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TRANSCRIPTIONAL INDUCTION OF IMMUNE PATHWAY GENES AND SUBSEQUENT ANTIBACTERIAL ACTIVITY IN THE DIGESTIVE TRACT OF THE CAT FLEA (*CTENOCEPHALIDES FELIS*).

by

KATIE WEBER

(Under the direction of Lisa D. Brown)

ABSTRACT

Fleas (Order Siphonaptera) are ectoparasites that serve as vectors of several human diseases (cat scratch disease, flea-borne spotted fever, murine typhus, and plague). Typically, these pathogens are acquired through the ingestion of a host-derived bloodmeal; thus, the lumen of the gut is the first barrier encountered by imbibed pathogens. During the early stages of infection, microbes come into contact with surrounding midgut epithelial cells which trigger a series of host responses to combat local infection. Specifically, pathogen recognition leads to intracellular signaling and an increase in the production of antimicrobial peptides (AMPs) via the IMD and Toll immune pathways. While the immune responses of other disease vectors have been examined, relatively little is known about how fleas respond immunologically to the pathogens they vector. To investigate the role of these immune signaling pathways in the defense against bacterial pathogens, we measured the relative mRNA levels of genes comprising the IMD and Toll pathways, as well as the two AMP genes *Attacin* and *Defensin*, in digestive tract of cat fleas (*Ctenocephalides felis*). At 4 and 24 hours post-exposure to an infected bloodmeal (*Bartonella henselae, Serratia marcescens*, or *Micrococcus luteus*), relative mRNA levels of select immune genes were measured using quantitative PCR (qPCR). Additionally, we measured the antimicrobial activity of proteins isolated from the digestive tracts of naïve and infected fleas.

Overall, our data suggest that in response to the two model bacterial species (Gram-negative *S. marcescens* and Gram-positive *M. luteus*) the local immune response in the cat flea is exclusively under the control of the IMD pathway, but that the flea-borne pathogen, Gram-negative *B. henselae* does not influence transcriptional induction of these genes. However, exposure to all bacterial species increased the antimicrobial activity of cat flea gut proteins at 24 hours. Ultimately, our findings provide critical insights as to how cat fleas modulate local immune responses against bacterial species in their gut.

INDEX WORDS: Siphonaptera, Pulicidae, Insect immunity, Epithelial immunity, Antimicrobial peptides, Immune signaling pathways

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B.S., Florida State University, 2021

A Thesis Submitted to the Graduate Faculty of Georgia Southern University in Partial Fulfillment of the Requirements for the Degree

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COLLEGE OF SCIENCE AND MATHEMATICS

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DEDICATION

For TD.

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INTRODUCTION

1.1 Transmission biology of flea-borne pathogens

Fleas (Order Siphonaptera) are hematophagous insects that serve as vectors for several disease-causing bacteria, including *Yersina pestis* (bubonic plague), *Rickettsia typhi* (murine typus), *Rickettsia felis* (flea-borne spotted fever), and *Bartonella henselae* (cat scratch disease). Fleas are holometabolous insects with a larval, pupal, and adult stage. Larvae feed on blood proteins found in adult flea excrement, while adults feed exclusively on vertebrate blood (mainly mammals and birds). Pathogen transmission to adults occurs horizontally when fleas ingest bacteremic vertebrate blood (Eisen et al., 2006; Chomel and Kasten, 2010; Reif et al., 2011). Transmission of *Rickettsia* species has also been demonstrated to occur between infected and naive fleas while cofeeding on a healthy host (Brown et al., 2015). During the larval stage, some pathogens can be transmitted vertically through a transovarial route (adult female to offspring) or a non-transovarial route (larval consumption of contaminated blood proteins found in adult excrement) (Farhang-Azad et al., 1985; Azad et al., 1992; Morick et al., 2013). Once inside the flea, flea-borne pathogens (mainly Gram-negative bacteria) colonize and replicate in the gut. Some flea-borne pathogens such as *Y. pestis* form protective biofilms in the foregut of *Xenopsylla cheopis* (rat flea) to aid in persistence and increase the likelihood of vertebrate transmission. As biofilm mass increases to encompass the proventriculus, transmission via regurgitation during blood feeding can occur (Jarrett et al., 2004; Bland et al., 2018). Other intracellular flea-borne pathogens, such as *Rickettsia* species colonize the midgut epithelium of *Xenopsylla cheopis* (rat flea) and *Ctenocephalides felis* (cat flea), while *B. henselae* colonizes the midgut lumen of the cat flea (Adams et al., 1990; Higgens et al., 1996; Foil et al., 1998).

Transmission of *B. henselae* to humans is known to occur via the introduction of bacteremic flea feces into breaks in the skin that are either self-inflicted following a flea bite or mediated through cat scratch (Mazurek et al., 2018). On the other hand, transmission of *Rickettsia felis* is hypothesized to occur from flea to human via flea bite (Macaluso et al., 2008; Thepparit et al., 2013). Though much is known about the transmission of these flea-borne pathogens, much remains unknown about how fleas respond to these infections.

1.2 Insect innate immune defense mechanisms

Unlike vertebrate animals, insects lack an adaptive immune system and rely exclusively on the innate immune system to defend against pathogens. Insect immune responses are categorized into two branches, either cellular – which is modulated by phagocytic immune cells called hemocytes – or humoral – which includes defense mechanisms such as prophenoloxidase induced melanization, or lysis/ disruption of cellular processes via the production of antimicrobial peptides (AMPs) and reactive oxygen species (ROS). Upon pathogen entry into the gut, a local humoral immune response is activated through the recognition of pathogen associated molecular patterns (PAMPs) that bind to pattern recognition receptors (PRR) on epithelial cells. Once a foreign molecule has been recognized, intracellular signaling and effector pathways increase production of antimicrobial peptides (AMPs) via the immune deficiency (IMD) and Toll pathways (Hillyer, 2016). Much of what is known about these signaling pathways was originally discovered in the Dipteran model species *Drosophila melanogaster* (fruit fly). Traditionally, the IMD pathway is activated in response to an infection with Gram-negative bacteria, and the Toll pathway to Gram-positive bacteria (De Gregorio et al., 2002).

1.3 Characterization of arthropod IMD and Toll pathways

Activation of the IMD signaling cascade occurs when the transmembrane receptor protein PGRP-LC recognizes DAP-type peptidoglycan released by replicating Gram-negative bacteria (Kaneko T. et al., 2004) (Fig. 1). The downstream activation of the adaptor protein IMD, as well as kinases TAB2 and TAK1 leads to activation of the IKK complex allowing for the phosphorylation of the transcription factor Relish (Rutschmann et al., 2000). The association of IMD with Fadd and the caspase Dredd then leads to the cleavage of the phosphorylated Relish into Rel-68 and Rel-49 (Georgel et al., 2001; Naitza et al., 2002; Stoven et al., 2003; Wiklund et al., 2009). Upon translocation of Rel-68 into the nucleus, the expression of AMP genes and the subsequent production of these effector molecules occurs (Stöven et al., 2000; Stöven et al., 2003; Hanson et al., 2019). The Toll pathway is activated indirectly by the secreted, extracellular peptidoglycan receptor protein PGRP-SA, upon recognition of Lys-type peptidoglycan found on the surface of Gram-positive bacteria (Bischoff et al., 2004; Yu et al., 2022) (Fig. 2). Subsequently, binding initiates a serine protease cascade that leads to the cleavage of the ligand Spätzle. Binding of Spätzle to the transmembrane receptor Toll initiates an intracellular signaling cascade involving the adaptor proteins Myd88 and tub, and the protein kinase pll. When the signaling complex is turned on, cactus, an inhibitor bound to the transcription factor dif/dorsal, is degraded allowing for the translocation of dif/dorsal into the nucleus and subsequent transcription of AMPs (Ip et al., 1993; Weber et al., 2003; Hanson et al., 2019; Yu et al., 2022) (Fig. 2). Activation of the Toll signaling pathway has been demonstrated to occur within the fat body and hemocoel of *D. melanogaster* (Schmid MR et al., 2014); however, there has been no indication that Toll signaling is involved in epithelial immunity. In contrast, IMD signaling has

been demonstrated to occur within the gut, fat body, and hemocytes (immune cells) of *D. melanogaster* (Liehl et al., 2006; Nehme et al., 2007; Yu et al., 2022).

While gene composition of the IMD pathway tends to be highly conserved between *D. melanogaster* and holometabolous blood-feeding insects such as mosquito spp. (*Anopheles gambiae, Aedes aegypti*), an incomplete or nonfunctional IMD pathway has been described for several hemimetabolous insects (kissing bugs, bed bugs) as well as some arachnids (ticks and mites). For example, the IMD pathway of *Rhodnius prolixus* (kissing bug), originally thought to be missing several key components, was found to be modified, containing orthologs for most genes, apart from the *IMD* gene, which appears to be missing (Salcedo-Porras et al., 2019). Genomic studies have revealed the absence of the *IMD* gene in several other hemimetabolous blood-feeding insects including *Cimex lectularius* (bed bug) as well as multiple triatomine species (*T. pallidopennis, T. dimidiata* and *T. infestans*) (Benoit et al. 2016; Zumaya-Estrada et al., 2018). The IMD pathway in *Pediculus humanus humanus* and *Pediculus humanus capitis* (body and head lice, respectively) was found to be nonfunctional, missing essential upstream components including PGRPs, IMD, and Fadd (Kim et al., 2011). Similarly, the arachnid *Ixodes scapularis* (deer tick) also lacks PGRPs, IMD, Dredd, and Fadd (Severo et al, 2013; Palmer et al., 2015; Gulia-Nuss et al., 2016) that have been classically defined in *D. melanogaster*; however, the IMD pathway remains functional (Shaw et al., 2017), suggesting a noncanonical composition or alternative mode of activation. Though the Toll pathway is highly conserved in many hematophagous arthropods, a modified or incomplete Toll pathway has been described in others. For example, in mosquito spp. *A. aegypti* and *An. gambiae*, the transcription factor Dif appears to be missing. Rather, the Dorsal orthologue REL1 is present to functionally compensate

for the discrepancy (Barillas-Mury et al., 1996; Shin et al., 2005; Nene et al., 2007). Similarly, in *I. scapularis*, presence of the *Dif* gene has not yet been reported (Fogaça et al., 2021).

