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Comparison of the Humoral Immune Response following Both Bacterial Challenge and RNAi of Major Factors on Proliferation of *Bartonella quintana* in the Human Louse

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Comparison of the Humoral Immune Response following Both Bacterial Challenge and RNAi of Major Factors on Proliferation of *Bartonella quintana* in the Human Louse

A Thesis Presented

By

JAKE MANUEL ZINA

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ABSTRACT

COMPARISON OF THE HUMORAL IMMUNE RESPONSE FOLLOWING BOTH BACTERIAL CHALLENGE AND RNAI OF MAJOR FACTORS ON PROLIFERATION OF *BARTONELLA QUINTANA* IN THE HUMAN LOUSE

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Human body lice, *Pediculus humanus humanus*, and head lice, *Pediculus humanus capitis*, have been hematophagous ectoparasites of humans for thousands of years. Despite being ecotypes, only body lice are known to transmit bacterial diseases to humans, and it appears that lower humoral and cellular immune responses allow body lice to possess a higher vector competence. We previously observed that the transcription level of the *defensin 1* gene was up-regulated only in head lice following oral challenge of *Bartonella quintana*, a causative agent of trench fever, and also that body lice excreted more viable *B. quintana* in their feces. In this study, we first investigated this differential immune response by performing RNAi to knockdown *defensin 1* by dsRNA injection. *B. quintana* was orally infected 72 h after injection and proliferation was compared at 2 hours (day 0) and day 4 post-infection. At day 0, bacterial cell numbers increased 1.5-fold in *defensin 1* (*Def1(-)*) knocked down head lice compared with non-knocked down, *pQE30*-dsRNA injected, head lice control. At day 4, *Def1(-)* knocked down head lice had 2.55-fold more bacterial cells than control head lice and 1.65-fold greater than body lice, indicating that defensin 1 was active in reducing *B. quintana* cell number in non-knocked down head lice. Second,

the levels of cytotoxic reactive oxygen species (ROS) generated by the epithelial cells of the alimentary tract were measured using two general indicators of ROS in both body and head lice at day 1 and day 4 following *B. quintana* challenge. Challenged body lice showed a 42% and 34% increase in ROS, whereas head lice showed a 70% and 22% increase at day 1 using CM-H₂DCFDA and HPF as general indicators, respectively. On day 4, all challenged lice showed similar ROS levels except for body lice which maintained their ROS levels (40% increase using CM-H₂DCFDA). Head lice are likely to have multiple immune and/or non-immune factors that suppress *B. quintana* proliferation, and the production of sustained ROS levels and/or the single knockdown of *Defensin 1* is not enough to increase *B. quintana* proliferation in head lice to that seen in body lice.

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CHAPTER 1

INTRODUCTION

1.1 *Bartonella quintana*

The World Health Organization (WHO) has reported that vector-borne diseases account for more than 17% of all infectious diseases, causing more than 700,000 deaths annually worldwide (World Health Organization, 2017). Vectors are living organisms that possess the ability to transmit diseases between organisms, mainly from human to human, or from animals to humans. The majority of these vectors are insects that feed on blood: ingesting a blood meal containing a disease-causing microorganism from the infected host and passing it on to their subsequent host either via another blood meal or from infected feces. *Bartonella quintana*, a Gram-negative bacteria capable of causing trench fever, an infection in humans typically resulting in a relapsing fever, endocarditis, and vascular proliferative lesions, can be fatal unless correctly diagnosed and treated with antibiotic therapy (Cockerell *et al.*, 1987). Trench fever is only one example of bartonellosis, a group of infectious diseases produced by bacteria of the genus *Bartonella*. A number of small outbreaks of bartonellosis were reported amongst the homeless in Seattle, Washington, and elsewhere in the early 1990s (Jackson *et al.*, 1996; Brouqui & Raoult, 2006). Trench fever continues to reemerge in people who are homeless, poor, and disadvantaged amongst the urban population, especially those who are immune compromised, such as alcoholics and patients infected with human immunodeficiency virus (Hotez, 2008).

1.2 Lice Ecotypes

The body louse, *Pediculus humanus humanus*, is an obligatory human ectoparasite and can pose serious public health concerns by serving as an insect vector for several infectious diseases - one of them being the aforementioned trench fever. Body lice along with conspecific head louse, *Pediculus humanus capitis*, spend most of their lives either directly on or in close proximity to their human hosts. These two ecotypes have adapted to occupying different niches on the human body while exclusively feeding on human blood: body lice inhabit clothing and visit human skin only to feed, whereas head lice are confined to the human scalp where they feed and reside there for their entire life (Buxton, 1946). Body and head lice are morphologically indistinguishable, aside from body lice being slightly larger than head lice when feeding normally on their human host. It is unsure whether they interbreed in the wild, but fertile offspring can be generated from crossing under laboratory conditions (Bacot, 1917). Evolutionarily, it is estimated the body louse originated from the head louse around the time when humans began wearing clothing roughly 40,000-70,000 years ago (Kirkness *et al.*, 2011).

1.3 Vector Competence & *Bartonella* Transmission

Despite differing in their choice of habitat on human hosts, of particular interest is their difference in vector competence in transmitting bacterial pathogens. Vector competence is defined as the capacity of a vector to allow the development and/or transmission of a microbial pathogen/parasite inside of its body. Many environmental, behavioral, and biological factors influence the relationship between a vector, a pathogen, and its vertebrate host. How insects gain

or lose vector competence and the mechanisms underlying these evolutionary processes are poorly understood in vector biology.

Body lice are vectors for infectious diseases such as epidemic typhus (*Rickettsia prowazekii*), relapsing fever (*Borrelia recurrentis*), and trench fever (*B. quintana*) in humans. All three of the pathogens that body lice are capable of transmitting are Gram-negative bacteria (Durden & Mullen, 2002, Lounibos, 2002). In the case of *B. quintana*, once it is introduced into the gut of a louse by blood feeding on an infected human host, the bacteria colonize and attach to the surface of epithelial cells of the alimentary tract and replicate (Ito & Vinson, 1965). Lice have a behavior of excreting urine and feces during feeding and viable *B. quintana* in louse fecal matter can be transferred to the human bloodstream via scratches or wounds on human skin. *B. quintana* can also form a biofilm-like structure in the feces, allowing *B. quintana* survival for up to a year. Thus, infected louse feces are the most common means for *B. quintana* transmission to humans (Kostrzewski, 1949; Seki *et al.*, 2007) In 2009, *B. quintana* was detected in 33% of the body lice recovered from the homeless in California, suggesting that *B. quintana* is still prevalent in the human population (Bonilla *et al.*, 2009). Unlike body lice, the conspecific head lice are not known to efficiently transmit bacterial diseases to humans. However, it has been suggested that head lice can serve as a vector based on the findings that *B. quintana* is detected in some head louse populations collected in Nepal and in the United States (Bonilla *et al.*, 2009; Sasaki *et al.*, 2006). Additionally, Previte *et al.* (2014) demonstrated that the numbers of bacteria cells were similar in body and head lice during the early stages of infection by *B. quintana* (0-3 days post-infection), indicating that at this time head lice may transmit this bacteria in a manner similar to body lice. It should be noted that *B. quintana* in head lice feces has been recently shown to not

be viable, meaning that head lice would have much lower or essentially no vector competence (Kim *et al.*, 2017). Since the insect immune response plays a key role in the insect-pathogen interaction, the difference in vector competence between body lice and head lice may be governed, in part, by eliciting different immune responses. Direct comparison of the molecular and physiological aspects of their immune system would dramatically expand our knowledge of how lice became vectors for human diseases, and how body lice evolved from their ancestral head lice progenitor to become a very efficient vector.

1.4 Immune Response

Since body and head lice possess virtually the same genome (Kirkness *et al.*, 2010; Kang *et al.*, 2015), determination of the differential immune responses against microbial challenge should efficiently elucidate the difference in vector competence between body and head lice. Unlike vertebrates, the insect immune response relies solely on the innate immune system, which has been extensively studied using various model insects such as *Drosophila melanogaster* (Christophides *et al.*, 2004; Hoffmann & Reichhart, 2002; Hultmark, 2003). The study of innate immunity in insects has not only improved our understanding of the insect immune response, but has also advanced human immunology as well. The discovery of Toll-like receptors as pattern recognition receptors (PRR) were first found in *D. melanogaster*, and later found in humans (Hoffmann *et al.*, 1996; Baxter *et al.*, 2017). Aside from physical barriers to infection at epithelial surfaces, innate immunity is composed of both cellular and humoral immune responses, each with their own ability to sense a diverse array of pathogens and effectively rid these pathogens upon recognition (Beutler, 2004). Cellular immune responses, such as

phagocytosis and encapsulation of invading microorganisms by hemocytes, and humoral immune responses, such as the initiation of enzyme cascades and secretion of antimicrobial peptides (AMPs) into the hemolymph (fluid portion of insect blood), are common amongst arthropod species (Baxter *et al.*, 2017; Lavine & Strand, 2002).

