

1 Insect immune specificity in a host-parasite model

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8

9 Abstract

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

23

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

31

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

39

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

48

49 Understanding the source of insect immune specificity is an intriguing issue [16,17]. Such
50 interactions can lead to the evolution and maintenance of genetic variation in natural populations
51 [18]. On the practical side, many diseases of humans and their domesticated species use
52 invertebrates, especially insects, as vectors [19]. Any effort to control these diseases will require a
53 better understanding of the relationship between host and parasite.

54

55 Here, we expand our previous study and carry out a transcriptome-wide analysis of gene expression
56 in bees during *C.bombi* infection. We have carried out three analyses, comparing a) expression in
57 infected and non-infected bees 24 hours after infection by *Crithidia bombi* b) expression at 24 and
58 48 hours after *C.bombi* infection and c) looked for differential gene expression associated with the
59 host-parasite genotype-genotype interaction at 24 hours after infection. Enrichment analysis was
60 also carried out on expression data to see which categories of molecules are differentially regulated
61 during infection. The results confirm our previous findings of up-regulation in antimicrobial peptide
62 expression and provide a comprehensive overview of changes in and the specificity of gene
63 expression after exposure to 2 strains of *C.bombi*.

64

65

66 **Methods**

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

72 ***Sample Collection***

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

80 ***Infections***

81 To prepare *C. bombi* isolates, faeces was collected from workers of naturally infected colonies, and
82 mixed with 50% diluted Meliose to create a standardized dose of 500 *Crithidia* cells per µl of
83 inoculum. Previous studies had shown that such inocula, prepared from different colonies, are
84 genotypically different [8] and generate specific responses in novel hosts [7]. We infected a sample
85 of workers from each of K and Q bumblebee colonies (representing different host lines) with an
86 inoculum of faeces from each of the two wild infected colonies (6 and 8 *Crithidia* strain). We also
87 collected uninfected controls. Bees were four days old at the time of infection. After infection bees
88 were kept in colony x strain groups (1–3 individuals depending on day collected) and fed *ad libitum*.
89 24 hours or 48 hours post infection the bees were sacrificed by freezing in liquid nitrogen. They
90 were then stored at –80°C.

91 ***RNA sample preparation and sequencing***

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

99

100 ***Statistical analysis***

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

106

107 Differential expression analysis was performed using the edgeR (3.4.0) package [23] in R (3.0.1)
108 [24]. Normalization factors were computed using the TMM technique, after which tagwise
109 dispersions were calculated and subjected to a generalized linear model (GLM). Resulting *p* values
110 were subjected to Benjamini–Hochberg multiple testing correction to derive FDRs; only transcripts
111 with a FDR < 0.05 were considered for further analysis. Three separate GLMs were carried out,
112 one looked for transcripts that are differentially expressed upon infection with *Crithidia* at 24 hours
113 post-infection (~0+colony+infect(yes/no)) infect here are bees either infected with strain 6 or 8, one
114 looking at the gene expression difference between 24 hours and 48 hours post strain 6 infection
115 (~0+colony + time) and a further GLM that looked for transcripts that were expressed in a specific
116 pattern at 24 hours post-infection (~0+colony*strain).

117

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

121

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

125

126

127 **Results**

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

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

137

138 *Peritrophic membrane:*

139 The peritrophic matrix (PM) forms a layer composed of chitin and glycoproteins that lines the
140 insect midgut lumen [27]. The PM facilitates digestion and forms a protective barrier to prevent the
141 invasion of ingested pathogens [27,28]. Fibrillin 1 (BTT14121_1), a venom protein precursor
142 (BTT32193_1), Neurotrypsin (BTT07956_1), Peritrophin-1-like (BTT01709_1, BTT22959_1,
143 BTT37215_1, BTT42262_1) and four chitinase transcripts (Chitinase 3: BTT23997_1
144 BTT38724_1, Chitinase 4 BTT20684_1, BTT23469_1) are downregulated upon infection.
145 Fibrillins are extracellular matrix macromolecules, ubiquitous in the connective tissues [29].
146 BTT32193_1 was classed as a venom protein, but was also very similar to Chitinase 3 (blastx $e =$
147 $1e^{-16}$). Chitinases modulate the structure and porosity of the PM [30]. Neurotrypsin is a serine
148 protease expressed in the nervous system [31]. However in the protease domain it shares similarities
149 with Sp22D, a chitin binding serine protease [32]. The chitin fibrils of the PM are assembled into a
150 wide cross-hatched pattern connected by peritrophins [30]. A second group made up of Peritrophin-
151 1 (BTT05886_1, BTT20661_1) and 3 further chitinase transcripts (Chitinase 2 :BTT23246_1,
152 Chitinase 3: BTT39163_1, Chitinase 4: BTT05313_1) is upregulated. Figure 2 shows the

