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Investigation of Differential Vector Competence of *Bartonella quintana* in Human Head and Body Lice

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**INVESTIGATION OF DIFFERENTIAL VECTOR COMPETENCE OF *Bartonella*
quintana IN HUMAN HEAD AND BODY LICE**

A Thesis Presented

by

DOMENIC J. PREVITE

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

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Molecular and Cellular Biology

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DEDICATION

To my family for their love and support through the entirety of my education

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ABSTRACT

DIFFERENTIAL VECTOR COMPETENCE OF *Bartonella quintana* IN HUMAN
HEAD AND BODY LICE

SEPTEMBER 2012

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Directed by: Professor John M. Clark, Ph.D.

Human head and body lice are obligatory hematophagous ectoparasites that belong to a single species *Pediculus humanus*. Only body lice, however, are vectors of the infections gram-negative bacteria *Bartonella quintana*. Due to their near identical genomes, yet differential vector competency, head and body lice provide an ideal model system to study an insects ability to gain or lose vector competency. Using our *in vitro* louse rearing system, we have infected both head and body lice with a blood containing *B. quintana* in order to detect differences in *B. quintana* proliferation between head and body lice as well as transcriptional regulation of immune-related genes. *B. quintana* proliferates rapidly in body lice after 6 days post-infection, but declines in head lice after 4 days post-infection, possibly explaining, in part, the differential vector competence between the two insects.

A transcriptome analysis using whole lice followed by qPCR verification of head and body lice immune-related genes was then conducted using uninfected, versus *B. quintana* infected lice to identify potential genes involved in vector competence. The

immune-related genes Defensin 1, Fibrinogen-related protein and Spaetzle, were differentially regulated between head and body lice and were identified as potential targets for future research.

Previously studied immune-related genes, PGRP, Defensin 1 and Defensin 2 transcription levels were also assessed in body louse midgut using qPCR following *B. quintana* infection. In this case, *B. quintana* infection did not result in significant effects on the transcript levels of these genes in midgut tissue. Overall transcriptional profiles of head and body lice genomes were notably different, including difference in the expression of 18.3 % of immune related genes, a finding that strongly supports the contention that immune system differences between head and body lice are the primary reason for difference in vector capacity.

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CHAPTER 1

INTRODUCTION

1.1 *Bartonella quintana*

Bartonella quintana is a slow growing, hemotropic, gram-negative bacterium that infects humans as their reservoir host. *B. quintana* is the causative agent for trench fever, a major epidemic, which affected millions in the world wars that has since subsided with the use of antibiotics (Byam and Lloyd, 1920). Today this epidemic is re-emerging as urban trench fever in the homeless and poor, especially those who are immune-compromised such as alcoholics and those infected with human immunodeficiency virus (Hotez, 2008). Trench fever, also known as 5 day fever, is defined as infection of human blood by *B. quintana* and typically includes severe head aches, pain in the legs, weakness, nausea, anorexia, abdominal pain and insomnia. It can also cause more serious conditions such as bacillary angiomatosis and endocarditis (Byam and Lloyd, 1920). *B. quintana* was recognized as being vectored between humans primarily through human body lice (*Pediculus humanus corporis*) as early as 1920 (Byam and Lloyd, 1920).

B. quintana grows inside the gut lumen of body lice and has no affect on lice viability (Weyer, 1960). *B. quintana* proliferation has been quantified in body lice over a 17 day period where growth over the first 6 days of the experiment following infection was stagnant and significant proliferation was not seen until day 7 (Seki *et al.*, 2007). From day 7 until day 15 of the experiment, *B. quintana* increases by over three orders of magnitude (Seki *et al.*, 2007). *B. quintana* multiplies throughout the life cycle of the louse, and remain in the lice feces as they are excreted (Kostrzewski, 1949) *B. quintana*

survives remarkably well in lice feces and can remain viable as long as one year (Kostrzewski, 1949). The bacteria are thought to be inoculated into the human blood stream through the feces of lice when an infested person scratches themselves, abrading the skin and allowing penetration through the skin (Raoult and Roux, 1999). *B. quintana* eventually travels to the blood stream where it colonizes inside of erythrocytes (Harms and Dehio, 2012). The erythrocytes are then taken up by the lice during feeding and the cycle starts again (Harms and Dehio, 2012). To date, however, there is only limited research on the molecular interactions involving *B. quintana* and the human body and head louse.

1.2 Human body and head lice

Human body (*Pediculous humanus humanus*) and head (*Pediculous humanus capitis*) lice are obligatory hematophagous ectoparasites that have thrived solely on human blood for 5-7 million years (Light *et al.*, 2003; Reed *et al.*, 2004). Lice are hemimetabolous insects, described by their nymphal instars compared to larva-pupa-adult transformations of holometabolous insects. Body lice evolved from conspecific head lice when humans started wearing clothing roughly 40,000-70,000 years ago (Kittler *et al.*, 2003). As suggested by their names, these two ecotypes occupy different niches on the human body. Head lice localize on the scalp and lay eggs on the shafts of human hair whereas body lice are found on the body only temporarily while blood feeding and return to the clothing when not feeding. Typically, body lice lay their eggs on clothing rather than on hair (Buxton, 1946). Morphologically, head and body lice share minor differences, most notably that body lice are larger than head lice. This larger size is

thought to allow for a bigger blood meal necessary for body lice since they are off of the human body more frequently and must survive longer periods of time without a blood meal than head lice (Reed *et al.*, 2004). Head lice take more frequent blood meals than do body lice and remain on the scalp continuously (Alpatov and Nasjukaova, 1955). In nature, head and body lice are not known to interbreed due to spatial separation (Busvine, 1978). Under laboratory conditions, however, they can interbreed to produce viable and fertile offspring (Bacot, 1917). Like all hematophagous insects, both ecotypes maintain an obligate bacteria endosymbiont, *Candidatus Riesia pediculicola*, which localize in various louse mycetomes and provide lice with essential B-vitamins that are lacking in their nutritionally-deficient human blood diet (Allen *et al.*, 2007; Perotti *et al.*, 2007).

The debate of the status of head and body lice as distinct species has been ongoing for many years. Some argue that head and body lice are no more than subspecies/ecotypes and are the result of intra-species variation (Nuttall, 1920. Ferris, 1935). Others believe that body lice are too similar to be a distinct species but instead are a species in the making, whereas some believe that differences in head and body lice are more than sufficient to consider them completely distinct species based upon their morphological and ecological characteristics (Busvine, 1944; Buxton, 1946; Fahrenholz, 1916; Shaefer, 1978). Depending on the definition used for species (i.e., biological, ecological, morphological, evolutionary, phylogenetic), one can argue they are distinct species or they are simply morphotypes of the same species.

The body louse genome has been recently fully sequenced and annotated and has revealed that the body lice has the smallest known genome of any insect (108-110 Mb) (Kirkness *et al.*, 2010). It encodes only 10,773 protein coding genes, 161 tRNAs, 57

microRNAs and 90% of the protein encoding genes share homology with sequences in other known insect species (Kirkness *et al.*, 2010).

Several different molecular analyses of speciation between head and body lice have produced a more definite, but not absolute, answer, which indicates that head and body lice are not distinct species but morphotypes/ecotypes of one species. Phylogenetic and population summary statistics conducted by Leo *et al.*, (2002), Kittler *et al.*, (2003), Reed *et al.*, (2004) and Barker *et al.*, (2005) have concluded that head and body lice are the same species. The most recent study, comparing transcriptional profiles of head and body lice directly for the first time using expressed sequence tagging (EST), showed yet again that genetically head and body lice are part of the same species with nucleotide diversity ranging from only 0.1-1.3% (Olds *et al.*, 2012).

Although the general consensus is that head and body lice are simply morphotypes within the same species, there are distinct physiological differences between the two that have major medical and social implications. The main physiological difference between the two morphotypes, is that only the body lice is known to be a vector of human disease (Lounibos, 2002). These diseases include epidemic typhus, relapsing fever, and trench fever, which are the result of infection by the gram-negative bacteria *Rickettsia prowazekii*, *Borrelia recurrentis*, and *Bartonella quintana*, respectively (Eremeeva *et al.*, 2007; Andersson and Dehio, 2000; Raoult and Roux, 1999). Because the genomes are statistically identical, differences in transcriptomes, proteomes and epigenetics are likely responsible for vector competency. The issue of gaining or losing vector competency in insects and host/pathogen interaction thus far is poorly understood. For this purpose, head and body lice present an ideal model system,

particularly in the study of the differences in their immune systems and how these differences influence vector competency (Kim *et al.*, 2011).

The human body louse genome is substantially smaller when compared to *D. melanogaster* and has only 93 immune response related genes (1/2~1/3 of other insects) (Kirkness *et al.*, 2010). Because human body lice feed almost exclusively on sterile blood, louse exposure to foreign pathogens is far less extensive than *D. melanogaster* and this process is believed to have resulted in a reduced number of immune response genes necessary for body lice to survive over evolutionary time (Kim *et al.*, 2011).