1.5 Antimicrobial Peptides

The effector cells of the cellular immune response are hemocytes, which respond to the recognition of foreign microorganisms to clear them from the host. A major component of the humoral immune system are secreted AMPs, which are naturally occurring molecules created by multicellular organisms to directly kill bacteria, yeasts, fungi, and even cancer cells (Zhang & Gallo, 2016). The majority of these synthesized AMPs are inactive precursor proteins or pro-peptides, and upon proteolysis become active effector peptides. Hundreds of these insect peptides with antimicrobial activities have been purified or identified (Yi & Huang *et al.*, 2014). In contrast to mammalian antimicrobial peptides, insects, such as *D. melanogaster*, produce peptides during an immune response that are active against a more limited range of microorganisms. The three general groups in the *D. melanogaster* humoral immune response, characterized after their main microbial targets, are: 1) antifungal peptides, 2) peptides active against Gram-negative bacteria, and 3) peptides active against Gram-positive bacteria (Ezekowitz & Hoffman, 2003). These immune defense systems are thought to interact, but the extent to which they behave and their nature after initiation of an immune response is poorly understood. Arthropod AMPs, specifically defensin, are generally active against Gram-positive bacteria, but they can also be effective against Gram-negative bacteria, fungi, yeasts, and

parasites (Dimarcq *et al.*, 1998; Yi & Huang *et al.*, 2014). For example, a defensin peptide purified from the hemolymph of the hard tick, *Amblyomma hebraeum*, displayed antibacterial activity against both Gram-positive and -negative bacteria (Lai *et al.*, 2004). The specificity of these synthesized peptides to foreign pathogens in insects is unclear, but their function in immune response is likely dependent on the membrane composition of the target microbe and cell type that allow these molecules to become active (Yi & Huang *et al.*, 2014). Of particular interest are the defensins, which have been shown to play an important role in host defense. The term “defensin” defines several peptide families based on functional (host defense) and structural (a compact cysteine-stabilized β -sheet structure) similarities, with the mode of action differing both within and from different families (Antcheva *et al.*, 2013; Dimarcq *et al.*, 1998). They occur in multigene families and are synthesized as prepropeptides and processed depending on the context. Expression is constitutive in some cases, but in others it has been shown to be induced by bacteria / bacterial components or by proinflammatory cytokines in humans (Hazlett & Wu, 2010). Invertebrate defensins have been isolated from the hemolymph of insects, arachnids, and mollusks (Froy & Gurevitz, 2003), with insect defensins capable of being induced by bacterial components for release from the fat body into the hemolymph, surface epithelia, and in the midgut. The function of the defensins has been suggested to both prevent infection of blood-sucking insects by blood-borne pathogens, as well as to protect the stored blood meal from microbial attack. Defensins have been shown to possess antimicrobial action *in vitro*, usually interacting with the microbial cell wall to create membrane permeabilization (Antcheva *et al.*, 2013; Ganz, 2003; Dimarcq *et al.*, 1998). A definite mode of action is still unclear in insects, however insect defensins have a common structural feature (an amino-terminal loop, an α -helix,

and a two-stranded antiparallel β -sheet) with vertebrate defensins, and it is suggested they have a similar mode of action.

1.6 Immune Genes of Body and Head Lice

The differences in the immune response between body and head lice were initially investigated in previous studies using two model bacteria: a Gram-positive (*Staphylococcus aureus*) and a Gram-negative (*Escherichia coli*) bacteria (Kim *et al.*, 2011; Kim *et al.*, 2012). Kim *et al.* showed that body lice may have lower immune responses against both Gram-positive and Gram-negative bacteria when compared with head lice (Kim *et al.*, 2011, 2012). A total of 93 immune-related genes were identified in both body and head lice, which is substantially fewer than in other insects (Kim *et al.*, 2011). Many gene families in the humoral immune system are considerably reduced in number or absent in the body louse genome. For genes related to pathogen recognition, only one type of peptidoglycan recognition protein (PGRP) was found in lice, whereas beta-glucan binding protein was not identified. In addition, the *immune deficiency* (*Imd*) gene and *Fas-Associated protein with Death Domain* (*FADD*) gene, which encode its adaptor protein in the Imd pathway, were not present. In contrast, all components in the Toll, Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) and c-Jun N-terminal kinase pathways were conserved. Amongst the various kinds of AMPs, only two types of defensins (defensin 1 and defensin 2) were annotated. Comparisons of the transcriptome (Olds *et al.*, 2012) and genome (Kang *et al.*, 2015) of body and head lice previously demonstrated that they both possess virtually identical genetic backgrounds and the same set of immune-related genes with nucleotide diversity ranging from only 0.1–1.3% in the coding region. Genome-wide

analysis of the whole body transcriptomes of body and head lice following *B. quintana* oral challenge revealed that some immune-related genes associated with the Toll pathway (i.e., *fibrinogen-like protein*, *spaetzle*, *defensin 1*, *serpin*, *scavenger receptor A* and *apolipoprotein 2*) were differentially expressed between body and head lice (Previte *et al.*, 2014).

1.7 Transcription Levels of Immune Genes in Body and Head Lice

The relative transcript levels of six representative immune-related genes involved in epithelial immunity were compared in a previous study performed by Kim *et al.* (2012): a single recognition protein (*PGRP*) and five major effector genes [*defensin 1*, *defensin 2*, *dual oxidase (Duox)*, *lysozyme* and *prophenoloxidase (PPO)*]. Transcriptional profiling of these representative immune genes in the alimentary tract tissue showed that the basal transcription levels of *PGRP*, *defensin 1*, and *defensin 2* were higher in the alimentary tract of head vs. body lice, while the others showed no apparent alteration in transcription (Kim *et al.*, 2012). Furthermore, none of the immune-related genes, except for the lysozyme gene, was significantly up or down regulated in the alimentary tract tissues of body vs. head lice following oral infection of the Gram-negative bacteria *Escherichia coli*. Kim *et al.* (2017) further studied these differences using the same six representative immune genes, but instead looked at the transcript levels in the gut tissue over time after a single oral challenge with *B. quintana*. The transcription level of *defensin 1* in the dissected alimentary tract was significantly upregulated by oral challenge only in head lice 4 days and 8 days post-infection, while the transcript levels of all five remaining genes were not significantly altered between either louse ecotype over time. In addition to lower basal

transcription levels of *defensin 1*, *defensin 2*, and *PGRP*, the transcription of *defensin 1* was not induced in body lice following *B. quintana* oral challenge, which was opposite to the head lice response to oral challenge. Therefore, the lack of induction of *defensin 1*, as well as the lower basal transcription levels of *PGRP* and *defensin 1* and *2* in body lice appear to be mechanisms consistent with a reduced immune response to *B. quintana* in body versus head lice. Considering that there is no cellular immune reaction in gut tissues, but only in the hemolymph, these findings suggest that selectively induced transcription following bacterial challenge, as well as constitutive up-regulation of some immune genes in head lice, can contribute to the rapid defense and enhanced immune capacity of head lice against intestinal bacterial infection, a response not seen with body lice.

1.8 Bacterial Oral Challenge & Transmission

In preliminary studies, Kim *et al.* (2017) compared the propagation and excretion in feces of green fluorescent protein (GFP)-expressing *B. quintana* (GFP-*B. quintana*) to that of wild-type *B. quintana* (WT-*B. quintana*) (Previte *et al.*, 2014) in both body and head lice following oral challenge. Despite ingesting the same amount of blood in a single sufficient feeding session, Kim *et al.* (2017) showed that the number of GFP-*B. quintana* per louse was higher on average in body lice than in head lice at 1, 3, 6, 9, and 12 days post-challenge. GFP-*B. quintana* proliferation declined for 3 days post-challenge, but subsequently increased in the alimentary tract of both body and head lice in a manner similar to the proliferation pattern of WT-*B. quintana* in the whole body studied by Previte *et al.*, 2014. The proliferation profiles of both GFP-*B. quintana* and WT-*B. quintana* reported by Kim *et al.* and Previte *et al.*, respectively,

showed that two phases of proliferation (i.e., an initial phase of decline in the number of bacteria until 2–4 days post-challenge and the second phase of increase thereafter) were commonly observed, particularly in body lice (Kim *et al.*, 2017; Previte *et al.*, 2014). A similar pattern was also observed in the initial study by Seki *et al.* (2007), however, this initial study showed *B. quintana* cell number remained at a stationary phase after reaching a maximum level, unlike the decline observed in the proliferation profile after 9 days post-challenge by both Kim *et al.* (2017) and Previte *et al.* (2014). The reason for this difference is unknown but it may be due, in part, to the accumulation of various factors in the two different experimental settings used. Differences in various experimental factors, including the frequency of blood feeding, the inoculating titer of *B. quintana* for oral challenge, the feeding systems, the strains of *B. quintana* used, the age and strains of lice used, and many more may have affected the dynamics of *B. quintana* excretion and proliferation.

1.9 Bacterial Oral Challenge & Excretion

Knowing that the majority of the pathogenic bacteria vectored by body lice are transmitted to humans via contaminated feces from infected lice, Kim *et al.* (2017) also observed the viability of *B. quintana* in the feces of both body and head lice. Excretion of GFP-*B. quintana* did not differ significantly between the infected body and head lice over a 15 day interval as judged by the estimated copy number of bacterial *16S-23S ribosomal RNA (rRNA)* gene present as a PCR product in the feces. Although the number of GFP-*B. quintana* cells was derived from the estimated copy number of *16S-23S rRNA* gene in the PCR product, this estimate does not separate viable whole cells from fragmented (non-viable) cells. Despite the

lack of significance in the estimated copy number between body lice and head lice in a unit amount (1 mg) of feces as judged by quantitative polymerase chain reaction (qPCR), fluorescence microscopy was performed by Kim *et al.* (2017) to assess the viability of *B. quintana* in the feces. A noticeably more consistent and intense GFP-*B. quintana* fluorescence was shown in body lice feces compared to head lice feces over time. Body lice excreted a significantly larger number of viable GFP-*B. quintana* in their feces, particularly during the early stage of infection (i.e., 1~3 days post-challenge), as determined by the viability index (Kim *et al.*, 2017). This finding suggests that the proportion of live and viable bacteria in the excreted feces infected by GFP-*B. quintana* is significantly larger in body lice than in head lice up to 11 days post-challenge. Combining the ability of *B. quintana* to both significantly proliferate in the alimentary tract and to be more viable in excreted body lice feces than in head lice feces, these mechanisms offer potential insight into the enhanced infectivity of the body louse, although this suggestion is just speculation at this time.

1.10 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are compounds generated in a variety of biological and metabolic processes in organisms and are important for different cellular functions. ROS are capable of influencing cellular processes such as cell proliferation and differentiation, cell death, signaling cascades, and expression of genes (Genestra, 2007). Some ROS are free radicals, which are highly reactive, primarily due to their presence of unpaired electrons that makes them more willing to obtain electrons to attain stability. Examples of these are superoxide ($O_2^{\cdot-}$), oxygen radical ($O^{\cdot-}_2$), hydroxyl (OH^{\cdot}), alkoxyradical (RO^{\cdot}), peroxy radical (ROO^{\cdot}), nitric oxide

(nitrogen monoxide; NO \cdot) and nitrogen dioxide (NO $_2$) Some ROS are not free radicals, such as: hydrogen peroxide (H $_2$ O $_2$), hypochlorous acid (HOCl), nitrous acid (HNO $_2$), organic peroxides (ROOH), aldehydes (HCOR), and peroxyxynitrite (ONOOH). These and many more non-radical species are capable of being converted to free radical species via natural reactions in living organisms (Phaniendra *et al.*, 2014).

