Insect immune specificity in a host-parasite model

C.E. Riddell[1], J.D. Lobaton Garces[1], S. Adams[2], D. Twell[1] & E.B. Mallon[1]

[1]Department of Biology, University of Leicester, U.K.

[2] School of Life Sciences, University of Warwick, U.K.

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, 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 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* depending on the genotype-genotype interactions of the given bumblebee colony and *Crithidia* strain.

Ecological studies with a number of natural, co-evolving host-parasite systems have shown that resistance to a parasite is highly variable in invertebrates, in part determined by the interaction of the genotypes of the host and the parasite (Schmid-Hempel 2001; Carius *et al.* 2001; Lambrechts 2010). Such interactions can lead to the evolution and maintenance of genetic variation in natural populations (Hamilton *et al.* 1981). Many human diseases use insects as vectors (Jacobslorena & Lemos 1995). Any effort at control will require a better understanding of this relationship between host and parasite.

How this level of specificity is generated is unclear. Genotype-genotype interactions 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 (Hauton & Smith 2007). It may be that such outcomes can be explained by other processes. For example, the bumblebee, *Bombus terrestris/*trypanosome *Crithidia bombi* system is a well studied example of these ecological host x parasite genotype-genotype interactions (Schmid-Hempel *et al.* 1999; Schmid-Hempel & Reber-Funk 2004). Yet 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 (Koch & Schmid-Hempel 2012).

Still there is evidence that the immune system must have a role in generating this host-parasite specific response. A number of studies have found differential immune genes 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.

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) looking 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 (Riddell *et al.* 2009). 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 (Schmid-Hempel & Reber-Funk 2004) and generate specific responses in novel hosts (Schmid-Hempel *et al.* 1999). 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.

86 ***RNA sample preparation and sequencing***

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

94

95 ***Statistical analysis***

96 The reference transcriptome was downloaded from
97 http://www.nematodes.org/downloads/databases/Bombus_terrestris/ (Colgan *et al.* 2011).
98 Functional annotation related to the transcriptome was obtained using the BLAST2GO package
99 (Götz *et al.* 2008). Alignment was done using GSNAP (version 2012-07-20) (Wu & Nacu 2010).
100 Only reads that mapped uniquely were selected for further analysis. Counts were generated per
101 transcript for each sample.

102

103 Differential expression analysis was performed using the edgeR (3.4.0) package (McCarthy *et al.*
104 2012) in R (3.0.1) (R Core Team 2013). Normalization factors were computed using the TMM
105 technique, after which tagwise dispersions were calculated and subjected to a generalized linear
106 model (GLM). Resulting *p* values were subjected to Benjamini–Hochberg multiple testing
107 correction to derive FDRs; only transcripts with a FDR < 0.05 were considered for further analysis.
108 Three separate GLMs were carried out, one looked for transcripts that are differentially expressed
109 upon infection with *Crithidia* at 24 hours post-infection (~0+colony+infect(yes/no)) infect here are
110 bees either infected with strain 6 or 8, one looking at the gene expression difference between 24
111 hours and 48 hours post strain 6 infection (~0+colony + time) and a further GLM that looked for

112 transcripts that were expressed in a specific pattern at 24 hours post-infection (~0+colony*strain).

113

114 Using Blast2Go, we then carried out an enrichment analysis (Fisher exact test) on each of these lists

115 of differentially expressed genes to see which GO terms are overrepresented relative to the entire

116 genome. We then used REVIGO to summarize and visualise these terms (Supek *et al.* 2011).

117

118 For each of the lists of differentially expressed transcripts we also carried out a blastx analysis

119 against the insect innate immunity database (IIID) (Brucker *et al.* 2012). We used the BLOSUM62

120 matrix with a word size of 3. The results were filtered to only contain hits with an *E*-value <1e-10, a

121 bit score ≥30,

122

123

124 **Results**

125 ***Genes differentially expressed at 24 hours post-infection***

126 31,843 unique transcripts were mapped to the transcriptome. 489 transcripts were found to be
127 differentially expressed 24 hours post-infection (FDR < 0.05), 324 were downregulated and 165
128 upregulated. Reannotating the transcripts using Blast2GO (blastx against the nr database with e <
129 0.001), 109 had no BLAST hits. A further 68 had uninformative BLAST hits (anonymous predicted
130 protein). The remaining 312 were used in the enrichment analysis. Figure 1 shows a summary of the
131 enriched GO terms found (Fisher's test p < 0.05). Defense response (GO:0006952, FDR = 0.047)
132 and chitin metabolism (GO:0006030, FDR = 0.032) were the only processes significantly enriched
133 at a more stringent level (FDR < 0.05).