In *D. melanogaster*, the IMD and Toll pathways are generally considered to function independently, however, cross regulation has been noted. Cross-activation is hypothesized to occur through either independent recognition events in which binding sites exist for both transcription factors (Dif/Dorsal, Relish) at the target promoter region or through the formation and subsequent binding of homo/heterodimers (Han and Ip, 1999; Hedengren-Olcott et al., 2004; Lemaitre and Hoffmann, 2007; Tanji et al., 2010). Several lines of evidence suggest that other insect Toll and IMD pathways may also function synergistically to induce transcription of effector (AMP) genes. The generation of transgenic strains of *A. aegypti* overexpressing REL2 resulted in increased resistance to infection with both Gram-negative (*E. cloacae, Pseudomonas aeruginosa*) and -positive bacteria (*Staphylococcus aureus*). Moreover, subsequent knockdown of the REL2 induced a decrease in resistance of *A. aegypti* to both Gram-negative and -positive bacteria. Taken together, REL2 appears to play an important role in the response to both Gram types of bacteria (Antonova et al., 2009). Another study found that the AMP gene *Gambicin* is synergistically regulated by the IMD and Toll pathway in *A. aegypti* (Zhang et al., 2017). Other RNAi experiments conducted in *R. prolixus* knocking down the ortholog *rpRelish* prior to infection with the Gram-positive bacterium *Staphylococcus aureus* demonstrated an overall decrease in AMP expression suggesting the potential for cross-regulation (Salcedo-Porras et al., 2019). Crosstalk has also been proposed in *T. pallidipennis*. For example, expression of lysozyme B, an AMP associated with the Toll pathway, was found to have decreased following the silencing of *tpRelish* and subsequent infection with Gram-negative *Escherichia coli*. The

same was noted with respect to Defensin B following bacterial challenge with Gram-positive *Micrococcus luteus* (Alejandro et al., 2022).

Although the IMD and Toll pathways in *D. melanogaster* and other insect models are well defined, few studies have examined these immune signaling pathways in fleas. The Toll pathway has yet to be characterized in flea spp., however, the IMD pathway appears to be a critical player in the response of fleas to the Gram-negative pathogens *Yersinia pestis* and *Rickettsia typhi*. Briefly, Bland et al., 2020 examined the transcriptomic response of the rat flea (*X. cheopis*) to *Y. pestis*. At 4 hours post-infection, increased transcript levels of *Relish*, *PRGP-LC*, *PGRP-LB*, and *Cactus* were found (Bland et al., 2020). Rennoll et al. (2017) examined the role of the IMD pathway in the local humoral response of *C. felis* against *R. typhi*. Using *C. felis* 1KITE transcripts to search for homology between *C. felis* and *D. melanogaster*, the authors found that the IMD and Toll pathways are conserved between the two organisms. To determine the involvement of the IMD pathway in the response to *R. typhi* infection, the researchers used RNAi to suppress expression of the genes *Imd* and *Relish*. The *R. typhi* burden was significantly higher in fleas with decrease expression of *IMD* and *Relish* genes relative to that of control fleas.

1.4 Pathway association, pathogen spectrum, and transcriptional regulation of AMPs across hematophagous arthropods.

Antimicrobial peptide constitution, pathogen spectrum, and pathway association have been found to be highly variable amongst hematophagous disease vectors. Genomic studies conducted in *An. gambiae* and *A. aegypti* uncovered the presence of 5 different classes of AMPs: Gambicin, Cecropins, Defensins, Attacin, and Diptericin (Christophides et al., 2002; Xiao et al., 2014). RNA interference experiments implementing gene knockdowns of *IMD* and *Rel2*, a

Relish orthologue, in the mosquito cell line Aag2 prior to bacterial challenge found a significant decrease in overall AMP expression. In contrast, gene knockdowns of Toll pathway components *Myd88*, *Toll* and *Rel1* resulted in little to no decrease in AMP expression. Thus, it is hypothesized that most *A. aegypti* AMPs are predominantly regulated by the IMD pathway (Zhang et al., 2017). Furthermore, the AMP Defensin was isolated from the hemolymph of *A. aegypti* following hemocoel injection with Gram-negative (*E. coli*) and Gram-positive (*M.s luteus*) bacteria (Lowenberger et al., 1995). Genomic sequencing of *C. lectuliarius* revealed the presence of Defensin-like peptides, Diptericin-like peptides, and Lysozymes (Benoit et al., 2016). Expression of these AMPs were subsequently confirmed in *C. lectuliarius* hemocytes (Potts et al., 2020). Though the mechanisms that regulate AMP expression in *C. lectuliarius* have yet to be fully elucidated, it is known that the putative *clDefensin* has strong activity against several Gram-negative and -positive bacteria in the midgut (Kaushal et al., 2016; Meraj et al., 2022). In both soft and hard tick spp., Defensins have been demonstrated to have broad spectrum activity against both Gram-negative and -positive bacteria. For example, in the midgut of the soft tick *Ornithodoros moubata*, Defensin A had activity against Gram-positive bacteria, however, Os and OsC, short peptide derivatives of Defensin isoform OsDef2 in *Ornithodoros savignnyi*, showed antimicrobial activity against both Gram-negative and -positive bacteria (Nakajima et al., 2003; Prinsloo et al., 2013; Wu et al., 2022). Furthermore, in hard tick spp. such as *Ixodes persulacatus*, persulcatusin – a Defensin unique to the tick midgut – exhibited strong antimicrobial activity against methicillin-resistant and vancomycin-resistant strains of *Staphylococcus aureus* (Miyoshi et al., 2017). Meanwhile, in the hard tick *Dermacentaor silvarum*, *Ds-Defensin* was found to be highly expressed in the midgut and salivary glands post-immune challenge with both Gram-types. Moreover, subsequent isolation and purification of Ds-

Defensin peptides for use in an antimicrobial activity assay yielded complementary results (Wang et al., 2015). Antimicrobial peptides that have been identified in *R. prolixus* and *T. pallidipennis* include Lysozymes, Defensins, and Prolixicin (Ursic-Bedoya et al., 2007). In *R. prolixus*, Lysozyme A, as well as Defensins A and B, have been demonstrated to have activity in the midgut against the Gram-positive bacterium *S. aureus*. Meanwhile, expression of Defensin C, an AMP primarily involved in the response to Gram-positive bacteria in *D. melanogaster,* was induced by the Gram-negative bacterium *E. coli* in *R. prolixus.* (Vieira et al., 2014). Prolixicin has also been demonstrated to have activity against Gram-negative bacteria in both the midgut and fat body of *R. prolixus* (Ursic-Bedoya et al., 2011). Taken together, this high variability suggests that AMPs are differently expressed in response to bacterial infection amongst hematophagous insects.

Evidence of differential expression of AMPs in fleas has been shown in several studies. Transcriptomic analysis of the digestive tract of *X. cheopis* at 4 hours after bacterial challenge with *Y. pestis* revealed Attacin to be the most upregulated AMP transcript, whereas *Defensin A* the most downregulated. In contrast, *Defensin* levels were found to be indifferent in the midgut amongst control and *R. typhi* infected groups in *C. felis* (Dreher-Lesnick et al. 2010). Moreover, *Defensin-2* (*Cf*-726) transcript levels were found to have increased in the salivary glands of *C. felis* following bacterial challenge with *R. felis* (Danchenko et al., 2021). One study suggests that the effectiveness of AMPs can depend on bacterial structure/properties. The *Cecropin* "*Cheopin*" derived from *X. cheopis* has been demonstrated to have high activity against the Gram-negative bacteria *E. coli* and *Pseudomonas aeruginosa* but little to no activity against the *Y. pestis* strain KIM6+ (Mathew et al., 2021). Moreover, thermal adaptation of *Y. pestis* KIM6+ to the flea's internal temperature confers a structural change in Lipid A, resulting in positively charged

surface molecules that in turn repel AMPs, which are also cationic (Rebeil et al., 2004; Anisimov et al., 2005; Knirel et al., 2005; Knirel et al., 2012).

Though pioneering studies have been conducted, there is still much to be uncovered about the roles of flea immune signaling pathways and AMPs in the response to flea-borne pathogens. To date, no studies have examined the immune signaling response in the fat body, gut, or hemocytes of *C. felis* following bacterial challenge with the flea-borne pathogen *B. henselae* nor Gram-positive bacterial spp. Additionally, the role AMPs in *C. felis* immunity have not yet been fully elucidated. Recent sequencing of the *C. felis* genome (2020) has provided the means to overcome this gap in knowledge. As demonstrated in several other hematophagous arthropods, we hypothesize that different bacterial pathogens will elicit distinct immune signaling responses in the gut of *C. felis.* We also hypothesize that a bacterial challenge will increase the antimicrobial activity in the *C. felis* digestive tract. To test these hypotheses, the relative expression levels of genes comprising the IMD and Toll pathways, as well as effector (AMP) genes, were measured following immune challenge with Gram-negative and Gram-positive bacteria. Additionally, we measured the antimicrobial activity of the flea digestive tract *in vitro* by isolating proteins from infected individuals.

1.5 Purpose of study

In their natural environments, insects are commonly challenged with pathogens of different classes (*e.g.*, fungi, bacteria, viruses, and parasites). A meta-analysis conducted in *D. melanogaster* introducing these different types of pathogens, found differentially expressed immune genes, suggesting that there could be pathogen specificity in insect immune responses (Waring et al., 2022). Comparing gene expression profiles across Gram-negative pathogens,

between Gram-negative and -positive pathogens, as well as between laboratory model species and flea-borne pathogens, could provide insights as to whether there are differences in the flea immune response to different types of pathogens. Ultimately, understanding how immune responses are modulated in *C. felis* across different pathogens could provide a way to intervene with and/or effectively decrease vector-host pathogen transmission.