Due to their non-specific, highly reactive nature, ROS are potentially toxic to the host and should thus be generated transiently and/or kept localized. The majority of ROS produced intracellularly originates in the mitochondrial respiratory chain, where the release of these products into the cytosol is tightly regulated to maintain the homeostasis of redox reactions and other important signaling pathways of the cell (Bae *et al.*, 2011). Other sources of ROS in any organism are microsomal oxidation of xenobiotics (synthetic chemical foreign to the body) and phagocytosis (Farooqui *et al.*, 2011). Microsomes created by reforming pieces of the endoplasmic reticulum (ER) in the laboratory have been shown to contain enzymes of the cytochrome *P450* monooxygenase (CYPs) system. CYPs are enzymes present in all kingdoms of life, and they act to catalyze oxidation of endogenous and xenobiotic compounds (using heme as a cofactor) while simultaneously generating ROS (Nelson, 2009). Xenobiotic clearance depends on many factors, but the two main phases of elimination are the introduction of polar groups (with the use of CYP enzymes) and/or the conjugation of molecules with water-soluble ligands (catalyzed by UDP-glucosidase, sulfotransferase, and glutathione S-transferase enzymes) (Farooqui *et al.*, 2011). It is also important that hosts have the capability to protect themselves from oxidative damage by detoxifying these potentially hazardous compounds from cells. Enzymes such as NADPH oxidase, superoxide dismutase (SOD), and myeloperoxidase help to

produce some of the most common ROS: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hypochlorous acid ($HOCl$), respectively. Metabolites created via an array of possible reactions can either be directly antimicrobial, or can yield other metabolites that are indirectly capable of having the same effect once being altered following further reactions. For example, hydrogen peroxide is capable of producing highly reactive halides after being generated by myeloperoxidase. Hypochlorite (OCl^-) is an example of one of these reactive halides that not only has the potential to directly act on microbes, but can also react with superoxide to yield another important antimicrobial species: hydroxyl radical (OH^\cdot) (Beutler, 2004).

1.11 ROS – Fenton Reaction

An important catalytic process that is capable of forming ROS is the Fenton reaction. The Fenton and Fenton-like reactions take place naturally in many different systems, such as in the environment for ROS generation in atmospheric clouds and in aquatic environments such as rivers and oceans, as well as in the human body with potential connections to aging, neurodegenerative diseases, cardiovascular diseases, and cancers (Chen, 2019). The Fenton reaction describes the formation of hydroxide (OH^-), hydroxyl radical (OH^\cdot), and ferric iron (II) (Fe^{3+}) via the reaction between ferrous iron (II) (Fe^{2+}) and hydrogen peroxide (H_2O_2) under acidic conditions (Dziubla and Butterfield, 2016). H_2O_2 produced by aerobic respiration is capable of oxidizing the reduced metal ion, Fe^{2+} , producing OH^- and OH^\cdot , two highly reactive oxygen species. These ROS can then serve as intermediates for other reactions, react with biomolecules to form additional free radicals, or even continue to generate more ROS (Tripathi *et al.*, 2018). Imbalances caused by these reactions, and the inability for biological systems to

detoxify these reactive intermediates, can lead to oxidative stress - which has the potential to cause free-radical mediated damage to proteins, membranes, and DNA (Tripathi *et al.*, 2018).

The Fenton reaction is of particular interest in hematophagous (blood-feeding) insects due to the uptake of large amounts of iron from the blood of the host via an ingested blood meal. Hematophagous arthropods include Diptera (mosquitoes, flies, and biting midges), Hemiptera (bed bugs and assassin bugs), Phthiraptera (lice), and Siphonaptera (fleas). When the hemoglobin protein in blood is digested by hematophagous insects, a significant amount of heme (containing iron) is released, which now serves as a pro-oxidant capable of inducing oxidative stress by producing hydroxyl radicals through the Fenton reaction, thus increasing the internal sources of ROS (Champion and Xu, 2017). It has been reported that higher systemic levels of ROS in mosquitos increase mosquito survival following bacterial challenge, thus showing the potential that the generation of ROS has as an effective mediator of antimicrobial defense in the arthropod gut (Molino-Cruz *et al.*, 2008). Understanding that excess ROS can lead to cellular damage, it is vital to mitigate the massive pulse of oxidative stress accompanied by blood feeding while simultaneously retaining the ability to combat infection through oxidative bursts (Graca-Souza *et al.*, 2006).

1.12 ROS – Oxidative Burst / Phagocytosis

Following the ingestion of microbes, an oxygen-dependent oxidative burst (also known as ‘respiratory burst’) occurs, rapidly releasing ROS to interfere with invading microbes and to expel them before causing a potential infection (Flaherty, 2012). This respiratory burst has only been shown *in vitro* in phagocytic cells of the western honey bee (*Apis mellifera*) when

stimulated by protein kinase C (PKC), thus generating ROS in the insect's hemocytes via a phagocyte oxidase system (Richardson *et al.*, 2018). Midgut cells play an essential role in the innate immune response of insects, as they constitute their most exposed interface to potential pathogens. As the primary infection route of *B. quintana* is to enter the alimentary tract of the louse via an infected blood meal, the primary immune response following feeding is the release of AMPs and a reactive oxygen species (ROS)-based 'oxygen burst.' This increase in the amount of ROS is generated by alimentary tract epithelial tissue, one of the major sites for the humoral immune response of the louse's innate immune system. Kim *et al.* (2011) has shown evidence suggesting that the immune system of body lice relies partially on phagocytosis, which implied the existence and function of phagocytic cells in these organisms. They showed that body and head lice, once injected with either *S. aureus* or *E. coli*, contained phagocytic cells in the abdominal region of the louse due to a combative immune response. This cellular immune response was delayed and showed a smaller phagocytosis index for both lice when exposed to *S. aureus*. During early immune response to *E. coli* (within 120 minutes after injection), body lice cellular immune response onset showed an 8-fold lower phagocytosis index than head lice (Kim *et al.*, 2011). This lowered ability of body lice to fight off bacterial infection enables them to become a better vector for diseases when compared to head lice, allowing for a more susceptible pathogen reservoir and efficient method of transmission. Although little is known about the cells of the immune response in body and head lice alike, it is possible these cells could have mechanistic similarities to that of the immune cells of mammals. Cells that are able to ingest particles, generate ROS, and then dispose of bacteria, are often considered phagocytes. Upon phagocytosis in some immune cells of mammals (i.e. neutrophils and macrophages), the

production of free radicals by the action of NADPH-oxidase through the cytochrome system has been implicated as a major microbe-killing defense mechanism (Graca-Souza *et al.*, 2006; Bergin *et al.*, 2005).

1.13 ROS - Blood Feeding

In 2017, Kim *et al.* also aimed to elucidate the effects that ROS had following the imbibing of a blood meal, and a potential means of antimicrobial defense via a respiratory burst. Kim *et al.* (2017) compared the ROS levels generated by epithelial cells in the alimentary tract tissue using three indicators that detect different types of ROS. It was found that the levels of ROS were not significantly different between starved and blood-fed lice, indicating that imbibing a blood meal did not affect ROS levels either in body or head lice (Kim *et al.*, 2017). While blood feeding rapidly decreased ROS through a mechanism involving heme-mediated activation of PKC in *Aedes aegypti* (Oliveira *et al.* 2011), the level of ROS in the gut was not significantly different in starved versus blood-fed lice as found by Kim *et al.* (2017). Nonetheless, they found that the net ROS levels were significantly higher in head versus body lice regardless of their feeding status, suggesting that the oxidative-based killing caused by ROS generation may also contribute to the suppressed proliferation of *B. quintana* in head lice (Kim *et al.*, 2017). Considering all of the aforementioned results, it is important to investigate the epithelial tissue-specific humoral immune responses and determine if any differences exist between body and head lice that may contribute to the differential proliferation of *B. quintana* in the gut and feces of lice.

In the following thesis research, we test the hypotheses that the difference in vector competence between body and head lice is due, in part, to eliciting different immune responses - particularly through an increased level of antimicrobial peptides and ROS in the head louse, reducing its vector competence. The knockdown of *defensin 1* by RNAi in lice should enhance the ability for either louse ecotype to harbor bacteria in their respective alimentary tracts. It is hypothesized that body lice are better vectors for *B. quintana* due to their lowered ability to combat early stages of infection as efficiently as head lice. In the present study, the role of defensin 1 in this process is assessed along with the ability of each louse ecotype to generate ROS to control bacteria proliferation and minimize infection. The following thesis research also tests the hypothesis that a diminished ROS-based immune response in body lice compared to head lice contributes to a greater proliferation of *B. quintana* in the gut, thus making the body louse a more superior vector for trench fever by excreting more viable bacteria through their feces. A ROS-based “oxygen burst” is postulated to occur more prominently in head than in body lice during early stages of infection. The amount of ROS is measured during initial stages (2 hours) and a later stage of infection (day 4) to observe the effects that the changing levels of ROS may have on proliferation of *B. quintana* in the alimentary tracts of both louse ecotypes. From these investigations, insights into the differential immune responses of body versus head lice should help elucidate the specific roles that AMPs (specifically defensin 1) and that of ROS production play during the early stages of imbibing an infected blood meal, and overall the vector competence these ecotypes possess.

CHAPTER 2

MATERIALS & METHODS

2.1 Lice Rearing

The San-Francisco (SF-BL) strain of body louse (*P. h. humanus*) and the Bristol (BR) strain of head louse (*P. h. capitis*), which were originally collected in San Francisco, California and Bristol, UK, respectively, have been reared on an *in vitro* membrane feeding system on human blood obtained from the US Red Cross (Yoon *et al.*, 2006). Lice colonies were maintained under the conditions of 30°C, 70-80% relative humidity, and 16L:8D in a rearing chamber. To compare the bacteria proliferation following oral challenge, 2-or-3-day old females were used for all experiments.

