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Short communication

Infection with the trypanosome *Crithidia bombi* and expression of immune-related genes in the bumblebee *Bombus terrestris*

Helge Schlüns [a,*], Ben M. Sadd [b], Paul Schmid-Hempel [b], Ross H. Crozier [a]

[a] School of Marine and Tropical Biology, Centre for Comparative Genomics, James Cook University, Townsville, Queensland 4811, Australia
[b] Institute for Integrative Biology, Experimental Ecology, Eidgnössische Technische Hochschule (ETH) Zentrum, CHN, CH-8092 Zurich, Switzerland

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ABSTRACT

Social bees and other insects are frequently parasitized by a large range of different microorganisms. Among these is *Crithidia bombi* (Kinetoplastida: Trypanosomatidae), a common gut parasite of bumblebees, *Bombus* spp. (Insecta: Apidae). Bumblebees are important pollinators in commercial and natural environments. There are clear detrimental effects of *C. bombi* infections on the fitness of bumblebees. However, little has been known about how the bee's immune system responds to infections with trypanosome parasites. Here, we study the immune response of *Bombus terrestris* on infection by *C. bombi*. We measured the expression of four immune-related genes (*Hemomucin*, *MyD88*, *Relish*, and *TEP7*) using RT-qPCR in adult *B. terrestris* workers that were either healthy or infected with the trypanosome parasite *C. bombi*. The potential recognition gene *Hemomucin* was significantly upregulated in the infected bees. Further, there was substantial and significant variation in all four genes among different bumblebee colonies irrespective of infection status.

1. Introduction

Parasitism is a frequent threat faced by most living organisms [1], and has important consequences for host fitness. There are a number of well-characterised host–parasite systems, especially for arthropod hosts [2,3]. It is clear from studies on these systems that hosts are not passive on infection, and show varying levels of resistance to parasites [4].

One host–parasite system that has been particularly well characterised in terms of its evolutionary ecology is that of the bumblebee host, *Bombus* spp., and the trypanosome parasite *Crithidia bombi* [5]. *C. bombi* belongs to the family Trypanosomatidae, which comprises unicellular eukaryotic kinetoplastid flagellates [6]. Many trypanosomes, such as *C. bombi*, have a single host cycle, occurring only in insects [6]. *C. bombi* is a widespread natural parasite of bumblebees, with prevalences of between 10% and 30% being common [7]. In *Bombus terrestris*, it has been shown that this gut-infecting parasite can have severe fitness implications, particularly relating to survival of queens over hibernation, colony founding, and the subsequent reproductive fitness of colonies [8,9]. Furthermore, infection of workers of another bumblebee species, *B. impatiens*, results in lower foraging efficiency [10]. This effect is important, as bumblebees are key pollinators in both commercial and natural environments [11]. Indeed, the importance of these pollinators has been raised given the recent concerns of diminishing honeybee populations [12]. Furthermore, the potential role of parasites in worldwide bumblebee declines [13] means that a good knowledge of the *Bombus–Crithidia* system is important for bumblebee conservation.

While host defense has been well characterized in some insects based on model systems and general immune elicitors [14,15], less is known about the molecular pathways involved in host defense in natural host–parasite interactions (with the exception of those of medical importance [16]). Two important signaling pathways involved in immunity in insects are the Toll and the Imd pathways [14,17,18]. The penultimate step (before effector molecules are synthesized, e.g. antimicrobial peptides) in both the Toll and the Imd pathway is the activation of nuclear factor κB-like transcription factors (NF-κB) termed Dorsal and Relish, respectively. Both the Toll and the Imd signaling pathway are stimulated on trypanosome infection in tsetse flies [19]. Furthermore, antimicrobial effector molecules of these pathways have been shown to be upregulated in dipterans on trypanosome infection [20–23]. To our knowledge there is no evidence that these two signaling pathways are involved in activating immune effector molecules other than antimicrobial peptides [14,15].

Recognition of pathogens is facilitated by members of various protein families such as PGRPs or the family of thioester-containing proteins (TEPs) [24]. Another molecule possibly functioning as a recognition receptor is Hemomucin, a surface

* Corresponding author. Tel.: +61 7 4781 5582; fax: +61 7 4725 1570.
E-mail address: helge.schluns@jcu.edu.au (H. Schlüns).

