A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Comparison of Immune Responses between Human Body Louse, *Pediculus humanus humanus*, and Head Louse, *P. h. capitis*: Insights into Vector Competence Difference

면역반응 비교 연구를 통한 몸니와 머릿니의 질병매개력 차이 규명

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ABSTRACT

The human body louse, *Pediculus humanus humanus*, and the head louse, *Pediculus humanus capitis*, are hematophagous ectoparasites of humans. Although both body and head lice belong to a single species, only body lice are known to transmit several bacterial diseases, including trench fever, through its feces. This difference in vector competence is assumed to be due to their different immune reactions. Here, the differences in the immune response between body and head lice were investigated by measuring the proliferation rates of two model bacteria, a Gram-positive *Staphylococcus aureus* and a Gram-negative *Escherichia coli*, following dermal challenge. Body lice showed a significantly reduced immune

response compared to head lice particularly to *E. coli* at the early stage of the immune challenge. The immune reactions in the alimentary tract were also compared between body and head lice following oral challenge of *E. coli*. Head lice suppressed the growth of *E. coli* effectively at the early stage of infection, resulting in gradual reduction of *E. coli* number in alimentary tract tissues. In contrast, the number of *E. coli* steadily increased in alimentary tract tissues of body lice.

The proliferation profiles of *Bartonella quintana*, the causative agent of trench fever, inside louse body and its excretion patterns were also investigated in the two louse subspecies following oral challenge with *B. quintana*-infected blood meal. GFP-expressing *B. quintana* cells gradually proliferated until 9 days after infection and the number was significantly higher in body lice than in head lice. The numbers of *B. quintana* detected in feces from infected lice were almost the same and steadily decreased over time in both body and head lice. Nevertheless, the viability of *B. quintana*, as determined by fluorescence, was significantly higher in body louse feces, especially at 1 day post-infection and this tendency lasted until no *B. quintana* is excreted. These findings demonstrate that body lice allow more extensive proliferation of *B. quintana* inside the alimentary tract and excrete feces containing more viable *B. quintana* following ingestion of infected blood meal, which primarily attribute to their higher vector competence.

As the first step to elucidate the molecular basis of reduced immune responses in body lice, immune-related genes were annotated from the genomes of body and head lice. A total of 93 immune-related genes were identified in both body and head lice, suggesting that both body and head lice have the same immune components. Many gene families in the humoral immune system were considerably reduced in number or absent in the body louse genome. In case of the genes related with pathogen recognition, only one type of membrane-binding peptidoglycan recognition protein (PGRP) was annotated whereas beta-glucan binding protein (BGBP) was not found. In addition, Imd and its adaptor protein FADD in the Imd pathway were not identified. In contrast, all components in the Toll, JAK/STAT (Janus kinase and Signal Transducer and Activator of Transcription) and JNK (c-Jun N-terminal kinase) pathways were preserved. Among the various kinds of antimicrobial peptides (AMP), only two types of defensin were annotated. The Louse PGRP, defensin 1 and defensin 2 showed 99.3, 99.1 and 98.2% similarity between body and head lice.

The differences in the cellular and humoral immune response between body lice were investigated. Body lice exhibited a significantly lower phagocytotic activity against *E. coli* than head lice, whereas the phagocytosis against *S. aureus* differed only slightly between body and head lice. Transcriptional profiling of representative genes involved in the humoral immune response revealed that both

body and head lice showed an increased immune response to *S. aureus* but little to *E. coli* following dermal challenge. Nevertheless, the basal transcription levels of major immune genes, such as PGRP, Thioester containing protein (TEP) 1, TEP2, Scavenger Receptor CI (SRCI), Dual oxidase (Duox) in whole body level were significantly lower in body versus head lice.

The epithelial cell-specific immune responses in the alimentary tract tissues were compared between body and head lice following bacteria oral challenge. Interestingly, the basal transcription levels of PGRP and defensins, which are the sole components of recognition and effector in the humoral immune response, respectively, were lower in body lice than in head lice. Defensin 1 was up-regulated by *B. quintana* oral challenge in head lice but not in body lice, whereas no differenece was observed by *E. coli* oral challenge. Thus, such non-inducible immune genes, along with the lower basal transcription levels of PGRP and defensins in body lice, were primarily responsible for the reduced immune response of body lice to Gram-negative bacteria including *B. quintana*. In addition, the level of cytotoxic reactive oxygen species (ROS) generated by epithelial cells, especially the hydroxyl radical and superoxide, was significantly lower in body lice than in head lice regardless of blood feeding although there was no difference in the transcription level of Duox. These results suggest that both the lower basal transcription level of immune-related genes, which is not inducible by *B. quintana*,

in conjuction with the lower amount of ROS in the alimentary tract of body lice reduce their immune responses, thereby allowing invading *B. quintana* to proliferate and excreting more viable bacteria in feces, which result in their higher vector competence compared to head lice.

Key words: *Pediculus humanus humanus*, *Pediculus humanus capitis*, Human louse, vector competence, immune response, *Bartonella quintana*

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ABBREVIATIONS

AMP	Antimicrobial peptide	PBS	Phosphate buffered saline
BGBP	Beta-glucan binding protein	PGRP	Peptidoglycan recognition protein
Ct	Threshold cycle	PO	Phenoloxidase
Duox	Dual oxidase	PPO	Prophenoloxidase
EF	Elongation factor	qPCR	Quantitative real-time PCR
FITC	fluorescein isothiocyanate	RACE	Rapid amplification of cDNA ends
GNBP	Gram-negative binding protein	ROS	Reactive oxygen species
IMD	Immune deficiency	SR	Scavenger receptor
JAK	Janus kinase	STAT	Signal transducer and activator of transcription
JNK	c-Jun N-terminal kinase	TEP	Thioester containing protein
NCBI	National center for biotechnology information		

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INTRODUCTION

The body louse, *Pediculus humanus humanus*, is an obligatory human ectoparasite and poses a serious public health threat by transmitting several diseases to humans, including epidemic typhus, relapsing fever and trench fever (Lounibos, 2002; Raoult and Roux, 1999). Body lice along with conspecific head lice, *Pediculus humanus capitis*, have a unique life history by spending virtually their entire life cycle either directly on or in close proximity to their human hosts and feeding exclusively on human blood. As suggested by their names, these two subspecies occupy different niche on the human body: body lice are found on clothing and visit human skin only when they feed (once or twice a day) whereas head lice confined to the human scalp, lay eggs on the hair shaft and feed every few hours (Buxton, 1946). Morpholigically, body lice are known to be larger than head lice, most notably that in the lengh of the tibia on the second pair of legs (Busvine, 1978). They do not interbreed in the wild, but fertile offsprings can be generated from crossing under the laboratory condition (Bacot, 1917). Body lice originated from conspecific head lice when humans began to wear clothing roughly 40,000-70,000 years ago (Kirkness et al., 2011), but they differ in the choice of habitat on human hosts and in their vector competence in transmitting bacterial pathogens, such as *Bartonella quintana*, *Rickettsia prowazekii*, and

Borrelia recurrentis. Since body lice transmit such diseases, they pose considerable health concerns particularly during times of social turmoil, such as economic downturns, war, and natural disasters. Unlike body lice, the conspecific head lice are not known to transmit the bacterial diseases to humans.

Vector competence is defined as the capacity of a vector to allow the development and transmission of a microbial pathogen inside of its body (Beerntsen et al., 2000). As a component of vectorial capacity that includes all of the environmental, behavioral, and biological factors influencing the interaction between a vector, a pathogen and a vertebrate host, vector competence is limited in genetic factors that influence the vector-pathogen relationship. How insects gain or lose vector competence is a crucial issue in vector biology, but its mechanisms and evolutionary processes are poorly understood. Since the insect immune response plays a key role in insect-pathogen interaction, the difference in vector competence is most likely governed by the different response of the immune system. Determination of the differential immune responses upon microbial challenges, therefore, would be of primary importance in elucidating the differences in vector competence between body and head lice. Since both body and head lice possess virtually the same genome, direct comparisons of the molecular and physiological aspects of immune system between body and head lice will drastically expand our understanding on how lice become vectors of

human diseases and how the vector competence of body lice have evolved from their ancestral head lice.

Unlike vertebrates, insect immune responses are solely conferred by the innate immune system, which has been extensively studied using various model insects, particularly *Drosophila melanogaster* (Christophides et al., 2004; Hoffmann and Reichhart, 2002; Hultmark, 2003). The innate immune system of *D. melanogaster* consists of humoral and cellular defense components (Aggarwal and Silverman, 2008). The humoral defense begins with the recognition of the pathogen, which leads to the expression of various antimicrobial peptides as final effector molecules via several pathways, including Toll, Immune Deficiency (Imd), and Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) (Aggarwal and Silverman, 2008; Ferrandon et al., 2007). In the cellular defense system, invading pathogens are recognized and destroyed by phagocytosis, encapsulation and melanization with the help of specialized cells such as plasmatocytes, lamellocytes and crystal cells, respectively (Lemaitre and Hoffmann, 2007). These antimicrobial peptides and specialized cells help to clear invading pathogens, enabling insects to survive in various infectious environments (Stuart and Ezekowitz, 2008).

Genome-wide sequencings and comparative genomic studies on immune-related genes have facilitated the discovery of novel components associated with

the innate immune system in insects (Rubin et al., 2000). Comparative studies on immune-related genes have been conducted in several other non-drosophilid model insects, including *Bombyx mori* (Tanaka et al., 2008), *Anopheles gambiae* (Christophides et al., 2002), *Apis mellifera* (Evans et al., 2006) and *Tribolium castaneum* (Zou et al., 2007). All these insects have the same basic architecture of innate immune system as *D. melanogaster* but there is a great deal of diversities in their individual immune components.

The body louse genome is the smallest (ca. 108 Mb) among insect genomes sequenced to date and contains only 10,773 annotated genes, which is notably fewer than other insects except for *A. mellifera* (Kirkness et al., 2011). Since lice feed exclusively on human blood, which is a largely sterile food, and have a simpler parasitic life history on their human hosts compared to free living insects, they likely encounter a less diverse array of pathogens, including bacteria. Based on this assumption, the numbers of genes related with immune pathways are expected to be reduced in human lice. This aspect of the body louse genome, however, has not been addressed to date (Kirkness et al., 2011). Although body lice originated from head lice relatively recently, thus sharing very similar genetic backgrounds, unique genetic aspects in immune pathways must exist to explain the distinct differences in vector competence between these two species. Since recent transcriptome-scale comparative analysis demonstrated that both body and

head lice are conspecific by having virtually the same genetic background with nucleotide diversity ranging from only 0.1-1.3% (Olds et al., 2012), the apparent differences in vector competence between body and head lice appear to be grounded on post-genomic differences, such as differential transcriptomes, proteomes and epigenetics. At the same time, characterization of the genes that influence immunity would allow the elucidation of the molecular and cellular basis of differential vector competence in insects and may provide important insights into the evolutionary process of vector competence.

Bartonella quintana is one of Gram-negative bacteria transmitted by body lice. *B. quintana* is the causative agent of trench fever, the common name for the acute febrile syndrome, which affected millions living under extreme conditions during World War I (Spach et al., 1995). *B. quintana* infection typically causes relapsing fever, endocarditis, and vascular proliferative lesions and it can be fatal unless correctly diagnosed and treated with antibiotic therapy (Cockerell et al., 1987). Small outbreaks of bartonellosis were reported among the homeless in Seattle, Washington and elsewhere at the beginning of the 1990s (Brouqui and Raoult, 2006; Jackson et al., 1996). Although the emergence of trench fever subsided with the use of antibiotics, this disease is re-emerging in the people who are homeless, indigence, and disadvantaged urban population, especially the immune-compromised such as alcoholics and the patients infected with human

immunodeficiency virus (Hotez, 2008). Recently, it is reported that *B. quintana* was detected from 33.3% of the body lice recovered from the homeless in California, suggesting this bacteria is still prevalent in human population (Bonilla et al., 2009).

Once *B. quintana* introduced to louse by blood feeding from a patient, the bacteria replicate, colonize and attach to the surface of epithelial cells of alimentary tract (Ito and Vinson, 1965). Lice have a habit of excreting during feeding, and viable *B. quintana* in louse fecal matter can be transferred to the bloodstream via scratches in the skin. Therefore, louse feces is the most common vehicle for *B. quintana* transmission to humans, where *B. quintana* forms a biofilm-like structure, thereby enabling their long survival upto a year (Kostrzewski, 1949; Seki et al., 2007). The primary infection route of pathogenic bacteria, including *B. quintana,* is to enter the alimentary tract of the louse via an infected blood meal, hence, the first immune response barrier following feeding is likely to be the release of antimicrobial peptides (AMPs) and the reactive oxygen species (ROS)-based ‘oxygen burst’ generated by alimentary tract epithelial tissue, one of major site for the humoral immune response of the louse's innate immune system. Considering this scenario, it is necessary to investigate the epithelial tissue-specific humoral immune responses and determine the differences between body and head lice as a potential cause of their differential vector competence.

In this study, differential immune responses to a model Gram-positive (*Staphylococcus aureus*) or a negative bacteria (*Escherichia coli*) were determined between body and head lice using a bacteria proliferation assay following dermal challenge. To compare the immune responses in the alimentary tract tissue, the bacterial proliferation profiles were determined in body and head lice following oral infection with *E. coli* as an indicator of differential immune responses. The profiles of propagation inside lice and excretion of orally-infected *B. quintana* were also compared. Immune-related genes were annotated from the body louse genome and newly sequenced head louse genome, and transcription profiles of immune-related genes in whole body and alimentary tract tissue were compared between body and head lice following bacterial infection. In addition, the transcriptional profiles of several representative genes involved in humoral immune responses were subsequently examined to determine any differences in their basal transcriptional levels as a potential reason for the increased vector competence seen in body lice. Differences in phagocytosis between the two louse species were also determined. Based on differential immune responses, I presented evidences explaining, at least in part, the differences in vector competence between body and head lice.

CHAPTER I

Comparison of Bacteria Proliferation and Excretion between Body and Head Lice

I-1. Comparison of Bacteria Proliferation between Body and Head Lice Following Dermal and Oral Challenges

Abstract

The human body louse, *Pediculus humanus humanus*, and the head louse, *Pediculus humanus capitis*, are hematophagous ectoparasites of humans. Although both body and head lice belong to a single species, only body lice are known to transmit several bacterial diseases to humans. This difference in vector competence is assumed to be due to their different immune reactions. Here, the differences in the immune response between body and head lice were investigated by measuring the proliferation rates of two model bacteria, a Gram-positive *Staphylococcus aureus* and a Gram-negative *Escherichia coli*, following dermal challenge. Body lice showed a significantly reduced immune response compared to head lice particularly to *E. coli* at the early stage of the immune challenge. The immune reactions in the alimentary tract were also compared between body and head lice following oral challenge of *E. coli*. Head lice suppressed the growth of *E. coli* effectively at the early stage of infection, resulting in gradual reduction of *E. coli* number in alimentary tract tissues. In contrast, the number of *E. coli* steadily

increased in alimentary tract tissues of body lice. Taken together, these data indicate that body lice have lower immune responses against both Gram-positive and -negative bacteria compared to head lice.

1. Materials and Methods

1.1. Lice rearing

The USDA strain of body louse (*P. h. humanus*) and the BR strain of head louse (*P. h. capitis*), which was originally collected in Bristol, UK, have been reared on *in vitro* membrane feeding system (Yoon et al., 2006). Lice colonies were maintained under the conditions of 30℃, 70-80% RH and 16L:8D in a rearing chamber. To compare the bacteria proliferation following challenge, 2-or 3-day-old female adults were used for all experiments.

1.2. Bacteria proliferation assay following dermal challenge

The two bacteria (a Gram-positive, penicillin-resistant *S. aureus* and a Gram-negative, ampicillin-resistant *E. coli*) strains used for immune challenge were obtained from KCCM (Korean Culture Centers of Microorganisms, Seoul, Korea). Bacteria stocks of *S. aureus* or *E. coli* were streaked on a nutrient agar plate (DIFCO) or a Luria broth (LB) agar plate, respectively. A single colony was picked and cultured in 2 ml Brain Heart Infusion (BHI) for *S. aureus* and LB for *E. coli* at 37℃ until an optical density (OD_{600}) of 1.0 was obtained (ca. 9 h) in the presence of appropriate antibiotics (62.5 ng/ml penicillin or 100 μg/ml ampicillin, respectively). Bacteria cultures were centrifuged at 10,000 g for 20 s, the pellets

resuspended in 2 ml of BHI or LB, respectively, and diluted 50 fold to a density of 2.0 x 10^7 cells/ml for injection.

Based on the average difference in the body size (volume) between the two louse species (1.44 ± 0.049 µl for body lice and 0.79 ± 0.026 µl for head lice), different volumes of bacteria suspension in either BHI or LB (46 nl or 23 nl for body or head lice, respectively) were injected ventrally into the 2nd abdominal segment of a female louse using nano injector (Nano Liter 2000, World Precision Instrument, Sarasota, FL, USA). Individually injected lice were transferred into 300 ml of BHI or LB at 0, 60, 120, 240 and 360 min post-injection immediately after surface-sterilization by immersion into 70% ethanol. The cuticle on the lateral side of an injected louse was cut longitudinally using microscissors and the louse left in the medium for 5 min. The louse body was then gently squeezed in order to release the remaining hemolymph into the BHI or LB medium. The eluted hemolymph in media (ca. 300 µl) was spread on either a penicillin (62.5 ng/ml)-nutrient agar plate or an ampicillin (100 µg/ml)-LB agar plate. Following overnight incubation at 37℃, the colony number was counted. The original bacteria suspensions (46 or 23 nl) were diluted in 300 ul BHI or LB and directly spread on an appropriate plate with antibiotics as a positive control. Lice injected with either sterile BHI or LB only were used as negative controls.

All statistical analysis was performed using the SPSS 22.0 software (SPSS

Inc., Chicago, IL, USA). Mean and standard deviation were calculated for each data set. One-way ANOVA was used to determine difference of colony number at different time point and two-way ANOVA was applied to detect the difference between body and head lice.

1.3. Bacteria proliferation assay following oral challenge

E. coli was chosen as a model Gram-negative bacterial pathogen and cultured in Luria broth at 37 ℃ until an optical density of 1.0 was obtained. A 2 ml bacteria culture was centrifuged and the pellet was resuspended in 100 µl phosphate buffered saline (PBS). The bacterial suspension was mixed with 2 ml human whole blood for oral infection. Female body and head lice were starved for 9 h and fed with the *E. coli*-inoculated blood using the artificial membrane-based rearing units for ca. 30 min to ensure sufficient feeding. Over the following days, the orally-infected lice were fed with uninfected blood.

The ventral aspect of the abdominal cuticle from an individual louse was cut longitudinally using microscissors and it was placed into a microcentrifuge tube containing 30 µl of ice-cold PBS/Tween20 buffer (1x PBS with 0.1% Tween 20, pH 7.4) at 0, 12, 24, 48 and 72 h after infection. The louse was left in the buffer for 2 min to allow the hemolymph to dilute into the buffer solution. The whole alimentary tract was removed and homogenized in PBS/Tween20 buffer. To

release genomic DNA (gDNA) from *E. coli* and louse tissue effectively, the diluted hemolymph and the homogenate were sonicated (Powersonic 410, HwashinTech, Korea), respectively, and then centrifuged at 10,000 g for 15 min. To semi-quantify *E. coli* proliferation, the *E. coli* 16s rRNA gene was PCR-amplified using a 10 μl aliquot from the supernatant from the diluted hemolymph and the alimentary tract tissue homogenate as gDNA templates, respectively. The louse elongation factor 1α (EF1α) gene was also amplified as a reference for the tissue handling, DNA preparation and data normalization.

Table 1. Sequences of the primers used for *E. coli* quantification.

Species	Gene		Sequence (5'→3')	Product size (bp)
P. humanus	EF1α	F[a]	GTCACCATTGGAAGATGTCG	138
		R[b]	GAATTCGATGGAGTCGCGTG	
E. coli	16S rRNA	F	GGGAGAACTTACCTCGGAAA	142
		R	AGCGGCACAAGCAGAATGAT	

[a] Forward primer.
[b] Reverse primer.

2. Results and Discussion

2.1. Proliferation of Bacteria following dermal challenge

To investigate the differences in the immune responses between body and head lice, proliferation rates of *S. aureus* and *E. coli* following bacterial challenge were measured (Fig. 1). In case of body lice, *S. aureus* proliferated rapidly until 120 min post-challenge (One-way ANOVA, $p=0.001$) and then declined ($p=0.02$), suggesting the initiation of a delayed immune response. Interestingly, *E. coli* also rapidly proliferated in body lice but its proliferation did not decline over post-challenge time ($p<0.05$). In head lice, neither *S. aureus* nor *E. coli* proliferated over time following injection ($p>0.05$), and no bacteria were detected after 360 min post-challenge. Taken together, both *S. aureus* and *E. coli* proliferated significantly in body versus head lice following dermal challenge (Two-way ANOVA, $p<0.001$), indicating that body louse have a reduced immune response compared to head louse, particularly to *E. coli* at the early stage of the immune response following bacterial challenge.

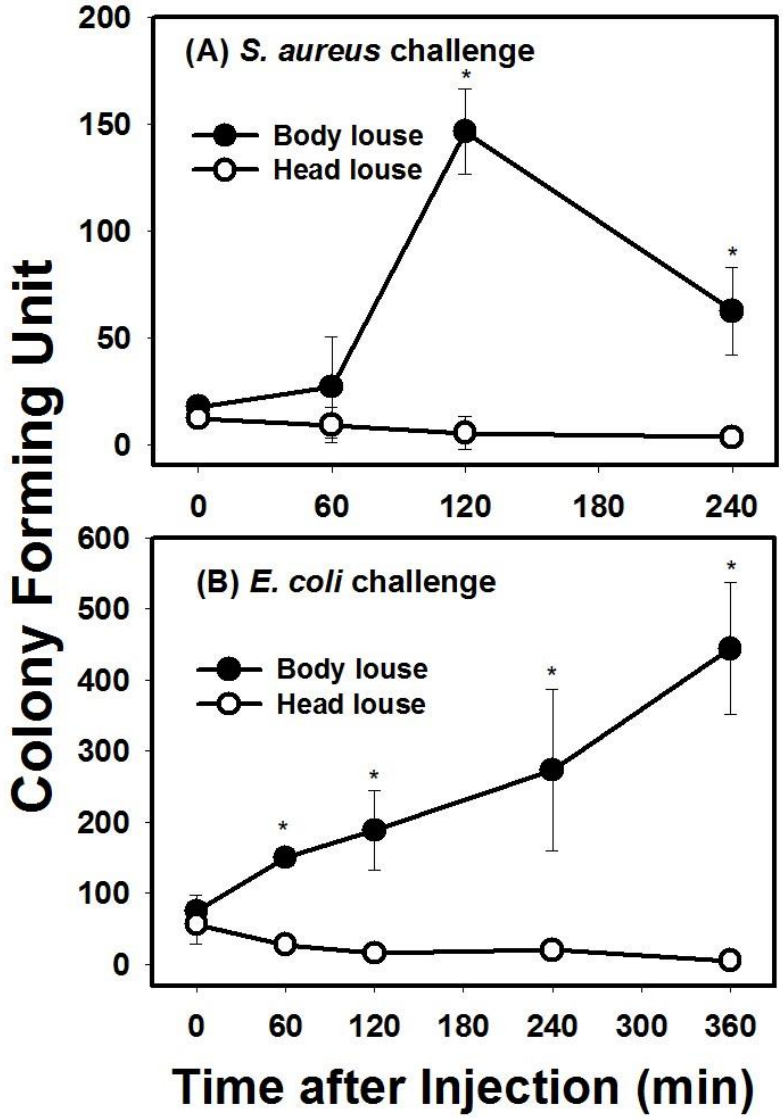


Figure 1. Time course of bacteria proliferation (colony forming unit) inside body and head lice injected with equal concentrations of *Staphylococcus aureus* (A) and *Escherichia coli* (B). Symbols with star mark (*) indicate statistically significant difference between body and head lice ($p<0.05$). Error bars indicate standard deviation of the mean.

2.2. Proliferation of Bacteria following oral challenge

Following oral infection, *E. coli* was strongly detected in the homogenates of alimentary tract tissues of body lice over the entire 72 h post-feeding interval as judged by the intensity of the PCR band (Fig. 2). This finding suggests that *E. coli* was continuously proliferating in the alimentary tract of body louse. In contrast, *E. coli* in head lice was detected strongly only at 0 h post-feeding interval with the intensity of the PCR band decreasing as the post-feeding time interval increased. These findings indicate that *E. coli* proliferation in gut tissue was suppressed in head lice.

These results have likewise been reported that both the Gram-negative human pathogenic bacteria, *Bartonella quintana* (Seki et al., 2007) and *Rickettsia prowazekii* (Houhamdi et al., 2002), proliferated in the digestive tract of the body lice, suggesting that the humoral immune response of alimentary tract tissue in body lice may have also been reduced to these bacteria. No *E. coli* was detected in any hemolymph samples, indicating that *E. coli* invading the alimentary tract did not pass through gut epithelia to hemocoel in either head or body lice.

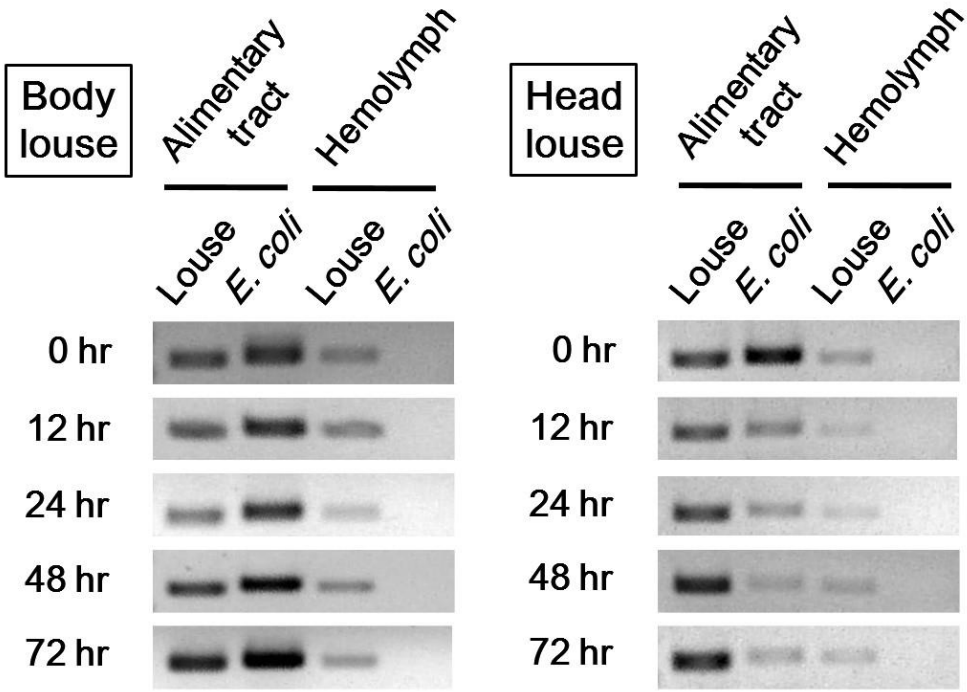


Figure 2. Proliferation profiles of *Escherichia coli* in the alimentary tract tissues of orally infected body and head lice. Louse EF1α and *E. coli* 16s rRNA were used as marker genes for the proper tissue preparation and the proliferation of *E. coli*, respectively.

I-2. Comparison of Proliferation and Excretion of *Bartonella quintana* between Body and Head Lice Following Oral Challenge

Abstract

The body and head lice (*Pediculus humanus humanus* and *Pediculus humanus capitis*, respectively) are hematophagous ectoparasites of humans but only the body louse is known to transmit bacterial diseases, including trench fever, through its feces. The proliferation profiles of *Bartonella quintana*, the causative agent of trench fever, inside louse body and its excretion patterns were investigated in the two louse subspecies following oral challenge with *B. quintana*-infected blood meal. GFP-expressing *B. quintana* cells proliferated over a period of 9 days post-infection with the number of cells significantly higher in body versus head lice. The numbers of *B. quintana* detected in feces from infected lice were almost the same and steadily decreased over time in both body and head lice. Nevertheless, the viability of *B. quintana*, as determined by fluorescence, was significantly higher in body louse feces, especially at 1 day post-infection and this tendency lasted until no *B. quintana* is detected in feces. These findings demostrate that

body lice allow more extensive proliferation of *B. quintana* inside the alimentary tract and excrete feces containing more viable *B. quintana* following ingestion of infected blood meal, which primarily attribute to their higher vector competence.

1. Materials and Methods

1.1. Lice rearing

The SF strain of body louse collected from San Francisco and the BR strain of head louse collected from Bristol, UK have been maintained in the same condition using *in vitro* rearing system (Yoon et al., 2006). Females (2- to 4-days post adult emergence) of both body and head lice were used for all experiments.

1.2. **B. quintana** *culture*

The green fluorescence protein (GFP)-expressing *B. quintana* strain, originally obtained from Dr. Jane Koehler (University of California-San Francisco), was maintained in a biosafety level 2 facility at the University of Massachusetts-Amherst. *B. quintana* from frozen stocks was cultured on chocolate agar plates in candle extinction jars at 37℃ for 10 days and then passed to fresh plates for additional 5-7 days culture using sterile loops (Zhang et al., 2004).