The innate immune response system of insects has a variety of anti-microbial pathways including, the Immune Deficiency pathway (IMD), Toll pathway, and Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway, which have been studied extensively and mostly in *D. melanogaster*. These immune pathways ultimately lead to the regulation of insect humoral immune response, including effector genes encoding the anti-microbial peptides (AMPs), Defensin 1 and Defensin 2. These immune pathways also regulate the cellular immune response in insects including the effector genes nodular and prophenyloxidase (PPO), which affect the louses phagocytic response.

Interestingly, human lice lack a functional IMD pathway, the major humoral immune pathway in dipterans and other higher order insects for selective protection against gram-negative bacteria. Human lice contain only one bacterial pattern recognition protein, (PGRP) compared to 13 in *D. melanogaster*. The louse PGRP has domains I, II and III common to many insect PGRPs and is the recognition protein thought to be responsible for upregulating the Toll pathway (Kim *et al.*, 2011). While

many insects, such as *D. melanogaster*, have many PGRPs specific to either gram-positive or gram-negative, lice PGRP is thought to sense both types of bacteria in that it has the highest sequence identity (30.5%) to the gram-negative sensing PGRP-B in *Ostrinia nubilalis*, and second highest sequence identity (28.9%) to the gram-positive sensing PGRP-LA in *Tribolium castaneum* (Khajuria *et al.*, 2011).

The primary infection route of pathogenic bacteria to human lice is through an infected blood meal that is ingested initially into the louse alimentary track. The first line of louse defense therefore is thought to be the use of AMPs excreted into the gut lumen. Human lice have only two functional genes encoding AMPs, Defensin 1 and Defensin 2, and these AMPs are thought to be up regulated via PGRP as part of the Toll pathway (Kim *et al.*, 2011).

Recently, a study was published using septic infection of two model bacteria, a gram-positive *Staphylococcus aureus* and a gram-negative *Escherichia coli*. Transcriptional profiling by qRT-PCR of the immune genes PGRP, Defensin 1 and Defensin 2 in whole lice revealed that both head and body lice elicited an increased humoral immune response to *S. aureus* by upregulating the transcription of Defensin 1 and 2 genes. *E. coli* elicited a no such response and only the head louse PGRP gene was up-regulated following infection over a 48 hour time period (Kim *et al.*, 2011). Although limited transcriptional immune response was seen between head and body lice upon *E. coli* infection, the *E. coli* population steadily increased over 48 hours in body lice whereas in head lice the population steadily decreased (Kim *et al.*, 2011).

A further study was conducted using oral infection of *E. coli* in blood, and subsequent transcriptional profiling of immune related genes in alimentary track tissue,

the first line of defense against bacterial ingestion (Kim *et al.*, 2012). Under uninfected conditions, head lice appear to maintain PGRP and Defensin 1 and 2 genes at higher basal transcription levels (3.7-fold, 6.1-fold and 2.3-fold), respectively compared to body lice, in order to respond quickly to invading bacteria (Kim *et al.*, 2012). Once again *E. coli* rapidly proliferated in body lice gut tissue, whereas *E. coli* steadily decreased in head lice over a 48 hour time period (Kim *et al.*, 2012). Although there was a significant up-regulation of PGRP, Defensin 1 and Defensin 2 upon *E. coli* infection in body lice, the reduced basal level of effector Defensin genes below the critical level needed for rapid response may allow for rapid proliferation of *B. quintana* (Kim *et al.*, 2012).

Previous studies on the interactions of human lice and bacteria have used the model gram-negative bacteria *E. coli* in order to study proliferation and transcriptional regulation on a limited number of genes. Our current studies using *B. quintana*-fed lice observed differences between head and body lice including the proliferation of *B. quintana*, transcriptional regulation of louse immune related genes, as well as possible interaction with the louse endosymbiont *Reisia*, in order to better elucidate the mechanisms by which body lice have evolved to become a pathogenic vector, while head lice have not. The primary hypothesis behind body lice gaining vector competency of gram-negative bacteria is the reduction of the humoral immune response over evolutionary time in order to maintain a large population of their gram-negative endosymbiont, *Reisia*, which in turn provide more B-vitamins and energy to support the larger body louse living off its human host for longer periods of time.

In this thesis, we will infect human lice with *B. quintana* and quantify the number of *B. quintana* cells in head and body lice over time in order to identify differences in *B.*

quintana proliferation in head versus body lice. We will also infect human lice with *B. quintana* and look at the whole genomic transcriptome profiles of head and body lice at 8 days post-infection, in order to identify possible candidate genes involved in vector competency. Finally, we will look at the transcriptional profiles of PGRP, Defensin 1 and Defensin 2 in alimentary tract tissues following *B. quintana* infection, in order to validate previous studies done using the model gram-negative bacteria *E. coli*.

CHAPTER 2

***B. quintana* PROLIFERATION IN HEAD AND BODY LICE**

2.1 *B. quintana* culture

The JK31 strain of *B. quintana*, originally obtained from Dr. Jane Koehler (University of California-San Francisco), was maintained in a biosafety level 2 (BSL2) facility at the Environmental and Molecular Toxicology Laboratory at the University of Massachusetts-Amherst. Frozen *B. quintana* stock was cultured on chocolate agar plates following Dr. Koehler's protocols (37 °C, culture plates in CO₂ jars) for 7 to 10 days. *B. quintana* was then passed to a fresh chocolate agar plate and cultured for an additional 4 to 5 days before use.

2.2 Body and head lice colonies

A field population of human body louse (Frisco-BL) was originally collected from 9 homeless individuals in San Francisco (December, 2008) by Dr. Jane Koehler's research group and a head louse population (BR-HL) was collected from Bristol, United Kingdom (Yoon et al., 2004). Both colonies were maintained on the *in vitro* rearing system with human blood supplemented with a penicillin/streptomycin solution at 1 µl/ml (Sigma Chemicals, St. Louis, MO) (Yoon *et al.*, 2006). Late third instars or first day adults were fed on non-antibiotic blood for 3 days prior to an experiment to ensure antibiotics are purged from adult lice.

2.3 Louse infection with *B. quintana* via a blood meal

B. quintana was harvested from a single chocolate agar plate using a sterile loop and suspended into 1 ml PBS (0.1 M, pH 7.2, 1 plate/ml PBS). Spectrophotometric readings (OD₆₀₀) were taken for *B. quintana* suspended in PBS to approximate cell counts per ml of blood. *B. quintana* cells were pelleted by centrifugation at 1000g for 4 minutes, and resuspended in 100 µl of fresh PBS to remove residual media. A 10 µl aliquot of the suspension was serially diluted into M199 media (Sigma Chemicals) supplemented with glutamine, sodium pyruvate and 20% fetal bovine serum (M199S), and dilutions (10⁻⁴-10⁻⁸ ml PBS suspension/ml M199S) were plated in triplicate for CFU/ml blood determination. The remaining suspension was then mixed with 4 ml of human blood without antibiotics to obtain a titer of approximately 1 x 10⁷ CFU/ml (Kosoy *et al.*, 2004) and used to fill the blood reservoir of each feeding unit. Approximately 100 lice, which had been starved for 6 hours, were fed on *B. quintana*-infected blood using the *in vitro* rearing system for 18 hours (overnight). Following feeding, lice were transferred to a new rearing unit with non-infected blood for the remainder of the experiments. Mortality data was also recorded for all experiments.

2.4 Detection of *B. quintana* proliferation by quantitative PCR (qRT-PCR)

Five *B. quintana*-infected lice were collected at various time points [0 (immediately after taking a blood meal), 2, 4, 6, 8, 10, 12 days] post-infection and the number of *B. quintana* cells/louse determined by qPCR. gDNA was extracted using Qiagen DNeasy blood and tissue kit according to manufacturer's instructions (Qiagen,

Valencia CA). qRT-PCR was performed to quantify cell counts with a SYBR-green power mix using the StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad CA) with Bq-ITS-F and Bq-ITS-R primers (Table 2), which amplified an 89 base pair fragment in the 16S-23S ribosomal RNA gene of *B. quintana* (Seki *et al.*, 2007). Five non-infected lice from each colony were also sampled to ensure there was no background *B. quintana* contamination in the colony. The number of amplified DNA copies were calculated based on Ct values of a standard curve of known copy numbers of a 134 base pair fragment containing the target 89 base pair fragment. gDNA extraction was normalized using qPCR with primers against the louse voltage-sensitive sodium channel alpha-subunit gene (Table 2). All experiments were standardized to the lowest number of *B. quintana* cells per louse at the 0 day time point in order to normalize the proliferation curve. Two-sided Student's t-tests were used to compare the number of *B. quintana* cells in head and body lice at each time point using Microsoft Excel ($P < 0.05$).

