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SHORT COMMUNICATION

Differential gene expression in the honeybee head after a bacterial challenge

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Summary
Bidirectional interactions between the immune and nervous systems are well established in vertebrates. Insects show similar neuro-immune-behavioral interactions to those seen in vertebrates. Using quantitative real-time PCR, we present evidence that gene expression in the honeybee head is influenced by activation of the immune system 8 h after a bacterial challenge with *Escherichia coli*. Seven genes were selected for quantitative analysis in order to cover both typical functions of the head such as exocrine secretion (*mrjp3* and *mrjp4*) and olfactory processes (*obp17*) as well as more general processes such as structural functions (mlc2 and *paramyosin*), stress response (*ERp60*) and energy housekeeping (*enolase*). In this way, we show at the molecular level that the immune system functions as a sensory organ in insects—as it does in vertebrates—which signals to the head that a bacterial infection is present, and leads to regulation of expression of several genes in the head by a yet unidentified mechanism.

Introduction

Bidirectional communication between the nervous system and the immune system has been extensively demonstrated in vertebrates [1–4]. Upon microbial challenge, the immune system signals the central nervous system. Immune cells have been shown to synthesize and release compounds that can directly and/or indirectly produce changes in neuronal

Abbreviatons: LPS, lipopolysaccharide; RT, reverse transcription; Ta, annealing temperature.

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activity [3,5], which can result in coordinated changes in behavior in some cases. In turn, the nervous system can alter immune function either directly, or indirectly via the endocrine system. Both nervous and endocrine system can synthesize and release factors that can bind to receptors found on immune cells [6,7]. Thus the immune system and the nervous system are involved in functionally relevant cross-talk, with homeostasis being the main function whereby the immune system functions as a sensory organ and signals to the central nervous system, and whereby the nervous system monitors immune status and actively respond to microbial challenge [8].

Interactions between the nervous system and the immune system are not unique to vertebrates. It has been shown that insects show similar neuro-immune-behavioral interactions to those seen in vertebrates. Activation of an immune response can result in illness-induced anorexia, behavioral fever, changes in reproductive behavior and decrease in learning ability in a range of insect species [9].

However, the molecular and physiological understanding of bidirectional neuro-immune interactions in insects is far behind on the understanding in vertebrates. Further insights into these interactions in insects are interesting because they have a less complicated, and hence easier to functionally dissect, system than vertebrates. Insects have a smaller central nervous system and their immune system lacks many of the complicating features of vertebrate immunity, such as the ability to produce antibodies [10]. In this context, the released genomic sequence of the honeybee [11] offers several possibilities to further explore the neuro-immune connections. Also, evidence for neuro-immune-behavioral interactions have already been presented in the honeybee. Mallon and colleagues [12] reported the reduced ability in associative learning of non-infected honeybees whose immune system was challenged by the non-pathogenic immunogenic lipopolysaccharide (LPS). The same group extended these findings in another hymenopter. LPS challenge of protein-starved bumblebees displayed impairment in odor/caloric-reward associative learning [13]. Evidence was also presented for increased food consumption after an immune response in the bumblebee [14]. In a proteomic study, we presented molecular evidence that activation of the immune system by bacterial challenge down-regulates the expression of proteins in the honeybee head possibly involved in exocrine secretion, learning and memory formation, odor sensing and visual functioning. Bacterial challenge also influences the expression of other proteins in the head such as structural proteins, proteins involved in signal transduction, energy housekeeping and stress response [15].

Using quantitative real-time PCR in this study, we present evidence that expression of several genes in the honeybee head is influenced by activation of the immune system 8 h after a bacterial challenge with *Escherichia coli*. We studied the genes *mrjp3* and *mrjp 4*, implicated in exocrine secretion from the hypopharyngeal glands, and *obp17*, implicated in olfactory processes, both typical functions of the head. We also studied genes of which the products are involved in more general processes in the head such as *ERp60*, implicated in the stress response, *mlc2* and *paramyosin*, both implicated in structural functions, and *enolase*, implicated in energy housekeeping. For all these genes, a differential protein expression in the honeybee head was already demonstrated after bacterial challenge in an earlier proteomic study [15]. In this way, we show that the immune system is able to signal to the head that a bacterial infection is present, which leads to regulation of expression of several genes in the head by a yet unidentified mechanism. This transcriptomic study is important because it gives information on whether the regulation of the affected genes is on the transcriptional or rather at the post-transcriptional level, information that was not available in our earlier proteomic study.

