Pathogens and Disease, 79, 2021, ftab020

doi: 10.1093/femspd/ftab020
Advance Access Publication Date: 26 March 2021
Research Article

RESEARCH ARTICLE

Dynamic gene expression in salivary glands of the cat flea during *Rickettsia felis* infection

Monika Danchenko*, Hanna J. Laukaitis and Kevin R. Macaluso†

Department of Microbiology and Immunology, University of South Alabama College of Medicine, 610 Clinic Drive, Mobile, AL 36688, USA

*Corresponding author: Department of Microbiology and Immunology, University of South Alabama College of Medicine, 610 Clinic Drive, Mobile, AL 36688, USA. Tel: +1 (251) 460-1878; E-mail: mdanchenko@southalabama.edu

One sentence summary: This study demonstrated gene expression patterns of immunity-related and flea-specific transcripts in the salivary glands of the cat flea in response to *Rickettsia felis* infection.

Editor: Jason Carlyon
†Kevin R. Macaluso, http://orcid.org/0000-0001-8537-6100

ABSTRACT

The cat flea, *Ctenocephalides felis*, is an arthropod vector capable of transmitting several human pathogens including *Rickettsia* species. Earlier studies identified *Rickettsia felis* in the salivary glands of the cat flea and transmission of rickettsiae during arthropod feeding. The saliva of hematophagous insects contains multiple biomolecules with anticlotting, vasodilatory and immunomodulatory activities. Notably, the exact role of salivary factors in the molecular interaction between flea-borne rickettsiae and their insect host is still largely unknown. To determine if *R. felis* modulates gene expression in the cat flea salivary glands, cat fleas were infected with *R. felis* and transcription patterns of selected salivary gland-derived factors, including antimicrobial peptides and flea-specific antigens, were assessed. Salivary glands were microdissected from infected and control cat fleas at different time points after exposure and total RNA was extracted and subjected to reverse-transcriptase quantitative PCR for gene expression analysis. During the experimental 10-day feeding period, a dynamic change in gene expression of immunity-related transcripts and salivary antigens between the two experimental groups was detected. The data indicated that defensin-2 (*Cf-726*), glycine-rich antimicrobial peptide (*Cf-83*), salivary antigens (*Cf-169* and *Cf-65*) and deorphanized peptide (*Cf-75*) are flea-derived factors responsive to rickettsial infection.

Keywords: cat flea; *Rickettsia*; salivary gland; gene expression

INTRODUCTION

Fleas are small (0.5–8 mm), apterous insects with both sexes of adult fleas being hematophagous. The cat flea, *Ctenocephalides felis*, is an ectoparasite ubiquitously found throughout the world with public health and veterinary importance related to both the direct impact of feeding and as a vector for several infectious microorganisms, including obligate intracellular *Rickettsia* species (Dryden and Rust 1994; Rust 2017). In addition to transmission of *Rickettsia typhi*, an emerging rickettsial agent, *Rickettsia felis*, was first identified by electron microscopy in the midgut epithelium of the cat flea (Adams, Schmidtmann and Azad 1990). Since its initial discovery, *R. felis* has been found in a variety of hematophagous arthropods. However, *R. felis* has been most frequently detected in the cat flea and, until recently, it was the only confirmed biological vector and reservoir of *R. felis* (Reif and Macaluso 2009; Brown and Macaluso 2016).

Received: 29 January 2021; **Accepted:** 24 March 2021

Flea-borne rickettsial transmission to a vertebrate host occurs via feeding as well as infectious arthropod feces. There is strong evidence that *R. felis* is transmitted horizontally by the cat flea primarily through infectious saliva (Legendre and Macaluso 2017). Previous studies demonstrated the microorganism within the salivary glands of fleas by transmission electron microscopy, PCR and immunofluorescence assay (Macaluso *et al.* 2008; Thepparit *et al.* 2013). Furthermore, naïve cats exposed to infected cat fleas seroconverted and rickettsial DNA was detected by PCR in the blood of those cats (Wedincamp and Foil 2000). While feeding on the host organism, bloodsucking insects secrete proteins to facilitate pathogen survival and transmission. In addition to known anticlotting, vasodilatory and anti-inflammatory effectors, insect saliva contains some unique proteins with unknown functions (Sakhon *et al.* 2013). For the cat flea, the salivary glands and the secreted factors play a pivotal role in blood-feeding.

To elucidate salivary factors associated with cat fleas, a cDNA library of the salivary glands was generated from adult cat fleas, annotated and compared to the rat flea *Xenopsylla cheopis*, which was investigated by similar means (Andersen *et al.* 2007). Sialomes of the cat and rat flea have in common a FS-I/H antigen family and the deorphanized 8-Cys families of peptides, which are unique to these organisms. The glycine-rich peptide and a novel short peptide family were found only in the cat flea (Ribeiro *et al.* 2012). While groups of immunity-related and flea-specific antigens have been identified in the cat flea salivary glands, their exact role in pathogen infection and transmission is unknown. Likewise, modification of the cat flea midgut transcriptional response to rickettsial infections has been demonstrated in other systems (Dreher-Lesnick *et al.* 2010; Rennoll *et al.* 2018), yet the relationship between the gene expression patterns and transmission is unresolved. Toward understanding the *Rickettsia* and flea interaction, this study was designed to assess differential expression of selected salivary gland transcripts by quantitative reverse transcription PCR (RT-qPCR) in *R. felis*-challenged cat fleas. Transcription analysis indicates that infection with *R. felis* affects gene transcription of flea-specific salivary factors in a non-uniform manner, suggesting a complex interplay between vector feeding and rickettsial infection.

MATERIALS AND METHODS

Source of fleas and *Rickettsia*

Adult cat fleas were purchased from Elward II Laboratory colony (EL) and maintained under standardized conditions within an artificial host system as previously described (Wade and Georgi 1988). Prior to use in assays, a portion of unfed fleas was tested to verify the absence of *R. felis* infection using a species-specific quantitative PCR (qPCR; Odhiambo *et al.* 2014).