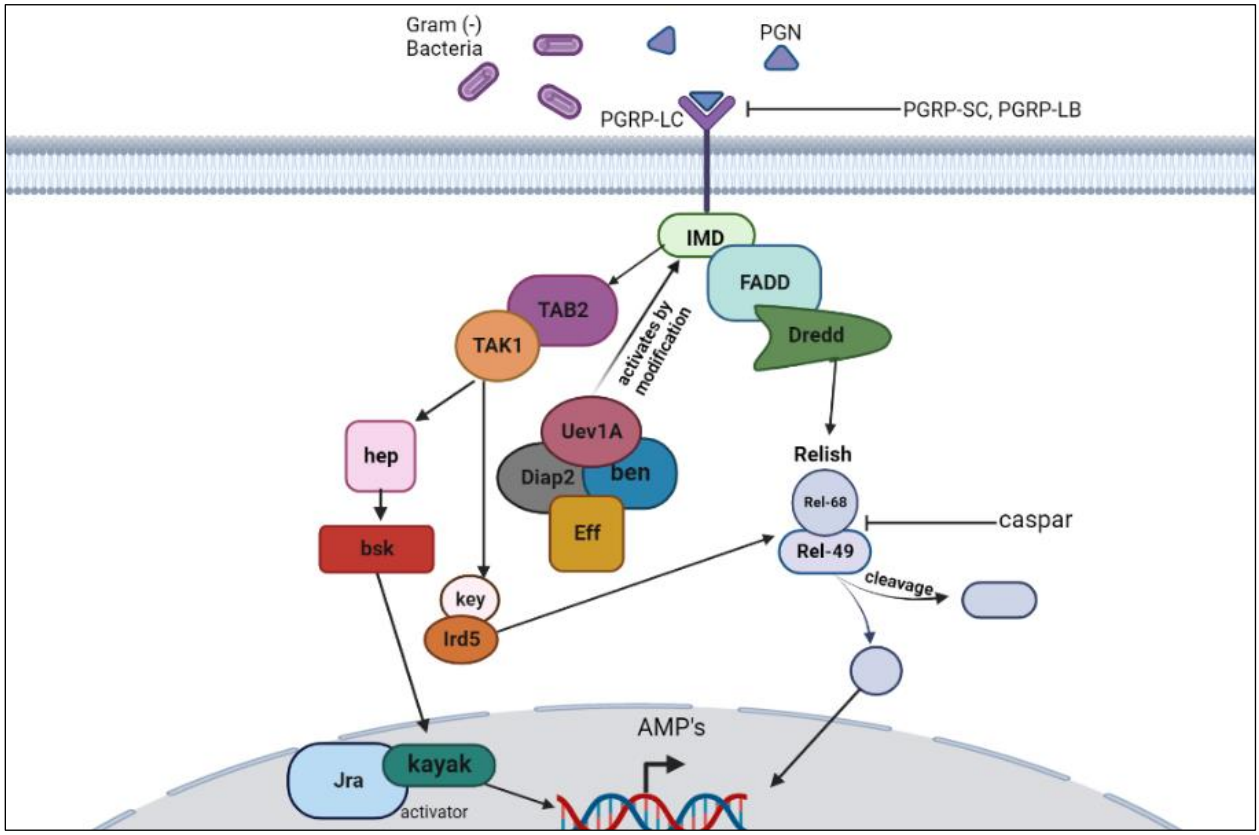


Figure 1. ***IMD signaling pathway in the Dipteran model species Drosophila melanogaster.*** The IMD pathway is activated in response to the detection of microbial cell wall components. *PGRP-LC* recognizes the diaminopimelic acid (DAP)-type peptidoglycan from Gram-negative bacteria. *PGRP-LC* recruits the adaptor *IMD*, which interacts with *FADD* and binds the apical caspase *Dredd*. This caspase then cleaves the transcription factor *Relish*, and the Rel domain translocates into the nucleus. *Relish* is phosphorylated by the *IKK* signaling complex, which is activated by *TAK1* and its adaptor *TAB2* in an IMD-dependent manner. Activation of the IMD pathway ultimately leads to the expression of AMP genes such as *Attacin*.

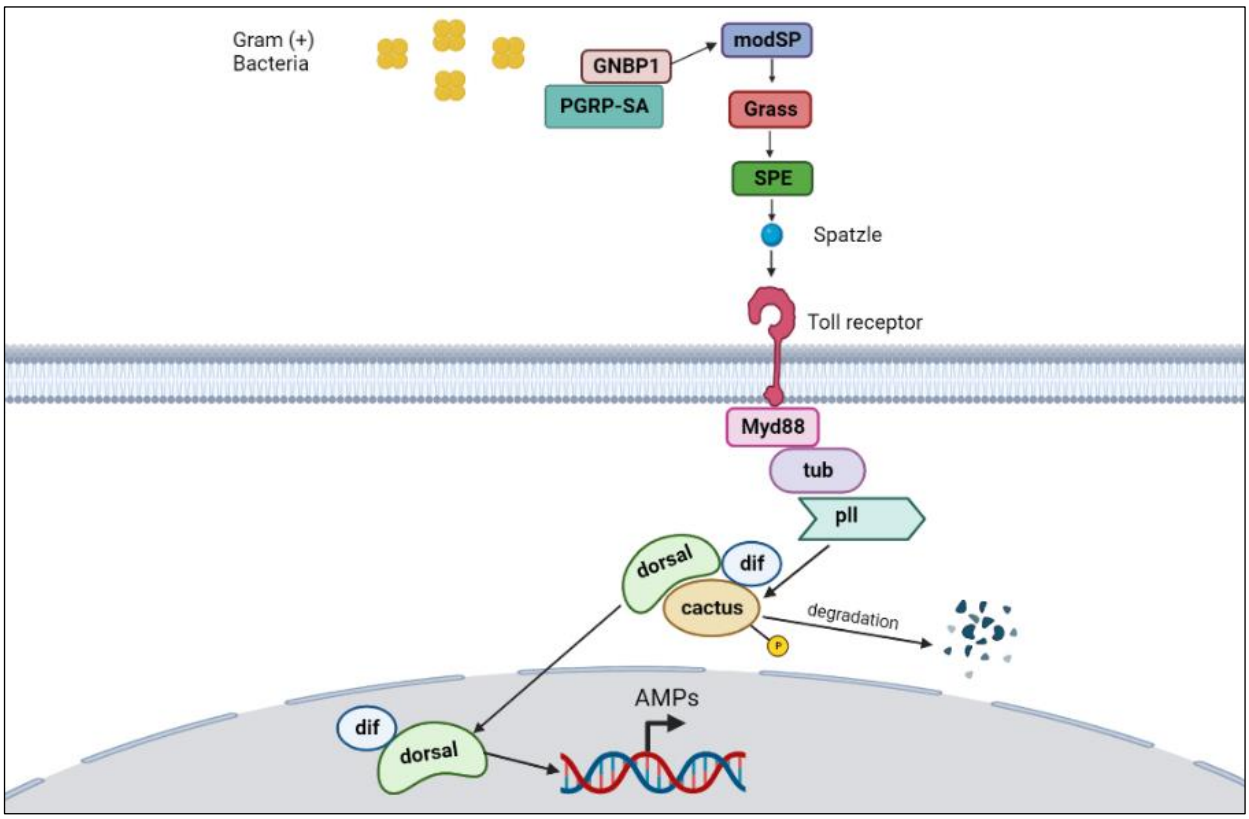


Figure 2. ***Toll signaling pathway in the Dipteran model species Drosophila melanogaster***. The Toll pathway is activated in the presence of microbial cell wall components. Activation occurs indirectly by *PGRP-SA* upon recognition/binding of Lys-type peptidoglycan found on the surface of Gram-positive bacteria. Once bound, a serine protease cascade is activated, resulting in the cleavage of the ligand Spätzle. Upon cleavage, Spätzle can then bind to the transmembrane receptor *Toll* and initiate an intracellular signaling cascade by the association of the adaptor proteins *Myd88* and *tub*, and the protein kinase *pll*. When the signaling complex is turned on, *cactus*, an inhibitor bound to the transcription factor *dif*/*dorsal*, is degraded allowing for the translocation of *dif*/*dorsal* into the nucleus and subsequent transcription of AMP genes.

CHAPTER 2
METHODS

2.1 Flea rearing and maintenance:

Cat fleas (*C. felis* Bouché), originally sourced from Elward II Laboratory (Soquel, CA, USA), were collected from colonies maintained at Georgia Southern University as previously described (Brown et al., 2021). Because Elward II Laboratory is no longer in service, the colony was supplemented with additional cat fleas from EctoServices (Henderson, NC, USA) as needed. A portion of these new cat fleas were tested to verify the absence of *B. henselae* infection with the use of quantitative real-time polymerase chain reaction (qPCR) analyses. Adult fleas were fed defibrinated bovine blood (Hemostat Laboratories, Dixon, CA, USA) using an artificial feeding system (Wade and Georgi, 1988). Immature stages were incubated until adult emergence at 25 °C and ≥85% relative humidity. To increase adult female survival rates, both sexes were utilized for feeding experiments; however, subsequent analyses were performed on adult females only.

2.2 Bacterial cultures:

The Gram-negative bacterial pathogen *Bartonella henselae* (Brenner et al., 1993; Regnery et al., 1996) (ATCC 49882) was grown by inoculating 1 mL of tryptic soy broth (TSB) with 1 mL of a thawed vial of previously frozen primary stock culture. The suspension was then added to a commercially prepared tryptic soy agar (TSA) slant with 5% sheep blood from Carolina Biological Supply Company or a freshly prepared TSA slant with 5% bovine blood from our laboratory. These biphasic slants were incubated using one of two methods: (1) a candle extinction jar placed inside a shaking incubator at 37°C and 200 rpm for approximately 3

days, or (2) a Ziplock bag with CO_2 gas generators (MGC AnaeroPack$^{@}$ System) inside a standard microbiological incubator at 37°C for approximately 10 days. Prior to use in experiments, the broth pool of the biphasic slant was pelleted by centrifugation (15,000 x *g* for 3 minutes) and resuspended in TSB. The Gram-negative bacterium *Serratia marcescens* (D1, Carolina Biological Supply Company) and Gram-positive bacterium *Micrococcus luteus* (Carolina Biological Supply Company) were grown overnight in a shaking incubator at 25°C and 350 rpm in nutrient broth.

2.3 Flea bacterial infections:

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). Once an optical density of approximately $OD_{600} = 5$ was reached, bacterial cultures were pelleted by centrifugation and resuspended in 600 µL of heat-inactivated blood (56°C for 10 minutes). For *S. marcescens* and *M. luteus*, absolute doses were determined by plating serial dilutions of the treatment culture on nutrient agar, growing them for 48 hours at 25°C, and then counting the resultant colony forming units (CFUs). Absolute doses of *B. henselae* were determined using qPCR and serial dilutions of a plasmid containing a portion of the citrate synthase gene of *Bartonella* to generate a standard curve. The plasmid was previously generated by Integrated DNA Technologies, Inc. (Coralville, IA, USA) using a 93-base pair fragment of *B. henselae* citrate synthase gene and the pIDTSmart (Amp) vector. Genomic DNA (gDNA) was extracted from the treatment culture using the PureLink^{TM} Genomic DNA Mini Kit (Invitrogen^{TM}, Waltha, MA) according to the manufacturer's instructions and eluted in 50 µL PureLink^{TM} Genomic Elution Buffer. A negative environmental control (DNA extraction reagents without biological

samples) was utilized for each DNA extraction process. Real-time quantitative PCR (qPCR) assays for detection of the citrate synthase gene of *Bartonella* were performed as described previously (Durden et al. 2021, Brown et al. 2022).