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glycoprotein that was suggested to be involved in inducing an immune response [25]. In *Drosophila melanogaster*, apart from being synthesized by hemocytes, Hemomucin is found in the several parts of the gut (proventriculus, midgut, and peritrophic membrane) [25]. This spatial location and Hemomucin's potential role in immunity make it an interesting candidate for defense against a gut-infecting parasite such as *C. bombi*.

While it is unclear exactly how the bumblebee immune system responds to infection by *Crithidia*, a few links between infection and host immunity have been established. When infected, *B. terrestris* show increased activity of the pro-phenoloxidase system (PO) [26]. Phenoloxidase has a core role in insect immunity and its activity results in the production of melanin [27]. Interestingly, PO activity was measured in the hemocoel while *Crithidia* only reside in the gut [26]. This suggests signaling between the gut and other tissues, and similar findings have been reported in dipterans infected with trypanosome parasites [20,21]. Furthermore, a recent study suggests differential upregulation of antimicrobial peptides presumably belonging to the Imd pathway of bees under infection by *C. bombi* [28].

The aim of the work presented here is to characterize better the response of *B. terrestris* on infection by *C. bombi*. To do this, we study the expression of four putative immune-related genes in adult *B. terrestris* workers infected with the trypanosome parasite *C. bombi*. The genes chosen were one gene each from the Toll and the Imd signaling pathway (*MyD88* and *Relish*, respectively) and two further potential recognition/effector genes (*Hemomucin* and *TEP7*).

2. Materials and methods

2.1. Bumblebee colonies and C. bombi

Bumblebee workers of the species *B. terrestris* were used in this study. Worker bees were sourced from four healthy colonies set up from queens collected in northwestern Switzerland in the spring of 2008. Bees were kept at 26 ± 1 °C under red light, with pollen and sugar water (ApiInvert®) provided *ad libitum*. Worker bees were collected when they eclosed as adults and isolated individually. These bees were allocated either to a control or to a treatment group. Seven days after eclosion the bees were starved of sugar water for 2.5 h, and then presented with 10 μl of *Crithidia* sugar water solution (1000 cells/μl) to take up *per os* (Treatment) or 10 μl of *Crithidia* free sugar water (Control). Experimental infections occurred between 14.30 h and 15.30 h. Bees were snap-frozen in liquid nitrogen 10 days after experimental infection and stored at −80 °C. Infection intensities were measured in 4–8 workers from each colony by dissecting the gut, and counts of parasite cells adjusted to number of parasite cells per bee.

The *C. bombi* isolate used in this experiment (08.068) was sourced from a queen collected in spring 2008 (Switzerland). For experimental infections, this isolate was pre-grown in vitro, with culturing carried out as described by Salathé-Zehnder [29].

2.2. RNA isolation and cDNA synthesis

RNA was extracted from whole abdomens of worker bumblebees using the Micro-to-Midi Total RNA Purification System (Invitrogen). Absence of genomic DNA was ensured as described previously [30]. First-strand cDNAs were synthesized using SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen) according to the recommendations of the supplier. 750 ng of total RNA was used per individual bee in the reverse transcription reactions in a total volume of 20 μl. Following an *E. coli* RNase H treatment the cDNAs of individual bees were diluted (1 in 20).

2.3. Genes and primers

Four candidate genes were chosen, one gene each from the Toll and the Imd signaling pathway (*MyD88* and *Relish*, respectively), two further potential recognition/effector genes (*Hemomucin* and *TEP7*) and a reference gene (ribosomal protein S5, *RPS5*). Sequences were acquired from a cDNA library [31]. They all show significant similarities to honeybee and other insect genes (reciprocal blast: BLASTx and tBLASTn $<1e{-}20$). Primers were designed using Oligo 4.0 for Macintosh [32] and OligoCalc [33].

