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Differential gene expression in laboratory strains of human head and body lice when challenged with *Bartonella quintana*, a pathogenic bacterium

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Abstract

Human head and body lice are obligatory hematophagous ectoparasites that belong to a single species, *Pediculus humanus*. Only body lice, however, are vectors of the infectious Gram-negative bacterium *Bartonella quintana*. Because of their near identical genomes, yet differential vector competence, head and body lice provide a unique model system to study the gain or loss of vector competence. Using our *in vitro* louse-rearing system, we infected head and body lice with blood containing *B. quintana* in order to detect both differences in the proliferation of *B. quintana* and transcriptional differences of immune-related genes in the lice. *B. quintana* proliferated rapidly in body lice at 6 days postinfection, but plateaued in head lice at 4 days postinfection. RNAseq and quantitative real-time PCR validation analyses determined gene expression differences. Eight immunoresponse genes were observed to be significantly different with many associated with the Toll pathway: Fibrinogen-like protein, Spaetzle, Defensin 1, Serpin, Scavenger receptor A and Apolipoporhrin 2. Our findings support the hypothesis that body lice, unlike head lice, fight infection from *B. quintana* only at the later stages of its proliferation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Primers used for quantitative real-time PCR (qPCR) experiments (1–3) and qPCR validation of immunoresponse genes (4–21).

Keywords

disease vector; trench fever; insect; ectoparasite; Pediculus humanus humanus; Pediculus humanus capitis

Introduction

Human head lice (*Pediculus humanus capitis*) and body lice (*Pediculus humanus humanus*) are obligatory hematophagous ectoparasites that have thrived solely on human blood for 5–7 million years (Reed *et al*., 2004; Light *et al*., 2008). Body lice evolved from conspecific head lice when humans started wearing clothing ~40 000–70 000 years ago (Kittler *et al*., 2003; Raoult *et al*., 2008; Veracx and Raoult, 2012). Head and body lice are thought to be very closely related species or even ecotypes of the same species (Leo *et al*., 2002; Li *et al*., 2010; Olds *et al*., 2012). Although head and body lice do not interbreed in the wild (Busvine, 1948; Schaefer, 1978), fertile F_1 hybrids have been reported under laboratory conditions (Mullen & Durden, 2009). They differ, however, in some very well characterized life-history patterns/traits. Head lice live and feed on the human scalp and females attach their eggs to the base of hair shafts. In contrast, body lice live mostly off of their human hosts in clothing, feed upon body regions other than the scalp and secure their eggs to clothing (Light *et al*., 2008). Another noticeable phenotypic difference is that body lice are larger in size than head lice (Light *et al*., 2008; Bonilla *et al*., 2009).

One of the most important physiological differences between head and body lice is that head lice do not transmit human diseases, whereas body lice are vectors of the following bacterial diseases to humans: trench fever caused by *Bartonella quintana*, relapsing fever caused by *Borrelia recurrentis*, and epidemic typhus caused by *Rickettsia prowazekii* (Light *et al*., 2008; Bonilla *et al*., 2009). Whether or not an insect can be a competent disease vector is probably influenced, in part, by their innate immune response.

The innate immune response system of insects has a variety of antimicrobial pathways including, the immune deficiency (IMD) pathway, Toll pathway and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, which have been studied extensively in *Drosophila melanogaster*. These immune pathways ultimately lead to the regulation of the insect's humoral [e.g. effector genes encoding the antimicrobial peptides (AMPs), Defensin 1 and Defensin 2] and cellular [e.g. effector genes such as those encoding nodular and prophenyloxidase (PPO), which affect the louse's haemocytic (macrophages) response] immune responses.

To date, several studies have compared the genes in the innate immune systems of the mosquito (Christophides *et al*., 2002), honeybee (Evans *et al*., 2006), flour beetle (Zou *et al*., 2007) and silkworm (Tanaka *et al*., 2008), and highlight both similarities and striking differences in gene families. In a similar comparison, both head and body lice were found to lack the immune response genes, IMD, GNBP and FADD, but still show differential immune responses to Gram-positive and -negative bacteria (Kim *et al*., 2011). When challenged with *Staphylococcus aureus*, a Gram-positive bacterium, a similar pattern of phagocytic activity in head and body lice was seen. A different pattern of phagocytic activity

was seen, however, when head and body lice were challenged with *Escherichia coli*, a Gram-negative bacterium. In this case, the head louse immune system responded much more aggressively against the Gram-negative bacteria, even in the absence of a functional IMD pathway (Kim *et al*., 2011).

B. quintana is a slow-growing, haemotropic, Gram-negative bacterium that infects humans and uses them as their reservoir host. *B. quintana* is the causative agent for trench fever, an epidemic disease that affected millions before the use of antibiotics (Byam & Lloyd, 1920; Brouqui & Raoult, 2006). *B. quintana* was recognized as being vectored between humans primarily through contact with the faeces of infected human body lice as early as 1920 (Byam & Lloyd, 1920). Today, this epidemic is re-emerging as urban trench fever in the homeless and the poor, especially those who are immunocompromised, such as alcoholics and those infected with human immunodeficiency virus (Hotez, 2008). Trench fever, also known as 5-day fever, is defined as the infection of human blood by *B. quintana* and typical symptoms include severe headaches, pain in the legs, weakness, nausea, anorexia, abdominal pain and insomnia. It can also cause more serious conditions such as bacillary angiomatosis and endocarditis (Byam & Lloyd, 1920; Harms & Dehio, 2012).

