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* (Schlüns *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

49 and 48 hours after *C.bombi* infection (24 versus 48 hour) and 3) searching for differential gene
50 expression associated with the host-parasite genotype-genotype interaction at 24 hours post
51 infection (Specificity). Enrichment analysis was also carried out on expression data to see which
52 categories of molecules are differentially regulated during infection. The results confirm our
53 previous findings of up-regulation in antimicrobial peptide expression and provide a comprehensive
54 overview of changes in and the specificity of gene expression after exposure to 2 strains of
55 *C.bombi*.
56

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/ (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.*

2012) in R (3.0.1) (R Core Team 2013). 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 (specifcity)(~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 = $1e\text{-}^{16}$). 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

153 :BTT23246_1, Chitinase 3: BTT39163_1, Chitinase 4: BTT05313_1) is upregulated. Figure 2
154 shows the correlation of expression patterns between these sixteen transcripts related to chitin
155 metabolism. There is some clustering, but not of any clear functional groups. Taken together this
156 differential expression suggests an important role for the repair or restructuring of the peritrophic
157 matrix in the bumblebees' response to *Crithidia*.
158
159 When the BLAST searches against the IIID and nr databases were combined, we found that 89
160 transcripts relate to canonical insect immune genes. We describe them in the order receptors, serine
161 proteases, signalling pathways and effectors (Schmid-Hempel 2005).
162
163 *Receptors:*
164 The Down syndrome cell adhesion molecule (Dscam), a pattern recognition receptor has come to
165 the forefront of research into insect immune specificity as thousands of different splice forms have
166 been identified and it is associated with insect immunity (Smith *et al.* 2011). We found five
167 downregulated transcripts annotated as immunoglobulin superfamily (*Dscam* included in hit list)
168 (BTT03519_1, BTT08682_1, BTT15814_1, BTT26724_1, BTT27678_1) and one upregulated
169 transcript (BTT03519_1).
170
171 *Serine proteases:*
172 Serine proteases are important proteolytic enzymes in many molecular pathways. When these serine
173 proteases are no longer needed, they are inactivated by serine protease inhibitors (Zhao *et al.* 2012).
174 CLIP domain serine proteases mediate insect innate immunity (Zou *et al.* 2006). Twenty one
175 transcripts related to serine proteases, serine protease homologues or serine protease inhibitors were
176 differentially expressed upon infection (see Table 1). Lipophorin receptor 2 (downregulated
177 BTT34617_1) binds with serpins to aid in their encytocytosis (Soukup *et al.* 2009).
178

179 *Signalling pathways:*

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

190

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

197

198 The downregulated Drumstick (BTT13062_1) interacts with the JAK/STAT pathway during its'
199 development role (Johansen *et al.* 2003), but we could not find any information about its immune
200 role. Two transcripts (BTT11590_1, BTT14205_1) of *Puckered* were downregulated. *Puckered*,
201 which codes for a dual specificity phosphatase, is a key regulator of the c-Jun-N-terminal kinase
202 (JNK) immune pathway (Karkali & Panayotou 2012). Mpk2/p38a (downregulated BTT05769_1) is
203 involved in the JNK Pathway and JAK/STAT Pathway. Heat-shock factor activation by p38 is a
204 recently discovered part of antimicrobial reactions in flies (Chen *et al.* 2010). We found two heat