2.2 *Bartonella quintana* Culture

The Gram-negative human pathogenic bacteria, *Bartonella quintana*, used for immune challenge was obtained from Dr. Jane Koehler (University of California-San Francisco, School of Medicine) and was maintained in a Biosafety Level 2+ facility at the University of Massachusetts-Amherst (Institutional Review Board E1404/001-002). Frozen stocks of wild-type strain *B. quintana* JK31 (deposited at www.BEIresources.org #NR-31832; Zhang *et al.*, 2004) were cultured on nutrient chocolate agar plates and stored in candle extinction jars at 35°C for 10 days until passing onto fresh plates for an additional 5-7 days of growth using sterile inoculating loops (Zhang *et al.*, 2004).

2.3 Oral Infection of Lice using *Bartonella quintana*

B. quintana cells were harvested from the chocolate agar plates by rinsing the surface of the plate with 1 ml of phosphate-buffered saline (PBS; pH 7.4). Bacterial cells were pelleted by centrifugation at 1,000 g for 4 min and washed twice with PBS. The pellet was resuspended in 100 µl PBS, and a 5 µl aliquot was taken for serial dilution. Each serial dilution was plated onto chocolate agar plates in triplicate for *B. quintana* enumeration. The numbers of colony forming units (CFUs) were counted 10 days after culture. Spectrophotometric readings [Optical density at 600 nm (OD₆₀₀)] were used to determine the approximate number of bacteria cells prior to louse infection. The remaining bacterial suspension was mixed with 3 ml of human whole blood (The American National Red Cross, Massachusetts Region) to a final titer of $\sim 1 \times 10^7$ CFU/ml (Kosoy *et al.*, 2004) before dividing equally amongst louse feeding systems. Both female body and head lice were starved for 15 h and fed with *B. quintana*-inoculated blood using the artificial membrane-based rearing system for 1 h to ensure a single sufficient feeding. Over the succeeding days, the orally-infected lice were fed with uninfected whole human blood and maintained until used in experiments. For *defensin 1* knockdown, dsRNA-introduced female head lice were used at 72 hours after injection (see section 2.4).

2.4 Gene Knockdown by RNA Interference (RNAi)

To synthesize double-stranded RNAs (dsRNAs), total RNA was extracted from 5 lice using TRI reagent (MRC, Cincinnati, OH, USA) according to the manufacturer's protocol. Total RNA samples were treated with DNase1 (TAKARA Biotechnology, Japan) to remove contaminated genomic DNA (gDNA). cDNA was synthesized from total RNA (2.5 µg) using the

Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) and diluted to 5 ng/ μ l to use as templates for qPCR. A *defensin 1* transcription template (337 bp, nucleotide #34-370) from the cDNA of body lice and a pQE30 transcription template (422 bp) from pQE30 UA vector (Qiagen Korea, Osong, Korea) were generated by PCR amplification using gene-specific primers with the T7 promoter sequence (TAATACGACTCACTATAGGG) attached at their 5' ends. The PCR products were purified and concentrated using the PCR SV mini kit (GeneAll Biotechnology, Osong, Korea). dsRNAs were synthesized from the PCR product using the MEGAscript T7 transcription kit (Ambion, Austin, TX) according to manufacturer's instructions and purified by ethanol precipitation. After resuspension in nuclease-free water, the dsRNA was quantified with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE), and its purity and quantity were examined by agarose gel electrophoresis with a Low DNA Mass Ladder (Invitrogen). The dsRNAs were diluted individually in nuclease-free water to 2 μ g/ μ l, and 46 nl of each dsRNA (92 ng) were injected into the 2nd abdominal segments of either body or head lice females using a nano-injector (Nano Liter 2000, World Precision Instrument, FL). Lice were injected individually to ensure dsRNA retention in the hemolymph of the laterally pierced cuticle of the abdomen. Injected lice were then transferred to the *in vitro* membrane feeding system and fed whole human blood for 3 days before oral infection (see section 2.3).

To confirm gene knockdown, ten injected lice were randomly selected at each time points and total RNA extracted using PureLink RNA Mini Kit (Invitrogen) with DNaseI on-column digestion according to the manufacturer's instructions. The first-strand cDNA was synthesized from total RNA using SuperScript III reverse transcriptase (Invitrogen), diluted to 5 ng/ μ l and used as template for qPCR of *defensin 1*. The qPCR was performed in a StepOne Plus Real Time

PCR System (Applied Biosystems, Darmstadt, Germany) using the following cycling conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, 56°C for 20 s, 60°C for 30 s and serial increase of 0.2°C per 1 s from 45 to 95°C for melting curve analysis. The reaction mixtures contained 1X Power SYBR Green PCR Master Mix (Applied Biosystems) and 0.5 pmol primers for *defensin 1* genes or *RpL13A* as a reference gene. The primer sets used for the qPCR are shown in Table 1. Quantification of relative transcript level of a gene was conducted based on the original concept of $2^{-\Delta C_t}$ (Pfaffl, 2001). qPCR was conducted with two technical replicates to adjust intra-PCR variation.

2.5 Standard Protocol for *Bartonella quintana* Quantification (using the original methods designed by Dr. Ju Hyeon Kim, Seoul National University, Republic of Korea)

Wild type *B. quintana* grown on a chocolate agar plate was harvested using the PBS method described above (see ‘Oral Infection of Lice using *B. quintana*’ section). The bacterial suspension was centrifuged at 1000 *g* for 4 min and the pellet resuspended in tissue lysis buffer for gDNA extraction using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. A 134-bp gDNA fragment of *B. quintana 16S-23S rRNA* intergenic spacer region was generated by PCR from the extracted gDNA, from which an 89-bp nested fragment amplified using the internal reference gene, *RpL13A* (*Ribosomal Protein L13A*), was PCR-amplified using a nested primer set. The PCR product was visualized on agarose gel using ethidium bromide staining, purified using a QIAquick Gel Extraction Kit (Qiagen) and cloned into the pGEM-T easy vector (Promega, Madison, WI, USA). After sequencing to confirm the product specificity, the positive plasmids were linearized with Sall

(Koschem, Seoul, Korea), purified using a QIAquick PCR Purification Kit (Qiagen) and quantified using a Nanodrop spectrophotometer (Nanodrop Technologies). Six serial dilutions of the linearized plasmids, ranging from 1 ng/μl to 10 fg/μl, were used as standard DNA for qPCR. The copy number of the *16S-23S rRNA* gene in each standard DNA sample was calculated from the amount and molecular mass of the linearized plasmid using a DNA molecular weight calculator (<http://www.currentprotocols.com/WileyCDA/CurPro3Tool/toolId-8.html>). A standard curve of the threshold cycle (Ct) values vs. the copy numbers was generated using qPCR and used to calculate the total copy numbers of the *16S-23S rRNA* gene in the target gDNA template. As a single *B. quintana* bacterium contains two copies of the *16S-23S rRNA* gene (Seki *et al.*, 2007), the number of *B. quintana* bacteria was calculated by dividing the estimated copy number of the *16S-23S rRNA* gene by 2.

2.6. Comparison of ROS Levels in Alimentary Tract Tissues

Female body and head lice were starved for 15 h prior to experiments and half of the total number of each type of louse were then fed *B. quintana*-infected or uninfected human blood using the *in vitro* rearing system until their alimentary tracts were full of blood by visual inspection. The alimentary tracts of starved and blood-fed lice were dissected in ice-cold PBS (pH 7.4) and the gut contents removed by washing in PBS prior to use in all experiments. To determine the types and levels of ROS produced, a number of detection methods were used. For the simultaneous detection of a wide array of ROS, the dissected alimentary tracts were incubated with a 10 μM solution of 5-(and-6)-chloromethyl-2', 7'-dichloro-dihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA; Invitrogen) as the general oxidant-sensitive fluorophore

for 20 min at room temperature in the dark. After incubation, the alimentary tracts were washed with PBS and homogenized using a glass micro homogenizer (Wheaton Industries, Millville, NJ, USA). The homogenates were centrifuged at 12,000 *g* for 1 min and fluorescence [Excitation (Ex) at 500 nm; Emission (Em) at 520 nm] measured with a GeminiXS spectrofluorometric plate reader (Molecular Devices, Sunnyvale, CA, USA). For the hydroxyphenyl fluorescein (HPF) assay, which also detects a similar range of ROS as CM-H₂DCFDA, the dissected alimentary tracts were incubated in 5 μ M HPF (Invitrogen) solution for 60 min at room temperature in the dark. After incubation, the tissues were homogenized and centrifuged at 12,000 *g* for 3 min and fluorescence (Ex at 490 nm; Em at 515 nm) measured using a UV max spectrofluorometric plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.7 Statistical Analysis

All statistical analyses were performed using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). The means and standard deviations (SD) were calculated for each data set. Statistical differences of mean values ($p < 0.05$) were determined by ANOVA followed by Tukey's post hoc test and Student's *t*-tests.