Experimental fleas were placed into four groups and starved overnight prior to feeding on an infectious bloodmeal. Each group was exposed to one of the following treatments: (1) untreated blood, (2) blood infected with *B. henselae*, (3) blood infected with *S. marcescens*, or (4) blood infected with *M. luteus*. Fleas were allowed to feed on each bloodmeal for a duration of either 4 or 24 hours. To quantify infection intensity for *B. henselae* per experimental trial, a subset of fleas (n = 5) was used for gDNA extractions and qPCR as described above for *Bartonella* treatment cultures. As a control, qPCR assays were used for detection of the flea 18s rRNA gene to confirm the presence of *C. felis* template gDNA (Durden et al., 2021, Brown et al., 2022). To measure infection intensity for *S. marcescens* and *M. luteus* per experimental trial, a subset of fleas (n = 5) were individually homogenized in PBS and plated on nutrient agar. Plates were incubated as described above and the number of CFUs were counted. Three independent trials were conducted per treatment group, and data are presented as (1) mean infectious dose/infection load and (2) infection prevalence. Mean infectious dose and infection loads of *B. henselae* were measured in copy number of the citrate synthase gene of *Bartonella* per 200μL bacterial culture or flea, respectively. Mean infectious dose and infection load of *S. marcescens* and *M. luteus* were measured in CFUs per 200 μL bacterial culture or flea, respectively. Infection prevalence was defined as the percentage of fleas infected with bacteria.

2.4 Quantification of immune pathway genes by qPCR:

Nine target genes were selected based on their function in the IMD and Toll pathways in *D. melanogaster*: *PGRP-LC, PGRP-LB, IMD, Relish, Attacin, PGRP-SA, Toll, Cactus,* and *Defensin* (Table 2). Primer sets were generated for the cat flea from transcript sequences obtained through NCBI databases as previously described (Table 1) (Brown et al. 2021). The previously validated *GAPDH* and *RPL19* genes were used as an endogenous control (McIntosh et al., 2016). All primer sequences used in this study are listed in Table 2. At 4- and 24- hours post-treatment, whole guts dissected from at least 30 fleas were pooled and homogenized with a sterile plastic pestle in 400µL of TRIzol™ reagent (Invitrogen™). Total RNA was isolated using the Direct-zol™ RNA Miniprep Plus kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. The RNA concentration was measured using a NanoDrop 2000 spectrophotometer (ThermoFisherScientific™), and 0.5 µg of RNA was used as a template for complementary DNA (cDNA) synthesis using the High-Capacity RNA-to cDNA™ kit (Applied Bio-systems, Foster City, CA USA) according to the manufacturer's instructions. Real-time quantitative PCR reactions were premixed in a 96-well plate containing 2X PowerUp™ SYBR™ Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.5 µM of the forward primer, 0.5M of the reverse primer, PCR-grade H_2O (Invitrogen UltraPure™ Distilled DNase/RNase-free water) and 5 µL of template cDNA. For negative controls, 5 µL of no RT reactions was performed to confirm the absence of gDNA, and PCR-grade H_2O was used in place of template. Ten microliters of each reaction were dispensed in triplicate into MicroAmp® Fast Optical 96-well reaction plates and ran on a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) at default thermal cycling conditions. To determine relative mRNA levels, an 8-point standard curve was used to interpolate the sample quantity of

the gene of interest (GOI) and the reference genes (GAPDH, RPL-19) for both infection and control groups. The GOI sample quantity was then normalized by comparison to the geometric means of the reference gene sample quantities (GOI /Geomean [reference genes]) (Brown et al., 2021). The results are presented as the mean ± standard error of the mean (SEM) mRNA levels of GOIs from cDNA generated from 3 independent trials.

2.5 Antimicrobial activity assay of flea gut contents:

The antimicrobial activity of flea gut contents was assessed *in vitro* using an assay adapted from Vieira et al. (2014). Following the same methods described above, fleas were exposed to one of the following treatments: (1) untreated blood, or (2) blood infected with bacteria (*B. henselae*, *S. marcescens*, or *M. luteus*). At 4- and 24-hours post-exposure, whole guts dissected from at least 35 fleas were pooled and homogenized in 200 µL of PBS plus 20 µL of a protease inhibitor (Sigma-Aldrich, Catalog No. P2714-1BTL) (Shin et al., 2010; Kalsy et al., 2020). To ensure the complete removal of treatment bacteria, the flea gut homogenate was centrifuged at 1400 rpm and 4°C, and the supernatant was filtered through a 0.45 µm hydrophilic PVDF membrane followed by a 0.22 µm hydrophilic PVDF membrane. Protein concentrations of each flea gut sample were measured using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific™) with bovine serum albumin standards according to the manufacturer's instructions. Assay reactions were pre-mixed in microcentrifuge tubes containing 30 µL of gut sample (1/4 dilution), 66 µL of PBS, 15 µL of *M. luteus*, and 7.5 µL of NB. Alternatively, for control wells, 30 µL of PBS was used in place of the gut sample. The mixtures were then aliquoted in triplicate into the wells of a 96-well half-area microtiter plate. The contents of the 96-well plate were then incubated for 24 hours at 25°C in a shaking incubator at 350 rpm. The contents of each well were diluted 1:100 in PBS, spread on nutrient agar plates, and incubated at room temperature (25°C)

for 5 days. Antimicrobial activity was then measured by comparing the resultant CFUs of *M. luteus* produced from experimental and control wells.

2.6 Statistical analyses:

All Data (gene expression, protein concentrations, and antimicrobial activity) were analyzed by ANOVA, followed by Tukey's multiple comparison test in GraphPad Prism version 10.2.2 (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $p \leq 0.05$.

Immune signaling pathway/ effector molecules	**Gene name (abbreviation) [organism]**	***D. melanogaster* Protein or Gene ID**	***C. felis* Gene ID**	***C. felis* Transcript ID**	***C. felis* Genomic ID**
IMD	Peptidoglycan-recognition protein LC-like (*PGRP-LC*) [*C. felis*]	NP_001163397.1	LOC113378741	XM_026619320.1	NW_020538040.1
	Immune deficiency (*IMD*) [*D. melanogaster*]	NM_001299643.1	-	GAYP02008970.1	NW_020536166.1
	Relish (*Rel*) [*D. melanogaster*]	NM_057746.4	-	GAYP02012752.1	NW_020537730.1
	Peptidoglycan-recognition protein LB-like (*PGRP-LB*) [*C. felis*]	NP_731575.1	LOC113382894	XM_026621143.1	NW_020539724.1
Toll	Peptidoglycan-recognition protein SA-like (*PGRP-SA*) [*C. felis*]	NP_001285128.1	LOC113373503	XM_026613796.1	NW_020537758.1
	Protein toll-like (*Toll*) [*C. felis*]	NP_001262995.1	LOC113376322	XM_026616231.1	NW_020538040.1
	NF-kappa-B inhibitor cactus-like (*Cactus*) [*C. felis*]	NP_476943.1	LOC113385240	XM_026623073.1	NW_020539725.1
Effector molecules	Attacin-A-like (*Attacin*) [*C. felis*]	NP_523745.1	LOC113388596	XM_026625965.1	NW_020539726.1
	Defensin-like (*Defensin A*) [*C. felis*]	XP_016929506.1	LOC113385115	XM_026622926.1	NW_020539725.1

Table 1. Gene names, *D. melanogaster* Protein or Gene IDs, and NCBI accessions for either *C. felis* putative or non-putative transcripts, genes, and corresponding *C. felis* genome accessions.

Gene name (abbreviation)	Target organism	Gene function	Forward primer (5'→3')	Reverse primer (5'→3')	Annealing temperature (°C)
Peptidoglycan-recognition protein LC-like (*PGRP-LC*)	*C. felis*	Pattern recognition receptor	GTACTGGCGTT TTGCCTTGT	CCACCCATTCT TGACGAGAT	56.9
Immune deficiency (*IMD*)	*C. felis*	Adaptor-enables death domain binding	TGCAAAATGTC AATCGAGGA	GGCCTAACTGG AGCATCATC	54.5
Relish (*Rel*)	*C. felis*	Transcription factor	TGCTTTTGGAT CGTTCAGTG	GCGATCAGAAC CCAATATACG	55.5
Peptidoglycan-recognition protein LB-like (*PGRP-LB*)	*C. felis*	Negative regulator	ATGGCTGCGAT TAGGACATT	CACCACATGCT CCAGGAATA	55.3
Peptidoglycan-recognition protein SA-like (*PGRP-SA*)	*C. felis*	Pattern recognition receptor	TGAAGGTGCAG GATGGTATAAA	CAAGTGCTCCT AGTGCAACG	57.7
Protein toll-like (*Toll*)	*C. felis*	Transmembrane receptor-enables cytokine and TIR domain binding	TTGTCGAATGA ACTGGTGCG	ACATACCGTCA AACACCCCT	58
NF-kappa-B inhibitor cactus-like (*Cactus*)	*C. felis*	Negative regulator	TCAGGAGCCAT CTGTCATTG	CTTTGCACGCC TCTGTCATA	56.4
Attacin-A-like (*Attacin*)	*C. felis*	Encode peptides that have antimicrobial activity	GCAGACCAGGC AGAAAAGAC	CTGAAGACCTC CTCCGACAG	58.3
Defensin-like (*Defensin A*)	*C. felis*	Encode peptides that have antimicrobial activity	CGGGTTAGTGT TGGTGGTTT	GAGATATGCAC CGAGCATTG	55
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	*C. felis*	Glycolysis	ACCCAAAAGAC TGTGGATGG	CGGAATGACTT TGCCTACAG	57.4
Ribosomal protein L19 (*RPL-19*)	*C. felis*	Encode structural cytoplasmic ribosomal proteins	TACAGCTAATG CCCGTACACC	TTCAACAAACG CCTCAGGAC	58.1
18S rRNA	*C. felis*	Encodes a structural subunit involved in protein synthesis	TGCTCACCGTT TGACTTGG	GTTTCTCAGGC TCCCTCTCC	60
Citrate synthase (*CS*)	*B. henselae*	Citric acid cycle	GTGCTAATCCA TTTGCATGTAT T	GTAACATTTTT AGGCATGCTTC ATTA	60

Table 2. Gene function, forward and reverse primers used for qPCR, and annealing temperature of each primer.