Materials and methods

Bacterial challenge and sample collection

Newly emerged (up to 1 day old) Carniolan honeybee workers (*Apis mellifera carnica*) were collected from hives of the experimental apiary in Ghent. A group of 100 newly emerged worker bees was taken from the same colony. A set of 50 worker bees was chosen *ad random* and was pricked in the abdomen (between second and third tergite) with a sterile needle dipped in a bacterial suspension of *E. coli* NCTC 9001 (three fresh colonies, overnight grown on nutrient-agar plate, suspended in 500 μl sterile physiological solution containing 15 mM NaCl, 75 mM KCl, 3 mM $CaCl_2$, 10 mM $MgCl_2$, 55.5 mM glucose, 15 mM sucrose and 55.5 mM fructose). A control group, containing the same number (50) of worker bees, was pricked in the same way but with a sterile needle dipped in the described physiological solution. After pricking, each group was put in an identical laboratory cage and incubated in the same incubator at 34 °C and 70% humidity rate. Both groups received the same amount of water and sugar water in identical sterile glass reservoirs.

Eight hours after bacterial challenge, animals were anesthetized by chilling. In both groups, the whole head of each worker was separated from the body by cutting precisely at the end of the thoracal tagmatum using a pair of scissors. Each whole head was stored separately in 250 μl RNA*later* solution (Ambion) at −20 °C and used for total RNA isolation.

RNA isolation and cDNA synthesis

Total RNA was isolated using the RNeasy Mini kit (Qiagen) followed by on-column DNase digestion with the RNase-free DNase set (Qiagen) according to the manufacturer's instructions. RNA was eluted using 40 μl RNase-free water and stored at −80 °C. After a minus reverse transcription control PCR with primers for *actin* [16], to verify for successful removal of all contaminating DNA, first-strand cDNA was synthesized from 5 μl total RNA using the RevertAid H Minus First Strand cDNA synthesis kit (Fermentas) following the manufacturer's instructions. The cDNA of the honeybee head was diluted five times with Tris–HCl (pH 8, 10 mM).

Gene selection and primer design

Seven candidate genes were selected for quantitative analysis in order to cover both typical functions of the head (*mrjp3*, *mrjp4* and *obp17*) as well as more general processes (*ERp60*, *mlc2*, *paramyosin* and *enolase*). In addition, expression of the protein products of these genes has been shown to be differentially regulated in the honeybee head 8 h after bacterial challenge in an earlier proteomic study [15]. Primers were designed for these seven genes using Primer3 [17], taking into account possible secondary structures of the amplicon (Mfold) [18]. Primer conditions were optimized by determining the optimal annealing temperature (Ta) and primer concentration, and amplicons were sequenced for verification using a Perkin Elmer Applied Biosystems 3130XL automated DNA sequencer with 50 cm capillaries filled with POP-7 polymer (Amersham Biosciences). Table 1 summarizes the information on the primers, including the Genbank accession numbers of the corresponding genes.

Real-time quantitative PCR

All real-time quantitative PCR reactions were performed in a 15 μl reaction volume on the iCycler iQ Real-Time PCR detection system (Bio-Rad) using the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) spiked with fluorescein calibration dye (Bio-Rad) and 125 nM of each specific primer. The PCR program consisted of an initial 2 min UDG incubation step at 50 °C, followed by a 2 min denaturation at 95 °C. Next, 45 cycles consisting of 20 s of denaturation at 95 °C and 40 s of combined annealing/extension at the optimal annealing temperature during which fluorescence was measured. This was followed by the measurement of fluorescence during a melting curve in which the temperature increased from 70 to 95 °C in sequential steps of 0.5 °C for 10 s. This insured the detection of one gene-specific peak and the absence of primer dimer peaks. To determine PCR efficiencies, relative standard curves were constructed using five-fold dilution series of pooled cDNA (bacteria-challenged+control) included in each run.

