Insect immune specificity in a host-parasite model

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Abstract

Ecological studies routinely show host-parasite genotype-genotype interactions in insect systems. The mechanisms behind these interactions are less clearly understood. Using the bumblebee *Bombus terrestris* / trypanosome *Crithidia bombi model system*, we have carried out a transcriptome-wide analysis of gene expression in bees during *C. bombi* infection. We have performed three analyses, comparing expression in infected and non-infected bees 24 hours after infection by *Crithidia bombi*, expression at 24 and 48 hours after *C.bombi* infection and finally looked for differential gene expression associated with the host-parasite genotype-genotype interaction at 24 hours after infection. We found a large number of genes differentially regulated belonging to numerous canonical immune pathways. These genes include receptors, signaling pathways and effectors. We found a possible interaction between the peritrophic membrane and the insect immune system in defense against *Crithidia.* Most interestingly we found differential expression of *Dscam* depending on the genotype-genotype interactions of the given bumblebee colony and *Crithidia* strain.

Invertebrate immunity consists of a suite of complex recognition proteins and signalling pathways that regulate the induction of effector molecules against broad classes of parasites such as bacteria, fungi, viruses and microparasites [1]. Ecological studies of co-evolving host-parasite systems have shown that resistance to a parasite is highly variable in invertebrates, in part determined by interaction of the genotypes of the host and the parasite [2]. Studies with a number of natural, co-evolving host-parasite systems show that the specific combination of host and parasite genotype can predict susceptibility to specific strains of parasite [3–5].

However, how this level of specificity is generated is unclear. Specificity quantified by ecological measures of disease resistance (e.g., host mortality, fecundity and infection rate) cannot explicitly test whether the immune response produces this level of specificity [6]. For example, the bumblebee, *Bombus terrestris* / trypanosome, *Crithidia bombi* is a well studied example of these ecological host x parasite genotype-genotype interactions [7,8]. Even here it has been shown that independent of host genotype, specific isolates of gut microbiota from different hosts are protective against particular parasite genotypes [9].

Still there is evidence that the immune system must have a role in both protecting bumblebees against *Crithidia* and in generating a host-parasite specific response. A number of studies have found differential candidate immune genes expression in response to *Crithidia* [10–13]. Recently we have shown increased *Crithidia* loads in bees whose expression of antimicrobial peptides was knocked down by RNAi [14]. We have also shown that bees from different host genotypes induce differential expression of antimicrobial peptides, according to the strain of *C. bombi* they had been infected with [15], that is we found specificity in the immune response as measured by a limited number of effectors.

Understanding the source of insect immune specificity is an intriguing issue [16,17]. Such interactions can lead to the evolution and maintenance of genetic variation in natural populations [18]. On the practical side, many diseases of humans and their domesticated species use invertebrates, especially insects, as vectors [19]. Any effort to control these diseases will require a better understanding of the relationship between host and parasite.

Here, we expand our previous study and carry out a transcriptome-wide analysis of gene expression in bees during *C.bombi* infection. We have carried out three analyses, comparing a) expression in infected and non-infected bees 24 hours after infection by *Crithidia bombi* b) expression at 24 and 48 hours after *C.bombi* infection and c) looked for differential gene expression associated with the host-parasite genotype-genotype interaction at 24 hours after infection. Enrichment analysis was also carried out on expression data to see which categories of molecules are differentially regulated during infection. The results confirm our previous findings of up-regulation in antimicrobial peptide expression and provide a comprehensive overview of changes in and the specificity of gene expression after exposure to 2 strains of *C.bombi*.

Methods

The samples used during this experiment are taken from Riddell *et al*. 2009 [15]. We have chosen samples that showed a reciprocal pattern of expression for the three antimicrobial peptides (AMPs) tested in that paper. These were colony K5 (called K from now on) and Q1 (Q) and strains 6 and 8. K-8 showed a high AMP expression, Q-8 a low expression level, Q-6 a high level and K-6 a low level of AMP expression.

Sample Collection

Experiments were carried out on one commercially reared bumblebee colony from Koppert Biological Systems U.K. (Colony K) and one colony from wild caught queens (Colony Q). All parasite isolates used originated from wild queens collected in Spring 2008 in the botanical gardens, University of Leicester. Experiments began when the colonies had a minimum of thirty workers, approximately four weeks old. Between observations, colonies were fed ad libitum with pollen (Percie du sert, France) and 50% diluted glucose/fructose mix (Meliose – Roquette, France). Before and during the experiments colonies were kept at 26°C and 60% humidity in constant red light.

Infections

To prepare *C. bombi* isolates, faeces was collected from workers of naturally infected colonies, and mixed with 50% diluted Meliose to create a standardized dose of 500 *Crithidia* cells per µl of inoculum. Previous studies had shown that such inocula, prepared from different colonies, are genotypically different [8] and generate specific responses in novel hosts [7]. We infected a sample of workers from each of K and Q bumblebee colonies (representing different host lines) with an inoculum of faeces from each of the two wild infected colonies (6 and 8 *Crithidia* strain). We also collected uninfected controls. Bees were four days old at the time of infection. After infection bees were kept in colony x strain groups (1–3 individuals depending on day collected) and fed *ad libitum*. 24 hours or 48 hours post infection the bees were sacrificed by freezing in liquid nitrogen. They were then stored at −80°C.