205 shock protein transcripts (BTT23758_2, BTT37030_1) and one other (BTT17701_1) that were
206 downregulated and upregulated respectively. These are all involved in the JAK/STAT pathway.
207
208 *Effectors:*
209 In our previous paper (Riddell *et al.* 2011) we found that antimicrobial peptides were upregulated at
210 24 hours post-infection. We would expect the same trend here. Indeed, we found that five
211 transcripts for defensin (BTT06274_2, BTT8490_1, BTT10405_1, BTT14019_1, and
212 BTT42034_1) and three hymenoptaecin transcripts (BTT18071_1, BTT24170_1, BTT24170_2)
213 were all upregulated. An apidaecin precursor (BTT33652_1) was downregulated. Apidaecin has
214 recently been shown to be expressed in bumblebees (Colgan *et al.* 2011). The downregulated beta-
215 amyloid-like protein (BTT20240_1) has been shown to be an antimicrobial peptide in mammals
216 (Soscia *et al.* 2010). Hemolectin (BTT15326_1, upregulated) is a clotting protein known to have a
217 role against gram-negative bacteria (Lesch *et al.* 2007).
218
219 Reactive oxygen species (ROS) are generated by respiration in the mitochondria or as part of the
220 immune response (Molina-Cruz *et al.* 2008). P450 cytochromes are oxidases acting terminally in
221 monooxygenase systems (Felix & Silveira 2012). Some are regulated in response to infection
222 possibly either as direct immune responders (Vlachou *et al.* 2005), producing nitric oxide (NO) or
223 other reactive oxygen radicals or as part of the host detoxification process decreasing oxidative
224 stress after an infection (Molina-Cruz *et al.* 2008). A number of *cytochromes P450* were
225 differentially expressed 24 hours post infection. Ten cytochrome p450 transcripts (*Cyp4p3*:
226 BTT05294_1, BTT20848_1, BTT22253_1, BTT23317_1, BTT32674_1, *cytochrome P450 4g15*:
227 BTT23811_1, BTT32459_1, *cytochrome P450 6k1*: BTT35547_1, BTT40653_1, *cytochrome P450*
228 *6a14*: BTT38445_1) were found to be downregulated. Three other *cytochrome P450* transcripts
229 (*Cyp4p3*: BTT21216_1, BTT35543_1, *cytochrome P450 315a1*: BTT26726_1) were upregulated.
230 Several other cytochromes (cytochrome b: BTT20524_1, BTT39776_1, BTT41896_1, and

231 cytochrome c: BTT05255_2) were downregulated.

232

233 Numerous other actors in the production of ROS were found to be differentially expressed. *TPX4*
234 (BTT13285_1), coding for a Thioredoxin-dependent peroxidase, which detoxifies H_2O_2 was
235 downregulated. This gene was found be differentially expressed during *Plasmodium* infection in
236 *Anopheles gambiae* (Baton *et al.* 2009). Calcineurin (BTT08150_1, BTT26273_1) was found to be
237 downregulated 24 hours post-infection, which agrees with our previous findings (Riddell *et al.*
238 2011). In infected *D. melanogaster* larvae, NO signals are enhanced by Calcineurin to promote
239 induction of robust immune responses via the Imd signalling pathway (Dijkers & O'Farrell 2007).

240

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

246

247 *Miscellaneous:*

248 Major royal jelly protein (BTT05317_2, BTT36365_1 upregulated) has been shown to have
249 antimicrobial properties and to be expressed in response to bacterial infection in honeybees
250 (Scharlaken *et al.* 2007; Buttstedt *et al.* 2013). Vitellogenin (downregulated BTT36006_1) is a
251 potent regulator of the immune response in honeybees (Amdam *et al.* 2004). Several orthologs of
252 putative *Drosophila* immune loci were differentially expressed 24 hours post-infection (CG12505:
253 BTT00934_1, CG18348: BTT04397_1, CG7296: BTT15035_1, BTT18395_1, CG8791:
254 BTT18908_1, CG5527: BTT35653_1, Fst: BTT11511_1). The downregulated CG4393
255 (BTT05817_1) is weakly analogous to TNF receptor associated factor 3 (TRAF3) that mediates
256 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 GNBP, BTT03533_1 and downregulated *GNBP1-2* 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).

334

335 *Effectors:*

336 Five transcripts relate to the AMPs *defensin* (BTT06274_2, BTT42034_1) and hymenoptaecin
337 (BTT18071_1, BTT24170_1, BTT24170_2). They were all upregulated. An apidaecin precursor
338 (BTT20828_1) was upregulated. Hemolectin had three downregulated transcripts (BTT14194_1,
339 BTT17013_1, BTT26614_1) and one upregulated (BTT15326_1). Argonaute-2, a RNA-silencing
340 endonuclease, is involved in antiviral defense in insects (downregulated BTT02374_1) (van Rij *et*
341 *al.* 2006).

