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 not 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, 1) comparing expression in infected and non-infected bees 24 hours after infection by *Crithidia bombi*, 2) comparing expression at 24 and 48 hours after *C. bombi* infection and 3) most importantly searching 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 related to numerous canonical immune pathways. These genes include receptors, signaling pathways and effectors. We discovered a possible interaction between the peritrophic membrane and the insect immune system in defense against *Crithidia*. Most interestingly we found differential expression of *Dscam* related transcripts depends on the genotype-genotype interactions of the given bumblebee colony and *Crithidia* strain.
Invertebrate ecological studies have found infection outcomes within a given host-parasite system are variable. Part of this variance is determined by the interaction of the genotype of the host and the genotype of the parasite (Schmid-Hempel 2001; Carius et al. 2001; Lambrechts 2010). That is this interaction between host and parasite is specific (Schmid-Hempel 2005). How is this level of specificity generated? An obvious answer would be an interaction between the parasite and the host's immune response. We cannot take this for granted. Various ecological measures of disease outcome have been used to quantify genotype-genotype interactions. These measures include host mortality, fecundity and infection rate. Such measures cannot test directly if it is the immune response that produces this level of specificity (Hauton & Smith 2007). It may be other non-immune processes could explain such outcomes.

The bumblebee, Bombus terrestris/trypanosome Crithidia bombi system displays host x parasite genotype-genotype interactions (Schmid-Hempel et al. 1999; Schmid-Hempel & Reber-Funk 2004). There is evidence that the immune system has a role in generating this host-parasite specific response. A number of studies have found differential immune gene expression in response to Crithidia (Schröns et al. 2010; Riddell et al. 2011; Brunner et al. 2013; Barribeau & Schmid-Hempel 2013). We found increased Crithidia loads in bees whose expression of antimicrobial peptides was knocked down by RNAi (Deshwal & Mallon 2014). We have even shown that bees from different host genotypes induce differential expression of antimicrobial peptides (AMPs), according to the strain of C. bombi they had been infected with (Riddell et al. 2009), that is we found specificity in the immune response itself. A recent paper using RNA-Seq found numerous genes are differentially expressed in a genotype-genotype fashion (Barribeau et al. 2014).

Here, we carry out a transcriptome-wide analysis of gene expression in bees during C. bombi infection. We have carried out three analyses, comparing 1) expression in infected and non-infected bees 24 hours after infection by Crithidia bombi (Infected versus uninfected) 2) expression at 24
and 48 hours after *C. bombi* infection (24 versus 48 hour) and 3) searching for differential gene expression associated with the host-parasite genotype-genotype interaction at 24 hours post infection (Specificity). 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 as previously described (Riddell et al. 2009). We have chosen samples that displayed 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). Faecal samples from these colonies were checked under a light microscope to ensure there was no Crithidia bombi present (Mallon et al. 2003). 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 (Schmid-Hempel & Reber-Funk 2004) and generate specific responses in novel hosts (Schmid-Hempel et al. 1999). However we can not rule out the possibility that we may be confounding our results with the addition of hidden infections or gut microbiota from the donor colonies. 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. Bees were collected over several days and distributed across treatment groups (Moret & Schmid-Hempel 2009). After infection bees were kept in colony x strain groups (1–3 individuals depending on day collected) and fed *ad libitum*. Twenty four hours or 48 hours post infection the bees were sacrificed by freezing in liquid nitrogen and stored at −80°C.

**RNA sample preparation and sequencing**

Total RNA was extracted from 23 individual homogenised abdomens using Tri-reagent (Sigma-Aldrich, UK). Samples (Colony-Strain-Timepoint (number of replicates)) were K-6-24 (3), K-6-48 (3), K-8-24 (3), K-8-48 (3), K-Uninfected (2), Q-6-24 (3), Q-6-48 (3), Q-8-24 (2), Q-uninfected (1). 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/](http://www.nematodes.org/downloads/databases/Bombus_terrestris/) (Colgan *et al.* 2011). Functional annotation related to the transcriptome was obtained using the BLAST2GO package (Götz *et al.* 2008). Alignment was done using GSNAP (version 2012-07-20) (Wu & Nacu 2010). 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 (McCarthy *et al.*
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 (Infected versus uninfected) (~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 (24 versus 48 hours)(~0+colony + time) and a further GLM that looked for transcripts that were expressed in a specific pattern at 24 hours post-infection (specificity)(~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 (Supek et al. 2011).