RNA sample preparation and sequencing

Total RNA was extracted from 23 individual homogenised abdomens using Tri-reagent (Sigma-Aldrich, UK). Any residual contaminants were removed from the RNA using the RNeasy mini kit (Qiagen, UK) and manufacturer's RNA clean-up protocol. To remove residual genomic DNA, RNA samples were treated with DNase (Sigma-Aldrich, UK). TruSeq RNA-seq libraries were made from the 23 samples at NBAF Edinburgh. Sequencing was performed on an Illumina HiSeq®2000 instrument (Illumina, Inc.) by the manufacturer's protocol. Multiplexed 50 base single-read runs were carried out yielding an average of 12M reads per sample.

Statistical analysis

The reference transcriptome was downloaded from http://www.nematodes.org/downloads/databases/Bombus_terrestris/ [20]. Functional annotation related to the transcriptome was obtained using the BLAST2GO package [21]. Alignment was done using GSNAP (version 2012-07-20) [22]. Only reads that mapped uniquely were selected for further analysis. Counts were generated per transcript for each sample.

Differential expression analysis was performed using the edgeR (3.4.0) package [23] in R (3.0.1) [24]. Normalization factors were computed using the TMM technique, after which tagwise dispersions were calculated and subjected to a generalized linear model (GLM). Resulting *p* values were subjected to Benjamini–Hochberg multiple testing correction to derive FDRs; only transcripts with a FDR < 0.05 were considered for further analysis. Three separate GLMs were carried out, one looked for transcripts that are differentially expressed upon infection with *Crithidia* at 24 hours post-infection (~0+colony+infect(yes/no)) infect here are bees either infected with strain 6 or 8, one looking at the gene expression difference between 24 hours and 48 hours post strain 6 infection (~0+colony + time) and a further GLM that looked for transcripts that were expressed in a specific pattern at 24 hours post-infection (~0+colony*strain).

Using Blast2Go, we then carried out an enrichment analysis (Fisher exact test) on each of these lists of differentially expressed genes to see which GO terms are overrepresented relative to the entire genome. We then used REVIGO to summarize and visualise these terms [25].

For each of the lists of differentially expressed transcripts we also carried out a blastx analysis against the insect innate immunity database (IIID) [26]. We used the BLOSUM62 matrix with a word size of 3. The results were filtered to only contain hits with an *E*-value <1e-10, a bit score ≥30,

Results

Genes differentially expressed at 24 hours post-infection

31,843 unique transcripts were mapped to the transcriptome. 489 transcripts were found to be differentially expressed 24 hours post-infection (FDR < 0.05), 324 were downregulated and 165 upregulated. Reannotating the transcripts using Blast2GO (blastx against the nr database with e < 0.001), 109 had no BLAST hits. A further 68 had uninformative BLAST hits (anonymous predicted protein). The remaining 312 were used in the enrichment analysis. Figure 1 shows a summary of the enriched GO terms found (Fisher's test p < 0.05). Defense response (GO:0006952, FDR = 0.047) and chitin metabolism (GO:0006030, FDR = 0.032) were the only processes significantly enriched at a more stringent level (FDR < 0.05).

Peritrophic membrane:

The peritrophic matrix (PM) forms a layer composed of chitin and glycoproteins that lines the insect midgut lumen [27]. The PM facilitates digestion and forms a protective barrier to prevent the invasion of ingested pathogens [27,28]. Fibrillin 1 (BTT14121_ 1), a venom protein precursor (BTT32193_1), Neurotrypsin (BTT07956_1), Peritrophin-1-like (BTT01709_1, BTT22959_1, BTT37215_1, BTT42262_1) and four chitinase transcripts (Chitinase 3: BTT23997_1 BTT38724_1, Chitinase 4 BTT20684_1, BTT23469_1) are downregulated upon infection. Fibrillins are extracellular matrix macromolecules, ubiquitous in the connective tissues [29]. BTT32193_1 was classed as a venom protein, but was also very similar to Chitinase 3 (blastx e = $1e\text{-}^{16}$). Chitinases modulate the structure and porosity of the PM [30]. Neurotrypsin is a serine protease expressed in the nervous system [31]. However in the protease domain it shares similarities with Sp22D, a chitin binding serine protease [32]. The chitin fibrils of the PM are assembled into a wide cross-hatched pattern connected by peritrophins [30]. A second group made up of Peritrophin-1 (BTT05886_1, BTT20661_1) and 3 further chitinase transcripts (Chitinase 2 :BTT23246_1, Chitinase 3: BTT39163_1, Chitinase 4: BTT05313_1) is upregulated. Figure 2 shows the