1.3. Oral infection of lice with **B. quintana**

B. quintana cells were harvested from a chocolate agar plate by rinsing the plate surface with 1 ml of PBS (pH 7.4), Cells were pelleted by centrifugation at 1,000 g for 4 min, washed twice with PBS and resuspended in 100 μl PBS. A 5 μl

aliquot of bacterial suspension was serially diluted and then plated on chocolate agar plates in triplicate for *B. quintana* enumeration. The number of CFUs was counted 10 days after culture in candle extinction jars at 37℃. Spectrophotometric readings (OD_{600}) were used to determine the approximate number of cells prior to use. The remaining *B. quintana* suspension was added to human whole blood to a final titer of ~1 x 10^7 CFU/ml (Kosoy et al., 2004). To obtain the same titer of *B. quintana* for both body and head lice, the infected blood was mixed together and divided equally and used to make two artificial feeding chambers. Body and head lice were starved for 8 h and fed with the *B. quintana*-inoculated blood using the feeding chambers for a single sufficient feeding (ca. 1 h). The infected lice were then transferred to a new feeding chambers containing fresh non-infected blood and maintained until used in experiments.

1.4. Standard protocol for B. quintana *quantification*

B. quintana on a chocolate agar plate was harvested using the PBS method described above. The bacterial suspension was centrifuged at 1,000 g for 4 min and the pellet was resuspended in tissue lysis buffer for gDNA extraction using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). A 134-bp gDNA fragment of *B. quintana* 16S-23S ribosomal RNA (rRNA) gene was

generated by PCR from the extracted gDNA, from which an 89-bp nested fragment was PCR-amplified using a nested primer set (Table 1). The PCR product was visualized on agarose gel using ethidium bromide staining, purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA), 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 SalI (Koschem, Seoul, Korea), purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and quantified usinga Nanodrop spectrophotometer (Nanodrop Technologies, Oxfordshire, UK). Six serial dilutions of the linearized plasmids, ranging from 1 ng/μl to10 fg/μl, were used as standard DNA for quantitative real-time PCR (qPCR). The copy number of 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 versus the copy numbers was generated and used to calculate the total copy numbers of 16S-23S rRNA gene in the target gDNA template. Since a single *B. quintana* cell contains two copies of 16S-23S rRNA gene (Seki et al., 2007), the number of *B. quintana* cell was calculated by dividing the estimated copy number of 16S-23S rRNA gene by 2.

Table 1. Sequences of the primers used for *B. quintana* quantification

Species	Gene		Sequence (5'→3')	Product size (bp)
P. humanus	RpS3	F[a]	GCGAGAATTGGCTGAAGATG	131
		R[b]	GAACGACAGAAGTCAACTCC	
B. quintana	16S-23S rRNA (standard)	F	GTCCTCCCTCTCTTATGAGG	398
		R	AACCAAATGGATAAGCGCCATA	
	16S-23S rRNA (qPCR)	F	GAGATAATGCCGGGGAAGGT	100
		R	GACTTGAACCTCCGACCTCA	

[a] Forward primer.
[b] Reverse primer.

1.5. B. quintana *proliferation assay*

Body and head lice were orally challenged by feeding on a blood meal infected with GFP-expressing *B. quintana* as described above. The *B. quintana*-challenged lice were reared with non-infected blood and collected at every 3-day post-challenge interval. The gDNA was extracted using a DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to manufacturer's protocol. qPCR was performed to determine the number of *B. quintana* in a single louse body using the LightCycler 96 System (Roche Diagnostics, Mannheim, Germany) with the following cycling conditions: 10 min at 95℃, followed by 45 cycles of 10 s at 95℃, 10 s at 58℃ and 10 s at 72℃. The specificity of qPCR was confirmed by melting curve analysis. Reactions contained 1X FastStart Essential DNA Green

Master (Roche, Bayern, Germany) and 0.5 μM of primer pairs. Fragments of gDNA from infected lice and standard plasmids were amplified with nested primer pairs of *B. quintana* 16S-23S rRNA gene as before and louse ribosomal protein S3 (RpS3) gene was used as an internal reference for normalization (Table 1). A standard curve of the Ct values from *B. quintana* standard plasmids versus the estimated cell number calculated from a 2:1 ratio between DNA copy number and *B. quintana* cell (see section 1.4) was generated and used to estimate the cell number of *B. quintana* in each louse sample.

1.6. B. quintana *detection from the feces of infected lice*

Feces were collected at 1- or 2-day intervals from both body and head lice infected with GFP-expressing *B. quintana* using the method described above, and gDNA was extracted from 1 mg of feces using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The number of *B. quintana* cell in the same weight of feces was calculated as described above (see section 1.4). The remaining feces were used to take a digital fluorescence image using a phase-contrast microscope equipped with FITC filter (Diaphot-TMD; Nikon, Garden City, NY, USA) to measure the fluorescence from GFP of live bacteria as an index for the viability of *B. quintana* in feces. Net fluorescence intensity of the images was measured using the ImageJ

program (NIH, Image Processing and Analysis in Java, http://rsb.info.nih.gov/ij/). Fluorescence images taken from feces of non-infected lice were used as background images. The fluorescence index of each image was determined by multiplying the area-value with the mean-value and then dividing by the feces area value of the image. Viability index was derived with following equation: Viability index = (P – C)/C, where P is the fluorescence index at post-challenge times and C is the fluorescence index of control feces from non-challenged louse at the same times.

1.7. Statistical analysis

All statistical analyses were performed using the SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Mean and standard deviation were calculated for each data set. Kolmogorov-Smirnov test was conducted to confirm whether the data was normally distributed and then statistical differences of mean values were determined by ANOVA followed by Tukey or Dunnett's T3 post hoc test and Student's t test.

2. Results

2.1. Proliferation of B. quintana

To investigate whether the proliferation profile of GFP-expressing *B. quintana* in lice is the same as that of the wild-type *B. quintana* strain observed previously (Previte et al., 2014), both body and head lice were orally infected with GFP-expressing *B. quintana* and the number of bacteria inside a single louse was determined for 12 days following oral challenge (Fig. 1). The number of *B. quintana* declined until 3 days post-challenge and increased after 3 days in both body and head lice (ANOVA, Dunnett's T3 test, $p<0.05$). Proliferation *B. quintana* reached a maximum level (3.4×10^7 cells per louse) and then declined (ANOVA, Dunnett's T3 test, $p<0.05$). Nevertheless, the average number of *B. quintana* per louse was significantly higher in body lice: 12.1-fold ($t=11.1$; d.f., 4; $p<0.001$); 3.0-fold ($t=3.5$; d.f., 10; $p=0.005$); 4.5-fold ($t=3.0$; d.f., 8; $p=0.02$); 5.3-fold ($t=5.9$; d.f., 5; $p=0.002$) and 2.0-fold ($t=4.5$; d.f., 7; $p=0.003$) at 1, 3, 6, 9 and 12 days post-challenge, respectively. There were no statistically significant differences in the mortality responses between head and body lice following *B. quintana* infection (data not shown).

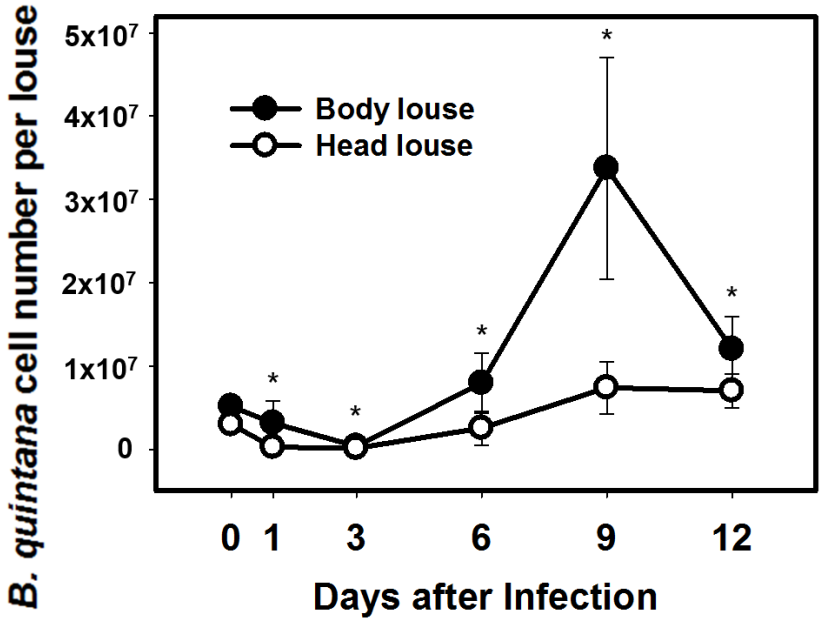


Figure 1. Time course of *B. quintana* proliferation in body and head lice following oral challenge. Body lice showed significantly more *B. quintana* cell number at every time point than head lice. Symbols with star mark (*) indicate statistically significant difference between body and head lice ($p<0.05$). Error bars indicate standard deviation of the mean.

2.2. Excretion of **B. quintana** *from orally infected lice*

Because the pathogenic bacteria vectored by body lice, including *B. quintana*, are transmitted to human by contaminated feces from infected lice, the number of bacteria cells in feces from infected lice was compared between body and head lice. qPCR showed that both body and head lice excreted almost the same amount of *B. quintana* cells, following a single feeding of infected blood (ANOVA, Tukey, $p>0.05$) (Fig. 2). However, it is noteworthy that since the number of *B. quintana* cells was derived from the estimated copy number of 16S-23S rRNA gene in the PCR product, the estimate does not separate viable whole cells from fragmented (non-viable) cells.

To compare the viability of *B. quintana* in feces, the fluorescence of GFP-expressing bacteria in feces was detected by fluorescence microscopy and quantified as a viability index. Although the number of *B. quintana* in feces did not differ significantly between the infected body and head lice over the entire 15 days post-challenge interval as judged by the 16S-23S rRNA gene, a dramatic difference was observed in the fluorescence intensity between body and head lice. Viability index of *B. quintana* decreased rapidly in both body and head lice over time, but body lice showed a consistently and significantly higher viability index over time when compared with head lice until 11 days post-challenge (ANOVA, $p<0.02$) (Fig. 2 and 4). These findings indicate that the proportion of live bacteria

in the excreted feces infected by *B. quintana* is significantly larger in body versus head lice.

The fluorescence intensity of *B. quintana* in head louse feces was not significantly different from that of non-infected control feces after 11 days post-challenge ($p>0.05$). Viable *B. quintana* were not detected in the feces of either body or head lice after 13 days post-challenge.

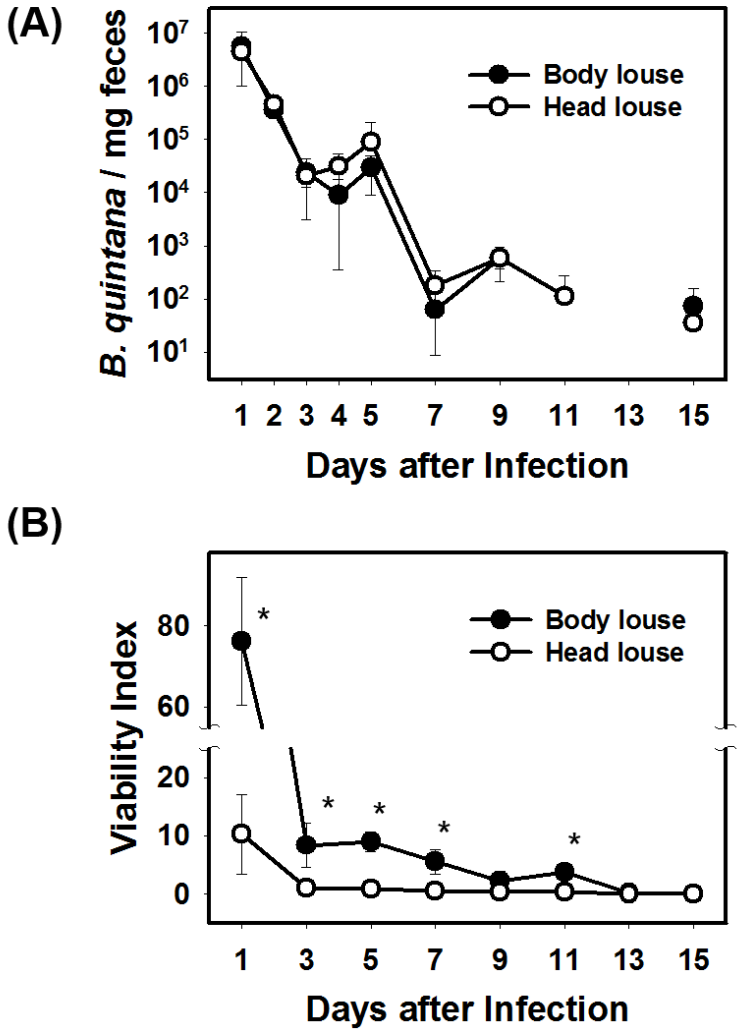


Figure 2. (A) Time course of *B. quintana* number in feces of body and head lice following a single oral challenge. (B) Time course of *B. quintana* viability as determined by fluorescence. Symbols with star mark (*) indicate statistically significant difference between body and head lice ($p<0.05$). Error bars indicate standard deviation of the mean.

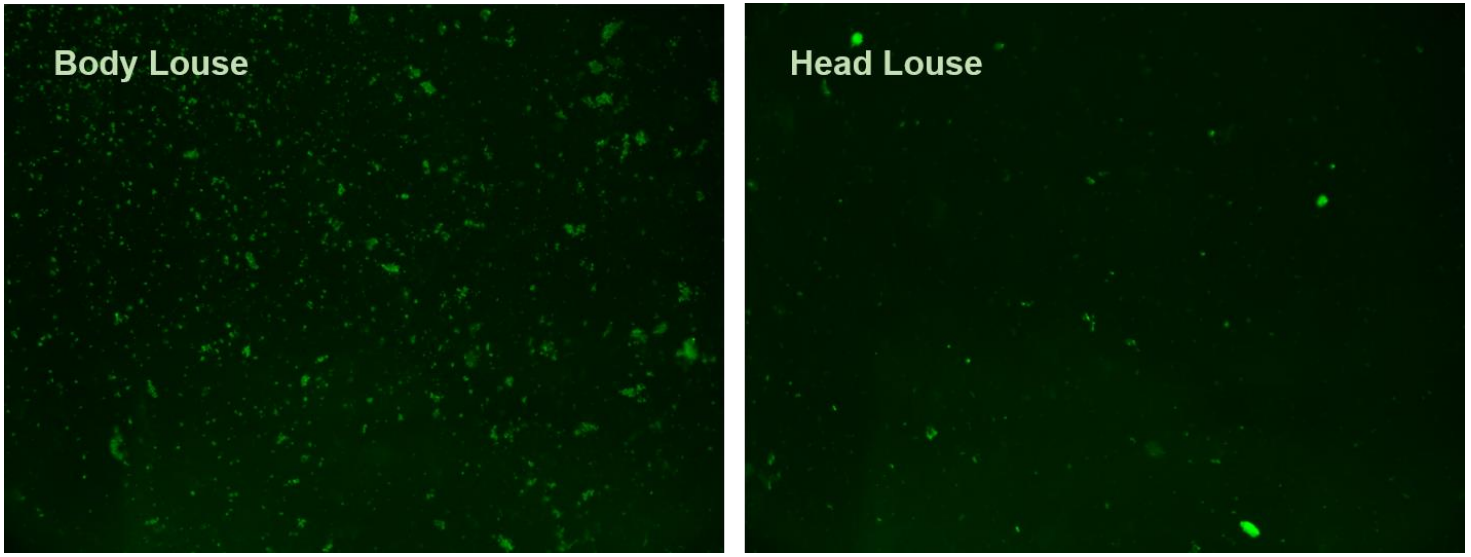


Figure 3. Fluorescence microscopic images of free *B. quintana* in PBS. GFP-expressing *B. quintana* came off from feces of orally-infected body and head lice (1 day after infection).

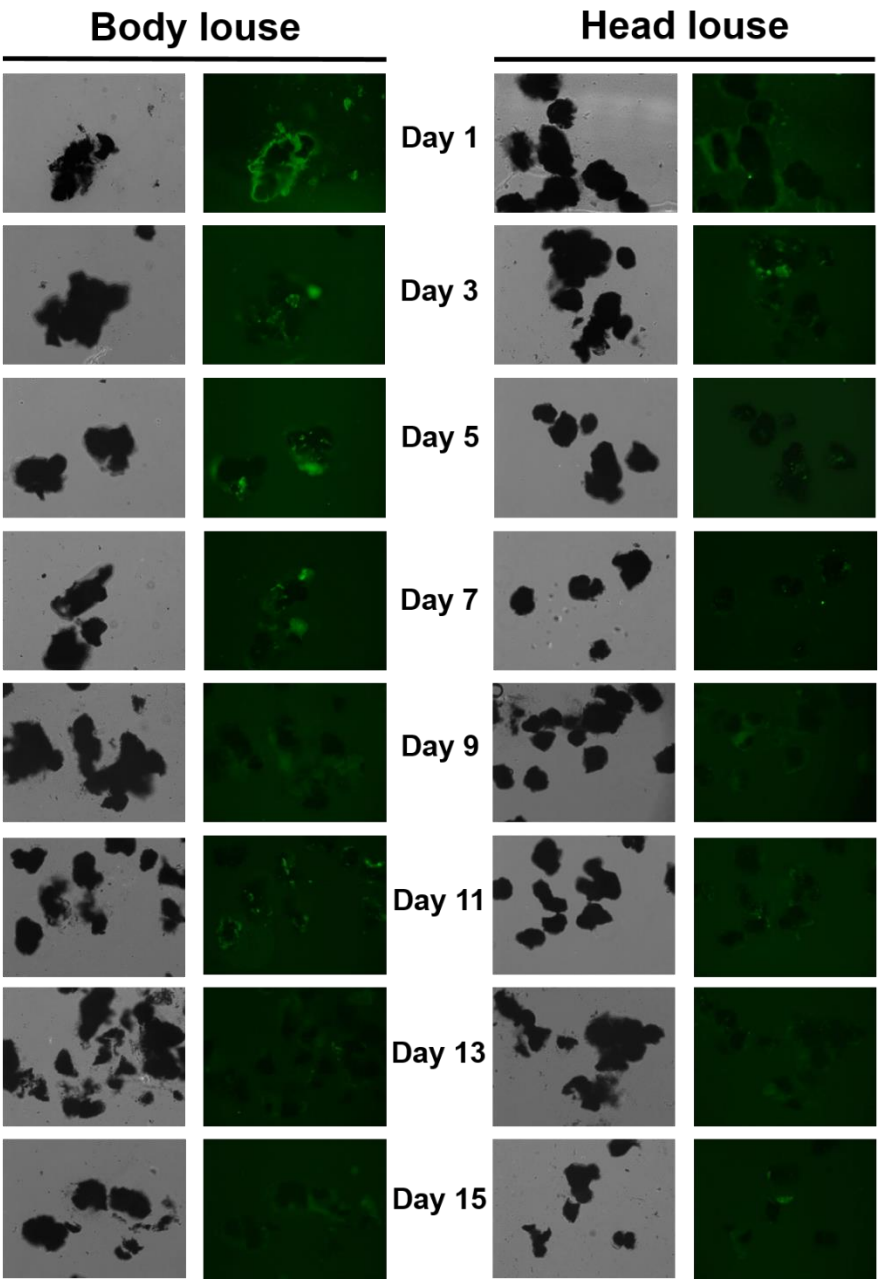


Figure 4. Representative light and fluorescence microscopic images of GFP-expressing *B. quintana* on louse feces in a time seires.

3. Discussion

In this study, the proliferation profiles of *B. quintana* and their viability in louse feces were compared between *B. quintana*-infected body and head lice. The number of *B. quintana* in lice increased and was significantly higher in body versus head lice over the entire period of 12 days. Additionally, the proliferation profiles of GFP-expressing *B. quintana* were similar to those of two previous reports using wild-type *B. quintana* in that two phases of proliferation (i.e., an initial phase of decline until 2-4 days post-challenge and the second phase of increase thereafter) were commonly observed particularly in body lice (Previte et al., 2014; Seki et al., 2007). The proliferation decline observed at the early stage of infection by *B. quintana* suggests that the humoral immune response in the alimentary track is actively engaged in the suppression of *B. quintana* proliferation and further plays a crucial role in shaping the second phase of proliferation in both body and head lice. In this study using GFP-expressing *B. quintana*, however, the *B. quintana* cell number declined after 9 days post-challenge in body lice unlike the previous observations using wild-type *B. quintana*, where *B. quintana* proliferation remained at the stationary phase after reaching maximum levels (Previte et al., 2014; Seki et al., 2007). This phenomenon may be due, in part, to the possible differences in the innate vitality between GFP-expressing *B. quintana* and wild-type *B. quintana.*

Despite the significantly higher proliferation rate of *B. quintana* in body lice, the number of *B. quintana* in a unit amount (1 mg) of feces was not significantly different between infected body and head lice over time as judged by qPCR. This finding suggests that body lice may have a greater capacity for harboring *B. quintana* inside their gut lumens compared with head lice and excrete only a small portion of proliferated *B. quintana.* In contrast, head lice likely have a lower harboring capacity and thus excrete most of proliferated *B. quintana*, thereby resulting in numbers similar to that of *B. quintana* excreted by body lice. Nevertheless, body lice excreted a significantly larger number of viable *B. quintana* in their feces particularly during the early stage of infection (i.e., 1~3 days post-challenge) as judged by the viability index. Since *B. quintana* is transmitted to humans via fecal contamination of skin abrasions or bite wounds from infected lice, the amount of viable *B. quintana* in louse feces should be a determining factor in vector competence. Thus, the higher vector competence of body lice is, in part, due to the augmented proliferation and excretion of viable pathogenic bacteria, including *B. quintana*. Our finding also suggests the importance of early stage of infection as a critical time period determining the difference in vector competence between body and head lice.

The number of *B. quintana* cells in unit amount (mg) of feces decreased in general over time following oral infection. This finding appears contrast to the

pervious report (Seki et al., 2007), in which *B. quintana* cells in feces per a single louse increased over time after the initial reduction until 4 days post-infection. It is unclear yet for this contradictory results but it can be speculated that the difference is caused by the combination of various factors in different experimental settings. In this study, the infected lice were allowed to feed continuously uninfected human blood using the feeding chamber unlike the previous study (Seki et al., 2007), in which lice were fed for a limited duration (20 min) a day. If the more frequent blood feeding and increase in feeding amount can raise the harboring capacity for *B. quintana* by some reason not yet known, the relative fraction of proliferated *B. quintana* to be excreted could be decreased as observed in this study. In addition, differences in various experimental factors, including the inoculating titer of *B. quintana* for oral infection, the feeding systems, the strains of *B. quintana* used, the age and strains of lice used, etc., may affect the different results on the dynamics of *B. quintana* excretion between the two studies.

Given that orally-infected *B. quintana* cannot pass through 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 humoral immune response in alimentary tract is the primary immune factor against invading pathogenic bacteria following an infected blood

meal. The humoral immune reactions against *B. quintana* in alimentary tract tissue, such as transcriptional regulation of immune-related genes and reactive oxygen species (ROS) level, are described in chapter III.

It is noteworthy that head lice have been suggested to be a vector like body lice based on the findings that *B. quintana* is detected in some head louse populations collected in Nepal and the US (Bonilla et al., 2009; Sasaki et al., 2006). However, as demonstrated in this study, simple detection of *B. quintana* in head lice via qPCR does not necessarily indicate their potential as a vector unless the titer or viability of *B. quintana* in head lice is also evaluated.

CHAPTER II

Annotation of Immune-related Genes from the Body and Head Lice Genomes and Molecular Characterization of Major Immune Components

II-1. Annotation of Immune-related Genes from the Genomes of Body and Head Lice

Abstract

Annotation of representative immune-related genes in the genomes of body and head lice revealed that both body and head lice have the same immune components. A total of 93 immune-related genes were identified in both body and head lice and the number is substantially fewer compared with other insects. Many gene families in the humoral immune system were considerably reduced in number or absent in the body louse genome. In case of the genes related with pathogen recognition, only one type of peptidoglycan recognition protein (PGRP) was annotated whereas beta-glucan binding protein (BGBP) was not found. In addition, Imd and its adaptor protein FADD in the Imd pathway were not identified. In contrast, all components in the Toll, JAK/STAT and JNK (c-Jun N-terminal kinase) pathways were preserved. Among the various kinds of antimicrobial peptides (AMP), only two types of defensin were annotated. The simplified immune system in human lice appears to be due to their parasitic life cycle and feeding only on relatively sterile human blood. The Louse PGRP,

defensin 1 and defensin 2, as the major immune genes involved in humoral immune response, showed 99.3, 99.1 and 98.2% similarity between body and head lice.

1. Materials and Methods

1.1. Annotation of immune-related genes from the body louse

Representative immune-related genes from *Bombyx mori* (Tanaka et al., 2008), *Drosophila melanogaster* (Christophides et al., 2002), *Anopheles gambiae* (Christophides et al., 2002), *Apis mellifera* (Evans et al., 2006), *Tribolium castaneum* (Zou et al., 2007), and other annotated insect genes in the NCBI protein server (http://www.ncbi.nlm.nih.gov/sites/entrez) were used as queries to search the body louse genome. Using these queries, the Phum U1.2 peptide database (http://phumanus.vectorbase.org/Tools/BLAST/) was searched by Blastp (Altschul et al., 1997). Protein sequences showing higher matches (e-value < 10^{-5}) were first retrieved and the Blastp search for the PhumU1.2 peptide database was repeated until no other newly listed proteins were found by using the body louse sequences as queries. Predicted proteins were analyzed by PROSITE (http://www.expasy.ch/prosite/) and Pfam (http://pfam.sanger.ac.uk/) to detect conserved domain structures required for assigning specific function. Structurally confirmed proteins in body lice were used as queries for NCBI Blast Search, and their phylogenetic relationships with other proteins determined. Signal peptides and transmembrane domains were predicted by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP) (Bendtsen et al., 2004) and SOSUI

(http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html), respectively

1.2. Head louse genome sequencing and immune gene annotation

1.2.1. Head lice rearing

A highly inbred BR-HL strain of head lice was used for the whole genome sequence analysis. The BR-HL strain was originally collected in Bristol, UK, and has been reared on the *in vitro* rearing system (Yoon et al., 2006).

1.2.2. Genomic DNA extraction

Genomic DNA was extracted from approximately 500 newly hatched first instar nymphs before their first blood meal using DNeasy blood & tissue kit (Qiagen, Hilden, Germany). Both quality and quantity of gDNA were analyzed by gel electrophoresis and Quant-iT™ PicoGreen® dsDNA Quanititation reagent (Invitrogen, Carlsbad, CA, USA).

1.2.3. Whole genome sequencing

Genome Analyzer IIx (Illumina, San Diego, CA, USA) with a mean length of 101 bp paired-end and GS FLX Titanium (Roche, Indianapolis, CA, USA) using a 3 kb library were used for genome sequencing according to the manufacturer's recommendations at the National Instrumentation Center for Environmental

Management (NICEM, Seoul, Korea) and DNA Link, Inc. (Seoul, Korea), respectively. The resulting sequence data were mapped to 8,588 contigs of the body louse genome. Unmapped reads were used for creating *de novo* assembly. All analyses and statistics for *de novo* assembly and reference mapping were performed using CLC Genomics Workbench (CLC bio, Aarhus, Denmark). The gene coding regions were predicted by GeneMark-ES version 2.3a (Ter-Hovhannisyan et al., 2008) in the *de novo* assembled contigs. Predicted protein-encoding genes were analyzed by BLASTP and the ones, which showed significant BLAST similarity (e-value < 10^{-5}) to proteins from other organisms in the non-redundant (NR) database at the National Center for Biotechnology Information (NCBI), were annotated. Comparison of the immune-related genes between head and body lice were conducted by BLAST-searching of all genes of each species against opposite species genome database on the condition of < 10^{-4} e-value.

1.3. Cloning of cDNA encoding PGRP and defensins

Full length cDNA sequences were determined by SMART RACE Kit (Clontech, Mountain View, CA, USA) with a slight modification. Total RNA was extracted from 10 adult lice using 200 μl of TRI reagent (MRC, Cincinnati, OH, USA) according to the manufacturer's instruction. Double-stranded cDNA for

Rapid Amplification of cDNA Ends (RACE) was synthesized from the total RNA following DNaseI (Takara biotechnology, Japan) treatment using the SMART Scribe reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with SMARTII A Oligo (5'-AAGCAGTGGTATCAACGCAGAGT-ACGCGGG-3'), 5'-CDS primer A ([5'-$(T)_{25}$VN-3' (N=A,C, G or T; V=A, G or C)]) or 3'-CDS primer A (5'-AAGCAGTGGTATCAACGCAGAGTAC$[T]_{30}$VN-3' [N=A, C, G or T; V=A, G or C]). To determine full transcript sequences of PGRP, Defensin 1 and defensin 2, specific primers for 5' and 3' RACE were designed based on the partial nucleotide sequences obtained from vectorbase (http://www.vectorbase.org) for body lice or genome sequencing data for head lice (Table 1). Using the 5'- and 3'-RACE cDNAs as templates, PCR was conducted with gene specific primers and Universal Primer Mix (UPM, Clontech) by denaturing at 95℃ for 2 min, followed by 25 cycles of 95℃ for 30 s, 68℃ for 30 s and 72℃ for 2 min. Nested PCR was performed with the dilution of primary PCR product as a template, gene specific primers and the Nested Universal Primer A (NUP, Clontech). The PCR program was 95℃ for 2 min, followed by 20 cycles of 95℃ for 30 s, 68℃ for 30 s and 72℃ for 2 min. All PCRs were carried out using Advantage 2 polymerase mix (Clontech). The PCR products were cloned into pGEM-T easy vector (Promega, Madison, WI, USA) and subjected to sequencing. Contig assembly was performed using the SeqMan program (DNAStar, Madison, WI, USA) to generate a full-

length transcript.

