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Comparison of the Proliferation and Excretion of *Bartonella quintana* between Body and Head Lice Following Oral Challenge

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Abstract

Human body and head lice are highly related hematophagous ectoparasites but only the body louse has been shown to transmit *Bartonella quintana*, the causative agent of trench fever. The mechanisms by which body lice became a vector for *B. quintana*, however, are poorly understood. Following the oral challenge, green fluorescence protein-expressing *B. quintana* proliferated over 9 days post-challenge with the number of bacteria being significantly higher in whole body versus head lice. The numbers of *B. quintana* detected in feces from infected lice, however, were approximately the same in both lice. Nevertheless, the viability of *B. quintana*, was significantly higher in body louse feces. Comparison of immune responses in alimentary tract tissues revealed that basal transcription levels of peptidoglycan recognition protein and defensins were lower in body lice and the transcription of *defensin 1* was up-regulated by oral challenge with wild-type *B. quintana* in head but not in body lice. In addition, the level of cytotoxic reactive oxygen species generated by epithelial cells was significantly lower in body lice. Although speculative at this time, the reduced immune response is consistent with the higher vector competence seen in body versus head lice in terms of *B. quintana* infection.

Keywords

Human lice; Body louse; Head louse; Trench fever; *Bartonella quintana*; Immune response; ROS; Alimentary tract; Bacterial challenge

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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 bacterial diseases to humans (Lounibos 2002; Raoult and Roux 1999). The body louse originated from the conspecific head louse, *Pediculus humanus capitis*, when humans began to wear clothing roughly 40,000–70,000 years ago (Kirkness et al. 2010), but they differ in the choice of habitat on human hosts and in their vector competence in transmitting Gram-negative bacterial pathogens, such as *Rickettsia prowazekii, Borrelia recurrentis* and *Bartonella quintana*, which respectively cause epidemic typhus, relapsing fever and trench fever. Because 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 these bacterial diseases to humans although *B. quintana* infection has been found in some head louse populations (Angelakis et al. 2011a; Angelakis et al. 2011b; Bonilla et al. 2009; Sasaki et al. 2006; Veracx and Raoult 2012).

Vector competence is defined as the capacity of a vector to allow the development and transmission of a microbial pathogen (Beerntsen et al. 2000). How insects gain or lose vector competence is a crucial issue in the field of vector biology, however, the molecular mechanisms and evolutionary processes are poorly understood. Since the insect immune response plays a key role in insect-pathogen interactions, we hypothesize here that the differences in vector competence may involve, in part, the dissimilarities in the response of their immune systems to bacterial challenge. The differences in the immune response between body and head lice were initially investigated in the previous studies using two model bacteria, a Gram-positive *Staphylococcus aureus* and a Gram-negative *Escherichia coli*, which showed that body lice may have lower immune responses against both Gram-positive and -negative bacteria when compared with head lice (Kim et al. 2011; Kim et al. 2012).

A total of 93 immune-related genes were identified in both body and head lice and this number is substantially fewer compared with other insects (Kim et al. 2011). Many gene families in the humoral immune system were considerably reduced in number or absent in the body louse genome. For genes related to pathogen recognition, only one type of peptidoglycan recognition protein (PGRP) was found in lice, whereas beta-glucan binding protein (BGBP) was not identified. In addition, the Imd (immune deficiency) gene and its adaptor protein FADD gene in the Imd pathway were not present. 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 2 types of defensin (defensin 1 and defensin 2) were annotated.

Transcriptional profiling of major representative immune genes in the alimentary tract identified no apparent alteration of transcription in three important genes for the humoral immune responses (i.e., *PGRP, defensin 1* and *defensin 2*) following *E. coli* oral challenge (Kim et al. 2012). Nevertheless, the basal transcription levels of these genes were higher in the alimentary tract of head versus body lice (Kim et al. 2012). Genome-wide analysis of the whole body transcriptomes of body and head lice following *B. quintana* oral challenge,

however, revealed that some immune-related genes associated with the Toll pathway (i.e., *fibrinogen-like protein, spaetzle, defensin 1, serpin, scavenger receptor A* and *apolipoporhrin 2*) were differently expressed between body and head lice (Previte et al. 2014). Considering that there is no cellular immune reaction in gut tissues, these findings suggest that the selectively induced transcription following bacteria challenge, as well as constitutive up-regulation of some immune genes in head lice, can contribute to the rapid defense and enhanced immune capacity of head lice against intestinal bacterial infection.

B. quintana infection in humans typically causes recurring 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). Trench fever continues to re-emerge in people who are homeless, indigent, and disadvantaged among the urban population, especially those who are immune-compromised such as alcoholics and patients infected with human immunodeficiency virus (Hotez 2008). Recently, it was reported that *B. quintana* was detected from 33.3% of the body lice recovered from the homeless in California, suggesting that *B. quintana* is still prevalent in the human population (Bonilla et al. 2009). Once *B. quintana* is introduced into the gut of a louse by blood feeding on an infected patient, the bacteria replicate, colonize and attach to the surface of epithelial cells of the alimentary tract (Ito and Vinson 1965). Lice have a behavior of excreting urine and feces during feeding and viable *B. quintana* in louse fecal matter can be transferred to the human blood stream via scratches or wounds in the skin. *B. quintana* can also form a biofilm-like structure in the feces, allowing *B. quintana* survival for up to a year. Thus, infected louse feces are the most common means for *B. quintana* transmission to humans (Kostrzewski 1949; Seki et al. 2007).