342

343 *Eater* encodes a transmembrane receptor involved in phagocytosis in *Drosophila* (Kocks *et al.*
344 2005). A transcript (BTT11132_1) relating to *Eater* was upregulated. The melanisation process
345 component Dopa decarboxylase (BTT19093_1) was upregulated. Another enzyme involved in
346 melanisation, laccase was found to be downregulated (BTT20241_1, BTT33633_1) (Arakane *et al.*
347 2005).

348

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

352

353 *Miscellaneous:*

354 A small number of transcripts were related to chitin metabolism. SCRASP1 has a chitin-binding
355 domain that has been hypothesized to sense chitin in response to injury and to transduce signals via
356 the serine protease domain (Blumberg *et al.* 2013). We found an upregulated transcript related to
357 *SCRASP 1* (BTT41923_1). A peritrophin precursor was also upregulated (BTT10727_1). As was a
358 chitinase 3 transcript (BTT23246_1).

359

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

393 factors downstream of the activation of the Toll and IMD pathways (Dong *et al.* 2012). Our results
394 suggest that *Dscam* related genes may be important in differentiating strains of the trypanosome
395 *Crithidia bombi*.
396
397 We found a number of genes associated with chitin metabolism. Through several pathways chitin
398 metabolism is fundamental to invertebrate immunity (Beckerman *et al.* 2013). As an aside, an
399 intriguing hypothesis is that chitin metabolism is the nexus through which defense against predators
400 and against parasites are traded-off (Beckerman *et al.* 2013). Our data suggests that the peritrophic
401 matrix may be fundamental in the bee's defence against *Crithidia.* The peritrophic matrix acts as an
402 immunological barrier against trypanosomes. Tsetse flies with an underdeveloped PM have lower
403 levels of refractoriness to trypanosome infections (Weiss *et al.* 2013). This is due to a premature
404 immune response; the trypanosomes get through the PM quicker and stimulate the immune
405 response at an earlier stage compared to refractory flies.
406
407 A similar study has recently been published (Barribeau *et al.* 2014). Although they find genotype x
408 genotype interactions in gene expression, it is in a much smaller number of genes. We hypothesise
409 that our much larger catalogue of genes, including *Dscam*, is due to our experimental design. We
410 used a 2x2 analysis of preselected reciporcal host-parasite interactions. This increased our
411 likelihood of detecting differential expression using RNA-seq compared to a less directed sample
412 collection.
413
414 In this paper we have shown that the expression of immune genes is associated with specific
415 interactions between different host and parsite genotypes in this bumblebee / trypanosome model. In
416 future RNAi work we will knockdown candidate genes thereby altering these specific interactions
417 to directly examine their biological significance.

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602

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)*.