134

135 *Peritrophic membrane:*

136 The peritrophic matrix (PM) forms a layer composed of chitin and glycoproteins that lines the
137 insect midgut lumen (Kuraishi *et al.* 2011). The PM facilitates digestion and forms a protective
138 barrier to prevent the invasion of ingested pathogens (Lehane 1997; Kuraishi *et al.* 2011). Fibrillin 1
139 (BTT14121_ 1), a venom protein precursor (BTT32193_1), Neurotrypsin (BTT07956_1),
140 Peritrophin-1-like (BTT01709_1, BTT22959_1, BTT37215_1, BTT42262_1) and four chitinase
141 transcripts (Chitinase 3: BTT23997_1 BTT38724_1, Chitinase 4 BTT20684_1, BTT23469_1) are
142 downregulated upon infection. Fibrillins are extracellular matrix macromolecules, ubiquitous in the
143 connective tissues (Isogai *et al.* 2003). BTT32193_1 was classed as a venom protein, but was also
144 very similar to Chitinase 3 (blastx e = $1e\text{-}^{16}$). Chitinases modulate the structure and porosity of the
145 PM (Dinglasan *et al.* 2009). Neurotrypsin is a serine protease expressed in the nervous system
146 (Gschwend *et al.* 1997). However in the protease domain it shares similarities with Sp22D, a chitin
147 binding serine protease (Danielli *et al.* 2000). The chitin fibrils of the PM are assembled into a wide
148 cross-hatched pattern connected by peritrophins (Dinglasan *et al.* 2009). A second group made up
149 of Peritrophin-1 (BTT05886_1, BTT20661_1) and 3 further chitinase transcripts (Chitinase 2

150 :BTT23246_1, Chitinase 3: BTT39163_1, Chitinase 4: BTT05313_1) is upregulated. Figure 2
151 shows the correlation of expression patterns between these sixteen transcripts related to chitin
152 metabolism. There is some clustering, but not of any clear functional groups. Taken together
153 however, this differential expression suggests an important role for the repair or restructuring of the
154 peritrophic matrix in the bumblebees' response to *Crithidia*.
155

156 When the BLAST searches against the IIID and nr databases are combined, eighty nine transcripts
157 relate to canonical insect immune genes. We describe them in the order receptors, serine proteases,
158 signalling pathways and effectors (Schmid-Hempel 2005).

159

160 *Receptors:*
161 The Down syndrome cell adhesion molecule (Dscam), a pattern recognition receptor has come to
162 the forefront of research into insect immune specificity as it has been found to have thousands of
163 different splice forms and is associated with insect immunity (Smith *et al.* 2011). We found five
164 downregulated transcripts annotated as immunoglobulin superfamily (*Dscam* included in hit list)
165 (BTT03519_1, BTT08682_1, BTT15814_1, BTT26724_1, BTT27678_1) and one upregulated
166 transcript (BTT03519_1).
167

168 *Serine proteases:*
169 Serine proteases are important proteolytic enzymes in many molecular pathways. When these serine
170 proteases are no longer needed, they are inactivated by serine protease inhibitors (Zhao *et al.* 2012).
171 CLIP domain serine proteases mediate insect innate immunity (Zou *et al.* 2006). Twenty one
172 transcripts related to serine proteases, serine protease homologues or serine protease inhibitors were
173 differentially expressed upon infection (see Table 1) . Lipophorin receptor 2 (downregulated
174 BTT34617_1) binds with serpins to aid in their encytocytosis (Soukup *et al.* 2009).
175