Rickettsia felis strain LSU (passage 3) was propagated in an *Ixodes scapularis*-derived cell line (ISE6), maintained in a modified L15B growth medium in a humidified incubator with 5% CO_2 at 32°C. Cellular infection was examined by Diff-Quik staining as described previously (Pornwiroon *et al.* 2006).

Cat flea infection and experimental design

A total of 400 fleas (sex ratio 1:1) were allowed to feed on heat-inactivated bovine blood (HemoStat Laboratories, Dixon, CA, USA) for 24 h. Following pre-feeding, cat fleas were starved for 6 h and subsequently given access to either a *R. felis*-infected bloodmeal or a bloodmeal containing uninfected ISE6 cells (control). For the *R. felis*-infection assay, rickettsiae were enumerated with the *Bac*Light Viability Kit (Invitrogen, Molecular Probes, Eugene, OR, USA) as described previously (Sunyakumthorn *et al.* 2008) and diluted to an inoculation dose of 3×10^{10} rickettsiae/mL (Brown *et al.* 2015). For the control treatment, intact uninfected ISE6 cells were prepared in an identical manner (Reif *et al.* 2011). Experimental fleas were allowed to feed on the *R. felis*-infected or control bloodmeal for 24 h, after which they continued feeding on defibrinated bovine blood for the duration of the study.

Fifty cat fleas were collected from each experimental group at 1, 3, 5, 7 and 10 days post-exposure (dpe). Upon collection, cat fleas were cleaned by sequential surface washing with 10% bleach for 2 min, 70% ethanol for 2 min, followed by rinsing with sterile distilled water three times. Female and male fleas were anesthetized on a chill table (BioQuip, Rancho Dominguez, CA, USA), and intact salivary glands and other tissues were microdissected in 0.01 M sterile phosphate-buffered saline (PBS) pH 7.2 on a glass depression slide. A total of two independent assays were carried out for each experimental group.

Extraction of nucleic acids from salivary glands

At each time point, pools of 100 salivary glands (per sex) were placed in 800 μL of TRIzol Reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA), and total RNA was extracted from salivary glands following a modified phenol-chloroform isolation protocol (Pandit, Cooper-White and Punyadeera 2013). The RNA pellet was resuspended in 25 μL of ultrapure RNase-free water (Invitrogen, Life Technologies, Grand Island, NY, USA). Subsequently, RNA samples were treated with DNase I (Promega, Thermo Fisher Scientific, Vilnius, Lithuania) according to the manufacturer's instructions. The quantity and quality of the extracted RNA samples were evaluated with ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Reverse transcription was performed on 200 ng of extracted RNA from each sample in 20 μL reaction volumes using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Complementary DNA was amplified using random hexamer primers. To confirm the absence of DNA contamination, no-reverse transcriptase controls were included. All samples were stored at −20°C until further processing.

Total DNA was isolated from the pooled salivary gland samples after nucleic acid separation with TRIzol Reagent according to the manufacturer's instructions with modifications. Briefly, 100 μL of TE buffer (Invitrogen, Thermo Fisher Scientific, Vilnius, Lithuania) and 150 μL of chloroform (Fisher Scientific, Fair Lawn, NJ, USA) were added to the interphase containing DNA, and samples were thoroughly vortexed and centrifuged for 10 min at 14 000 × *g* at 4°C. The aqueous phase (200 μL) was transferred into a new microcentrifuge tube and precipitated with a double volume of absolute ethanol (Fisher Scientific, Fair Lawn, NJ, USA), GlycoBlue Coprecipitant (Invitrogen) and 5 M ammonium acetate (Invitrogen, Thermo Fisher Scientific, Vilnius, Lithuania) overnight at −80°C. DNA samples were then pelleted, washed with 70% ethanol and centrifuged again for 10 min at 10 000 × *g* at 4°C. Salivary gland DNA samples were air-dried for 30 min at room temperature and resuspended in 15 μL of TE buffer.

Selection of flea salivary molecules and design of oligonucleotides

We selected a subset of flea-specific and immunity-related factors previously identified in the uninfected cat flea sialotranscriptome (Ribeiro *et al.* 2012). Transcripts were chosen based

on their predicted functional classification (Table 1). Oligonucleotide sets were derived from the cat flea sialotranscriptome sequences available on GenBank (BioProject 165049), designed using the Primer3 software (Rozen and Skaletsky 2000) and evaluated using the Beacon Designer software (http://premierbiosoft.com/). The specificity of each TaqMan type probe and primer pair was assessed with Primer Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The oligonucleotide primers and probes (Table 1) were synthesized by Integrated DNA Technologies.

Representative qPCR products for each transcript were verified by gel analysis and cloned into pCR4-TOPO vector (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. M13 forward and M13 reverse primers were used in the sequencing reaction to obtain sequence data for each insert. Amplicons were analyzed using the BioEdit Sequence Alignment Editor software (Hall 1999).

Gene expression analysis

The salivary gland-derived transcripts obtained by cDNA synthesis were subjected to qPCR for gene expression analysis. Components of qPCR, including iTaq Universal Probes Supermix (Bio-Rad, Hercules, CA, USA), 200 nM of each forward and reverse primer, 300 nM of each fluorogenic probe, nuclease-free water and 5 μL of cDNA template, or serial dilutions of standards, were pre-mixed in 35 μL volumes in 96-well plates and aliquoted in triplicate of 10 μL reactions to 384-well plates. Amplifications were carried out in a LightCycler 480 II Instrument (Roche Life Sciences, Indianapolis, IN, USA). The amplification conditions were as follows: an initial denaturation step at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing and elongation at 58°C for 60 s, with fluorescence acquisition in single mode.

To correct experimentally induced differences between samples, normalization was performed. The endogenous reference gene, elongation factor 1-α (*Cf-Ef*), was previously reported as a reliable candidate for the gene expression analyses in the cat flea (McIntosh *et al.* 2016). A fluorescent hydrolysis probe (5′TGC CTT CCA AAC CCA TGT GTG TC 3′) was additionally designed for the qPCR assay, based on the *Cf-Ef* transcript sequence (#XM_026608272) available in GenBank.