The mRNA level of each gene was measured in 10 separate randomly selected heads of the bacteria-challenged group (biological replicates) and in 10 separate randomly selected heads of the control group (biological replicates). Each PCR reaction of a head was run in duplicate (technical replicates). Thus, 48 real-time PCR reactions were performed for each gene, including three no-template controls and a five-fold dilution series with five measuring points.

Using the PCR base line subtracted analysis mode of the iCycler iQ 3.0 software, baseline and threshold values were first automatically determined for all reactions. Afterwards, threshold values have been manually set using the log RFU axis, to ensure comparability between data. Then all data have been reanalyzed. The average cycle threshold value of each duplicate reaction was converted to relative quantities using PCR efficiencies.

Table 1 Information of the primers used for real-time PCR.

Gene	Genbank accession number	Primer sequence (5′→3′)	Amplicon length (bp)	Ta[a] (°C)	% Identity[b]	*E*[c] (%)
mrjp3	NM_001011601	TAAACGCCACCACAGGAATG GGAAGTCAATCGATGGAAGG	149	61	97	106
mrjp4	NM_001011610	CGAAGATTGCTCTGGAATCG ATCGTGCGGTATCTCGACTTG	175	61	97	99
ERp60	XM_623279	ACTCTTGCTAAAGTTGATTGTACAG TAGATGCTGGACCAACTTGTG	178	61	99	81
mlc2	XM_393371	GCCGAATTTAAAGAGGCATTC TTCATCGTCATCCTGACCTG	216	61	99	103
paramyosin	XM_393281	ACGAACGTCGTCTGACTGAG TTGTTCTTGTTCGCAACGTC	196	61	98	97
enolase	XM_625053	GGTGATGAAGGTGGTTTTGC GATGCAGCAACATCCATACC	125	61	95	101
obp17	NM_001040207	GGGCAAGATCAATATGGACGAC AGGTGAACATTCGGCAAG	172	61	93	96
actin	AB023025	TGCCAACACTGTCCTTTCTG AGAATTGACCCACCAATCCA	155	61	99	104
GAPDH	XM_393605	GATGCACCCATGTTTGTTTG TTTGCAGAAGGTGCATCAAC	203	61	100	93
RPL13a	XM_623810	TGGCCATTTACTTGGTCGTT GAGCACGGAAATGAAATGGT	191	61	98	90
RPS18	XM_625101	GATTCCCGATTGGTTTTTGA CCCAATAATGACGCAAACCT	149	61	97	88

[a]Optimal annealing or elongation temperature in the PCR program for a specific primer.
[b]Percentage homology of the sequenced amplicon with the corresponding reference sequence from Genbank.
[c]Measure of the real-time PCR reaction efficiency (calculated by the standard curve method).

Normalization of real-time data

The genes *GAPDH*, *RPS18*, *actin* and *RPL13a*, evaluated by geNorm [19] and Normfinder [20] software, are appropriate reference genes for the honeybee head in the context of bacterial challenge as described by Scharlaken and colleagues [21]. The geometric mean of the relative quantities of these four reference genes was used to calculate an accurate normalization factor. Each relative quantity was divided by the respective normalization factor to obtain a normalized value for each transcript.

Statistical analysis

Statistical significance was assessed by Mann–Whitney *U*-test when comparing normalized data from bacteria-challenged and control worker bees. *P* values less than 0.05 corrected for multiple testing using the false discovery rate (FDR) method, devised by Benjamini and Hochberg [22], were considered significant. Mann–Whitney *U*-test was performed using SPSS v 15.0 for Windows (SPSS, Inc.).

Results

The RNA expression levels of seven genes were determined 8 h after bacterial challenge with *E. coli* in the head of the honeybee and compared to the RNA expression levels in the heads of control bees using real-time PCR. Gene-specific amplification was confirmed for the seven primer pairs by sequencing, by a single peak in melt-curve analysis and a single band with the expected size in agarose gel electrophoresis. No primer-dimer formation was detected and the standard curves derived from five-fold serial dilutions of pooled cDNA from the head (bacteria-challenged/control bees) gave correlation coefficients greater than 0.98 and efficiencies between 81% and 106%. The quantitative real-time data of both groups (bacteria-challenged and control bees) were normalized to the geometric mean of the four best reference genes as described in our previous study [21].