176 *Signalling pathways:*

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

187

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

194

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

202 shock protein transcripts (BTT23758_2, BTT37030_1) and one other (BTT17701_1) that were
203 downregulated and upregulated respectively. These are all involved in the JAK/STAT pathway.
204
205 *Effectors:*
206 Our previous paper (Riddell *et al.* 2011) found that antimicrobial peptides were upregulated at 24
207 hours post-infection. We would expect the same to be true here. Indeed, we found 5 transcripts for
208 defensin (BTT06274_2, BTT8490_1, BTT10405_1, BTT14019_1, and BTT42034_1) and 3
209 transcripts for hymenoptaecin (BTT18071_1, BTT24170_1, BTT24170_2), all upregulated. An
210 apidaecin precursor (BTT33652_1) was downregulated. Apidaecin has recently been shown to be
211 expressed in bumblebees (Colgan *et al.* 2011). The downregulated beta-amyloid-like protein
212 (BTT20240_1) has been shown to be an antimicrobial peptide in mammals (Soscia *et al.* 2010).
213 Hemolectin (BTT15326_1, upregulated) is a clotting protein known to have a role against gram
214 negative bacteria (Lesch *et al.* 2007).
215
216 Reactive oxygen species (ROS) are generated by respiration in the mitochondria or as part of the
217 immune response (Molina-Cruz *et al.* 2008). P450 cytochromes are oxidases acting terminally in
218 monooxygenase systems (Felix & Silveira 2012). Some are regulated in response to infection
219 possibly either as direct immune responders (Vlachou *et al.* 2005), producing nitric oxide (NO) or
220 other reactive oxygen radicals or as part of the host detoxification process decreasing oxidative
221 stress after an infection (Molina-Cruz *et al.* 2008). A number of *cytochromes P450* were
222 differentially expressed 24 hours post infection. Ten cytochrome p450 transcripts (*Cyp4p3*:
223 BTT05294_1, BTT20848_1, BTT22253_1, BTT23317_1, BTT32674_1, *cytochrome P450 4g15*:
224 BTT23811_1, BTT32459_1, *cytochrome P450 6k1*: BTT35547_1, BTT40653_1, *cytochrome P450*
225 *6a14*: BTT38445_1) were found to be downregulated. Three other *cytochrome P450* transcripts
226 (*Cyp4p3*: BTT21216_1, BTT35543_1, *cytochrome P450 315a1*: BTT26726_1) were upregulated.
227 Several other cytochromes (cytochrome b: BTT20524_1, BTT39776_1, BTT41896_1, and

228 cytochrome c: BTT05255_2) were downregulated.

229

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

238

239 We found downregulation of sortilin-related receptor-like (BTT31654_1). In mammals, sortilin aids
240 in phagocytosis (Wähe *et al.* 2010). Two downregulated transcripts (BTT35021_1, BTT08756_1)
241 were matched to *croquemort. Croquemort,* which codes for a scavenger receptor is a key part of
242 the Imd pathway but in its apototic phagocytosis role not its immune one (Franc *et al.* 1999).
243 Annexin IX (downregulated BTT02025_1) has been shown to be induced by septic injury in
244 *Drosophila*. It is thought to encode for an anticoagulant (Gregorio *et al.* 2001).

245

246 *Miscellaneous:*

247 Major royal jelly protein (BTT05317_2, BTT36365_1 upregulated) has been shown to have
248 antimicrobial properties and to be expressed in response to bacterial infection in honeybees
249 (Scharlaken *et al.* 2007; Buttstedt *et al.* 2013). Vitellogenin (downregulated BTT36006_1) is a
250 potent regulator of the immune response in honeybees (Amdam *et al.* 2004). Several orthologs of
251 putative *Drosophila* immune loci were found to be differentially expressed 24 hours post-infection
252 (CG12505: BTT00934_1, CG18348: BTT04397_1, CG7296: BTT15035_1, BTT18395_1,
253 CG8791: BTT18908_1, CG5527: BTT35653_1, Fst: BTT11511_1). The downregulated CG4393

254 (BTT05817_1) is weakly analogous to TNF receptor associated factor 3 (TRAF3) that mediates
255 signal transduction involved in mammalian immune responses. Downregulated BTT37289_1 codes
256 for a putative fatty acyl-CoA reductase.
257