2.5 Results of *B. quintana* infection and profliteration

A total of 5 replicated experiments were completed for both head and body lice, however, not all replicates had lice samples taken from identical time points post-infection. Head lice experiments had 5 replicates at time points 0, 2 and 4 days, 4 replicates at 6 days, and 3 replicates at 8, 10 and 12 days. Body lice had 5 replicates at time point 0, 4 replicates at 2 days, 5 replicates at 4 days, 4 replicates at 6 days, 5 replicates at 8 days and 3 replicates at 10 and 12 days. A statistically similar amount of *B. quintana* cells per ml of blood was used for each experiment. For head lice, an

average of $4.47 \times 10^8 \pm 1.09 \times 10^8$ CFU/ml blood was used in each of the five experiments. Body lice replicates were fed an average of $3.77 \times 10^8 \pm 1.34 \times 10^8$ CFU/ml blood. These values are not significantly different ($P > 0.05$). It was also determined that there were no differences in the mortality response between head and body lice following *B. quintana* infection (data not shown).

B. quintana cell counts per head or body louse were both normalized to 9.45×10^4 at the 0 day time point post-infection in order to compare cell counts between head and body lice (Fig. 1).

Head lice at 2 days post-infection had an average of $1.34 \times 10^5 \pm 6.06 \times 10^4$ *B. quintana* cells per louse (1.41 \pm 0.64-fold change over 0 day time point); 4 days post-infection had an average of $9.84 \times 10^4 \pm 5.95 \times 10^4$ *B. quintana* cells per louse (1.04 \pm 0.62-fold change over 0 day time point); 6 days post-infection had an average of $4.95 \times 10^4 \pm 2.46 \times 10^4$ *B. quintana* cells per louse (0.52 \pm 0.26-fold change over 0 day time point); 8 days post-infection had an average of $1.78 \times 10^4 \pm 5.26 \times 10^3$ *B. quintana* cells per louse (0.19 \pm 0.056-fold change over 0 day time point); 10 days post-infection had an average of $1.23 \times 10^4 \pm 3.98 \times 10^3$ *B. quintana* cells per louse (0.13 \pm 0.042-fold change over 0 day time point); and 12 days post-infection had an average of $1.56 \times 10^4 \pm 2.33 \times 10^3$ *B. quintana* cells per louse (0.16 \pm .025-fold change over 0 day time point).

Body lice at 2 days post-infection had an average of $6.15 \times 10^4 \pm 3.04 \times 10^4$ *B. quintana* cells per louse (0.65 \pm 0.33-fold change over 0 day time point); 4 days post-infection had an average of $1.75 \times 10^5 \pm 7.32 \times 10^4$ *B. quintana* cells per louse (1.86 \pm 0.80-fold change over 0 day time point); 6 days post-infection had an average of $1.52 \times 10^5 \pm 4.78 \times 10^4$ *B. quintana* cells per louse (1.61 \pm 0.62-fold change over time point 0);

8 days post-infection had an average of $1.13 \times 10^6 \pm 2.74 \times 10^5$ (9.58 ± 3.53 -fold change over 0 day time point); 10 days post-infection had an average of $2.69 \times 10^6 \pm 6.52 \times 10^5$ (32.46 ± 6.90 -fold change over 0 day time point); 12 days post-infection had an average of $2.88 \times 10^6 \pm 4.49 \times 10^5$ (30.46 ± 4.75 -fold change over 0 day time point). The cell count in the last two time points post-infection (10 and 12 days) were not significantly different from each other ($P > 0.05$).

When comparing *B. quintana* cell counts per louse in head versus body lice at each time point individually, body lice showed significantly more *B. quintana* cells per louse at 8 days (63.7-fold), 10 days (219.3-fold) and 12 (184.8-fold) days post infection ($P < 0.05$; Fig. 1)

2.6 Discussion of *B. quintana* infection and proliferation

The proliferation of *B. quintana* in body lice was similar to that previously reported by Seki *et al* (2007), where proliferation was stagnant until day 6 post-infection and significant proliferation did not occur until between 6 and 8 days post-infection. Head lice demonstrated relatively stagnant proliferation until 4-6 days post infection, and from there the cell count of *B. quintana* declined steadily. This evidence, although not definitive, suggests that the ability of body lice to vector *B. quintana*, whereas head lice do not, is due to the ability of the bacteria to grow inside of the body louse gut following the ingestion of an infected blood meal. Proliferation in the body louse gut would likely lead to an increased number of viable bacteria in the feces and subsequently a greater chance for humans to be exposed and infected.

The root cause behind increased proliferation in body versus head lice is still largely unknown. Based on the large differences in the transcriptional profiles of immune-related genes between head and body lice, the current hypothesis is that body lice have relaxed their innate immune system during their evolution from head lice in order to maintain a larger amount of their gram-negative endosymbiont *Reisia*, which subsequently provides body lice with more B-vitamins and energy allowing the louse to stay off of the human host for longer periods of time and occupy the relatively new niche of human clothing. The ramification of this process is that bacteria acquired in an infected blood meal are also allowed to proliferate, increasing the vector competency of the human body louse.

Differences in the transcript level of immune-related genes appear to be involved in gram-negative bacterial proliferation. Under uninfected conditions, the body louse has lower basal transcript levels of PGRP and Defensin 1 genes (3.7-fold, 6.1-fold, respectively) compared to head lice as determined by qRT-PCR (Kim *et al.*, 2012). Decreased levels of PGRP and Defensin 1 could allow for the rapid proliferation of *B. quintana* at earlier time points in body lice and possibly result in the production of biofilms to protect itself from the louse innate immune system. The decreased innate immune response could also allow for more *B. quintana* to breach the alimentary tract epithelial tissue and invade the hemocoel, possibly allowing for enhanced *B. quintana* growth in the combined presence of an attenuated phagocytic response.

These previous studies only looked at a small subset of humoral immune-related genes and one cellular immune response gene, prophenyloxidase (PPO), under uninfected conditions and 8 hours post-infection with *E. coli*. The subsequent experiments

described later in this thesis look to determine the root causes of bacterial proliferation in body versus head lice by looking at all immune related genes, both humoral and cellular, before and after infection with the natural pathogen *B. quintana*.

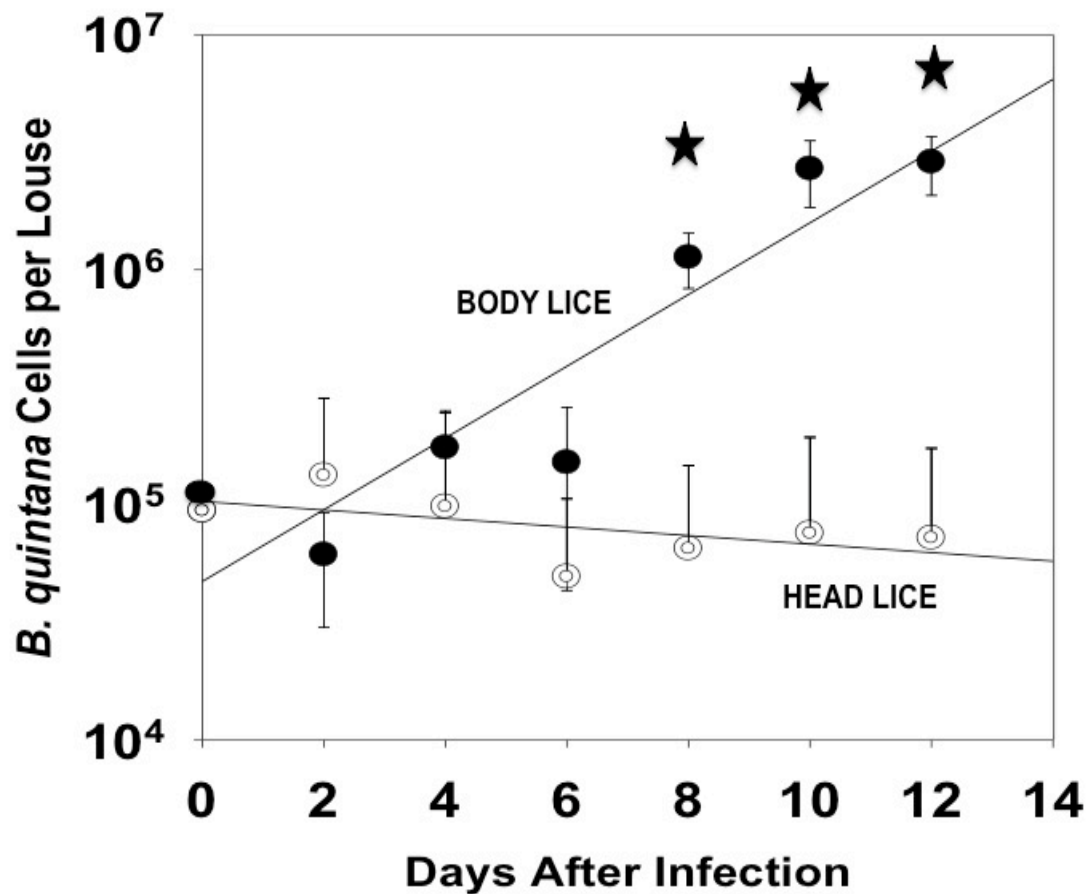


Figure 1. *B. quintana* proliferation in body and head lice. Adult female 1-4 day old body (San Francisco strain) and head (Bristol strain) lice maintained on the *in vitro* rearing system (Yoon *et al.*, 2006) were fed on human blood inoculated with *B. quintana* harvested from 7-10 day old plates, at approximately 1×10^7 CFU/ml blood (Seki *et al.*, 2007). After an overnight feed lice were transferred to uninfected blood. 5 lice were collected on 2 day intervals, and genomic DNA extracted to determine *B. quintana* proliferation, with primers amplifying an 89 base pair fragment in the 16S-23S rRNA gene of *B. quintana* (Seki *et al.*, 2007). Cell counts were normalized to the lowest 0 day cell count as well as relative lice gDNA detected by louse sodium channel primers.