Using the Mann–Whitney *U*-test, four of the seven tested genes showed *P* values less than 0.05 in differential gene expression (mrjp3, ERp60, *paramyosin* and *enolase*; see Figure 1). When *P* values were corrected for multiple testing by the FDR method [22], the same four genes displayed a statistically significant *P* value (see Table 2). The RNA levels of *mrjp3* and *ERp60* were higher in the bacteria-challenged worker bees compared to the control bees. The same effect in the honeybee head was observed for *obp17* after bacterial

Table 2 Statistics of the differential expression of seven genes in the honeybee head 8 h after bacterial challenge.

Gene	*U* value[a]	*P* value[b]	Rank[c] (i)	FDR-corrected *P* value[d]
enolase	2	0.0008*	1	0.0071
paramyosin	9	0.002*	2	0.0143
mrjp3	15	0.008*	3	0.0214
ERp60	20	0.023*	4	0.0286
obp17	28	0.096	5	0.0357
mlc2	32	0.174	6	0.0429
mrjp14	32	0.174	7	0.05

[a]*U* value as determined by Mann–Whitney *U*-test using SPSS v 15.0 for Windows.
[b]Observed *P* value = $P_{(i)}$; asterisk (*) indicate statistical significance, this means $P_{(i)}$ < FDR corrected *P* value.
[c]The rank sorts *P* values from smallest to largest.
[d]False discovery rate (FDR) is the expected proportion of false discoveries among the discoveries.

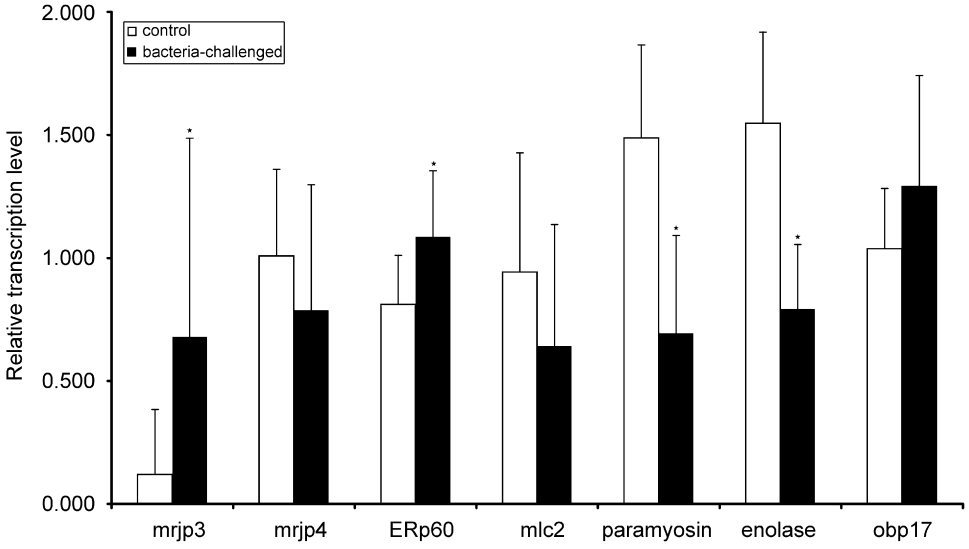

Figure 1 Real-time PCR analysis of genes in the head of the honeybee after pricking with a needle dipped in a physiological solution (control) and after pricking with a needle dipped in a bacterial suspension (bacteria-challenged). Asterisks (*) mark statistically significant differences (based on Mann–Whitney *U*-test with *P* value <0.05 corrected for multiple testing by false discovery rate) after bacterial challenge. Error bars represent standard deviations.

challenge but no significant *P* values could be obtained. On the other hand, *enolase* and *paramyosin* displayed lower transcript levels in the heads of bacteria-challenged workers compared to the control bees. Lower transcript levels were also observed in the honeybee head for *mrjp4* and *mlc2* after bacterial challenge but again without obtaining significant *P* value.