CHAPTER 3
RESULTS

3.1 Flea infection prevalence and intensity

For each set of experiments, fleas were orally infected with the Gram-negative, flea-borne pathogen *B. henselae*, or two model bacteria, Gram-negative *S. marcescens* and Gram-positive *M. luteus.* Fleas were allowed to feed on the infectious bloodmeal for either 4 or 24 hours, and the following data were combined from six independent trials (Table 3). At 4 hours post-exposure, infection prevalence (percentage of fleas infected with bacteria) was 67%, 70%, and 70% for *B. henselae*, *S. marcescens*, and *M. luteus*, respectively, and 57%, 97%, and 63% for the same bacterial species at 24 hours post-exposure. Infection intensity for *B. henselae* at 4 and 24 hours (average copy number of the citrate synthase gene of *Bartonella* per infected flea) was 25,912 copies (± 9,946 SEM) and 1,220 copies (± 168 SEM), respectively. Infection intensity for *S. marcescens* and *M. luteus* (average number of CFUs per *S. marcescens*- or *M. luteus*-infected flea) was 989 CFUs (± 556 SEM) and 255 CFUs (± 72 SEM) at 4 hours, respectively, and 872 CFUs (± 233 SEM) and 1,235 CFUs (± 486 SEM) at 24 hours, respectively.

3.2 Select genes of the IMD pathway, but not the Toll pathway, are upregulated in response to laboratory strains of bacteria only

To characterize the immune signaling pathways in the flea digestive tract, we measured transcript levels of select effector and IMD/Toll pathway genes following 4 hours and 24 hours post-exposure to an infected bloodmeal with either *B. henselae*, *S. marcescens*, or *M. luteus*. The results for the IMD pathway are presented in the following order: activation molecules (*PGRP-LC*, *IMD*, *Relish*), effector molecules (*Attacin*, *Defensin*), and regulatory molecules (*PGRP-LB*).

Similarly, the results for the Toll pathway are presented as activation molecules (*PGRP-SA*, *Toll*) and regulatory molecules (*Cactus*). At 4 hours post-exposure, differences in relative mRNA levels of molecules involved in the IMD pathway were only observed in fleas that fed on an *S. marcescens*-infected bloodmeal. Specifically, *Relish*, *Attacin*, and *PGRP-LB* were 1.9, 7.5, and 2.7-fold higher in this treatment group than untreated fleas (Fig. 3C; Tukey's: $p = 0.0157$, Fig. 3E; Tukey's: $p < 0.0001$, Fig. 3F; Tukey's: $p = 0.0158$, respectively). Levels of *PGRP-LC*, *IMD*, and *Defensin* were not affected by an *S. marcescens*-infected bloodmeal at this time point. Again, *B. henselae* and *M. luteus* treatments did not affect mRNA levels in the IMD pathway at 4 hours post-exposure. Furthermore, none of the treatments affected the relative mRNA levels of molecules involved in the Toll pathway at this time point.

At 24 hours post-exposure, differences in relative mRNA levels of molecules involved in the IMD pathway were observed in fleas that fed on *S. marcescens*- and *M. luteus*-infected bloodmeals. Specifically, *IMD* was 1.3 and 1.7-folder higher in fleas exposed to *S. marcescens* and *M. luteus*, respectively, compared to untreated fleas (Fig. 3B; Tukey's: $p= 0.0425$, Tukey's: $p = 0.0003$, respectively). Additionally, *Defensin* and *PGRP-LB* were 5.0-fold higher in fleas exposed to *S. marcescens* than control fleas (Fig. 3D; Tukey's: $p = 0.0031$, Fig. 3F; Tukey's: $p = 0.0030$, respectively). Levels of *PGRP-LC*, *Relish*, and *Attacin* were not affected by either *S. marcescens*- and *M. luteus*-infected bloodmeals. Like above, *B. henselae* did not affect mRNA levels in the IMD pathway at 24 hours post-exposure, and none of the treatments affected the relative mRNA levels of molecules involved in the Toll pathway at this time point. Overall, these data show three effects for each type of bacterium: (1) the Gram-negative, flea-borne pathogen *B. henselae* does not stimulate transcriptional induction of either immune signaling pathway, (2) the Gram-negative, model species *S. marcescens* increases expression of numerous genes in the

IMD pathway only, and (3) the Gram-positive, model species *M. luteus* induces expression of an IMD-specific target gene (*IMD*), but does not stimulate transcriptional induction of the Toll pathway.

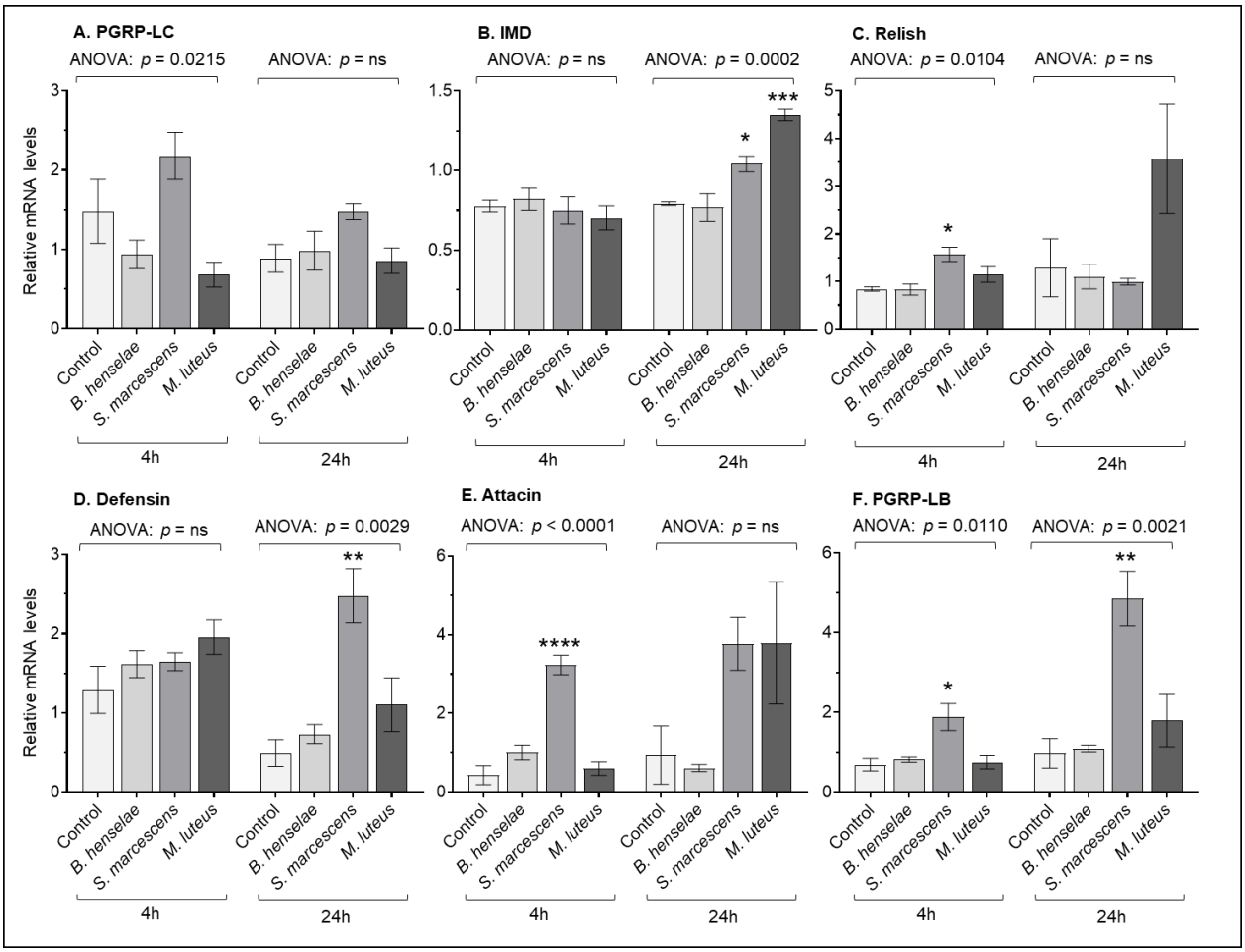
3.3 Antimicrobial activity increases at 24 hours post-immune challenge

To complement the gene expression data, we examined the antimicrobial activity of proteins isolated from the digestive tracts of naïve and infected fleas at 4- and 24-hours post-exposure to *B. henselae, S. marcescens*, and *M. luteus*. The concentration of protein isolated from flea digestive tracts at 4 hours post-exposure to *S. marcescens* was significantly lower than the concentrations of protein isolated from the digestive tracts of naïve fleas (Fig. 5; Tukey's: $p = 0.0263$), as well as fleas infected with *M. luteus* and *B. henselae* (Fig. 5; Tukey's: $p = 0.0025$ and $p = 0.0071$, respectively). However, no significant differences in protein concentrations were noted between groups at 24 hours post-infection. Despite the significant differences observed between protein concentrations, no significant differences in the antimicrobial activity of gut contents were noted between naïve fleas and infected fleas at 4 hours post-exposure (Fig. 6). In contrast, antimicrobial activity significantly increased in flea digestive tracts at 24 hours post-exposure to *B. henselae*, *S. marcescens*, and *M. luteus* relative to naïve fleas (Fig. 6; ANOVA: $p < 0.0001$). Specifically, fleas exposed to *B. henselae* for 24 hours secreted proteins that were 99% more toxic against bacteria relative to those from naïve fleas (Fig. 6; Tukey's: $p < 0.0001$). Similarly, proteins isolated from fleas exposed to *S. marcescens* or *M. luteus* for 24 hours were 98% more lethal against bacteria than those collected from naïve fleas (Fig. 6; Tukey's: $p < 0.0001$ and $p < 0.0001$, respectively). Overall, the data shows that proteins isolated from the digestive tracts of fleas exposed to an infectious bloodmeal (*B. henselae, S. marcescens*, and *M.*