To quantify copies of each transcript of interest in the flea salivary gland samples, 10-fold serial dilutions of pCR4-TOPO plasmids containing a single-copy portion of the individual corresponding nucleotide target sequence were used to generate a standard curve. The relative abundance of transcripts, coding selected salivary factors in the *R. felis*-infected fleas, was compared at each time point to the transcription level in control cat fleas that fed on the uninfected ISE6-containing bloodmeal. The relative expression of each target gene of interest (*goi*) was normalized to the reference gene in each sample (*goi*/*Cf-Ef*) and compared to the ratio of *goi*/*Cf-Ef* in uninfected control fleas. The gene expression levels of the controls were set as a baseline and were used to determine relative change in gene expression during *R. felis*-infections. Mean fold changes and standard deviations from two biological replicates were calculated.

Molecular detection of *R. felis* in fleas

To quantify the *R. felis* load in treated cat fleas, total genomic DNA was extracted from dissected organs and individual flea bodies devoid of salivary glands from each collection point using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Bacterial load in salivary gland pools and individual flea carcasses was determined by qPCR as mentioned above (Odhiambo *et al.* 2014). The quantity of rickettsiae in the samples was calculated as a ratio of rickettsial *ompB* gene to the copy number of a single copy flea-specific gene (*Cf-Ef*), providing the *R. felis* genomic equivalent per host cell. Quantitative PCR analyses used the plasmid pCR4-TOPO-*CfEf* or -*ompB* as a standard template to create serial 10-fold dilutions. To confirm the identity of *R. felis* detected by qPCR in the flea salivary glands, standard PCR with the sequencing of the rickettsial 17-kDa antigen gene was performed (Jiang *et al.* 2013). A 440 bp segment amplified with the primers R17k.31F and R17k.469R was assembled using the BioEdit Sequence Alignment Editor software (Hall 1999), and the obtained sequences were compared with those available in the GenBank database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). An environmental control (extraction reagents without a biological sample) and negative control (ultrapure water instead of a template) were utilized for each DNA extraction and PCR methods.

Statistical analyses

For statistical analyses, a two-way analysis of variance (ANOVA) was performed with Fisher's LSD test (within each time point comparing groups) or Tukey's test (within each group comparing change over time). For all comparisons, a *P*-value of <0.05 was considered significantly different. Analyses were done using Prism 8 software (GraphPad Software, San Diego, CA, USA).

RESULTS

In an attempt to survey differentially expressed salivary gland transcripts of the cat flea, the transcriptional profiles were compared between *R. felis*-infected and uninfected controls. Prior to each experiment, a portion ($n = 11$ per sex) of newly emerged fleas were confirmed negative for *R. felis* by qPCR. For the experiments, 25 females and 25 males were dissected at each collection time point. The tested samples included total RNA and DNA extracted from pooled tissues at 1, 3, 5, 7 and 10 dpe. The dissected salivary glands were tested by species-specific qPCR for the presence of *R. felis* to evaluate the bacterial burden in the pooled tissue samples. *Rickettsia felis* was detected in the cat flea salivary glands, both in females and males, at each time point, with the highest load ($1.51 \times 10^5 \pm 8.56 \times 10^4$ rickettsiae/10^6 flea cells) at 3 dpe. Viability of bacteria in the salivary gland samples was confirmed by amplification of rickettsial *ompB* from cDNA synthesized from pooled salivary gland total RNA extracts at all time points (Table S1, Supporting Information). The presence of *R. felis* in the salivary glands was also confirmed by conventional PCR and sequencing, using the 17-kDa antigen gene primer set. Amplicons were identical to the published sequences for *R. felis* LSU (Pornwiroon *et al.* 2006). Likewise, the remaining carcasses of representative individuals were tested by qPCR in the same manner as the salivary gland samples to determine the infection prevalence and the bacterial load in the cat fleas exposed to an *R. felis*-infected bloodmeal (Table S2, Supporting Information). The infection prevalence in flea carcasses ranged from 72.7 to 100%, with extraction and PCR controls negative in each trial.

Based on the previously published sialotranscriptome of the cat flea, two immunity-related (*Cf-726* and *Cf-83*) and four flea-specific factors (*Cf-169*, *Cf-65*, *Cf-12* and *Cf-75*) transcription was assessed by RT-qPCR assays in the salivary glands (Table 1).

Table 1. List of oligonucleotides designed for transcriptional analysis.

Class	Transcript	Oligonucleotide sequence (5′ → 3′)	Amplicon size (bp)	Annotation (Accession #)
Antimicrobial peptides	*Cf-726*	F: TGT TCG CCA CCG TTC AAT CC R: CGC AAG TAA CCC GCT TCT GT P: GGA GAG CCA GTT GAG CAG TTG GT	144	Defensin-2; arthropod antibacterial defensin family (#JW050225)
	Cf-83	F: CAA GGT CAT GGT CAA GGT CAT R: CCT CCA GGT TTG TGA TGG TT P: CGG TGG TGG TCA TGG TGG TCA T	125	Glycine-rich protein; unique for the cat flea, similar to attacin/holotricin-3 (#JW050193)
Flea-specific antigens	*Cf-169*	F: CAG ATT CAG ATT CCG ATG CAA A R: TCT GGG AGG TCT ACA ATA ACA AA P: CGA GGT GGA GAT GAT GCT GGA TGT	116	FS-I salivary antigen 1; unique flea antigen (#JW050190)
	Cf-65	F: CCA AGA TGG AAG AAA TAA TCA GGA R: TGG TGC TTC ACA GTA ACA ATG P: TGG CGG TTG TGG AAA TGG TGG T	130	FS-H salivary antigen 3; unique flea antigen (#JW050226)
	Cf-12	F: TGC TTT AGT AGT TGT CCA TTG TGT R: ACT TAT TTG GTG TCG TCC TCC T P: GGT AAA CAG TAT AGC GAA TGC TCC A	85	Flea-specific secreted salivary protein (#JW050235)
	Cf-75	F: AAG GGC TTC TGG TGT TAC TG R: TCC TTT CTT GGC ACT ACA TTC A P: TCA GGG CAA CCT ACA AGT TCC AGA	121	Deorphanized peptide; flea-specific secreted salivary protein (#JW050195)

F—forward primer; R—reverse primer; P—TaqMan probe.