Table 1. Sequences of the primers used for RACE

Gene	Type	Sequence (5'→3')
PGRP	3' RACE	CAGACGAATTTCAACGGTCCTGTGAC
	3' RACE nested	CGAACACGCGACTCCATCGAATTC
Defensin 1	5' RACE	TCAATGATCCCCAAGGTGTGCTAGC
	5' RACE nested	TTGGCATCTTCTTCACCGGCGACA
	3' RACE	GCTTTTGTTTCTGCCGTGCCYGTAC
	3' RACE nested	GCGATGCGGATTTTCAAGTTGAAG
Defensin 2	5' RACE	GCAATACAATGAGCAGCACATGCAG
	5' RACE nested	CAAGTTGCTCTCCTGAATCTTCCTC
	3' RACE	CTTCGTCGTGGCATCAGGTTTACCG
	3' RACE nested	ACCGTCGACGTTGAACCRATTCCC

1.4. Sequence analysis

Alignment of amino acid sequences and phylogenetic analysis of immune-related genes were performed using MegAlign (DNAStar, Madison, WI, USA) and MEGA6 software, respectively (Tamura et al., 2013). The phylogenetic tree was constructed by Neighbor-Joining (Vingron and Argos, 1989) and p-distance methods with 10,000 replicates for bootstrap value. The three-dimensional (3D) structure modeling of defensins were conducted based on the structure of the

insect defensin A (Cornet et al., 1995) and *Anopheles* defensin (Landon et al., 2008) peptides in the Protein Data Bank (PDB). The sequences of louse genes were submitted to the molecular modeling server of the SWISS-MODEL (Automated Comparative Protein Modeling Server) (Arnold et al., 2006) for structure prediction, and 3D structures were analyzed using the UCSF Chimera (Pettersen et al., 2004). The calculated isoelectric point (pI), transmembrane region and signal peptide cleavage site were predicted by Compute pI/Mw tool (http://web.expasy.org/compute_pi/) (Gasteiger et al., 2005), TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0) (Krogh et al., 2001) and SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/), (Petersen et al., 2011) respectively. For the prediction of transcription factor binding sites in 5' upstream region of genes, two different motif discovery programs were used: TFSearch (http://www.cbrc.jp/research/db/TFSEARCH.html) and Match (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi). The 1000-bp long putative regulatory region of target gene (800-bp upstream plus 200-bp downstream genomic DNA sequences from the transcription starting site) was used for transcription factor binding site prediction. The sequences were obtained from the vectorbase (http://www.vectorbase.org) and the head louse genome sequence data for body and head lice, respectively.

1.5. Fluorescence in situ *hybridization*

Fluorescence *in situ* hybridization (FISH) was conducted to analyze the distribution of defensin 1 and defensin 2 genes in louse alimentary tract tissues. cDNA fragments of 343 bp defensin 1 and 355 bp defensin 2 were amplified by PCR using the following conditions: a single denaturation cycle at 95℃ for 2 min and 35 cycles of 95℃ for 30 s, 58℃ (defensin 1) or 57℃ (defensin 2) for 30 s and 68℃ for 1 min. The PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and then the digoxigenin (DIG)-labeled sense and antisense RNA probes were generated using SP6 and T7 RNA polymerase, respectively. All procedures were performed according to the previously published methods with slight modifications (Morris et al., 2009). Whole alimentary tracts dissected from female lice were fixed with 0.1% diethylpyrocarbonate (DEPC)-treated 4% paraformaldehyde (PFA) and washed in DEPC-treated PBT (PBS with 0.1% Tween-20, pH 7.4). Hybridization to the probes was performed at 58℃ for 20 h. After hybridization, the samples were washed with 5X SSC at 63℃ for 10 min and then with 0.2X SSC at 63℃ for 30 min. After treatment with 1X blocking agent (Roche Molecular Biochemicals, Indianapolis, IN) in maleic acid, defensin 1 and defensin 2 signals were visualized using the horseradish peroxidase (POD) coupled to anti-DIG (defensin 1) or anti-FITC (defensin 2) antibodies (Roche Molecular Biochemicals, Indianapolis, IN) at a 1:1000 dilution.

POD signals were detected using the tyramide signal amplification (TSA) system (Thermo Scientific). The digital images taken using Zeiss Laser Scanning Microscope (LSM) 710 (Carl Zeiss, Oberkochen, Germany) were processed using the Zeiss LSM image browser to make projection images and aligned using Adobe Photoshop (Adobe, San Jose, CA, USA).

Table 2. Sequences of the primers used for *in situ* hybridization

Gene		Sequence (5'→3')	Product size (bp)
Defensin 1	F[a]	TTTTTCAAAAATGAACGGATTAACGATTG	343
	R[b]	ATTCATCTTCTGCATCTGCAAATTC	
Defensin 2	F	AAAATGAACGGTTTGAATTTGATTATTAT	355
	R	TTCATTTTCGACAGCGGCAAAC	

[a] Forward primer.
[b] Reverse primer.

2. Results

2.1. Annotation of immnue-related genes

A list of immune-related genes predicted in the body louse genome was presented in Table 3. The number of immune-related genes in each category was compared with other insects, including *B. mori* (Tanaka et al., 2008), *D. melanogaster* (Christophides et al., 2002), *A. gambiae* (Christophides et al., 2002), *A. mellifera* (Evans et al., 2006) and *T. castaneum* (Zou et al., 2007) (Table 3). A total of 93 immune-related genes were identified from the body louse genome, which is comparable to *A. mellifera* but is a much smaller number (1/2~1/3) than that identified in other insects. Many gene families in the humoral immune system were considerably reduced in number or absent in the body louse genome. In case of the genes related with pathogen recognition, only one type of peptidoglycan recognition protein (PGRP) was annotated, which has a transmembrane domain. The genes homologous to beta-glucan binding protein (BGBP) were not found. In addition, some components in the Imd pathway (Imd and its adaptor protein FADD) were not identified. In contrast, all components in the Toll, JAK/STAT and JNK (c-Jun N-terminal kinase) pathways were preserved. Among the various kinds of antimicrobial peptides (AMP) known in insects, only two defensin genes (defensin 1 and defensin 2) were identified.

All the immune related genes that had been identified in the body louse genome were annotated in the head louse genome (Table 3). No head louse-specific immune genes were detected, and the average nucleotide sequence identity in the 1:1 orthologous genes between head and body lice was 96.8% (Table 4).

Table 3. Comparison of the immune-related genes annotated from the genomes of *Pediculus humanus* (*P. hum*), *Bombyx mori*[a] (*B. mor*), *Drosophila melanogaster*[b] *(D. mel*), *Anopheles gambiae*[b] (*A.gam*), *Apis mellifera*[c] (*A. mel*) and *Triolium castaneum*[d] (*T. cas*)

Category	Gene Name	*P. hum*	*B. mor*	*D. mel*	*A. gam*	*A.mel*	*T. cas*
Recognition	PGRP	1	12	13	7	4	6
	GNBP	**x**[e]	4	3	7	2	3
	Fibrinogen-related protein	2	3	14	61	2	7
	C-type lectin	9	21	34	25	10	16
	Hemocytin	1	1	1	x	1	x
	Galectin	3	4	6	8	2	3
	TEP	3	3	6	15	3	4
	NimrodA	1	1	1	1	1	1
	NimrodB	x	1	5	1	1	1
	NimrodC	x	2	4	2	2	2
	SR-A	4	4	5	5	3	4
	SR-B	10	13	12	15	9	16
	SR-C	1	1	4	1	1	1
	Draper	1	1	1	1	1	1
	Dscam	1	1	1	1	1	1
	Duox	1	x	1	1	1	x
Modulator	CLIP serine protease	6	15	37	41	18	26
	Serpin	16	26	30	17	5	31
Toll pathway	Spätzle	3	3	6	6	2	6
	Toll	6	14	9	10	5	10
	MyD88	1	1	1	1	1	1
	Tube	1	1	1	1	1	1
	Pelle	1	1	1	1	1	1
	TRAF2	1	1	1	1	1	1
	ECSIT	1	1	1	1	1	1
	Cactus	1	1	1	1	3	1
	Dif/Dorsal	1	1	2	1	2	2
IMD pathway	Imd	**x**	1	1	1	1	1
	Dredd	1	1	1	1	1	1
	TAK1	1	1	1	1	1	1
	FADD	**x**	1	1	1	1	1
	TAB2	1	1	1	1	1	1
	IAP2	1	1	1	1	1	1
	IKK beta	1	1	1	1	1	1
	Relish	1	1	1	1	2	2

Table 3. Comparison of the immune-related genes annotated from the genomes of *Pediculus humanus* (*P. hum*), *Bombyx mori*[a] (*B. mor*), *Drosophila melanogaster*[b] *(D. mel*), *Anopheles gambiae*[b] (*A.gam*), *Apis mellifera*[c] (*A. mel*) and *Triolium castaneum*[d] (*T. cas*). (continued)

Category	Gene Name	*P. hum*	*B. mor*	*D. mel*	*A. gam*	*A.mel*	*T. cas*
JNK pathway	Hem	1	1	1	1	1	1
	JNK (Basket)	1	1	1	1	1	3
	Kay	1	1	1	1	1	1
	Jun	1	1	1	1	1	1
JAK/STAT pathway	Domeless	1	1	1	1	1	
	JAK	1	x	1	1	1	1
	STAT	1	1	1	2	1	1
Effectors	PPO	1	2	3	9	1	3
	Noduler	1	1	1	1	1	1
	Defensin	2	1	1	4	2	4
	Other AMPs	**x**	29	19	6	4	8
Total		93	184	240	268	108	181

[a] (Tanaka et al., 2008)

[b] (Christophides et al., 2002)

[c] (Evans et al., 2006)

[d] (Zou et al., 2007)

[e] x: not found

Table 4. List of immune-related genes annotated from the genomes of body and head lice

Gene Name	Number	Body louse ID	Head louse ID	Identity (%)	E value
Recognition	**38**				
PGRP	1	PHUM581030	HLORF11059	99.3	0
GNBP (BGBP)	x[a]				
Fibrinogen-related protein	2	PHUM562660	HLORF10623	99.4	0
		PHUM500950	HLORF09263	99.3	2.1E-81
C-type lectin	9	PHUM467750	HLORF08594	100	3.0E-170
		PHUM248020	HLORF04183	95.4	3.9E-156
		PHUM458550	HLORF08320	86.4	1.5E-05
		PHUM390090	HLORF06700	99.4	3.1E-105
		PHUM509080	HLORF09507	97.7	0
		PHUM150830	HLORF02473	99.3	0
		PHUM151070	HLORF02487	100	1.6E-141
		PHUM280850	HLORF04774	100	4.5E-78
		PHUM489310	HLORF08916	94.2	1.2E-161
Hemocytin	1	PHUM474690	HLORF08712	96.3	0
Galectin	3	PHUM402330	HLORF07000	98.5	0
		PHUM275780	HLORF04708	99.5	1.4E-111
		PHUM051550	HLORF00913	94.2	0
TEP	3	PHUM375050	HLORF06425	85.8	0
		PHUM289860	HLORF04974	99.3	0
		PHUM289710	HLORF04971	97.9	0
Nimrod A	1	PHUM522270	HLORF09845	94.5	0
Nimrod B	x				

Table 4. List of immune-related genes annotated from the genomes of body and head lice (continued)

Gene Name	Number	Body louse ID	Head louse ID	Identity (%)	E value
Nimrod C	x				
Draper	1	PHUM049590	HLORF00866	99.3	0
Dscam	1	PHUM602300	HLORF11620	44.2	0
Duox	1	PHUM454140	HLORF08183	99.1	0
Scavenger receptor A	4	PHUM454890	HLORF08199	93.3	0
		PHUM602700	HLORF11644	98.4	0
		PHUM066640	HLORF01137	100	0
		PHUM534870	HLORF10080	98.8	0
Scavenger receptor B	10	PHUM603690	HLORF11674	99.0	0
		PHUM424210	HLORF07465	99.8	0
		PHUM569600	HLORF10779	96.2	0
		PHUM569610	HLORF10780	100	0
		PHUM365540	HLORF06255	99.2	0
		PHUM569120	HLORF10757	97.5	0
		PHUM351630	HLORF05958	99.6	0
		PHUM351640	HLORF05959	99.8	0
		PHUM365690	HLORF06260	99.6	0
		PHUM563930	HLORF10646	99.4	0
Scavenger receptor C	1	PHUM356530	HLORF06042	99.5	8.7E-132
Modulator	**22**				
CLIP serine protease	6	PHUM501910	HLORF09292	99.4	0
		PHUM360690	HLORF06138	92.6	0
		PHUM451100	HLORF08107	98.5	2.1E-148

Table 4. List of immune-related genes annotated from the genomes of body and head lice (continued)

Gene Name	Number	Body louse ID	Head louse ID	Identity (%)	E value
CLIP serine protease (continued)	**6**	PHUM192460	HLORF03113	98.6	0
		PHUM571420	HLORF10835	99.2	5.5E-145
		PHUM027570	HLORF00524	99.8	0
Serpin	16	PHUM220550	HLORF03612	98.2	0
		PHUM432060	HLORF07679	99.5	0
		PHUM291200	HLORF04991	98.3	0
		PHUM291170	HLORF04988	97.4	0
		PHUM492620	HLORF09064	99.2	0
		PHUM108970	HLORF01738	98.9	4.8E-155
		PHUM108960	HLORF01738	90.7	1.1E-91
		PHUM106690	HLORF01704	97.1	0
		PHUM106570	HLORF01703	98.7	1.9E-174
		PHUM106460	HLORF01702	86.8	0
		PHUM075870	HLORF01225	99.8	0
		PHUM221060	HLORF03625	91.6	0
		PHUM600840	HLORF11548	97.5	0
		PHUM291190	HLORF04990	97.7	0
		PHUM291180	HLORF04989	96.0	0
		PHUM311330	HLORF05347	98.4	0
Toll pathway	**16**				
Spätzle	3	PHUM596260	HLORF11408	99.6	0
		PHUM332090	HLORF05632	99.6	6.2E-139
		PHUM057390	HLORF00994	88.7	0

Table 4. List of immune-related genes annotated from the genomes of body and head lice (continued)

Gene Name	Number	Body louse ID	Head louse ID	Identity (%)	E value
Toll	6	PHUM529420	HLORF09977	99.3	0
		PHUM081740	HLORF01330	99.9	0
		PHUM480550	HLORF08823	95.8	0
		PHUM108410	HLORF01728	100	0
		PHUM107160	HLORF01719	99.0	0
		PHUM006690	HLORF00161	98.5	0
MyD88	1	PHUM536290	HLORF10117	98.0	0
Tube	1	PHUM194370	HLORF03155	99.6	0
Pelle	1	PHUM518290	HLORF09774	98.1	0
TRAF2	1	PHUM129280	HLORF02091	98.5	0
ECSIT	1	PHUM075600	HLORF01219	99.5	0
Cactus	1	PHUM345810	HLORF05855	99.2	0
Dorsal	1	PHUM534140	HLORF10065	99.5	0
Imd pathway	**6**				
IMD	x				
Dredd	1	PHUM574530	HLORF10867	98.7	0
TAK1	1	PHUM125410	HLORF01932	98.6	0
FADD	x				
Tab2	1	PHUM433350	HLORF04584	93.9	0
IAP2	1	PHUM080100	HLORF01300	95.7	0
IKK beta(IRD5)	1	PHUM605130	HLORF11731	98.3	0
Relish	1	PHUM424590	HLORF07494	94.4	0

Table 4. List of immune-related genes annotated from the genomes of body and head lice (continued)

Gene Name	Number	Body louse ID	Head louse ID	Identity (%)	E value
JNK pathway	**4**				
Hem	1	PHUM588610	HLORF11246	100	0
JNK(Basket)	1	PHUM128040	HLORF02026	39.0	0
Kay	1	PHUM237480	HLORF03977	100	3.7E-151
Jun(jra)	1	PHUM379500	HLORF06548	99.2	1.7E-69
JAK/STAT pathway	**3**				
Domeless	1	PHUM374950	HLORF06422	96.3	0
JAK	1	PHUM202560	HLORF03268	100	0
STAT	1	PHUM335200	HLORF05714	99.2	0
Effector	**4**				
PPO	1	PHUM448900	HLORF08027	99.7	0
Noduler	1	PHUM249370	HLORF04228	98.8	1.0E-90
Defensin	2	PHUM365700	HLORF12652	100	0
		PHUM595870	HLORF11388	97.0	4.9E-52
Other AMPs	x				
Total	**93**				

[a] not found

2.2. Molecular characterization of PGRP and defensins

2.2.1. Sequence analysis of PGRP

Both body louse PGRP (BLPGRP) and head louse PGRP (HLPGRP) were composed of 546 amino acid residues of 62.3 kDa and 61.2 kDa, respectively. They showed a 99.3% similarity both in nucleotide and amino acid sequences (Fig 1). Unlike the short-type PGRPs, which are small extracellular proteins (~200 amino acids, 19-20 kDa), louse PGRPs belong to the long-type PGRP. Louse PGRPs were determined to have one transmembrane domain in N-terminal and two PGRP domains, each of which can be divided into three PGRP subdomains I, II and III based on the conserved homology in C-terminal, but no signal peptide was detected (Fig 1, 2). The NCBI Blast search and phylogenetic analysis showed that louse PGRP sequences are closely related to the I-alpha type PGRP identified from *Zootermopsis nevadensis* (Isoptera) with a 38% similarity (Fig 4).

Seven transcription factor binding sites that are specific to five different transcription factors (BR-C Z4-like, Elf-1-like, Hb-like, Dfd-like and Ttk 69k-like) were observed in the putative regulatory region (800-bp upstream and 200-bp downstream from the transcription start site). No differences in the binding motifs in the regulatory region were found between BLPGRP and HLPGRP (Fig 5).

Figure 1. Comparison of deduced amino acid sequences of PGRP from body and head lice. The dotted line box indicates PGRP domains. Substrate binding site are marked by asterisks. BL, body louse; HL, head louse.

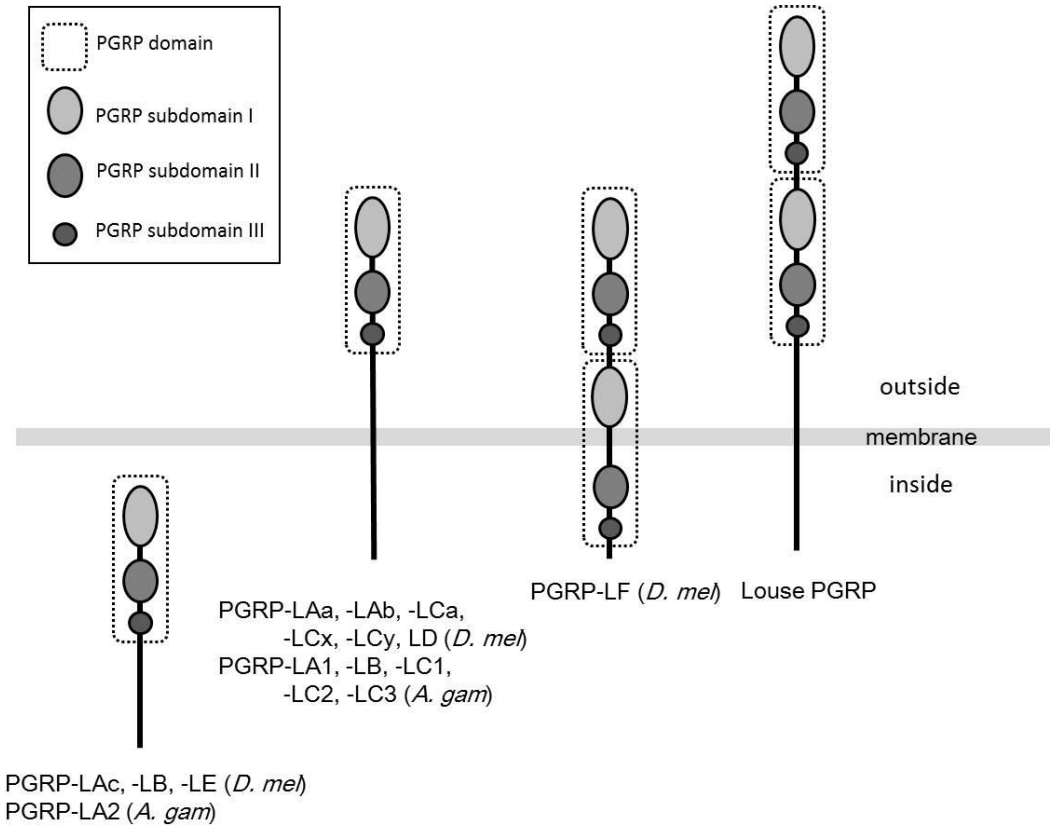


Figure 2. Representative insect long type PGRPs and louse PGRP: predicted domains and cellular locations. *D. mel*, *Drosophila melanogaster*; *A. gam*, *Anopheles gambiae* (Dziarski, 2004).

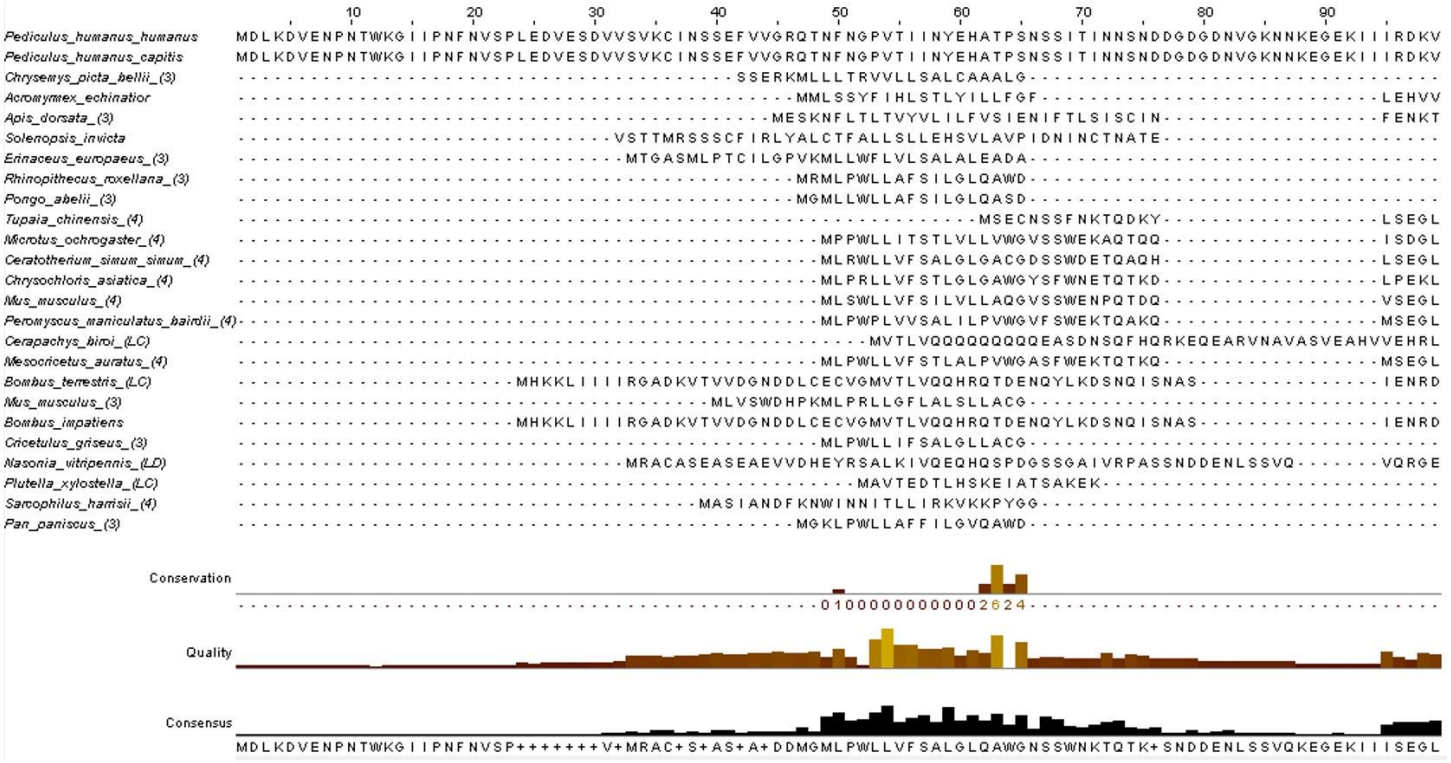


Figure 3. Deduced amino acid sequence alignment of PGRPs from body louse (*Pediculus humanus humanus*) and head louse (*Pediculus humanus capitis*) and other species. PGRP sequences showing higher similarity with those of louse were selected using BLAST search. PGRP subdomains in domain 2 are marked by color boxes (green, domain III; pink, domain II; blue, domain I).

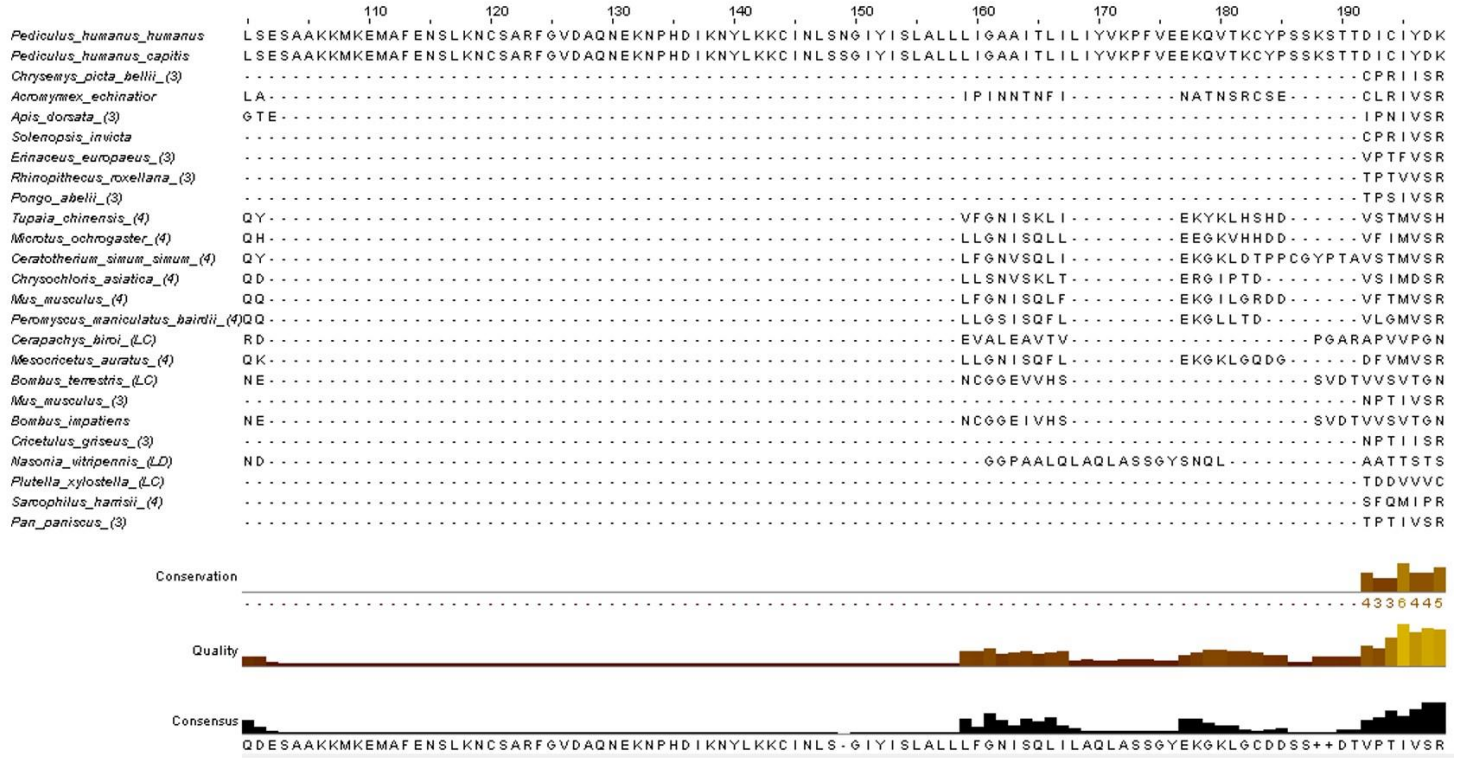


Figure 3. Deduced amino acid sequence alignment of PGRPs from body louse (*Pediculus humanus humanus*) and head louse (*Pediculus humanus capitis*) and other species. PGRP sequences showing higher similarity with those of louse were selected using BLAST search. PGRP subdomains in domain 2 are marked by color boxes (green, domain III; pink, domain II; blue, domain I). (continued)