CHAPTER 3

RESULTS

3.1 Comparison of *Bartonella quintana* Proliferation following RNAi of Major Factors

It has been shown previously that the amount of *defensin 1* transcript increased in both body and head lice following *E. coli* oral infection, suggesting this peptide's inducible role in humoral immune suppression of Gram-negative bacteria (Kim *et al.*, 2012). First, RNAi was conducted as above and validated using *defensin 1*-dsRNA injection to knockdown the transcription level of this gene in lice used in the current thesis research (Fig. 1). To investigate the differences in the immune responses between body and head lice due to infection, proliferation of *B. quintana* following RNA interference (RNAi) knockdown of *defensin 1* was measured (Fig. 2). In the case of *defensin 1*-knockdown head lice (Def1(-)), *B. quintana* number was maintained at a slightly higher level (2.69×10^7 and 1.28×10^7 *B. quintana* cell number per louse at day 0 and day 4, respectively) than in control (*pQE30*-dsRNA) head lice (1.79×10^7 and 5.00×10^6 *B. quintana* cell number per louse at day 0 and day 4, respectively). A 1.5-fold (50%) increase and a 2.5-fold (155%) increase in *B. quintana* number was seen in Def1(-) head lice at day 0 and day 4, respectively, indicating that knockdown of *defensin 1* allowed increased proliferation of *B. quintana* in head lice. As expected, the levels of *B. quintana* in both non-knockdown and knockdown head lice were lower (3.9-fold and 2.6 fold decrease, respectively) than that measured in body lice (7.02×10^7 *B. quintana* cell number per louse) at 2 hours (day 0) post-infection. It is of interest, however, that at 4 days post-infection, the Def1(-) head lice had an increased amount of *B. quintana* (1.28×10^7 *B. quintana* cell number per louse) when

compared to body lice (7.74×10^6 *B. quintana* cell number per louse), a 1.65% increase in bacterial cell number per louse. Since there is an initial decrease in the levels of *B. quintana* during the early stages (0-4 days) following infection, which is then followed by an exponential increase of bacteria number in both head and body lice over the next 10-11 days (Previte *et al.*, 2014), the slight increase in levels of *B. quintana* seen in Def1(-) head lice over both body lice and non-knockdown head lice at day 4 post-infection could likely lead to a substantial increase in *B. quintana* at day 11 post-infection when proliferation peaks.

Although not statistically significant ($p < 0.05$), defensin 1 appears to play a role in the proliferation of *B. quintana* in both head and body lice and knockdown of *defensin 1* leads to increased, although slight, proliferation of *B. quintana* in Def1(-) head lice during the early stages of infection. Other immune factors such as peptidoglycan recognition protein (PGRP), defensin 2, prophenoloxidase (PPO), dual oxidase (Duox), and Lysozyme also need to be further studied. These genes were previously found to serve as representative immune-related genes because they are responsible for pathogen recognition, downstream signal cascade events (particularly in the Toll pathway), and the direct killing of invading pathogens (Kim *et al.*, 2012). Further understanding of how these genes function together upon bacterial infection will lead to a more complete explanation for the enhanced bacterial proliferation of *B. quintana* in body lice when compared to head lice during the early stages of infection and during exponential growth. Of particular interest would be the determination of whether the additional genetic factors work either additively or synergistically in shaping the proliferation curve of *B. quintana*.

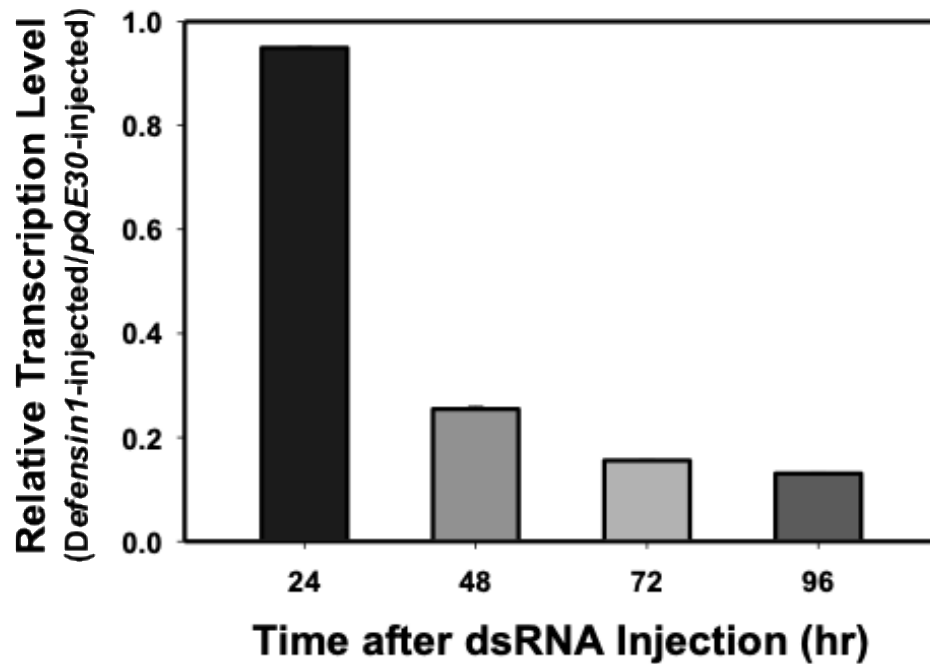


Figure 1. Relative transcription level of *Defensin 1*-dsRNA injected head lice compared with *pQE30*-dsRNA injected control head lice over time. Transcription level is relative to *Ribosomal Protein L13a (RpL13A)*, which was used as a reference gene for normalizing mRNA transcription. RNAi was validated by measuring transcription level at 24, 48, 72, and 96 hours after injection of dsRNA.

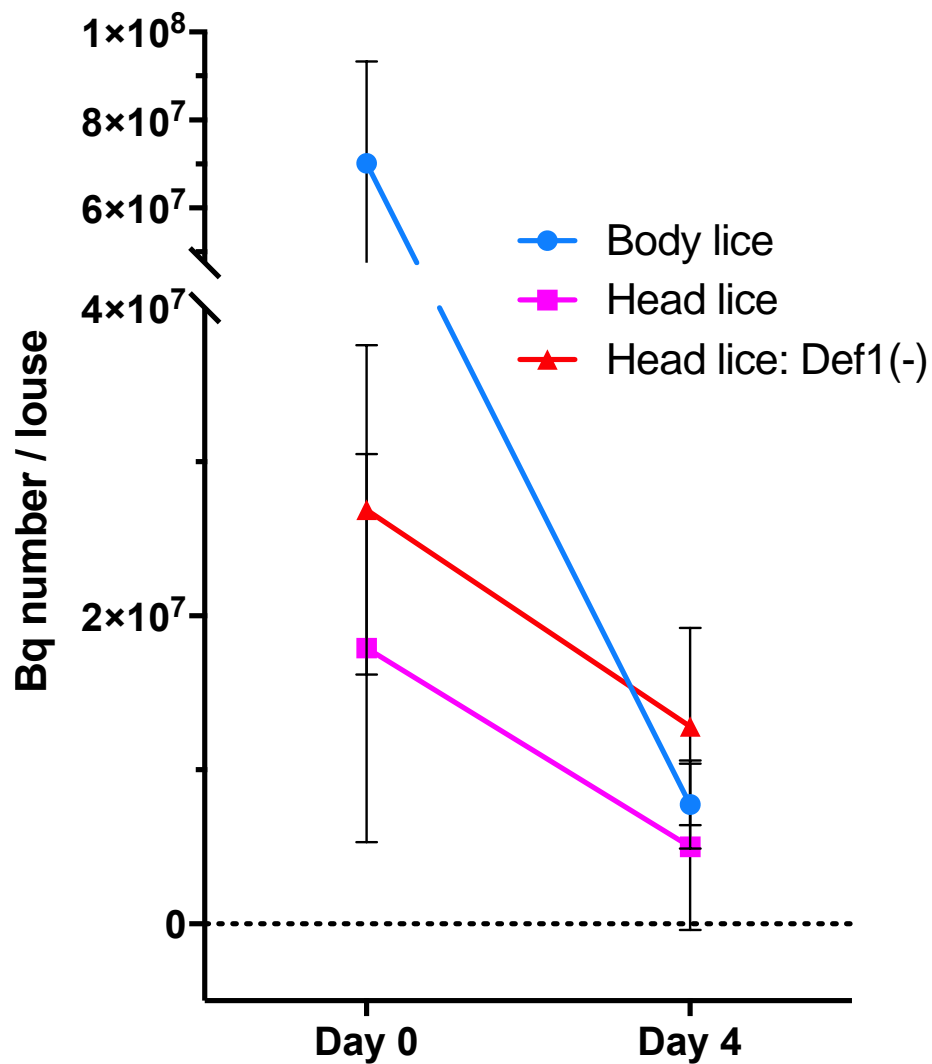


Figure 2. *Bartonella quintana* cell number per louse following oral challenge in both head and body lice, 2 hours (Day 0) and 96 hours (Day 4) after ingestion of an infected blood meal. Both body and head lice were injected with *pQE30*-dsRNA to serve as controls for comparison, while *defensin 1*-knockdown head lice (Head lice: Def1(-)) were injected with *defensin 1*-dsRNA for RNAi. Injection was performed 72 hours prior to oral infection of *B. quintana*. The dashed line serves as reference for the absence of bacteria. Error bars indicate standard deviation of the mean [Two-way ANOVA, Tukey's test].

In summary, it has been previously reported that the transcription level of the *defensin 1* gene was up-regulated only in head lice following oral challenge of *B. quintana* (Kim *et al.*, 2017). As the other five representative immune genes (*PGRP*, *defensin 2*, *PPO*, *Duox*, and *Lysozyme*) chosen in the same study had no alteration in transcription levels in either louse ecotype (Kim *et al.*, 2017), it was important to conduct gene knockdown experiments to test the importance of this gene in question. RNAi was validated using qPCR by measuring transcription levels at 24, 48, 72, and 96 hours following injection (Fig. 1). Transcription level analysis showed that after 48 hours following injection, the level of *defensin 1* compared to the reference gene (*RpL13A*) showed greater than 1.7-fold decrease in gene expression, with even greater decrease at 72- and 96-hours post-injection (Fig. 1).

Following oral infection, *B. quintana* was strongly detected in the whole-body homogenates of body lice (7.02×10^7 *B. quintana* cell number per louse) initially at 2 hours (day 0) post-infection (Fig. 2). At the same time point, head lice had less *B. quintana* in the whole-body homogenates (1.79×10^7 *B. quintana* cell number per louse), a 3.9-fold reduction. In *defensin 1* (*Def1(-)*) knocked down head lice, however, the bacteria cell number increased to 2.69×10^7 compared with non-knocked down head lice (1.79×10^7), a 1.5-fold increase, indicating that *defensin 1* was active in reducing *B. quintana* cell number in non-knocked down head lice (Fig. 2).