The primers used for quantitative PCR are as follows: *Relish* forward 5′-CAGCAGTAAAAATCCCCGAC-3′, and reverse 5′-CAGCACGAATAAGTGAACATA-3′; *TEP7* forward 5′-CTTGTCCCGTATGTATGGAGTT-3′, and reverse 5′-ACTGTAAACAGGAGCAATTTGG-3′; *Hemomucin* forward 5′-AGCATTCCCAGATTTAGCACT-3′, and reverse 5′-TAACAGTTGATTTCGGAGGTA-3′; *MyD88* forward 5′-TTGCCTTCTGAAAATGGATTAC-3′, and reverse 5′-TTGCTGTTGCCAAACTGTTA-3′; *RPS5* forward 5′-AATTATTTGGTCGTTGGAATTG-3′, and reverse 5′-TAACGTCCAGCAGAATGTGGTA-3′. The amplicons are between 115 bp and 192 bp long.

2.4. Quantitative PCR

Quantitative PCRs were performed using Platinum® SYBR® Green SuperMix-UDG (Invitrogen). Each 20 μl qPCR reaction contained 5 μl of cDNA, 1 μl BSA (1 mg/ml), 3 mM of $MgCl_2$, 1 mM of dNTPs, and 0.2 μM of each primer. A CAS-1200™ robot (Corbett Life Sciences) aliquoted cDNAs and PCR master mixes. The qPCRs were carried out on a RotorGene 6000 thermal cycler (Corbett Life Sciences). A fixed protocol was applied for all PCRs (2 min at 50 °C; 2 min at 95 °C; 45 cycles of 15 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C). A melt-curve analysis was performed after cycling (50–99 °C) to check for potential non-target amplifications. Two replicates were run for each individual cDNA and for each gene.

Transcription levels of the genes of interest were normalized against the ribosomal protein S5 gene as this gene has been demonstrated to show consistent expression across different life stages and disease status in bees [17,34,35] and has been used in several studies on bee immunity [17,28,30,34,35]. There is only a single nucleotide difference between the primers of the normalizer gene previously applied in qPCR in honeybees and the bumblebee primers used here. The threshold cycles (Ct) were determined by using the automatic threshold function of the RotorGene 6000 software (version 1.7) (Corbett Life Sciences). The mean Ct value of the two replicates was converted into gene expression taking into account the efficiencies of the PCRs as described earlier [30]. The efficiencies were obtained from standard curves, i.e., a dilution series of pooled cDNAs was included in each run for every primer pair.

2.5. Statistical analysis

Separate ANOVAs were used to analyze the expression of each of the immune genes. The colony of origin and infection status of each individual were included as fixed effects in the models, and the interaction between the two included if it significantly improved the fit of the model. Where appropriate, the response variable of expression was transformed to meet the assumptions of normality and homogeneity of variances (*Relish*: $y^{0.35}$; *MyD88*: $y^{0.14}$). All analyses were carried out in R2.6 for Mac [36].

3. Results

The bumblebees in all four colonies had large numbers of *C. bombi* parasites in their guts 10 days after experimental infection

Table 1
ANOVA tables for gene expression in each of the four investigated genes.

Factor	MS	*F*	d.f.	*p*
(a) *Hemomucin*				
Infection	0.0039	9.18	1	0.0048**
Colony	0.0040	9.23	3	0.0002**
Infection × colony	0.0013	3.04	3	0.043*
Residuals	0.0004		32	
(b) *MyD88* (transformed $y^{0.14}$)				
Infection	0.0103	2.59	1	0.116
Colony	0.0252	6.31	3	0.0015**
Residuals	0.0040		35	
(c) *Relish* (transformed $y^{0.35}$)				
Infection	0.0147	4.53	1	0.041*
Colony	0.0225	6.93	3	0.001**
Infection × colony	0.0143	4.38	3	0.011**
Residuals	0.0033		32	
(d) *TEP7*				
Infection	0.0078	2.84	1	0.101
Colony	0.0177	6.43	3	0.001**
Residuals	0.0028		35	

* Significant at 0.05.
** Significance level adjusted for testing of multiple genes [37].

with *C. bombi*. The average (median) number of parasites in colony A was 320,000 [range 180,000–10,240,000] ($n = 5$), in colony B 160,000 [range 60,000–1,760,000] ($n = 5$), in colony C 55,000 [range 10,000–6,320,000] ($n = 4$), and in colony D 300,000 [40,000–1,440,000] ($n = 8$).