B. quintana proliferates inside the gut lumen of body lice and has no effect on lice viability (Weyer, 1960). *B. quintana* proliferation has been quantified in body lice over a 17-day period where growth over the first 6 days of the experiment following infection was stagnant and significant proliferation was not seen until day 7 (Seki *et al*., 2007). From day 7 until day 15 of the experiment, *B. quintana* cell numbers increased by over three orders of magnitude (Seki *et al*., 2007). *B. quintana* multiplies throughout the life cycle of the body louse and contaminates their faeces, which are excreted (Kostrzewski, 1949). *B. quintana* survives remarkably well in faeces and can remain viable for as long as 1 year (Kostrzewski, 1949). The bacteria infect humans when an infested person scratches the louse bite, abrading the skin and allowing the infected faeces to make contact with the blood stream (Raoult & Roux, 1999). *B. quintana* eventually colonizes erythrocytes (Harms & Dehio, 2012). Lice then take up the infected erythrocytes, during feeding, and the cycle completes (Harms & Dehio, 2012). To date, however, little is known regarding the molecular interactions involving *B. quintana* and the human body and head louse.

In the present study, we infected human head and body lice with *B. quintana*, by feeding them an infected blood meal, and quantified the number of *B. quintana* cells that were present over time in order to determine any differences in *B. quintana* proliferation after infection. We also examined the whole genomic transcriptome profiles of head and body lice at 8 days post-infection in order to identify possible candidate genes involved in vector competence. With the body louse genome being fully annotated and with the availability of relatively complete body and head louse transcriptomes (Kirkness *et al*., 2010; Olds *et al*., 2012), we were able to ascertain transcriptome differences, giving us insights into why body lice vector *B. quintana* and head lice do not. To accomplish this goal, we measured the differential gene expression using an Illumina platform and subsequent RNAseq analysis with quantitative real-time PCR (qPCR) validation in both head and body lice after infection with *B. quintana*.

Results

Bartonella quintana *infection and proliferation*

All infection and proliferation experiments were replicated five times for both head and body lice. Depending on the number of lice available at the start of each experiment and the higher mortality seen with older lice, louse samples taken from identical timepoints post-infection varied from 3 to 5 (Table 1). Head lice experiments had five replicates at timepoints 0, 2 and 4 days, four replicates at 6 days, and three replicates at 8, 10 and 12 days. Body lice had five replicates at timepoint 0, four replicates at 2 days, five replicates at 4 days, four replicates at 6 days, five replicates at 8 days and three replicates at 10 and 12 days. A statistically similar amount of *B. quintana* cells per ml of blood was used for each experiment. For head lice, a mean of $4.47 \times 10^8 \pm 1.09 \times 10^8$ colonyforming units (CFU)/ml blood was used in each of the five experiments. Body lice replicates were fed a mean of $3.77 \times 10^8 \pm 1.34 \times 10^8$ CFU/ml blood. These values were not significantly different ($P > 0.05$). *B. quintana* cell counts per head or body louse were both normalized to 9.45×10^4 at the 0 day timepoint post-infection (lowest 0 day cell count) in order to compare cell counts between head and body lice (Fig. 1). It was also determined that there were no differences in the mortality response between head and body lice after *B. quintana* infection (data not shown).

Head lice at 2 days post-infection had a mean of $1.34 \times 10^5 \pm 6.06 \times 10^4$ *B. quintana* cells per louse (1.41 ± 0.64-fold change from the 0-day timepoint), at 4 days post-infection had a mean of $9.84 \times 10^4 \pm 5.95 \times 10^4$ *B. quintana* cells per louse (1.04 ± 0.62-fold change from the 0-day timepoint), at 6 days post-infection had a mean of $4.95 \times 10^4 \pm 2.46 \times 10^4$ *B. quintana* cells per louse (0.52 ± 0.26-fold change from the 0-day timepoint), at 8 days post-infection had a mean of $1.78 \times 10^4 \pm 5.26 \times 10^3$ *B. quintana* cells per louse (0.19 ± 0.056-fold change from the 0-day timepoint), at 10 days post-infection had a mean of $1.23 \times 10^4 \pm 3.98 \times 10^3$ *B. quintana* cells per louse (0.13 ± 0.042-fold change from the 0-day timepoint), and at 12 days post-infection had a mean of $1.56 \times 10^4 \pm 2.33 \times 10^3$ *B. quintana* cells per louse [0.16 ± 0.025-fold change from the 0-day timepoint (Fig. 1)].

Body lice at 2 days post-infection had a mean of $6.15 \times 10^4 \pm 3.04 \times 10^4$ *B. quintana* cells per louse (0.65 ± 0.33-fold change from the 0-day timepoint), at 4 days post-infection had a mean of $1.75 \times 10^5 \pm 7.32 \times 10^4$ *B. quintana* cells per louse (1.86 ± 0.80-fold change from the 0-day timepoint), at 6 days post-infection had a mean of $1.52 \times 10^5 \pm 4.78 \times 10^4$ *B. quintana* cells per louse (1.61 ± 0.62-fold change from the 0-day timepoint), at 8 days post-infection had a mean of $1.13 \times 10^6 \pm 2.74 \times 10^5$ (9.58 ± 3.53-fold change from the 0-day timepoint), at 10 days post-infection had a mean of $2.69 \times 10^6 \pm 6.52 \times 10^5$ (32.46 ± 6.90-fold change from the 0-day timepoint), at 12 days post-infection had a mean of $2.88 \times 10^6 \pm 4.49 \times 10^5$ [30.46 ± 4.75-fold change from the 0-day timepoint (Fig. 1)]. The cell counts at the last two timepoints post-infection (10 and 12 days) were not significantly different from each other ($P > 0.05$).

When comparing *B. quintana* cell counts per louse in head vs body lice at each timepoint individually, body lice showed significantly more *B. quintana* cells per louse at 8 days

(63.7-fold change), 10 days (219.3-fold change) and 12 days (184.8-fold change) post-infection ($P < 0.05$; Fig. 1).

Sequence analysis and read mapping

Mapping of Illumina read data against the 10 992 body louse gene models (including miRNAs) resulted in a mean of 283× coverage from all three biological replicates. RNAseq analysis resulted in a mean of 68 367 908 total reads with a mean of 47 067 912 reads uniquely mapped.

