANOVA: $p < 0.0001$). Specifically, the antibacterial activity of gut contents was lowest in 2-day-old fleas (*i.e.*, highest number of *M. luteus* recovered from the assay) and increased by 91% between 2- and 5-day-old fleas (Fig. 1B, Tukey's: $p <$

0.0001). The antibacterial activity was similar between 5- and 7-day old fleas (Fig. 1B, Tukey's: $p = 0.8252$), and then decreased by 446% between 7- and 14-day-old fleas (Fig. 1B, Tukey's: $p < 0.0001$). Together, these data suggest that increased protein concentration in the flea digestive tract correlates with increased antibacterial activity, but the strength of antibacterial activity declines with age.

Discussion

In the current study, we observed increasing protein concentration within the flea gut with age. Additionally, the antimicrobial assay demonstrated that ingesting a blood meal induces expression of immunological responses within the flea gut that limit growth of *M. luteus*. Finally, our results indicate that, although protein concentrations increased with age, the strength of antibacterial activity ultimately declines. This pattern parallels the performance of the immune systems in most biological organisms, *i.e.* immune functions decline with age. For example, Viera *et al.* (2014) shows similar results with increasing concentrations of antimicrobial peptides in *Rhodnius prolixus*, another hematophagous species, following consumption of blood meals over a period of time. Specifically, blood meals induced the expression of the antimicrobial peptides within *R. prolixus* that were free from bacterial infection, demonstrating that the proteins were produced as a consequence of feeding. Taken together, blood-feeding in *C. felis* induces the expression of proteins within the gut that combat microbial infection.

The Toll and IMD pathways play a vital role in the innate immune responses of various organisms (De Gregorio E et al. 2002). The Toll pathway is notably activated in response to Gram-positive bacteria, while IMD activates in response to Gram-negative bacteria. These pathways generate antimicrobial peptides and are especially important for

immune defense in insects. Renoll *et al.* (2018) demonstrated similarities between the *Drosophila* and *C. felis* genome, indicating a level of conservation between the IMD and Toll pathways between the two organisms. Downregulating IMD transcription in *C. felis* with the use of siRNA resulted in an increased *Rickettsia typhi* infection within the flea midgut. Similarly, Brown *et al.* (2021) highlighted the role of reactive oxygen species (ROS) in *C. felis*, which also serves as a primary defense mechanism for the *Drosophila* in the event of an oral infection. Production of H_2O_2 was shown to increase in *C. felis* infected with Gram-negative *Serratia marcescens*, and when the fleas were fed blood meals combined with an antioxidant to reduce the presence of ROS, bacterial infection significantly increased. Overall, *Drosophila* is a valuable system to validate *C. felis* immunological studies due to the high degree of similarities.

Conclusion

Ctenocephalides felis has demonstrated the ability to produce antimicrobial proteins in their gut as a result of blood feeding, regardless of the presence of a pathogen. Protein expression increased over time alongside the feeding period, which initially inhibited bacterial growth, and waned off with flea age. Similarities between *Drosophila* and *C. felis* could provide insight into the immune mechanisms of the flea, which serve as a reservoir for dangerous pathogens.

Citations

Centers for Disease Control and Prevention. (2020a, January 23). *For Veterinarians*. Centers for Disease Control and Prevention. https://www.cdc.gov/bartonella/veterinarians/index.html

Centers for Disease Control and Prevention. (2020b, August 13). *Common fleas of the United States*. Centers for Disease Control and Prevention. https://www.cdc.gov/fleas/fleas_of_the_us.html

Brown LD, Immunity of fleas (Order Siphonaptera), Developmental & Comparative Immunology, Volume 98, 2019, Pages 76-79, ISSN 0145-305X, https://doi.org/10.1016/j.dci.2019.03.019.

Brown LD, Maness Ryne, Hall Clark, Gibson JD, Reactive oxygen species-mediated immunity against bacterial infection in the gut of cat fleas (*Ctenocephalides felis*), Insect Biochemistry and Molecular Biology, Volume 136, 2021, 103620, ISSN 0965-1748,https://doi.org/10.1016/j.ibmb.2021.103620.

Bland DM, Martens CA, Virtaneva K, Kanakabandi K, Long D, Rosenke R, et al. (2020) Transcriptomic profiling of the digestive tract of the rat flea, *Xenopsylla cheopis*, following blood feeding and infection with Yersinia pestis. PLoSNegl Trop Dis 14(9): e0008688

De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B. The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. EMBO J. 2002 Jun 3;21(11):2568-79. doi: 10.1093/emboj/21.11.2568. PMID: 12032070; PMCID: PMC126042.

Dillon RJ, Dillon VM. The gut bacteria of insects: nonpathogenic interactions. Annu Rev Entomol. 2004;49:71-92. doi: 10.1146/annurev.ento.49.061802.123416. PMID: 14651457.

Dreher-Lesnick, S. M. Ceraul, S. C. Lesnick, J. J. Gillespie, J. M. Anderson, R. C. Jochim, J. G. Valenzuela, A. F. Azad. Analysis of Rickettsia typhi-infected and uninfected cat flea (*Ctenocephalides felis*) midgut cDNA libraries: deciphering molecular pathways involved in host response to R. typhi infection. Insect Molecular Biology (2010) 19(2), 229–241.

Rennoll SA, Rennoll-Bankert KE, Guillotte ML, Lehman SS, Driscoll TP, Beier- Sexton M, Rahman MS, Gillespie JJ, Azad AF. 2018. The cat flea (*Ctenocephalides felis*) immune deficiency signaling pathway regulates Rickettsia typhi infection. Infect Immun 86:e00562-17. https://doi.org/10.1128/ IAI.00562-17.

Rust MK. Recent Advancements in the Control of Cat Fleas. Insects. 2020 Sep 29;11(10):668. doi: 10.3390/insects11100668. PMID: 33003488; PMCID: PMC7600267

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal Biochem. 1985 Oct;150(1):76-85. doi: 10.1016/0003-2697(85)90442-7. Erratum in: Anal Biochem 1987 May 15;163(1):279. PMID: 3843705.

Vieira, C.S., Waniek, P.J., Mattos, D.P. *et al.* Humoral responses in *Rhodnius prolixus*: bacterial feeding induces differential patterns of antibacterial activity and enhances mRNA levels of antimicrobial peptides in the midgut. *Parasites Vectors* **7**, 232 (2014). https://doi.org/10.1186/1756-3305-7-232

Yu Zhang, Yu Nie, Yuan-Ping Deng, Guo-Hua Liu, Yi-Tian Fu. The complete mitochondrial genome sequences of the cat flea *Ctenocephalides felis* (Siphonaptera: Pulicidae) support the hypothesis that *C. felis* isolates from China and USA were the same *C. f. felis* subspecies, Acta Tropica, Volume 217, 2021, 105880, ISSN 0001-706X, https://doi.org/10.1016/j.actatropica.2021.105880.