153 correlation of expression patterns between these sixteen transcripts related to chitin metabolism.
154 There is some clustering, but not of any clear functional groups. Taken together however, this
155 differential expression suggests an important role for the repair or restructuring of the peritrophic
156 matrix in the bumblebees' response to *Crithidia*.
157
158 When the BLAST searches against the IIID and nr databases are combined, eighty nine transcripts
159 relate to canonical insect immune genes. We describe them in the order receptors, serine proteases,
160 signalling pathways and effectors [16].
161
162 *Receptors:*
163 The Down syndrome cell adhesion molecule (Dscam), a pattern recognition receptor has come to
164 the forefront of research into insect immune specificity as it has been found to have thousands of
165 different splice forms and is associated with insect immunity [33]. We found five downregulated
166 transcripts annotated as immunoglobulin superfamily (*Dscam* included in hit list) (BTT03519_1,
167 BTT08682_1, BTT15814_1, BTT26724_1, BTT27678_1) and one upregulated transcript
168 (BTT03519_1).
169
170 *Serine proteases:*
171 Serine proteases are important proteolytic enzymes in many molecular pathways. When these serine
172 proteases are no longer in need, they are inactivated by serine protease inhibitors [34]. CLIP domain
173 serine proteases mediate insect innate immunity [35]. 8 transcripts corresponded to clip serine
174 proteases (CLIPA6: BTT20125_1, CLIP A7: BTT07313_1, BTT31897_1, CLIPD5: BTT10579_1,
175 BTT10912_1, BTT18247_1 BTT25711_1, BTT06803_1). All were downregulated. Another
176 immune related serine protease SP27 (BTT08108_1, BTT38696_1) was also downregulated. The
177 serine protease homologue SPH54 (BTT06125_1) was downregulated. SP35 (BTT05300_1), SP24
178 (BTT03436_1) and a different SPH 54 transcript (BTT01977_1) were upregulated. Seven

179 transcripts (spn4: BTT04130_1, BTT40693_1, BTT41025_1, BTT41461_1, NEC-like:
180 BTT31997_1, and SRPN10: BTT04508_1, BTT20259_1) referring to serine protease inhibitors
181 were downregulated. The *necroti*c (*nec*) gene encodes the serine protease inhibitor Nec. This
182 controls a proteolytic cascade which activates the innate immune response to fungal and Gram
183 positive bacterial infections [36]. Lipophorin receptor 2 (downregulated BTT34617_1) binds with
184 serpins to aid in their encytocytosis [37].
185
186 *Signalling pathways:*
187 We found a transcript for *Spatzle* (BTT19738_1) downregulated at this time point. Activation of the
188 Toll immune pathway requires the activation of *Spatzle* [1]. MyD88 (upregulated BTT15687_1) is a
189 death domain-containing adaptor activated by Toll leading to the activation of *Pelle*. *Dorsal*
190 (BTT25273_1) was also downregulated. The nuclear translocation of Dorsal, a member of the NF-
191 kB family, in the Toll pathway induces the expression of many immune genes. We found an
192 upregulated transcript (BTT09662_1) for *Helicase89B* part of the Toll and Imd Pathway. It is
193 required downstream of NF-kB for the activation of AMP genes in *Drosophila melanogaster* [38].
194 *ird5* codes for a catalytic subunit of an IkappaB kinase that cleaves Relish. Relish (Imd pathway) is
195 an essential regulator of antimicrobial peptide gene induction. We found *ird5* (BTT03904_1) to be
196 downregulated 24 hours post-infection.
197
198 In mammals semaphorins are crucially involved in various aspects of the immune response [39]. A
199 semaphorin-5A-like transcript (BTT01850_1) was downregulated 24 hours post-infection.
200 Semaphorin regulates the activity of Ras-family small GTPases [39]. A Ras-like protein11B
201 transcript (BTT05368_1) was also down regulated. The Ras/MAPK pathway was found to be
202 essential for the suppression of the Imd immune pathway in *Drosophila* [40].
203
204 The downregulated Drumstick (BTT13062_1) interacts with the JAK/STAT pathway during its'

development role [41], but we could not find any information about its immune role. Two transcripts (BTT11590_1, BTT14205_1) of *Puckered* were downregulated. *Puckered*, which codes for a dual specificity phosphatase, is a key regulator of the c-Jun-N-terminal kinase (JNK) immune pathway [42]. Mpk2/p38a (downregulated BTT05769_1) is involved in the JNK Pathway and JAK/STAT Pathway. Heat-shock factor activation by p38 is a recently discovered part of antimicrobial reactions in flies [43]. We found two heat shock protein transcripts (BTT23758_2, BTT37030_1) and one other (BTT17701_1) that were downregulated and upregulated respectively. These are all involved in the JAK/STAT pathway.

Effectors:

Our previous paper [10] found that antimicrobial peptides were upregulated at 24 hours post-infection. We would expect the same to be true here. Indeed, we found 5 transcripts for defensin (BTT06274_2, BTT8490_1, BTT10405_1, BTT14019_1, and BTT42034_1) and 3 transcripts for hymenoptaecin (BTT18071_1, BTT24170_1, BTT24170_2), all upregulated. An apidaecin precursor (BTT33652_1) was downregulated. Apidaecin has recently been shown to be expressed in bumblebees [20]. The downregulated beta-amyloid-like protein (BTT20240_1) has been shown to be an antimicrobial peptide in mammals [44]. Hemolectin (BTT15326_1, upregulated) is a clotting protein known to have a role against gram negative bacteria [45].