Analysis of differential gene expression

Considering all 10 992 genes, 23, 19, 552 and 954 genes were significantly differentially expressed for the four two-way comparisons, respectively: control (uninfected) head lice (HLC) vs treated (infected) head lice (HLT), control body lice (BLC) vs treated body lice (BLT), HLC vs BLC and HLT vs BLT, respectively (Table 2). To determine if genes were acting in a similar fashion when compared between ecotypes, log2 fold change values for genes that were found to be significant in both the HLC vs BLC and HLT vs BLT two-way comparisons ($n = 437$) were plotted against one another (Fig. 2). Most genes showed a similar fold change pattern regardless if they were from uninfected or infected lice. Only five genes showed a different fold change pattern when compared between uninfected and infected lice. Two genes were hypothetical proteins, but PHUM427700 (Apolipoporhrin 2/Apolipoprotein-D), PHUM365700 (Defensin 1) and PHUM595870 (Defensin 2) were immunoresponse genes.

Of the 93 immunoresponse genes found in the genomes of head and body lice and of an additional 12 genes associated with the immune response, only 9 and one (PPO), respectively, were found to be significantly different in negative binomial analysis in the two-way comparisons (Table 3). qPCR was conducted on these 10 significant genes and an additional six genes of interest for a total of 16 immunoresponse genes. qPCR showed significance in a total of eight genes in 18 two-way comparisons (Table 3).

In the HLC vs HLT comparison, no genes were found to be significantly differentially transcribed using either of the analysis methods (i.e. negative binomial and qPCR in combination; Fig. 2A) and only the two defensin genes (Defensin 1 and 2) showed significance in the negative binomial analysis.

Overall, five genes showed a significant increase in transcription in BLT vs BLC samples using both or one of the two analyses compared with a single gene (Defensin 2) that was overtranscribed in BLC as determined by the qPCR analysis. Apolipoporhin 2 transcription was found to be significantly higher in BLT vs BLC samples using both the negative binomial and qPCR analyses (Fig. 2B). Defensin 2 transcription was significant in both negative binomial (higher in BLT than in BLC) and qPCR (higher in BLC than in BLT) analyses, but the results of these analyses were contrasting.

When comparing HLC with BLC, six genes were significant in both the negative binomial and qPCR analyses (Fig. 3A). Four genes (Apolipoporhin 2, Defensin 1, Scavenger receptor A and Serpin) were over-expressed in HLC vs two genes over-expressed in BLC

(Fibrinogen-like Protein and Spaetzle). Overall, 10 genes were significantly over-expressed in both or one of the analyses, with five genes over-expressed in HLC vs six genes in BLC.

When comparing HLT with BLT, three genes were significantly differentially transcribed as concluded from both analysis methods. Fibrinogen-like protein and Spaetzle were significantly over-transcribed in BLT, and Scavenger receptor A was over-transcribed in HLT (Fig. 3B). These results were similar to those obtained for uninfected lice where Fibrinogen-like Protein and Spaetzle were overtranscribed in BLC, whereas Scavenger receptor A was over-transcribed in HLC. Overall, 12 genes were found to be significantly over-transcribed by both or one of the analysis methods, with five genes over-transcribed in HLT vs seven genes over-transcribed in BLT. As was the case for the BLC vs BLT comparison, Defensin 2 was significant in both analysis methods (qPCR and negative binomial), but each resulted in a different conclusion. qPCR suggests Defensin 2 is over-transcribed in treated head lice whereas negative binomial suggests Defensin 2 was overtranscribed in treated body lice. (Fig. 4A, B).

Discussion

Bartonella quintana *infection and proliferation*

The proliferation of *B. quintana* in body lice was similar to that previously reported by Seki *et al*. (2007), where the number of *B. quintana* per louse remained relatively constant until day 6 post-infection and then significant proliferation occurred between 6 and 8 days post-infection. Conversely, head lice showed little or no proliferation between 4 and 6 days post-infection, and from this time the cell count of *B. quintana* remained steady. These results, although not definitive, suggest that the ability of body lice, but not head lice, to vector *B. quintana*, is attributable, in part, to the ability of the bacteria to grow inside of the body louse gut (and possibly its hemocoel) following the ingestion of an infected blood meal. Proliferation in the body louse gut would probably lead to an increased number of viable bacteria in the faeces and subsequently to a greater chance for humans to be exposed to and infected with *B. quintana*. The mechanisms allowing proliferation in body vs head lice, however, are still largely unknown.

Differential gene transcription before and after B. quintana *infection*

Head and body lice share virtually the same genetic background, including all 93 immunoresponse genes. Both organisms lack the recognition gene, GNBP and both IMD and FADD from the IMD pathway. Because there are no genes that are present in one but not the other organism (Olds *et al*., 2012), differences in gene transcription could give rise, in part, to the difference in vector competence observed between head and body lice.

Of the hundreds of genes found differentially transcribed using the negative binomial analysis, a gene ontology analysis revealed no enrichment categories of significance in terms of biological differences of these organisms (data not shown). A few metabolic enrichment categories were identified, but none of these seem to explain the differences in vector competence. We identified six immunoresponse genes, however, that did show significant differential transcription in the negative binomial analyses and were confirmed with qPCR

when comparing between the two ecotypes. Differential expression of these types of genes would certainly lead to differential shaping of the innate immune response in lice and may have a role in explaining the differences in vector competence between head and body lice.