All four genes showed higher expression in the infected bees across all four colonies by about 20% relative to the control group (*Hemomucin* 26%; *MyD88* 18%; *Relish* 16%; *TEP7* 21%; with standard errors 5–12%). However, only *Hemomucin* is statistically significantly upregulated in the infected bees after adjusting significance levels by applying the false discovery rate method for multiple tests [37]. *Relish* also showed a strong trend of upregulation in infected bees, but was not significant after the adjustment for multiple testing. All four bumblebee colonies differed significantly (after adjusting for multiple tests) in gene expression in all four genes irrespective of infection status (Table 1 and Fig. 1). There was a significant interaction (after adjusting for multiple tests) between infection status and colony origin in *Relish* expression but not in any of the other three genes studied (Table 1).

4. Discussion

Social insects (e.g. social bees and wasps, ants, and termites) can be attacked by many pathogenic microorganisms and other parasites [7,18]. Insect hosts are capable of mounting an immune response when infected. In our experiment, we observed large differences in the expression of immune-related genes even among age-controlled worker bees from the same colony despite the fact that bumblebee workers are highly related ($r = 0.75$) and hence genotypically very similar. Such large variability is not unusual [4], but it makes the detection of upregulation due to infection difficult. Despite this variation within colonies, the four ANOVAs (for the four different genes) demonstrated significant differences in gene expression among colonies irrespective of infection status. Moreover, the ANOVA analyzing *Hemomucin* showed this immune-related gene was significantly upregulated when the bees were infected with the trypanosome parasite *C. bombi*.

The significant upregulation of *Hemomucin* demonstrates that, irrespective of the reasons behind within and among colony variation in gene expression, *C. bombi* parasite infections enhance expression of this immune-related gene. Binding of Hemomucin to a lectin was found to be correlated with the expression of an antimicrobial peptide gene (*cecropin A1*) in *Drosophila* [25]. More recently, Aguilar et al. [38] demonstrated that infection of the bacterium *Salmonella typhimurium* in *Anopheles gambiae* induces