Differential expression patterns of the selected salivary gland-specific transcripts between *R. felis*-infected and uninfected control cat fleas were observed. Likewise, changes in transcription dynamics of individual genes during the feeding period at each time point were identified (Fig. 1). Interestingly, an overall down-regulation of flea-specific transcript abundance in *Rickettsia*-infected cat fleas at 1 dpe was detected. Over the course of feeding, the majority of tested salivary transcript expression levels were elevated in response to the *R. felis* challenge.

In this study, an increase in gene expression of both antimicrobial peptides (AMPs) *Cf-726* and *Cf-83* was observed (Fig. 1, panel A). There was a consistent increase in *Cf-726* transcript levels in the challenged group compared to the control group at all time points assessed. The transcript abundance differed most significantly at 10 dpe (6.23-fold; $P = 0.0064$). The expression of the antimicrobial peptide *Cf-83* was significantly upregulated at 3 dpe in the salivary glands of infected cat fleas, mirroring the highest rickettsial burden in these tissues (7.16-fold; $P = 0.0334$). This result suggests that the flea reacts to rickettsial infection with an antimicrobial response.

The gene expression of salivary antigens *Cf-169* and *Cf-65* from the infected cat fleas was significantly altered compared to the uninfected controls (Fig. 1, panel B). Similar to the transcript *Cf*-83, the greatest difference in transcription for *Cf-169* and *Cf-65* was observed at 3 dpe (1.76-fold; $P = 0.0033$, 2.30-fold; $P = 0.0013$, respectively), when the recorded bacterial load in the salivary glands was highest. The salivary antigen 1 (*Cf-169*) was significantly down-regulated at 1 dpe (3.32-fold; $P = 0.0320$), with a significant change in the transcript abundance of *Cf-169* between 1 dpe and 10 dpe ($P = 0.0013$). Although a decrease in gene expression of the salivary antigen 3 (*Cf*-65) at 1 dpe was followed by an increase in the transcript abundance at later time points, the variation in transcription was not significant.

Based on the obtained RT-qPCR data, the flea-specific transcript *Cf-12* was abundantly expressed in the salivary glands of both *R. felis*-infected and uninfected fleas. However, no significant change in expression between treatment or time points was detected (Fig. 1, panel C). The gene expression of the deorphanized flea-specific transcript *Cf-75* was dynamic in the presence of *R. felis* (Fig. 1, panel C). One day after exposure, a significant decrease of *Cf-75* was detected in the salivary glands of *R. felis*-infected fleas (4.50-fold; $P = 0.0337$). Moreover, significant differences in the transcriptional level between *R. felis*-infected and uninfected salivary glands were identified (1 dpe to 7 dpe; $P = 0.0195$ and 1 dpe to 10 dpe; $P = 0.0128$). Combined, the current study identified unique transcriptional patterns in response to rickettsial infection.

DISCUSSION

Rickettsia felis is an emerging human pathogen, the etiological agent of flea-borne spotted fever (Schriefer *et al.* 1994). This microorganism has been increasingly identified worldwide as a cause of disease, with human cases reported on every continent except for Antarctica (Angelakis *et al.* 2016). The clinical symptoms of *R. felis* infection are similar to those of other rickettsioses, including high fever, headache, myalgia and maculopapular rash (Abdad, Stenos and Graves 2011; Blanton and Walker 2017). Severe clinical manifestations due to *R. felis* infection have also been reported (Zavala-Velázquez *et al.* 2000; Lindblom, Severinson and Nilsson 2010), including two fatal cases of acute meningoencephalitis in Indonesia (Mawuntu *et al.* 2020). A recent study demonstrated by culture isolation, immunofluorescence and PCR that domestic dogs can act as vertebrate reservoir hosts for *R. felis* (Ng-Nguyen *et al.* 2020). *Rickettsia felis* was demonstrated to be horizontally transmitted from infected dogs to naïve cat fleas, as well as from naturally infected cat fleas to seronegative dogs. However, the rickettsemia level in the dogs, nor the actual inoculation dose a flea may be exposed to, were not determined. As cat fleas are the primary flea species on domestic dogs, the transmission cycle of *R. felis* between vector and vertebrate host requires further examination.

Bioactive molecules in the salivary glands of hematophagous arthropods have evolved to enable feeding and novel compounds of putative secreted molecules, some of which appear to be genus or species-specific, have been discovered via salivary