Since the primary infection route of *B. quintana* is to enter the alimentary tract of the louse via an infected blood meal, the first immune response following feeding is likely the release of AMPs and reactive oxygen species (ROS)-based ‘oxygen burst’ generated by alimentary tract epithelial tissue, one of major sites for the humoral immune response of the louse's innate immune system. Considering this scenario, it is important to investigate the epithelial tissue-specific humoral immune responses and determine if any differences exist there between body and head lice that may contribute to the differential proliferation of *B. quintana* in the gut and feces of lice.

In this study, the profile of propagation and excretion of green fluorescence protein (GFP)-expressing *B. quintana* (GFP-*B. quintana*) following oral challenge were compared between body and head lice. The propagation profile of GFP-*B. quintana* was also compared to that of wild-type *B. quintana* (WT-*B. quintana*) (Previte et al. 2014) and the viability of GFP-*B. quintana* in louse feces was determined. The transcriptional profiles were examined to compare any differences in the basal transcriptional levels and the inducibility of several representative genes involved in humoral immune responses in the alimentary tract, the primary infection site of *B. quintana*, after an infected blood meal. The levels of ROS, an additional humoral immune factor, were also measured and compared to identify any additional basis for the differential proliferation of *B. quintana* seen in body versus head lice.

Results

Proliferation of GFP-*B. quintana* following oral challenge

To investigate whether the proliferation profile of GFP-*B. quintana* in lice is the same as that of the WT-*B. quintana* strain observed previously (Previte et al. 2014), both body and head lice were orally infected with GFP-*B. quintana* and the numbers of bacteria inside a single louse were determined for 12 days following oral challenge (Fig. 1). The amount of ingested blood by a single sufficient feeding was not significantly different between body and head lice (0.89 ± 0.19 μl for a body louse and 0.85 ± 0.14 μl for a head louse; 1.058 g/ml human blood; Student's t-test, $p=0.49$). The number of GFP-*B. quintana* declined for 3 days post-challenge, and subsequently increased significantly in both body and head lice (ANOVA, Dunnett's T3 test, $p<0.05$). Proliferation of GFP-*B. quintana* reached a maximum level (3.4×10^7 cells per louse) in body lice, and then declined ($p<0.05$). The average number of GFP-*B. quintana* per louse was significantly higher in body lice versus head lice: 12.1-fold ($p<0.001$); 3.0-fold ($p=0.005$); 4.5-fold ($p=0.02$); 5.3-fold ($p=0.002$) and 2.0-fold ($p=0.003$) at 1, 3, 6, 9 and 12 days post-challenge, respectively. The overall proliferation pattern of GFP-*B. quintana* was similar to that of WT-*B. quintana* (Previte et al. 2014). There were no statistically significant differences in the mortality between body and head lice following GFP-*B. quintana* challenge or between infected and control lice (0–2% during whole experimental period), which is in accordance with a previous report (Fournier et al. 2001).

Excretion of GFP-*B. quintana* from orally infected lice

Because the pathogenic bacteria vectored by body lice, including *B. quintana*, are largely 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. Results obtained using quantitative real-time PCR (qPCR) showed that both body and head lice excreted almost the same number of GFP-*B. quintana* cells following a single feeding of infected blood (ANOVA, $p>0.05$) (Fig. 2). Because the number of GFP-*B. quintana* cells was derived from the estimated copy number of *16S–23S ribosomal RNA* (*rRNA*) gene in the PCR product, the estimate does not separate viable whole cells from fragmented (non-viable) cells.

In order to compare the viability of *B. quintana* in feces from body versus head lice, the fluorescence of GFP-*B. quintana* bacteria in feces was detected by fluorescence microscopy and quantified as a viability index. Although the number of GFP-*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 estimated copy number of the *16S–23S rRNA* gene, a noticeable difference was observed in the fluorescence intensity between body and head lice. Comparatively, the viability index of GFP-*B. quintana* decreased rapidly in both body and head lice, however, body lice showed a consistently and significantly higher (6.4~10.6-fold) viability index over time when compared with head lice until 11 days post-challenge (ANOVA, $p<0.02$) (Fig. 2B and Supplementary Fig. 1). These findings indicate that the proportion of live bacteria in the excreted feces infected by GFP-*B. quintana* is significantly larger in body versus head lice.

The fluorescence intensity of GFP-*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 GFP-*B. quintana* were not detected in the feces of either body or head lice after 13 days post-challenge.

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

To determine whether *B. quintana* ingestion triggers humoral immune responses in alimentary tract tissue, the transcriptional changes in major immune genes were evaluated over time following *B. quintana* oral challenge. A total of six representative immune genes, including a single recognition protein (*PGRP*) and major effector genes [*defensin 1, defensin 2, Dual oxidase* (*Duox), lysozyme* and *Prophenoloxidase* (*PPO*)] were selected.