For each of the lists of differentially expressed transcripts we also carried out a blastx analysis against the insect innate immunity database (IIID) (Brucker et al. 2012). 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 and a bit score ≥30.
Results

Genes differentially expressed at 24 hours post-infection (Infected versus uninfected)

31,843 unique transcripts were mapped to the transcriptome. 489 transcripts were found to be differentially expressed 24 hours post-infection (FDR < 0.05), including 324 downregulated and 165 upregulated transcripts (See supplementary data for complete list). 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 (Kuraishi et al. 2011). The PM facilitates digestion and forms a protective barrier to prevent the invasion of ingested pathogens (Lehane 1997; Kuraishi et al. 2011). 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 (Isogai et al. 2003). BTT32193_1 was classed as a venom protein, but was also very similar to Chitinase 3 (blastx e = 1e-16). Chitinases modulate the structure and porosity of the PM (Dinglasan et al. 2009). Neurotrypsin is a serine protease expressed in the nervous system (Gschwend et al. 1997). However in the protease domain it shares similarities with Sp22D, a chitin binding serine protease (Danielli et al. 2000). The chitin fibrils of the PM are assembled into a wide cross-hatched pattern connected by peritrophins (Dinglasan et al. 2009). A second group made up of Peritrophin-1 (BTT05886_1, BTT20661_1) and 3 further chitinase transcripts (Chitinase 2...
Chitinase 3: BTT39163_1, Chitinase 4: BTT05313_1) is upregulated. Figure 2 shows the correlation of expression patterns between these sixteen transcripts related to chitin metabolism. There is some clustering, but not of any clear functional groups. Taken together this differential expression suggests an important role for the repair or restructuring of the peritrophic matrix in the bumblebees' response to *Crithidia.*

When the BLAST searches against the IIID and nr databases were combined, we found that 89 transcripts relate to canonical insect immune genes. We describe them in the order receptors, serine proteases, signalling pathways and effectors (Schmid-Hempel 2005).

**Receptors:**

The Down syndrome cell adhesion molecule (Dscam), a pattern recognition receptor has come to the forefront of research into insect immune specificity as thousands of different splice forms have been identified and it is associated with insect immunity (Smith *et al.* 2011). We found five downregulated transcripts annotated as immunoglobulin superfamily (*Dscam* included in hit list) (BTT03519_1, BTT08682_1, BTT15814_1, BTT26724_1, BTT27678_1) and one upregulated transcript (BTT03519_1).

**Serine proteases:**

Serine proteases are important proteolytic enzymes in many molecular pathways. When these serine proteases are no longer needed, they are inactivated by serine protease inhibitors (Zhao *et al.* 2012). CLIP domain serine proteases mediate insect innate immunity (Zou *et al.* 2006). Twenty one transcripts related to serine proteases, serine protease homologues or serine protease inhibitors were differentially expressed upon infection (see Table 1). Lipophorin receptor 2 (downregulated BTT34617_1) binds with serpins to aid in their encytocytosis (Soukup *et al.* 2009).
Signalling pathways:

We found a transcript for Spatzle (BTT19738_1) downregulated at this time point. Activation of the Toll immune pathway requires the activation of Spatzle (Lemaitre & Hoffmann 2007). MyD88 (upregulated BTT15687_1) is a death domain-containing adaptor activated by Toll leading to the activation of Pelle. Dorsal (BTT25273_1) was also downregulated. The nuclear translocation of Dorsal, a member of the NF-kB family, in the Toll pathway induces the expression of many immune genes. We found an upregulated transcript (BTT09662_1) for Helicase89B part of the Toll and Imd Pathway. It is required downstream of NF-kB for the activation of AMP genes in Drosophila melanogaster (Yagi & Ip 2005). ird5 codes for a catalytic subunit of an IkappaB kinase that cleaves Relish. Relish (Imd pathway) is an essential regulator of antimicrobial peptide gene induction. We found ird5 (BTT03904_1) to be downregulated 24 hours post-infection.