153 correlation of expression patterns between these sixteen transcripts related to chitin metabolism.
154 There is some clustering, but not of any clear functional groups. Taken together however, this
155 differential expression suggests an important role for the repair or restructuring of the peritrophic
156 matrix in the bumblebees' response to *Crithidia*.

157

158 When the BLAST searches against the IID and nr databases are combined, eighty nine transcripts
159 relate to canonical insect immune genes. We describe them in the order receptors, serine proteases,
160 signalling pathways and effectors [16].

161

162 *Receptors:*

163 The Down syndrome cell adhesion molecule (Dscam), a pattern recognition receptor has come to
164 the forefront of research into insect immune specificity as it has been found to have thousands of
165 different splice forms and is associated with insect immunity [33]. We found five downregulated
166 transcripts annotated as immunoglobulin superfamily (*Dscam* included in hit list) (BTT03519_1,
167 BTT08682_1, BTT15814_1, BTT26724_1, BTT27678_1) and one upregulated transcript
168 (BTT03519_1).

169

170 *Serine proteases:*

171 Serine proteases are important proteolytic enzymes in many molecular pathways. When these serine
172 proteases are no longer in need, they are inactivated by serine protease inhibitors [34]. CLIP domain
173 serine proteases mediate insect innate immunity [35]. 8 transcripts corresponded to clip serine
174 proteases (CLIPA6: BTT20125_1, CLIP A7: BTT07313_1, BTT31897_1, CLIPD5: BTT10579_1,
175 BTT10912_1, BTT18247_1 BTT25711_1, BTT06803_1). All were downregulated. Another
176 immune related serine protease SP27 (BTT08108_1, BTT38696_1) was also downregulated. The
177 serine protease homologue SPH54 (BTT06125_1) was downregulated. SP35 (BTT05300_1), SP24
178 (BTT03436_1) and a different SPH 54 transcript (BTT01977_1) were upregulated. Seven

179 transcripts (spn4: BTT04130_1, BTT40693_1, BTT41025_1, BTT41461_1, NEC-like:
180 BTT31997_1, and SRPN10: BTT04508_1, BTT20259_1) referring to serine protease inhibitors
181 were downregulated. The *necrotic (nec)* gene encodes the serine protease inhibitor Nec. This
182 controls a proteolytic cascade which activates the innate immune response to fungal and Gram
183 positive bacterial infections [36]. Lipophorin receptor 2 (downregulated BTT34617_1) binds with
184 serpins to aid in their encytocytosis [37].

185

186 *Signalling pathways:*

187 We found a transcript for *Spatzle* (BTT19738_1) downregulated at this time point. Activation of the
188 Toll immune pathway requires the activation of *Spatzle* [1]. MyD88 (upregulated BTT15687_1) is a
189 death domain-containing adaptor activated by Toll leading to the activation of *Pelle*. *Dorsal*
190 (BTT25273_1) was also downregulated. The nuclear translocation of Dorsal, a member of the NF-
191 kB family, in the Toll pathway induces the expression of many immune genes. We found an
192 upregulated transcript (BTT09662_1) for *Helicase89B* part of the Toll and Imd Pathway. It is
193 required downstream of NF-kB for the activation of AMP genes in *Drosophila melanogaster* [38].
194 *ird5* codes for a catalytic subunit of an IkappaB kinase that cleaves Relish. Relish (Imd pathway) is
195 an essential regulator of antimicrobial peptide gene induction. We found *ird5* (BTT03904_1) to be
196 downregulated 24 hours post-infection.