Basal transcription levels of *PGRP, defensin 1* and *defensin 2* were significantly lower (3.7-, 3.0- and 1.9-fold; $p=0.02$, 0.007 and 0.03, respectively) in body versus head lice whereas no statistically significant differences were found in the remaining three genes tested (Fig. 3). The transcription of *defensin 1* was significantly up-regulated by oral challenge only in head lice (ANOVA, Tukey, $p<0.05$). The transcription levels of other genes were not altered in either louse ecotype over time (Fig. 4). 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 ($p=0.005$ and $p=0.001$). 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 ($p=0.01$) and 4.4-fold ($p=0.02$) higher in head lice at 4 and 8 days post-challenge, respectively.

ROS level in alimentary tract tissue

To compare ROS levels generated by epithelial cells in alimentary tract tissue, three indicators that detect different types 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 imbibing a blood meal did not affect ROS levels either in body or head lice ($p=0.59$ for body lice, $p=0.30$ for head lice) (Fig. 5). Regardless of blood feeding or not, higher levels of ROS (1.3-fold in starved lice, $p=0.002$ versus 1.1-fold in fed lice, $p=0.004$) were always detected in head louse epithelial cells as determined 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 hydroxypheny fluorescein (HPF), which is also a general ROS indicator, but not sensitive to the light-induced oxidation. Head lice showed 2.3-fold ($p=0.006$) and 2.5-fold ($p=0.004$) 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 nitro blue tetrazolium (NBT), was not significantly different between body and head lice ($p=0.45$ for starved lice, $p=0.27$ for fed lice).

Discussion

In this study, the proliferation profiles of GFP-*B. quintana* in whole lice and the viability of GFP-*B. quintana* in louse feces were compared between body and head lice orally challenged with *B. quintana*-contaminated blood meals. The number of GFP-*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-*B. quintana* were similar to those of two previous reports using WT-*B. quintana* in that two phases of proliferation (i.e., an initial phase of decline in the number of bacteria until 2–4 days post-challenge and the second phase of increase thereafter) were commonly observed, particularly in body lice (Previte et al. 2014; Seki et al. 2007). The proliferation decline observed at the early stage of infection by both WT- and GFP-*B. quintana* suggests that the humoral immune response in the alimentary tract is actively engaged in the suppression of *B. quintana* proliferation and further may play a role in shaping the second phase of proliferation in both body and head lice. In this study using GFP-*B. quintana*, and in our previous study using WT-*B. quintana* (Previte et al. 2014), however, the *B. quintana* cell number declined after 9 days post-challenge in body lice unlike the initial study by Seki et al, where *B. quintana* proliferation remained at the stationary phase after reaching maximum levels (Previte et al. 2014; Seki et al. 2007). The reason for this difference is unknown but it may be due, in part, to the possible differences in the blood feeding system and protocol, where the infected lice were allowed to feed continuously on uninfected human blood using the in vitro feeding chamber in our study whereas lice were fed for a limited duration (20 min) only once a day in the initial study (Seki et al. 2007). Perhaps the multiple blood meals can increase the ROS-based oxygen bursts, thereby suppressing *B. quintana* proliferation more effectively in our experimental setting.

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. Nevertheless, body lice excreted a significantly larger number of viable GFP-*B. quintana* in their feces, particularly during the early stage of infection (i.e., 1~3 days post-challenge), as determined by the viability index. Because *B. quintana* is largely transmitted to humans via fecal contamination of skin abrasions or bite wounds from infected lice, the greater numbers of viable *B. quintana* in body louse feces could play a role in the enhanced infectivity of the body louse although this suggestion is speculation at this time.

The number of *B. quintana* cells per unit amount (1 mg) of feces decreased in general over time following oral challenge. This finding appears in contrast to the reports by Seki et al. (Seki et al. 2007), in which *B. quintana* cells in feces from a single body louse increased over time after the initial reduction in the first 4 days post-challenge. The reason for this contradictory result is unclear, but it can be speculated that the difference is caused by the accumulation of various factors in the two different experimental settings used. Differences in various experimental factors, including the frequency of blood feeding, the inoculating titer of *B. quintana* for oral challenge, the feeding systems, the strains of *B. quintana* used, the age and strains of lice used, etc., may affect the dynamics of *B. quintana* excretion in the two different studies.

Given that *B. quintana* cannot pass through alimentary tract tissue and remain in the gut lumen or attached to 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 the alimentary tract is the primary immune factor against invading pathogenic bacteria following an infected blood meal. To elucidate what molecular factors may be associated with the different excretion patterns of viable *B. quintana* in body versus head lice, the transcriptional profiles of six main humoral immune-related genes in alimentary tract tissue 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 again in this study (Fig. 3). Given that there is no cellular immune response in alimentary tract tissue, it appears that the lower basal transcription levels of *PGRP* and *defensin* 1 in body lice may have allowed *B. quintana* to survive in larger numbers in the gut than in head lice at the early stage of infection. Recent comparisons of the transcriptome (Olds et al. 2012) and genome (Kang et al. 2015) demonstrated that body and head lice possess virtually identical genetic backgrounds and the same set of immune-related genes with nucleotide diversity ranging from only 0.1–1.3% in the coding region. 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 a non-coding region(s), including *cis*-/*trans*-regulatory elements and/or miRNAs.