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

259 43 transcripts were found to be differentially expressed between 24 hours post-infection and 48
260 hours post-infection. Of these 17 had no BLAST hits. A further six had uninformative BLAST hits
261 (anonymous predicted protein). The remaining 20 were used in the analysis. Defense response was
262 the only GO term significantly enriched (FDR= 0.00015), with seven transcripts. Three transcripts
263 correspond to Hymenoptaecin (BTT18071_1, BTT24170_1,BTT24170_2). They were all
264 upregulated. This suggests a continuing strong AMP production 48 hours after infection. This
265 agrees with other immune assays in bumblebees (Korner & Schmid-Hempel 2004). Argonaute-2, a
266 RNA-silencing endonuclease, is involved in antiviral defense in insects (downregulated
267 BTT02484_1) (van Rij *et al.* 2006). GstD8, a glutathione S-transferase, is involved in the
268 detoxification process (upregulated BTT04810_1) (Gerardo *et al.* 2010). Dopa decarboxylase
269 (upregulated BTT28048_1) converts L-dopa to dopamine during the melanisation process (Ferdig *et*
270 *al.* 2000). SCR-B9 (upregulated BTT40924_1) codes for a scavenger receptor protein. Scavenger
271 receptor proteins have been found to be microbial pattern recognition receptors in flies (Rämet *et al.*
272 2001).

273 **Genes differentially expressed depending on host genotype – parasite genotype interactions**

274 There were 591 differentially expressed transcripts (FDR < 0.05). Reannotating the transcripts
275 using Blast2GO (blastx against the nr database with e < 0.001), 150 had no BLAST hits. A further
276 64 had uninformative BLAST hits (anonymous predicted protein). There were 109 transcripts that
277 had previously been found to be differentially expressed at 24 hours post infection. Figure 3 shows
278 a multidimensional scaling (MDS) plot of the samples based on the expression of these 591 genes.

279 It can be clearly seen that the 11 samples are grouped into their colony-strain interaction.

280

281 Of the 591 transcripts, 132 were upregulated and 459 were downregulated. Up or downregulation
282 does not have the same meaning here as in the infected versus uninfected model were there was a
283 clear baseline (uninfected). Depending on how you order the GLM we could get the reciprocal
284 result. Our model used colony K strain 8 as the final contrast. From our previously published qPCR
285 data (Riddell *et al.* 2009), we know the colony K strain 8 interaction displayed the highest levels of
286 AMPs (effectors). Therefore when we say a transcript is upregulated, we mean it is upregulated in
287 this high immune response interaction.

288

289 As with the infection data, we combined the BLAST searches against the IIID and nr databases.
290 Ninety transcripts correspond to canonical insect immune genes. We again describe them in the
291 order receptors, serine proteases, signalling pathways and effectors (Schmid-Hempel 2005).

292

293 *Receptors:*

294 Two transcripts were associated with gram negative binding proteins (upregulated GNBP,
295 BTT03533_1 and downregulated *GNBP1-2* BTT35513_1) Although, as their name suggests,
296 GNBPs are most associated with defense against gram negative bacteria, they have been show to
297 have a role in respond to *Plasmodium* infections (Tahar *et al.* 2002). C-type lectins (CTLs) bind
298 carbohydrates and mediate processes including pathogen recognition (Cirimotich *et al.* 2010).
299 CTL4 is agonist to *Plasmodium* infections in mosquitoes (Cirimotich *et al.* 2010). A CTL4
300 transcript (BTT29328_1) was found to be downregulated.