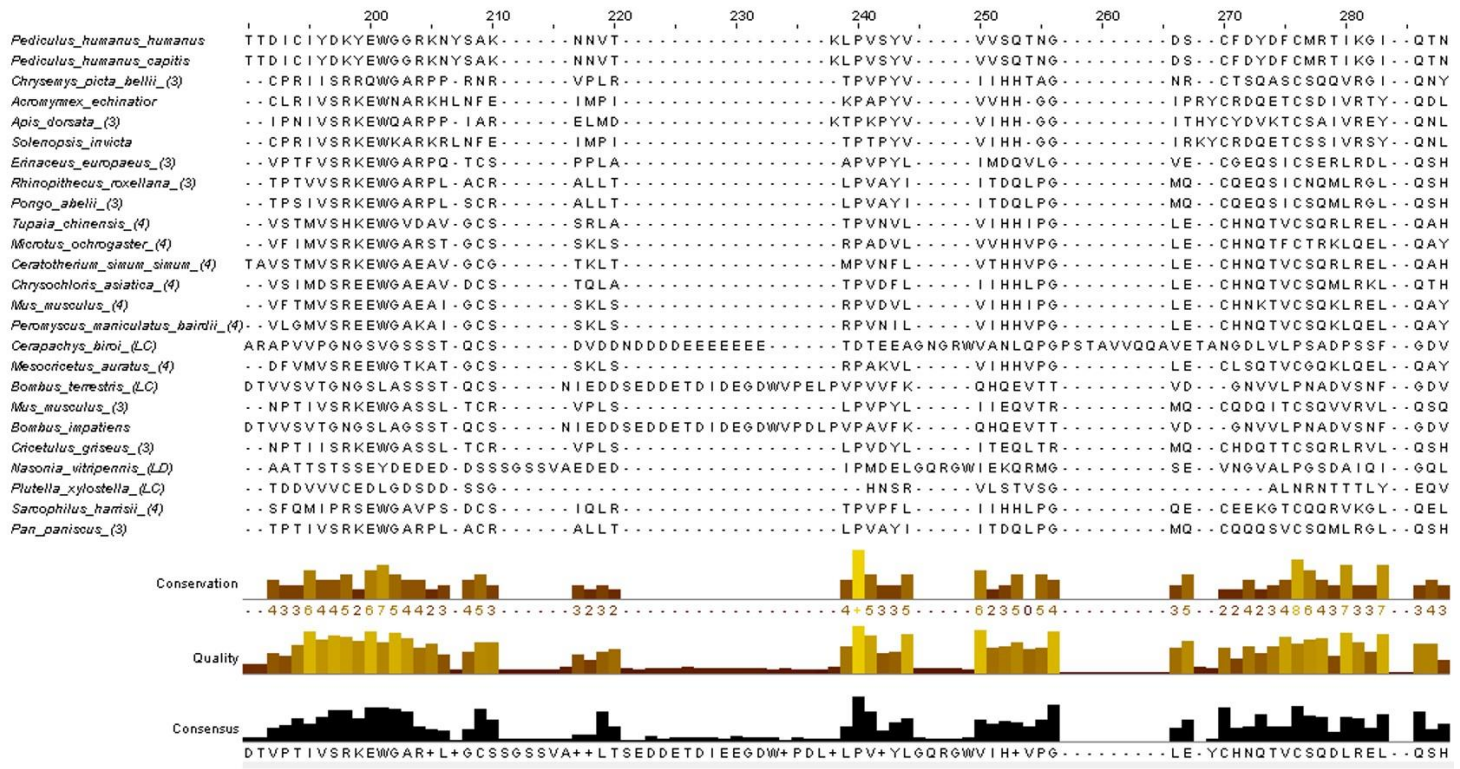
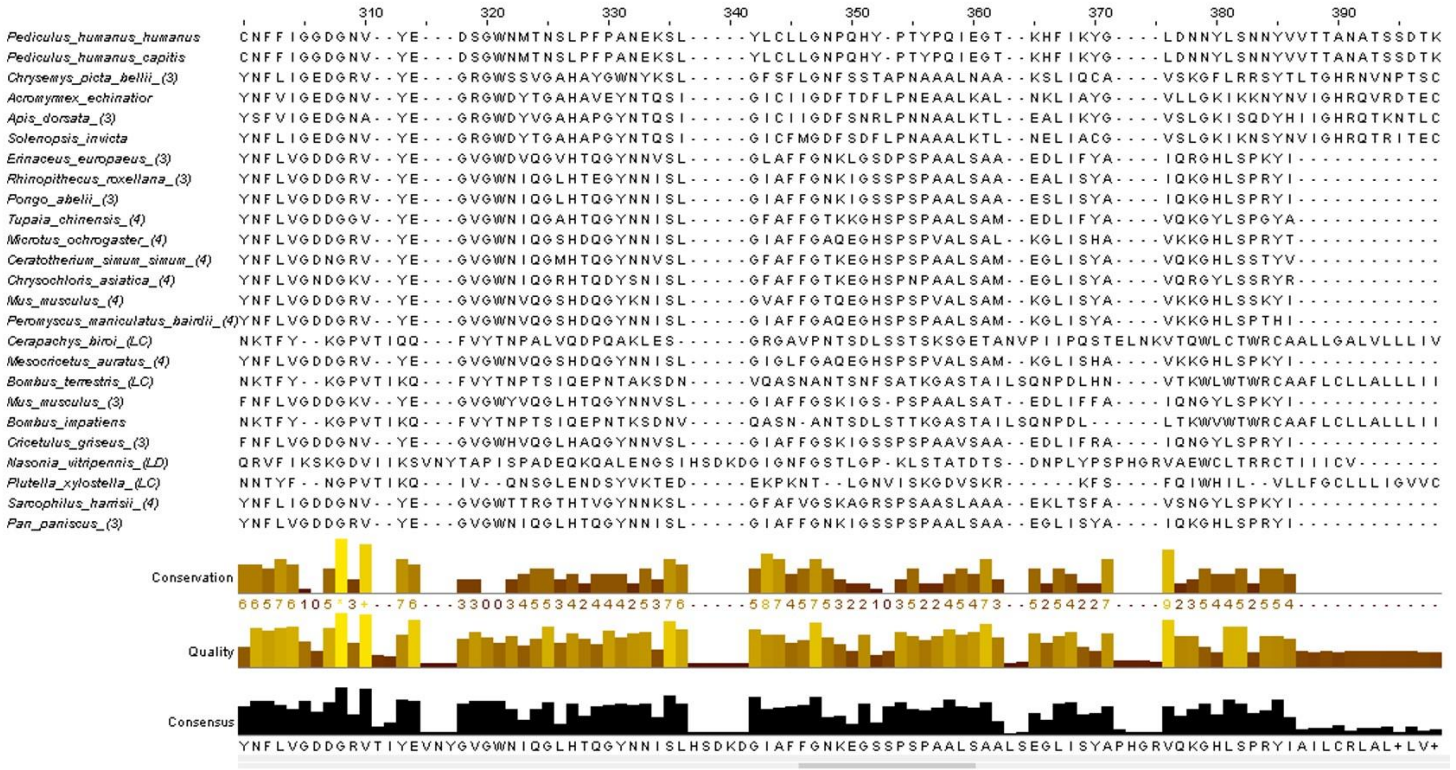


Figure 3. Deduced amino acid sequence alignment of PGRPs from body louse (*Pediculus humanus humanus*) and head louse (*Pediculus humanus capitis*) and other speices. PGRP sequences showing higher similarity with those of louse were selected using BLAST search. PGRP subdomains in domain 2 are marked by color boxes (green, domain III; pink, domain II; blue, domain I). (continued)



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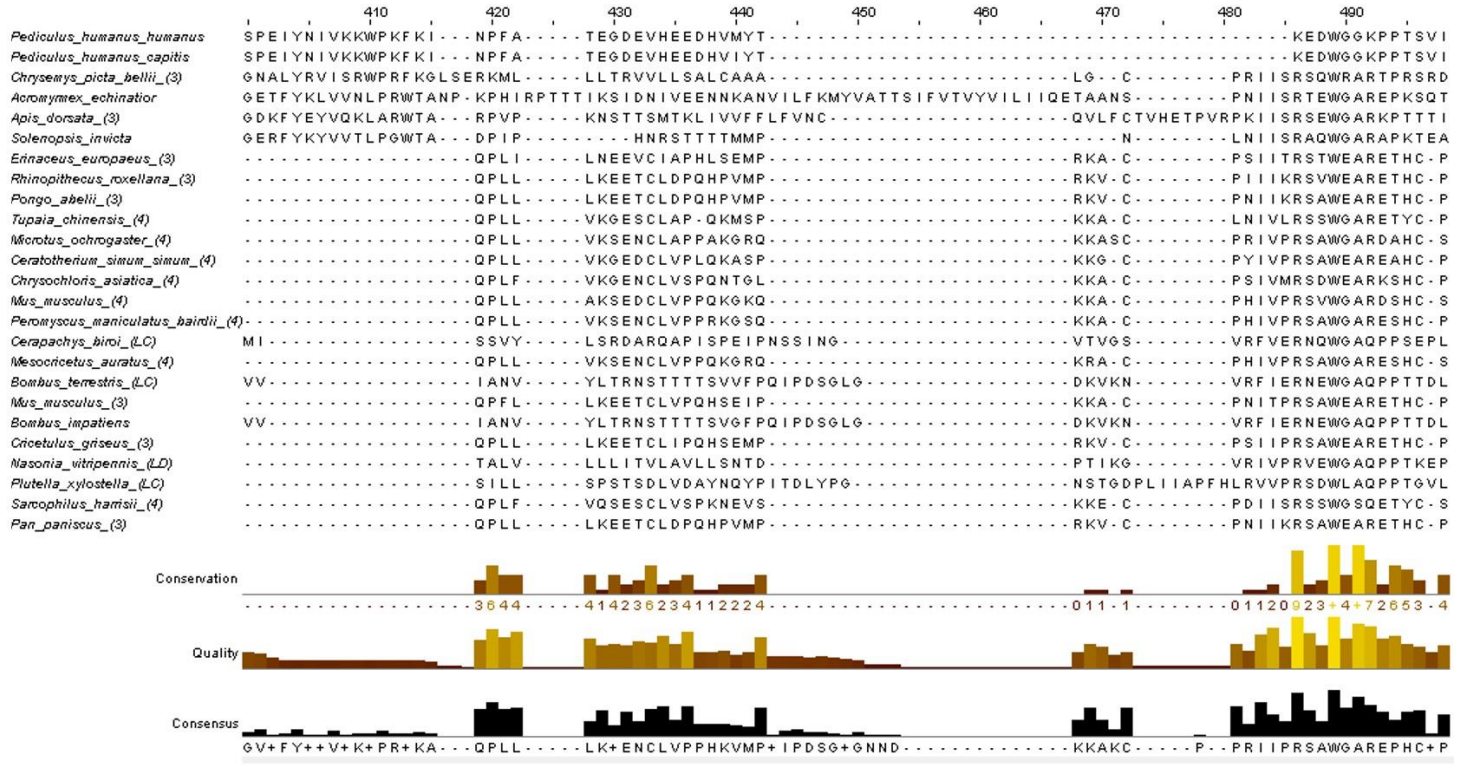


Figure 3. Deduced amino acid sequence alignment of PGRPs from body louse (*Pediculus humanus humanus*) and head louse (*Pediculus humanus capitis*) and other speices. PGRP sequences showing higher similarity with those of louse were selected using BLAST search. PGRP subdomains in domain 2 are marked by color boxes (green, domain III; pink, domain II; blue, domain I). (continued)

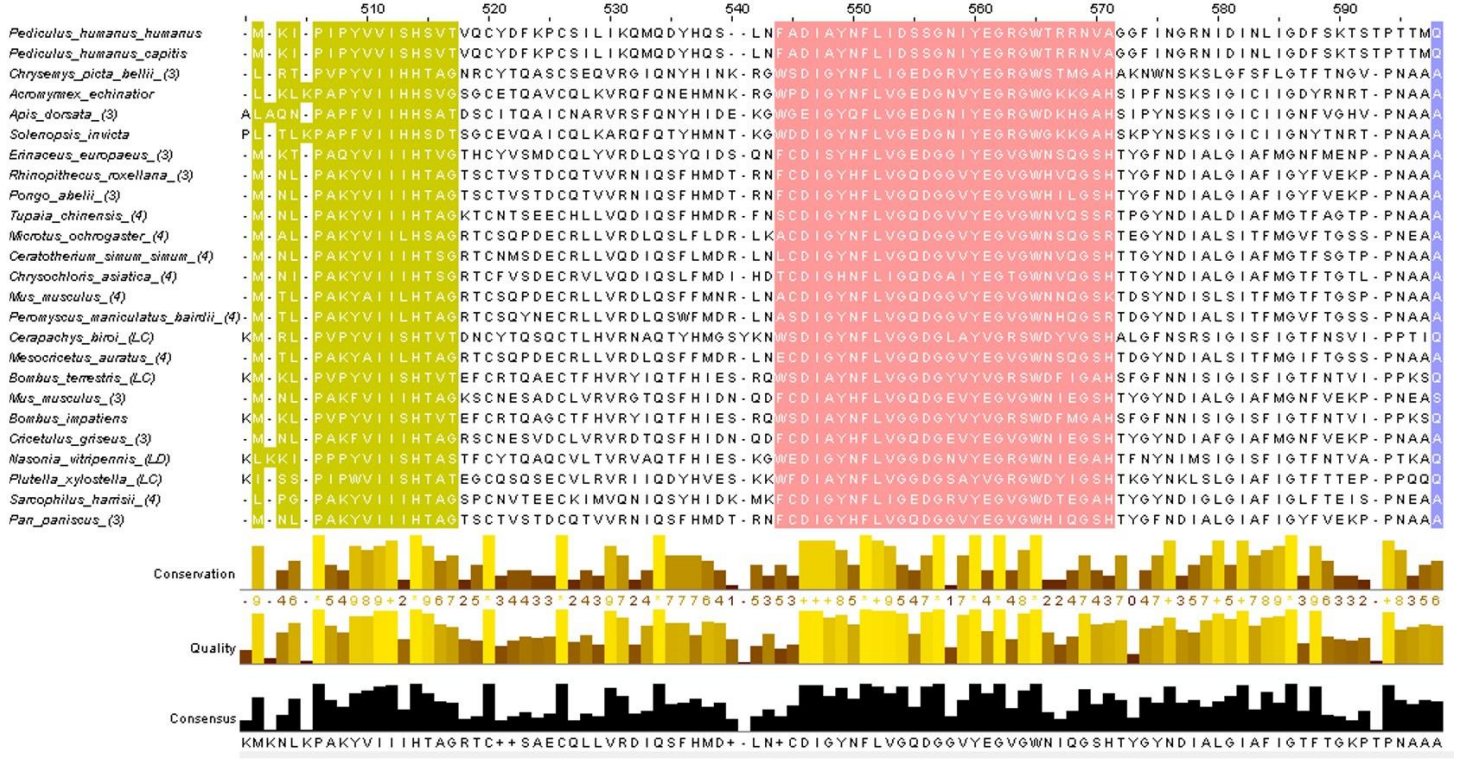


Figure 3. Deduced amino acid sequence alignment of PGRPs from body louse (*Pediculus humanus humanus*) and head louse (*Pediculus humanus capitis*) and other speices. PGRP sequences showing higher similarity with those of louse were selected using BLAST search. PGRP subdomains in domain 2 are marked by color boxes (green, domain III; pink, domain II; blue, domain I). (continued)

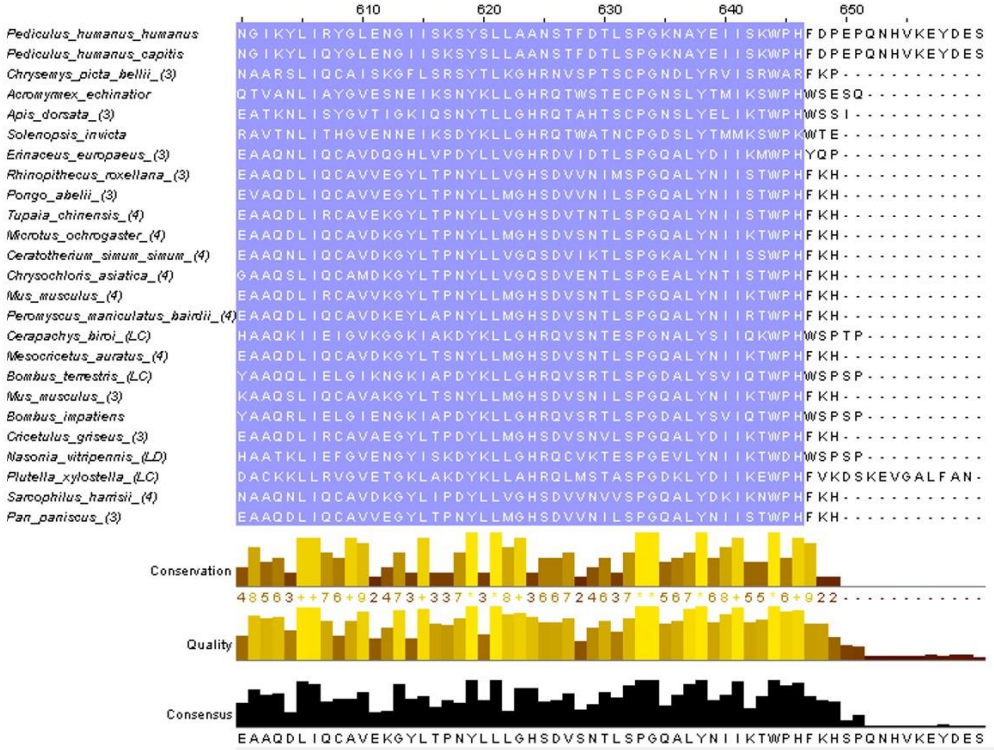


Figure 3. Deduced amino acid sequence alignment of PGRPs from body louse (*Pediculus humanus humanus*) and head louse (*Pediculus humanus capitis*) and other speices. PGRP sequences showing higher similarity with those of louse were selected using BLAST search. PGRP subdomains in domain 2 are marked by color boxes (green, domain III; pink, domain II; blue, domain I). (continued)

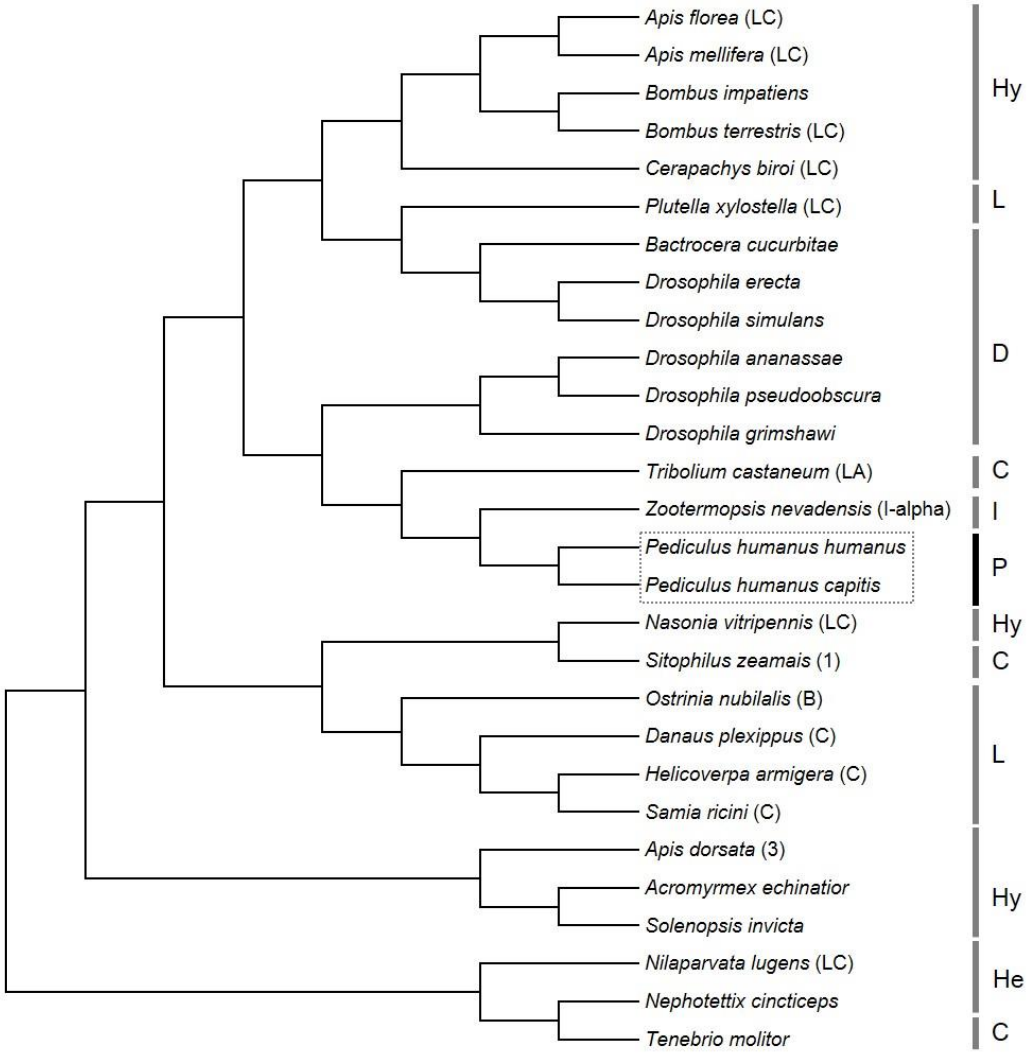


Figure 4. Phylogenetic tree of PGRPs from lice and other insects. Hy, Hymenoptera; L, Lepidoptera; D, Diptera; C, Coleoptera; I, Isoptera; P, Phthiraptera; He, Hemiptera.

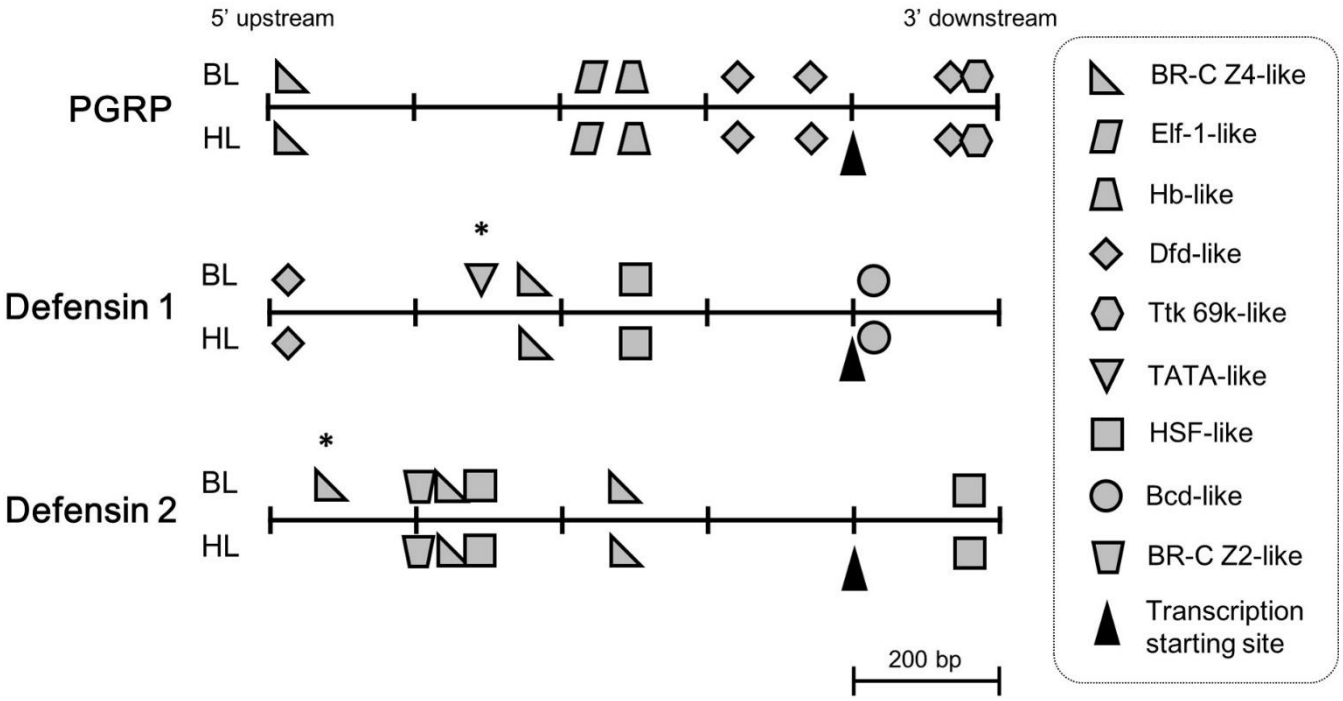


Figure 5. The potential transcription factor binding motifs observed in the putative regulatory region (800-bp upstream and 200-bp downstream from the gene transcription start site). Different shapes represent different potential motifs. The upper side is body louse (BL) sequence and the bottom side is head louse (HL) sequence. Different motifs between body and head lice were marked by asterisk.

2.2.2. Sequence analysis of defensin 1 and defensin 2

Both body louse defensin 1 (BLDef1) and head louse defensin 1 (HLDef1) were composed of 109 amino acids containing N-terminal signal peptide and 44 residues of C-termianl mature peptide (4.8 kDa) and their deduced amino acid sequences were identical (Fig. 6A). The mature peptide of louse defensin 1 contained six cysteine residues at positions 69, 86, 90, 100, 105 and 107, which create three disulfide bonds to form defensin-specific structure (Fig. 6C). The pI of mature defensin 1 was 9.86.

Both body louse defensin 2 (BLDef2) and head louse defensin 2 (HLDef2) were composed of 116 amino acids, among which two residues were different at 30^{th} (glutamine for BLDef2 vs. arginine for HLDef2) and 108^{th} (tyrosine for BLDef2 vs. aspartic acid for HLDef2) positions (Fig. 6B) but only the latter existed in mature peptide. Louse defensin 2 also has signal peptide cleavage site and defensin-specific six cysteine residues were also conserved at positions 75, 91, 95, 107, 112 and 114 in the mature peptide of 46 amino acids (Fig. 6C). The pIs of mature peptide were estimated to be 9.64 and 9.50 for BLDef2 and HLDef2, respectively. Louse defensin 1 and defensin 2 showed 48.1% and 69.6% similarities in full-length and mature peptide, respectively.

Insect defensins with high sequence similarity to louse defensin 1 and defensin 2 were collected (38 insect species including 13 Diptera, 5 Coleoptera,

14 Hymenoptera, 5 Hemiptera and 1 Tysanura) and aligned (Figure 8). The invertebrate defensin domain, namely the mature peptide region, was conserved in various insect species, whereas the 5' leader sequences were variable, resulting in low sequence homology. In the phylogenetic tree generated from 40 defensin-related sequences from insects, louse defensin 1 and defensin 2 were clustered with that of *Graminella nigrifrons* (Hemiptera) (Fig 14).

Analysis of 3D structure showed that both defensin 1 and defensin 2 have one α-helix (His^{83}-Lys^{93} of defensin1 and Asn^{87}-Ile^{96} of defensin 2) and only defensin 2 has two β-sheets (β1: Gly^{104}-Cys^{107}; β2: Cys^{112}-Arg^{115}) (Fig. 9). One different amino acid in defensin 2 was located in a loop between 2 β-sheets, which caused a slight distortion of the loop but overall structures of BLDef2 and HLDef2 were identical.

Four transcription factor binding sites (Dfd-like, BR-C Z4-like, HSF-like and Bcd-like) were observed in the putative regulatory region (800-bp upstream and 200-bp downstream from the transcription start site) of louse defensin 1 and only body louse has TATA-like binding site at 520-bp upstream (Fig 5). In case of defensin 2, six (BLDef2) or five (HLDef2) binding sites that are specific to three different transcription factors (BR-C Z4-like, BR-C Z2-like and HSF-like). Body louse has an additional BR-C Z4-like binding site at 719-bp upstream of transcription starting site of BLDef2 (Fig. 11).

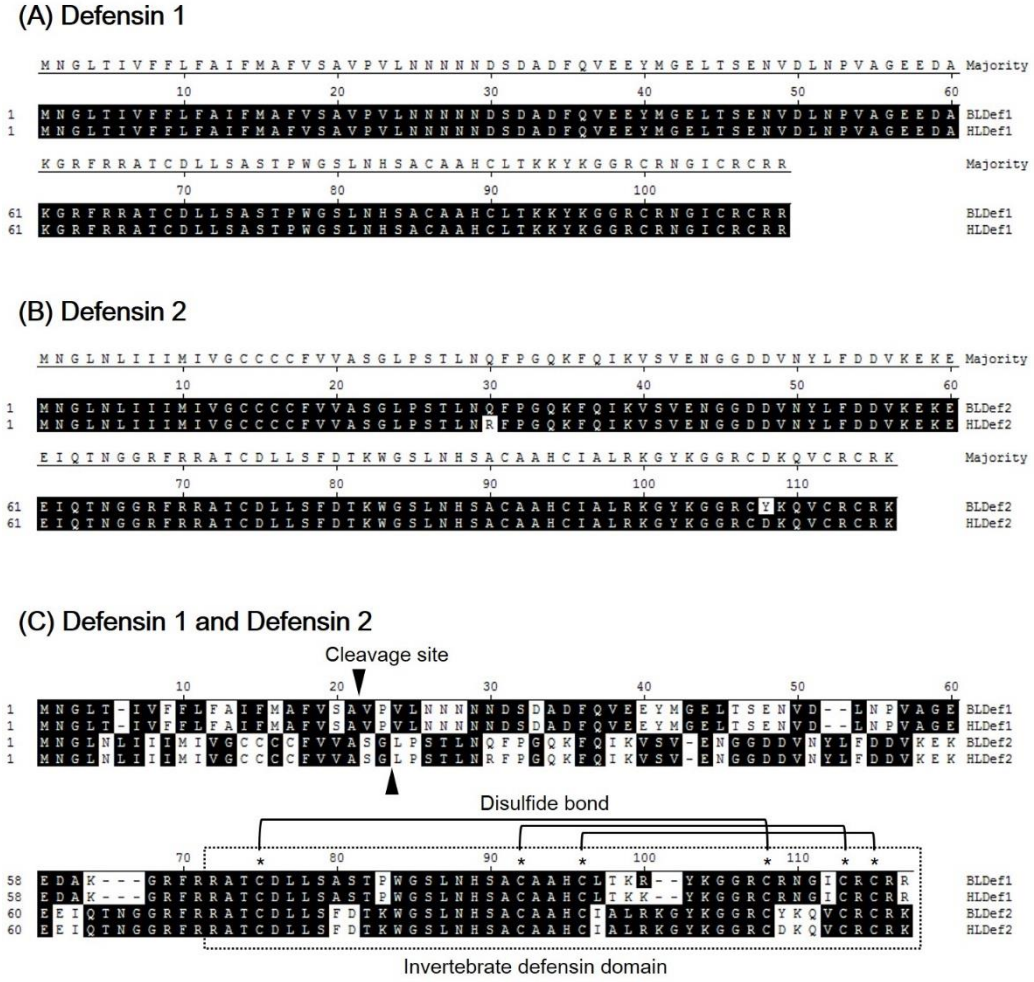


Figure 6. Comparison of deduced amino acid sequences of defensin 1 and defensin 2 from body and head lice. The dotted box (C) indicates the invertebrate defensin domain. The asterisks (*) indicate the cysteine residues that are conserved in defensins and inversed triangles (▼) show the location of signal peptide cleavage site. BL, body louse; HL, head louse.