Three genes, Fibrinogen-like Protein, Spaetzle and Scavenger receptor A were confirmed by both analysis methods in both ecotype comparisons (HLC vs BLC and HLT vs BLT), with consistently higher transcript levels found in both BLC and BLT samples for Fibrinogen-like Protein and Spaetzle and consistently higher transcript levels in both HLC and HLT samples for Scavenger receptor A. Fibrinogen-like Protein, which is part of the cellular innate immune response, is a common bacterial recognition protein conserved in invertebrates and mammals, and its role is to directly tag microbial sugar moieties for phagocytic degradation in blood serum or haemolymph (Faik *et al*., 2011). The transcript levels of Fibrinogen-like Protein were significantly greater in BLC when compared with HLC (2.00-fold) and this level increased when BLT was compared with HLT (2.77-fold). There was also a *B. quintana* infection effect in body lice, as BLT resulted in 1.34-fold more transcript than that detected in BLC. By 8 days post-infection, head lice had killed or contained the invading *B. quintana* whereas this bacterium was still proliferating and spreading in body lice. Because this protein targets bacteria for endocytosis by haemocytes, it seems likely that body lice were responding significantly to the later stages of infection when proliferation has reached a maximum, probably allowing the *B. quintana* invasion into the hemocoel.

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

The transcript level of Spaetzle was 1.64-fold higher in BLC compared with HLC. At 8 days post-infection, BLT was 2.30-fold higher than HLT, supporting the hypothesis that as *B. quintana* proliferates in the gut and invades the hemocoel, it elicits an immune response, specifically by up-regulation of the Toll pathway. Also, BLT was 1.4-fold greater than BLC, which once again supports the hypothesis that as *B. quintana* proliferates it enters the hemocoel and elicits a humoral immune response.

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

The transcript level of Scavenger receptor A was 1.66-fold greater in HLC than in BLC and was 1.75-fold greater in HLT than in BLT. It has been previously shown that head lice have more rapid and active phagocytes that result in higher phagocytic activity than do body lice, specifically directed against its Gram-negative endosymbiont, *Riesia*, during its migration from the stomach mycetome to the filarial mycetome (Perotti *et al*., 2007). The over transcription of Scavenger receptor A in head lice may be a possible reason for the

heightened phagocyte activity in head lice and another possible factor in the head louse's ability to attenuate *B. quintana* initial proliferation, a finding consistent with its reduced vector competence.

The Defensins are effector genes within the Toll pathway, which are translated into AMPs that are produced in almost all epithelial and immune-related cells. Defensins secreted by epithelial cells in the alimentary tract are one of the first humoral immune defence barriers against foreign pathogens (Kim *et al*., 2012). Transcription of Defensin 1 was also found to be significantly increased in HLC vs BLC samples by both analyses. qPCR results indicated that HLC maintained the transcript level of Defensin 1 at a value 1.67-fold higher compared with BLC, a finding that correlated with previous studies (Kim *et al*., 2012). This value was reduced to 1.19-fold at 8 days post-infection (HLT vs BLT) but was still significantly elevated in HLT. In head lice, it appears that *B. quintana* is either contained in the gut or has been killed by the increased amount of Defensin 1 present in the gut owing to the inherent higher basal transcription level of this gene. At 8 days post-infection, Defensin 1 transcripts increased 2.23-fold in BLT compared with BLC. It is likely that at 8 days post-infection, the proliferation of *B. quintana* has caused Toll-pathway up-regulation, inducing Defensin 1 production in body lice. This late humoral immune response may be one of the reasons why *B. quintana* does not kill body lice post-infection.

Like Defensin 1, Apolipoporhin 2 was also significantly over-expressed in HLC compared with the BLC sample according to both analyses. Apolipoporhin 2 is a general stress-reducing anti-oxidant and is shown to be age-dependently up-regulated in the human brain, as well as in patients with Alzheimer's disease (Kalman *et al*., 2000; Loerch, 2008). When over-expressed in *D. melanogaster*, it also increases the fly lifespan, as well as functioning as a lipid antioxidant conferring resistance to oxidative stress (Walker *et al*., 2006).

The transcript level of Apolipoporhin 2 was 1.69-fold greater in HLC than in BLC samples. The head louse under uninfected conditions is thought to keep its humoral (AMPs) and cellular (phagocytic) immune responses constitutively up-regulated to rapidly and effectively deal with any *B. quintana* obtained in an infected blood meal. Sustaining this enhanced immune response would probably use more energy, which would create oxidative stress in the form of reactive oxygen species and necessitate the constitutive up-regulation of Apolipoporhin 2. Interestingly, a 2.19-fold increase in its transcript level was also seen in BLT vs BLC. Because Apolipoporhin 2 functions as a general stress-reducing agent, transcription of it is probably increased as a result of the oxidative stress associated with increased *B. quintana* invasion into the hemocoel following its proliferation in the gut. Under infected conditions and hemocoel invasion, the body louse appears to up-regulate its humoral (Spaetzle, Defensin 1) and phagocytic (Fibrinogen-like Protein) immune responses, probably causing oxidative stress and necessitating the subsequent increased transcription of the stress-reducing Apolipoporhin 2 at the later stages of *B. quintana* infection and proliferation.

While there are still many questions as to the exact mechanisms leading to the enhanced vector competence of body vs head lice, our present results demonstrate that a number of immunoresponse genes are differentially transcribed in a manner consistent with an altered

innate immune response, which could potentially be involved in the vector competence differences observed; however, it is important to note that other molecular pathways and factors may be involved in, or even may play a greater role in, vector competence, as compared with the immune system genes/proteins. The relative roles that these different processes play in vector competence may potentially be resolved using a reverse genetics strategy/strategies. For example, in order to further explore this complex interaction, a RNA interference (RNAi) knockdown approach might be helpful to elicit gene function; body and head lice contain the necessary RNAi machinery for RNAi knockdown (Pittendrigh *et al*., 2011) and we have previously demonstrated RNAi knockdown can be accomplished (Yoon *et al*., 2011). In addition, differences in phagocytic response between head and body lice must be explored. The introduction of green fluorescent proteinlabelled *B. quintana* into the hemocoel of head and body lice, coupled with a phagocytosis activity assay, would enable a comparison of the cellular immune response in head and body lice. Thus, the present study provides results that will allow researchers to investigate the next steps in deciphering this complex interaction. It should also be pointed out that, although the low level of antibiotics that were used to rear both the head and body lice in the present study did not significantly affect the specific developmental parameters examined, the antibiotic treatment may have altered the associated microbiomes of these lice and possibly affected their endosymbionts; alterations that clearly could have affected the ability of either the body or head lice to vector *B. quintana*.