197

198 In mammals semaphorins are crucially involved in various aspects of the immune response [39]. A
199 semaphorin-5A-like transcript (BTT01850_1) was downregulated 24 hours post-infection.
200 Semaphorin regulates the activity of Ras-family small GTPases [39]. A Ras-like protein11B
201 transcript (BTT05368_1) was also down regulated. The Ras/MAPK pathway was found to be
202 essential for the suppression of the Imd immune pathway in *Drosophila* [40].

203

204 The downregulated Drumstick (BTT13062_1) interacts with the JAK/STAT pathway during its'

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

213

214 *Effectors:*

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

223

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

231 *P450 4g15*: BTT23811_1, BTT32459_1, *cytochrome P450 6k1*: BTT35547_1, BTT40653_1,
232 *cytochrome P450 6a14*: BTT38445_1) were found to be downregulated. Three other *cytochrome*
233 *P450* transcripts (*Cyp4p3*: BTT21216_1, BTT35543_1, *cytochrome P450 315a1*: BTT26726_1)
234 were upregulated. Several other cytochromes (*cytochrome b*: BTT20524_1, BTT39776_1,
235 BTT41896_1, and *cytochrome c*: BTT05255_2) were downregulated.

236

237 Numerous other actors in the production of ROS were found to be differentially expressed. *TPX4*
238 (BTT13285_1), coding for a Thioredoxin-dependent peroxidase, was downregulated. This gene was
239 found to be differentially expressed during *Plasmodium* infection in *Anopheles gambiae* [49].
240 Thioredoxin-dependent peroxidase detoxifies H₂O₂. Calcineurin (BTT08150_1, BTT26273_1) was
241 found to be downregulated 24 hours post-infection. This agrees with our previous findings [10]. In
242 infected *D. melanogaster* larvae, NO signals are enhanced by Calcineurin to promote induction of
243 strong, robust immune responses via the Imd signalling pathway [50].

244

245 We found downregulation of sortilin-related receptor-like (BTT31654_1). In mammals, sortilin aids
246 in phagocytosis [51]. Two downregulated transcripts (BTT35021_1, BTT08756_1) were matched to
247 *croquemort*. *Croquemort*, which codes for a scavenger receptor is a key part of the Imd pathway
248 but in its apoptotic phagocytosis role not its immune one [52]. Annexin IX (downregulated
249 BTT02025_1) has been shown to be induced by septic injury in *Drosophila*. It is thought to encode
250 for an anticoagulant [53].

251

252 *Miscellaneous:*

253 Major royal jelly protein (BTT05317_2, BTT36365_1 upregulated) has been shown to have
254 antimicrobial properties and to be expressed in response to bacterial infection in honeybees [54,55].
255 Vitellogenin (downregulated BTT36006_1) is a potent regulator of the immune response in
256 honeybees [56]. Several orthologs of putative *Drosophila* immune loci were found to be

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

262

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

264 43 transcripts were found to be differentially expressed between 24 hours post-infection and 48
265 hours post-infection. Of these 17 had no BLAST hits. A further six had uninformative BLAST hits
266 (anonymous predicted protein). The remaining 20 were used in the analysis. Defense response was
267 the only GO term significantly enriched (FDR= 0.00015), with seven transcripts. Three transcripts
268 correspond to Hymenoptaecin (BTT18071_1, BTT24170_1, BTT24170_2). They were all
269 upregulated. This suggests a continuing strong AMP production 48 hours after infection. This
270 agrees with other immune assays in bumblebees [57]. Argonaute-2, a RNA-silencing endonuclease,
271 is involved in antiviral defense in insects (downregulated BTT02484_1) [58]. GstD8, a glutathione
272 S-transferase, is involved in the detoxification process (upregulated BTT04810_1) [59]. Dopa
273 decarboxylase (upregulated BTT28048_1) converts L-dopa to dopamine during the melanisation
274 process [60]. SCR-B9 (upregulated BTT40924_1) codes for a scavenger receptor protein.
275 Scavenger receptor proteins have been found to be microbial pattern recognition receptors in flies
276 [61].