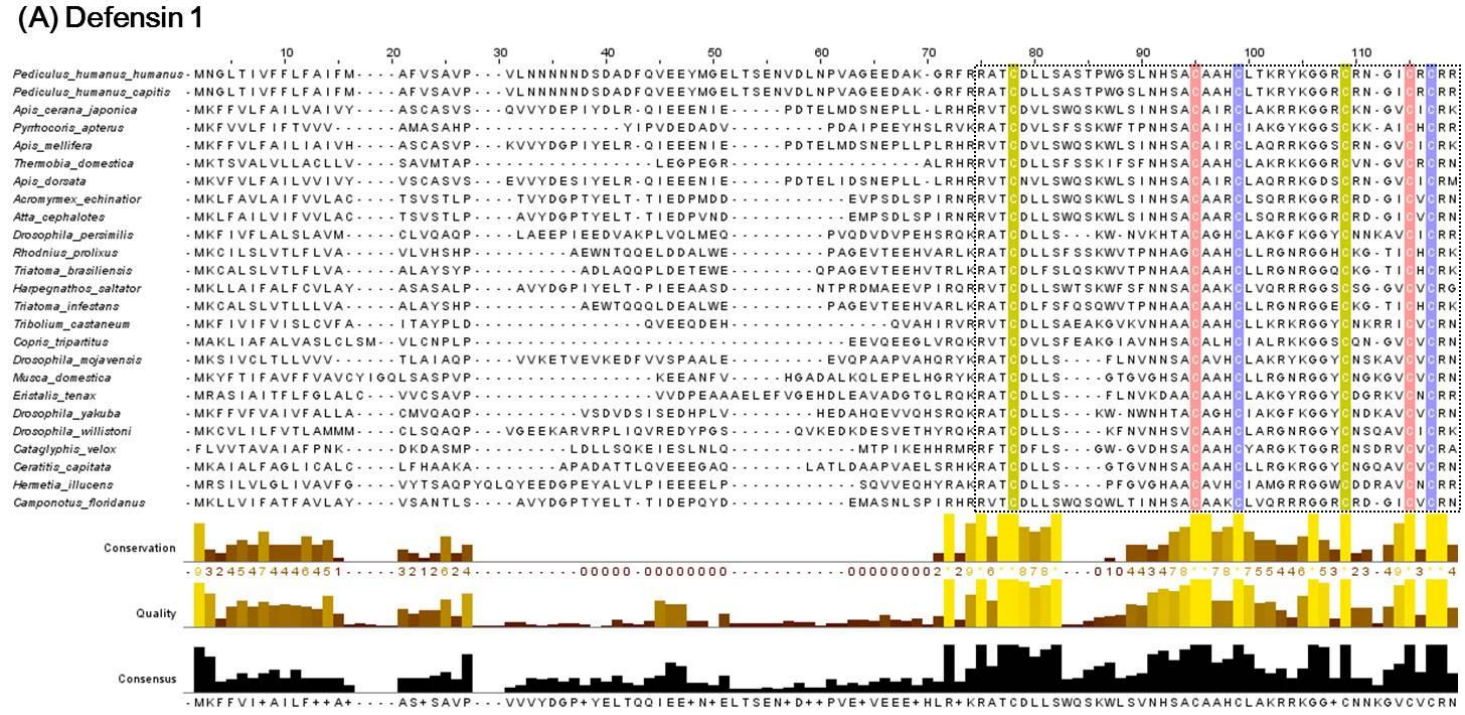


Figure 7. Deduced amino acid sequence alignment of defensin 1 from body louse (*Pediculus humanus humanus*) and head louse (*Pediculus humanus capitis*) and other insects. Defensin sequences showing higher similarity with those of louse were selected using BLAST search. Defensin-specific six cysteine residues are marked by color strips. Dotted box indicates invertebrate defensin domain.

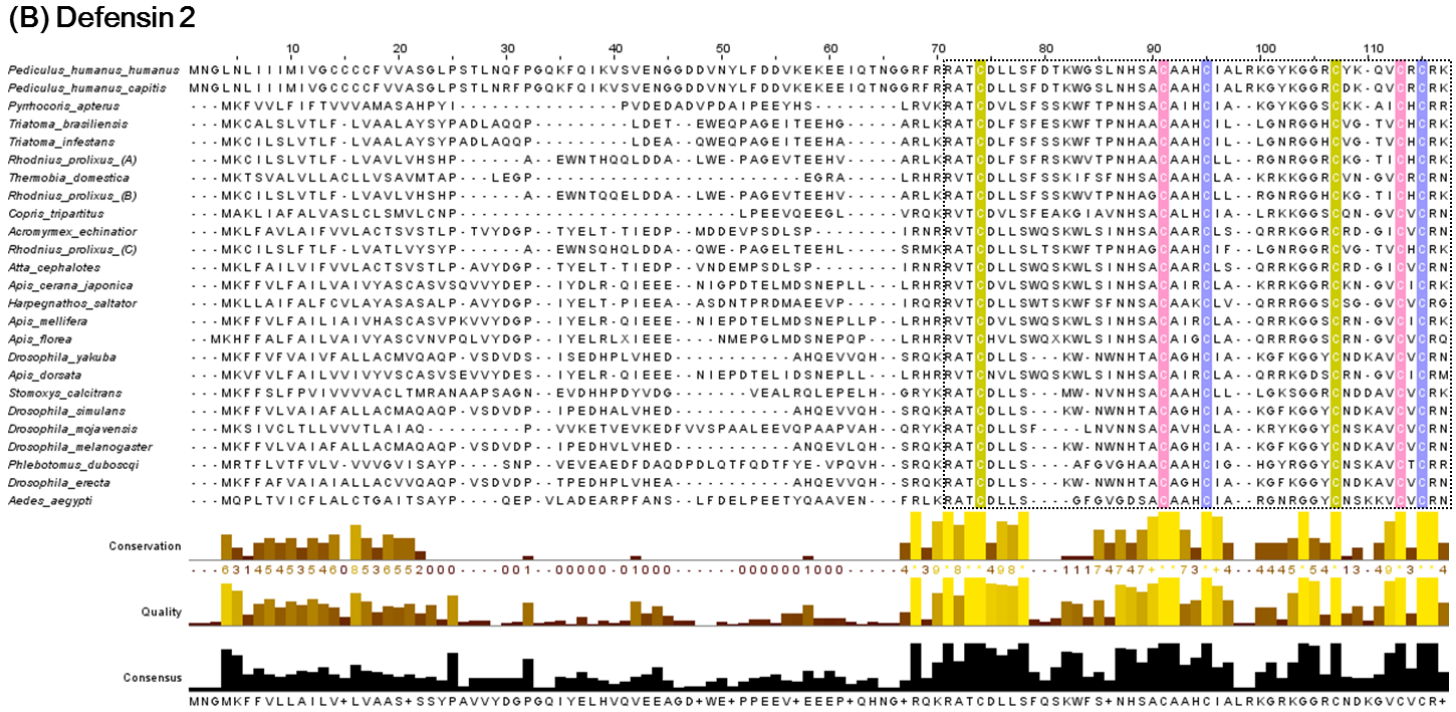


Figure 7. Deduced amino acid sequence alignment of defensin 2 from body louse (*Pediculus humanus humanus*) and head louse (*Pediculus humanus capitis*) and other insects. Defensin sequences showing higher similarity with those of louse were selected using BLAST search. Defensin-specific six cysteine residues are marked by color strips. Dotted box indicates invertebrate defensin domain.

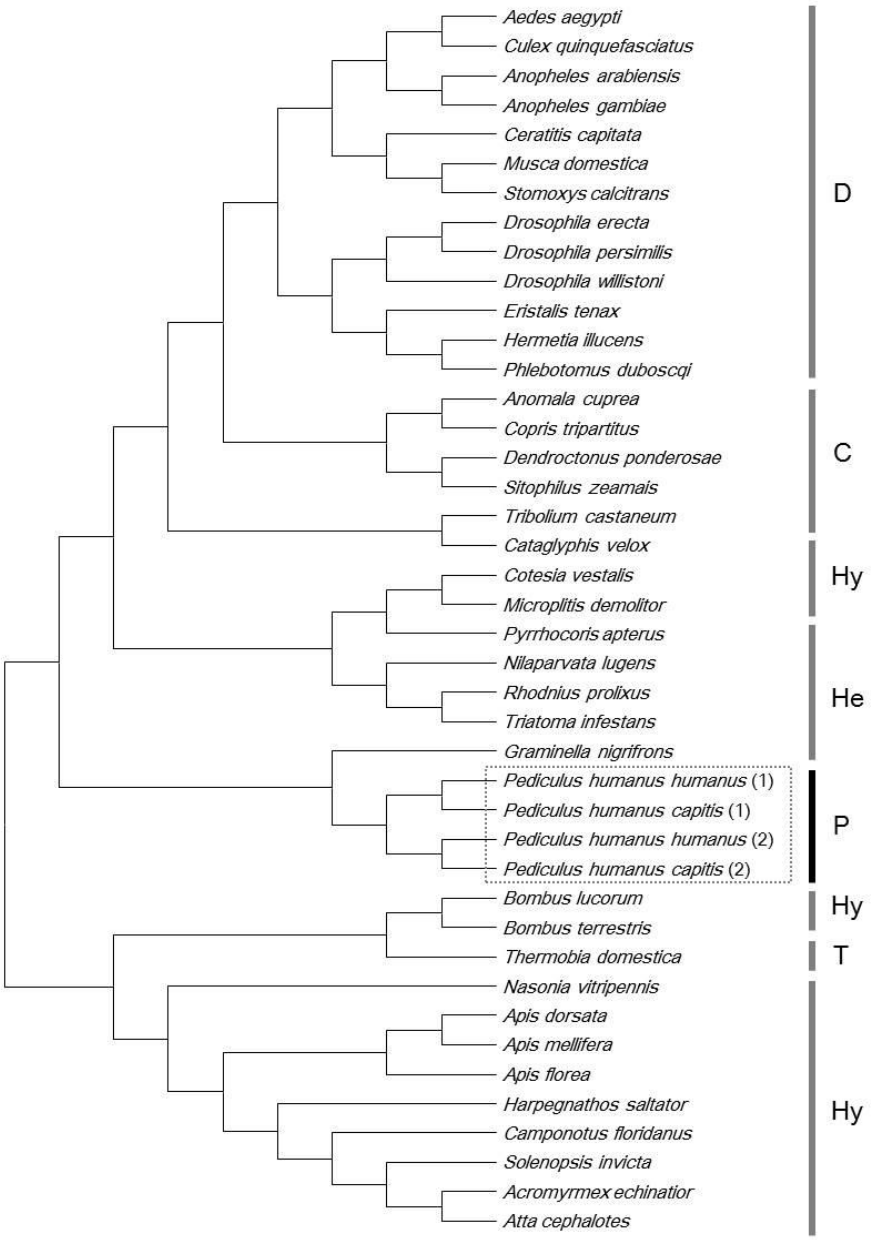


Figure 8. Phylogenetic tree of defensins from human lice (1, defensin 1 vs. 2, defensin 2) and other insects. D, Diptera; C, Coleoptera; Hy, Hymenoptera; He, Hemiptera; P, Phthiraptera; T, Thysanura.

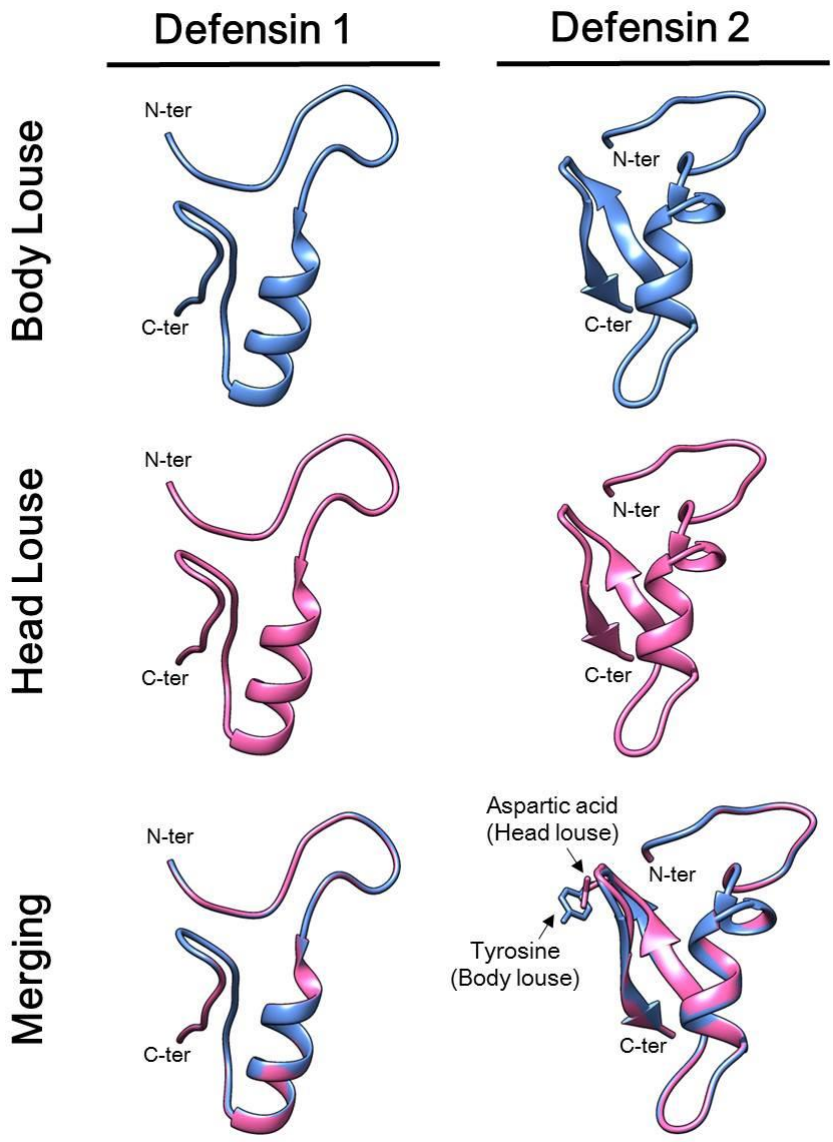


Figure 9. Three-dimensional protein structures of louse defensin 1 (left panel) and defensin 2 (right panel). Side chains of different amino acids between BLDef2 and HLDef2 were shown. N-ter, N-terminal; C-ter, C-terminal.

2.2.3. Histochemical analysis of defensin 1 and defensin 2

FISH using defensin-specific probes revealed the characteristic localization patterns of antimicrobial peptides (Fig 10). The red fluorescence signals of defensin 1 were observed in midgut of lice but not in other parts of alimentary tract including malpighian tubes. In contrast, the defensin 2 mRNA signals was exclusively observed in elongated cells of tracheoles attached on alimentary tract, but not in epithelial cells of gut tissue (data not shown). These data indicate that defensin 1 and defensin 2 are differentially expressed in different tissues. Defensin 1 appears to be responsible for the immune reaction against infected pathogen via alimentary tract, whereas defensin 2 involved in the immune protection against pathogen invading via air way.

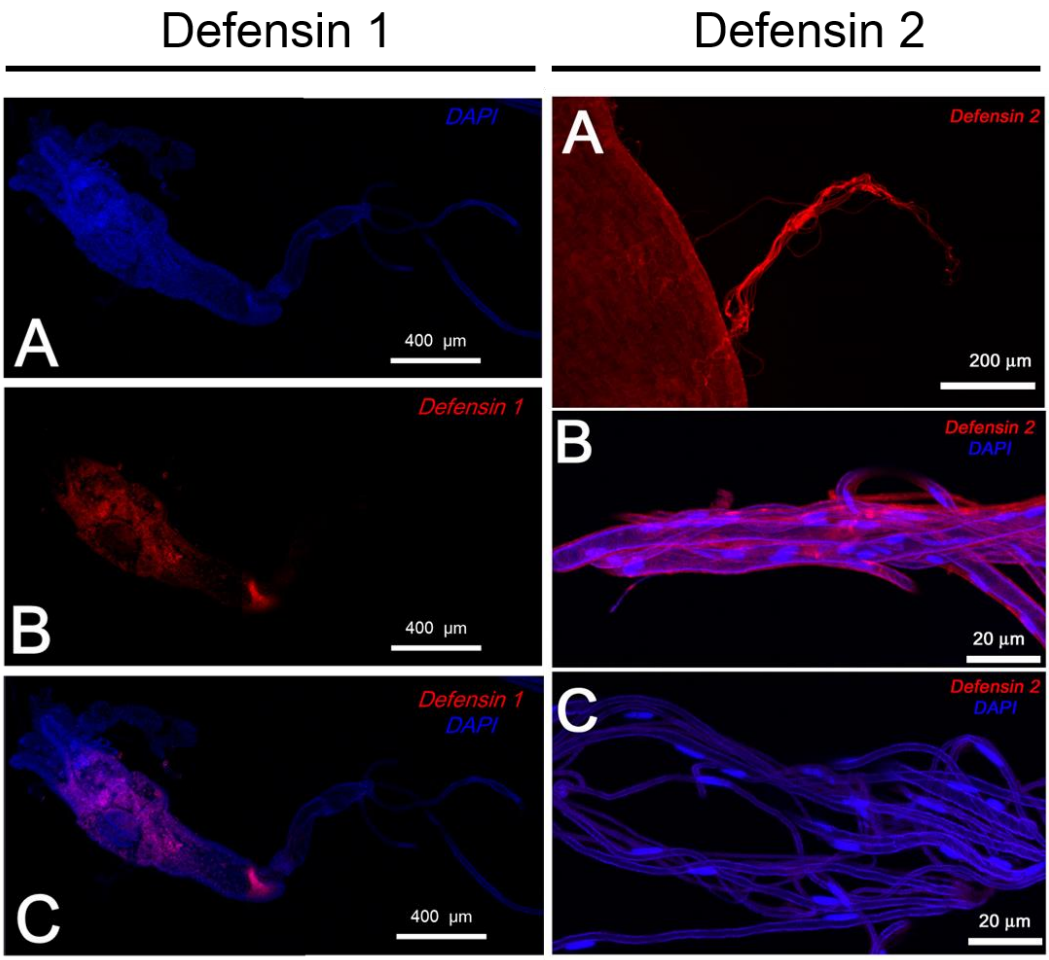


Figure 10. Expression of defensin 1 (left panel) and defensin 2 (right panel) in alimentary tract and tracheole. Red and blue colors indicate defensin 1 or defensin 2 mRNA and DAPI-stained cell nuclei, respectively. Left panel: A, DAPI-staining; B, defensin 1 expression; C, merged image. Right panel: A, tracheole attached on midgut; B, defensin 2 expression; C, control.

3. Discussion

Like other insects, human body and head lice possess common pathways for both the humoral inducible immune system (except for the Imd pathway) and the cellular immune system. The number of genes involved in humoral immune defense was particularly reduced in human lice with only one pattern recognition protein and two types of defensin found. In addition, neither BGBP nor Imd and its adapter protein, FADD, were present. The simplified immune system with a reduced number of components in body lice appears to be due to their parasitic life cycle and feeding only on relatively sterile human blood. In support of this contention, *D. melanogaster* larvae, which live in more varied and less sanitized environments, possess 16 pattern recognition proteins and 20 antimicrobial peptides for humoral defense (Lemaitre and Hoffmann, 2007). In contrast, *A. mellifera* inhabits in a more sanitized condition due to social defense strategies, including mutual grooming and organized maintenance of hive (Tarpy, 2003), and possesses a reduced number of immune-related genes, including six recognition proteins and six antimicrobial peptides, etc (Evans et al., 2006).

Human lice possess symbiotic Gram-negative bacteria in their stomach disc, *Candidatus* Riesia pediculicola that is known to be essential for the production of nutritional components (Sasaki-Fukatsu et al., 2006). Riesia is absolutely required

by the louse for the supply of pantothenic acid (vitamin B5) which cannot be obtained from human blood as only feeding source. Considering that human lice have incomplete Imd pathway that plays a key role to trigger immune reaction against Gram-negative bacteria, it seems that lice could protect their endosymbiotic bacteria from their innate immune reactions. Similarly, absence of functional Imd pathway is also reported in pea aphid (Gerardo et al., 2010), asian citrus psyllid (Reese et al., 2014), *Rodnius prolixus*, and *Ixodes scapularis* (unpublished data), which have primary endosymbiotic bacteria and transmit Gram-negative bacteria to plant or human. With this in mind, experiments that examine the relationship between the imcomplete humoral immune system lacking Imd pathway and the vector competence would provide a better understanding to study about arthropod vector physiology.

The louse PGRP is one of long-type PGRPs and has characteristics as the membrane-bound receptor like PGRP-LC of *D. melanogaster* and *A. gambiae*. In contrast, other PGRPs have a signal peptide to be secreted or do not have a signal peptide, thus being either intracellular or secreted by another mechanisms (Dziarski and Gupta, 2006). Interestingly, unlike most of PGRPs that contain only 1 PGRP domain, louse PGRPs have a unique features in that the two PGRP domains are extracellularly located via a transmembrane domain (Fig 2) and no apparent sign of alternative splicing is observed (data not shown).

In general, the specificity of the antimicrobial responses depends on the properties of individual PGRPs. *Drosophila* PGRP-LC, which has the highest sequence identity with louse PGRPs, shows specificity for meso-diaminopimelic acid (DAP)-type peptidoglycans present in Gram-positive bacilli and Gram-negative bacteria, whereas PGRP-SA is specific for Lysine (Lys)-type peptidoglycans present in most other Gram-positive bacteria (Royet and Dziarski, 2007). The Toll pathway, a main immune pathway, is triggered by PGRP-SA-Gram-negative binding protein (GNBP)-1 complex that recognizes the Lys-type peptidoglycan, whereas activation of PGRP-LC by DAP-type peptidoglycan binding triggers the IMD pathway, another main immune pathway. In addition, PGRP-LC may have a role in phagocytosis of Gram-negative but not of Gram-positive bacteria (Ramet et al., 2002). Contary to the well-known functions of PGRPs in *Drosophila*, the role of louse PGRP is unclear because the molecular components expected to interact with PGRP (i.e., Imd and FADD) are absent in lice. However, among the invertebrate PGRPs with their functions revealed, the *Ostrinia nubilalis* PGRP-B, which shows the highest sequence identity (30.5%) to louse PGRP, is up-regulated only after Gram-negative bacteria challenge (Khajuria et al., 2011), whereas the *Tribolium castaneum* PGRP-LA with the second highest sequence identity (28.9%) responds to Gram-positive bacteria. The intermediate properties along with the uniqueness of PGRP in human lice suggest

that it may function as a general receptor for both Gram-positive and -negative bacteria although it is speculative to make a prediction without experimental basis to determine the relative affinity of louse PGRP to different peptidoclycans.

Insect defensin is an antibacterial peptide with a high molecular identity and a characteristic six-cysteine/three-disulfide bridge pattern. All insect defensins have the same cysteine pairing (i.e., Cys1-Cys4, Cys2-Cys5 and Cys3-Cys6) (Froy and Gurevitz, 2003; Hetru et al., 1998), which was also observed in both louse defensin 1 and defensin 2. Although more than 170 antimicrobial peptides have been found in insects, body and head lice have only one type of antimicrobial peptide, defensin. Insect defensins are generally active against Gram-positive bacteria, but they are also effective against a few Gram-negative bacteria, fungi, yeasts and *Plasmodium* (Dimarcq et al., 1998; Hoffmann and Hetru, 1992). As is the case of PGRP, it is likely that these two louse defensins possess antimicrobial activity against a broad spectrum of pathogens in different organs (i.e. gut, fat body, trachea, hemocyte, etc.) considering that human lice have only defensins as antimicrobial peptides unlike other arthropods. Similar cases, in which defensin shows activity against Gram-negative bacteria, have been previously reported in a variety of arthropods. For example, a defensin peptide purified from hemolymph of hard tick, *Amblyomma hebraeum*, displayed antibacterial activity against both Gram-positive and -negative bacteria (Lai et al., 2004). In case of cotton leafworm,

Spodoptera littoralis, both the immunized hemolymph and the purified defensin exhibited significant activities against Gram-positive and -negative bacteria (Seufi et al., 2011). In addition, defensins found in an African malaria mosquito, *Anopheles gambiae* (Vizioli et al., 2001), an European bumblebee, *Bombus pascuorum* (Rees et al., 1997), a jewel wasp, *Nasonia vitripennis* (Ye et al., 2010) and a cattle tick, *Haemaphysalis longicornis*, (Zhou et al., 2007) also showed antibacterial activity against both Gram-negative and -positive bacteria. Experiments that examine the antimicrobial activity spectrum of louse defensins would be necessary to explain how body and head lice manage their immune system with only one type of antimicrobial peptide.

In general, insect defensins have an N-terminal loop and an α-helix followed by an antiparallel β-sheet structure connected by disulfide bonds (Cornet et al., 1995; Ganz, 2003). Louse defensin 2 showed these unique structures, but louse defensin 1 seems to have only an α-helical fragment and the three defensin-specific cysteine pairs without β-sheet (Fig 9). In addition, louse defensins showed the high pI values like other insect defensins that exert the antibacterial activity through the interaction between the positively charged peptides and the negatively charged bacterial membrane components (i.e., polysaccharide and lipopolysaccharide in Gram-positive and –negative bacteria, respectively (Bulet et al., 1999).

Understanding the immune system of a human disease transmitting vector is of great importance because it largely determines the vector competence. The immune defense cascade against bacterial pathogens, such as *B. quintana*, *R. prowazekii* and *B. recurrentis*, in body lice, however, still remains unknown. Nevertheless, the annotation of body louse genes related with the immune defense system should provide basic information for understanding molecular aspects of vector competence. Since body lice have a much reduced number of immune-related genes compared to other insects, transcriptional profiling for the representative immune-related genes can be achieved in a more efficient manner.

All of the three pathogens that body lice transmit (*B. quintana*, *R. prowazekii* and *B. recurrentis*) are Gram-negative bacteria (Lounibos, 2002; Raoult and Roux, 1999). As demonstrated in body lice, the lack of the components required for a functional Imd pathway and an impaired cellular immune reaction may result in a reduced immune defense against Gram-negative pathogenic bacteria, allowing propagation of invading pathogens and eventually rendering lice a vector for pathogen transmission to their human host. Since head lice share a great deal of genetic background with body lice but do not transmit diseases, a comparative investigation of these immune-related genes would provide invaluable insights into the differences in vector competence between these two closely-related lice species. Although a comparative study on differential vector competence in two

phylogenetically related species of mosquitoes, *Anopheles quadriannulatus* and *A. gambiae*, were conducted previously (Habtewold et al., 2008), precise cross-comparison of immune-related genetic componentswas not feasible due to their substantial genetic differences. With this in mind, body and head lice sharing almost identical genetic background should provide a much better model system to study vector competence and its evolution.

CHAPTER III

Comparison of Cellular and Humoral Immune Responses between Body and Head Lice

III-1. Comparison of Cellular and Humoral Immune Responses between Body and Head Lice Following Dermal Challenge

Abstract

The differences in the cellular and humoral immune response between body lice, *Pediculus humanus humanus*, and head lice, *Pediculus humanus capitis*, were investigated following *S. aureus* and *E. coli* challenge as model Gram-positive and -negative bacteria. Body lice exhibited a significantly lower phagocytotic activity against *E. coli* than head lice, whereas the phagocytosis against *S. aureus* differed only slightly between body and head lice. Transcriptional profiling of representative genes involved in the humoral immune response revealed that both body and head lice showed an increased immune response to *S. aureus* but little to *E. coli*. Likewise, no apparent alteration of transcription was observed in alimentary tract tissue following *E. coli* oral challenge. Nevertheless, the basal transcription levels of major immune genes were higher in body versus head lice. These findings suggest that the greater immune responses in head lice against *S. aureus* is due to humoral immune reactions and those against *E. coli* is largely due to enhanced phagocytosis and higher constitutive transcription levels of major

immune genes.

1. Materials and Methods

1.1. Lice rearing

The USDA strain of body louse (*P. h. humanus*) has been reared on live rabbits for 10-20 min per day. Lice were fed human blood for 2 h per day on the *in vitro* membrane feeding system (Yoon et al., 2006) for 3-4 days prior to all experiments to provide the same feeding conditions as used for head lice. The BR strain of head lice (*P. h. capitis*) was originally collected in Bristol, UK, and has been reared on the *in vitro* membrane feeding system. Lice colonies were maintained under conditions of 30℃, 70-80% RH and 16L:8D in a rearing chamber. 2 to 4-day-old and 5 to 7-day-old female of both body and head lice were used for transcription profiling and for *in vivo* phagocytosis assay, respectively.