Similar to the response following *E. coli* challenge (Kim et al. 2012), *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 the head lice response. Therefore, the lack of the inducibility of *defensin 1* as well as the lower basal transcription levels of *PGRP* and *defensins* in body lice appear to be mechanisms consistent with a reduced immune response to *B. quintana* in body versus head lice. The analysis of whole body transcriptome also revealed that defensin 1 was upregulated in *B. quintana*-challenged head lice (Previte et al. 2014), supporting the contention that defensin 1 may be an important immune response component not only in the alimentary tract but in other tissues involved in the immune reaction. Because both body and head lice lack a functional Imd pathway that plays a key role in triggering immune reaction against Gram-negative bacteria, it remains to be elucidated which immune pathway mediates the up-regulated transcription of *defensin 1* following *B. quintana* challenge in head lice. 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.

It still remains to be elucidated, however, 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 (data not shown). Other possibilities include differences in the miRNA-mediated post-transcriptional regulation in the not-yet-identified *trans*-acting components and in the epigenetic factors, such as methylation. In addition, the effects of obligate endosymbionts, such as *Candidatus*

Riesia pediculicola, on host immune responses also need to be investigated as a potential factor governing the vector competence.

The generation of ROS is one of the key mediators of antimicrobial defense in the arthropod gut (Molina-Cruz et al. 2008). While blood feeding rapidly decreased ROS through a mechanism involving heme-mediated activation of protein kinase C (PKC) in *Aedes aegypti* (Oliveira et al. 2011), the level of ROS in the gut was not significantly different in starved versus blood-fed lice. Nonetheless, the net ROS levels were significantly higher in head versus body lice regardless of their feeding status, suggesting that the oxidative-based killing caused by ROS generation may also contribute to the suppressed proliferation of *B. quintana* in head lice. Considering that the constitutive level of the *Duox* gene was not significantly 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 groups. Therefore, it has been hypothesized that the Hbp-coat of *B. quintana* likely serves as a potent antioxidant barrier due to the intrinsic peroxidase activity of Hbp, thereby providing tolerance to the ROS generated in the gut of vector arthropods (Battisti et al. 2006; Harms and Dehio 2012). Nevertheless, the significantly lower level of ROS in the alimentary tract of body lice is consistent with increased proliferation and viability of *B. quintana*.

Finally, it has been suggested that head lice, like body lice, may also be a vector based on the findings that *B. quintana* was detected in some head louse populations collected in Nepal and the US (Bonilla et al. 2009; Sasaki et al. 2006). As demonstrated in this study, however, simple detection of DNA from *B. quintana* in head lice via qPCR does not necessarily indicate their potential as a vector, which requires living bacteria.

Conclusions

In this study, we presented data identifying differences between the humoral immune response of body versus head lice that were consistent with the increased rate of proliferation in the gut and numbers of viable *B. quintana* in the feces of body lice. In the alimentary tract of body lice, we observed lower basal transcription levels of major immune genes, their lack of inducibility following *B. quintana* challenge and a reduced level of ROS when compared to head lice. All of these differences, demonstrated uniquely in body lice, are consistent with a reduced immune response in the alimentary tract of body lice. Although speculative at this time, the higher proliferation in the gut and the excretion of more viable *B. quintana* in feces are factors that may contribute to the higher vector competence of body versus head lice. To prove this point, however, gene knockdown experiments will need to be done in head lice showing that without the gene products of the key immune response genes identified here, the proliferation and viability of *B. quintana* will increase to levels similar to that found in body lice. If successful, head lice with these key genes knocked down should become as infective as body lice using a surrogate mammal model such as rabbit.

Experimental Procedures

Lice rearing

The San Francisco strain of body lice (SF-BL) and the Bristol strain of head lice (BR-HL) have been reared using an *in vitro* rearing system (Yoon et al. 2006) under the environmental conditions of 30°C, 70% relative humidity and 16 h/8 h light/dark in rearing chambers (IRB No. E1404/001-002). Females (2- to 4-days post adult emergence) of both body and head lice were used for all experiments.

B. quintana culture

The wild-type strain (*B. quintana* JK31, deposited at www.BEIresources.org #NR-31832) (Zhang et al. 2004) and the GFP-expressing isogenic strain of *B. quintana* JK31 (Lee and Falkow 1998; Park et al. 2001) were maintained in a biosafety level 2 facility. *B. quintana* from frozen stocks was cultured on chocolate agar plates in candle extinction jars at 37°C for 10 days and then passed to fresh plates for an additional 5–7 days of growth prior to use (Zhang et al. 2004).

Oral challenge of lice with *B. quintana*

Both WT- and GFP-*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 colony forming units (CFUs) was counted 10 days after culture in candle extinction jars at 37°C. Spectrophotometric readings (OD_{600}) were used to determine the approximate number of bacteria prior to use. The remaining *B. quintana* suspension was added to human whole blood (Korean Red Cross, Seoul, Korea) to a final titer of ~1×10^7 CFU/ml (Kosoy et al. 2004). To obtain the same titer of *B. quintana* for both body and head louse experiments, the infected blood was mixed, divided equally and used to make two *in vitro* feeding chambers. Body and head lice were starved for 8 h and then fed with the *B. quintana*-inoculated blood using the feeding chambers to provide a single sufficient feeding (ca. 1 h). Lice were visually inspected to ensure that a blood meal had been taken. The infected lice were then transferred to new feeding chambers containing fresh non-infected blood and maintained until used in experiments. For each experiment, 20 body and head lice were used and each experiment was replicated 6~7 times.