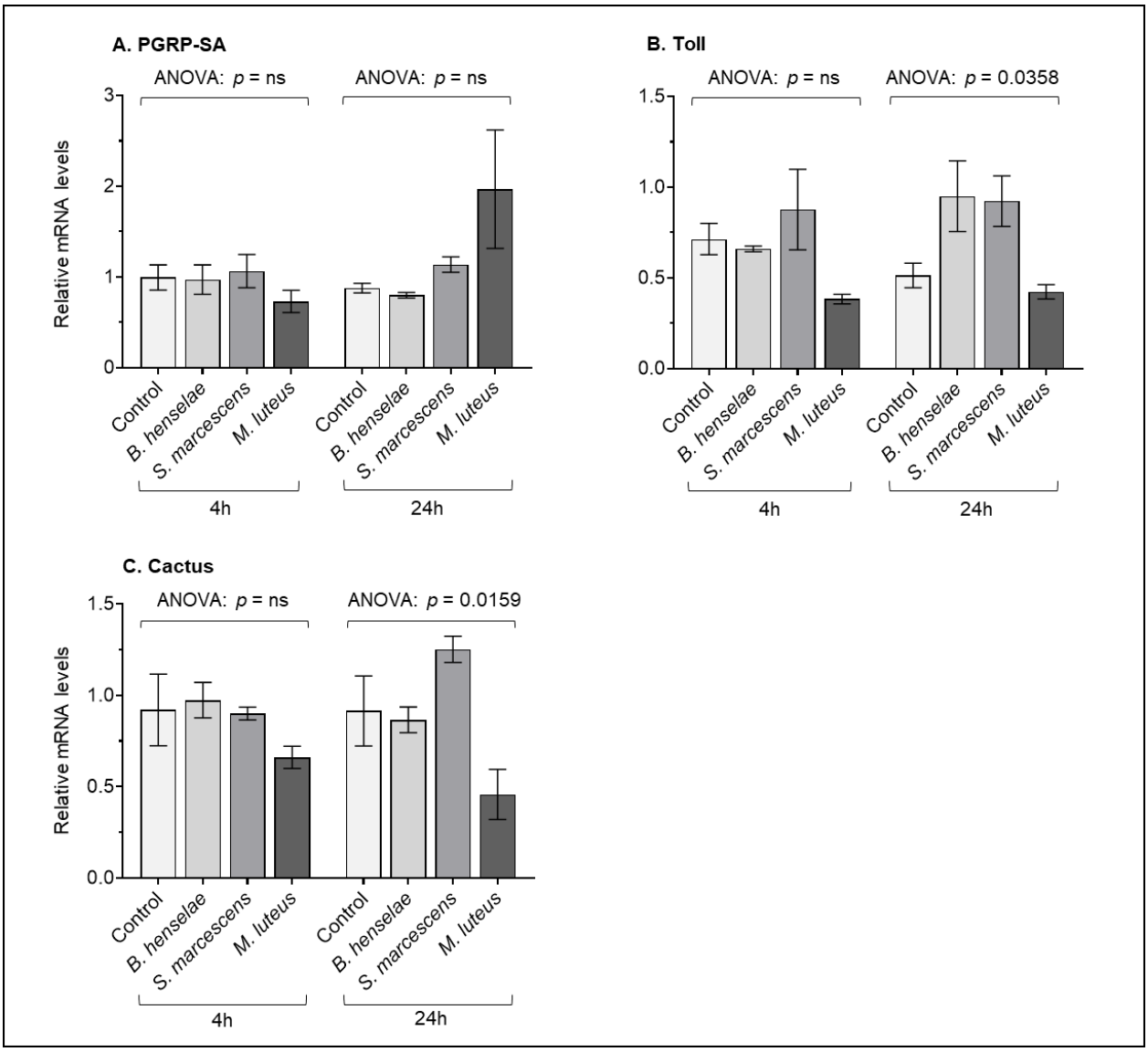
luteus) for 24 hours exhibit increased antimicrobial activity relative to the proteins isolated from naïve fleas.

Bacteria	Experiment	Mean infectious dose (±SEM)	Hours post-exposure	Mean infection load/flea (± SEM)	Prevalence (%)	n
Bartonella henselae	**Antimicrobial activity**	**3.90E+09 (±8.62E+08)**	**4**	**25912 (±9946)**	**67**	**15**
	Gene expression	4.09E+08 (±1.25E+08)	24	1245 (±189)	93	15
	Antimicrobial activity	1.58E+09 (±7.37E+08)	24	1.10E+03 (±4.31E+02)	**20**	15
	Combined	**9.96E+08 (±4.35E+08)**	**24**	**1220 (±168)**	**57**	**30**
Micrococcus luteus	Gene expression	5.13E+07 (±8.47E+06)	4	151 (±134)	60	15
	Antimicrobial activity	3.79E+07 (±7.19E+06)	4	333 (±74)	80	15
	Combined	**4.46E+07 (±5.80E+06)**	**4**	**255 (±72)**	**70**	**30**
	Gene expression	2.49E+06 (±1.26E+06)	24	1791 (±660)	87	15
	Antimicrobial activity	3.45E+07 (±8.34E+06)	24	31 (±8)	40	15
	Combined	**1.85E+07 (±8.097E+06)**	**24**	**1235 (±486)**	**63**	**30**
Serratia marcescens	Gene expression	2.36E+08 (±6.24E+07)	4	2370 (±1360)	53	15
	Antimicrobial activity	3.37E+07 (±5.67E+06)	4	153 (±106)	87	15
	Combined	**1.35E+08 (±5.32E+07)**	**4**	**998 (±556)**	**70**	**30**
	Gene expression	6.59E+07 (±4.32E+07)	24	1065 (±430)	93	15
	Antimicrobial activity	7.74E+06 (±3.61E+06)	24	693 (±212)	100	15
	Combined	**3.68E+07 (±2.72E+07)**	**24**	**872 (±233)**	**97**	**30**

Table 3. Infectious dose administered, infection prevalence (%), and mean infection loads (±SEM) of *B. henselae*, *S. marcescens,* and *M. luteus* at 4 and 24 hours post-exposure. Mean infectious dose and infection loads of *B. henselae* were measured in copy number of the citrate synthase gene of *Bartonella* per 200µL of bacterial culture or flea, respectively. Mean infectious dose and infection loads of *S. marcescens* and *M. luteus* were measured in colony forming units (CFUs) per 200 µL of bacterial culture or flea, respectively. Three independent trials were conducted for each experiment at each time point, and the combined data reported is the mean across all experiments (6 independent trials). Infection intensity of *B. henselae* at 4 hours post-exposure for the gene expression analyses was not recovered.



Figures 3A-F. ***Relative mRNA levels of genes comprising the IMD pathway, effector genes Attacin and Defensin, and the IMD regulatory molecule PGRP-LB in the digestive tracts of either naïve (control) fleas or fleas that have been orally infected with either Bartonella henselae, Serratia marcescens, or Micrococcus luteus.*** Data are shown as the mean (± SEM) relative mRNA levels from 3 independent trials combined. The data were analyzed by ANOVA followed by Tukey's Multiple Comparison Test in GraphPad Prism V10.2.2. Significant differences in relative mRNA levels are depicted as they relate to the control group. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$.



Figures 4A-C. ***Relative mRNA levels in genes comprising the Toll pathway & the Toll regulatory molecule Cactus in the digestive tracts of either naïve (control) fleas or fleas that have been orally infected with either Bartonella henselae, Serratia marcescens, or Micrococcus luteus.*** Data are shown as the mean (± SEM) relative mRNA levels from 3 independent trials combined. The data were analyzed by ANOVA followed by Tukey's Multiple Comparison Test in GraphPad Prism V10.2.2. Significant differences in relative mRNA levels are depicted as they relate to the control group. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$.