Experimental procedures

Bacterial strain and culture conditions

The JK31 strain of *B. quintana*, isolated from a patient with human immunodeficiency virus who had blood infection and bacillary angiomatosis (Zhang *et al*., 2004), was maintained in a biosafety level 2 facility at the Environmental and Molecular Toxicology Laboratory at the University of Massachusetts-Amherst. Frozen *B. quintana* stock was cultured on chocolate agar plates at 37 °C, in CO_2 jars for 7–10 days (Zhang *et al*., 2004). *B. quintana* was then passed to a fresh chocolate agar plate and cultured for an additional 4 to 5 days before use. Bacteria were harvested from the plate by adding 1 ml of phosphate-buffered saline (PBS; 0.1 M, pH 7.2) with an automatic pipettor and resuspending the bacteria by repetitively pumping the solution 5–10 times. CFUs per µl values were then estimated using the dilutions of bacterial suspension in PBS (10 µl, 10^4-to 10^7-fold dilutions) plated on the chocolate agar plates.

Body and head lice colonies

A field population of human body louse (Frisco-BL) was originally collected from nine homeless individuals in San Francisco (December, 2008) by Dr Jane Koehler's research group and a head louse population (Bristol-head lice; BR-HL) was collected from Bristol, UK (Yoon *et al*., 2004). Both colonies were maintained on the *in vitro* rearing system with human blood supplemented with a penicillin/streptomycin solution at 1 µl/ml (Sigma Chemicals, St. Louis, MO, USA; Takano-Lee *et al*., 2003; Yoon *et al*., 2006). The amount of antibiotic used only suppressed bacterial growth in the blood meal so feeding cups only had to be changed every 24 h, not every 12 h as would be necessary without antibiotics. By

24 h, there will be bacterial growth in the blood meal and any longer times would lead to an increase in mortality. Because lice take only small blood meals their endosymbionts are exposed to even less antibiotic than the bacteria growing in the blood meal. The differences in developmental time, egg hatch and fecundity have also been examined and no significant differences in lice reared *in vivo* compared with lice reared on the *in vitro* rearing system with antibiotic at these low concentrations were found (Takano-Lee *et al*., 2003; Yoon *et al*., 2006). Lastly, late third instars or first day adults were fed on antibiotic-free blood for 3 days before an experiment to ensure purging of antibiotics from adult lice.

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

Bartonella quintana were harvested from a single chocolate agar plate using a sterile loop and suspended into 1 ml PBS (0.1 M, pH 7.2, 1 plate/ml PBS). Spectrophotometric readings (OD_{600}) were taken for *B. quintana* suspended in PBS to approximate cell counts per ml of blood. *B. quintana* cells were pelleted by centrifugation at 1000 *g* for 4 min, and resuspended in 100 μl of fresh PBS to remove residual media. A 10-μl aliquot of the suspension was serially diluted into M199 media (Sigma Chemicals), supplemented with glutamine, sodium pyruvate and 20% fetal bovine serum (M199S), and dilutions (10^{-4}–10^{-8} ml PBS suspension/ml M199S) were plated in triplicate for CFU/ml blood determination. The remaining suspension was then mixed with 4 ml of human blood without antibiotics to obtain a titre of ~1×10^7 CFU/ml (Kosoy *et al*., 2004) and used to fill the blood reservoir of each feeding unit. Approximately 100 lice, which had been starved for 6 h, were fed on *B. quintana*-infected blood using the *in vitro* rearing system for 18 h (overnight). Following feeding, lice were transferred to a new rearing unit with non-infected blood for the remainder of the experiments. Mortality data were also recorded for all experiments. Five replicated experiments for both head and body lice were carried out.

Detection of B. quintana *proliferation by qPCR*

Five *B. quintana*-infected and uninfected lice from both colonies were collected at various timepoints [0 (immediately after taking a blood meal), 2, 4, 6, 8, 10, 12 days] post-infection and the number of *B. quintana* cells/louse was determined by qPCR. Genomic DNA was extracted using Qiagen DNeasy blood and tissue kit according to manufacturer's instructions (Qiagen, Valencia, CA, USA). qPCR was performed to quantify cell counts with a SYBR-green power mix using the StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and the Bq-ITS-F and Bq-ITS-R primer set (Table S1), which amplified an 89-base pair (bp) fragment in the 16S-23S ribosomal RNA gene of *B. quintana* (Seki *et al*., 2007). Non-infected lice from each colony were also sampled to ensure there was no background *B. quintana* contamination. The number of amplified DNA copies was calculated based on Ct values of a standard curve of known copy numbers of a 134-bp fragment containing the target 89-bp fragment amplified using the RpL13A-rt-F and RpL13A-rt-R primers (Seki *et al*., 2007). Genomic DNA extraction was normalized using qPCR with primers against the louse voltage-sensitive sodium channel α-subunit gene, amplified using the L-SC-F and L-SC-R primers (Table S1). All experiments were standardized to the lowest number of *B. quintana* cells per louse at the 0 day timepoint in order to normalize the proliferation curve. Two-sided Student's *t*-tests were used to compare

the number of *B. quintana* cells in head and body lice at each timepoint using Microsoft Excel ($P < 0.05$).