Discussion

There are few studies on immune–neural interactions in insects. Given the wealth of information on the insect immune system and on the nervous system, the combination of these two fields is important for those studying insects. In mammals, neural–immune bidirectional connections are important for the proper functioning of the immune system. Although immune–neural links may have an ancient evolutionary basis [3,23,24], their implications for behavior seem to vary across insect species. For example, activating the immune system resulted in a decline in feeding in the caterpillar, *Manduca sexta* [25], while triggering the bumblebees immune system leads to increased food consumption [14]. These variabilities may limit the generalization of results but will provide new insights. Moreover, the better understanding of neural–immune interactions in insects may improve our ability to fully comprehend the proper functioning of insect immune systems.

In mollusks, some of the evidence for immune–neural interactions comes from studies examining how parasites and pathogens influence host behavior. Changes in host behavior after infection imply a change in neural function and therefore could indicate an underlying immune–neural connection. The difficulty with this line of evidence is that the change in behavior may be due to some mechanism correlated with infection as opposed to being caused by an immune-derived factor [26,27]. Studies that report (behavioral) changes using an artificial immune challenge (e.g. injection with LPS or pricking with a needle dipped in a bacterial suspension) on the other hand provide good evidence that immune-derived factors indeed can influence neural function.

It is interesting to know that some compounds we found in the head, of which the expression is significantly changed after bacterial challenge, also show changes in expression in other body parts. For example, the protein expression of enolase [28–30] and paramyosin [30] was also significantly changed in the hemolymph of immune-challenged fruit flies, *Drosophila melanogaster*. This might point to a similar regulatory mechanism. For instance, by the NF-κB pathways (Toll and Imd) or the JAK/STAT pathway. Honeybees appear to have maintained each of the known insect immune-related pathways [31]. It is possible that these pathways also regulate the expression of other genes than the genes coding for immune effector proteins.

Due to the fact that bacteria-challenged and control bees were put in two different laboratory cages (but in the same incubator), we cannot exclude the presence of a cage effect on the observed differential gene expression data. However, putting both groups in different cages is necessary as a measure to prevent contamination of the control group and to obtain reliable results. Since we penetrate the body cavity with a needle, there is a chance for horizontal transmission of bacteria when control bees and bacteria-challenged bees are in the same cage. There is a chance that a small amount of hemolymph exposed to bacteria is present on the abdomen after pricking, which increases the risk of infecting control bees.

Using Mann–Whitney *U*-test with FDR correction, four genes (*enolase*, *paramyosin*, *mrjp3* and *ERp60*) show statistically significant *P* values (see Table 2) in the differential gene expression after bacterial challenge. Although the remaining data are only suggestive of a pattern of up- or down-regulation in the honeybee head after bacterial challenge due to the lack of statistical confirmation, they are still valuable because the seven genes were already implicated by earlier differential proteomic work [15]. In addition, the genes *enolase*, *paramyosin* and *mrjp3* displayed strong up- or down-regulation of protein expression in the earlier proteomic study in contrast to the other four tested genes, which displayed moderate up- or down-regulation. This suggests that it is rather difficult to obtain statistically significant data using quantitative real-time PCR when dealing with intermediate or small differences in expression.

We presented evidence that activation of the immune system by bacterial challenge significantly down-regulates the expression of *enolase* and up-regulates the expression of mrjp3 in the honeybee head 8 h after a bacterial challenge. In addition, the transcript levels after a bacterial challenge of the genes *mrjp3* and *enolase* are in agreement with the protein expression [15] and point to a regulation at the transcriptional level. Also the RNA levels of the genes *mrjp4* and *mlc2* seem to be in agreement with their protein expression levels, but here statistical evidence is lacking. For *mrjp4* this is perhaps due to the lowest expression level of the mRNA compared with other *mrjp* mRNAs in the heads of worker bees [32]. Little is known about the *mrjp* genes or their protein products. They are secretory proteins with N-terminal hydrophobic regions common to eukaryotic signal peptides. MRJPs occur in the hypopharyngeal glands as the major component of the larval bee queen food, also known as royal jelly (RJ). At least one *mrjp* (*mrjp1*) is also expressed in the mushroom bodies of the honeybee brain, implicating this gene in behavior [33]. Another MRJP (MRJP9) was found to be a new venom compound [34]. This suggests that MRJPs can be multifunctional, performing a nutritional role as a component of RJ as well as executing additional roles in various tissues including the brain. Schmitzova et al. [35] also do not exclude other roles for MRJP3 and MRJP4, especially because these two proteins contain lower overall content of essential amino acids. A characteristic feature of the MRJP3 protein is the presence of a region with a variable number of tandem repeats located at the C-terminus [36]. It is also possible that such repeats are implicated in protein–protein interactions, indicating a more complex regulatory function. The up-regulation of MRJP3 after bacterial challenge strongly suggests an additional role in the head after immune challenge. Enolases have been characterized as highly conserved cytoplasmic glycolytic enzymes that catalyze the formation of phosphoenolpyruvate from 2-phosphoglycerate, the second of the two high-energy intermediates that generate ATP in glycolysis. Our result about