301

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

305 BTT22598_1, BTT22819_1, BTT23339_1, BTT24070_1, IGFn3-7: BTT08109_1, BTT09498_1)
306 and one was upregulated (IGFn3-8: BTT03519_1). *Dscam* and most of the other Ig superfamily
307 transcripts cluster together in the top right of figure 4, suggesting they are similarly expressed.
308
309 *Serine proteases:*
310 28 transcripts related to serine proteases, serine protease homologues or serine protease inhibitors
311 were differentially expressed (see Table 2).
312
313 *Signalling pathways:*
314 The Toll-like receptor *18Wheeler* (BTT35732_1) was upregulated as was *Toll 10* (BTT09386_1).
315 18Wheeler has been shown to be important in the anti gram-negative immune response in
316 *Drosophila* larvae (Ligoxygakis *et al.* 2002). *Dorsal 1A* (BTT04010_1), a transcription factor that
317 is a fundamental part of the Toll pathway, was downregulated. A transcript for *Spatzle 1-2* was
318 downregulated (BTT10679_1).
319
320 The tyrosine kinase *Pvr* (BTT04822_1), which inhibits JNK activation (Bond & Foley 2009) was
321 downregulated. *Jun*, a transcription factor of the JNK pathway was downregulated (BTT13636_1).
322 *Mpk2/p38a* (downregulated BTT16580_1) and *MAPKKK9* (downregulated BTT04404_1) are
323 mitogen-activated protein kinases involved in the JNK Pathway and JAK/STAT pathways. We
324 found two heat shock protein transcripts (BTT17371_1, BTT22195_1) and one other (BTT17701_1)
325 that were downregulated and upregulated respectively. These are all involved in the JAK/STAT
326 pathway. *Akt 1* (downregulated BTT14188_1) is part of the insulin/insulin-like growth factor 1
327 signaling (IIS) cascade. IIS plays a critical role in the regulation of innate immunity. Activation of
328 *Akt* signaling leads to a decrease in malaria infection intensity in mosquitoes (Corby-Harris *et al.*
329 2010).
330

331 *Effectors:*

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

338

339 *Eater* encodes for a transmembrane receptor involved in phagocytosis in *Drosophila* (Kocks *et al.*
340 2005). A transcript (BTT11132_1) relating to *Eater* was upregulated. The melanisation process
341 component Dopa decarboxylase (BTT19093_1) was upregulated. Another enzyme involved in
342 melanisation, laccase was found to be downregulated (BTT20241_1, BTT33633_1) (Arakane *et al.*
343 2005).

344

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

348

349 *Miscellaneous:*

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

355

356 *Retinoid and fatty-acid-binding protein (RfaBp)* (BTT07678_1) was downregulated. RfaBp was

357 found to be upregulated upon injection of LPS in *Drosophila* during a proteomic study (Vierstraete
358 *et al.* 2004). *Notch* (upregulated BTT09545_1) is involved in the specification of crystal cells in
359 *Drosophila melanogaster* (Mukherjee *et al.* 2011). Several orthologs of putative *Drosophila*
360 immune loci were found to be differentially expressed (CG5527: BTT08512_1, CG12505:
361 BTT00934_1, CG13323: BTT38025_1, BTT38087_1, CG17560: BTT02877_1 downregulated,
362 BTT05845_1 upregulated, CG18348: BTT20843_1)
363

364 **Discussion**

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

369

370 We confirmed the importance of antimicrobial peptides in the specific defense against *Crithidia*
371 (Riddell *et al.* 2009, 2011; Deshwal & Mallon 2014). It is also clear that several other effectors
372 including ROS and phagocytosis may be important. Several immune pathways seem to be
373 important in the anti-*Crithidia* response. These include the Toll, Imd and JAK/STAT pathways.
374 Toll especially seems to be important in a specific immune response.

375

376 There are a larger proportion of receptor transcripts found in the specificity analysis (3.2% 19/591)
377 compared to the infection analysis (1.2% 6/489). This is not surprising, as we would expect a
378 specific immune response to a given strain to be based mainly on how it is recognised. Although
379 several receptors, including GNBPs and lectins, are differentially expressed, the most exciting
380 discovery is the large number of transcripts related to *Dscam*. The Down syndrome cell adhesion
381 molecule (Dscam), a pattern recognition receptor has come to the forefront of research into insect
382 immune specificity as it has been found to have thousands of different splice forms and is
383 associated with insect immunity (Smith *et al.* 2011). In the fruit fly *Drosophila*, silencing of *Dscam*
384 retards the insect's capacity to engulf bacteria by phagocytosis (Watson *et al.* 2005). In *Anopheles*,
385 the Dscam splice forms produced in response to parasite exposure differs between bacteria and
386 *Plasmodium* and between *Plasmodium berghei* and *Plasmodium falciparum* (Dong *et al.* 2006).
387 This has been tempered by the finding that Dscam diversity does not increase with exposure to
388 increasing heterogeneity of *Plasmodium* falciparum genotypes (Smith *et al.* 2011). Recently it has
389 been shown that *Dscam* specificity is mediated by specific splice-factors transcription downstream