1.2. In vivo *phagocytosis assay*

The *in vivo* phagocytosis assay was based on the previous reported method (Moita et al., 2005) with modifications. Stocks of heat-killed and fluorescein isothiocyanate (FITC)-labeled (Alexa Fluor 488) *S. aureus* and *E. coli* (Invitrogen, Carlsbad, CA, USA) were suspended in 1 ml PBS (pH 7.4) and sonicated (Branson Sonifier 450, Branson Ultrasonics, Danbury, CT, USA) to obtain a

homogenous suspension. Live lice were secured ventral side up with double-sided sticky tape to the top surface of a glass microscope slide and kept on ice to suppress phagocytosis. Homogenously suspended FITC-labeled *S. aureus* or *E. coli* (46 or 23 nl for body or head lice, respectively) were injected into the ventral abdomen (posterior 2nd segment) of a louse using a nano injector. The bacteria-injected lice were held at room temperature for 60, 120 and 240 min to allow phagocytosis to resume, and then placed again on ice. Lice were then injected with a 5-fold, PBS-diluted, trypan blue solution (Invitrogen/GIBCO, 92 nl or 46 nl for body or head lice, respectively) to quench the fluorescence from non-phagocytosed bacteria (Elrod-Erickson et al., 2000). Immediately after the injection of trypan blue, a digital fluorescence image (10x magnification) was taken and stored using a phase-contrast microscope equipped with FITC filter (Invitrogen, Carlsbad, CA, USA). Fluorescence images taken from lice simultaneously injected with bacteria and trypan blue were used as background images to estimate the net fluorescence intensity. Each phagocytosis analysis was repeated six times.

The fluorescence images of the lateral abdominal region (96 x 145 and 70 x 112 pixels for body and head lice, respectively) were cropped from the original picture of a single louse to remove the background fluorescence from other parts of body using Adobe Photoshop (Adobe, San Jose, CA, USA). Fluorescence

intensity of the cropped images was measured using ImageJ program (NIH, Image Processing and Analysis in Java, http://rsb.info.nih.gov/ij/). TIFF files were transformed into 8 bit color type and fluorescence intensity was determined by adjustment of the threshold. Total intensity was derived by multiplying the Area-value with the Mean-value, and the phagocytosis index was estimated with following equation: Phagocytosis index = (Ft – Fc)/Fc, where Ft is Fluorescence at post-injection time and Fc is Fluorescence immediately following injection)

1.3. Transcription profiling of louse immune-related genes in whole body following dermal challenge

The tip of a fine needle (Shiga insect pin 100 white, Nissei Trading Co. Ltd., Tokyo, Japan) was dipped into an aliquot (100 µl) of PBS-diluted bacteria culture (*S. aureus* or *E. coli*) and shaken gently 3-4 times. The immune response experiments were conducted by ventrally piercing the 2nd abdominal segment of a female louse with the bacteria-contaminated needle. The inoculated lice were maintained at 30℃ and 70-80% RH for 8 h without feeding. The bacterial challenge experiments were performed with 3-6 biological replicates (20 lice per replicate).

Following bacterial challenge, total RNA was extracted from 12 live lice per replicate using TRI reagent (MRC, Cincinnati, OH, USA) according to the

manufacture's protocol. Total RNA samples were treated with DNaseI (TAKARA biotechnology, Japan) to remove contaminated genomic DNA. cDNA was synthesized from total RNA (2.5 μg) using the Superscript III reverse transcriptase (Invitrogen, Clasbad, CA) and diluted to 5 ng/ml to use as templates for the qPCR.

Among the 93 immune-related genes, genes responsible for pathogen recognition and downstream signal cascade, particularly in Toll pathway, and genes involved in direct killing of invaded pathogens were selected as representative marker genes to investigate the humoral and cellular immune responses following bacterial challenge. Primers of Elongation factor 1 α (EF1α) as a reference gene and 19 immune-related genes [PGRP, thioester containing protein (TEP) 1-3, hemocytin, scavenger receptor CI (SR-CI), Spätzle 1-2, Toll-1-6, defensin 1-2, dual oxidase (Duox), prophenoloxidase (PPO) and noduler] were designed to have similar properties in terms of length and GC content (Table 1). Priming site sequences in both lice species were confirmed to be identical by sequencing. The qPCR reaction mixture (20 μl) contained 5 pmol gene-specific primers, 0.5 U 2X DyNAmo HS SYBR Green master mix (Finnzyme, Espoo, Finland), and 5 μl of cDNA template. qPCR was performed using the Opticon 3 thermal cycler (MJ Research, Waltham, MA) with following program: 95℃ for 15 min, 35 cycles of 95℃ for 10 s, 58℃ for 20 s, 72℃ for 20 s and serial

increase per 0.2℃ for 1 s from 45℃ to 95℃ for melting curve analysis. Quantification of transcript level or relative copy number of a gene was conducted using the relative quantification method based on the original concept of $2^{-\Delta Ct}$ methods (Pfaffl, 2001).

Significant differences of transcription level between body and head lice were tested using Student's *t* test. The level for accepted statistical significance was $p < 0.05$.

Table 1. Sequences of the primers used for qPCR of immune-realted genes

Gene		Sequence (5'→3')	Product size (bp)
EF1α	F[a]	CAAGAAGCTGTGCCAGGAGA	142
	R[b]	GCACAATGACCTGAGCAAGG	
PGRP	F	GTCACCATTGGAAGATGTCG	138
	R	GAATTCGATGGAGTCGCGTG	
TEP1	F	CTACGACTCAAGCAGAAGAGC	142
	R	AACGGTTGGACTCGCTCTTTG	
TEP2	F	TTTCCAAAGCCGTGTCCTGG	136
	R	GTGATATTCCGGGAACCCAG	
TEP3	F	GGAAGCTTTATCCGAACCGAA	138
	R	TTTGGTCGTTACGTTAGCCTG	
Hemocytin	F	GGAAGGTCTTTGCGGCACTT	129
	R	GATGTTGGCGCACATGGAAT	
SR-CI	F	GATGCACGATTTCGATTGGAG	138
	R	AAACGGGGCTGTAAAGTCTTG	
Spätzle 1	F	TGAAATCGTCGGCGTACCTG	129
	R	CGTACGTCATCATTTCCCTCTTGA	
Spätzle 2	F	TCATCCTCCACCACCACCAC	135
	R	TCGCAAGCATCTACTCTTCCGT	
Toll-1	F	CACGGTTACGTGTCGGGAAA	139
	R	TCTGAGTCCGTCGAAACCGA	
Toll-2	F	AATACCCACGGGAGCATTGG	104
	R	TCGGACTGAATATGTCGGTTTC	
Toll-3	F	GTCGTGGTCGATGGAAATTCG	138
	R	CGAACGCTGCTCTCTTTATCCG	
Toll-4	F	CAACGACCTCACACAACTCATAGTT	108
	R	CCAAAACGACGAGGTGAAGC	

Table 1. Sequences of the primers used for qPCR of immune-realted genes (Continued).

Gene		Sequence (5'→3')	Product size (bp)
Toll-5	F	TGCACTTGTGCCAAAGGGAT	130
	R	CGCGTACGTGCATTGGATTT	
Toll-6	F	GTACCCACCGGAGGCATAGC	107
	R	CGACGCAAAGCACTGAAACC	
Defensin 1	F	GGGAGAACTTACCTCGGAAA	142
	R	AGCGGCACAAGCAGAATGAT	
Defensin 2	F	TGGAGGAAGATTCAGGAGAGC	127
	R	GCAACGTCCACCTTTGTAACC	
Duox	F	CGAAGATGAAGGTGAAGGAGG	144
	R	CCGTCGCCATCTTTATCGAC	
PPO	F	TTACGCATTATCGGTCGCCA	144
	R	CTCGGATCTAAAACGCTCGTG	
Noduler	F	GGAGATGTAGCCGTTGGTAA	149
	R	TCTGATAGACCTTCAGGTGC	

[a] Forward primer.
[b] Reverse primer.

2. Results

2.1. Comparison of phagocytic activity between body and head lice

Typical fluorescence images of body and head lice injected with FITC-labeled *S. aureus* (Fig. 1A) or *E. coli* (Fig. 1B) are given over time. Individual phagocytes engulfing injected bacteria particle (thereby emitting fluorescence) were first observed in head louse from 10 min post-injection onward with immobilized phagocyte clusters appearing in the lateral sides of the abdomen from 60 min post-injection onward (see yellow arrows). Although free phagocytes engulfing bacteria in hemocoel were also detected (see the red arrow), the time course of phagocytosis was determined by measuring the fluorescence images of the lateral abdominal region, where most phagocyte clusters were located since overall fluorescence generated from the fast circulating phagocytes at the initial stage of phagocytosis was weak and difficult to capture (Fig. 1).

Following FITC-labeled *S. aureus* injection, body lice showed a significant delay (120 min) in phagocytotic activity compared to head louse (Fig. 1A). Although maximum activity may not have been achieved in the current study, phagocytotic activity measured at 240 min post-injection in body louse was 2.1-fold lower than in head louse, but this trend was not statistically significant ($p=0.09$). Head lice also exhibited a more rapid onset of phagocytosis that reached

a higher level than in body lice.

Injection of FITC-labeled *E. coli* also resulted in a different time course of phagocytosis between body and head lice (Fig. 1B). In body lice, the onset of phagocytosiswas delayed compared with head lice. Although the maximum level of phagocytosis may not have been achieved, the phagocytotic activity at 240 min post-injection was 2.3-fold lower in body than head louse. The differences inphagocytosis between body and head lice were more apparent during the early immune response where the phagocytosis index value of body louse was 8.4-fold lower ($t=9.0$; d.f., 8 ; $p<0.001$) than that of head lice at 120 min post-injection. As judged also by the phagocytosis index, overall phagocytotic activity against *E. coli* was approximately 6-fold higher than that against *S. aureus* in both louse species.

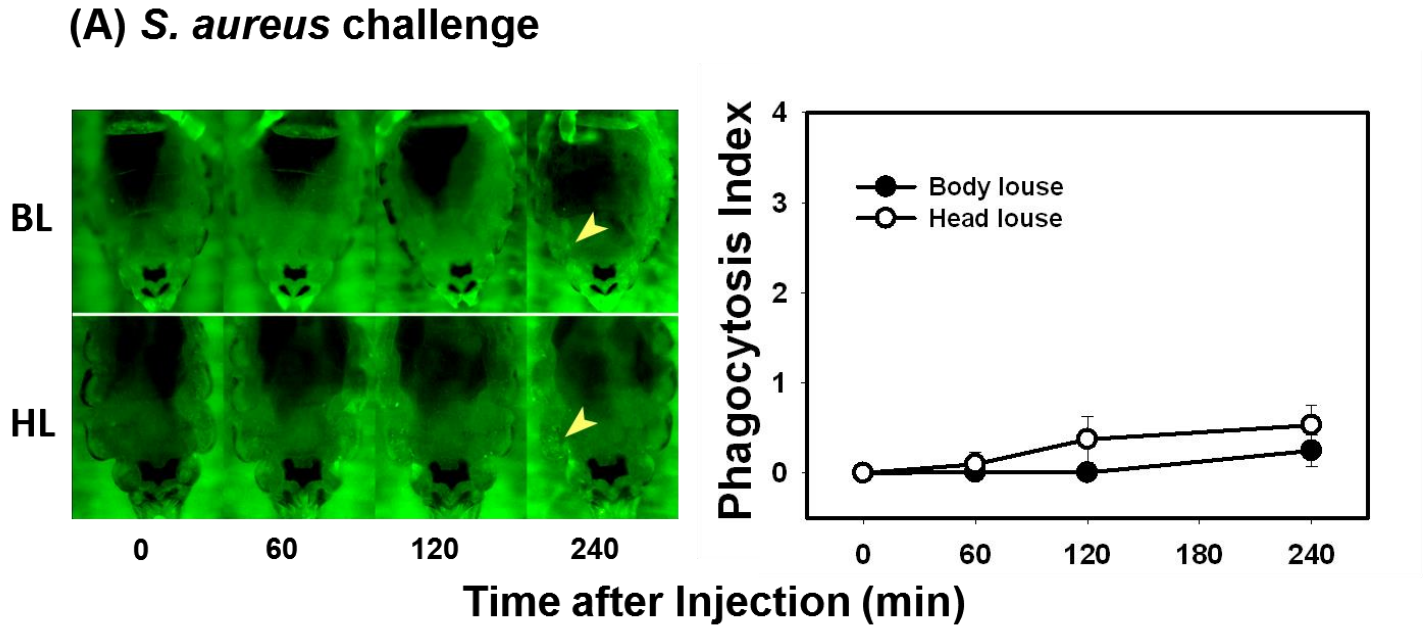


Figure 1. Representative fluorescence microscopic images of abdominal region of the lice injected with FITC-labeled *Staphylococcus aureus* (A) or *Escherichia coli* (B) and the time course of phagocytosis as determined by phagocytosis index. The fluorescence images were obtained from a single louse in a time series. Yellow and red arrows in the left panels indicate the typical phagocyte clusters immobilized in the lateral region of abdomen and free phagocyte engulfing bacteria in hemocoel, respectively. The size of body louse images was reduced 1.5-fold to make it similar to that of head lice. BL, body louse; HL, head louse. Error bars indicate standard deviation of the mean.

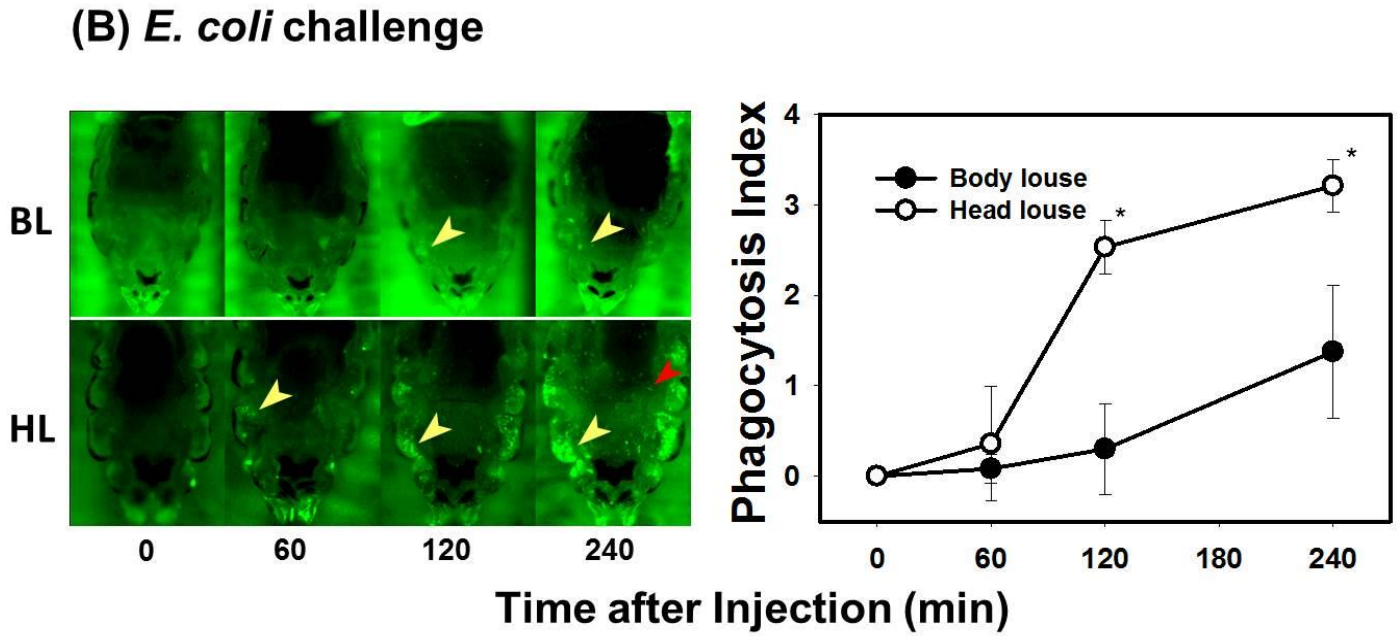


Figure 1. Representative fluorescence microscopic images of abdominal region of the lice injected with FITC-labeled *Staphylococcus aureus* (A) or *Escherichia coli* (B) and the time course of phagocytosis as determined by phagocytosis index. The fluorescence images were obtaine from a single louse in a time series. Yellow and red arrows in the left panels indicate the typical phagocyte clusters immobilized in the lateral region of abdomen and free phagocyte engulfing bacteria in hemocoel, respectively. The size of body louse images was reduced 1.5-fold to make it similar to that of head lice. BL, body louse; HL, head louse. Error bars indicate standard deviation of the mean. (continued)

2.2. Transcriptional profiling of immune-related genes in whole body

To determine the humoral immune response differences between body and head lice, transcription levels of humoral immune-related genes in females were evaluated following bacterial challenge. A total of 19 representative genes, which included members in each immune response, were selected (PGRP, TEP1-3, hemocytin, SR-CI, Spätzle 1-2, Toll-1-6, defensin 1-2, Duox, PPO and noduler). All of the head louse orthologs corresponding the 19 representative genes were cloned and their sequences verified. Sequence comparison revealed no sequence differences in the priming regions for qPCR between body and head lice (data not shown).

Among the 19 representative genes, only PGRP, defensin 1 and defensin 2 were over-transcribed in the females of both body and head lice following *S. aureus* challenge (Fig. 2A). The transcriptional profiles of the other genes were not significantly altered. The relative transcription level of PGRP in infected head lice was 1.5-fold higher when compared to infected body lice ($t=5.8$; df, 4; $p=0.004$). The transcript level of defensin1 in both infected body and head lice was similar (1.5-fold for head and 1.6-fold for body lice). The transcript level of defensin 2, however, was 11.1-fold higher in challenged head lice when compared to unchallenged control head lice. The transcript level of defensin 2 was lso increased in challenged body lice, but was significantly less (3-fold) when

compared to challenged head louse. The transcript levels of most of other immune-related components in the Toll pathway, such as Spätzle and Toll, and for the hemocyte aggregation (hemocytin) (Kotani et al., 1995) and nodule formation (noduler) (Gandhe et al., 2007) were virtually unchanged in both challenged body and head lice. Interestingly, the transcript levels were 2.3-2.8 and 1.3-3.2-fold decreased in the cases of Toll-2 and PPO in both challenged louse species, respectively.

The transcriptional profiles of the 19 representative immune-related genes in female body and head lice following *E. coli* challenge are shown in Fig. 2B. Unlike the case of *S. aureus* challenge, none of the genes were significantly over-transcribed following *E. coli* challenge in either body or head louse. Several genes, including defensin 1 and PPO, actually had transcript levels that were significantly less than their respective unchallenged controls ($p=0.002$-0.059). Although not significantly different, Toll-2 also showed reduced transcript levels ($p>0.05$). PGRP, the recognition gene for humoral immune response, showed a relative transcript level of 0.9-fold in both challenged body and head lice ($p=0.051$-0.059). The transcript levels of intermolecules in the Toll pathway likewise were not increased as seen in *S. aureus* challenge. The transcript level of Defensin 1 was decreased in both louse species. The transcript level of defensin 2 was decreased in head louse but was increased 1.47-fold in body lice ($p=0.048$).

The basal transcription level of 19 major immune genes from non-challenged lice was also compared (Fig 2C). Among the genes, the transcription levels of only 5 genes were significantly different between non-challenged body and head lice. PGRP as the pathogen recognition protein that triggers Toll pathway and Duox related to ROS reaction were expressed more in head lice (1.5-fold; $t=-4.1$; d.f., 10; $p=0.002$ and 2.9-fold; $t=-8.9$; d.f., 4; $p<0.001$). Interestingly, the basal transcription levels of opsonization and recognition genes related to phagocytosis, such as TEP1 (1.9-fold; $t=-8.9$; d.f., 10; $p<0.001$) TEP2 (1.7-fold; $t=-2.6$; d.f., 9; $p=0.03$) and SR-CI (2.0-fold; $t=-5.8$; d.f., 10; $p<0.001$), were also higher in head lice.

In summary, enhanced humoral immune responses were induced in both body and head lice following *S. aureus* challenge. However, the similar induced immune responses were not observed following *E. coli* challenge. In cross-comparison between body and head lice, higher basal transcription levels of several immune genes were observed in head lice.

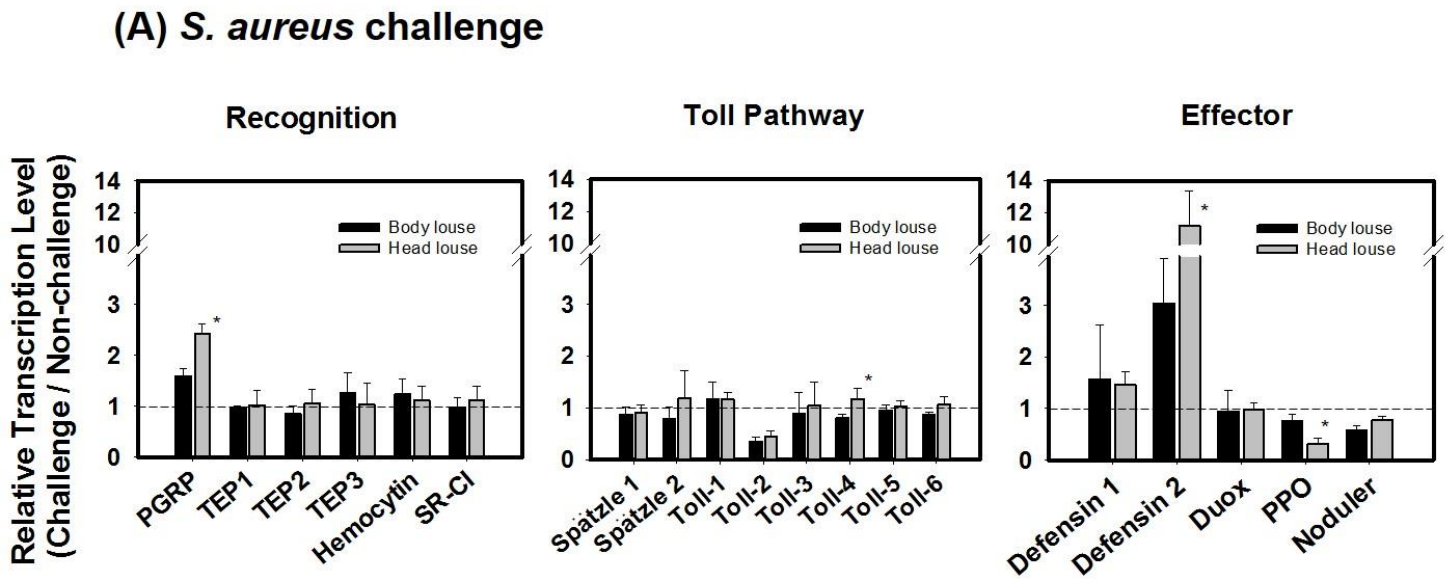
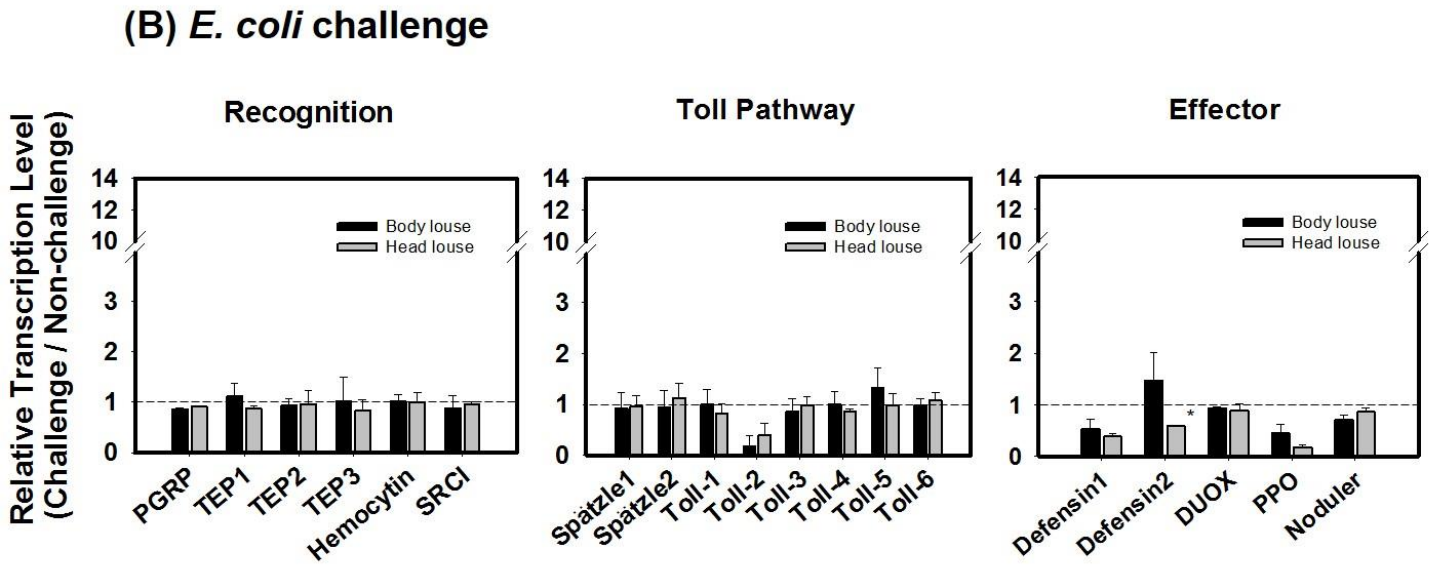


Figure 2. Comparison of the relative transcription level of 19 representative immune-related genes following challenges by *Staphylococcus aureus* (A) and *Escherichia coli* (B) or basal transcription level (C) in body and head lice. Bars with star mark (*) indicate statistically significant difference between body and head lice ($p<0.05$). Error bars indicate standard deviation of the mean. The dotted line (value=1) corresponds to an identical relative transcription level between control and challenged lice.



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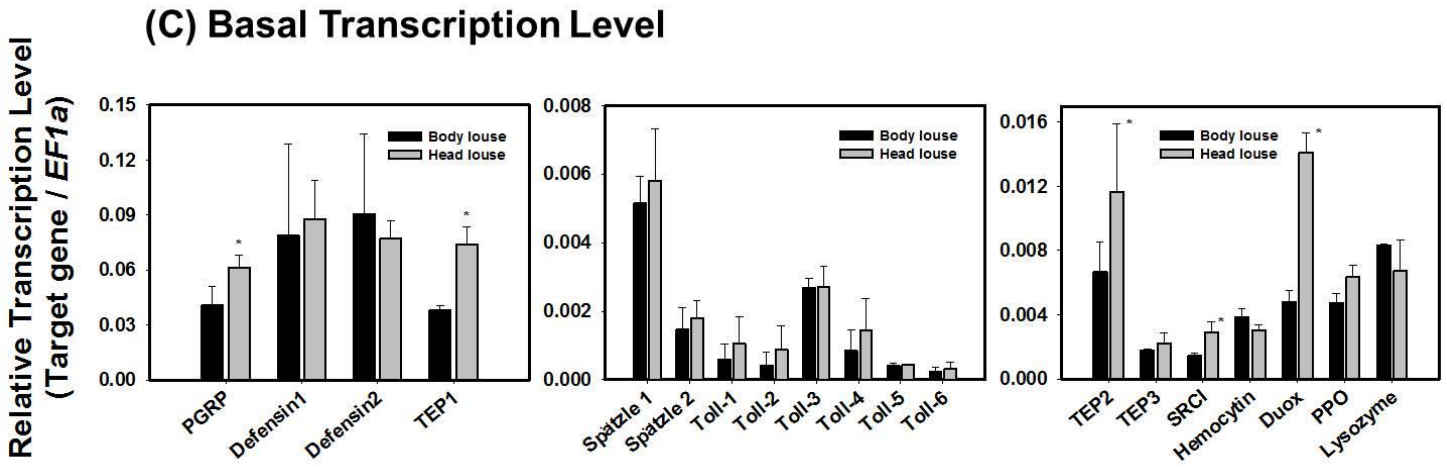

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3. Discussion

3.1. Differences in cellural immune responses

Body lice elicited a significantly delayed and lower level of phagocytosis to both *S. aureus* and *E. coli*. Similar to this finding, a delay of phagocytotic activity in body lice during endosymbiont migration has also been reported (Perotti et al., 2007). The rapid proliferation of *E. coli* inside body lice without any suppression (Chapter I, Fig. 1B) is likely attributed to the delayed and lower cellular immune response and to the lack of the Imd pathway (Chapter II). Similarly, the rapid onset of the proliferation peak of *S. aureus* in body lice seen at 120 min post-injection (Chapter I, Fig. 1A) can be explained by the delayed cellular immune response, whereas the suppression of *S. aureus* proliferation afterward is likely due to the initiation of the humoral immune response, particularly the functional Toll pathway. In contrast, through a rapidly responding and strong cellular immune defense against *E. coli*, head lice appears to suppress the initial proliferation of *E. coli* completely (Chapter I, Fig. 1B), thereby not allowing the subsequent proliferation despite the lack of the Imd pathway. Head lice also appear to suppress *S. aureus* proliferation efficiently by having both an increased cellular response and a functional humoral immune system.

It remains to be elucidated what factors are involved in the delayed and lower

level of phagocytotic activity seen in body louse. Since preliminary examination showed that the phagocyte number in unit volume of hemolymph is not different between body and head lice (data not shown), the differential phagocytotic activity is more likely associated with certain factors inside the pathways. Complete analysis of the components in the phagocytosis pathways from pathogen recognition to signal transduction would be required to elucidate the factors responsible for the differences between body and head lice. The absence of some Imd pathway components may contribute, at least in part, to the delayed and diminished phagocytosis response in body lice. Considering that TEP covalently binds to the surface of invading pathogens, activates immune responses such as phagocytosis, lysis and melanisation and is required for promotion of phagocytosis of both Gram-positive and –negative bacteria (Blandin and Levashina, 2004), the higher basal transcription levels of TEP1 and TEP2 likely play a role in rapid cellular immune reaction of head lice. Similary, the transcription level of SR-CI, which binds to both Gram-positive and –negative bacteria and is required for optimal phagocytosis (Ramet et al., 2001), is higher in head lice. Taken together, it appears that up-regulated expression of recognition genes related to phagocytosis could explain, at least in part, the enhanced phagocytic activity observed in head lice. Some differences in phagocytosis regulating factors, including eicosanoid pathway (Stanley et al., 2009), may also

be involved in the differential phagocytic activity between body and head lice.