As seen in earlier studies, cell numbers decrease substantially over the first four days post-infection before rebounding exponentially (Previte *et al.* 2014). In the current experiment, the *B. quintana* cell numbers were found to decrease to similar levels in body (7.74×10^6 *B. quintana* cell number per louse) and in non-knocked down head lice (5.00×10^6 *B. quintana* cell

number per louse) at 96 hours (day 4) post infection (Fig. 2). Although not significant, the cell number in non-knocked down head lice was 1.6-fold less than in body lice at this time point. In head lice injected with *defensin 1*-dsRNA, *B. quintana* cell numbers also decreased to 1.28×10^7 *B. quintana* cell number per louse, but were 2.6-fold greater than non-knocked down head lice and 1.7-fold greater than body lice at day 4 (Fig. 2). Although these differences are not statistically significant, it does appear that the knockdown of *defensin 1* in head lice allows for increased proliferation of *B. quintana* compared with both non-knocked down head lice and body lice. ($p > 0.05$).

As reported by Previte *et al.* (2014), *B. quintana* cell number reaches a similar level per louse in both body and head lice after 4 days following infection. The proliferation profile of *B. quintana* following oral challenge likewise showed a decline for 3 days post-challenge in both body and head lice, and was subsequently followed by a significant increase in body lice and a slight increase in quantity in head lice immediately after 3 days (Kim *et al.*, 2014). These previous findings along with RNAi knockdown of *defensin 1* indicate that defensin 1 may not be the sole mediator of an early immune response in both of these louse ecotypes.

Given that orally-infected *B. quintana* cannot pass through the alimentary tract tissue and remain in the gut lumen and on the surface of epithelial cells (Ito and Vinson, 1965), it can be postulated that the cellular immune response is less important and that the humoral immune response is the primary immune factor against this invading pathogenic bacteria following ingestion of an infected blood meal. As reported by Previte *et al.* (2014) and Kim *et al.* (2017), the proliferation profile of *B. quintana* following oral challenge showed a decline for 3 days post-challenge, followed by a subsequent increase in both body and head lice. Kim *et al.* (2017)

also showed that the level of *defensin 1* transcript in head lice increased 3.2-fold relative to control (0 day) at 4 days post-challenge with *B. quintana*. For these reasons, the *B. quintana* cell number was observed following RNAi 4 days after oral challenge to see if head lice possessed a similar proliferation pattern to that of body lice following knockdown of this major effector gene. At both 2- and 96-hours post-infection, the numbers of *B. quintana* cells increased substantially in knocked down versus non-knocked down head lice and were greater than in body lice, indicating a role for defensin 1 in shaping the head louse immune response to this bacteria. Although not significant, the non-knocked down head lice have fewer *B. quintana* cells than body lice at 96 hours post infection and this difference may be amplified greatly due to the exponential growth seen for *B. quintana* in body lice over the next 6-7 days, leading to peak proliferation (Previte et al. 2014). Nonetheless, other representative immune genes (*PGRP*, *defensin 2*, *PPO*, *Duox*, and *Lysozyme*) could likely also be important and should be examined in the future.

3.2 Comparison of ROS Levels in Alimentary Tract Tissue following Infection with *Bartonella quintana* by Oral Challenge

As previously studied by Kim *et al.* (2017), ROS levels generated by epithelial cells in the alimentary tract tissue were compared between starved and blood-fed lice using three fluorescent indicators that test for a variety of ROS. Two of these indicators, 5-(and-6)-chloromethyl-2', 7'-dichloro-dihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) and hydroxyphenyl fluorescein (HPF), are general indicators and can test for a wide array of ROS. In the present study, the epithelial cell-specific immune response as determined by ROS production in the alimentary tract tissues were compared again between body and head lice, but in this case

following bacterial oral challenge using *B. quintana*. As determined by two general indicators of ROS, CM-H₂DCFDA and HPF, lice at day 1 following infection showed the greatest percent difference in ROS production of challenged lice relative to unchallenged lice. Orally challenged body lice showed a 42% (SD = 43%) and 34% (SD = 33%) increase in ROS whereas head lice showed a 70% (SD = 25%) and 22% (SD = 43%) increase in ROS at Day 1, using CM-H₂DCFDA and HPF, respectively. On day 4, however, orally challenged body lice showed a 40% (SD = 62%) increase and a 4% (SD = 8%) decrease in ROS using CM-H₂DCFDA and HPF, respectively. Orally challenged head lice showed a 0% (SD = 22%) change and 5% (SD = 40%) increase in ROS at day 4, using CM-H₂DCFDA and HPF, respectively. These results suggest that a large burst of ROS in both ecotypes is present immediately after oral infection. Although speculative, it can also be said that after 4 days body lice sustain a higher ROS production as bacterial numbers increase, whereas head lice return to a normal level of ROS production in the epithelial cells after combating most of the bacteria invading the alimentary tract at earlier time points.

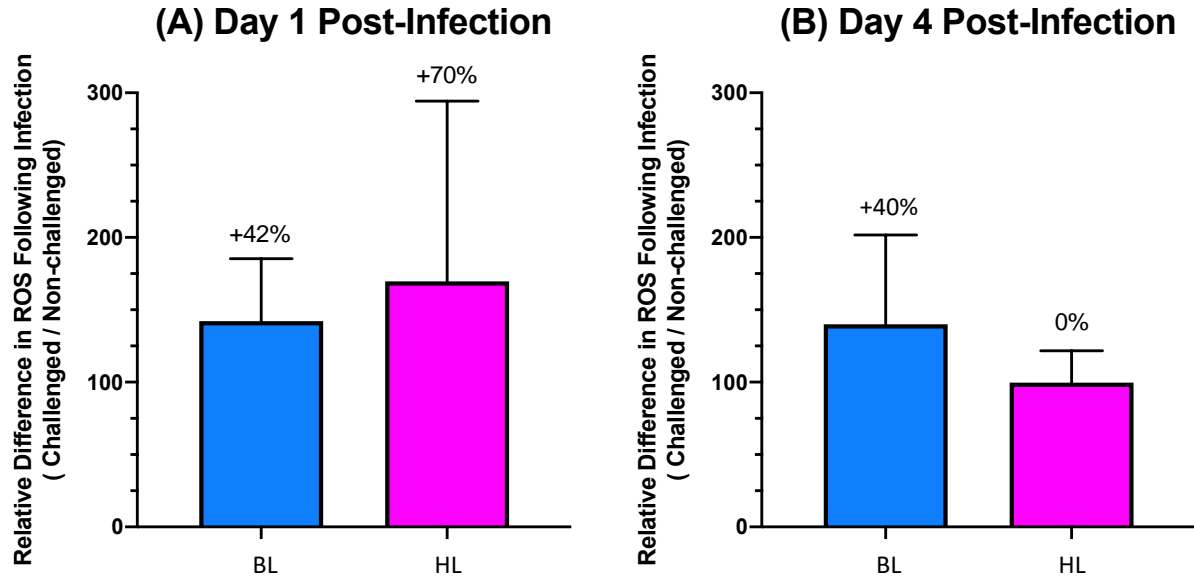


Figure 3. Comparison of the levels of general reactive oxygen species (ROS) produced using CM-H₂DFCDA in the alimentary tract tissue of both body and head lice following (A) 1 day post-infection, and (B) 4 days post-infection following oral challenge with *B. quintana*. Percent increase or decrease of ROS following *B. quintana* infection of orally challenged lice relative to uninfected, non-challenged lice is shown above the bars. N=4, n=20 lice used for each replication. Error bars indicate standard deviation of the mean [Two-way ANOVA, Tukey's test].

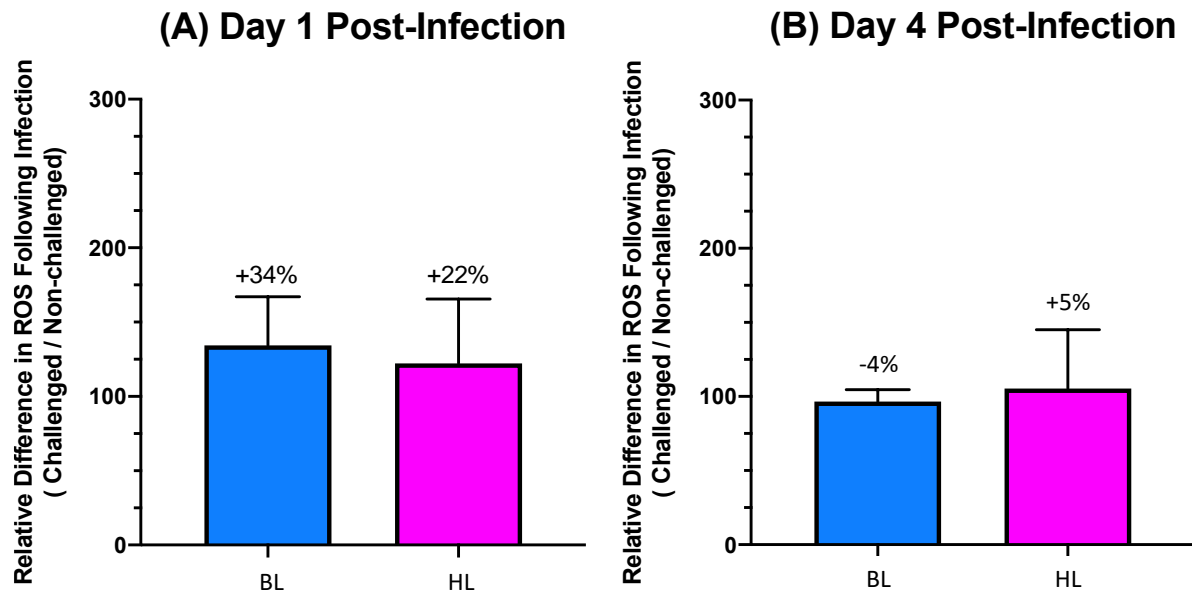


Figure 4. Comparison of the levels of general reactive oxygen species (ROS) produced using HPF in the alimentary tract tissue of body and head lice following (A) 1 day post-infection, and (B) 4 days post-infection. Percent increase or decrease of ROS following *B. quintana* infection of orally challenged lice relative to uninfected, non-challenged lice is shown above the bars. N=5, n=20 lice used for each replication. Error bars indicate standard deviation of the mean [Two-way ANOVA, Tukey's test].