CHAPTER 3

TRANSCRIPTOME ANALYSIS OF *B. quintana*-INFECTED HEAD AND BODY LICE

3.1 *B. quintana* culture

B. quintana, JK31 strain, originally obtained from Dr. Jane Koehler (University of California-San Francisco) was maintained in a biosafety level 2 (BSL2) facility at the Environmental and Molecular Toxicology Laboratory at the University of Massachusetts-Amherst. Frozen *B. quintana* stocks were cultured on chocolate agar plates following Dr. Koehler's protocols (37 °C, culture plates in CO₂ jars) for 7 to 10 days. *B. quintana* was then passed to a fresh chocolate agar plate and cultured for an additional 4 to 5 days before use.

3.2 Body and head louse strains

A field population of human body lice (Frisco-BL) originally collected from 9 homeless individuals in San Francisco (December, 2008) by Dr. Jane Koehler's research group and a head louse population (BR-HL) collected from Bristol, United Kingdom (Yoon *et al.* 2004) were both maintained on the *in vitro* rearing system with human blood supplemented with a 1:1 penicillin:streptomycin solution at 1 µl/ml, (Sigma Chemicals, St. Louis, MO) (Yoon *et al.* 2006). As late third instars or first day adults, lice were fed on non-antibiotic blood for 3 days before experimental usage to ensure antibiotics are purged and lice are 1-4 day old adults.

3.3 Louse infection with *B. quintana* via blood meal

B. quintana was harvested from 1 chocolate agar plate using a sterile loop, and suspended into 1 ml PBS (0.1 M, pH 7.2). OD₆₀₀ reading was taken for *B. quintana* suspended in PBS to determine cell counts. *B. quintana* cells were washed twice with 1 ml PBS with centrifugation (1000 g) in between washes. After a final spin, the supernatant was removed and the pellet was suspended in 100 µl of PBS by pipetting. 10 µl of the suspension was serially diluted into M199 media (Sigma) supplemented with glutamine, sodium pyruvate and 20% fetal bovine serum (M199S), and dilutions (10⁻⁴-10⁻⁸ ml PBS suspension/ml M199S) were plated in triplicate for CFU/ml blood determination. The suspension was centrifuged a final time, and the pellet was mixed with human blood to obtain a titer of approximately 1 x 10⁷ CFU/ml (Kosoy *et al.*, 2004) for feeding. Approximately 200 lice, which were starved for 6 hrs, were fed on *B. quintana*-infected blood or on non-infected blood (control) using the *in vitro* rearing system for 18 hrs (overnight). Fed lice were transferred into a clean rearing unit with uninfected blood and maintained for 8 days post-infection. Louse samples were collected at 8 days post infection (20 lice/experiment) and stored in liquid N₂. Collected louse samples from 3 replicated experiments (20 infected body lice (BLT) X 3, 20 infected head lice (HLT) X 3, 20 uninfected body lice (BLC) X 3, and 20 uninfected head lice (HLC) X 3) were packaged on dry-ice and shipped to the Pittendrigh laboratory (University of Illinois at Urbana-Champaign, UIUC) for transcriptome analysis.

3.4 RNA extraction and transcriptome analysis (done at UIUC)

Total RNA was extracted from lice using the Qiagen RNeasy Extraction kit (Qiagen, Valencia, CA) according to manufacturer's protocols. Samples were analyzed at the Keck Center/UIUC and sequenced on a single flow cell of the Illumina Genome Analyzer 2 using 100 pyrosequencing cycles and data obtained as .fastq files (Illumina Inc. San Diego, CA). Two samples were sequenced per lane for a total of 6 lanes with an average of 282x coverage. Raw sequence data from head and body louse RNAseq sequencing were analyzed using the CLC Genomics Workbench 4x (CLC BIO, Muehlta, Germany). Pre-processing involved trimming of nucleotides with quality scores of < 20 . The body louse gene models (Kirkness *et al.*, 2010) version 1.2, were downloaded from Vectorbase.org (<http://phumanus.vectorbase.org/GetData/Downloads/>) in the file "phumanus.TRANSSCRIPTS-PhumU1.2.fa.gz", unpacked, and uploaded into the CLC Genomics Workbench 4x. Processed head and body louse read data were independently mapped to the body louse gene models using the parameters: mismatch cost = 2, insertion and deletion cost = 3, and similarity $\geq 80\%$. Once mapping was completed for the 10,994 transcripts contained within the body louse gene models, a RNAseq analysis was conducted on each treatment and replicate for all transcripts, resulting in a table consisting of expression values based upon reads per kilobase of exon model per million mapped reads (RPKM; Mortzavi *et al.*, 2008). Each of these tables, 12 in total, were exported as .csv files and merged into a single Excel spreadsheet for statistical analysis.

For each transcript, RPKM values were square-root transformed to a Poisson distribution of count data (and a second analysis assuming a negative binomial distribution has also been performed with a similar list of important genes being

differentially expressed). Any RPKM value less than 1 was counted as a 0. The standard error mean was compared for all comparisons of 2 or 4 treatments, resulting in a list of p-values for each transcript. This list was then ordered from lowest to highest and using a cumulative p-value, all transcripts less than 0.05 were considered significant.

A second analysis using the same .csv files was run using a negative binomial distribution and analyzed with the R and DEseq software packages. Each transcript was analyzed in four 2-way comparisons (Head Lice Treated (HLT)-Head Lice Control (HLC), Body Lice Treated (BLT)-Body Lice Control (BLC), HLC-BLC and HLT-BLT). DEseq uses a geometric mean to adjust for uneven distribution of reads across replicates and treatments to allow for direct comparison of resultant values to calculate fold change and p-value. Subsequently, log₂ fold change and an adjusted p-value were calculated. To control for false discovery rate (FDR), the adjusted p-value was calculated with a Benjamini-Hochberg procedure (Benjamini and Hochberg , 1995).

After both analyses were run, immune genes that were significantly differentially transcribed in any of the two-way comparisons using either the 2-way Poisson distribution and/or negative binomial distribution analysis were selected. qRT-PCR was run with primers against these genes to verify the transcription levels in the genes selected and validate the use of one statistical method over the other.

3.5 Results of the body and head lice transcriptome analysis

3.5.1 Poisson Statistics. Transcriptome analysis using a Poisson distribution that compared the four 2-way treatments (HLT-HLC, BLT-BLC, HLC-BLC, HLT-BLT) resulted in 11 immune genes being differentially expressed in one of the four

comparisons. All 11 of the genes were differentially expressed between head and body lice under uninfected conditions. No genes showed changes in expression eight days post-infection. Eight were over-expressed in body lice compared to head lice including Fibrinogen-like-recognition protein, Spaetzle, Scavenger A, Cactus, Traf 2, Scavenger B, Clip serine and JNK basket. Three genes, including effector genes Defensin 1 and Nodular as well as Apolipoporphin 1, were over-expressed in head lice compared to body lice under uninfected conditions. Of these genes, only four, Fibrinogen-like protein, Spaetzle, Defensin 1 and Cactus, were verified as over-expressed using qRT-PCR.

3.5.2 Negative Binomial Statistics. The transcriptome analysis using the negative binomial distribution, again comparing the four 2-way treatments (HLT-HLC, BLT-BLC, HLC-BLC, HLT-BLT), resulted in 10 genes being differentially expressed in at least one of the four comparisons. Eight of the 10 genes showed differential expression between head and body lice under uninfected conditions. Spaetzle and Fibrinogen-like-protein again showed over-expression in body lice compared to head lice under uninfected conditions as well as PPO. Scavenger A, Defensin 1, Defensin 2, Apolipoporphin 2 and Serpin all were over-expressed in head lice compared to body lice under uninfected conditions. Of these only six, Fibrinogen-Like protein, Scavenger A, Spaetzle, Defensin 1, Apolipoporphin 2 and Serpin were verified using qRT-PCR.

Defensin 2 and Apolipoporphin 2 had more transcript in infected body lice compared to uninfected body lice. Only Apolipoporphin 2 was verified using qRT-PCR. Defensin 1 and Defensin 2 had more transcript in uninfected head lice compared to *B. quintana*-infected lice. Neither of these genes were verified in qRT-PCR.