390 of activation of the Toll and Imd pathways (Dong *et al.* 2012). Our results suggest that *Dscam* may
391 be important in differentiating strains of the trypanosome *Crithidia bombi*.
392
393 We found a number of genes associated with chitin metabolism. The peritrophic matrix may be
394 fundamental in the bee's defense against *Crithidia.* The peritrophic matrix acts as an immunological
395 barrier against trypanosomes. Tsetse flies with an underdeveloped PM have lower levels of
396 refractoriness to trypanosome infections (Weiss *et al.* 2013). This is due to a premature immune
397 response; the trypanosomes get through the PM quicker and stimulate the immune response at an
398 earlier stage compared to refractory flies.
399
400 A similar study has recently been published (Barribeau *et al.* 2014). Although they find geneotype x
401 genotype interactions in gene expression, it is in a much smaller number of genes. None of these
402 genes offer a potential model for generating specificity. We hypothesise that our much larger
403 catalogue of genes, including Dscam, is due to our experimental design. We used a 2x2 analysis of
404 preselected reciporcal host-parasite interactions. This increased our likelihood of detecting
405 differential expression using RNA-seq compared to a less directed sample collection.
406
407 Given that we have found that the bees own physiology, especially its immune response is vital in
408 both the defense against *Crithidia* and in explaining the host-parasite specificity, how do we
409 incorporate recent findings that the bees gut microbiota are important in exactly these phenomena
410 (Koch & Schmid-Hempel 2011, 2012). Gut microbiota impact the condition of the PM and gut
411 epithelium generally (Buchon *et al.* 2009a; Weiss *et al.* 2013). It has recently been suggested that
412 the components of the peritrophic matrix may be under the control of various immune pathways,
413 Imd (Buchon *et al.* 2009b) and STAT (Narasimhan *et al.* 2014) explicitly. Gut microbiota stimulate
414 these pathways keeping the PM intact. The intact peritrophic matrix then acts as a physical barrier
415 to colonization by parasites. Future work will focus on understanding the interactions of this

416 triumvirate of host genotype, parasite genotype and gut microbiota and their effect on disease
417 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 *(Supek* et al. *2011)*

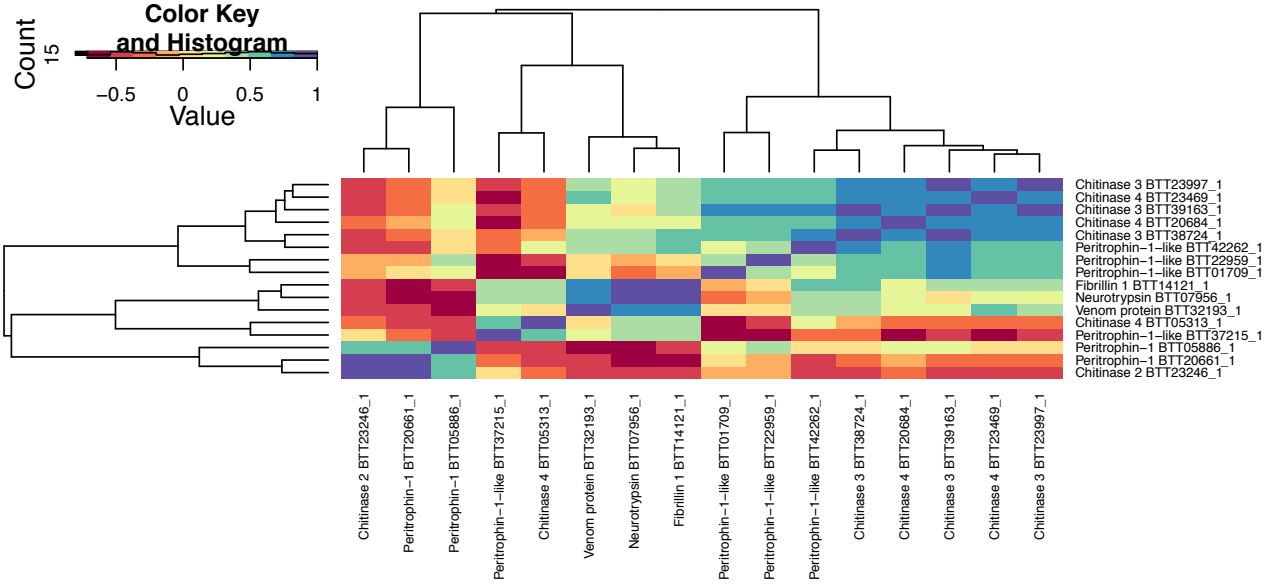

Figure 2. A heatmap showing the correlations of the expression patterns of the transcripts labelled as chitin metabolism genes that where differentially expressed twenty four hours after infection.