In summary, ROS levels generated by epithelial cells in the alimentary tract tissue of both body and head lice following oral challenge with *B. quintana* were measured using two fluorescent indicators, CM-H₂DCFDA and HPF, that detect different types of ROS were used. The levels of ROS were previously not found to be significantly different between starved and blood-fed lice under any of the experimental conditions tested (Kim *et al.* 2017). These findings indicated that ingestion of a blood meal did not affect ROS levels in body or head lice. These researchers did show, however, that regardless of blood feeding or not, higher net levels of ROS were always detected in head louse epithelial cells when compared to body louse epithelial cells, as determined by both of the general indicators above (Kim *et al.* 2017).

To determine whether bacterial challenge triggered a ROS-based humoral immune response in the alimentary tract tissue, both female body and head lice were either fed: 1) a normal blood meal (non-challenged lice), or 2) or a blood meal infected with *B. quintana* (challenged lice). Lice at Day 1 following infection showed the greatest percent difference of ROS in the alimentary tract of challenged lice relative to unchallenged lice (Fig. 3) as determined by CM-H₂DCFDA, which is a general indicator that can detect a wide array of ROS, including OH, ONOO⁻, OCl⁻, O₂⁻, H₂O₂, NO⁻ and ROO⁻. This percent difference was found to be greater in head lice (70% increase) than in body lice (42% increase), although there were no statistically significant differences ($p > 0.05$, Fig. 3). This increase in ROS in challenged versus unchallenged lice for both body and head lice is likely attributed to a large burst of ROS in both ecotypes up to 1 d after imbibing an infected blood meal - most likely due to bacterial recognition combined with the Fenton reaction using hemoglobin from the blood meal. Challenged body lice at day 4

following infection showed a 40% increase in the levels of ROS produced by the alimentary tract, whereas there was no change in the levels produced by head lice. This decrease in ROS in head lice, although not significant (70% versus 0%, $p>0.05$, Fig. 3), could be due to the enhanced ability of head lice to rid themselves of *B. quintana* in the early stages of infection, and thus return to a normal range of ROS in the absence of bacteria. Body lice, on the other hand, have significantly higher *B. quintana* cell numbers per louse even 1 d after infection, as previously observed by Kim *et al.* (2017) and in this present thesis (Fig. 2). The sustained increase of ROS 4 days following infection in challenged body lice could be due to their lowered innate immune response, in which they are likely combating ongoing infection by increasing their ROS production.

A similar trend was found when ROS was detected with HPF, which is also a general ROS indicator, but not sensitive to light-induced oxidation as is CM-H₂DCFDA. There was a large increase in the level of ROS at 1 d following infection in challenged lice relative to unchallenged lice, but when using this indicator, body lice showed a greater percent difference (Fig. 4). Due to insensitivity to light-induced oxidation, the ROS levels detected by HPF more likely reflect the amount of ROS generated in the epithelial cells of the louse. At Day 1 following infection, body lice showed more of an increase in levels (34% versus 22%, $p>0.05$, Fig. 4) of ROS detected by HPF in the bacterial-challenged lice than in the non-challenged lice when similarly compared with head lice. At Day 4 following infection, there was a relative decrease since 1 d in the amount of ROS produced by both body and head lice. Challenged body lice showed a 4% decrease and head lice showed a 5% increase in ROS relative to non-challenged (control) lice. It is possible that after 4 days following infection, both body and head lice have

combated with most of the bacteria invading the alimentary tract, and thus returned to a normal level of ROS production in the epithelial cells. This data does not conform to previously published results (Previte *et al.*, 2014) (Kim *et al.*, 2017) that show *B. quintana* increased substantially over time from Day 3 to Day 10 following infection.

CHAPTER 4

DISCUSSION

In the present study, data has been presented to further identify the differences between the humoral immune response of body versus head lice, following oral bacterial challenge, that have been previously investigated. To elucidate what molecular factors may be associated with the differential immune response following oral challenge by *B. quintana* in body versus head lice, proliferation patterns following infection and transcriptional profiles of immune-related genes were initially observed by Previte *et al.* (2014) and Kim *et al.* (2017). In terms of bacterial infection and proliferation, Previte *et al.* (2014) reported that the number of *B. quintana* per body louse remained relatively constant until 6 days post-infection, where significant proliferation occurred between 6- and 8-days post-infection. Head lice showed little or no proliferation between 4- and 6-days post-infection, and the cell count of *B. quintana* remained steady. Kim *et al.* (2017) showed a similar overall proliferation pattern, with average numbers of *B. quintana* per louse being significantly higher in body lice versus head lice as soon as 1-day post-infection. Kim *et al.* (2017) reported that body lice had significantly higher levels of *B. quintana* per louse at 1-day (12.1-fold), 3-days (3.0-fold), 6-days (4.5-fold), 9-days (5.3 fold), and 12-days (2.0-fold) post-challenge, respectively. Kim *et al.* (2017) also compared the generation of ROS in alimentary tract tissue, and found that not only did imbibing a non-infected blood meal have no effect on ROS levels in either body or head lice, but that higher levels of ROS (1.3-fold and 2.3-fold in starved lice, 1.1-fold and 2.5-fold in fed lice) were always detected in head louse epithelial cells using CM-H₂DCFDA and HPF indicators, respectively.

In the present thesis study following oral infection, *B. quintana* number was initially compared at 2 hours (Day 0) post-infection, and then later at 4 days post-infection (Fig. 2). At Day 0 post-infection, head lice had the least amount of *B. quintana* (1.79×10^7 *B. quintana* cell number per louse), a 3.9-fold reduction when compared to body lice control. There was no significant difference between treatments in this study, the Previte *et al.* (2014) study, or the Kim *et al.* (2017) study at 0-days post-infection. However, in this thesis study, in the *defensin 1* knocked down (Def1(-)) head lice, the bacterial cell number increased to 2.69×10^7 compared with non-knocked down head lice (1.79×10^7) at day 0, a 1.5-fold increase, indicating that defensin 1 was active in reducing *B. quintana* cell number in non-knocked down head lice (Fig. 2). The cell number in non-knocked down head lice was 1.6-fold less than in body lice at 4-days post-infection, still showing an increased amount of *B. quintana* in body lice versus head lice around this time point. Interestingly, in Def1(-) head lice, *B. quintana* cell numbers also decreased to 1.28×10^7 *B. quintana* cell number per louse but were 2.6-fold greater than non-knocked down head lice and 1.7-fold greater than body lice at Day 4 (Fig. 2). In the aforementioned study by Kim *et al.* (2017), transcription levels of six major immune-related genes (*PGRP*, *defensin 1*, *defensin 2*, *Duox*, *lysozyme*, and *PPO*) were also quantified following oral challenge, but transcription of *defensin 1* was the only gene significantly up-regulated in head lice at 4- and 8-days post-infection. Although the differences in the present study are not statistically significant, it does appear that the knockdown of *defensin 1* in head lice allows for increased proliferation of *B. quintana* compared with both non-knocked down head lice and body lice. ($P > 0.05$).

Kim *et al.* (2017) also reported that significantly lower levels of ROS in the alimentary tract of body lice were always found when compared to head lice when fed on uninfected blood using CM-H₂DCFDA and HPF indicators. This thesis study presents an oxidative-burst-like effect following the ingestion of an infected blood meal in both body and head lice 1 day after infection. At 1-day post-infection, both body lice (42% and 34% increase) and head lice (70% and 22% increase) showed an increase in ROS using CM-H₂DCFDA and HPF, respectively. On day 4, however, orally challenged body lice showed a 40% increase and a 4% decrease in ROS using CM-H₂DCFDA and HPF, respectively. Orally challenged head lice showed a 0% change and 5% increase in ROS at day 4, using CM-H₂DCFDA and HPF, respectively. This increase in ROS in challenged versus unchallenged lice, for both body lice and head lice, is likely attributed to a large burst of ROS in both ecotypes up to 1 day after bacterial recognition of an infected blood meal. Although this current study found that body lice had higher levels of ROS than head lice (not conforming with Kim *et al.* (2017)) on day 1 and day 4 post-infection, it is not known with certainty what function enhanced oxidative stress plays in either increasing or decreasing the vector competence of body versus head lice due to this discrepancy and the fact that the difference found were not statistically significant due to variation in the responses. Although speculative, it appears that after 4 days body lice sustain a higher ROS production as bacterial numbers increase, whereas head lice return to a normal level of ROS production in the epithelial cells after combating most of the bacteria invading the alimentary tract at earlier time points.

It was postulated that following RNAi-based knockdown of *defensin 1*, the knocked down head lice should exhibit an increase in *B. quintana*, which will be more similar to that of body lice. If found to be true, this would validate that defensin 1 is indeed important in the regulation of *B. quintana* proliferation in lice during the early stages of infection and in the shaping of the louse immune response. Although the current findings did not show statistically significant differences between the levels of *B. quintana* during early stages of infection in control (non-knocked down) head lice versus *defensin 1*-knockdown head lice, the numbers of *B. quintana* cells were substantially increased in knocked down head lice at both 0 and 4 days post-infection compared with non-knocked down control head lice and their numbers actually exceeded those seen in body lice at day 4 post-infection. It is therefore probable that the higher level of *defensin 1* in non-knocked down control head lice is responsible, in part, for the reduced number of *B. quintana* in the early stages of infection in head versus body lice. These results are certainly consistent with the hypothesis that *defensin 1*, which is constitutively over expressed in head versus body lice (Previte *et al.*, 2014; Kim *et al.*, 2017), is responsible, in part, in reducing the numbers of *B. quintana* cells in the alimentary tissue and subsequently reducing the number of viable bacteria in the feces, reducing infection. Conversely, the decreased level of *defensin 1* in the alimentary tissue of body lice would allow a decreased immune response, increased *B. quintana* proliferation, and a more viable amount bacteria in feces, leading to increased infection and enhanced vector competence.