Reactive oxygen species (ROS) are generated by respiration in the mitochondria or as part of the immune response [46]. P450 cytochromes are oxidases acting terminally in monooxygenase systems [47]. Some are regulated in response to infection possibly either as direct immune responders [48], producing nitric oxide (NO) or other reactive oxygen radicals or as part of the host detoxification process decreasing oxidative stress after an infection [46]. A number of *cytochromes P450* were differentially expressed 24 hours post infection. Ten cytochrome p450 transcripts (*Cyp4p3*: BTT05294_1, BTT20848_1, BTT22253_1, BTT23317_1, BTT32674_1, *cytochrome*

231 *P450 4g15*: BTT23811_1, BTT32459_1, *cytochrome P450 6k1*: BTT35547_1, BTT40653_1,
232 *cytochrome P450 6a14*: BTT38445_1) were found to be downregulated. Three other *cytochrome*
233 *P450* transcripts (*Cyp4p3*: BTT21216_1, BTT35543_1, *cytochrome P450 315a1*: BTT26726_1)
234 were upregulated. Several other cytochromes (cytochrome b: BTT20524_1, BTT39776_1,
235 BTT41896_1, and cytochrome c: BTT05255_2) were downregulated.
236
237 Numerous other actors in the production of ROS were found to be differentially expressed. *TPX4*
238 (BTT13285_1), coding for a Thioredoxin-dependent peroxidase, was downregulated. This gene was
239 found be differentially expressed during *Plasmodium* infection in *Anopheles gambiae* [49].
240 Thioredoxin-dependent peroxidase detoxifies H_2O_2. Calcineurin (BTT08150_1, BTT26273_1) was
241 found to be downregulated 24 hours post-infection. This agrees with our previous findings [10]. In
242 infected *D. melanogaster* larvae, NO signals are enhanced by Calcineurin to promote induction of
243 strong, robust immune responses via the Imd signalling pathway [50].
244
245 We found downregulation of sortilin-related receptor-like (BTT31654_1). In mammals, sortilin aids
246 in phagocytosis [51]. Two downregulated transcripts (BTT35021_1, BTT08756_1) were matched to
247 *croquemort. Croquemort*, which codes for a scavenger receptor is a key part of the Imd pathway
248 but in its apototic phagocytosis role not its immune one [52]. Annexin IX (downregulated
249 BTT02025_1) has been shown to be induced by septic injury in *Drosophila*. It is thought to encode
250 for an anticoagulant [53].
251
252 *Miscellaneous:*
253 Major royal jelly protein (BTT05317_2, BTT36365_1 upregulated) has been shown to have
254 antimicrobial properties and to be expressed in response to bacterial infection in honeybees [54,55].
255 Vitellogenin (downregulated BTT36006_1) is a potent regulator of the immune response in
256 honeybees [56]. Several orthologs of putative *Drosophila* immune loci were found to be

differentially expressed 24 hours post-infection (CG12505: BTT00934_1, CG18348: BTT04397_1, CG7296: BTT15035_1, BTT18395_1, CG8791: BTT18908_1, CG5527: BTT35653_1, Fst: BTT11511_1). The downregulated CG4393 (BTT05817_1) is weakly analogous to TNF receptor associated factor 3 (TRAF3) that mediates signal transduction involved in mammalian immune responses. Downregulated BTT37289_1 codes for a putative fatty acyl-CoA reductase.

Genes differentially expressed between 24 hours post-infection and 48 hours post-infection

43 transcripts were found to be differentially expressed between 24 hours post-infection and 48 hours post-infection. Of these 17 had no BLAST hits. A further six had uninformative BLAST hits (anonymous predicted protein). The remaining 20 were used in the analysis. Defense response was the only GO term significantly enriched (FDR= 0.00015), with seven transcripts. Three transcripts correspond to Hymenoptaecin (BTT18071_1, BTT24170_1,BTT24170_2). They were all upregulated. This suggests a continuing strong AMP production 48 hours after infection. This agrees with other immune assays in bumblebees [57]. Argonaute-2, a RNA-silencing endonuclease, is involved in antiviral defense in insects (downregulated BTT02484_1) [58]. GstD8, a glutathione S-transferase, is involved in the detoxification process (upregulated BTT04810_1) [59]. Dopa decarboxylase (upregulated BTT28048_1) converts L-dopa to dopamine during the melanisation process [60]. SCR-B9 (upregulated BTT40924_1) codes for a scavenger receptor protein. Scavenger receptor proteins have been found to be microbial pattern recognition receptors in flies [61].

Genes differentially expressed depending on host genotype – parasite genotype interactions

There were 591 differentially expressed transcripts (FDR < 0.05). Reannotating the transcripts using Blast2GO (blastx against the nr database with e < 0.001), 150 had no BLAST hits. A further 64 had uninformative BLAST hits (anonymous predicted protein). There were 109 transcripts that had previously been found to be differentially expressed at 24 hours post infection. Figure 3 shows

a multidimensional scaling (MDS) plot of the samples based on the expression of these 591 genes. It can be clearly seen that the 11 samples are grouped into their colony-strain interaction.