3.2. Differences in humoral immune responses in whole body

Following *S. aureus* challenge, both body and head louse females showed activation of the Toll pathway as judged by the up-regulation of PGRP and defensin 2. In general, both body and head lice exhibited similar patterns of immune gene induction following *S. aureus* challenge, suggesting that the humoral immune system, particularly the Toll pathway, is virtually identical in both body and head lice. Along with the fact that entire Toll pathway is well conserved in body lice (Chapter II), these findings suggest that both body and head lice have an efficient humoral immune system against *S. aureus*.

Following *E. coli* challenge, transcript levels of representative humoral immune-related genes (defensin 1 and defensin 2) did not increase significantly in either body or head lice. It can be speculated that this lack of immune response is due to the non-functional Imd pathway with missing components, such as Imd and FADD. The Imd pathway is activated during Gram-negative bacterial challenge and its signal cascade begins from the recognition of bacteria by receptor like PGRP-LC (Ferrandon et al., 2007). In body lice, since only one type of PGRP was found, it may be involved in recognizing both Gram-positive and -negative bacteria as described in Chapter II. Based on the qPCR results, transcript level of

PGRP increased following the challenge with either *S. aureus* or *E. coli* but the transcription of effector genes, such as defensin 1 or defensin 2, were not induced. This finding implies that signal relay is likely blocked by the absence of downstream signaling molecules, such as Imd, in the pathway. Taken together, it appears that both body and head lice have a less efficient humoral immune response against *E. coli*, as compared to *S. aureus*. In contrast, since no significant differences in the humoral immune responses to *E. coli* following challenge were found between two louse species, such a deficient humoral immune response mediated via Imd pathway to *E. coli* is not likely responsible for the differential vector competence between two louse species. However, instead of inducible humoral immune reactions, the higher basal transcription level of major immune components could contribute to the vector competence, in part.

III-2. Comparison of Humoral Immune Responses in Alimentary Tract Tissue between Body and Head Lice Following Oral Challenge

Abstract

The epithelial cell-specific immune responses in the alimentary tract tissues were compared between body and head lice following bacteria oral challenge. Interestingly, the basal transcription levels of PGRP and defensins, which are the sole components of recognition and effector in the humoral immune response, respectively, were lower in body lice than in head lice. Defensin 1 was up-regulated by *B. quintana* oral challenge in head lice but not in body lice, whereas no differenece was observed by *E. coli* oral challenge. Thus, such non-inducible immune genes, along with the lower basal transcription levels of PGRP and defensins in body lice, were primarily responsible for the reduced immune response of body lice to Gram-negative bacteria including *B. quintana*. In addition, the level of cytotoxic reactive oxygen species (ROS) generated by epithelial cells, especially the hydroxyl radical and superoxide, was significantly lower in body lice than in head lice regardless of blood feeding although there was no difference

in the transcription level of Duox. These results suggest that both the lower basal transcription level of immune-related genes, which is not inducible by *B. quintana*, in conjuction with the lower amount of ROS in the alimentary tract of body lice reduce their immune responses, thereby allowing invading *B. quintana* to proliferate and excreting more viable bacteria in feces, which result in their higher vector competence compared to head lice.

1. Materials and Methods

1.1. Lice rearing

The USDA and SF (San Francisco) strains of body lice and the BR strain of head lice have been reared using *in vitro* rearing system (Yoon et al., 2006) under the environmental conditions of 30℃, 70% RH and 16L:8D in rearing chambers. Females (2- to 4-day old) of both body and head lice were used for every experiment.

1.2. Transcription profiling of louse immune-related genes in alimentary tract tissue following **E. coli** *oral challenge*

Body and head louse females were orally infected with *E. coli* as described in Chapter I. Briefly, *E. coli* was cultured in Luria broth at 37℃ until an optical density of 1.0 was obtained. The bacteria culture (2 ml) was centrifuged and the pellet was resuspended in 100 μl PBS. The bacterial suspension was mixed with 2 ml human whole blood for oral infection. Female body and head lice were starved for 9 h and fed with the *E. coli*-inoculated blood using the artificial membrane-based rearing units for ca. 30 min to ensure sufficient feeding. Infected and uninfected control lice were maintained at 30℃ and 70% RH for 8 h without feeding.

Louse alimentary tract tissue was dissected out in ice-cold PBS and immediately placed in RNAlater solution (Ambion, Austin, TX) to fix the tissue and maintain the quality of RNA. Following dissection of all lice, RNAlater solution was removed carefully by pipetting and total RNA was extracted from the dissected tissue using TRI reagent (MRC, Cincinnati, OH, USA) according to the manufacture's protocol. cDNA was synthesized from DNaseI (TAKARA biotechnology, Japan)-treated total RNA using the Superscript III reverse transcriptase system (Invitrogen, Carlsbad, CA) and diluted to 5 ng/μl.

The relative transcript levels of 6 representative immune-related genes (PGRP, defensin 1, defensin 2, Duox, lysozyme and PPO) involved in epithelial immunity were determined using qCR with primers listed in Table 1. The qPCR reaction mixtures (20 μl) contained 10 μl 2X DyNAmo HS SYBR Green master mix (Finnzyme, Espoo, Finland), 5 μl of cDNA template and 1 μl each of 5 pmol primers. The reactions were carried out with Opticon 3 thermal cycler (MJ Research, Waltham, MA) under the following thermal conditions: 95℃ for 15 min, 35 cycles of 95℃ for 10 s, 58℃ for 20 s, 72℃ for 20 s. The specificity of qPCR was confirmed by melting curve analysis. All experiments were repeated independently 5-6 times, each with three biological replicates.

To search for an optimum reference gene for qPCR that shows the same transcription level in both body and head lice, the transcription levels of 7

housekeeping genes, such as EF1α, glutathione-S-transferase D, Ras-associated protein 1, Na+/K+ ATPase, succinate dehydrogenase, ribosomal protein L13A (RpL13A) and glyceraldehydes-3-phosphate dehydrogenase, were compared between body and head lice. Based on this preliminary result, RpL13A gene that exhibited an almost the same transcription level was chosen as the reference gene in order to normalize the transcript levels of immune-related genes. qPCR was repeated 3-6 times with independently extracted total RNA and each reaction was conducted with two technical replicates to adjust intra-PCR variation.

Significant differences of transcription level between body and head lice were tested using Student's *t* test. The level for accepted statistical significance was $p < 0.05$.

Table 1. Sequences of the primers used for qPCR of immune-realted genes

Gene		Sequence (5'→3')	Product size (bp)
RpL13A	F[a]	GTTAGGGGAATGCTTCCACAC	142
	R[b]	GGTCTAAGGCAGAGAACGCT	
PGRP	F	GTCACCATTGGAAGATGTCG	138
	R	GAATTCGATGGAGTCGCGTG	
Defensin 1	F	GGGAGAACTTACCTCGGAAA	142
	R	AGCGGCACAAGCAGAATGAT	
Defensin 2	F	TGGAGGAAGATTCAGGAGAGC	127
	R	GCAACGTCCACCTTTGTAACC	
Duox	F	CGAAGATGAAGGTGAAGGAGG	144
	R	CCGTCGCCATCTTTATCGAC	
Lysozyme	F	CTGGTGATGGTTTCAACGCG	137
	R	GGCGACGAAGAAGTTACCGT	
PPO	F	TTACGCATTATCGGTCGCCA	144
	R	CTCGGATCTAAAACGCTCGTG	

[a] Forward primer.
[b] Reverse primer.

1.3. Transcription profiling of louse immune-related genes in alimentary tract tissue following B. quintana *oral challenge*

A wild-type *B. quintana* strain was maintained in a biosafety level 2 facility at the University of Massachusetts-Amherst and Seoul National University. Frozen *B. quintana* stock was cultured on chocolate agar plates following Koehler's

protocols (37 °C, culture plates in candle extinction jars) for 10 days (Zhang et al., 2004). *B. quintana* was then passed to a fresh chocolate agar plate using sterile loops and cultured for an additional 5-7 days before use. *B. quintana* for experiments were harvested from a chocolate agar plate by rinsing the surface of plate with 1 ml of PBS (pH 7.4). The harvested *B. quintana* was washed twice by centrifugation at 1,000 g for 4 min and resuspended in 100 μl of fresh PBS to remove residual media. Spectrophotometric readings (OD_{600}) were taken for *B. quintana* suspended in PBS to approximate cell counts per ml of blood. The *B. quintana* bacterial suspension (5 μl) was serially diluted and then plated on chocolate agar plate in triplicate for *B. quintana* enumeration. The number of CFUs was counted after 10 days of culture in candle extinction jars at 37℃. OD_{600} was also measured to determine the approximate number of cells prior to use.

The remaining suspension was then mixed with 4 ml of human blood to obtain a titer of approximately 1 x 10^7 CFU/ml (Kosoy et al., 2004) and used to fill the blood reservoir of each feeding unit. To obtain the same titer of *B. quintana* for both body and head lice, the infected blood was mixed together and divided again and used to make artificial feeding chambers. Body and head lice were starved for 8 h and fed the *B. quintana*-inoculated blood using the feeding chambers via a single sufficient feeding (ca. 3 h). The infected lice were then transferred to a new

feeding chambers containing fresh non-infected blood and maintained until use for experiments.

Following the oral challenge with *B. quintana*, the entire alimentary tract of the infected lice was removed by dissection in sterile, ice-cold PBS at 1, 4 and 8 days post-infection. The dissected alimentary track was immediately placed in RNAlater solution (Ambion, Austin, TX, USA) to maintain the quality of RNA. Upon the completion of the dissection, all the alimentary tract tissues were homogenized in 100 μl of TRI reagent (MRC, Cincinnati, OH, USA) and total RNA was extracted according to the manufacturer's instruction. The first-strand cDNA was synthesized from the DNaseI (Takara biotechnology, Japan)-treated total RNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and diluted to 5 ng/μl to use as template for qPCR. The 6 major immune-related genes (PGRP, defensin 1, defensin 2, Duox, lysozyme and PPO) involved in pathogen recognition and direct defense were selected as representative genes to examine the humoral immune responses of epithelial cells of alimentary tract tissue. The qPCR was performed in a StepOne Plus Real Time PCR System (Applied biosystems, Darmstadt, Germany) using following cycling conditions: 95℃ for 10 min, 40 cycles of 95℃ for 15 s, 56℃ for 20 s and 60℃ for 30 s. The reaction mixtures contained 1X Power SYBR Green PCR Master Mix (Applied biosystems) and 0.5 μM primers for immune genes or *RpL13A* as a reference gene.

The primer sets used for the qPCR are shown in Table 1.

1.4. Comparison of ROS level in alimentary tract tissue of starved or blood fed lice

Female body and head lice were starved for 8 h prior to experiments and half of them were then fed human blood using the in vitro rearing system until their guts were full of blood by visual inspection (ca. 30 min). 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 CM-H_2DCFDA [5-(and-6)-chloromethyl-2',7'-dichloro-dihydrofluorescein diacetate, acetyl ester] (Invitrogen, Carlsbad, CA, USA) 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 (Ex: 500 nm; Em: 520 nm) measured with a GeminiXS spectrofluorometric plate reader (Molecular Devices, Sunnyvale, CA, USA).

For the hydroxypheny fluorescein (HPF) assay, which also detects a similar range of ROS as CM-H_2DCFDA, the dissected alimentary tracts were incubated in 5 μM of HPF (Invitrogen, Carlsbad, CA, USA) 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: 490 nm; Em: 515 nm) measured using a spectrofluorometric plate reader.

Nitro blue tetrazolium (NBT) was used to assess the generation of superoxide from epithelial cells based on previously described method with modification (Arumugam et al., 2000). Briefly, the dissected alimentary tracts were incubated in NBT reaction solution (0.7 mM NBT, 0.5 mM EDTA) at 37℃ for 60 min. Tissues were homogenized and centrifuged at 1,000 g for 10 min. After the supernatant was removed, the pellet was resuspended in 70% methanol. After another centrifugation at 1,000 g for 10 min, the pellet was resuspended in extraction solution (2M KOH + DMSO, 6:7 v/v) to dissolve insoluble formazan and then centrifuged again at 3,500 g for 20 min. The optical density of the supernatant at 630 nm was measured using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

1.5. Statistical analysis

All statistical analysis was performed using the SPSS 22.0 software (SPSS

Inc., Chicago, IL, USA). Mean and standard deviation were calculated for each data set and student's *t* test and two-way ANOVA followed by Tukey post hoc test were used to determine statistical differences.

2. Results

2.1. Transcriptional profiling of immune-related genes in alimentary tract tissue following **E. coli** *challenge*

Of the several representative immune-related genes chosen for the alimentary tract tissue-specific transcriptional profiling (Fig. 1), PGRP (3.7 fold; t=-2.7; d.f., 11; p=0.02), defensin 1 (3.0 fold; t=-4.7; d.f., 11; p=0.001), and defensin 2 (1.9 fold; t=-2.5; d.f., 11; p=0.028) had significantly higher basal (no bacteria challenge) transcription levels in head lice compared with body louse (Fig. 1B). The basal transcription levels of other genes, including Duox, lysozyme and PPO, did not significantly differ between body and head lice ($p>0.05$). 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* (Fig. 1C).

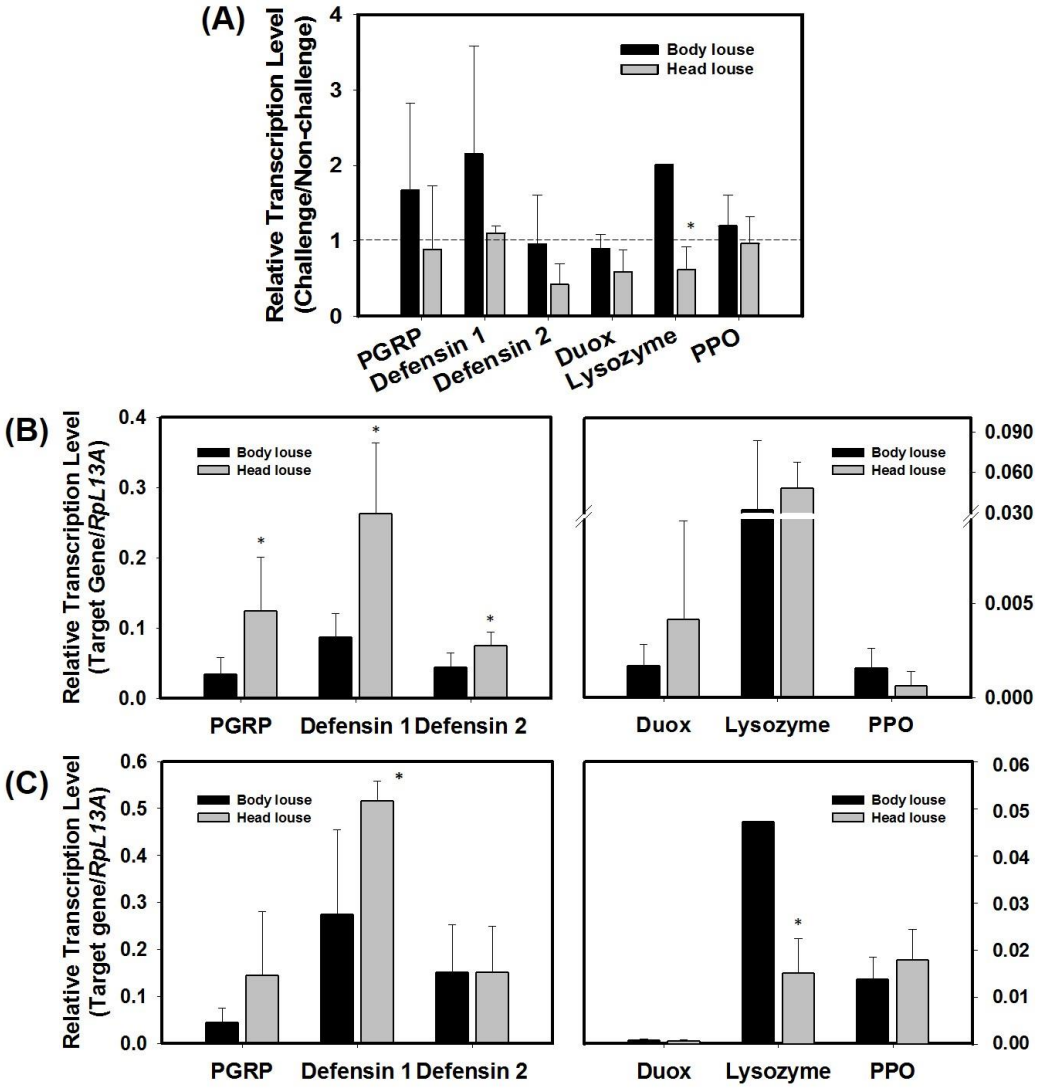

Figure 1. Comparison of the fold-changes of transcript by *E. coli* challenge (A) and the relative transcription level of representative immune-related genes in the alimentary tract tissues of non-challenged (B) or orally-challenged (C) between body and head lice. Bars with star mark (*) indicate statistically significant difference between body and head lice ($p<0.05$). Error bars indicate standard deviation of the mean.

2.2. Transcriptional profiling of immune-related genes in alimentary tract tissue following B. quintana *challenge*

To determine whether *B. quintana* ingestion triggers humoral immune responses in alimentary tract tissue, the change in major immune gene transcription was evaluated over time following *B. quintana* oral challenge. A total of 6 representative immune genes, including a single recognition protein and major effector genes, were selected (PGRP, defensin 1, defensin 2, Duox, lysozyme and PPO).

The higher basal transcription levels of PGRP and defensin 1 in head lice were confirmed again in the transcriptional profiling following *B. quintana* challenge. The transcription of defensin 1 was significantly up-regulated by oral challenge only in head lice (ANOVA, Tukey, $p<0.05$) but the transcription levels of other genes were not altered in either lice over time (Fig. 2). The level of defensin 1 transcript in head lice increased 3.2- and 3.0-fold relative to control (0 day) at 4 and 8 days post-challenge with *B. quintana*, respectively ($t=-3.5$; d.f., 10; $p=0.005$ and $t=-5.1$; d.f., 8; $p=0.001$). However, the transcription level of defensin 1 was not altered in body lice (ANOVA, Tukey, $p>0.05$) and the rate of increase was 12.5-fold ($t=-3.451$; d.f., 7; $p=0.01$) and 4.4-fold ($t=-3.359$; d.f., 6; $p=0.02$) higher in head lice at 4 and 8 days post-challenge, respectively.

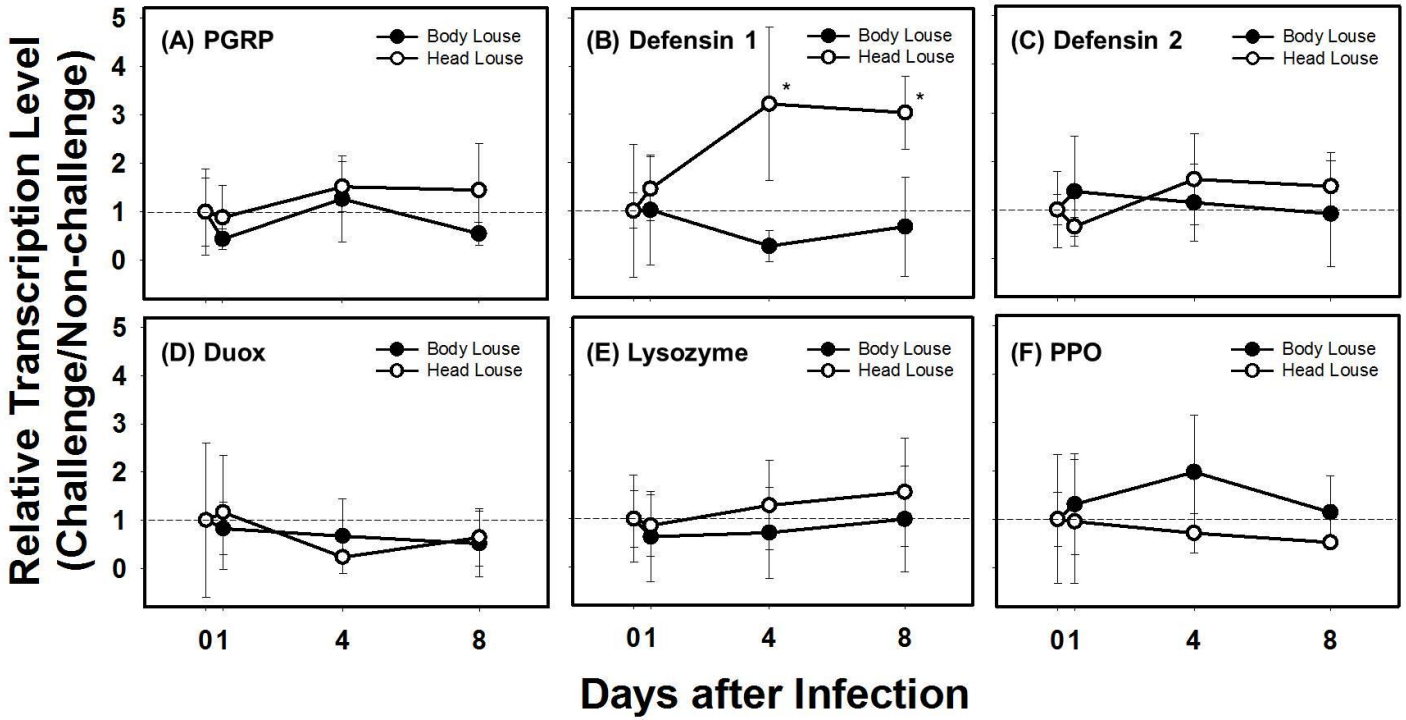

Figure 2. Comparison of the fold-changes of transcript of 6 major immune genes by *B. quintana* between body and head lice. Symbols with star mark (*) indicate statistically significant difference between body and head lice ($p<0.05$). Error bars indicate standard deviation of the mean. The dotted line (value=1) corresponds to an identical relative transcription level between control and challenged lice.

2.3. ROS level in alimentary tract tissue following **B. quintana** *oral challenge*

To compare ROS level generated by epithelial cells in alimentary tract tissue, three indicators that detect different ranges of ROS were used. The levels of ROS were not significantly different between starved and blood-fed lice under any of the experimental conditions, indicating that an imbibing a blood meal did not affect ROS levels either in body or head lice ($p>0.05$) (Fig 3). Regardless of blood feeding or not, higher levels of ROS (1.3-fold; $t=-6.8$; d.f., 4; $p=0.002$ in starved lice and 1.1-fold; $t=-6.1$, d.f., 4; $p=0.004$ in fed lice) were always detected in head louse epithelial cells as judged by CM-H_2DCFDA, which is a general indicator that can detect a wide array of ROS, including OH^-, $ONOO^-$, OCl^-, O_2^-, H_2O_2, NO^- and ROO^-. This phenomenon became more apparent when ROS was detected with HPF, which is also a general ROS indicator, but not sensitive to the light-induced oxidation. Head lice showed 2.3-fold ($t=-4.2$; d.f., 6; $p=0.006$) and 2.5-fold ($t=-5.9$; d.f., 4; $p=0.004$) higher levels of ROS detected by HPF in the starved or fed condition, respectively, when compared with body lice. Therefore, the ROS level detected by HPF more likely reflects the net amount of ROS generated in the epithelial cells. However, the amount of superoxide (O_2^-), specifically detected by NBT, was not significantly different between body and head lice ($p>0.05$).

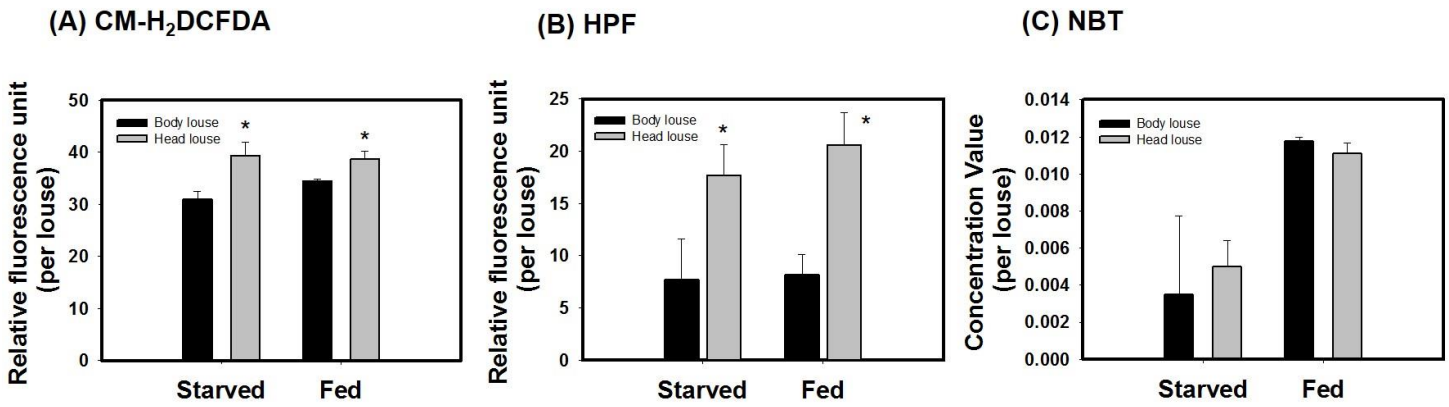

Figure 3. Comparison of generation of general reactive oxygen species using CM-H_2DCFDA (A) and HPF (B), superoixde using NBT (C) in the alimentary tract tissue between starved or fed body and head lice. Bars with star mark (*) indicate statistically significant difference between body and head lice ($p<0.05$). Error bars indicate standard deviation of the mean.

3. Discussion

There is only one PGRP gene in the genomes of either the head or body louse which has a transmembrane domain and 2 carboxy terminal PGRP domains. This structure is common to the insect membrane-spanning PGRPs, such as *Drosophila* PGRP LA, LC, LD and *Anopheles* PGRP LA, LB, LC. As described in Chapter II, the intermediate properties associated with the only PGRP gene in human louse suggest that it may function as a general receptor for both Gram-positive and -negative bacteria. Therefore, the significantly reduced basal transcription of PGRP in the alimentary tract tissues of body louse likely results in the down-regulation of downstream immune responses to Gram-negative bacteria, including *E. coli* and perhaps the human pathogenic bacteria.

Since defensin 1 and defensin 2 are the only AMPs in human lice, they are most likely also involved in the humoral suppression of Gram-negative bacteria. Thus, the significantly lower basal transcription of both defensin 1 and defensin 2 in the alimentary tract tissues likely reduces the initial immune response of body louse to Gram-negative bacteria (Fig. 1B). Although the amount of defensin 1 transcript increased both in body and head lice following *E. coli* oral infection, its total induced transcription level was still lower in body louse compared with head louse (Fig. 1C). In contrast, the total induced transcription level of defensin 2 did

not differ between body and head lice. Therefore, it appears that the louse defensin 1 is the main AMP used against Gram-negative bacteria, including *E. coli*, and the different transcription level of defensin 1, rather than defensin 2, partially contributes to the different proliferation rate of *E. coli* in the alimentary tract tissue of body versus head louse.

To elucidate what molecular factors may be associated with the different excretion pattern of viable *B. quintana* seen by body versus head lice, the transcriptional profiles of 6 main humoral immune-related genes were determined following oral challenge by *B. quintana*. The higher basal transcription levels of PGRP and defensin 1 in head versus body lice (Kim et al., 2012) were confirmed in this study (Fig. 3). Given that there is no cellular immune response in alimentary tract tissue, it seems that the lower basal transcription levels of PGRP and defensin 1 in body lice allowed *B. quintana* in the gut to survive better than in head lice. Recent comparisons of the transcriptome (Olds et al., 2012) and genome (Kang et al., 2015) demonstrated that both body and head lice possess virtually identical genetic background and the same set of immune-related genes with nucleotide diversity ranging from only 0.1-1.3% in the coding region. Thus, the apparent differences in basal transcription levels of some immune genes between body and head lice appear to be due to different gene regulation factors in non-coding region, including *cis*-/*trans*-regulatory elements and/or miRNAs.

Similar to the response following *E. coli* challenge, defensin 1 transcription was selectively up-regulated relative to defensin 2 only in head lice following oral challenge with *B. quintana*, which supports the hypothesis that defensin 1 may be a major AMP used against Gram-negative bacteria, including *B. quintana*. In addition to their lower basal transcription levels, transcription of defensin 1 was not induced in body lice following *B. quintana* oral challenge, which was opposite to head lice. Therefore, the lack of defensin 1 inducibility as well as the lower basal transcription levels of *PGRP* and defensins in body lice appear primarily responsible for the reduced immune response of body lice to *B. quintana*. Since both body and head lice lack a functional IMD pathway that plays a key role to trigger immune reaction against Gram-negative bacteria, it remains to be elucidated which immune pathway mediated the up-regulated transcription of defensin 1 following *B. quintana* challenge. Nevertheless, since both body and head lice possess only one pathogen recognition protein (i.e., PGRP), PGRP appears to be involved in the recognition of both Gram-positive and –negative bacteria.