Standard protocol for *B. quintana* quantification

GFP-*B. quintana* grown on a chocolate agar plate was harvested using the PBS method described above (see ‘Oral challenge of lice with *B. quintana*’ section). The bacterial suspension was centrifuged at 1,000 g for 4 min and the pellet resuspended in tissue lysis buffer for genomic DNA (gDNA) extraction using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). A 134-bp gDNA fragment of *B. quintana 16S–23S rRNA* intergenic spacer region was generated by PCR from the extracted gDNA, from which an 89-bp nested fragment 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 using a 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 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* bacterium contains two copies of *16S–23S rRNA* gene (Seki et al. 2007), the number of *B. quintana* bacteria was calculated by dividing the estimated copy number of *16S–23S rRNA* gene by 2.

GFP-*B. quintana* proliferation assay

Body and head lice were orally challenged by feeding on a blood meal infected with GFP-*B. quintana* as described above. The GFP-*B. quintana*-challenged lice were subsequently reared with non-infected blood and collected at intervals of every 3 days post-challenge. The gDNA was extracted using a DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the 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°C, followed by 45 cycles of 10 s at 95°C, 10 s at 58°C and 10 s at 72°C. The specificity of qPCR was confirmed by melting curve analysis. Reactions contained 1× 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 the *B. quintana* 16S–23S rRNA gene as before and louse *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 bacteria number calculated from a 2:1 ratio between DNA copy number and *B. quintana* cell (see 'Standard protocol for *B. quintana* quantification' section) was generated and used to estimate the number of *B. quintana* bacteria in each louse sample.

GFP-*B. quintana* detection in the feces of infected lice

Feces were collected at 1- or 2-day intervals from both body and head lice infected with GFP-*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* bacteria in 1 mg of feces was calculated as described above (see 'standard protocol for *B. quintana* quantification' section). 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 GFP fluorescence 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 (Kim et al. 2011).

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

Body and head lice were orally challenged with WT-*B. quintana* using the same method described above (see 'Oral challenge of lice with *B. quintana*' section). Following the oral challenge, the entire alimentary tract of the infected louse was removed by dissection in sterile, ice-cold PBS at 1, 4 and 8 days post-challenge. The dissected alimentary tract was immediately placed in RNAlater solution (Ambion, Austin, TX, USA) to maintain the quality of RNA. Upon the completion of the dissection, the alimentary tract tissues from each time group were homogenized in 100 μl of TRI reagent (MRC, Cincinnati, OH, USA) and total RNA 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), diluted to 5 ng/μl and used as template for quantitative real-time PCR (qPCR). The six 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°C for 10 min, 40 cycles of 95°C for 15 s, 56°C for 20 s, 60°C for 30 s and serial increase per 0.2°C for 1 s from 45°C to 95°C for melting curve analysis. The reaction mixtures contained 1× 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 2. Quantification of relative transcript level of a gene was conducted based on the original concept of $2^{-\Delta Ct}$ (Pfaffl 2001). qPCR was repeated 5–7 times with total RNA from independently infected lice and each was conducted with two technical replicates to adjust intra-PCR variation.

Comparison of ROS levels in alimentary tract tissues of starved versus blood-fed lice

Female body and head lice were starved for 8 h prior to experiments and half of each type of louse 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 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.

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°C 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 (2 M 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).