Since some insect defensins are known to be active against Gram-negative bacteria (Mandrioli *et al.*, 2003), down-regulation of defensin expression in female lice would be beneficial in reducing their immune response against their Gram-negative, primary

endosymbiotic bacteria, *Candidatus* *Riesia pediculicola* (*Riesia*). Both body and head lice keep their primary endosymbiotic bacteria in the mycetomes of their stomach disc, where the endosymbionts perform crucial roles in their survival (Perotti *et al.*, 2007). To transfer their endosymbionts to the next generation of oocytes, lice must allow them to cross the hemolymph from stomach disc to ampullae in the oviduct during the late 3rd instar to adult stages. During migration, the endosymbionts are exposed to the immune defenses (cellular immune system) of the host louse. It is still our contention that body lice, in evolving from head lice, reduced their humoral and cellular immune responses to protect their vertically-transmitted endosymbiotic bacteria, which are indispensable for louse nutrition and survival by providing vitamin B₅ that is not synthesized by the human louse itself. This reduced immune response in body lice is necessary because they needed to become bigger than head lice in order to occupy the relatively new niche of human clothing that first occurred some 40,000–70,000 years ago (Kittler *et al.*, 2003). The larger size of the body louse is necessary for them to take a larger blood meal and to become more mobile in order to successfully adapt to their new habitat of human clothes. To do this, body lice may have relaxed their innate immune response against their *Riesia* endosymbionts in order for them to multiply and supply the louse with more vitamin B₅ necessary for increased growth. Inadvertently, this reduction in the immune response also has allowed the invasion of other Gram-negative pathogenic bacteria. Based on this, it can be speculated that young, adult body lice need to reduce their immune defenses against the endosymbionts during early adulthood to allow efficient vertical transmission from the mycetome to the ovary, thereby resulting in the enhanced vector competence of body versus head lice.

The number of bacteria after 2 hours (day 0) should also be taken into account, as it is possible the bacterial burden in head lice may not have been raised to a number large enough to mount a significant immune response. Kim *et al.* (2017) reported that the level of *Defensin 1* transcript in head lice increased 3.2-fold relative to control (day 0) at 4 days post-challenge with *B. quintana*. Using *E. coli*, Kim *et al.* (2012) also showed that the total induced transcription level of *defensin 2* did not differ between body and head lice when challenged orally. Louse *defensin 1* is the main AMP used against Gram-negative bacteria, and the different transcription level of *defensin 1*, rather than *defensin 2*, partially contributes to the different proliferation rate of *B. quintana* in the alimentary tract tissue of body versus head lice (Kim *et al.*, 2012). It should be noted that the level of *defensin 1* transcript in the knockdown head lice may have had a more significant effect on *B. quintana* cell number several days following (days 6-12) and should be further studied.

The proliferation pattern of *B. quintana* during these later days of infection is still unclear. The most frequent studies using both GFP-*B. quintana* (Kim *et al.*, 2017) shows a sharp decline in body lice proliferation and no change in head lice proliferation after 9 days post-challenge. The study by Previte *et al.* (2014) using WT-*B. quintana* shows a steady plateau after 10 days post-challenge in both body and head lice. These studies are unlike the initial study by Seki *et al.* (2007) where *B. quintana* proliferation remains at a stationary phase until about day 7, and from there on the cell number increases significantly by over three orders of magnitude. The reason for this difference in the proliferation pattern is unknown, but it may be due, in part, to the differences in the blood-feeding system and/or feeding protocol used. Infected lice, which were fed on infected blood for 18 hrs (overnight) in the Previte *et al.* study (2014), were then allowed

to feed continuously on uninfected human blood for the remainder of the study using the *in vitro* feeding chamber in this and the most recent studies, whereas lice were fed for a limited duration (20 min) once a day in the initial study (Seki *et al.*, 2007). The present study most resembles the Kim *et al.* (2017) study, where lice were fed infected blood for 1 hr (enough to be fully engorged), and then were allowed to feed continuously on uninfected human blood for the remainder of the study. Perhaps the multiple bloodmeals also increased the ROS-based oxygen bursts, thereby suppressing *B. quintana* proliferation more effectively in this and the other experimental settings.

The generation of ROS is one of the key mediators of antimicrobial defense in the gut of blood-feeding insects (Molina-Cruz *et al.*, 2008). Both indicators (CM-H₂DCFDA and HPF) used in this study were also used in a previous study by Kim *et al.* (2017), and showed significantly higher net levels of ROS in head louse epithelial cells when compared to body louse epithelial cells. They also showed that imbibing a blood meal had no effect on the levels of ROS produced by either louse ecotype (Kim *et al.*, 2017). In the present study, the most apparent difference in ROS in the alimentary tract of orally challenged (+ *B. quintana*) versus non-challenged (-*B. quintana*) lice is seen at 1 d post-infection. Although the magnitude of the percent increase depends on the indicator used, it appears as though there is still a ROS-based increase during the first 24 hours (Day 1) post-infection. Sustained levels of ROS are detrimental to organisms, so it is plausible that by 96 hours (Day 4 post-infection) the levels are not as robust.

Unlike *E. coli* and other Gram-negative bacteria, *B. quintana* is known to possess several heme-binding proteins (Hbps) in its outer membrane surface, which bind to heme groups.

Therefore, it has been hypothesized that the Hbp-coat of *B. quintana* probably serves as a potent antioxidant barrier owing to the intrinsic peroxidase activity of Hbp, thereby providing tolerance to the ROS generated in the gut of vector arthropods (Battisti *et al.*, 2006; Harms & Dehio, 2012). Upon further investigation, the frequency of blood meals and the role of ROS-based oxygen bursts in the humoral immune response of these ectoparasites to *B. quintana* and other bacteria should be studied to better understand the relationship between increased ROS and its potential for decreased bacterial colonization / vector competence.

It has also been shown by Kim *et al.* (2011) that head lice exhibit a significantly higher phagocytotic activity against injected *E. coli* than body lice, suggesting that the greater immune response in head lice against injected *E. coli* is largely due to enhanced phagocytosis and not due to differences in the humoral immune response. Body lice elicited a significantly delayed and lower level of phagocytosis to both injected *S. aureus* and *E. coli* (Kim *et al.*, 2011). Body lice have also been shown to have a greater capacity for harboring *B. quintana* inside their gut lumens and excreting more viable bacteria in their feces when compared to head lice, as judged by fluorescence microscopy (Kim *et al.*, 2017). The viability of GFP-*B. quintana* bacteria in the feces of body versus head lice showed that the number of GFP-*B. quintana* did not differ significantly between the infected body and head lice over an entire 15-day post-challenge interval when measured by RT-qPCR, but a noticeable difference was observed in the fluorescence intensity between viable body and head lice (Kim *et al.*, 2017). Comparatively, the viability index of GFP-*B. quintana* decreased rapidly in both body and head lice; however, body lice showed a consistently and significantly higher viability index over time when compared with head lice until 11 days post-challenge. These findings indicate that the proportion of live bacteria

in the excreted feces infected by GFP-*B. quintana* is significantly larger in body versus head lice. The larger proportion of viable bacteria in excreted feces, paired with a reduced phagocytotic activity in body lice could be responsible, in part, for their increased vector competence during early stages of infection and could account for the larger amount of *B. quintana* cells found in body lice in the present study at day 0. To further test this hypothesis, however, phagocytosis analysis should be done with fluorescently labelled *B. quintana*, and both the abdomen and feces should be observed following oral challenge.

Genome-wide analysis of the whole body transcriptomes of body and head lice following *B. quintana* oral challenge revealed that a variety of immune-related genes associated with the Toll pathway (i.e., *fibrinogen-like protein*, *spatzle*, *defensin 1*, *serpin*, *scavenger receptor A* and *apolipoprotein 2*) were differentially expressed between body and head lice (Previte *et al.*, 2014). It has been previously shown that some immune-related genes (*PGRP*, *defensin 1*, *defensin 2*) had significantly higher basal (no bacteria challenge) transcription levels in head lice compared with body lice when orally challenged with *B. quintana* (Kim *et al.*, 2017). The basal transcription levels of other effector genes, including *Duox*, *Lysozyme*, and *PPO*, did not significantly differ between body and head lice. In contrast to the basal transcription levels, none of immune-related genes, except the lysozyme gene in body lice, was significantly up or down regulated in the alimentary tract tissues of either head or body lice following oral infection by *E. coli*. Comparative analyses of the body and head louse transcriptomes and proteomes, following *B. quintana* and differential bacterial challenges, will provide useful information to identify the molecular components that determine the vector competence of lice. Although the present study examines the effects of bacteria acquired during human blood feeding based on the membrane

feeding system previously mentioned, it is necessary to identify more immune components in the alimentary tract and to study the louse immune system under these realistic conditions.

Furthermore, it would be beneficial to study the dynamics of pathogen-lice interactions in other pathogenic bacteria, such as *Borrelia recurrentis*, to better understand the mechanisms underlying this relationship. To further show that body lice have a reduced immune response when compared to head lice, more of the aforementioned studies should be performed and immune factors assessed. Gene knockdown experiments will need to be conducted in head lice that show that without the gene products of the key immune response genes identified, the proliferation and viability of *B. quintana* increase to levels similar to that found in body lice. If successful, head lice with these key genes knocked down should become as infective as body lice using a surrogate mammal model such as the rabbit. Information on the bacteria-specific differences in immune responses obtained in such studies will provide fundamental insights into the factors determining the vector competence differences between these two louse species and the evolutionary processes of how body lice became a vector and head lice did not.

Table 1. Sequences of the primers used for transcription level quantification and for *Bartonella quintana* proliferation.

Gene		Sequence (5'→3')	Product Size (bp)
<i>Defensin 1</i> -dsRNA	F	TAATACGACTCACTATAGGGCAAAAATGAACGGATTAACGATTG	337
	R	TAATACGACTCACTATAGGGTAGATGATTCATCTTCTGCATC	
<i>Defensin 1</i>	F	GGGAGAACTTACCTCGGAAA	142
	R	AGCGGCACAAGCAGAATGAT	
<i>Ribosomal Protein L13A</i>	F	GTTAGGGGAATGCTTCCACAC	142
	R	GGTCTAAGGCAGAGAACGCT	

F, forward primer; R, reverse primer; qPCR, quantitative real-time PCR

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