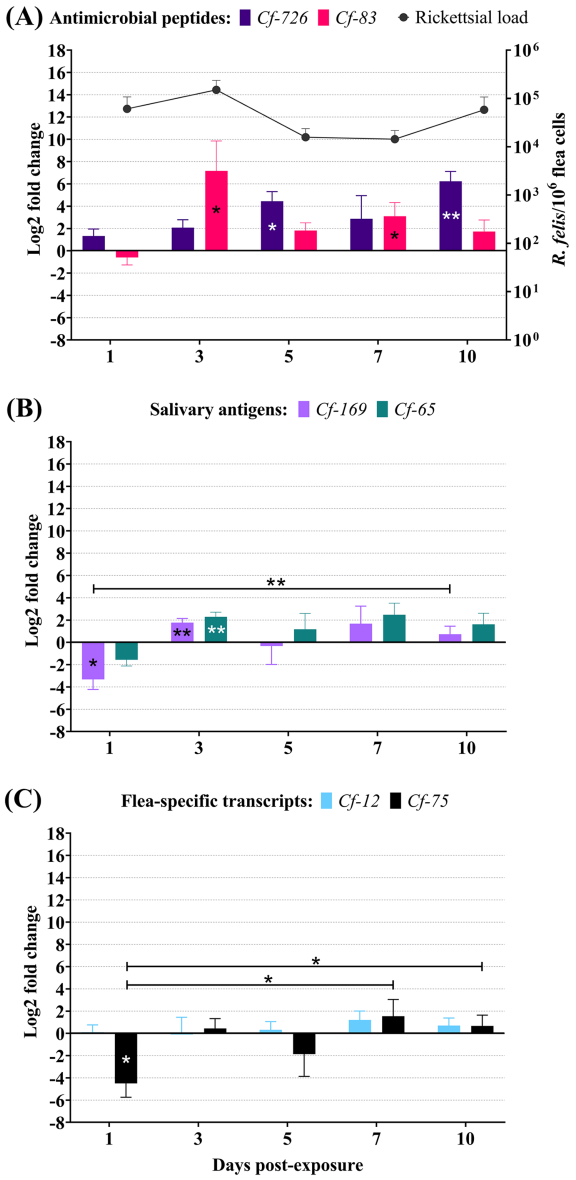

Figure 1. Gene expression of selected antimicrobial peptides (*Cf-83* and *Cf-726* in panel **A**), salivary antigens (*Cf-65* and *Cf-169* in panel **B**) and flea-specific salivary transcripts (*Cf-12* and *Cf-75* in panel **C**). Salivary glands were dissected from both control fleas (fed uninfected ISE6 cells) and fleas infected with *R. felis* at 1, 3, 5, 7 and 10 days after exposure. The relative abundance of transcripts in the infected fleas was compared to the control fleas at each time point. Data are normalized to the reference gene, elongation factor 1-α. For statistical analyses, two-way ANOVA was performed (*$P < 0.05$, **$P < 0.01$). Standard errors of the mean were calculated from two independent biological replicates. Asterisks in bars indicate statistical difference between infected and uninfected fleas at the same collection time point; asterisks above lines represent change over time within the infected group. The bacterial load in the pooled salivary glands was calculated as a ratio of a single copy flea-specific gene to the copy number of rickettsial *ompB* gene, as determined by qPCR.

transcriptome analyses (Andersen *et al.* 2007; Leitner, Costero-Saint Denis and Wali 2011). To enhance their chances of survival and subsequent transmission, vector-borne pathogens can take advantage of vector feeding as well as modulate the vector immune system by receptor-ligand and signaling pathway interactions or gene transcription regulation (Targett 2006; Fontaine *et al.* 2011; Bland *et al.* 2020). The current study aimed to characterize the transcriptional response of selected immunity-related and flea-specific antigens in the salivary glands of the cat flea in response to infection with *R. felis*. While *R. felis* infection of cat flea salivary glands has been examined microscopically (Macaluso *et al.* 2008), the current data provide valuable insight into the molecular interactions associated with infection.

Antimicrobial peptides are multifunctional components of the innate immune system in multicellular organisms. Insect AMPs defend against microorganisms via a variety of mechanisms such as membrane disruption/depolarization, inhibition of membrane protein synthesis, interference with metabolism, or targeting cytoplasmic components (Baxter, Contet and Krueger 2017; Wu, Patočka and Kuča 2018). In addition to direct microbial lysis, AMPs can bind and neutralize endotoxins and modulate the host immunity (Brogden 2005; Zhang and Gallo 2016). Although AMPs are mainly synthesized by the fat body and certain hemocytes (Bulet *et al.* 1999), multiple arthropod tissues are capable of the gene expression of AMPs, including the midgut, ovaries and salivary glands (Ceraul *et al.* 2007; Ribeiro *et al.* 2012; Sunyakumthorn *et al.* 2013; Zumaya-Estrada, Rodríguez and Rodríguez 2018; Rennoll *et al.* 2018).

Defensins are a family of arginine-rich AMPs with strong activity against a broad range of microorganisms. A common group of inducible AMPs, defensin structural features allow binding to the cell membrane to form pore-like defects, which facilitates the efflux of intracellular components (Ganz and Lehrer 1995). A typical defensin, transcript *Cf-726*, was identified in the sialotranscriptome of the cat flea and the predicted protein sequence has a signal peptide for secretion with the arthropod defensin antibacterial family domain of the Antimicrobial peptide database (Wang and Wang 2004). Infection of a tick, *Dermacentor variabilis*, with *Rickettsia montanensis* resulted in the activation of defensin-1 and -2, which is suggested to be a limiting factor of *R. montanensis* infection (Ceraul *et al.* 2007; Pelc *et al.* 2014). Consistent with studies in other arthropod vectors, elevated defensin-2 (*Cf-726*) transcription in the salivary glands of *R. felis*-infected cat fleas was observed. Although there was no direct correlation between AMP transcription and rickettsial load, the factors contributing to rickettsial infection in the salivary glands are not known. It is likely that cat fleas secrete rickettsiae back into the blood source over the course of feeding (Brown *et al.* 2015), and the activation of defensin had a negative effect on the rickettsial load; the balance of transmission biology and host response regulating *R. felis* burden in tissues remains an active area of investigation. In contrast with the findings in ticks and the current study, defensin transcription was not detected in the cat flea midgut in response to a *R. typhi* challenge (Dreher-Lesnick *et al.* 2010). Likewise, the infection of *Drosophila* cells *in vitro* with *R. typhi* induced the expression of a subset of AMP genes, but no significant increase in defensin transcript levels was detected (Rennoll *et al.* 2018). It can be expected that differences in immune stimulation are observed when comparing arthropod tissues (salivary glands vs. midgut) or species of *Rickettsia* (*R. felis* vs. *R. typhi*), similar to what has been observed in ticks (Sunyakumthorn *et al.* 2013). The specificity and inducibility of defensin family members in the cat flea during rickettsial infection requires further examination.