Of the 591 transcripts, 132 were upregulated and 459 were downregulated. Up or downregulation does not have the same meaning here as in the infected versus uninfected model were there was a clear baseline (uninfected). Depending on how you order the GLM we could get the reciprocal result. Our model used colony K strain 8 as the final contrast. From our previously published qPCR data [15], we know the colony K strain 8 interaction displayed the highest levels of AMPs (effectors). Therefore when we say a transcript is upregulated, we mean it is upregulated in this high immune response interaction.

As with the infection data, we combined the BLAST searches against the IIID and nr databases. Ninety transcripts correspond to canonical insect immune genes. We again describe them in the order receptors, serine proteases, signalling pathways and effectors [16].

Receptors:

Two transcripts were associated with gram negative binding proteins (upregulated GNBP, BTT03533_1 and downregulated *GNBP1-2* BTT35513_1) Although, as their name suggests, GNBPs are most associated with defense against gram negative bacteria, they have been show to have a role in respond to *Plasmodium* infections [62]. C-type lectins (CTLs) bind carbohydrates and mediate processes including pathogen recognition [63]. CTL4 is agonist to *Plasmodium* infections in mosquitoes [63]. A CTL4 transcript (BTT29328_1) was found to be downregulated.

One downregulated transcript was related to *Dscam* (BTT12755_1). A further fourteen downregulated transcripts were part of the Ig superfamily (IGFn3-1: BTT05561_1, BTT05581_1, BTT08682_1, BTT12655_1, BTT13442_1, BTT14516_1, BTT18750_1, BTT21156_1,

BTT22598_1, BTT22819_1, BTT23339_1, BTT24070_1, IGFn3-7: BTT08109_1, BTT09498_1) and one was upregulated (IGFn3-8: BTT03519_1). *Dscam* and most of the other Ig superfamily transcripts cluster together in the top right of figure 4, suggesting they are similarly expressed.

Serine proteases:

28 transcripts related to serine proteases, serine protease homologues or serine protease inhibitors were differentially expressed. Twelve serine protease transcripts were upregulated (*cSp3*: BTT35293_1, *Sp18*: BTT20808_1, *Tequilla/GRAL/Sp23*: BTT01709_1, BTT05886_1, BTT09081_1, BTT20661_1, BTT20725_1, BTT24359_1, BTT25071_1, *Sp27:* BTT40251_1, *Sp35:* BTT05300_1, *Sp40:* BTT15256_1). Six serine protease transcripts were downregulated (*cSP3*: BTT10579_1, BTT10912_1, BTT18247_1, BTT25711_1, *Sp28:* BTT20637_1, *Sp35: BTT10155_1*). Two serine protease homologues were downregulated (*Sph54:* BTT27769_1, *cSPH39*: BTT21868_1). One serine protease homologue was upregulated (*Sph56:* BTT17814_1) Six serine protease inhibitor transcripts were downregulated (*Spn 4:* BTT04130_1, *SRPN10*: BTT02607_1, BTT4508_1, BTT20259_1, BTT40693_1, Kunitz ser-protease inhibitor: BTT14993_1). The *necrotic* (*nec*) gene was upregulated (BTT35742_1).

Signalling pathways:

The Toll-like receptor *18Wheeler* (BTT35732_1) was upregulated as was *Toll 10* (BTT09386_1). 18Wheeler has been shown to be important in the anti gram-negative immune response in *Drosophila* larvae [64]. *Dorsal 1A* (BTT04010_1), a transcription factor that is a fundamental part of the Toll pathway, was downregulated. A transcript for *Spatzle 1-2* was downregulated (BTT10679_1).

The tyrosine kinase *Pvr* (BTT04822_1), which inhibits JNK activation [65] was downregulated. Jun, a transcription factor of the JNK pathway was downregulated (BTT13636_1). *Mpk2/p38a*

(downregulated BTT16580_1) and MAPKKK9 (downregulated BTT04404_1) are mitogen-activated protein kinases involved in the JNK Pathway and JAK/STAT pathways. We found two heat shock protein transcripts (BTT17371_1, BTT22195_1) and one other (BTT17701_1) that were downregulated and upregulated respectively. These are all involved in the JAK/STAT pathway. *Akt 1* (downregulated BTT14188_1) is part of the insulin/insulin-like growth factor 1 signaling (IIS) cascade. IIS plays a critical role in the regulation of innate immunity. Activation of *Akt* signaling leads to a decrease in malaria infection intensity in mosquitoes [66].

Effectors:

Five transcripts relate to the AMPs *defensin* (BTT06274_2, BTT42034_1) and hymenoptaecin (BTT18071_1, BTT24170_1, BTT24170_2). They were all upregulated. An apidaecin precursor (BTT20828_1) was upregulated. Hemolectin had three downregulated transcripts (BTT14194_1, BTT17013_1, BTT26614_1) and one upregulated (BTT15326_1). Argonaute-2, a RNA-silencing endonuclease, is involved in antiviral defense in insects (downregulated BTT02374_1) [58].