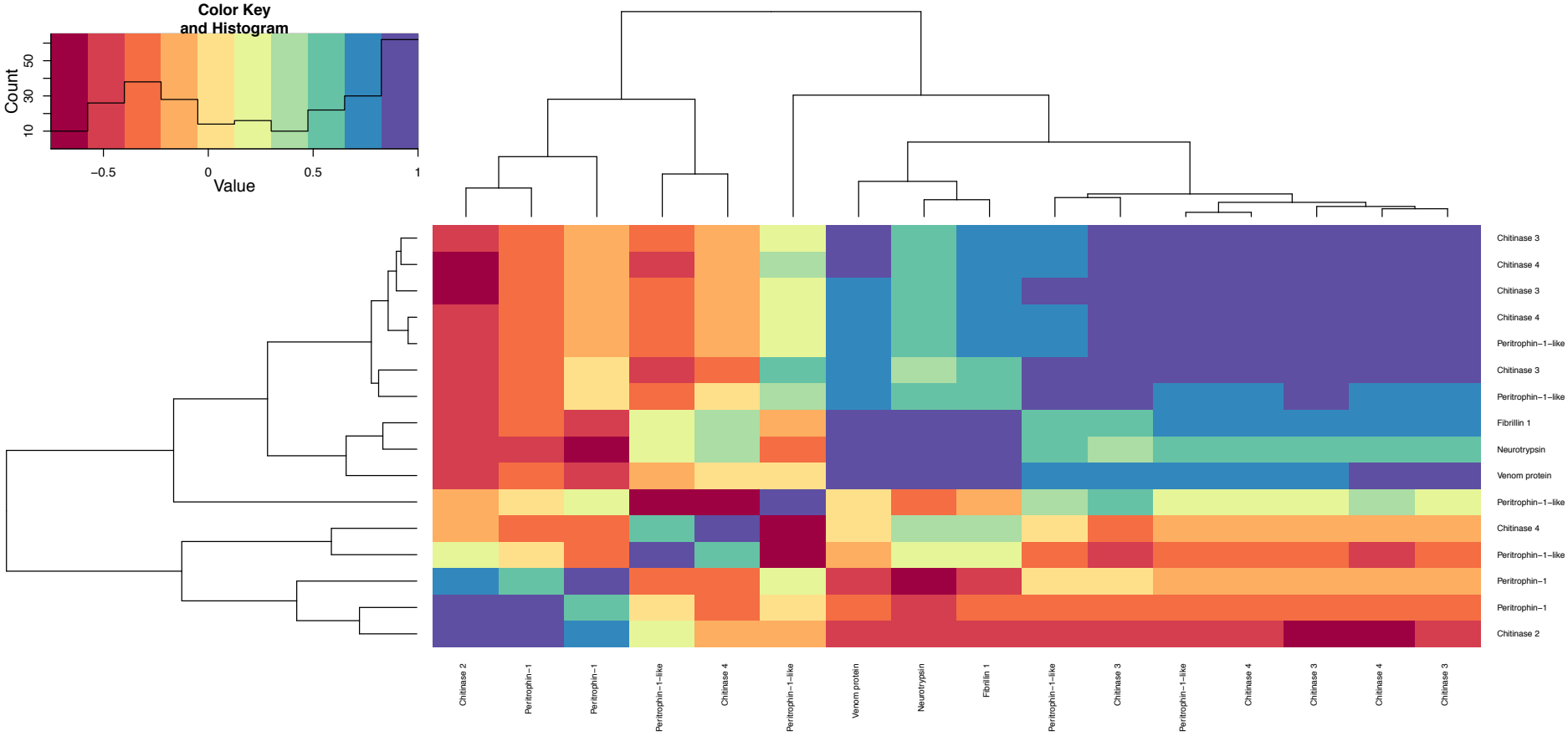

Figure 2. A heatmap showing the correlations of the expression patterns of the transcripts labelled as chitin metabolism genes that where differentially expressed twenty four hours post-infection compared to uninfected samples (infected versus uninfected).