The generation of ROS is one of key mediators of antimicrobial defense in arthropod gut (Molina-Cruz et al., 2008). While blood feeding immediately decreased ROS through a mechanism involving heme-mediated activation of protein kinase C (PKC) in *Aedes aegypti* (Oliveira et al., 2011), Nonetheless, the

net ROS levels were higher in head versus body lice regardless of their feeding status, suggesting that the oxidative killing caused by ROS generation may also contribute to the suppressed proliferation of *B. quintana* in head lice (Chapter I). Considering that the constitutive level of the Duox gene was not statistically different between these two louse subspecies, despite its higher mean value in head lice, it remains to be elucidated how less ROS was generated in the body louse gut. Unlike *E. coli* and other Gram-negative bacteria, *B. quintana* is known to possess several hemin-binding proteins (Hbps) in its outer membrane surface, which bind to heme. Therefore, it has been hypothesized that the Hbp-coat of *B. quintana* likely serves as a potent antioxidant barrier due to Hbp's intrinsic peroxidase activity, thereby providing tolerance to the ROS generated in the gut of vector arthropods (Battisti et al., 2006); (Harms and Dehio, 2012). Nevertheless, the significantly lower ROS in the alimentary track of body versus head lice likely results in a reduced protection against *B. quintana* proliferation and viability.

CONCLUSION

Body lice showed relatively less sensitive responses to Gram-positive *S. aureus*, Gram-negative *E. coli* and human pathogenic bacteria *B. quintana* compared to head lice. In addition, the viability of *B. quintana* in the feces from infected lice was significantly higher in body lice. These differences demonstrate that body lice have reduced immune responses to these bacteria compared with head lice. Nevertheless, both lice have the same immune components with one long-type PGRP (no short-type PGRP and BGBP), two defensins (no other antimicrobial peptide) and non-functional Imd pathway. The most apparent differences in the immune responses between body and head lice were found in their phagocytosis activity profiles. Despite the lack of humoral immune responses to Gram-negative bacteria, a more rapid and elevated phagocytotic response in head lice may provide sufficient protection against *E. coli* infection. In addition, body lice showed the lower basal transcription levels of PGRP, TEP1, TEP2, SRCI and Duox in whole body scale and PGRP and defensin 2 were up-regulated following *S. aureus* dermal challenge whereas no alteration was detected after *E. coli* challenge. Moreover, the reduced ROS generation and the lower basal transcription levels of major immune genes, such as PGRP, defensin 1 and defensin 2 were observed in alimentary tract tissue of body lice.

In summary, head lice appear to maintain immune genes at a higher basal level than that seen in body lice so that they can respond more quickly and efficiently to invading bacteria. In contrast, body lice appear to have evolved a reduced acute immune response by down-regulating major bacterial pattern recognition protein genes (i.e., PGRP) and effector genes (e.g., defensins). This relaxed immune response may be the inadvertent result of the necessity of protecting their endosymbiont, *Candidatus* Riesia pediculicola, which are indispensable for louse nutrition and survival by providing vitamin B5 that is not synthesized by the human louse itself. Protection of endosymbiont *C.* R. pediculicola may have been necessary because they needed to become bigger than head lice in order to occupy the relatively new niche of human clothing that first occured some 40,000–70,000 years ago (Kittler et al., 2004). The larger size of the body louse may have been a prerequisite for them to take a larger blood meal and to become more mobile in order to successfully adapt to their new habitat. By doing so, the minimized local immune response, mainly due to the reduced levels of immune effectors below a critical level necessary for the efficient killing of invading bacteria, may allow the rapid proliferation of bacteria only in the body louse, thereby resulting in the enhanced vector competence of body versus head lice.

However, it still remains to be elucidated how body lice suppress the transcription of main immune genes (i.e., PGRP and defensins). No apparent

differences in the transcription factor binding motifs in the putative regulatory domain of these immune genes suggest that such transcription differences are not directly regulated by transcription control. Thus, other possibilities include the differences in the miRNA-mediated post-transcriptional regulation, in the not-yet-identified *trans*-actining components and in the epigenetic factors, such as methylation. In addition, effects of endosymbionts on host immune responses also need to be investigated as a potential factor governing the vector competence.

LITERATURES CITED

Aggarwal, K., Silverman, N., 2008. Positive and negative regulation of the *Drosophila* immune response. Bmb Rep 41, 267-277.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25, 3389-3402.

Arnold, K., Bordoli, L., Kopp, J., Schwede, T., 2006. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22, 195-201.

Arumugam, M., Romestand, B., Torreilles, J., Roch, P., 2000. *In vitro* production of superoxide and nitric oxide (as nitrite and nitrate) by *Mytilus galloprovincialis* haemocytes upon incubation with PMA or laminarin or during yeast phagocytosis. Eur J Cell Biol 79, 513-519.

Bacot, 1917. A contribution to the bionomics of *Pediculus humanus* (vestimenti) and *Pediculus capitis*. Parasitology 9, 228-258.

Battisti, J.M., Sappington, K.N., Smitherman, L.S., Parrow, N.L., Minnick, M.F., 2006. Environmental signals generate a differential and coordinated expression of the heme receptor gene family of *Bartonella quintana*. Infect Immun 74, 3251-3261.

Beerntsen, B.T., James, A.A., Christensen, B.M., 2000. Genetics of mosquito vector competence. Microbiol Mol Biol R 64, 115-137.

Bendtsen, J.D., Nielsen, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol 340, 783-795.

Blandin, S., Levashina, E.A., 2004. Thioester-containing proteins and insect immunity. Mol Immunol 40, 903-908.

Bonilla, D.L., Kabeya, H., Henn, J., Kramer, V.L., Kosoy, M.Y., 2009. *Bartonella quintana* in body lice and head lice from homeless persons, San Francisco, California, USA. Emerg Infect Dis 15, 912-915.

Brouqui, P., Raoult, D., 2006. Arthropod-borne diseases in homeless. Century of Rickettsiology: Emerging, Reemerging Rickettsioses, Molecular Diagnostics, and Emerging Veterinary Rickettsioses 1078, 223-235.

Bulet, P., Hetru, C., Dimarcq, J.L., Hoffmann, D., 1999. Antimicrobial peptides in insects; structure and function. Dev Comp Immunol 23, 329-344.

Busvine, 1978. Evidence from double infestations for the specific status of human head and body lice (Anoplura). Syst Entomol 3, 1-8.

Buxton, P.A., 1946. The louse. Williams and Wilkins Co., Baltimore, p. 164.

Christophides, G.K., Vlachou, D., Kafatos, F.C., 2004. Comparative and

functional genomics of the innate immune system in the malaria vector *Anopheles gambiae*. Immunol Rev 198, 127-148.

Christophides, G.K., Zdobnov, E., Barillas-Mury, C., Birney, E., Blandin, S., Blass, C., Brey, P.T., Collins, F.H., Danielli, A., Dimopoulos, G., Hetru, C., Hoa, N.T., Hoffmann, J.A., Kanzok, S.M., Letunic, I., Levashina, E.A., Loukeris, T.G., Lycett, G., Meister, S., Michel, K., Moita, L.F., Muller, H.M., Osta, M.A., Paskewitz, S.M., Reichhart, J.M., Rzhetsky, A., Troxler, L., Vernick, K.D., Vlachou, D., Volz, J., von Mering, C., Xu, J.N., Zheng, L.B., Bork, P., Kafatos, F.C., 2002. Immunity-related genes and gene families in *Anopheles gambiae*. Science 298, 159-165.

Cockerell, C.J., Whitlow, M.A., Webster, G.F., Friedmankien, A.E., 1987. Epithelioid angiomatosis - a distinct vascular disorder in patients with the acquired-immunodeficiency-syndrome or aids-related complex. Lancet 2, 654-656.

Cornet, B., Bonmatin, J.M., Hetru, C., Hoffmann, J.A., Ptak, M., Vovelle, F., 1995. Refined three-dimensional solution structure of insect defensin A. Structure (London, England : 1993) 3, 435-448.

Dimarcq, J.L., Bulet, P., Hetru, C., Hoffmann, J., 1998. Cysteine-rich antimicrobial peptides in invertebrates. Biopolymers 47, 465-477.

Dziarski, R., 2004. Peptidoglycan recognition proteins (PGRPs). Mol Immunol 40, 877-886.

Dziarski, R., Gupta, D., 2006. The peptidoglycan recognition proteins (PGRPs). Genome Biol 7.

Elrod-Erickson, M., Mishra, S., Schneider, D., 2000. Interactions between the cellular and humoral immune responses in *Drosophila*. Curr Biol 10, 781-784.

Evans, J.D., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J.L., Jiang, H., Kanost, M., Thompson, G.J., Zou, Z., Hultmark, D., 2006. Immune pathways and defence mechanisms in honey bees *Apis mellifera*. Insect Mol Biol 15, 645-656.

Ferrandon, D., Imler, J.L., Hetru, C., Hoffmann, J.A., 2007. The *Drosophila* systemic immune response: sensing and signalling during bacterial and fungal infections. Nat Rev Immunol 7, 862-874.

Froy, O., Gurevitz, M., 2003. Arthropod and mollusk defensins - evolution by exon-shuffling. Trends Genet 19, 684-687.

Gandhe, A.S., John, S.H., Nagaraju, J., 2007. Noduler, a novel immune up-regulated protein mediates nodulation response in insects. J Immunol 179, 6943-6951.

Ganz, T., 2003. Defensins: Antimicrobial peptides of innate immunity. Nat Rev Immunol 3, 710-720.

Gasteiger, E., Hoogland, C., Gattiker, A., Wilkins, M.R., Appel, R.D., Bairoch, A., 2005. Protein identification and analysis tools on the ExPASy server. Springer.

Gerardo, N.M., Altincicek, B., Anselme, C., Atamian, H., Barribeau, S.M., De Vos, M., Duncan, E.J., Evans, J.D., Gabaldon, T., Ghanim, M., Heddi, A., Kaloshian, I., Latorre, A., Moya, A., Nakabachi, A., Parker, B.J., Perez-Brocal, V., Pignatelli, M., Rahbe, Y., Ramsey, J.S., Spragg, C.J., Tamames, J., Tamarit, D., Tamborindeguy, C., Vincent-Monegat, C., Vilcinskas, A., 2010. Immunity and other defenses in pea aphids, *Acyrthosiphon pisum*. Genome Biol 11.

Habtewold, T., Povelones, M., Blagborough, A.M., Christophides, G.K., 2008. Transmission blocking immunity in the malaria non-vector mosquito *Anopheles quadriannulatus* species A. Plos Pathog 4.

Harms, A., Dehio, C., 2012. Intruders below the radar: molecular pathogenesis of *Bartonella* spp. Clin Microbiol Rev 25, 42-78.

Hetru, C., Hoffmann, D., Bulet, P., 1998. Antimicrobial peptides from insects.

Hoffmann, J.A., Hetru, C., 1992. Insect defensins - inducible antibacterial peptides. Immunol Today 13, 411-415.

Hoffmann, J.A., Reichhart, J.M., 2002. *Drosophila* innate immunity: an evolutionary perspective. Nat Immunol 3, 121-126.

Hotez, P.J., 2008. Neglected infections of poverty in the United States of America. Plos Neglect Trop D 2.

Houhamdi, L., Fournier, P.E., Fang, R., Lepidi, H., Raoult, D., 2002. An experimental model of human body louse infection with *Rickettsia prowazekii*. J Infect Dis 186, 1639-1646.

Hultmark, D., 2003. *Drosophila* immunity: paths and patterns. Curr Opin Immunol 15, 12-19.

Ito, S., Vinson, J.W., 1965. Fine structure of *Rickettsia quintana* cultivated *in vitro* and in louse. J Bacteriol 89, 481.

Jackson, L.A., Spach, D.H., Kippen, D.A., Sugg, N.K., Regnery, R.L., Sayers, M.H., Stamm, W.E., 1996. Seroprevalence to *Bartonella quintana* among patients at a community clinic in downtown Seattle. J Infect Dis 173, 1023-1026.

Kang, J.S., Cho, Y., Kim, J.H., Kim, S.H., Yoo, S., Noh, S., Park, J., S., Y.K., Clark, J.M., Pittendrigh, B.R., Chun, J., Lee, S.H., 2015. Comparison of the genome profiles between head and body lice. J Asia-Pac Entomol 18, 377-382.

Khajuria, C., Buschman, L.L., Chen, M.S., Zurek, L., Zhu, K.Y., 2011. Characterization of six antibacterial response genes from the European corn borer (*Ostrinia nubilalis*) larval gut and their expression in response to bacterial challenge. J Insect Physiol 57, 345-355.

Kim, J.H., Yoon, K.S., Previte, D.J., Pittendrigh, B.R., Clark, J.M., Lee, S.H., 2012. Comparison of the immune response in alimentary tract tissues from body versus head lice following *Escherichia coli* oral infection. J Asia-Pac Entomol 15,

409-412.

Kirkness, E.F., Haas, B.J., Sun, W.L., Braig, H.R., Perotti, M.A., Clark, J.M., Lee, S.H., Robertson, H.M., Kennedy, R.C., Elhaik, E., Gerlach, D., Kriventseva, E.V., Elsik, C.G., Graur, D., Hill, C.A., Veenstra, J.A., Walenz, B., Tubio, J.M.C., Ribeiro, J.M.C., Rozas, J., Johnston, J.S., Reese, J.T., Popadic, A., Tojo, M., Raoult, D., Reed, D.L., Tomoyasu, Y., Kraus, E., Mittapalli, O., Margam, V.M., Li, H.M., Meyer, J.M., Johnson, R.M., Romero-Severson, J., VanZee, J.P., Alvarez-Ponce, D., Vieira, F.G., Aguade, M., Guirao-Rico, S., Anzola, J.M., Yoon, K.S., Strycharz, J.P., Unger, M.F., Christley, S., Lobo, N.F., Seufferheld, M.J., Wang, N.K., Dasch, G.A., Struchiner, C.J., Madey, G., Hannick, L.I., Bidwell, S., Joardar, V., Caler, E., Shao, R.F., Barker, S.C., Cameron, S., Bruggner, R.V., Regier, A., Johnson, J., Viswanathan, L., Utterback, T.R., Sutton, G.G., Lawson, D., Waterhouse, R.M., Venter, J.C., Strausberg, R.L., Berenbaum, M.R., Collins, F.H., Zdobnov, E.M., Pittendrigh, B.R., 2011. Genome sequences of the human body louse and its primary endosymbiont provide insights into the permanent parasitic lifestyle (vol 107, pg 12168, 2010). P Natl Acad Sci USA 108, 6335-6336.

Kittler, R., Kayser, M., Stoneking, M., 2004. Molecular evolution of *Pediculus humanus* and the origin of clothing (vol 13, pg 1414, 2003). Curr Biol 14, 2309-2309.

Kosoy, M., Mandel, E., Green, D., Marston, E., Childs, J., 2004. Prospective studies of *Bartonella* of rodents. Part I. Demographic and temporal patterns in population dynamics. Vector-Borne Zoonot 4, 285-295.

Kostrzewski, J., 1949. The epidemiology of trench fever. Bull Acad Pol Sci Biol 7, 233-263.

Kotani, E., Yamakawa, M., Iwamoto, S., Tashiro, M., Mori, H., Sumida, M., Matsubara, F., Taniai, K., Kadonookuda, K., Kato, Y., Mori, H., 1995. Cloning and expression of the gene of hemocytin, an insect humoral lectin which is homologous with the mammalian von-Willebrand-factor. Bba-Gene Struct Expr 1260, 245-258.

Krogh, A., Larsson, B., von Heijne, G., Sonnhammer, E.L.L., 2001. Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. J Mol Biol 305, 567-580.

Lai, R., Lomas, L.O., Jonczy, J., Turner, P.C., Rees, H.H., 2004. Two novel non-cationic defensin-like antimicrobial peptides from haemolymph of the female tick, *Amblyomma hebraeum*. Biochem J 379, 681-685.

Landon, C., Barbault, F., Legrain, M., Guenneugues, M., Vovelle, F., 2008. Rational design of peptides active against the gram positive bacteria *Staphylococcus aureus*. Proteins 72, 229-239.

Lemaitre, B., Hoffmann, J., 2007. The host defense of *Drosophila melanogaster*. Annu Rev Immunol 25, 697-743.

Lounibos, L.P., 2002. Invasions by insect vectors of human disease. Annu Rev Entomol 47, 233-266.

Moita, L.F., Wang-Sattler, R., Michel, K., Zimmermann, T., Blandin, S., Levashina, E.A., Kafatos, F.C., 2005. *In vivo* identification of novel regulators and conserved pathways of phagocytosis in *Anopheles gambiae*. Immunity 23, 65-73.

Molina-Cruz, A., Dejong, R.J., Charles, B., Gupta, L., Kumar, S., Jaramillo-Gutierrez, G., Barillas-Mury, C., 2008. Reactive oxygen species modulate anopheles gambiae immunity against bacteria and *Plasmodium*. J Biol Chem 283, 3217-3223.

Morris, C.A., Benson, E., White-Cooper, H., 2009. Determination of gene expression patterns using *in situ* hybridization to *Drosophila testes*. Nat Protoc 4, 1807-1819.

Olds, B.P., Coates, B.S., Steele, L.D., Sun, W., Agunbiade, T.A., Yoon, K.S., Strycharz, J.P., Lee, S.H., Paige, K.N., Clark, J.M., Pittendrigh, B.R., 2012. Comparison of the transcriptional profiles of head and body lice. Insect Mol Biol 21, 257-268.

Oliveira, J.H.M., Goncalves, R.L.S., Lara, F.A., Dias, F.A., Gandara, A.C.P., Menna-Barreto, R.F.S., Edwards, M.C., Laurindo, F.R.M., Silva-Neto, M.A.C., Sorgine, M.H.F., Oliveira, P.L., 2011. Blood meal-derived heme decreases ROS levels in the midgut of *Aedes aegypti* and allows proliferation of intestinal microbiota. Plos Pathog 7.

Perotti, M.A., Allen, J.M., Reed, D.L., Braig, H.R., 2007. Host-symbiont interactions of the primary endosymbiont of human head and body lice. Faseb J

21, 1058-1066.

Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods 8, 785-786.

Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C., Ferrin, T.E., 2004. UCSF chimera - A visualization system for exploratory research and analysis. J Comput Chem 25, 1605-1612.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29.

Previte, D., Olds, B.P., Yoon, K., Sun, W., Muir, W., Paige, K.N., Lee, S.H., Clark, J., Koehler, J.E., Pittendrigh, B.R., 2014. Differential gene expression in laboratory strains of human head and body lice when challenged with *Bartonella quintana*, a pathogenic bacterium. Insect Mol Biol 23, 244-254.

Ramet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B., Ezekowitz, R.A.B., 2002. Functional genomic analysis of phagocytosis and identification of a *Drosophila* receptor for *E. coli*. Nature 416, 644-648.

Ramet, M., Pearson, A., Manfruelli, P., Li, X.H., Koziel, H., Gobel, V., Chung, E., Krieger, M., Ezekowitz, R.A.B., 2001. *Drosophila* scavenger receptor CI is a pattern recognition receptor for bacteria. Immunity 15, 1027-1038.

Raoult, D., Roux, V., 1999. The body louse as a vector of reemerging human diseases. Clin Infect Dis 29, 888-911.

Rees, J.A., Moniatte, M., Bulet, P., 1997. Novel antibacterial peptides isolated from a European bumblebee, *Bombus pascuorum* (Hymenoptera, Apoidea). Insect Biochem Molec 27, 413-422.

Reese, J., Christenson, M.K., Leng, N., Saha, S., Cantarel, B., Lindeberg, M., Tamborindeguy, C., MacCarthy, J., Weaver, D., Trease, A.J., 2014. Characterization of the Asian citrus psyllid transcriptome. Journal of genomics 2, 54.

Royet, J., Dziarski, R., 2007. Peptidoglycan recognition proteins: pleiotropic sensors and effectors of antimicrobial defences. Nat Rev Microbiol 5, 264-277.

Rubin, G.M., Yandell, M.D., Wortman, J.R., Miklos, G.L.G., Nelson, C.R., Hariharan, I.K., Fortini, M.E., Li, P.W., Apweiler, R., Fleischmann, W., Cherry, J.M., Henikoff, S., Skupski, M.P., Misra, S., Ashburner, M., Birney, E., Boguski, M.S., Brody, T., Brokstein, P., Celniker, S.E., Chervitz, S.A., Coates, D., Cravchik, A., Gabrielian, A., Galle, R.F., Gelbart, W.M., George, R.A., Goldstein, L.S.B., Gong, F.C., Guan, P., Harris, N.L., Hay, B.A., Hoskins, R.A., Li, J.Y., Li, Z.Y., Hynes, R.O., Jones, S.J.M., Kuehl, P.M., Lemaitre, B., Littleton, J.T., Morrison, D.K., Mungall, C., O'Farrell, P.H., Pickeral, O.K., Shue, C., Vosshall, L.B., Zhang, J., Zhao, Q., Zheng, X.Q.H., Zhong, F., Zhong, W.Y., Gibbs, R., Venter, J.C., Adams, M.D., Lewis, S., 2000. Comparative genomics of the eukaryotes. Science 287, 2204-2215.

Sasaki-Fukatsu, K., Koga, R., Nikoh, N., Yoshizawa, K., Kasai, S., Mihara, M., Kobayashi, M., Tomita, T., Fukatsu, T., 2006. Symbiotic bacteria associated with stomach discs of human lice. Appl Environ Microb 72, 7349-7352.

Sasaki, T., Poudel, S.K.S., Isawa, H., Hayashi, T., Seki, N., Tomita, T., Sawabe, K., Kobayashi, M., 2006. First molecular evidence of *Bartonella quintana* in *Pediculus humanus capitis* (Phthiraptera : Pediculidae) collected from Nepalese children. J Med Entomol 43, 788-788.

Seki, N., Kasai, S., Saito, N., Komagata, O., Mihara, M., Sasaki, T., Tomita, T., Sasaki, T., Kobayashi, M., 2007. Quantitative analysis of proliferation and excretion of *Bartonella quintana* in body lice, *Pediculus humanus* L. Am J Trop Med Hyg 77, 562-566.

Seufi, A.M., Hafez, E.E., Galal, F.H., 2011. Identification, phylogenetic analysis and expression profile of an anionic insect defensin gene, with antibacterial activity, from bacterial-challenged cotton leafworm, *Spodoptera littoralis*. Bmc Mol Biol 12.

Spach, D.H., Kanter, A.S., Dougherty, M.J., Larson, A.M., Coyle, M.B., Brenner, D.J., Swaminathan, B., Matar, G.M., Welch, D.F., Root, R.K., Stamm, W.E., 1995. *Bartonella* (*Rochalimaea*) *quintana* bacteremia in inner-city patients with chronic-alcoholism. New Engl J Med 332, 424-428.

Stanley, D., Miller, J., Tunaz, H., 2009. Eicosanoid actions in insect immunity.

J Innate Immun 1, 282-290.

Stuart, L.M., Ezekowitz, R.A., 2008. Phagocytosis and comparative innate immunity: learning on the fly. Nat Rev Immunol 8, 131-141.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol 30, 2725-2729.

Tanaka, H., Ishibashi, J., Fujita, K., Nakajima, Y., Sagisaka, A., Tomimoto, K., Suzuki, N., Yoshiyama, M., Kaneko, Y., Iwasaki, T., Sunagawa, T., Yamaji, K., Asaoka, A., Mita, K., Yamakawa, M., 2008. A genome-wide analysis of genes and gene families involved in innate immunity of *Bombyx mori*. Insect Biochem Molec 38, 1087-1110.

Tarpy, D.R., 2003. Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth. P Roy Soc B-Biol Sci 270, 99-103.

Ter-Hovhannisyan, V., Lomsadze, A., Chernoff, Y.O., Borodovsky, M., 2008. Gene prediction in novel fungal genomes using an *ab initio* algorithm with unsupervised training. Genome Res 18, 1979-1990.

Vingron, M., Argos, P., 1989. A fast and sensitive multiple sequence alignment algorithm. Comput Appl Biosci 5, 115-121.

Vizioli, J., Richman, A.M., Uttenweiler-Joseph, S., Blass, C., Bulet, P., 2001.

The defensin peptide of the malaria vector mosquito *Anopheles gambiae*: antimicrobial activities and expression in adult mosquitoes. Insect Biochem Molec 31, 241-248.

Ye, J.L., Zhao, H.W., Wang, H.J., Bian, J.M., Zheng, R.Q., 2010. A defensin antimicrobial peptide from the venoms of *Nasonia vitripennis*. Toxicon 56, 101-106.

Yoon, K.S., Strycharz, J.P., Gao, J.R., Takano-Lee, M., Edman, J.D., Clark, J.M., 2006. An improved *in vitro* rearing system for the human head louse allows the determination of resistance to formulated pediculicides. Pestic Biochem Phys 86, 195-202.

Zhang, P., Chomel, B.B., Schau, M.K., Goo, J.S., Droz, S., Kelminson, K.L., George, S.S., Lerche, N.W., Koehler, J.E., 2004. A family of variably expressed outer-membrane proteins (Vomp) mediates adhesion and autoaggregation in *Bartonella quintana*. P Natl Acad Sci USA 101, 13630-13635.

Zhou, J., Liao, M., Ueda, M., Gong, H., Xuan, X., Fujisaki, K., 2007. Sequence characterization and expression patterns of two defensin-like antimicrobial peptides from the tick *Haemaphysalis longicornis*. Peptides 28, 1304-1310.

Zou, Z., Evans, J.D., Lu, Z.Q., Zhao, P.C., Williams, M., Sumathipala, N., Hetru, C., Hultmark, D., Jiang, H.B., 2007. Comparative genomic analysis of the *Tribolium* immune system. Genome Biol 8.

KOREAN ABSTRACT

면역반응 비교 연구를 통한
몸니와 머릿니의 질병매개력 차이 규명

서울대학교 대학원

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김주현

초록

몸니와 머릿니는 사람의 외부 기생성 흡혈곤충으로서 동종(*Pediculus humanus*)내 아종으로 분류되나, 오로지 몸니만이 사람에게 참호열, 재귀열, 발진티푸스 등의 세균성 질병을 옮기는 것으로 알려져 있으며 이는 몸니와 머릿니간의 서로 다른 면역력에 기인한 것으로 생각된다. 이러한 면역 반응의 차이를 확인하기 위하여 대표적인 그람양성균인 *Staphylococcus aures*와 그람음성균인 *Escherichia coli*를 체강 내 감염시킨 결과, 두 균 모두 몸니 내에서는 밀도가 증가하나 머릿니 내에서는 감염 초기부터 증식이 억제됨을 확인하였다. 인체병원성 세균이 주로 존재하는 경로인 소화관에서의 면역반응을

조사하기 위해 섭식을 통해 *E. coli*를 감염시킨 경우 역시 동일한 결과를 보였으며, 실제 참호열의 원인균인 *Bartonella quintana*를 이용한 비교에서도 마찬가지로 몸니 내에서 세균의 밀도가 높았다. 또한 감염된 이의 배설물에서 *B. quintana*의 존재 양상을 비교한 결과, 배설되는 세균의 양은 동일하나 그 중 생균수의 비율은 몸니에서 현저히 높게 관찰되었다. 이의 배설물에 존재하는 세균이 피부의 상처를 통해 인체 내로 침입하는 것이 몸니를 통한 질병 매개의 기작임을 고려할 때, 이러한 생균수의 차이는 몸니와 머릿니의 질병매개력 차이의 주요한 요인일 것으로 생각된다.

몸니의 낮은 면역반응에 대한 분자생물학적 연구의 기반으로서, 몸니와 머릿니의 유전체로부터 면역과 관련된 유전자를 조사하였다. 총 93개의 유전자가 밝혀졌으며 그 수와 종류는 몸니와 머릿니에서 동일하였다. 병원체의 인식에 관여하는 유전자는 세포막에 결합되어 있는 형태의 peptidoglycan recognition protein(PGRP) 1종이 유일하며 beta glucan binding protein(BGBP)은 존재하지 않았다. 체액성 면역에 관여하는 신호전달체계인 Toll, JAK/STAT, JNK pathway는 보존되어 있었으나 Imd pathway의 경우 주요한 구성 요소인 Imd와 FADD가 발견되지 않아 그 기능이 불완전할 것으로 추정된다. 다른 곤충과는 달리, 이의 유전체 내에서는 다양한 종류의 항균 펩타이드 중 2종의 defensin만이 존재하였다.

상기 면역관련 유전자를 기반으로 하여 몸니와 머릿니의 전반적인 면역 반응을 전 충체와 소화관의 두 측면에서 비교하였다. 세포성 면역의 비교를 위해

*S. aureus*와 *E. coli*를 혈강 내 주입하여 식균작용을 관찰한 결과, 머릿니에서 더 높은 반응이 나타났으며 *S. aureus*에 비해 *E. coli*에 대한 식균작용이 더 강하게 관찰되었다. 반면에 전 충체를 대상으로 주요 면역 관련 유전자의 전사량을 조사하였을 시 *S. aureus*에 의한 면역반응 증가가 더욱 높았다. 그러나 감염되지 않은 몸니와 머릿니를 비교했을 경우 PGRP, Thioester containing protein(TEP)1, TEP2, Scavenger receptor CI, Dual oxidase (Duox)의 기본 발현량이 몸니 내에서 더 낮게 관찰되었다. 이러한 양상은 소화관의 상피세포에서도 동일하게 발견되는데, 대표적인 체액성 면역 관련 유전자인 PGRP, defensin 1, defensin 2의 기본 발현량이 몸니 내에서 더 낮았으며 *B. quintana*의 섭식을 통한 감염 후 defensin 1의 전사량이 높아짐을 관찰하였다. 또한 세포독성이 있는 활성산소 역시 흡혈 여부와 관련없이 몸니 내에서 더 적게 발생하였다.

종합하자면, 면역과 관련된 유전자의 종류는 몸니와 머릿니에서 동일하나 주요 면역 유전자의 기본 발현량 및 활성산소 발생량의 감소로 인해 몸니의 전반적인 면역반응이 저하되고, 그에 따른 인체병원성 세균의 체내 증식과 배설물의 생균수 증가가 몸니의 높은 질병매개력에 대한 주요한 원인이 될 것으로 생각된다.

검색어: 몸니, 머릿니, 질병매개력, 곤충 면역 반응, 곤충 유전체, 참호열,

Bartonella quintana

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