the down-regulation of *enolase* expression—and possible lower energy production in the head—is in agreement with the hypothesis made by Riddell and colleagues [13] and Tyler and colleagues [14] about the immune response affecting learning [13] and food consumption [14] in bumblebees. They showed that the immune system needs high-energy resources while fighting the infection, with other systems being sacrificed in for its continuing efficiency. This hypothesis is supported by down-regulation of MRJP4 in a previous proteomic study [15] where it is suggested that gland secretion is sacrificed for the energy production/conservation strategy.

This study also shows that the differential transcript level in the head is in some cases not in agreement with protein expression 8 h after a bacterial challenge. The expression of the genes *paramyosin* (down-regulated), *ERp60* and *obp17* (up-regulated) also seem to be influenced by activation of the immune system, but the transcript levels of these genes after bacterial challenge are opposite to their protein expression. Protein levels of ERp60 and OBP17 were significantly lower in bacteria-challenged worker bees, whereas the ones of paramyosin were significantly higher [15]. Discrepancy between mRNA and protein abundance might be attributed to post-transcriptional regulatory mechanisms. It is well recognized that cells regulate gene expression and protein abundance separately and that a single transcript does not usually translate into a single protein. Protein abundance depends not only on transcription rates of the gene but also on additional control mechanisms, such as mRNA stability, translational regulation and protein degradation [37]. Preliminary analysis on the literature on these negatively correlated genes showed that higher mRNA levels and lower protein levels (such as for *ERp60* and *obp17* in this study) after treatment were observed when there was evidence for regulation of translation [38], while higher protein and lower mRNA levels (such as for *paramyosin* in this study) were observed for genes in which the mRNA was stored in the nucleus and released once protein levels had reached some threshold limit [39]. Incorporating knowledge of these discrepancies should facilitate the identification of those data for which the protein can be predicted from the transcriptome and those that should be treated with caution [40]. The *paramyosin* gene codes for a structural protein and *obp17* codes for a protein involved in olfactory processes. The honeybee *Erp60* gene codes for proteins that show similarity with a protein disulfide isomerase (PDI) family member but also with a 58 kDa glucose-regulated protein. Little is known about ERp60 in insects. *Drosophila* ERp60 polypeptides are similar to human PDI within almost all their domains, and ERp60 is in fact an active disulfide isomerase [41]. In other species, *ERp60* is implicated in the stress response and codes for a protein involved in oxidative protein folding.

In conclusion, this study shows that the expression of the genes *enolase*, *paramyosin*, *mrjp3* and *ERp60* in the head of the honeybee is significantly different after activation of the immune system by bacterial challenge. The transcript levels of the gene *mrjp3*, *ERp60* and *obp17* show (a pattern of) up-regulation of expression in the honeybee head 8 h after bacterial challenge, while the transcript levels of *enolase*, *paramyosin*, *mrjp4* and *mlc2* display (a pattern of) down-regulation after bacterial challenge. When compared to the proteomic data, expression of the genes *enolase*, *mrjp3*, *mrjp4* and *mlc2* seems to be regulated at the transcriptional level after bacterial challenge, while a post-transcriptional regulatory mechanism seems to be involved in the expression of *paramyosin*, *ERp60* and *obp17* because of discrepancies between mRNA and protein level in the head after bacterial challenge. These molecular data show that the honeybee immune system is able to function as a sensory organ which signals to the head that a bacterial infection is present. This should lead to differential expression of gene products in the honeybee head involved in general processes (such as *enolase*, *paramyosin* and *ERp60*) and in typical functions of the head (such as *mrjp3*).

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