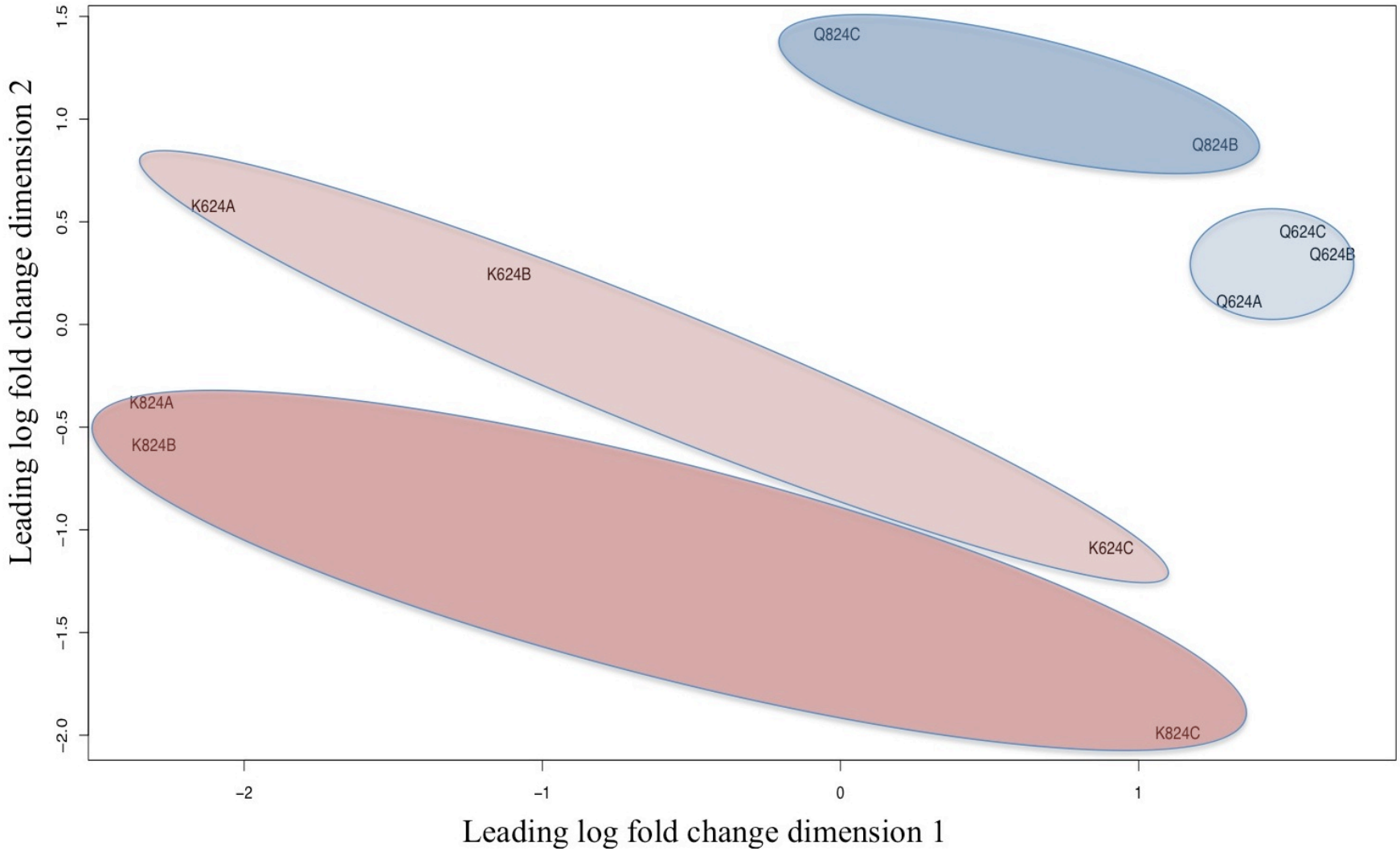

Figure 3. A multidimensional scaling (MDS) plot of the 11 samples used in the specificity analysis based on the expression of 591 differentially expressed transcripts. There are two colonies (K (red) and Q (blue)) and two *Crithidia* strains (6 (light) and 8 (dark)). A/B/C refers to replicates. Dimension 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.