In mammals semaphorins are crucially involved in various aspects of the immune response (Takamatsu & Kumanogoh 2012). A semaphorin-5A-like transcript (BTT01850_1) was downregulated 24 hours post-infection. Semaphorin regulates the activity of Ras-family small GTPases (Takamatsu & Kumanogoh 2012). A Ras-like protein11B transcript (BTT05368_1) was also downregulated. The Ras/MAPK pathway was found to be essential for the suppression of the Imd immune pathway in Drosophila (Ragab et al. 2011).

The downregulated Drumstick (BTT13062_1) interacts with the JAK/STAT pathway during its’ development role (Johansen et al. 2003), 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 (Karkali & Panayotou 2012). 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 (Chen et al. 2010). 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:**

In our previous paper (Riddell et al. 2011) we found that antimicrobial peptides were upregulated at 24 hours post-infection. We would expect the same trend here. Indeed, we found that five transcripts for defensin (BTT06274_2, BTT8490_1, BTT10405_1, BTT14019_1, and BTT42034_1) and three hymenoptaecin transcripts (BTT18071_1, BTT24170_1, BTT24170_2) were all upregulated. An apidaecin precursor (BTT33652_1) was downregulated. Apidaecin has recently been shown to be expressed in bumblebees (Colgan et al. 2011). The downregulated beta-amyloid-like protein (BTT20240_1) has been shown to be an antimicrobial peptide in mammals (Soscia et al. 2010). Hemolectin (BTT15326_1, upregulated) is a clotting protein known to have a role against gram-negative bacteria (Lesch et al. 2007).

Reactive oxygen species (ROS) are generated by respiration in the mitochondria or as part of the immune response (Molina-Cruz et al. 2008). P450 cytochromes are oxidases acting terminally in monooxygenase systems (Felix & Silveira 2012). Some are regulated in response to infection possibly either as direct immune responders (Vlachou et al. 2005), producing nitric oxide (NO) or other reactive oxygen radicals or as part of the host detoxification process decreasing oxidative stress after an infection (Molina-Cruz et al. 2008). 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 P450 4g15: BTT23811_1, BTT32459_1, cytochrome P450 6k1: BTT35547_1, BTT40653_1, cytochrome P450 6a14: BTT38445_1) were found to be downregulated. Three other cytochrome P450 transcripts (Cyp4p3: BTT21216_1, BTT35543_1, cytochrome P450 315a1: BTT26726_1) were upregulated. Several other cytochromes (cytochrome b: BTT20524_1, BTT39776_1, BTT41896_1, and...
cytochrome c: BTT05255_2) were downregulated.

Numerous other actors in the production of ROS were found to be differentially expressed. TPX4 (BTT13285_1), coding for a Thioredoxin-dependent peroxidase, which detoxifies H$_2$O$_2$ was downregulated. This gene was found be differentially expressed during *Plasmodium* infection in *Anopheles gambiae* (Baton et al. 2009). Calcineurin (BTT08150_1, BTT26273_1) was found to be downregulated 24 hours post-infection, which agrees with our previous findings (Riddell et al. 2011). In infected *D. melanogaster* larvae, NO signals are enhanced by Calcineurin to promote induction of robust immune responses via the Imd signalling pathway (Dijkers & O’Farrell 2007).

We found downregulation of sortilin-related receptor-like (BTT31654_1). In mammals, sortilin aids in phagocytosis (Wähe et al. 2010). Two downregulated transcripts (BTT35021_1, BTT08756_1) were matched to *croquemort*, which codes for a key scavenger receptor in the Imd pathway (Franc et al. 1999). Annexin IX (downregulated BTT02025_1) has been shown to be induced by septic injury in *Drosophila* and is thought to encode for an anticoagulant (Gregorio et al. 2001).