Eater encodes for a transmembrane receptor involved in phagocytosis in *Drosophila* [67]. A transcript (BTT11132_1) relating to *Eater* was upregulated. The melanisation process component Dopa decarboxylase (BTT19093_1) was upregulated. Another enzyme involved in melanisation, laccase was found to be downregulated (BTT20241_1, BTT33633_1) [68].

Cyp4p3 transcript BTT40653_1 was upregulated. Two previously unseen *Cyp4p3* transcripts (BTT05254_1, BTT20622_2) were upregulated and one (BTT36257_1) downregulated. *TPX4* (BTT13285_1) that codes for a Thioredoxin-dependent peroxidase was downregulated.

Miscellaneous:

A small number of transcripts were related to chitin metabolism. SCRASP1 has a chitin-binding

360 domain that has been hypothesized to sense chitin in response to injury and to transduce signals via
361 the serine protease domain [69]. We found an upregulated transcript related to *SCRASP 1*
362 (BTT41923_1). A peritrophin precursor was also upregulated (BTT10727_1). As was a chitinase 3
363 transcript (BTT23246_1).
364
365 *Retinoid and fatty-acid-binding protein (RfaBp)* (BTT07678_1) was downregulated. RfaBp was
366 found to be upregulated upon injection of LPS in *Drosophila* during a proteomic study [70]
367 (Vierstraete *et al.* 2004). *Notch* (upregulated BTT09545_1) is involved in the specification of
368 crystal cells in *Drosophila melanogaster* [71]. Several orthologs of putative *Drosophila* immune
369 loci were found to be differentially expressed (CG5527: BTT08512_1, CG12505: BTT00934_1,
370 CG13323: BTT38025_1, BTT38087_1, CG17560: BTT02877_1 downregulated, BTT05845_1
371 upregulated, CG18348: BTT20843_1)
372

Discussion

We present a comprehensive transcriptomic analysis of gene expression in this important model host-parasite system. We have identified a large number of bumblebee genes whose expression is changed upon infection with *Crithidia*. We have also found a large number of genes whose expression depends on the interaction between host and parasite genotypes that is show specificity.

We confirmed the importance of antimicrobial peptides in the specific defense against *Crithidia* [10,14,15]. It is also clear that several other effectors including ROS and phagocytosis may be important. Several immune pathways seem to be important in the anti-*Crithidia* response. These include the Toll, Imd and JAK/STAT pathways. Toll especially seems to be important in a specific immune response.

There are a larger proportion of receptor transcripts found in the specificity analysis (3.2% 19/591) compared to the infection analysis (1.2% 6/489). This is not surprising, as we would expect a specific immune response to a given strain to be based mainly on how it is recognised. Although several receptors, including GNBPs and lectins, are differentially expressed, the most exciting discovery is the large number of transcripts related to *Dscam.* The Down syndrome cell adhesion molecule (Dscam), a pattern recognition receptor has come to the forefront of research into insect immune specificity as it has been found to have thousands of different splice forms and is associated with insect immunity [33]. In the fruit fly *Drosophila*, silencing of *Dscam* retards the insect's capacity to engulf bacteria by phagocytosis [72]. In *Anopheles*, the Dscam splice forms produced in response to parasite exposure differs between bacteria and *Plasmodium* and between *Plasmodium berghei* and *Plasmodium falciparum* [73]. This has been tempered by the finding that Dscam diversity does not increase with exposure to increasing heterogeneity of *Plasmodium* falciparum genotypes [33]. Recently it has been shown that *Dscam* specificity is mediated by specific splice-factors transcription downstream of activation of the Toll and Imd pathways [74].

399 Our results suggest that *Dscam* may be important in differentiating strains of the trypanosome
400 *Crithidia bombi.*
401
402 We found a number of genes associated with chitin metabolism. The peritrophic matrix may be
403 fundamental in the bee's defense against *Crithidia*. The peritrophic matrix acts as an immunological
404 barrier against trypanosomes. Tsetse flies with an underdeveloped PM have lower levels of
405 refractoriness to trypanosome infections [75]. This is due to a premature immune response; the
406 trypanosomes get through the PM quicker and stimulate the immune response at an earlier stage
407 compared to refractory flies.
408
409 Given that we have found that the bees own physiology, especially its immune response is vital in
410 both the defense against *Crithidia* and in explaining the host-parasite specificity, how do we
411 incorporate recent findings that the bees gut microbiota are vital in exactly these phenomena [9,76].
412 Gut microbiota impact the condition of the PM and gut epithelium generally [75,77]. It has recently
413 been suggested that the components of the peritrophic matrix may be under the control of various
414 immune pathways, Imd [78] and STAT [79] explicitly. Gut microbiota stimulate these pathways
415 keeping the PM intact. The intact peritrophic matrix then acts as a physical barrier to colonization
416 by parasites. Future work will focus on understanding the interactions of this triumvirate of host
417 genotype, parasite genotype and gut microbiota and their effect on disease outcome.
418