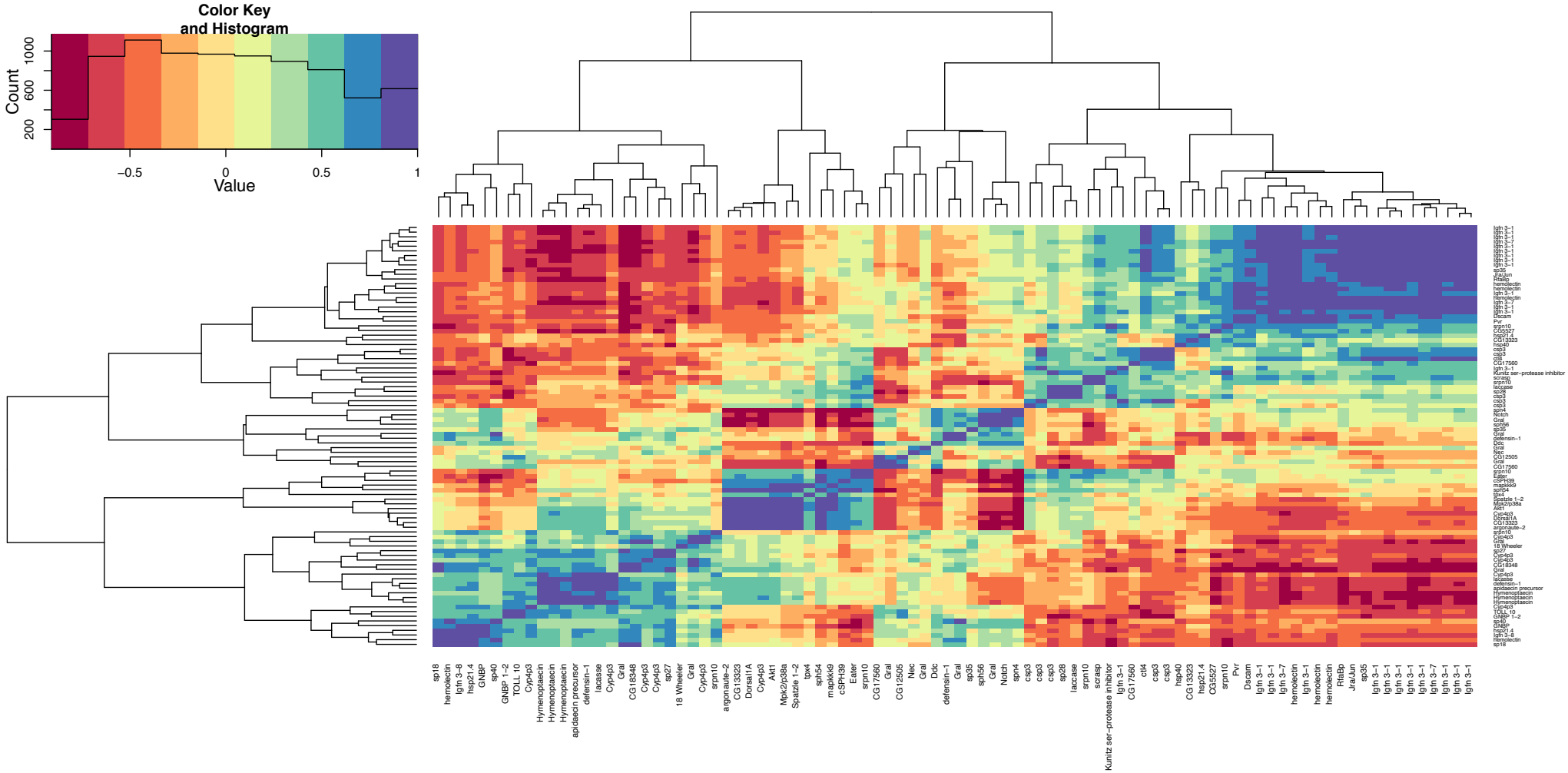

Figure 4. A heatmap showing the correlations of the expression patterns of the 90 transcripts labelled as immune genes in the analysis identifying genes differentially expressed depending on host genotype – parasite genotype interactions (Specifcity).

Serine Proteases	Upregulated	Downregulated
CLIPA6		BTT20125_1
CLIPA7		BTT07313_1, BTT31897_1
CLIPD5		BTT10579_1, BTT10912_1, BTT18247_1 BTT25711_1, BTT06803_1
SP24	BTT03436_1	
SP27		BTT08108_1, BTT38696_1
SP35	BTT05300_1	
Serine protease homologues	Upregulated	Downregulated
SPH54	BTT01977_1	BTT06125_1
Serine protease inhibitors	Upregulated	Downregulated
NEC-like		BTT31997_1
spn4		BTT04130_1, BTT40693_1, BTT41025_1, BTT41461_1
SRPN10		BTT04508_1, BTT20259_1

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).

Serine Proteases	Upregulated	Downregulated
cSp3	BTT35293_1	BTT10579_1, BTT10912_1, BTT18247_1, BTT25711_1
Sp18	BTT20808_1	
Sp27	BTT40251_1	
Sp28		BTT20637_1
Sp35	BTT05300_1	BTT10155_1
Sp40	BTT15256_1	
Tequilla/GRAL/Sp23	BTT01709_1, BTT05886_1, BTT09081_1, BTT20661_1, BTT20725_1, BTT24359_1, BTT25071_1	
Serine protease homologues	Upregulated	Downregulated
cSPH39		BTT21868_1
Sph54		BTT27769_1
Sph56	BTT17814_1	
Serine protease inhibitors	Upregulated	Downregulated
Kunitz ser-protease inhibitor		BTT14993_1
necrotic (nec)	BTT35742_1	
Spn 4		BTT04130_1
SRPN10		BTT02607_1, BTT4508_1, BTT20259_1, BTT40693_1

Table 2. List of transcriptions associated with serine proteases and serine protease inhibitorsfound to be differentially expressed in the specificity analysis.