In summary, only three genes, Fibrinogen-like protein, Spaetzle and Defensin 1, were constitutively over-expressed using both Poisson and negative binomial distributions and verified as over-expressed with qPCR. Fibrinogen-like protein and Spaetzle were overexpressed under uninfected conditions in body lice compared to head lice, and maintained this over expression 8 days post-infection. The AMP Defensin 1 was over expressed in head lice under uninfected conditions and maintained this overexpression 8 days post infection. The genes discussed in the following section (3.6) were all significantly different in at least one of the four comparisons using qRT-PCR techniques. All data is present in Table 1, Figure 2a and Figure 2b.

3.6 Discussion of the head and body lice transcriptome analysis

3.6.1 Fibrinogen-related protein (PHUM:500950). The Fibrinogen-related protein is likely a part of the cellular response in head and body lice that is up-regulated to deal with invading bacteria. It is a common bacterial recognition protein conserved in invertebrates and mammals who's most common role is directly tagging microbial sugar moieties for phagocytic degradation in blood serum or hemolymph (Faik *et al.*, 2001).

A BLAST search of the protein sequence resulted in a 65% identity match with Fibrinogen-related-proteins from *Drosophilla mojavensis*, *Drosophila virilis* and *Drosophila ananassae*, all of which included the important C-terminal domain, which is responsible for microbial sensing (Faik *et al.*, 2011). In *D. melanogaster*, it has 65% amino acid identity to a scabrous protein, which is a Fibrinogen-related-protein implicated in a variety of processes, which also contains the Fibrinogen C-terminal domain.

The transcript level in BLC was greater when compared to HLC (2.00-fold) and this level increased when BLT was compared to HLT (2.77-fold). There was also a *B. quintana* infection effect in body lice, as BLT resulted in 1.34-fold more transcript than detected in BLC. By the 8 day post-infection time point, head lice had killed off or contained the invading *B. quintana* whereas it was still proliferating and spreading in the body lice. Because this protein targets bacteria for endocytosis by phagocytes in the hemolymph, it seems likely that body lice were responding significantly to infection by increasing the transcript level of the Fibrinogen-related-protein, which would increase the cellular response by enhancing phagocytosis.

3.6.2 Defensin 1 (PHUM: 365700). The Defensins are effector genes within the Toll pathway, which are translated into AMPs that are produced in almost all immune-related and epithelial cells. Defensins secreted by epithelial cells in the alimentary track are one of the first humoral immune defense barrier against foreign pathogens (Kim *et al.*, 2012).

A BLAST search resulted in a 45% identity match to Defensin 1 in *Ctenocephalides felis* (Cat flea), 46% to Defensin 1 in *Apis cereana japonica* (Japanese honey bee), and 44% to Defensin 2 in *Apis mellifera* (European honey bee). A BLAST search against *D. melanogaster* resulted in a 36% identity match against its Defensin.

qRT-PCR results indicated that HLC maintained the transcript level of Defensin 1 at a value 14.2-fold higher compared to BLC, a finding that correlated with previous studies (Kim *et al.*, 2012). This value was reduced to 5.3-fold 8 days post-infection. The reduced basal level of Defensin 1 in body lice may allow for the initial proliferation of *B. quintana* within the gut lumen, or possibly allow for the formation of biofilms on

alimentary track epithelial cells, which could eventually lead to hemocoel invasion. After 8 days post-infection, however, Defensin 1 increased 2.23-fold in BLT compared to BLC. It is likely that at 8 days post-infection *B. quintana* has infected the hemocoel and has caused Toll-pathway up regulation inducing Defensin production in body lice. In head lice, *B. quintana* is either contained in the gut or has been killed off by the increased amount of Defensin 1 present in the gut due to the inherent high basal transcription level of this gene.

3.6.3 Spaetzle (PHUM: 596260). Spaetzle is an extracellular protein that is the ligand for the Toll receptor. In order to become active, extracellular recognition factors initiate serine protease cascades, which proteolytically cleave Spaetzle into its active form (Mizuguchi *et al.*, 1998). Once active, it binds to the Toll receptor, which leads to a cascade of intracellular events eventually leading to the transcription of Toll effector genes such as AMPs.

A BLAST search of the louse Spaetzle sequence resulted in matches to known Spaetzle proteins in *Acromyrmex echinator* (Leaf cutter ant), *Harpegnathos saltator* (Jumping ant) and *Camponotus floridanus* (Florida carpenter ant) at 42%, 38% and 37% amino acid sequence identity, respectively, as well as 30% sequence identity to *D. melanogaster* Spaetzle isoform 8.29.

The transcript levels of Spaetzle were 1.64-fold higher in BLC samples compared to HLC samples. At 8 days post-infection, BLT samples were 2.30-fold higher than HLT samples, supporting the contention that *B. quintana* must proliferate and invade the hemocoel to elicit an immune response, specifically by up-regulation of the Toll pathway.

Also, BLT samples were 2.23-fold greater than BLC samples, which once again supports the contention that *B. quintana* was entering hemocoel to elicit an immune response. Therefore, Spaetzle appears to be up-regulated by *B. quintana* in body lice resulting in increased production of Defensin 1 via the Toll pathway. Because the transcript level of Spaetzle is greater in BLC compared to HLC, it is theorized that a separate mechanism independent of the Toll pathway must be responsible for the constitutive basal over-expression of Defensin 1 in head lice (14.2-fold more in HLC versus BLC).

3.6.4 Serpin (PHUM:311330). Serpin, a serine protease inhibitor gene, is another gene that supports the idea of a separate mechanism independent of the Toll pathway, which is controlling the basal level constitutive overexpression of AMPs in uninfected head lice. This upstream regulator of the Toll pathway inhibits the cleavage of Spaetzle into its active form, ultimately down regulating the Toll pathway.

A BLAST search of the louse Serpin resulted in a 61% amino acid sequence identity to Serpins in *Ayrthosiphon pisum* (Pea aphid) and *Tribolium castaneum* (Red flower beetle) as well as 54% amino acid sequence identity to the *D. melanogaster* Serpin I4.

The transcript level of Serpin in HLC was 3.22-fold greater than in BLC samples. In theory, this process would result in reduced activation of the Toll pathway in HLC versus BLC, although Serpin function in insects is not well studied and the limited research done in *D. melanogaster* has shown Serpins have a wide variety of functions (Reichart *et al.*, 2011). Nevertheless, a reduction in Toll pathway activity should subsequently lead to a reduction in the production of AMPs. However, head lice maintain 14.2-fold higher

Defensin 1 transcript under uninfected conditions, a finding that again supports the hypothesis that head lice control their basal transcription level of AMPs by a mechanism separate from the induction of the Toll pathway.

3.4.5. Cactus (PHUM: 345810). Cactus is an intracellular protein that is downstream of the Toll receptor that acts as a Toll pathway inhibitor. Specifically it is an inhibitor of transcription factor NF κ B, which up-regulates effector genes such as AMPs (Wu and Anderson 1998). If Cactus is up-regulated, more will bind and inhibit the proteins Dif and Dorsal, which ultimately activate NF κ B and up-regulate AMPs, therefore Cactus up-regulation results in lower AMP transcript levels.

A BLAST search of the louse Cactus protein sequence resulted in matches to predicted NF κ B inhibitor Cactus isoform 1 in *Nasonia vitripennis* (Parasitoid wasp) with a 42% amino acid sequence identity, a predicted stress-induced phosphoprotein 1-like in *Megachile rotundata* with a 44% amino acid sequence identity and a known Cactus protein in *Apis adeniformis* (Black dwarf honey bee) with a 41% amino acid sequence identity. Louse Cactus protein is 47% similar to the Cactus zygotic protein in *D. melanogaster*.

The transcript level of Cactus in BLC samples was 1.38-fold greater when compared to the transcript levels in HLC samples. Since Cactus is further downstream than Spaetzle, it may have a more direct inhibitory effect on the production of AMPs, and could be part of the reason for the 14.2-fold increase in the transcript level of Defensin 1 in HLC versus BLC samples, although it is still likely a different mechanism may be used for the resulting higher constitutive basal expression of Defensin 1 in head lice. After 8

days post-infection, there is no statistical difference between the transcript levels of Cactus from BLT versus HLT samples, a finding that once again supports the idea that *B. quintana* upon reaching the hemocoel is causing body lice to down-regulate the immune inhibitory effects of Cactus thus, increasing its immune response by increasing Toll pathway activation.

3.6.6. Scavenger Receptor A (ScavA, PHUM:066640). ScavA is one of many receptors expressed on the outside of macrophages involved in the recognition of microbes for phagocytic degradation (Goh *et al.*, 2010). Although there is limited research done in insects, human Scav A has an affinity for gram-negative bacteria and targets *E. coli* for phagocytosis (Peiser *et al.*, 2000).