RNASeq of B. quintana*-infected head and body lice*

Approximately 200 lice from each colony, which were starved for 6 h, were fed on *B. quintana*-infected (treated) or on uninfected blood (control) using the *in vitro* rearing system for 18 h (overnight) as described in the previous section. Fed lice were transferred into a clean rearing unit with uninfected blood and maintained for 8 days post-infection. Louse samples were collected at 8 days post-infection (20 lice/experiment) and stored in liquid N_2. Collected louse samples from three replicated experiments (20 BLT × 3, 20 HLT × 3, 20 BLC × 3, and 20 HLC × 3) were packaged on dry ice and shipped to the Pittendrigh laboratory (University of Illinois at Urbana-Champaign) for transcriptome analysis.

Total RNA was extracted from each pooled sample using an RNA extraction kit (Qiagen) according to manufacturer's instructions. Samples were submitted to the Keck Center at the University of Illinois at Urbana-Champaign and sequenced on an Illumina Genome Analyzer 2 (GA2) using 50 pyrosequencing cycles. A total of 12 samples (four treatments × 3 replicates) were sequenced, over six lanes with two bar coded samples per lane using 100-bp paired end reads and data obtained as .fastq files.

Raw data processing and transcriptome analysis

Raw sequence read data from head and body louse cDNA pools were analysed using CLC GENOMICS WORKBENCH 4×. Pre-processing involved trimming of nucleotides with quality scores of <20 from Illumina GA2 reads. The body louse gene models version 1.2 were downloaded from Vectorbase.org. Processed head and body louse read data from each of the 12 samples were independently mapped to the body louse gene models using the parameters: mismatch cost = 2; insertion and deletion cost = 3; and similarity ≥80%.

RNAseq analysis was performed with CLC GENOMICS WORK-BENCH, modelled after Mortazavi *et al*. (2008) except that in each case, the 'reference genome' included only transcripts so as to allow direct comparison of individual transcripts from each treatment. RNAseq parameters on CLC GENOMICS WORKBENCH included paired distance of 180–250, minimum reads of 10, maximum mismatches of 2, minimum length fraction of 0.9 and minimum similarity fraction of 0.8. The data were treated as prokaryotic owing to omission of annotation with the transcript files we used for each organism. Values for each transcript were reported as unique reads mapped.

Assuming a negative binomial distribution, unique reads mapped were analysed using R (R Development Core Team, 2008) with DESEQ (Anders & Huber, 2010) software packages. Each transcript was analysed for (four) two-way comparisons (HLC vs HLT, BLC vs BLT, HLC vs BLC and HLT vs BLT). DESEQ uses a geometric mean to adjust for uneven distribution of reads across replicates and treatments to allow direct comparison of resultant values to calculate fold change and *P* value. Consequently log2 fold change and an adjusted *P* value are calculated. To control for false discovery rate, the adjusted *P* value was calculated with a Benjamini–Hochberg procedure (Benjamini & Hochberg, 1995). The

resulting list of transcripts was considered significantly differentially expressed based upon the two-way comparison performed.

Finally, a reverse transcriptase qPCR was run on 17 immunoresponse genes, 11 of which were found to be significant in the negative binomial analysis and six other genes that were not found to be significant but were of interest. Multiple primer sets were constructed for each of the 17 target genes to determine a single best primer set for qPCR (Table S1). From our 12 samples (three replicated experiments of four treatments), total RNA was extracted and cDNA synthesized using Promega GOScript reverse transcriptase system (Promega, Madison, WI, USA) under standard conditions. A cDNA of Elongation factor 1-α was also synthesized and used as a normalization control between samples. cDNA samples were diluted based upon the lowest concentration of Elongation factor 1-α so that concentrations were the same across all samples. Equalized samples were prepared for qPCR using the Promega GOTaq qPCR master mix and then analysed on the A&B Bio Systems Step One Plus real time PCR system. Each gene was run in triplicate. A mean and standard error was calculated for each gene based upon the values from the triplicate set of values and a statistical pairwise analysis was conducted, based upon the aforementioned two-way comparisons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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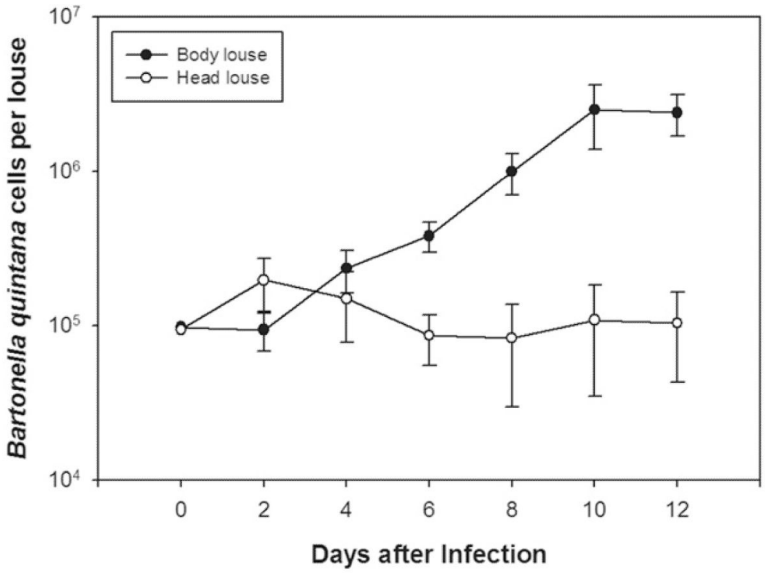

Figure 1.