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

278 There were 591 differentially expressed transcripts (FDR < 0.05). Reannotating the transcripts
279 using Blast2GO (blastx against the nr database with $e < 0.001$), 150 had no BLAST hits. A further
280 64 had uninformative BLAST hits (anonymous predicted protein). There were 109 transcripts that
281 had previously been found to be differentially expressed at 24 hours post infection. Figure 3 shows

282 a multidimensional scaling (MDS) plot of the samples based on the expression of these 591 genes.

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

284

285 Of the 591 transcripts, 132 were upregulated and 459 were downregulated. Up or downregulation

286 does not have the same meaning here as in the infected versus uninfected model where there was a

287 clear baseline (uninfected). Depending on how you order the GLM we could get the reciprocal

288 result. Our model used colony K strain 8 as the final contrast. From our previously published qPCR

289 data [15], we know the colony K strain 8 interaction displayed the highest levels of AMPs

290 (effectors). Therefore when we say a transcript is upregulated, we mean it is upregulated in this high

291 immune response interaction.

292

293 As with the infection data, we combined the BLAST searches against the IID and nr databases.

294 Ninety transcripts correspond to canonical insect immune genes. We again describe them in the

295 order receptors, serine proteases, signalling pathways and effectors [16].

296

297 *Receptors:*

298 Two transcripts were associated with gram negative binding proteins (upregulated GGBP,

299 BTT03533_1 and downregulated *GNBP1-2* BTT35513_1) Although, as their name suggests,

300 GNBP are most associated with defense against gram negative bacteria, they have been show to

301 have a role in respond to *Plasmodium* infections [62]. C-type lectins (CTLs) bind carbohydrates and

302 mediate processes including pathogen recognition [63]. CTL4 is agonist to *Plasmodium* infections

303 in mosquitoes [63]. A CTL4 transcript (BTT29328_1) was found to be downregulated.

304

305 One downregulated transcript was related to *Dscam* (BTT12755_1). A further fourteen

306 downregulated transcripts were part of the Ig superfamily (IGFn3-1: BTT05561_1, BTT05581_1,

307 BTT08682_1, BTT12655_1, BTT13442_1, BTT14516_1, BTT18750_1, BTT21156_1,

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

311

312 *Serine proteases:*

313 28 transcripts related to serine proteases, serine protease homologues or serine protease inhibitors
314 were differentially expressed. Twelve serine protease transcripts were upregulated (*cSp3*:
315 BTT35293_1, *Sp18*: BTT20808_1, *Tequilla/GRAL/Sp23*: BTT01709_1, BTT05886_1,
316 BTT09081_1, BTT20661_1, BTT20725_1, BTT24359_1, BTT25071_1, *Sp27*: BTT40251_1,
317 *Sp35*: BTT05300_1, *Sp40*: BTT15256_1). Six serine protease transcripts were downregulated
318 (*cSP3*: BTT10579_1, BTT10912_1, BTT18247_1, BTT25711_1, *Sp28*: BTT20637_1, *Sp35*:
319 *BTT10155_1*). Two serine protease homologues were downregulated (*Sph54*: BTT27769_1,
320 *cSPH39*: BTT21868_1). One serine protease homologue was upregulated (*Sph56*: BTT17814_1)
321 Six serine protease inhibitor transcripts were downregulated (*Spn 4*: BTT04130_1, *SRPN10*:
322 BTT02607_1, BTT4508_1, BTT20259_1, BTT40693_1, Kunitz ser-protease inhibitor:
323 BTT14993_1). The *necrotic (nec)* gene was upregulated (BTT35742_1).

324

325 *Signalling pathways:*

326 The Toll-like receptor *18Wheeler* (BTT35732_1) was upregulated as was *Toll 10* (BTT09386_1).
327 *18Wheeler* has been shown to be important in the anti gram-negative immune response in
328 *Drosophila* larvae [64]. *Dorsal 1A* (BTT04010_1), a transcription factor that is a fundamental part
329 of the Toll pathway, was downregulated. A transcript for *Spatzle 1-2* was downregulated
330 (BTT10679_1).