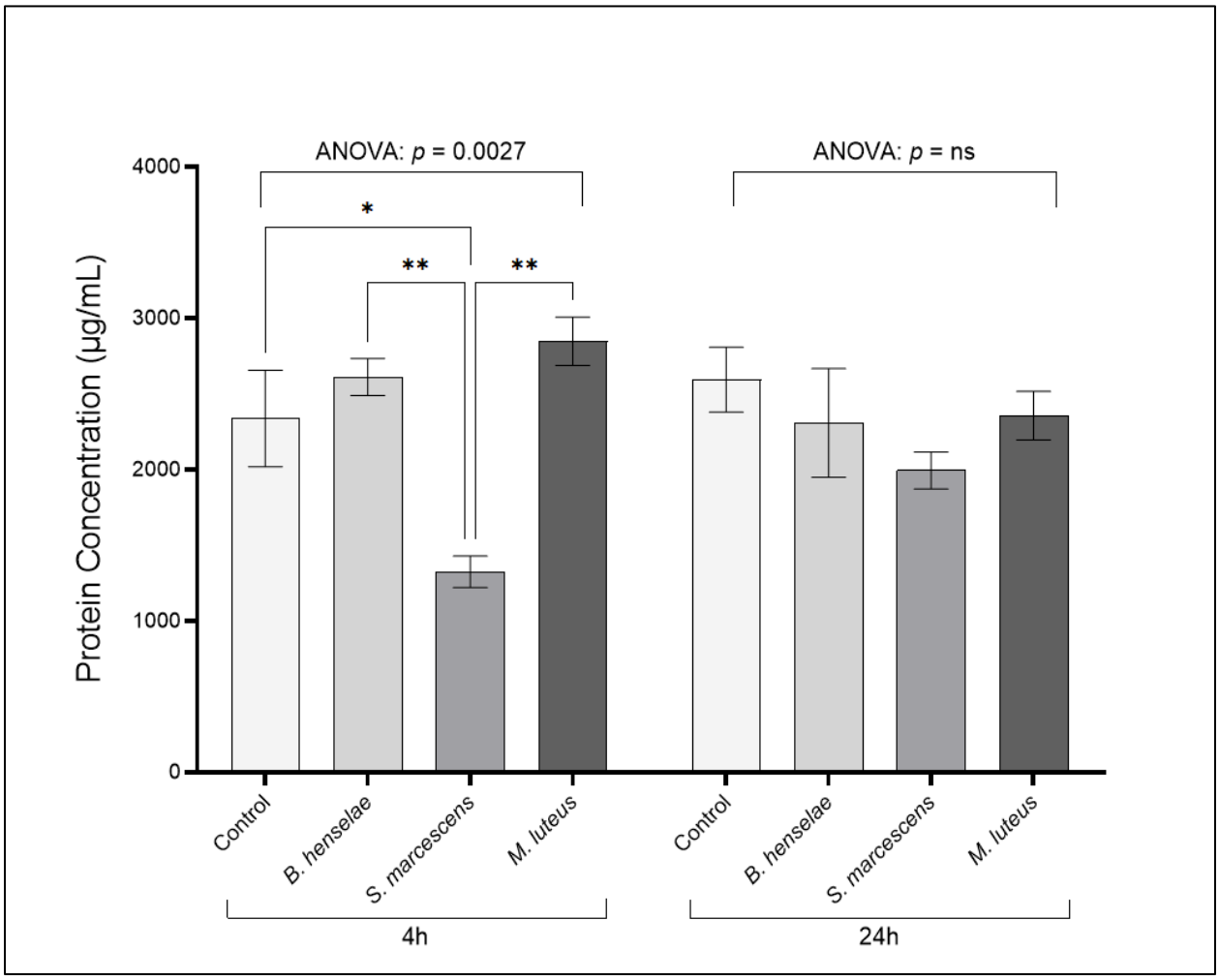


Figure 5. ***Relative concentration of proteins isolated from the digestive tracts of either naïve (control) or infected (B. henselae, S. marcescens, or M. luteus) fleas*.** Data are shown as the mean (± SEM) protein concentrations from 3 independent trials combined. The data were analyzed by ANOVA followed by Tukey's Multiple Comparison Test in GraphPad Prism V10.2.2. Significant differences in protein concentrations are depicted as they relate to one another * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$.

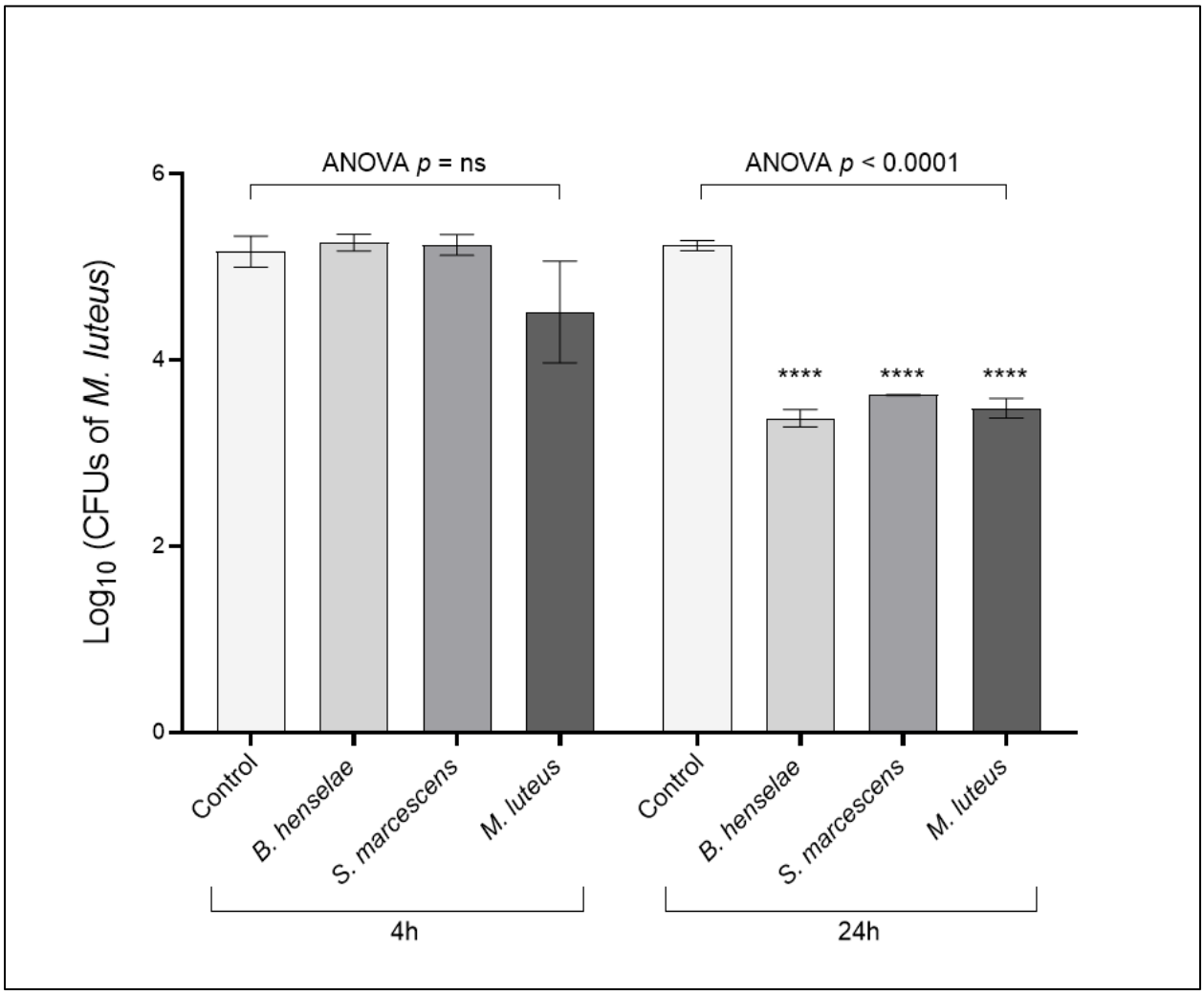


Figure 6. ***Antimicrobial activity of proteins isolated from infected and naïve flea digestive tracts against M. luteus*.** Antimicrobial activity was measured *in vitro* using proteins isolated from the digestive tracts of naïve and infected (*B. henselae, S. marcescens,* or *M. luteus*) fleas; gut proteins were tested against the bacterium *M. luteus* (OD_{600}=5) after a 24-hour incubation period. Data are shown as the mean (± SEM) CFUs from 3 independent trials combined. The data were analyzed by ANOVA followed by Tukey's multiple comparison test in GraphPad Prism V10.2.2. Significant differences in antimicrobial activity are depicted as they relate to the control group. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$.

CHAPTER 4
DISCUSSION

Cat fleas (*C. felis*) are a common ectoparasite encountered in the veterinary and medical fields, compared to other disease vectors; yet, how cat fleas respond immunologically to the etiological agents that they vector (*e.g.*, *Bartonella henselae*, *Rickettsia felis, Rickettsia typhi*) is poorly understood. Fleas primarily acquire infections through the ingestion of a host-derived bloodmeal; thus, the gut lumen is often the first barrier that pathogens encounter. As such, during the early stages of infection, bacterial pathogens are typically localized to the flea gut where they encounter midgut epithelial cells (Brown, 2019). In response to pathogen recognition, insects initiate humoral immune defenses such as the production of AMPs (Hillyer, 2016). The importance of AMPs in the defense against bacterial pathogens has been demonstrated in several hematophagous arthropods (triatomines, mosquitos, bed bugs, ticks) (Lowenberger et al., 1995; Christophides et al., 2002; Ursic-Bedoya et al., 2007; Vieira et al., 2014; Xiao et al., 2014; Benoit JB et al., 2016; Potts et al., 2020), including the closely related rat flea (*Xenopsyllya cheopis*) (Bland et al., 2020). In contrast, relatively little is known about the production of AMPs by the IMD and Toll pathways and the subsequent role of these AMPs in the defense against bacterial pathogens in the digestive tracts of cat fleas. The current study provides evidence that (1) the IMD pathway, but not the Toll pathway, is activated in response to laboratory strains of bacteria, (2) the flea-borne pathogen *B. henselae* does not elicit activation of either the IMD nor Toll pathway, and (3) there is associated antimicrobial activity as a consequence of these infections, regardless of pathway activation. Overall, our findings provide critical insights into the local humoral immune response mediated against bacterial pathogens in cat flea digestive tracts.

4.1. The role of the IMD pathway in the digestive tract of cat fleas

In the well-studied fruit fly (*D. melanogaster*), the IMD pathway starts by recognizing peptidoglycan in Gram-negative bacteria via the transmembrane receptor *PGRP-LC*. Similarly, in blood-feeding insects, *PGRP-LC* has been implicated in the activation of the IMD pathway in mosquitoes (*A. aegypti* and *An. gambiae*), triatomines (*R. prolixus*), and rat fleas (*X. cheopis*) (Wang et al., 2018; Salcedo-Porras et al., 2020; Bland et al., 2020). In the present study, the expression of *PGRP-LC* was not affected by either Gram-negative or Gram-positive bacteria. Because other genes in the IMD pathway were impacted by treatment, the mRNA levels of *PGRP-LC* relative to the control may indicate that a different immune receptor in the flea digestive tract regulates the IMD pathway. For example, in *D. melanogaster*, *PGRP-LE* & *PGRP-LA* also modulate the activation of the IMD pathway in the midgut (Neyen et al., 2012; Gendrin et al., 2013). Likewise, in mosquito spp., *PGRP-LA* positively regulates the IMD pathway (Gendrin et al., 2017). Although the putative transcript of *PGRP-LC* recovered from the *C. felis* genome appears to be conserved with that of *D. melanogaster*, further study is needed to characterize the function of the *PGRP-LC*-like immune receptor in the gut of the cat flea.

Next, in the IMD signaling cascade, PGRP-LC recruits the adaptor protein IMD, which associates with Fadd and Dredd to initiate the cleavage and subsequent translocation of the transcription factor Relish into the cell nucleus. In the current study, expression of *IMD* increased in the cat flea digestive tract at 24 hours post-exposure to the two laboratory model bacteria species (*S. marcescens* and *M. luteus*); whereas, increased expression levels of *Relish* were observed in the digestive tracts of cat fleas at 4 hours post-exposure to *S. marcescens* only. The importance of *IMD* and *Relish* in the IMD signaling cascade against flea-borne pathogens has been demonstrated in cat and rat fleas. For example, knockdown of *IMD* and *Relish* prior to oral

infection with the Gram-negative bacterium *R. typhi* led to an increase in bacterial load in the *C. felis* midgut relative to control fleas (Rennoll et al., 2017). In the rat flea (*X. cheopis*), *Relish* was upregulated in the digestive tract at 4-hours post-exposure to the Gram-negative bacterium *Y. pestis* (Bland et al., 2020).