Bartonella quintana proliferation in body and head lice. Adult female 1–4 day old body (San Francisco strain, Frisco BL) and head (Bristol strain, BR-HL) lice, maintained on the *in vitro* rearing system (Yoon *et al*., 2006), were fed on human blood inoculated with *B. quintana* harvested from 7–10 day old plates, at ~1 × 107 colony-forming unit/ml blood (Seki *et al*., 2007). After an overnight feed, lice were transferred to uninfected blood. Five lice were collected at 2-day intervals, and genomic DNA extracted to determine *B. quintana* proliferation, with primers amplifying an 89 base pair fragment in the 16S–23S rRNA gene of *B. quintana* (Seki *et al*., 2007). Cell counts were normalized to the lowest 0 day cell count as well as relative lice genomic DNA detected by louse sodium channel primers.

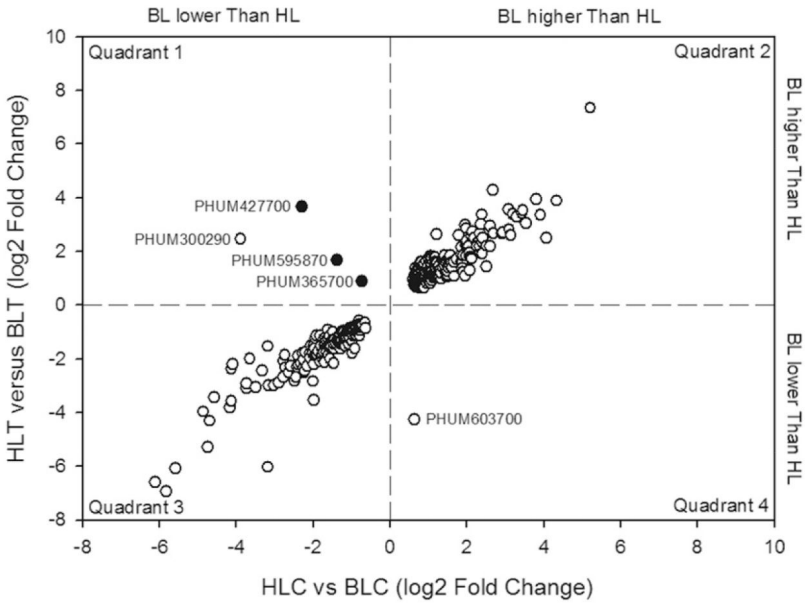

Figure 2.

Log2 fold change values plotted for the 437 genes common to both control head lice (HLC) vs control body lice (BLC) and treated head lice (HLT) vs treated body lice (BLT) two-way comparisons. Quadrant 1 contains genes where BLC had lower gene expression than HLC but BLT had higher gene expression than HLT. Quadrant 2 contains genes where BLC had higher gene expression than HLC but BLT had higher gene expression than HLT. Quadrant 3 contains genes where BLC had lower gene expression than HLC but BLT had lower gene expression than HLT. Quadrant 4 contains genes where BLC had higher gene expression than HLC but BLT had lower gene expression than HLT. Filled circles highlight the three immune response-related genes that fall into quadrant 1. HL, head lice; BL, body lice.

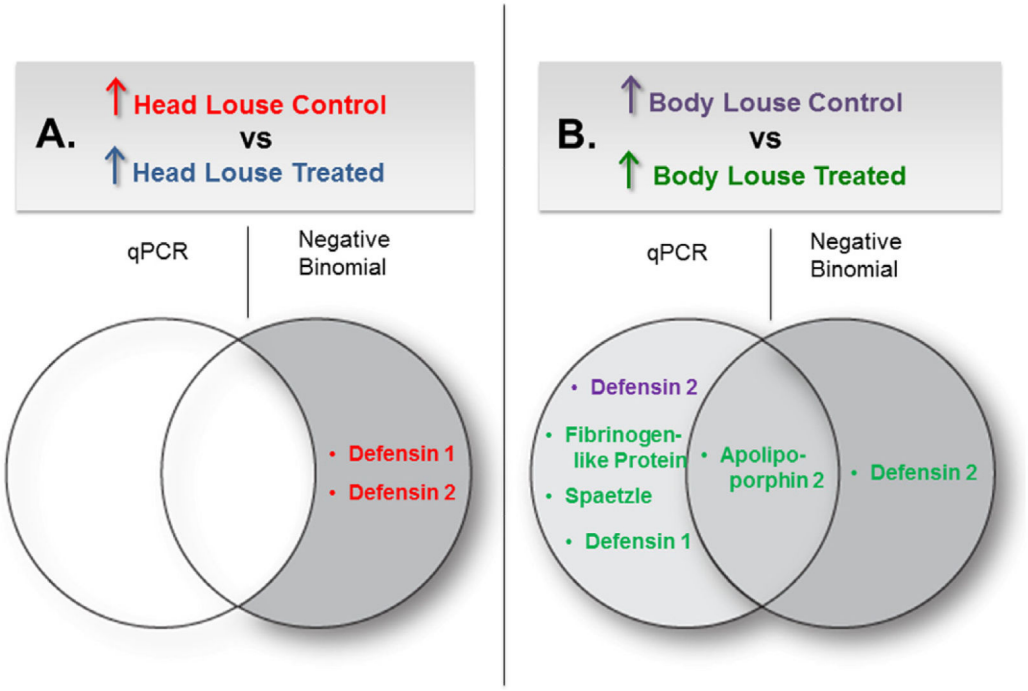

Figure 3.

Venn diagrams comparing statistically significant immune-related genes from transcriptome analysis when control (uninfected) head louse samples are compared with treated (infected) head louse samples (A) and when control body louse samples are compared with treated body louse samples (B). Gene transcription level in whole head and body lice under uninfected conditions and 8 days post-infection with *Bartonella quintana* were assessed using two methods: (1) bioinformatically using a negative binomial distribution, and (2) verified by quantitative real-time PCR (qPCR). Colours correspond to the immune-related gene and treatment under which it was found to be statistically significant ($P < 0.05$).