Another important group of AMPs are those with an overrepresentation of glycine residues in their amino acid sequence. They are effective mainly against Gram-negative bacteria (attacins, holotricin-2), although some (holotricin-3) show antimicrobial activity against Gram-positive bacteria and fungi as well (Hultmark *et al.* 1983; Lee *et al.* 1995; Carlsson *et al.* 1998). Attacins inhibit the biosynthesis of the bacterial outer membrane proteins thereby preventing cell division (Carlsson *et al.*

1991). The transcript *Cf-83* is a unique glycine-rich peptide with an antimicrobial domain in its primary structure found exclusively in the cat flea sialotranscriptome. The observed upregulation in gene expression pattern of *R. felis*-infected cat fleas suggests that the transcript *Cf-83* can play a role in response to bacterial infection of the salivary glands; however, the direct impact on *R. felis* remains unknown.

Flea bites often cause local cutaneous inflammatory reactions largely determined by salivary components. The oral secretions of the cat flea contain components responsible for producing a hypersensitivity reaction called flea allergy dermatitis (Trudeau *et al.* 1993). Infestation by the cat flea can cause severe cutaneous reactions and dermatitis; however, few studies have examined flea antigens (Greene, Penhale and Thompson 1993; Greene *et al.* 1993; Lee, Jackson and Opdebeeck 1997). The FS-I/FS-H antigen family refers to peptides annotated as flea antigens, consisting of a group of 15 related peptides that share signal peptides indicating secretion (Lee *et al.* 1999; Andersen *et al.* 2007). In total, two members of this family were recognized in the cat flea sialotranscriptome, namely the FS-I/FS-H antigen family member 1 (*Cf-169*) and the FS-I/FS-H antigen family member 3 (*Cf-65*). In the present study, changes in expression profiles of these salivary antigen transcripts were distinct in the cat flea, reflecting a different overall response to *R. felis* infection. Although the presence of these transcripts in the cat flea salivary glands indicates their role in blood-feeding, the biological function of salivary antigens is largely unknown, and the influence of rickettsial infection on flea feeding requires detailed examination.

Lastly, two flea-specific salivary transcripts, *Cf-12* and *Cf-75*, were selected for transcriptional analysis in the presented study. The predicted peptide sequence encoded by *Cf-12* belongs to a novel short protein family of yet unknown function. Although *Cf-12* was consistently expressed in the salivary glands during the feeding period, no significant changes were observed in the transcription levels in response to *R. felis* infection. In contrast, the transcript *Cf-75*, belonging to the deorphanized 8-cys peptide family and putatively identified as beta-neurotoxin Td2, which acts as a local anesthetic and potential plasminogen interacting protein (Tordai, Bányai and Patthy 1999; Ribeiro *et al.* 2012), displayed a dynamic transcriptional regulation in the infected salivary glands. Further functional characterization of these transcripts and a role for the subsequent protein in rickettsial infection and transmission is currently under investigation.

Upon inoculation with an infectious bloodmeal, rickettsial pathogens colonize the midgut and subsequently disseminate to the salivary glands, presumably via hemolymph. Migration of *R. felis* to the salivary glands was previously reported to take 7–14 days, as observed by immunofluorescence assay (Thepparit *et al.* 2013). Further studies revealed that transmission of *R. felis* via co-feeding occurs between cat fleas as soon as 12–24 h; however, rickettsiae were not detected in the salivary glands 1 dpe by qPCR or immunofluorescence assay; therefore, early-phase transmission was concluded to be mechanical as bacteria were released from contaminated mouthparts during probing events (Brown *et al.* 2016). Biological and mechanical transmission of pathogens is not mutually exclusive and co-occur in the same arthropod vector (Desquesnes *et al.* 2009). The utilization of both mechanisms might be very favorable depending on the transmission cycle of the pathogen. The current study demonstrated that infection of the salivary glands by rickettsiae occurred within 24 h and rickettsial DNA was also detected by qPCR in the hemolymph of infected cat fleas as early as 1 dpe (Table S3, Supporting Information). These results suggest that *R. felis* can cross the midgut epithelium and migrate through the hemocoel rapidly. The detection of rickettsial infection at earlier time points may be the result of the sensitivity of the *ompB* target sequence probe-based assay, which was determined to be 10 times greater than the 17-kDa antigen gene target sequence and SYBR Green assay used in the earlier study (Henry *et al.* 2007). Additional experiments focusing on the infection time course within the cat flea are required to detail the kinetics of pathogen biology in the cat flea vector.

The presence of infectious bacteria in the salivary glands of blood-feeding arthropods alone alters saliva composition, such as the selective down-regulation of salivary factors in *Borrelia*-infected ticks or the selective up-regulation of the tick salivary genes *salp15* and *salp16* by the spirochetes and *Anaplasma phagocytophilum*, respectively (Ramamoorthi *et al.* 2005; Sukumaran *et al.* 2006). Less is known with respect to intermittent feeding insects such as cat fleas, but bacterial infection of salivary glands may be important to investigate as an approach to interfere with successful flea feeding. Based on the gene expression data, we identified a differential response to rickettsial infection of cat flea salivary glands. Elucidating the molecular relationship between arthropods and *Rickettsia* is essential in deciphering the basis of vector competence, transmission events and the epidemiology of emerging and re-emerging rickettsial diseases.

AUTHORS CONTRIBUTION

MD: Development of the methodology; Planning and executing the experiments; Data analysis; Writing of the original manuscript.

HL: Cell culture maintenance and propagation of *Rickettsia*; Contribution to the data analysis.

KRM: Conceptualization; Contribution to experimental design and data analysis.

All authors discussed the results and agreed on the final manuscript.

ACKNOWLEDGMENTS

We thank Dr. Sean P. Riley and Dr. R. Ryan Wood for the helpful comments regarding experimental design and data analysis.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSPD* online.

FUNDING

This work was supported by the National Institutes of Health [AI122672 to K.R.M.].

Conflicts of Interest. None declared.

REFERENCE

Abdad MY, Stenos J, Graves S. *Rickettsia felis*, an emerging flea-transmitted human pathogen. *Emerg Health Threats J* 2011;**4**:7168.

Adams JR, Schmidtmann ET, Azad AF. Infection of colonized cat fleas, *Ctenocephalides felis* (Bouché), with a rickettsia-like microorganism. *Am J Trop Med Hyg* 1990;**43**:400–9.