Statistical analysis

All statistical analyses were performed using the SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). Mean and standard deviation (S.D.) were calculated for each data set. Kolmogorov-Smirnov test was conducted to confirm whether the data were 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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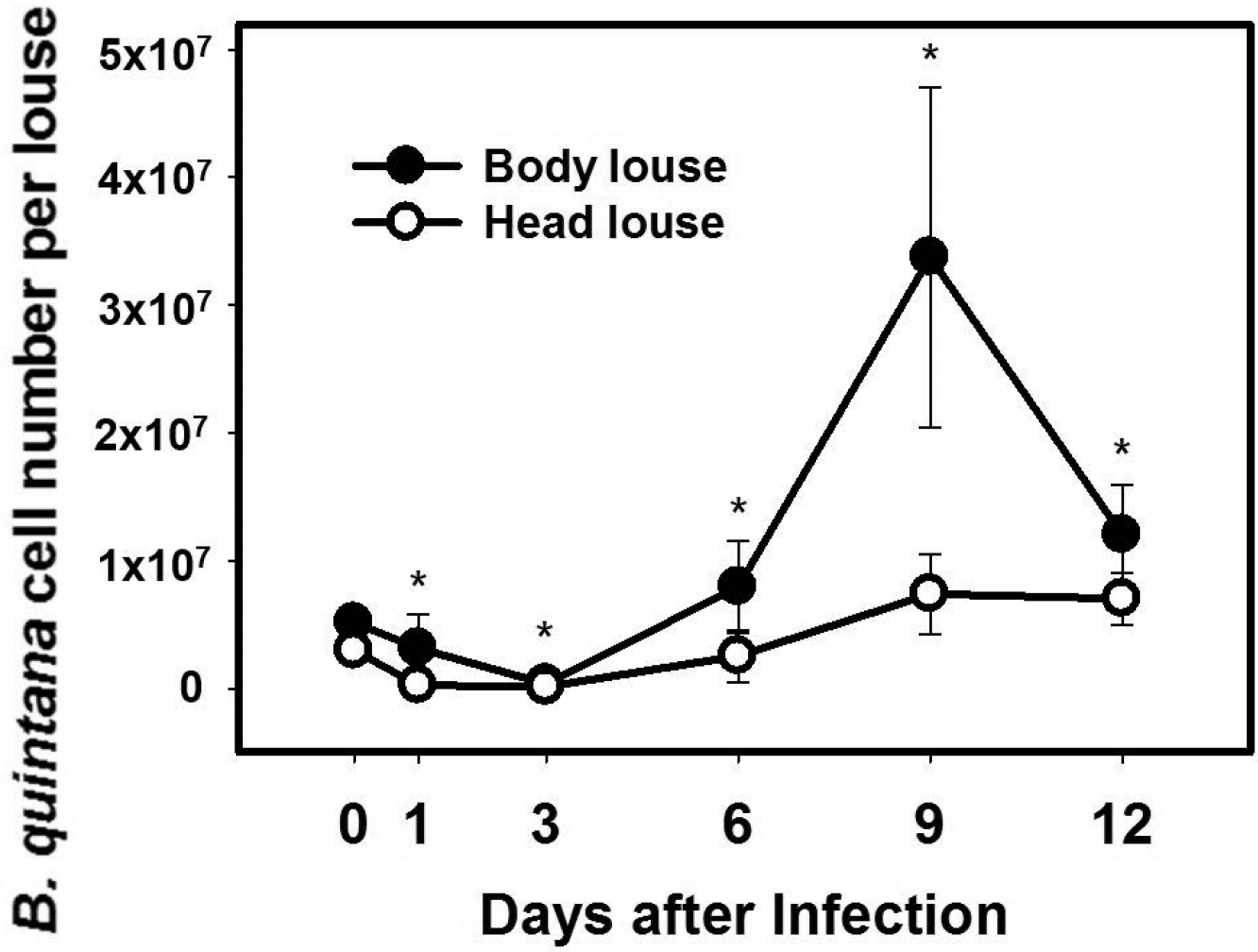

Figure 1.