331

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

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

341

342 *Effectors:*

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

348

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

353

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

357

358 *Miscellaneous:*

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

360 domain that has been hypothesized to sense chitin in response to injury and to transduce signals via
361 the serine protease domain [69]. We found an upregulated transcript related to *SCRASP 1*
362 (BTT41923_1). A peritrophin precursor was also upregulated (BTT10727_1). As was a chitinase 3
363 transcript (BTT23246_1).

364

365 *Retinoid and fatty-acid-binding protein (RfaBp)* (BTT07678_1) was downregulated. RfaBp was
366 found to be upregulated upon injection of LPS in *Drosophila* during a proteomic study [70]
367 (Vierstraete *et al.* 2004). *Notch* (upregulated BTT09545_1) is involved in the specification of
368 crystal cells in *Drosophila melanogaster* [71]. Several orthologs of putative *Drosophila* immune
369 loci were found to be differentially expressed (CG5527: BTT08512_1, CG12505: BTT00934_1,
370 CG13323: BTT38025_1, BTT38087_1, CG17560: BTT02877_1 downregulated, BTT05845_1
371 upregulated, CG18348: BTT20843_1)

372

373 **Discussion**

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

378

379 We confirmed the importance of antimicrobial peptides in the specific defense against *Crithidia*
380 [10,14,15]. It is also clear that several other effectors including ROS and phagocytosis may be
381 important. Several immune pathways seem to be important in the anti-*Crithidia* response. These
382 include the Toll, Imd and JAK/STAT pathways. Toll especially seems to be important in a specific
383 immune response.

384

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

399 Our results suggest that *Dscam* may be important in differentiating strains of the trypanosome
400 *Crithidia bombi*.

401

402 We found a number of genes associated with chitin metabolism. The peritrophic matrix may be
403 fundamental in the bee's defense against *Crithidia*. The peritrophic matrix acts as an immunological
404 barrier against trypanosomes. Tsetse flies with an underdeveloped PM have lower levels of
405 refractoriness to trypanosome infections [75]. This is due to a premature immune response; the
406 trypanosomes get through the PM quicker and stimulate the immune response at an earlier stage
407 compared to refractory flies.

408

409 Given that we have found that the bees own physiology, especially its immune response is vital in
410 both the defense against *Crithidia* and in explaining the host-parasite specificity, how do we
411 incorporate recent findings that the bees gut microbiota are vital in exactly these phenomena [9,76].
412 Gut microbiota impact the condition of the PM and gut epithelium generally [75,77]. It has recently
413 been suggested that the components of the peritrophic matrix may be under the control of various
414 immune pathways, Imd [78] and STAT [79] explicitly. Gut microbiota stimulate these pathways
415 keeping the PM intact. The intact peritrophic matrix then acts as a physical barrier to colonization
416 by parasites. Future work will focus on understanding the interactions of this triumvirate of host
417 genotype, parasite genotype and gut microbiota and their effect on disease outcome.