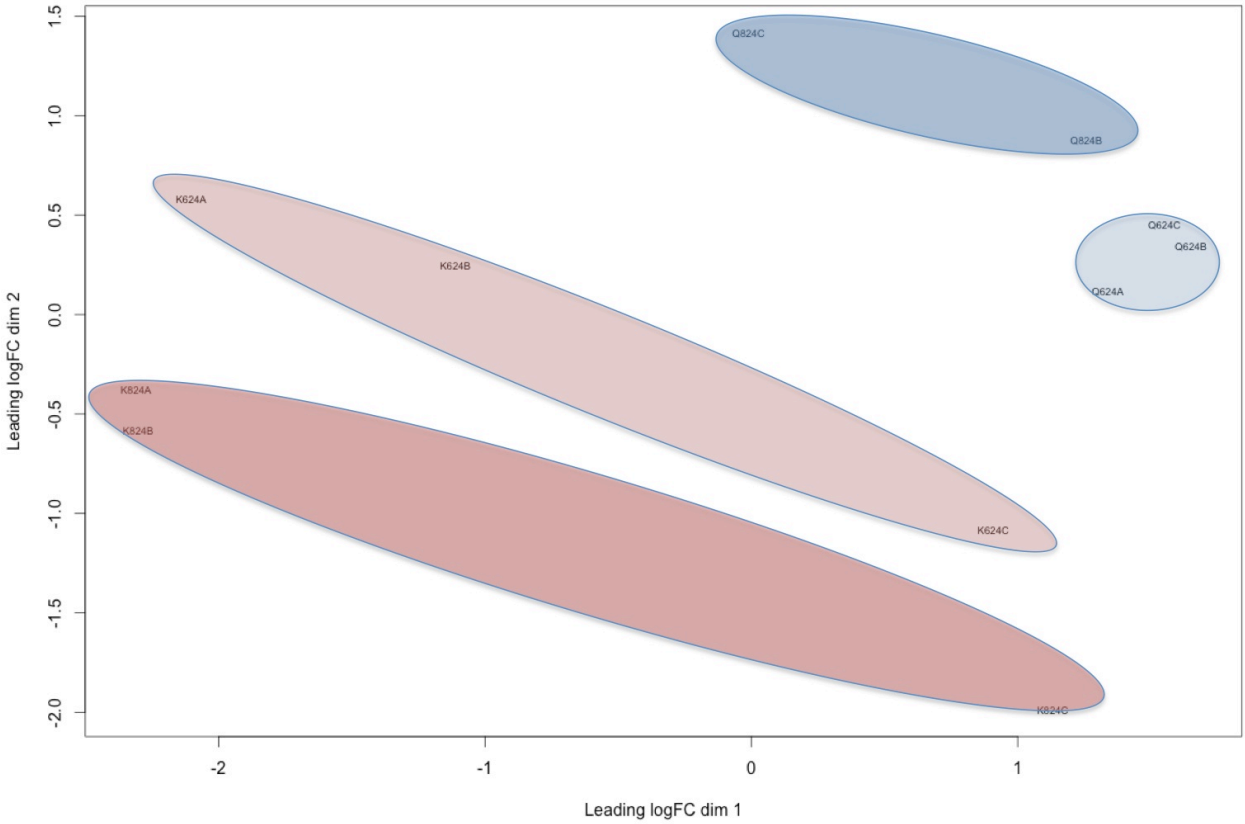

Figure 3. A multidimensional scaling (MDS) plot of the 11 samples used in the specificity analysis based on the expression of 591 differentially expressed transcripts. There are two colonies (K (red) and Q (blue)) and two *Crithidia* strains (6 (light) and 8 (dark)). Dimension 1 is the direction that 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