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

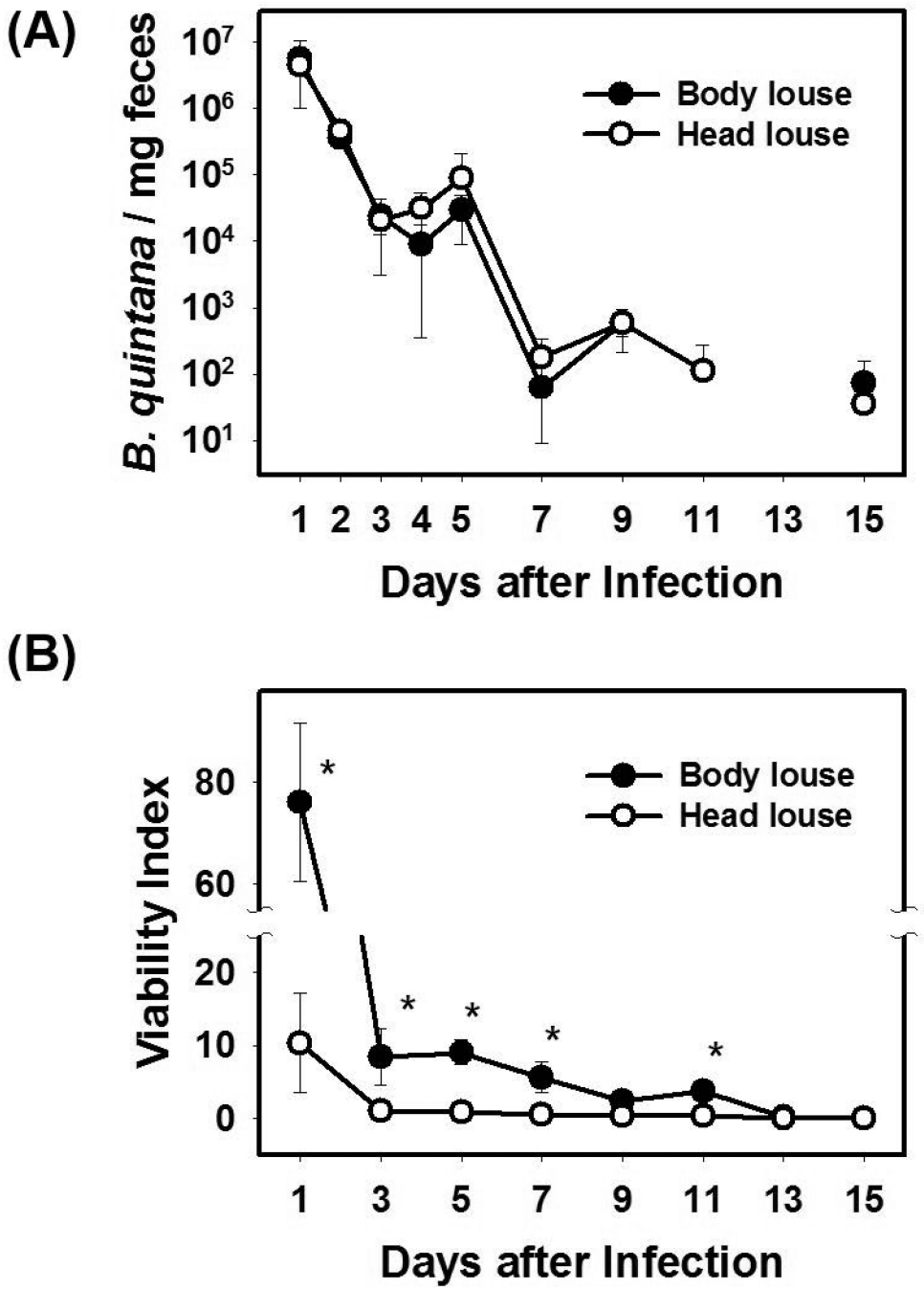

Figure 2.

(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 in louse feces as determined by fluorescence. Symbols with asterisk (*) indicate statistically significant difference between body and head lice ($p<0.05$). Error bars indicate standard deviation of the mean.

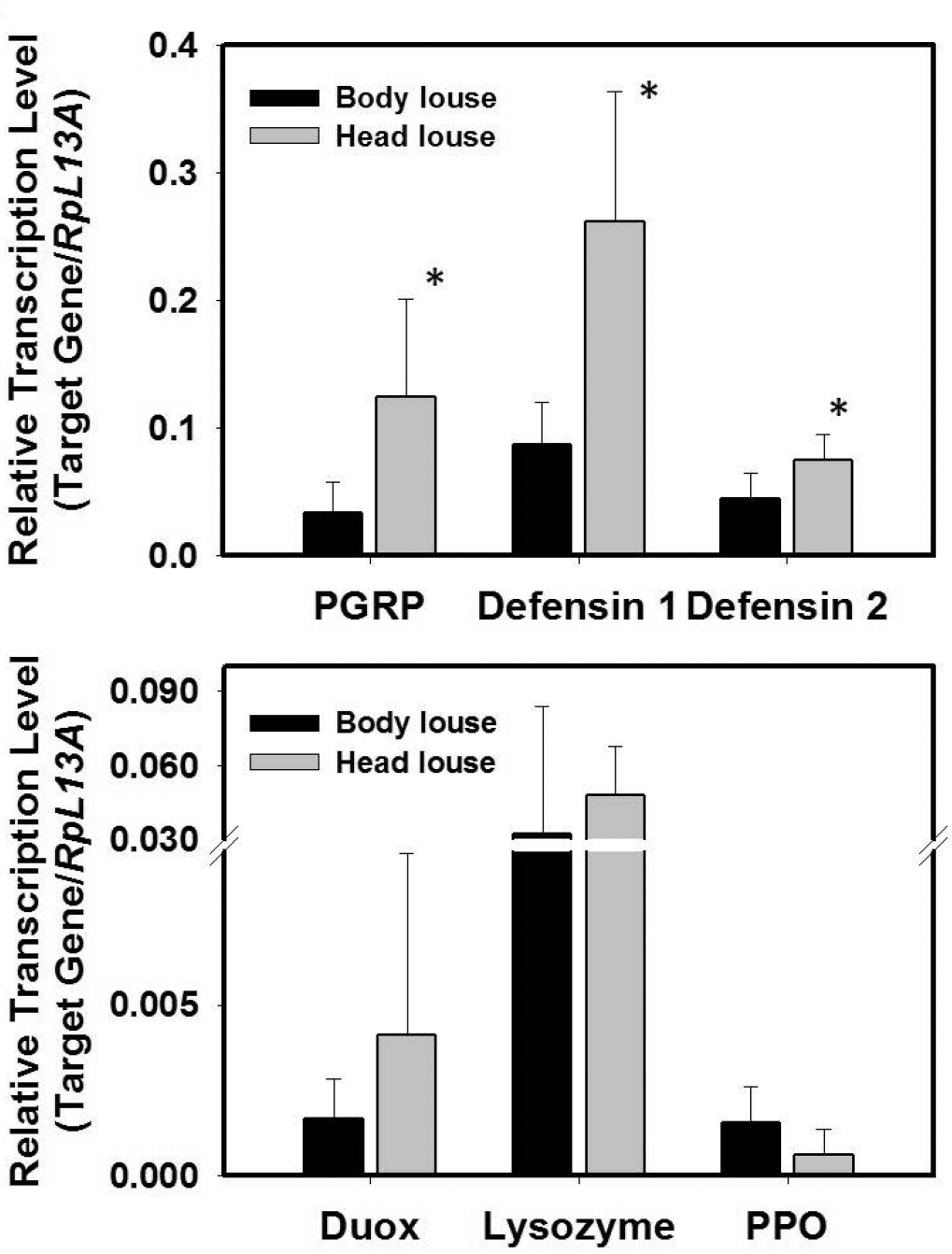

Figure 3.