**Miscellaneous:**

Major royal jelly protein (BTT05317_2, BTT36365_1 upregulated) has been shown to have antimicrobial properties and to be expressed in response to bacterial infection in honeybees (Scharlaken et al. 2007; Buttstedt et al. 2013). Vitellogenin (downregulated BTT36006_1) is a potent regulator of the immune response in honeybees (Amdam et al. 2004). Several orthologs of putative *Drosophila* immune loci were 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 (24 versus 48 hours)**

43 transcripts were 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. Defence 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 (Korner & Schmid-Hempel 2004). Argonaute-2, a RNA-silencing endonuclease, is involved in antiviral defence in insects (downregulated BTT02484_1) (van Rij *et al.* 2006). GstD8, a glutathione S-transferase, is involved in the detoxification process (upregulated BTT04810_1) (Gerardo *et al.* 2010). Dopa decarboxylase (upregulated BTT28048_1) converts L-dopa to dopamine during the melanisation process (Ferdig *et al.* 2000). 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 (Rämet *et al.* 2001).

**Genes differentially expressed depending on host genotype – parasite genotype interactions (Specificity)**

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. A
multidimensional scaling (MDS) plot of the samples based on the expression of these 591 genes. Reveals that the 11 samples group into their colony-strain interaction (Figure 3).

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 the GLM is ordered, the reciprocal result could be observed. Our model used colony K strain 8 as the final contrast. From our previously published qPCR data (Riddell et al. 2009), 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 (Schmid-Hempel 2005).

**Receptors:**

Two transcripts were associated with gram-negative binding proteins (upregulated GNB1-2 BTT35513_1). Although GNBPs are most associated with defense against gram-negative bacteria, they have been show to have a role in response to *Plasmodium* infections (Tahar et al. 2002). C-type lectins (CTLs) bind carbohydrates and mediate processes including pathogen recognition (Cirimotich et al. 2010). CTL4 is agonist to *Plasmodium* infections in mosquitoes (Cirimotich et al. 2010). 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 (see Table 2).

Signalling pathways:
The Toll-like receptor 18Wheeler (BTT35732_1) and Toll 10 (BTT09386_1) were both
upregulated. 18Wheeler has been shown to be important in the anti gram-negative immune response
in Drosophila larvae (Ligoxygakis et al. 2002). Dorsal 1A (BTT04010_1), a transcription factor
that is an important part of the Toll pathway, was downregulated. A transcript for Spatzle 1-2 was
also downregulated (BTT10679_1).

The tyrosine kinase Pvr (BTT04822_1), which inhibits JNK activation (Bond & Foley 2009) 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 (Corby-
Harris et al. 2010).
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) (van Rij et al. 2006).

Eater encodes a transmembrane receptor involved in phagocytosis in Drosophila (Kocks et al. 2005). A transcript (BTT11132_1) relating to Eater was upregulated. The melanisation component Dopa decarboxylase (BTT19093_1) was upregulated. Another enzyme involved in melanisation, laccase was found to be downregulated (BTT20241_1, BTT33633_1) (Arakane et al. 2005).

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 domain that has been hypothesized to sense chitin in response to injury and to transduce signals via the serine protease domain (Blumberg et al. 2013). We found an upregulated transcript related to SCRASP1 (BTT41923_1). A peritrophin precursor was also upregulated (BTT10727_1). As was a chitinase 3 transcript (BTT23246_1).
Retinoid and fatty-acid-binding protein (RfaBp) (BTT07678_1) was downregulated. RfaBp was found to be upregulated upon injection of LPS in Drosophila during a proteomic study (Vierstraete et al. 2004). Notch (upregulated BTT09545_1) is involved in the specification of crystal cells in Drosophila melanogaster (Mukherjee et al. 2011). Finally, several orthologs of putative Drosophila immune loci were found to be differentially expressed (CG5527: BTT08512_1, CG12505: BTT00934_1, CG13323: BTT38025_1, BTT38087_1, CG17560: BTT02877_1 downregulated, BTT05845_1 upregulated, CG18348: BTT20843_1).
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 are changed upon infection with Crithidia. We also found a large number of genes whose expression depends on the interaction between host and parasite genotypes and therefore show specificity.