The translocation of *Relish* into the cell nucleus initiates the transcription of several insect AMP genes, such as *Attacin* and *Defensin*. Although the functional essence of these peptides has been extensively studied in fly spp. (*D. melanogaster*, *Hermetia illucens, Bactrocera doralis*) and other holometablous insects, Attacins remain an understudied class of antimicrobial peptides across hematophagous arthropods. Attacins are most often associated with anti-Gram-negative activity; however, in some insects (*e.g.*, *Bactrocera doralis*, *Spodoptera exigua*), other isoforms of these peptides do not appear to discriminate against Gram-types (Buonocore et al., 2021). In the present study, the *C. felis Attacin* gene was upregulated in response to exposure to a Gram-negative *S. marcescens*-infected bloodmeal at 4 hours only. The expression of *Attacin* in *S. marcescens*-exposed groups returned to control group levels at 24 hours, and was not affected by the Gram-negative, flea-borne pathogen *B. henselae* or the Gram-positive bacterium *M. luteus*. In *D. melanogaster*, Defensins have often been implicated in the defense against Gram-positive bacteria; however, a recent study has demonstrated variants of Defensin to have activity against Gram-negative bacteria (Gao et al., 2024). This is in stark contrast with the Defensins of other Dipterans such as *A. aegypti*, as well as putative Defensins of some hemipterans and arachnids, which have consistently proven to have activity against both Gram-negative and Gram-positive bacteria (Lowenberger et al., 1995; Kaushal et al., 2016; Meraj et al., 2022; Wu et al., 2022). Antimicrobial peptides are diversely regulated by the IMD and/or Toll pathway(s) across hematophagous insects. Moreover, although potentially tissue-

specific, the co-regulation of Defensins by the IMD and Toll pathways has been implicated in several insect studies (Salcedo-Porras et al 2019; Meraj et al., 2021; Meraj et al., 2022; Alejandro et al., 2022). However, in *A. aegypti* Aag2 cells, AMPs including several isoforms of Defensin appear to be regulated by the IMD pathway in response to *E. coli* (Zhang R et al., 2017). Although *Defensin* expression is known to occur in the midgut of insects, it is unclear as to which pathways regulate *Defensin*. The present study revealed increased expression of *Defensin* in flea digestive tracts at 24 hours post-exposure to the Gram-negative bacterium *S. marcescens* only. Additionally, similar to above for *Attacin*, expression of *Defensin* was not affected by the Gram-negative, flea-borne pathogen *B. henselae* or the Gram-positive bacterium *M. luteus*.

Finally, *PGRP-LB* is released in the lumen, negatively regulating the IMD pathway. Here, *PGRP-LB* was found to be upregulated in fleas exposed to *S. marcescens* during early (4 hours post-exposure) and later stages of infection (24 hours post-exposure). Additionally, the expression of *PGRP-LB* increased between 4- and 24-hours post-exposure to an infected bloodmeal. In a study on rat fleas (*X. cheopis*), *PGRP-LB* was found to be upregulated in the digestive tracts of fleas challenged with *Y. pestis* at 4 hours post-exposure. However, increased expression of *PGRP-LB* was also demonstrated to occur as a response to blood feeding alone (Bland et al., 2020). Although further study is needed, the transcription of AMPs may decline due to the negative feedback loop initiated in part by *PGRP-LB* activation. Ultimately, as demonstrated originally in *D. melanogaster*, and later in many hematophagous insects, the upregulation of *PGRP-LB* may suggest that AMPs are tightly regulated in cat fleas to prevent an excess of damage to internal organs (Zaidman-Rémy et al., 2006; Royet and Dziarski, 2007; Bland et al., 2020).

4.2. The role of the Toll pathway in the digestive tract of cat fleas

Typically, Gram-positive bacteria solely activate the Toll signaling pathway. However, it has become apparent that the insect immune response mediated against Gram-positive bacteria can be co-regulated by the IMD and Toll pathway, although there is limited support that Toll signaling occurs in the gut epithelium (Schmid et al., 2014; Salcedo-Porras et al 2019; Alejandro et al., 2022). Moreover, the Toll pathway is not known to play a direct role in the gut immune response of fruit flies (*D. melanogaster*); however, in the digestive tract of the rat flea (*X. cheopis*), a negative regulator of the Toll pathway, *Cactus,* was upregulated in response to *Y. pestis* infection (Bland et al., 2020). As such, we investigated the expression of select Toll pathway genes (*PGRP-SA*, *Toll*) including the negative regulator *Cactus* in the digestive tract of the cat flea following bacterial challenge. Of the three Toll associated genes, no significant differences in expression levels were observed between infected and naïve fleas at any experimental time point. Therefore, the data suggests that the Toll signaling pathway does not participate in the response to infection with the Gram-negative bacterial pathogens *B. henselae* and *S. marcescens*, nor the Gram-positive bacterium *M. luteus* in the digestive tracts of cat fleas.

4.3. The associated antibacterial activity of proteins isolated from the digestive tract of cat fleas

Several studies have illustrated the complexity of the relationship between the transcription of AMPs and the associated antimicrobial activity across hematophagous insects. For example, in female bed bugs (*C. lectularius*), the expression of *clDefensins* peaked in the midgut at 12 hours post-injection with the Gram-negative bacterium *E. coli* and Gram-positive bacterium *Bacillus subtilis* followed by a steep decrease in expression at 24 hours post-injection; however, antimicrobial activity in the midgut was found to be substantially higher at 24 hours

post-injection with the aforementioned bacteria relative to 8 hours post-injection (Meraj et al., 2021). In *R. prolixus*, expression of *Defensin A*, *Defensin B*, and *Defensin C* decreased substantially at 7 days post-exposure to *S. aureus* and *E. coli*; however, in measuring the antimicrobial activity of midgut contents following infection, there was increased antimicrobial activity against *S. aureus in vitro* but not *E. coli* (Vieira et al., 2014). In the current study, despite the increase in mRNA levels of several IMD associated genes (including the downstream target *Attacin*), proteins isolated from flea digestive tracts at 4 hours post-exposure to an infected bloodmeal reflected similar antimicrobial activity against *M. luteus* to that of the control. On the other hand, proteins isolated from flea digestive tracts at 24-hours post exposure to an infected bloodmeal demonstrated potent antimicrobial activity against *M. luteus in vitro*. In conjunction with the increase in mRNA levels of *Defensin* at 24 hours, this could potentially highlight the anti-Gram-negative and- positive activity of Defensins, as demonstrated in other hematophagous insects (Lowenberger et al., 1995; Vieira et al., 2014; Kaushal et al., 2016; Meraj et al., 2022).

4.4. The flea immune response to oral infection with B. henselae

Although the flea immune response has been examined in response to *R. typhi* and *Y. pestis* infection, no studies have examined the flea immune response against *B. henselae* (Rennoll et al., 2018; Bland et al., 2020). To address this gap in knowledge, we examined the transcriptional and functional response of associated gut proteins to oral infection with *B. henselae*. Interestingly, we observed an increase in antimicrobial activity *in vitro*, despite no impact on the expression of genes comprising the IMD and Toll pathways or the effector genes *Attacin* and *Defensin*. Because *B. henselae* is a flea-borne pathogen, this bacterium may possess properties that interfere with the transcription/translation of select AMPs. In the body louse, transcriptional levels of select immune genes, including *Defensin 1*, *Defensin 2*, and *PGRP*, were

found to be similar to the control in the alimentary tract at 4- and 8-days post-exposure to *Bartonella quintana* (Kim et al., 2017). However, when the antimicrobial activity of recombinant Defensins 1 and 2 in body lice was measured *in vitro* against *B. quintana*, recombinant Defensin 2 displayed increased antimicrobial activity against this pathogen. Meanwhile, the results regarding the antimicrobial activity of recombinant Defensin 1 against *B. quintana* were not significant (Yoon et al., 2023). Additionally, other effector and IMD/Toll pathway genes may be involved in the response to *B. henselae* infection, or other humoral immune defenses may be at play, such as reactive oxygen species (Bland et al., 2020; Brown et al., 2021). Moreover, several studies have implicated digestive serine proteases in the response against flea-borne pathogens. For example, serine proteases (trypsin and chymotrypsin) have been found to be upregulated in response to challenge with *B. henselae* and *R. typhi* in *C. felis* as well as in the closely associated *X. cheopis* in response *to Y. pestis* (Dreher-Lesnick et al., 2010; Zhou et al., 2012; Bland et al., 2020; André et al., 2022).

Conclusion

There are several limitations to our study. First, the antimicrobial activity was measured *in vitro* against a Gram-positive bacterium. Because fleas primarily vector Gram-negative bacteria, measuring the antimicrobial activity of gut proteins derived from infected fleas against a Gram-negative bacterium would provide a more comprehensive viewpoint to this study (Vieira et al., 2014; Batista KKS et al., 2021). Second, to examine the relationship between the transcription and translation of AMPs, as well as the efficacy of AMPs against different bacterial pathogens, AMPs would need to be isolated/purified from flea digestive tract proteins. Alternatively, the immune contribution of AMP genes could be determined by silencing select AMP genes via RNAi prior to the immune challenge (Morejon and Michel, 2023). Third,

expression was examined in select effector/pathway genes; further study examining all immune genes comprising the IMD and Toll pathways, as well as all flea effector (AMP) genes, would provide a more comprehensive outlook as to how AMPs are regulated in response to bacterial infection. Finally, pre-screening fleas prior to dissections using a fluorescently labeled pathogen would circumvent the discrepancies observed between experiments regarding flea infection prevalence.

Collectively taken, this study contributes to the standing knowledge of immune signaling pathways and AMP-mediated responses against bacterial infection in hematophagous arthropods. The results from the current study suggest that immune signaling responses are induced in a highly specific and potentially time-dependent manner in response to invading bacterial pathogens in the flea digestive tract. Specifically, the IMD pathway is activated in response to both the Gram-negative bacterium *S. marcescens* and the Gram-positive bacterium *M. luteus*. Transcriptional activation of the IMD pathway has also occurred with respect to the flea-borne pathogens *Rickettsia typhi* (*C. felis*) and *Yersinia pestis* (*X. cheopis*) (Rennoll et al., 2017, Bland et al., 2020), however, *B. henselae* does not appear to induce a transcriptional response. Additionally, this study provides evidence that antimicrobial activity increases in the digestive tract of fleas after exposure to bacterial pathogens *S. marcescens*, *M. luteus*, and *B. henselae*. Ultimately, these findings provide critical insights as to how cat fleas modulate responses against bacterial species in the gut.

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