Andersen JF, Hinnebusch BJ, Lucas DA *et al.* An insight into the sialome of the oriental rat flea, *Xenopsylla cheopis* (Rots). *BMC Genomics* 2007;**8**:102.

Angelakis E, Mediannikov O, Parola P *et al*. *Rickettsia felis*: the complex journey of an emergent human pathogen. *Trends Parasitol* 2016;**32**:554–64.

Baxter RH, Contet A, Krueger K. Arthropod innate immune systems and vector-borne diseases. *Biochemistry* 2017;**56**:907–18.

Bland DM, Martens CA, Virtaneva K *et al*. Transcriptomic profiling of the digestive tract of the rat flea, *Xenopsylla cheopis*, following blood feeding and infection with *Yersinia pestis*. *PLoS NeglTrop Dis* 2020;**14**:e0008688.

Blanton LS, Walker DH. Flea-borne rickettsioses and rickettsiae. *Am J Trop Med Hyg* 2017;**96**:53–56.

Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?. *Nat Rev Microbiol* 2005;**3**:238–50.

Brown LD, Banajee KH, Foil LD *et al*. Transmission mechanisms of an emerging insect-borne rickettsial pathogen. *Parasit Vectors* 2016;**9**:237.

Brown LD, Christofferson RC, Banajee KH *et al*. Cofeeding intra- and interspecific transmission of an emerging insect-borne rickettsial pathogen. *Mol Ecol* 2015;**24**:5475–89.

Brown LD, Macaluso KR. An emerging flea-borne rickettsiosis. *Curr Trop Med Rep* 2016;**3**:27–39.

Bulet P, Hetru C, Dimarcq JL *et al*. Antimicrobial peptides in insects; structure and function. *Dev Comp Immunol* 1999;**23**:329–44.

Carlsson A, Engström P, Palva ET *et al*. Attacin, an antibacterial protein from *Hyalophora cecropia*, inhibits synthesis of outer membrane proteins in *Escherichia coli* by interfering with omp gene transcription. *Infect Immun* 1991;**59**:3040–5.

Carlsson A, Nyström T, de Cock H *et al*. Attacin–an insect immune protein–binds LPS and triggers the specific inhibition of bacterial outer-membrane protein synthesis. *Microbiology* 1998;**144**:2179–88.

Ceraul SM, Dreher-Lesnick SM, Gillespie JJ *et al*. New tick defensin isoform and antimicrobial gene expression in response to *Rickettsia montanensis* challenge. *Infect Immun* 2007;**75**:1973–83.

Desquesnes M, Biteau-Coroller F, Bouyer J *et al*. Development of a mathematical model for mechanical transmission of trypanosomes and other pathogens of cattle transmitted by tabanids. *Int J Parasitol* 2009;**39**:333–46.

Dreher-Lesnick SM, Ceraul SM, Lesnick SC *et al*. 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 Mol Biol* 2010;**19**:229–41.

Dryden MW, Rust MK. The cat flea: biology, ecology and control. *Vet Parasitol* 1994;**52**:1–19.

Fontaine A, Diouf I, Bakkali N *et al*. Implication of haematophagous arthropod salivary proteins in host-vector interactions. *Parasit Vectors* 2011;**4**:187.

Ganz T, Lehrer RI. Defensins. *Pharmacol Ther* 1995;**66**:191–205.

Greene WK, Carnegie RL, Shaw SE *et al*. Characterization of allergens of the cat flea, *Ctenocephalides felis*: detection and frequency of IgE antibodies in canine sera. *Parasit Immunol* 1993;**15**:69–74.

Greene WK, Penhale WJ, Thompson RC. Isolation and in vitro translation of messenger RNA encoding allergens of the cat flea, *Ctenocephalides felis*. *Vet Immunol Immunopathol* 1993;**37**:15–23.

Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 1999;95–98.

Henry KM, Jiang J, Rozmajzl PJ *et al*. Development of quantitative real-time PCR assays to detect *Rickettsia typhi* and *Rickettsia felis*, the causative agents of murine typhus and flea-borne spotted fever. *Mol Cell Probes* 2007;**21**:17–23.

Hultmark D, Engström A, Andersson K *et al*. Insect immunity. Attacins, a family of antibacterial proteins from *Hyalophora cecropia*. *EMBO J* 1983;**2**:571–6.

Jiang J, Maina AN, Knobel DL *et al*. Molecular detection of *Rickettsia felis* and *Candidatus Rickettsia asemboensis* in fleas from human habitats, Asembo, Kenya. *Vector Borne Zoonotic Dis* 2013;**13**:550–8.

Lee SE, Jackson LA, Opdebeeck JP. Salivary antigens of the cat flea, *Ctenocephalides felis felis*. *Parasit Immunol* 1997;**19**:13–19.

Lee SE, Johnstone IP, Lee RP *et al*. Putative salivary allergens of the cat flea, *Ctenocephalides felis felis*. *Vet Immunol Immunopathol* 1999;**69**:229–37.

Lee SY, Moon HJ, Kurata S *et al*. Purification and cDNA cloning of an antifungal protein from the hemolymph of *Holotrichia diomphalia* larvae. *Biol Pharm Bull* 1995;**18**:1049–52.

Legendre KP, Macaluso KR. *Rickettsia felis*: a review of transmission mechanisms of an emerging pathogen. *Trop Med Infect Dis* 2017;**2**.

Leitner WW, Costero-Saint Denis A, Wali T. Immunological consequences of arthropod vector-derived salivary factors. *Eur J Immunol* 2011;**41**:3396–400.

Lindblom A, Severinson K, Nilsson K. *Rickettsia felis* infection in Sweden: report of two cases with subacute meningitis and review of the literature. *Scand J Infect Dis* 2010;**42**:906–9.

Macaluso KR, Pornwiroon W, Popov VL *et al*. Identification of *Rickettsia felis* in the salivary glands of cat fleas. *Vector Borne Zoonotic Dis* 2008;**8**:391–6.