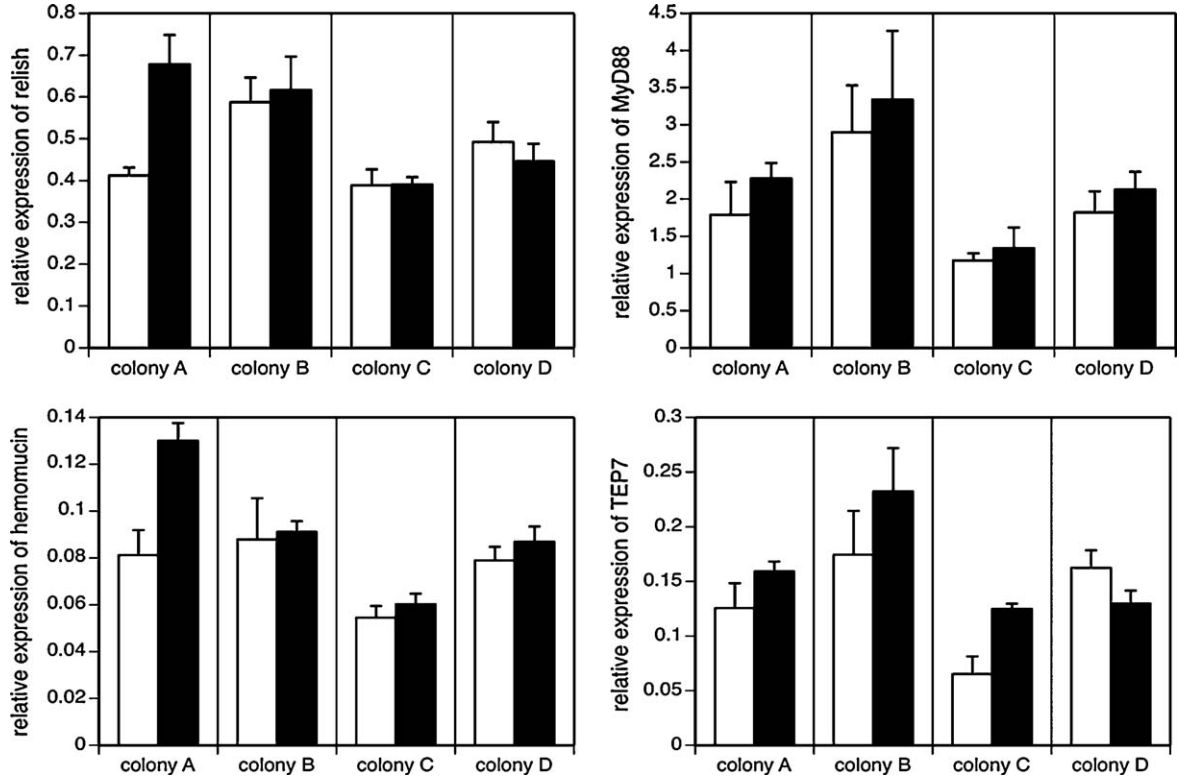

Fig. 1. Relative expression of the immune-related genes *Relish*, *Hemomucin*, *MyD88*, and *TEP7* in four bumblebee colonies (A–D). Empty bars: healthy bumblebees; filled bars: bumblebees infected with the trypanosome parasite *Crithidia bombi*; error bars: standard error of the mean.

transcription of a *Hemomucin*-like gene. There is some evidence that *Hemomucin* has been under recent positive selection in some populations of *Drosophila simulans* [39].

Relish is the only gene with a significant interaction between the factors "Colony" and "Infection" in the statistical analysis of gene expression (Table 1). This means that the response to infection, in terms of the change in *Relish* expression, differs between the genotypic units represented by colonies. Such interactions could, in part, be behind some of the genotype specific differences in infection intensities found in this host–parasite system [5]. Additionally, *Relish* shows a trend for upregulation on infection, but this result was not significant after adjusting significance levels for multiple tests. Future studies should address in more detail whether *Relish* is indeed involved in the immune response of bumblebees when infected with *C. bombi*. A role for *Relish* would seem plausible, as antimicrobial peptides presumably belonging to the Imd signaling pathway are upregulated under infection of *C. bombi* [28]. Further, *Relish* and the Imd signaling pathway play a role in the response of tsetse flies infected with trypanosomes [40].

Our results demonstrate substantial and significant variation in expression of immune-related genes among different bumblebee colonies irrespective of infection status. The experimental setup does not facilitate an analysis of potential causes for this variation, but whatever these are they will probably lead to differences in observed immune responses under field conditions. Variability in immune competence and its underlying genetic polymorphisms seem to be common in natural populations [41]. Moreover, in other experimental infections of *B. terrestris* with *C. bombi* it has been shown that the levels of resistance vary across host types, and that parasite resistance across host genotypes is not uniform, but rather there is an interaction between host-type and parasite-type in determining infection intensity [5]. Similarly to our results, variation in immune gene expression among colonies has also been reported in honeybees [42] and now in *B. terrestris*, too [28]. Interestingly, productivity of the honeybee colonies was negatively correlated with the extent of an immune response indicating a trade-off between the two traits [42]. Such trade-offs between immunity and other costly traits are at the centre of theories of ecological and evolutionary immunology [4].

The two genes for which we do not have significant evidence from our experiments of being differentially expressed on infection with *C. bombi* are *TEP7* (thioester-containing protein 7) and *MyD88* (myeloid differentiation primary response gene 88). *MyD88* codes for a protein of the Toll signaling pathway which interacts with Toll, Tube, and the Pelle kinase [43,44]. MyD88 possesses the conserved Toll/Interleukin-1 receptor-like motif (TIR) and further a death domain motif [43,44]. The other gene, *TEP7*, codes for a protein of the group of α_2-macroglobulins that are phylogenetically widespread [45]. α_2-Macroglobulins bind to proteinases and render them dysfunctional. TEPs binding to pathogens might also facilitate cell lysis or phagocytosis [24]. We suggest, however, that given the limited sample sizes of this study, our negative results should be interpreted with caution.

These results present a first insight into the immune pathway activation on infection of bumblebees by *Crithidia*. Given the economic and environmental importance of the bumblebee system, further understanding of the interactions with parasites is essential. This work lays a foundation for further research concerning host–parasite interactions and host defense.

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