When a BLAST search was run comparing protein sequences, matches to lysyl oxidase proteins from *Megachile rotundata*, *Daphnia pulex* (Water flea) and *Harpegnathos saltator* were the three top hits, all with 65% amino acid identity matches. These proteins have lysyl oxidase domains that encompass approximately the first 100 amino acids at the N-terminus, and the remaining approximately 300 amino acids are homologous to scavenger receptor proteins. The amino acid sequence of louse ScavA is 57% similar to a *D. melanogaster* protein that once again has both a lysyl oxidase domain as well as a scavenger receptor domain.

The transcript levels of ScavA was 1.53-fold greater in HLC versus BLC and was 1.33-fold greater in HLT versus BLT samples. It has been previously shown that head lice have more rapid and active phagocytes resulting in higher phagocytic activity than do body lice, specifically against its gram-negative endosymbiont *Reisia* during its migration

from the stomach mycetome to the filarial mycetome (Perotti *et al.*, 2007). The overexpression of ScavA in head lice may be a possible reason for the heightened phagocyte over-activity in head lice as well as another possible factor in the head louses ability to curb *B. quintana* proliferation.

3.6.7 Apolipoprotein 2 (Apolipoprotein D PHUM:427700) Apolipoprotein D is a general stress reducing anti-oxidant, and is shown to be age-dependently up-regulated in the human brain, as well as in Alzheimers patients (Kalman *et al.*, 2000; Loerch, 2008). When over-expressed in *D. melanogaster*, it also increases the flies lifespan, as well as function as a lipid antioxidant conferring resistance to oxidative stress (Walker *et al.*, 2006).

A BLAST search of louse Apolipoprotein D protein sequence resulted in matches to Apolipoprotein D from *Megachile rotundata*, *Harpegnathos saltador* and *Apis florea* (Red dwarf honey bee) with 57%, 49% and 48% amino acid identity match, respectively. Louse apolipoprotein D had a 36% amino acid identity match to a neural lazarrillo gene in *D. melanogaster*, which is an Apolipoprotein type protein that responds to oxidative stress.

The transcript level of Apolipoprotein D was 1.45-fold greater in HLC versus BLC samples. Interestingly, a 2.19-fold increase in its transcript level was seen in BLT versus BLC samples. Because Apolipoprotein D functions as a general stress reducing agent, it is likely up-regulated due to the oxidative stress associated with *B. quintana* invasion into the hemocoel after proliferation in the gut. The head louse under uninfected conditions must keep its immune and phagocytic response up-regulated to rapidly deal

with *B. quintana* infection likely using more energy in the process and creating oxidative stress. The body louse under infected conditions and hemocoel invasion must up-regulate its immune and phagocytic response causing oxidative stress and subsequent up-regulation of the stress-reducing Apolipoprotein D.

3.6.8 c-Jun NH₂ terminal kinase (JNK basket gene, PHUM:128040) JNK basket was not verified to be significantly different using qRT-PCR techniques, however it was an interesting gene that was differentially regulated when analyzed using 2-way Poisson statistical analysis. This gene is integral in the pathway leading to regeneration of gut epithelial tissues (Buchon *et al.*, 2009). When a BLAST search was run comparing protein sequence, there was a 95% match to stress-activated protein kinase JNK-like from *Camponotus floridanus* and *Megachile rotundata*, and 88% match to *D. melanogaster*.

The transcript level of the JNK-basket gene was greater in BLC compared to HLC as well as in BLT versus HLT samples. Its over-expression under uninfected and infected conditions may be required to regenerate damaged gut epithelial tissues following bacterial proliferation and subsequent invasion of *B. quintana* into the body louse hemocoel thereby reducing the bacterial induced mortality response.

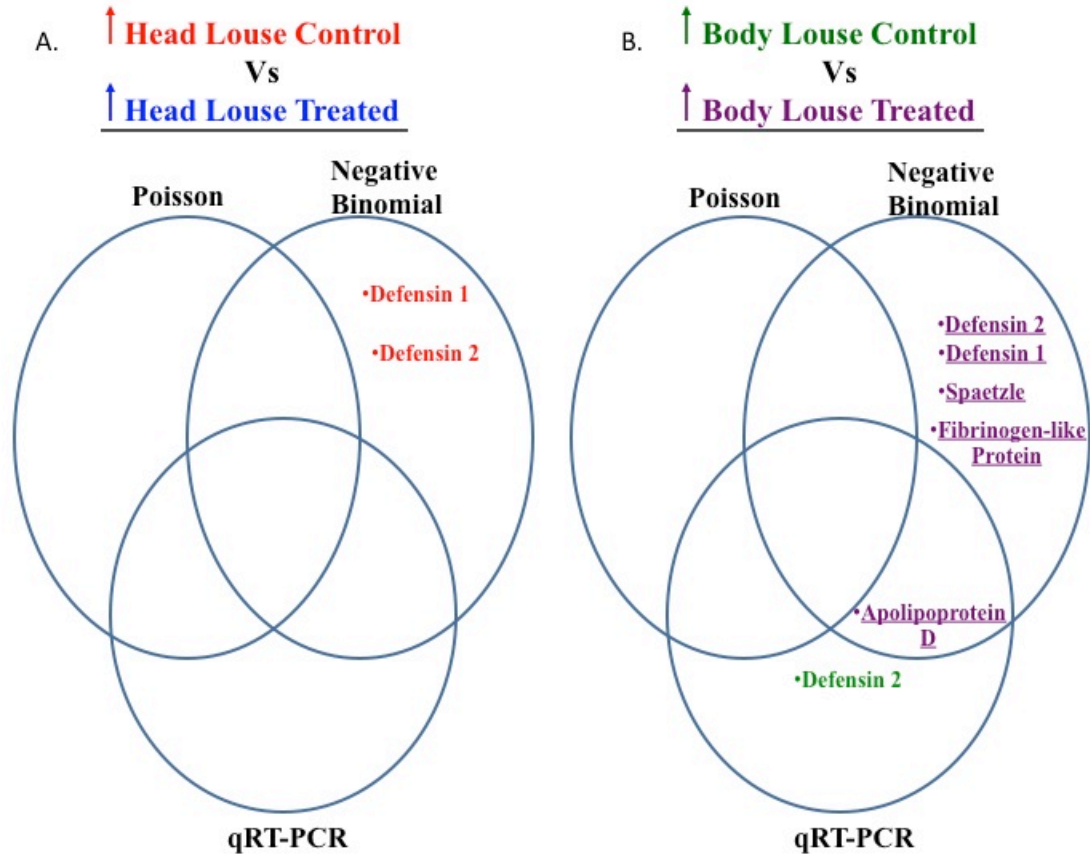


Figure 2. Venn diagrams comparing statistically significant immune-related genes from transcriptome analysis when head louse control samples are compared to head louse treated samples (A) and when body louse control samples are compared to body louse treated samples (B). Gene transcription level in whole head and body lice under uninfected conditions and 8 days post *B. quintana*-infection were assessed using two statistical methods, 2-way Poisson and negative binomial distributions, and verified by qRT-PCR. Above are statistically significant immune-related genes ($P < 0.05$).

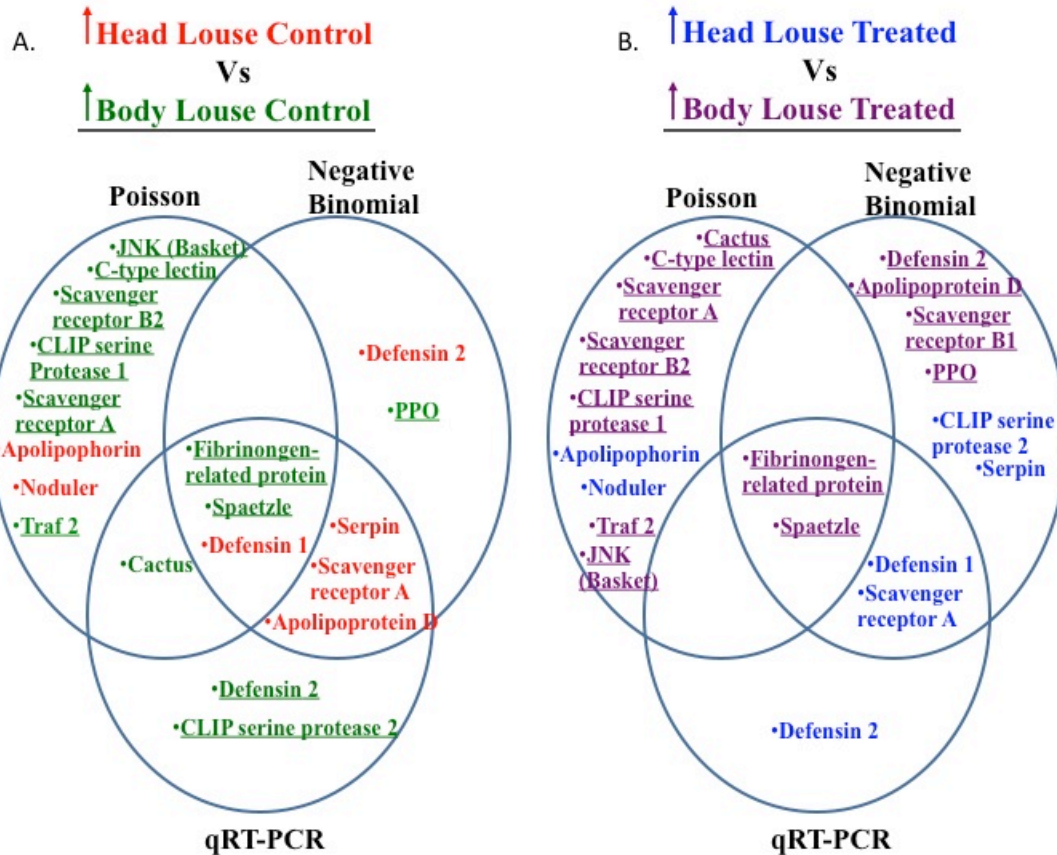


Figure 3. Venn diagrams comparing statistically significant immune-related genes from transcriptome analysis when head louse control samples are compared to body louse treated samples (A) and when head louse treated samples are compared to body treated treated samples (B). Gene transcription level in whole head and body lice under uninfected conditions and 8 days post *B. quintana*-infection were assessed using two statistical methods, 2-way Poisson and negative binomial distributions, and verified by qRT-PCR. Above are statistically significant immune-related genes ($P < 0.05$).