We confirmed the importance of antimicrobial peptides in the specific defence against Crithidia (Riddell et al. 2009, 2011; Deshwal & Mallon 2014). 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 it may be expected that a specific immune response to a given strain would 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 thousands of different splice forms are generated and it is associated with insect immunity (Smith et al. 2011). In the fruit fly Drosophila, silencing of Dscam retards the insect's capacity to engulf bacteria by phagocytosis (Watson et al. 2005). In Anopheles, the Dscam splice forms produced in response to parasite exposure differs between bacteria and Plasmodium and between Plasmodium berghei and Plasmodium falciparum (Dong et al. 2006).

This has been tempered by the finding that Dscam diversity does not increase with exposure to increasing heterogeneity of Plasmodium falciparum genotypes (Smith et al. 2011). Recently it has been shown that Dscam specificity is mediated by the transcriptional regulation of specific splicing...
factors downstream of the activation of the Toll and IMD pathways (Dong et al. 2012). Our results suggest that Dscam related genes may be important in differentiating strains of the trypanosome Crithidia bombi.

We found a number of genes associated with chitin metabolism. Through several pathways chitin metabolism is fundamental to invertebrate immunity (Beckerman et al. 2013). As an aside, an intriguing hypothesis is that chitin metabolism is the nexus through which defense against predators and against parasites are traded-off (Beckerman et al. 2013). Our data suggests that the peritrophic matrix may be fundamental in the bee’s defense against Crithidia. The peritrophic matrix acts as an immunological barrier against trypanosomes. Tsetse flies with an underdeveloped PM have lower levels of refractoriness to trypanosome infections (Weiss et al. 2013). This is due to a premature immune response; the trypanosomes get through the PM quicker and stimulate the immune response at an earlier stage compared to refractory flies.

A similar study has recently been published (Barribeau et al. 2014). Although they find genotype x genotype interactions in gene expression, it is in a much smaller number of genes. We hypothesise that our much larger catalogue of genes, including Dscam, is due to our experimental design. We used a 2x2 analysis of preselected reciprocal host-parasite interactions. This increased our likelihood of detecting differential expression using RNA-seq compared to a less directed sample collection.

In this paper we have shown that the expression of immune genes is associated with specific interactions between different host and parasite genotypes in this bumblebee / trypanosome model. In future RNAi work we will knockdown candidate genes thereby altering these specific interactions to directly examine their biological significance.
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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 (infected versus uninfected). This figure was produced using Revigo (Supek et al. 2011).
Figure 2. A heatmap showing the correlations of the expression patterns of the transcripts labelled as chitin metabolism genes that were differentially expressed twenty four hours post-infection compared to uninfected samples (infected versus uninfected).
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)). A/B/C refers to replicates. 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 leading log-fold-change is the average (root-mean-square) of the largest absolute log-fold-changes between each pair of samples. The samples are grouped into their colony-strain interaction.
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 (Specificity).
<table>
<thead>
<tr>
<th>Serine Proteases</th>
<th>Upregulated</th>
<th>Downregulated</th>
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</thead>
<tbody>
<tr>
<td>CLIPA6</td>
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<td>BTT20125_1</td>
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<td>CLIPA7</td>
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<td>SP27</td>
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<td>BTT05300_1</td>
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<td>Serine protease homologues Upregulated</td>
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<tr>
<td>SPH54</td>
<td>BTT01977_1</td>
<td>BTT06125_1</td>
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<td>Serine protease inhibitors Upregulated</td>
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<td>SRPN10</td>
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<td>BTT04508_1, BTT20259_1</td>
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</table>

Table 1. List of transcriptions associated with serine proteases and serine protease inhibitors found to be differentially expressed twenty four hours post infection with Crithidia bombi (Infected versus uninfected).
<table>
<thead>
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<th>Serine Proteases</th>
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<th>Serine protease homologues</th>
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<th>Serine protease inhibitors</th>
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<td>necrotic (nec)</td>
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<td>Spn 4</td>
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Table 2. List of transcriptions associated with serine proteases and serine protease inhibitors found to be differentially expressed in the specificity analysis.