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Figure 1 A summary of the enriched GO terms (based on Blast2Go annotation) found for differentially expressed genes at 24 hours post-infection compared to uninfected samples. This figure was produced using Revigo [25]

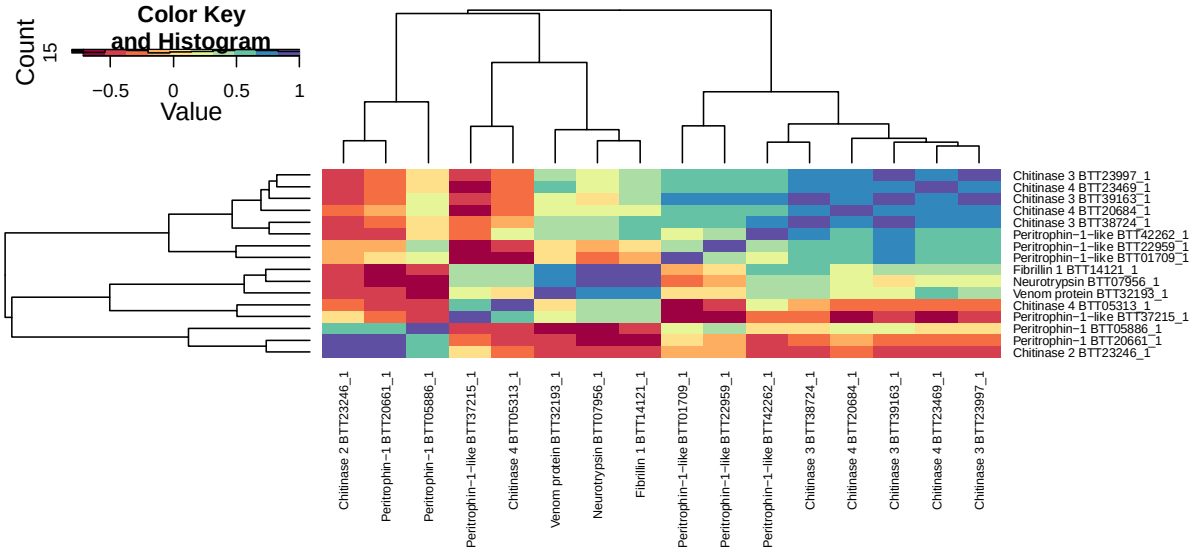

Figure 2. A heatmap showing the correlations of the expression patterns of the transcripts labelled as chitin metabolism genes that where differentially expressed twenty four hours after infection.