Table 1. Statistically relevant immune-related genes in head and body lice following infection by *B. quintana* and transcriptome analysis is determined by 2-way Poisson, negative binomial and verified using qRT-PCR techniques.

GeneName	Vector-Base ID	POISSON (RKPM)				BINOMIAL (Log2 Fold Change)				qPCR ($2^{\Delta\Delta Ct}$)			
		HLC-HLT	BLC-BLT	HLC-BLC	HLT-BLT	HLC-HLT	BLC-BLT	HLC-BLC	HLT-BLT	HLC-HLT	BLC-BLT	HLC-BLC	HLT-BLT
^b Fibrinogen-Like Protein	500950	-	-	^a -9.07	-8.20	-	-	1.49	1.25	-	1.34	2.00	2.77
Scavenger A	066640	-	-	-10.5	-9.73	-	-	-0.77	-0.93	-	-	1.53	1.33
Spaetzle	596260	-	-	-8.25	-9.69	-	-	0.83	1.04	-	1.40	1.64	2.30
Defensin 1	365700	-	-	8.50	-	-0.67	-	-0.74	-0.90	-	2.23	14.2	5.26
^c Defensin 2	595870	-	-	-	-	-1.47	1.58	-1.38	1.67	-	2.77	1.94	2.22
Apolipoporphin 2	427700	-	-	-	-	-	5.03	-2.29	3.66	-	2.19	1.45	-
Serpin	311330	-	-	-	-	-	-	-2.06	-1.51	-	-	3.23	-
Clip Serine	571420	-	-	-	-	-	-	-	-2.25	-	-	2.34	-
^d Cactus	345810	-	-	-10.9	-11.3	-	-	-	-	-	-	1.38	-
Scavenger B	351630	-	-	-	-	-	-	-	0.55	-	-	-	-
PPO	448900	-	-	-	-	-	-	0.68	0.89	-	-	-	-
Traf 2	129280	-	-	-37.4	-36.9	-	-	-	-	-	-	-	-
Nodular	249370	-	-	42.6	43.9	-	-	-	-	-	-	-	-
Apolipoporphin 1	145960	-	-	25.1	24.1	-	-	-	-	-	-	-	-
Scavenger B	569610	-	-	-12.0	-12.4	-	-	-	-	-	-	-	-
Clip serine	451100	-	-	-8.9	-9.2	-	-	-	-	-	-	-	-
JNK basket	128040	-	-	-20.7	-19.9	-	-	-	-	-	-	-	-

^aValue – Indicates over-expression in highlighted treatment. *All values listed are statistically significant (p<0.05)

^bGene Name- Indicates differential gene expression detected by both statistical methods and verified by qPCR

^cGene Name- Indicates differential gene expression detected by negative binomial statistics and verified by qPCR

^dGene name- Indicates differential gene expression detected by Poisson distribution statistics and verified by qPCR.

CHAPTER 4

TRANSCRIPTIONAL PROFILING OF MIDGUT IMMUNE-RELATED GENES FROM BODY LICE

4.1 Body louse strain

Body lice used for the transcriptional profiling of immune related-related genes from midgut tissues were from the Culpepper strain that has been maintained on rabbits at UMass-Amherst since 1999 (Culpepper, 1944). Unfed first instars were picked from the colony maintained on rabbits and put on the *in vitro* rearing system (Yoon *et al.* 2006). Lice were grown on human blood supplemented with a penicillin, streptomycin solution at 1 µl/ml (Sigma Chemicals) (Yoon, *et al.* 2006). Late third instars or first day adults were fed on non-antibiotic blood for 3 days before experimental usage to ensure antibiotics are purged and lice are 1-4 day old adults.

4.2 Transcriptional profiling of louse immune-related genes following *B. quintana* infection

Culpepper body lice were fed with blood infected with *B. quintana* as described above (Section 2.3). Twelve lice per replicate were collected for transcriptional profiling at 12 hours, 4 and 8 days following feeding on infected blood as well as control lice for reference. Louse gut tissue (alimentary tract) was excised by cutting the anus with microintraocular scissors (World Precision Instruments, Sarasota, FL) and teasing the gut out with forceps into RNAlater buffer. Total RNA was extracted using RNeasy mini kit according to manufacturer's instructions (Qiagen). Total RNA samples were treated with

DNase I (Invitrogen, Carlsbad, CA) to remove genomic DNA. cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) and diluted to 5 ng/ul for use in qRT-PCR experiments. qRT-PCR was performed in StepOnePlus Real-Time PCR system (Applied Biosystems) using Power Sybr Green PCR Master Mix (Applied biosystems) with the following thermal cycling program: 95 °C for 10 min, 35 cycles of 95 °C for 15 s, 60 °C for 1 min, and serial increase per 0.3 °C for 1 s from 50 °C to 95 °C for melting curve analysis. RpL13A was used as our reference gene and primers for PGRP and Defensin 1 and 2 are listed in Table 2. Quantification of transcript level or relative copy number of a gene was done using relative quantification methods based upon $2^{-\Delta C_t}$ (Pfaffl, 2001).

4.3 Results of the transcriptional profiling of body louse immune-related genes following *B. quintana* infection

A total of 3 biological replicates were run and samples collected at 12 hours, 4 days and 8 days post *B. quintana* infection. An average titer of $9.46 \times 10^7 \pm 4.56 \times 10^7$ CFU/ml blood was used for the three replicates. Mortality data was collected and there was no significant increase in mortality due to *B. quintana* infection (data not shown).

4.3.1 PGRP. PGRP had a relative transcript level of 0.325 ± 0.078 at 12 hours post *B. quintana* infection, a relative transcript level of 1.48 ± 0.876 at 4 days post *B. quintana* infection and a relative transcript level of 0.661 ± 0.095 at 8 days post *B. quintana* infection compared to uninfected lice (Fig. 3). None of the PGRP transcript levels were statistically different from uninfected body lice.

4.3.2 Defensin 1. Defensin 1 had a relative transcript level of 2.14 ± 1.15 at 12 hours post *B. quintana* infection, a relative transcript level of 1.72 ± 1.01 at 4 days post *B. quintana* infection and a relative transcript level of 1.42 ± 0.792 at 8 days post *B. quintana* infection compared to uninfected body lice (Fig. 3). None of the Defensin 1 transcript levels were statistically different from uninfected body lice.

4.3.3 Defensin 2. Defensin 2 had a relative transcript level of 1.27 ± 0.645 at 12 hours post *B. quintana* infection, a relative transcript level of 0.77 ± 0.520 at 4 days post *B. quintana* infection and a relative transcript level of 1.26 ± 0.95 at 8 days post *B. quintana* infection compared to uninfected lice (Fig. 3). None of the PGRP transcript levels were statistically different from uninfected body lice.

4.4 Discussion of the transcriptional profiling of body louse immune-related genes following *B. quintana* infection

4.4.1 PGRP. Transcript levels of the body louse PGRP gene were not significantly different from those determined from uninfected lice at 12 hours, 4 and 8 day time point samples taken at the 12 hour post-infection time point. The transcript level of PGRP in the sample (0.325-fold) does not correlate with the PGRP transcript level in the sample taken at the 8 hour post *E. coli* infection (~1.5-fold) previously reported by Kim *et al.*, (2012) in midgut tissue. After 8 days post-infection with *B. quintana*, PGRP transcript levels from midgut tissue was not significantly different from the level determined in

uninfected body lice, a finding that correlates with qRT-PCR findings from whole body lice 8 days post-infection with *B. quintana*. Together, these results indicate that PGRP may not be critical in *B. quintana* proliferation, although a comparable head lice data set would allow us to distinguish differences between head and body lice.