Mawuntu AHP, Johar E, Anggraeni R *et al*. *Rickettsia felis* identified in two fatal cases of acute meningoencephalitis. *PLoS NeglTrop Dis* 2020;**14**:e0007893.

McIntosh CH, Baird J, Zinser E *et al*. Reference gene selection and RNA preservation protocol in the cat flea, *Ctenocephalides felis*, for gene expression studies. *Parasitology* 2016;**143**:1532–42.

Ng-Nguyen D, Hii SF, Hoang MT *et al*. Domestic dogs are mammalian reservoirs for the emerging zoonosis flea-borne spotted fever, caused by *Rickettsia felis*. *Sci Rep* 2020;**10**:4151.

Odhiambo AM, Maina AN, Taylor ML *et al*. Development and validation of a quantitative real-time polymerase chain reaction assay specific for the detection of *Rickettsia felis* and not *Rickettsia felis*-like organisms. *Vector Borne Zoonotic Dis* 2014;**14**:476–81.

Pandit P, Cooper-White J, Punyadeera C. High-yield RNA-extraction method for saliva. *Clin Chem* 2013;**59**:1118–22.

Pelc RS, McClure JC, Sears KT *et al*. Defending the fort: a role for defensin-2 in limiting *Rickettsia montanensis* infection of *Dermacentor variabilis*. *Insect Mol Biol* 2014;**23**:457–65.

Pornwiroon W, Pourciau SS, Foil LD *et al*. *Rickettsia felis* from cat fleas: isolation and culture in a tick-derived cell line. *Appl Environ Microbiol* 2006;**72**:5589–95.

Ramamoorthi N, Narasimhan S, Pal U *et al*. The Lyme disease agent exploits a tick protein to infect the mammalian host. *Nature* 2005;**436**:573–7.

Reif KE, Kearney MT, Foil LD *et al*. Acquisition of *Rickettsia felis* by cat fleas during feeding. *Vector Borne Zoonotic Dis* 2011;**11**:963–8.

Reif KE, Macaluso KR. Ecology of *Rickettsia felis*: a review. *J Med Entomol* 2009;**46**:723–36.

Rennoll SA, Rennoll-Bankert KE, Guillotte ML *et al*. The Cat Flea (*Ctenocephalides felis*) immune deficiency signaling pathway regulates *Rickettsia typhi* infection. *Infect Immun* 2018;**86**.

Ribeiro JM, Assumpção TC, Ma D *et al*. An insight into the sialotranscriptome of the cat flea, *Ctenocephalides felis*. *PLoS One* 2012;**7**:e44612.

Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 2000;**132**:365–86.

Rust MK. The biology and ecology of cat fleas and advancements in their pest management: a review. *Insects* 2017;**8**:118.

Sakhon OS, Severo MS, Kotsyfakis M *et al*. A Nod to disease vectors: mitigation of pathogen sensing by arthropod saliva. *Front Microbiol* 2013;**4**:308.

Schriefer ME, Sacci JB, Dumler JS *et al*. Identification of a novel rickettsial infection in a patient diagnosed with murine typhus. *J Clin Microbiol* 1994;**32**:949–54.

Sukumaran B, Narasimhan S, Anderson JF *et al*. An *Ixodes scapularis* protein required for survival of *Anaplasma phagocytophilum* in tick salivary glands. *J Exp Med* 2006;**203**:1507–17.

Sunyakumthorn P, Bourchookarn A, Pornwiroon W *et al*. Characterization and growth of polymorphic *Rickettsia felis* in a tick cell line. *Appl Environ Microbiol* 2008;**74**:3151–8.

Sunyakumthorn P, Petchampai N, Grasperge BJ *et al*. Gene expression of tissue-specific molecules in ex vivo *Dermacentor variabilis* (Acari: ixodidae) during rickettsial exposure. *J Med Entomol* 2013;**50**:1089–96.

Targett GA. Parasites, arthropod vectors, and immune responses. *Parasit Immunol* 2006;**28**:117–9.

Thepparit C, Hirunkanokpun S, Popov VL *et al*. Dissemination of bloodmeal acquired *Rickettsia felis* in cat fleas, *Ctenocephalides felis*. *Parasit Vectors* 2013;**6**:149.

Tordai H, Bányai L, Patthy L. The PAN module: the N-terminal domains of plasminogen and hepatocyte growth factor are homologous with the apple domains of the prekallikrein family and with a novel domain found in numerous nematode proteins. *FEBS Lett* 1999;**461**:63–67.

Trudeau WL, Fernández-Caldas E, Fox RW *et al*. Allergenicity of the cat flea (*Ctenocephalides felis felis*). *Clin Exp Allergy* 1993;**23**:377–83.

Wade SE, Georgi JR. Survival and reproduction of artificially fed cat fleas, *Ctenocephalides felis* Bouché (Siphonaptera: pulicidae). *J Med Entomol* 1988;**25**:186–90.

Wang Z, Wang G. APD: the antimicrobial peptide database. *Nucleic Acids Res* 2004;**32**:590D–592.

Wedincamp J, Foil LD. Infection and seroconversion of cats exposed to cat fleas (*Ctenocephalides felis* Bouché) infected with *Rickettsia felis*. *J Vector Ecol* 2000;**25**:123–6.

Wu Q, Patočka J, Kuča K. Insect antimicrobial peptides, a mini review. *Toxins* 2018;**10**:461.

Zavala-Velázquez JE, Ruiz-Sosa JA, Sánchez-Elias RA *et al*. *Rickettsia felis* rickettsiosis in Yucatán. *Lancet North Am Ed* 2000;**356**:1079–80.

Zhang LJ, Gallo RL. Antimicrobial peptides. *Curr Biol* 2016;**26**:R14–9.

Zumaya-Estrada FA, Rodríguez MC, Rodríguez MH. Pathogen-insect interaction candidate molecules for transmission-blocking control strategies of vector borne diseases. *Salud Publica de Mexico* 2018;**60**:77–85.