418

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

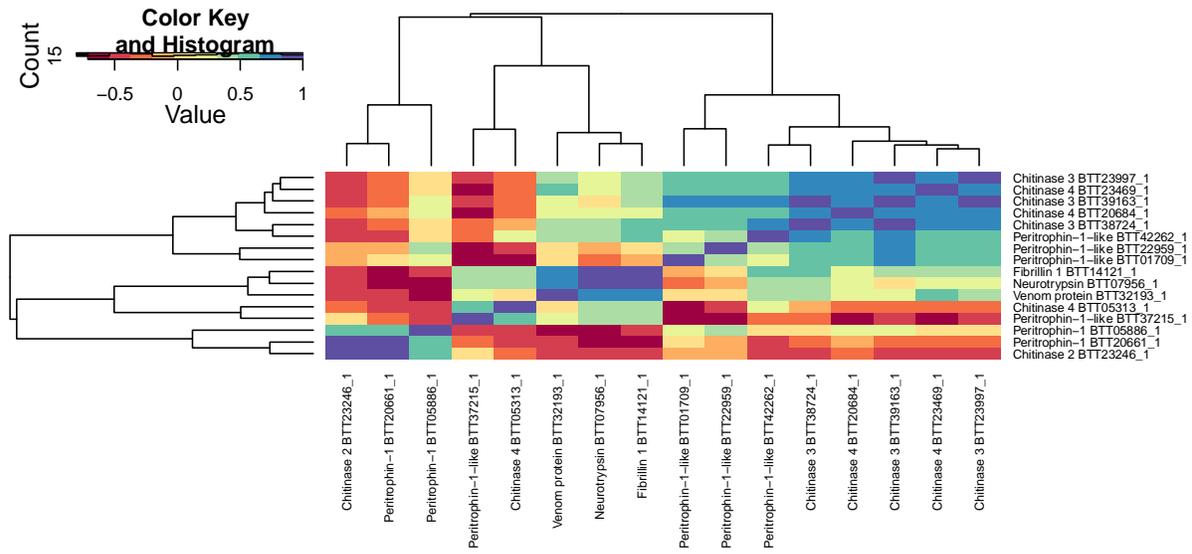


Figure 2. A heatmap showing the correlations of the expression patterns of the transcripts labelled as chitin metabolism genes that were differentially expressed twenty four hours after infection.

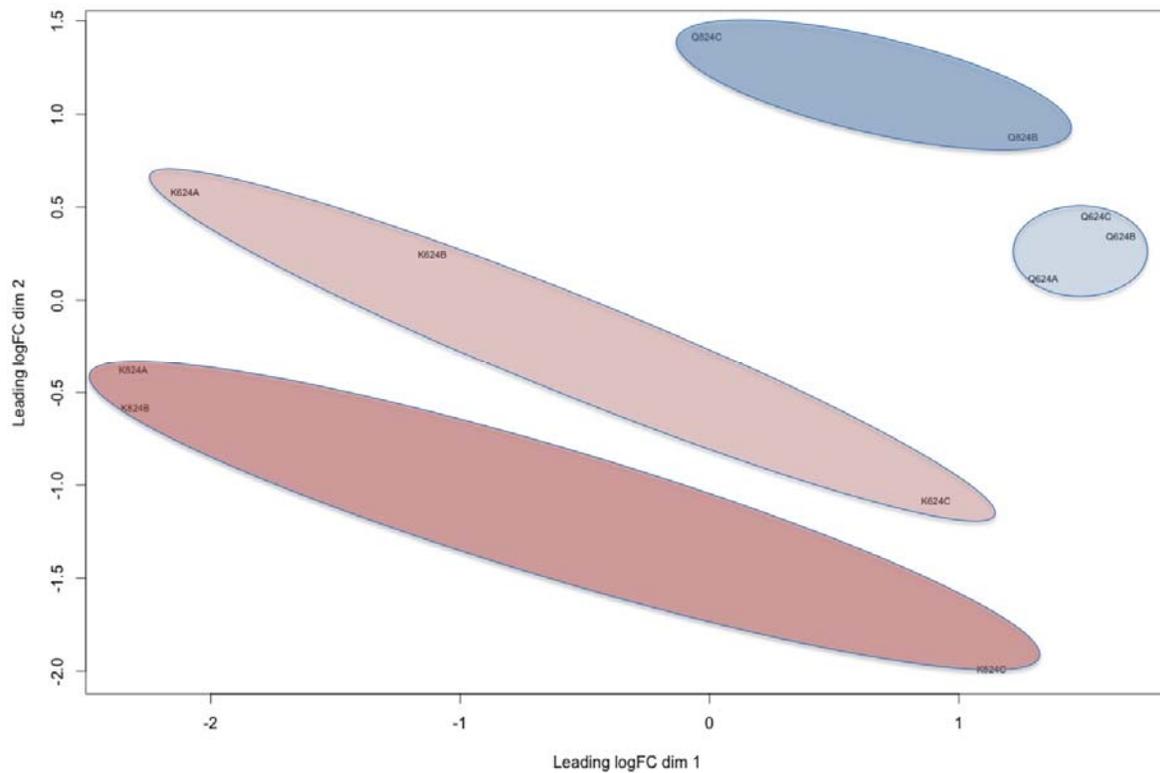


Figure 3. A multidimensional scaling (MDS) plot of the 11 samples used in the specificity analysis based on the expression of 591 differentially expressed transcripts. There are two colonies (K (red) and Q (blue)) and two *Crithidia* strains (6 (light) and 8 (dark)). Dimension 1 is the direction that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