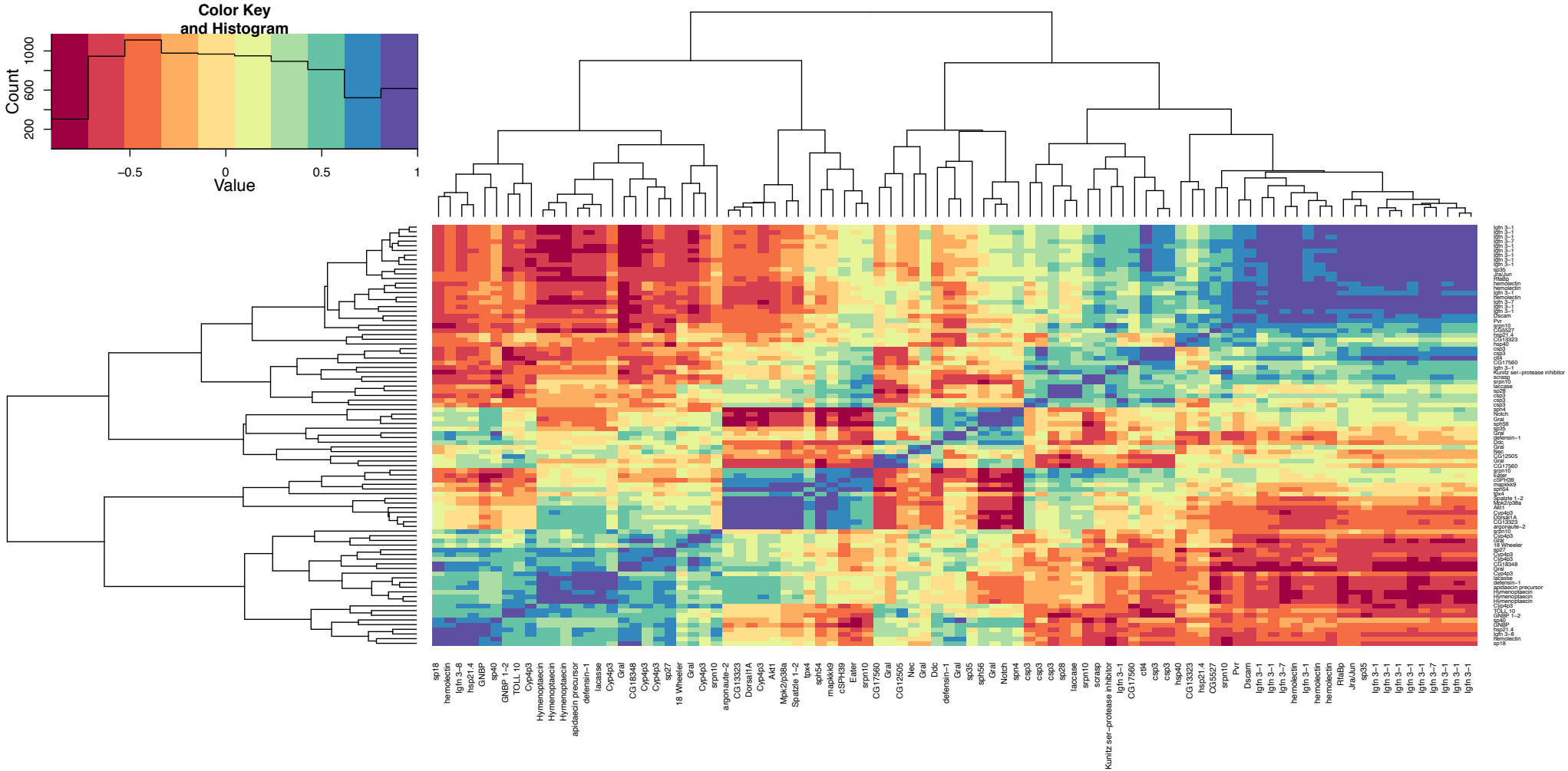

Figure 4. A heatmap showing the correlations of the expression patterns of the 90 transcripts labelled as immune genes in the analysis identifying genes differentially expressed depending on host genotype – parasite genotype interactions.

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.

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.