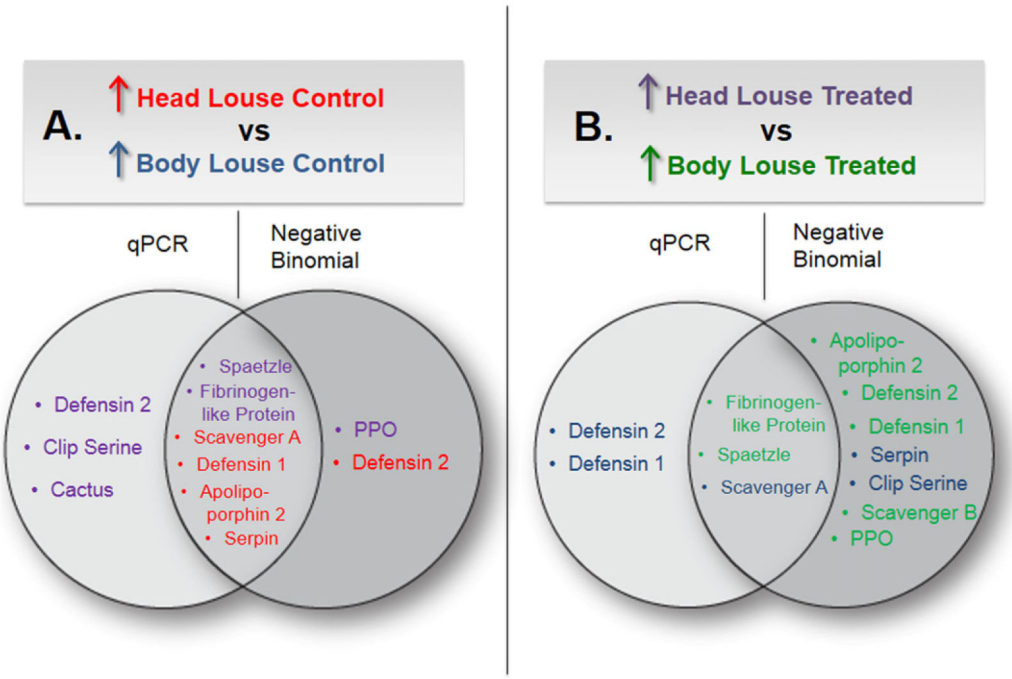

Figure 4.

Venn diagrams comparing statistically significant immune-related genes from transcriptome analysis when control (uninfected) head louse samples are compared with control body louse samples (A) and when treated (infected) head louse samples are compared with treated body louse samples (B). Gene transcription level in whole head and body lice under uninfected conditions and 8 days post-infection with *Bartonella quintana* were assessed using two methods: (1) bioinformatically using a negative binomial distribution, and (2) verified by quantitative real-time PCR (qPCR). Colours correspond to the immune-related gene and treatment under which it was found to be statistically significant ($P < 0.05$). PPO, prophenyloxidase.

Table 1

Number of replicates (five lice per replicate) taken, at each of the seven timepoints, post-infection with *Bartonella quintana*

Louse sample	Number of replicates per time post infection, days						
	0	**2**	**4**	**6**	**8**	**10**	**12**
Head	5	5	5	4	3	3	3
Body	5	4	5	4	5	3	3

Table 2

Statistically significant genes found to be differentially transcribed using the negative binomial analysis of the Illumina dataset for each two-way comparison

	Two-way comparisons			
Gene distribution	**HLC vs HLT**	**BLC vs BLT**	**HLC vs BLC**	**HLT vs BLT**
Total significant genes	23	19	546	945
Higher in treated	23	10	–	–
Higher in control	0	9	–	–
Higher in body lice	–	–	268	251
Higher in head lice	–	–	278	694

HLC, control head lice; HLT, treated head lice; BLC, control body lice; BLT, treated body lice.

Table 3

Statistically relevant immune response-related genes in head and body lice following infection by *Bartonella quintana* as determined by transcriptome analysis using two-way negative binomial analysis of RNAseq data and verified using quantitative real-time PCR

Gene description	Vectorbase ID PHUM	Negative binomial (Log2 fold change)				qPCR ($2^{\Delta\Delta Ct}$)			
		HLC-HLT	BLC-BLT	HLC-BLC	HLT-BLT	HLC[a]-HLT[b]	BLC-BLT	HLC-BLC	HLT-BLT
Fibrinogen-like Protein	500950	–	–	1.49	1.26	–	1.34	2	2.77
Scavenger A	66640	–	–	–0.77	–0.93	–	–	1.66	1.75
Spaetzle	596260	–	–	0.83	1.05	–	1.4	1.64	2.3
Defensin 1	365700	–0.69	–	–0.74	0.9	–	2.23	1.67	1.19
Defensin 2	595870	–1.47	1.58	–1.38	1.67	–	1.36	1.94	1.45
Apolipo-porphin 2	427700	–	5.04	–2.29	3.66	–	2.19	1.69	–
Serpin	311330	–	–	–2.06	–1.51	–	–	1.3	–
Clip Serine	571420	–	–	–	–2.25	–	–	2.34	–
Scavenger B	351630	–	–	–	0.55	–	–	–	–
PPO	448900	–	–	0.68	0.89	–	–	–	–
Traf 2	129280	–	–	–	–	–	–	–	–
Nodular	249370	–	–	–	–	–	–	–	–
Apolipo-porphin 1	154960	–	–	–	–	–	–	–	–
Scavenger B2	569610	–	–	–	–	–	–	–	–
Clip serine 2	451100	–	–	–	–	–	–	–	–
JNK basket	128040	–	–	–	–	–	–	–	–

PPO, prophenyloxidase; qPCR, quantitative real-time PCR; HLC, control head lice; HLT, treated head lice; BLC, control body lice; BLT, treated body lice.
[a]Value, over-expression in unhighlighted treatment. *All values listed are significant ($P < 0.05$).
[b]Value, over-expression in highlighted treatment. *All values listed are significant ($P < 0.05$).