4.4.2 Defensin 1. The transcript level of body louse Defensin 1 gene was not significantly different from uninfected body lice at any time point, although there was a noticeable trend of decreasing transcript from 12 hours (2.14-fold), to 4 days (1.72-fold) to 8 days (1.42-fold). The 12 hour post-infection time point (2.14-fold) correlated with results found by Kim *et al.*, (2012) in which louse midguts were removed 8 hours after orally-ingesting *E. coli* and Defensin 1 transcript was ~2-fold greater than in uninfected body lice, suggesting that *B. quintana* is causing an immediate up-regulation of AMPs in the body lice. Both the 8 day post-infection time point samples from midgut (1.42-fold) and the 8 day post-infection time point samples from whole lice (2.23-fold) had greater transcript levels than uninfected lice, indicating *B. quintana* is eliciting an effect of defensin 1 regulation, although the differences in the midgut data are not significant.

4.4.3 Defensin 2. The transcript levels of Defensin 2 were not significantly different from the levels determined in uninfected body lice samples at any of the time points post-infection. Defensin 2 transcript level at the 12 hour post-infection time point (1.27-fold) correlated with results found by Kim *et al.*, (2012) in which louse midguts were removed 8 hours after orally-ingesting *E. coli* and Defensin 2 transcript were approximately the same as uninfected lice, suggesting that *B. quintana* is causing little effect on Defensin 2 in body lice. The transcript level of Defensin 2 at 8 days post-infection from whole body

lice was 2.77-fold less in infected versus uninfected lice. This contradicts that obtained from midgut Defensin 2 at 8-days post-infection which was 1.26-fold greater than uninfected lice, although this value had an extremely large error bar associated with it. Overall, Defensin 2 transcript levels in previous experiments using *E.coli*-fed body lice and current experiments using *B. quintana*-fed body lice were inconsistent and showed no significant trend or infection effect, indicating Defensin 2 may not be integral in the vector competence of *B. quintana* in body lice.

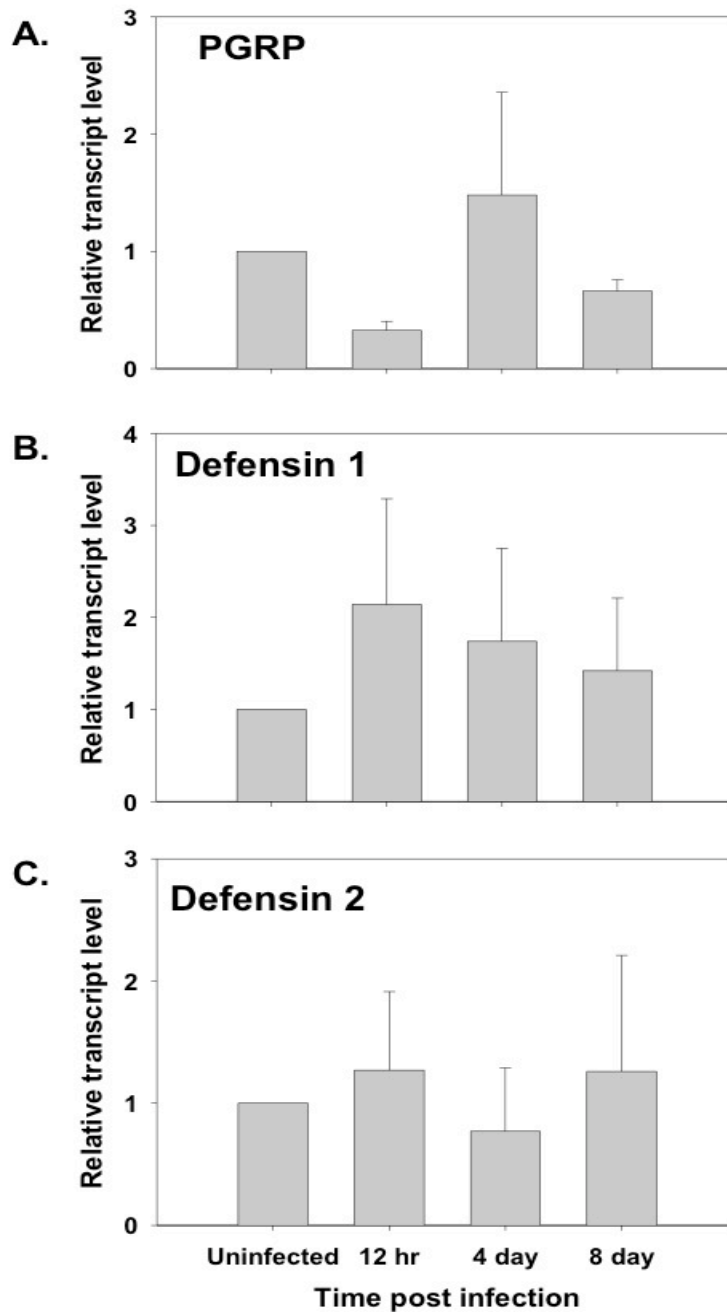


Figure 4. PGRP, Defensin 1 and Defensin 2 transcriptional profiling from louse alimentary tract. Adult female (1-4 day old) body (San Francisco strain) and head (Bristol strain) lice maintained on the *in vitro* rearing system (Yoon *et al.*, 2006) were fed on human blood inoculated with *B. quintana* harvested from 7-10 day old plates at approximately 1×10^7 CFU/ml blood (Seki *et al.*, 2007). After a 12 hour feed, lice were transferred to uninfected blood and 12 lice were collected at 12 hour, 4 day and 8 day time points. Lice alimentary tract was removed, total RNA was extracted, and qRT-qPCR was performed with primers against (A) PGRP, (B) Defensin 1 and (C) Defensin 2 genes with Rpl13A used as a housekeeping gene.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Our overall goal of this thesis was to ascertain differences in body and head lice that ultimately lead to the enhanced vector competence of *B. quintana* in body versus head lice. Our contention is that *B. quintana*'s ability to proliferate in body lice and its inability to proliferate in head lice is at least partially the cause for the differential vector capacity. We then sought to identify immune-related genes potentially responsible for proliferation in body versus head lice.

We examined the basal levels (uninfected) of immune-related gene transcription in whole body and head lice as well as the changes in transcription in 8 days post *B. quintana*-infected using two different statistical methods, 2-way Poisson and negative binomial, and verified with qPCR. Fibrinogen-related protein, Defensin 1 and Spaetzle were three immune-related genes that were differentially transcribed using the two different statistical methods and verified with qRT-PCR, making them potential genes involved in vector competence. *B. quintana* proliferation began at 3 days post-infection, showed rapid proliferation at 6 days post-infection in body lice and was at maximum at 8 days post-infection. In head lice, *B. quintana* population reached a maximum at 2 days post-infection and declined until 8 days post-infection although there was no statistical differences over time post infection. The 8 day post-infection time point may have not been the optimal time for transcriptome analysis, and a wider array of time points including 0, 2, 4 and 6 days post-infection, using immune responsive tissues, specifically the midgut tissues, may identify more genes responding to the proliferation of *B.*

quintana. Following the identification of potential genes playing a role in vector competence, potential hypotheses of gene function can be tested using RNAi knockdown approaches, a method that has previously been effective generating transcript knockdown in body lice (Yoon *et al.*, 2011). These methods, coupled with more extensive transcriptomic, proteomic, and metabolomic studies will allow us to elucidate the mechanisms involved in louse vector competence of *B. quintana*.

Based upon previous findings by Kim *et al.*, (2012) identifying PGRP, Defensin 1 and Defensin 2 as potential genes involved in vector competence, we examined transcription levels of these genes following *B. quintana* infection in body louse midgut. None of these genes appeared to be significant in our evaluation of midgut transcription. The discrepancy in results could be due to the use of different bacteria for infection. Previous results by Kim *et al.*, (2012) used *E. coli* infected blood whereas our experiments used *B. quintana*. Although levels although more replicates, and a comparable head lice data set of these same genes transcription levels would allow us to assess differences between head and body lice.

Overall transcriptional profiles of head and body lice genomes were notably different, including difference in the expression of 18.3 % of immune related genes, a finding that strongly supports the contention that immune system differences between head and body lice are the primary reason for difference in vector capacity.

Table 2. Primers used for qRT-PCR experiments

Gene Name	Primer Sequence
Bq-ITS-F	gccgccttcgtttctcttc
Bq-ITS-R	agtgcttccttaaagtccaag
L-SC-F	acgatgatgaagatgaagatgaagg
L-SC-R	caagaggagtagacgccaatc
RpL13A-rt-F	gtaggggaatgctccacac
RpL13A-rt-R	ggctaaggcagagaacgct
PGRP-rt-F	tgtcaccattggaagatgtcg
PGRP-rt-R	ttcgatggagtcgcgtgttc
Def1-rt-F	gggagaacttacctcggaag
Def1-rt-R	agcggcacaagcagaatgat
Def2-rt-F	atggcggcgacgatgttaat
Def2-rt-R	gggatccccattttgtatcgaa

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