Comparison of the relative transcription level of representative immune-related genes in the alimentary tract tissues of non-challenged lice. Bars with asterisk (*) indicate statistically significant difference between body and head lice ($p<0.05$). Error bars indicate standard deviation of the mean.

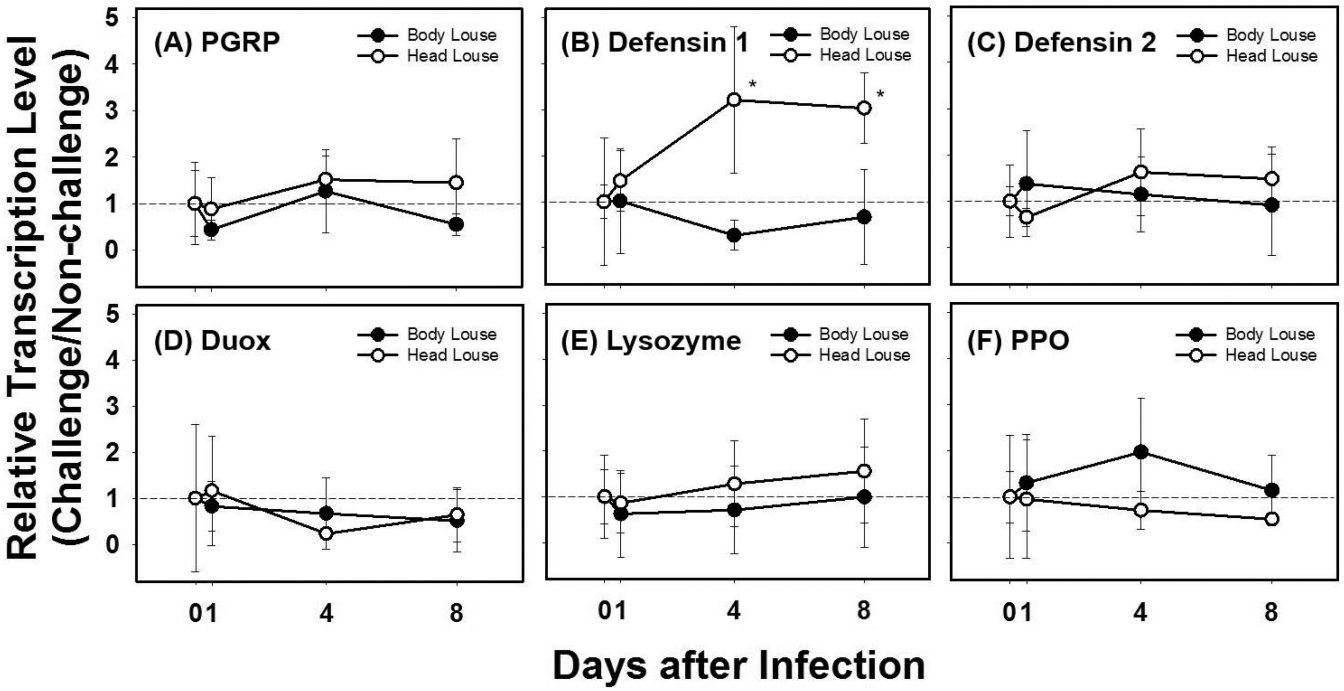

Figure 4.

Comparison of the fold-changes of transcript of 6 major immune genes between body and head lice after a single oral challenge with *B. quintana*. Symbols with asterisk (*) indicate statistically significant difference between body and head lice ($p<0.05$). Error bars indicate standard deviation of the mean.

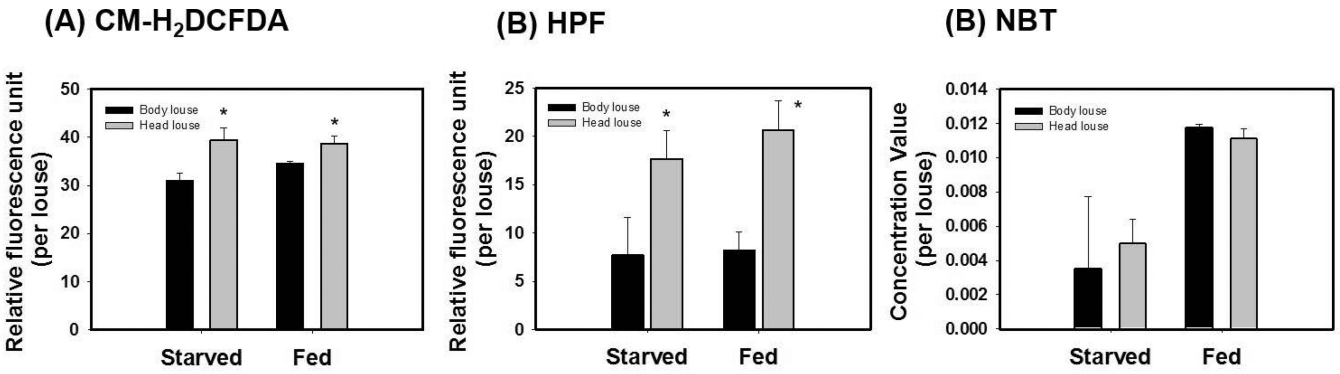

Figure 5.

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

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.

Table 2

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.