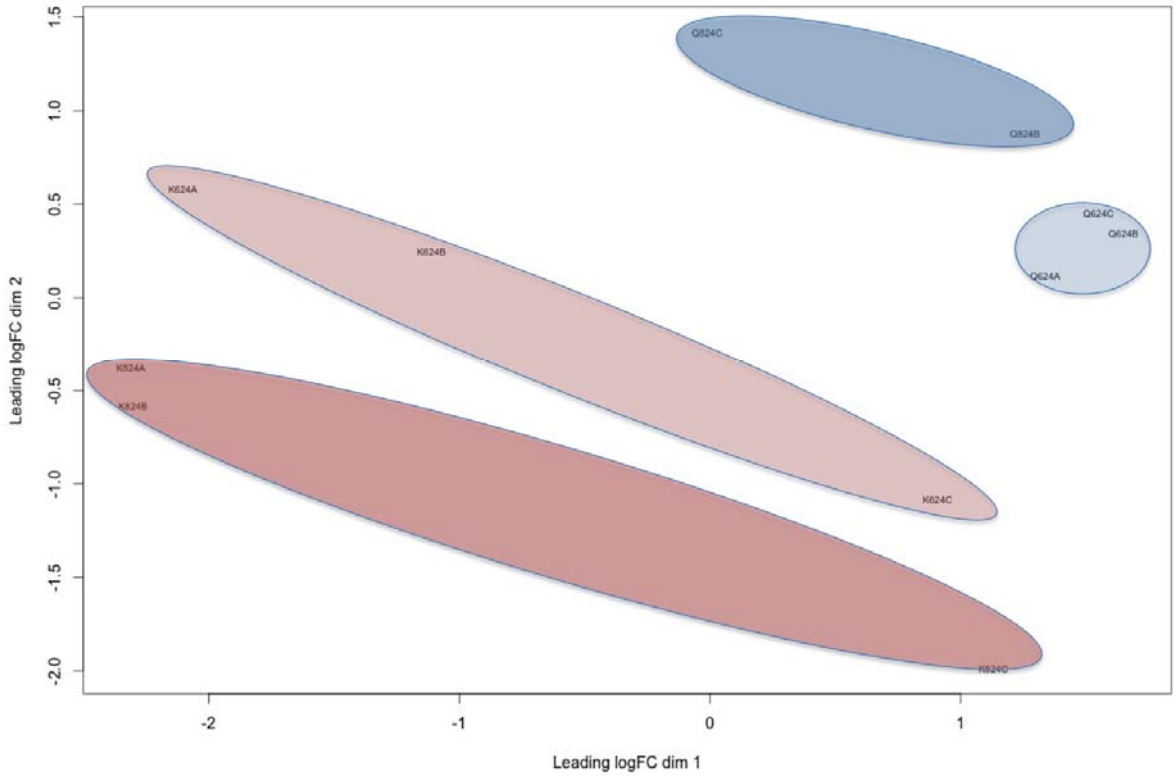

Figure 3. A multidimensional scaling (MDS) plot of the 11 samples used in the specificity analysis based on the expression of 591 differentially expressed transcripts. There are two colonies (K (red) and Q (blue)) and two *Crithidia* strains (6 (light) and 8 (dark)). Dimension 1 is the direction that best separates the samples. Dimension 2 is the next best direction, uncorrelated with the first, that separates the samples. The samples are clearly grouped into their colony-strain interactio

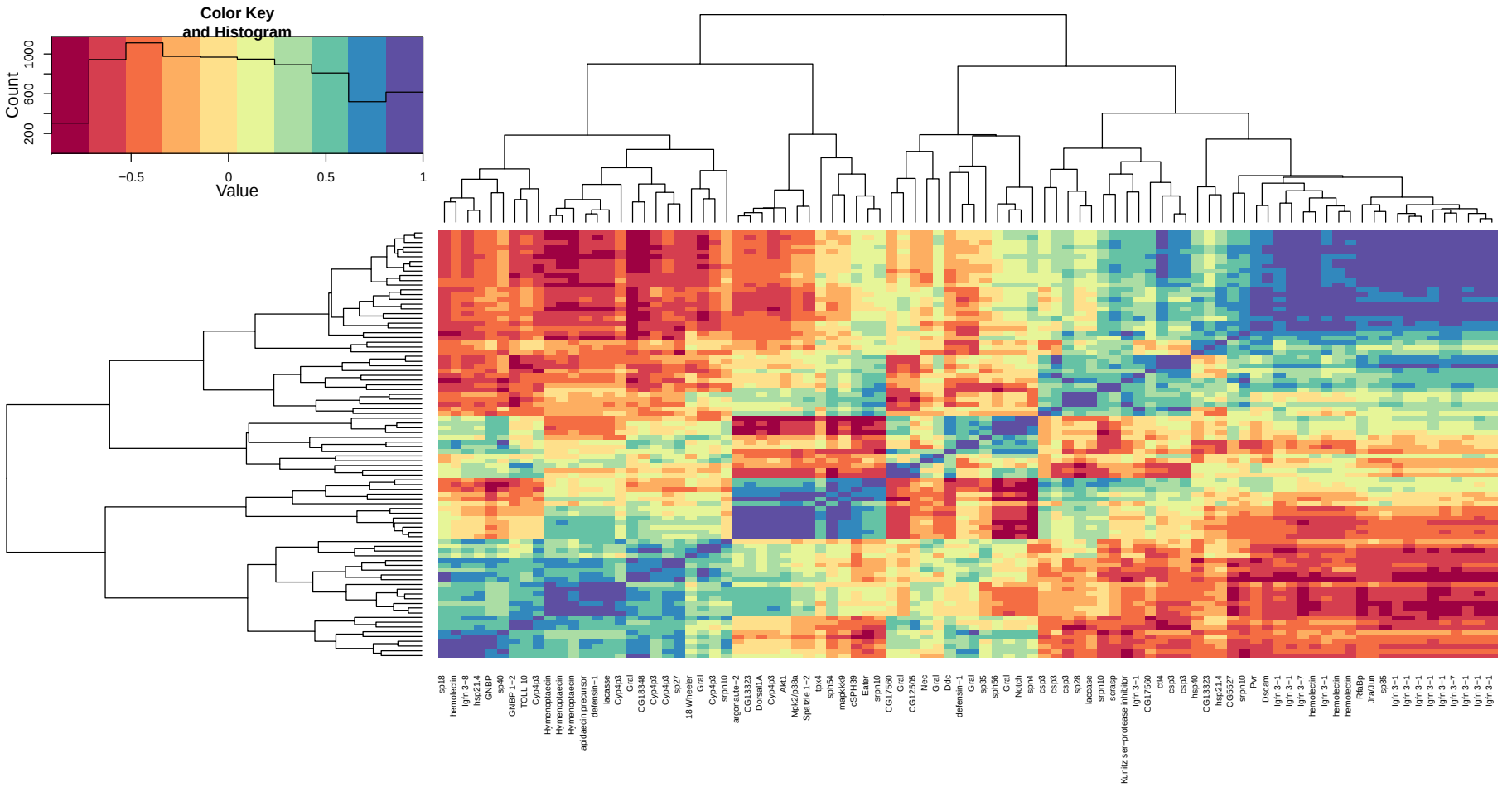

Figure 4. A heatmap showing the correlations of the expression patterns of the 90 transcripts labelled as immune genes in the analysis identifying genes differentially expressed depending on host genotype – parasite genotype interactions.