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The effect of entomopathogenic fungal culture filtrate on the immune response and haemolymph proteome of the large pine weevil, *Hylobius abietis*

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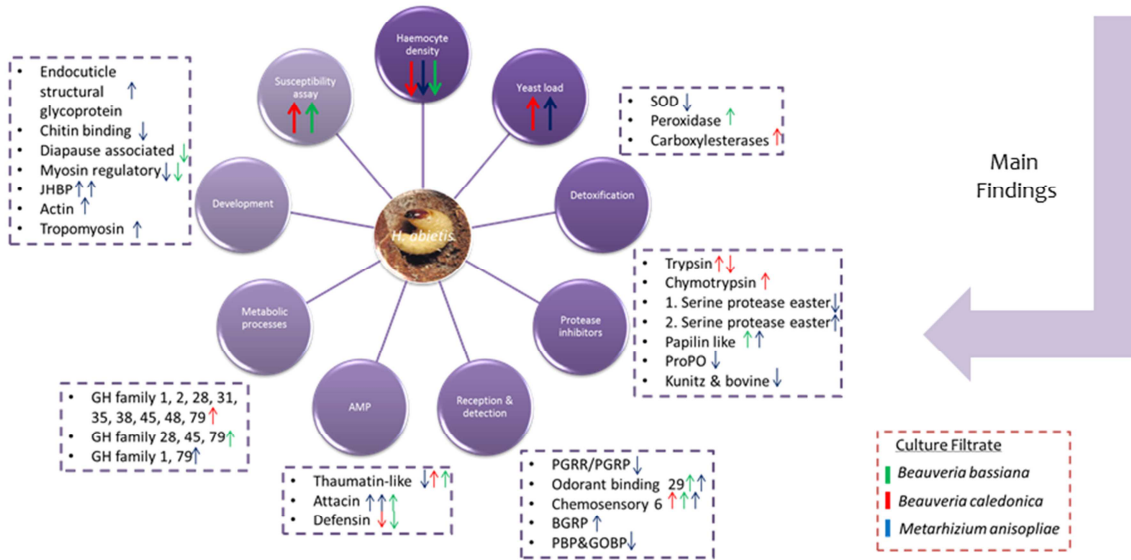
EPF culture



Inject larvae

Haemocyte density assay
Yeast load assay
Double challenge assay
Haemolymph proteomics

Comparative Analyses



ACCEPTED MANUSCRIPT

1 **The effect of entomopathogenic fungal culture filtrate on the immune response and**
2 **haemolymph proteome of the large pine weevil, *Hylobius abietis*.**

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12
13 **Abstract**

14 The large pine weevil *Hylobius abietis* L. is a major forestry pest in 15 European countries,
15 where it is a threat to 3.4 million hectares of forest. A cellular and proteomic analysis of the
16 effect of culture filtrate of three entomopathogenic fungi (EPF) species on the immune
17 system of *H. abietis* was performed. Injection with *Metarhizium brunneum* or *Beauvaria*
18 *bassiana* culture filtrate facilitated a significantly increased yeast cell proliferation in larvae.
19 Larvae co-injected with either *Beauvaria caledonica* or *B. bassiana* culture filtrate and
20 *Candida albicans* showed significantly increased mortality. Together these results suggest
21 that EPF culture filtrate has the potential to modulate the insect immune system allowing a
22 subsequent pathogen to proliferate. Injection with EPF culture filtrate was shown to alter the
23 abundance of protease inhibitors, detoxifying enzymes, antimicrobial peptides and proteins
24 involved in reception/detection and development in *H. abietis* larvae. Larvae injected with *B.*
25 *caledonica* culture filtrate displayed significant alterations in abundance of proteins involved
26 in cellulolytic and other metabolic processes in their haemolymph proteome. Screening EPF
27 for their ability to modulate the insect immune response represents a means of assessing EPF
28 for use as biocontrol agents, particularly if the goal is to use them in combination with other
29 control agents.

30 **Keywords**

31 Large pine weevil, entomopathogenic fungi, proteomic, transcriptome, immunomodulation,
32 biocontrol

33 **Abbreviations**

34 PO, phenoloxidase; PPO, prophenoloxidase; EPF, entomopathogenic fungi; PCA, principal
35 component analysis; AMP, antimicrobial peptide; LFQ, label free quantification; GH,
36 glycoside/glycosyl hydrolase

37 **1. Introduction**

38 There is increasing interest in the exploitation of entomopathogenic fungi (EPF), particularly
39 *Beauveria* spp and *Metarhizium* spp., for the biological control of insect pests. Biocontrol
40 agents can be deployed where use of chemical pesticides is restricted or where resistance has
41 developed. The large pine weevil *Hylobius abietis* L. is a major forestry pest in 15 European
42 countries, where it is a threat to 3.4 million hectares of forest (Långström and Day 2004).
43 Until recently, young trees were protected with cypermethrin or alpha cypermethrin as a
44 control measure, but is no longer permitted in forests certified as sustainably managed. All
45 stages of *H. abietis* are susceptible to strains of *Metarhizium* and *Beauveria* in laboratory
46 assay (Ansari and Butt, 2012), but the performance of EPF in field trials has been
47 disappointing (Williams et al., 2013). Failure of biocontrol agents to live up to expectations
48 in the field is not uncommon. One approach to improving the success of biocontrol is to
49 deploy a combination of agents against the pest. Synergistic interactions, where the success
50 of the combination is greater than that of the individual agents, are frequently reported
51 between EPF and other pathogens including nematodes (Ansari *et al.* 2006; 2008; Anbesse,
52 2008) and bacteria (Wraight and Ramos, 2005; Sayed and Behle, 2017). Synergy may result
53 from the combined agents rendering the host more susceptible through modulating its
54 immune system, prolonging developmental stages or by the two treatments acting on
55 different components of the host population (Lacey *et al.* 2015).

56 The ability to modulate the immune response of an insect rendering it more susceptible to
57 other pathogens would have great significance for integrated pest management. Both insects
58 and their pathogens must constantly improve their defence and virulence, respectively, to
59 survive (Wojda 2016; Joop and Vilcinskis, 2016). The insect immune system is composed of
60 the cellular and humoral defences (Hoffmann 1995). Humoral defences include antimicrobial

61 peptides (AMPs), production of reactive forms of oxygen and nitrogen, soluble effector
62 molecules and cascades that regulate clotting and melanisation of insect haemolymph (Strand
63 2008). Cellular defences encompass haemocyte mediated defences (Lavine and Strand 2002).
64 There is an overlap between humoral and cellular defences in the recognition of pathogens;
65 many humoral factors regulate the activity of haemocytes and haemocytes produce many
66 humoral defence molecules such as defence peptides and stress proteins (Strand 2008,
67 Grizanova et al. 2014, Wojda 2016).

68 Host colonization by EPF requires the ability to cope with host immune defences and extract
69 nutrients from the host (Gillespie et al., 2000) which is achieved through immune evasion by
70 cryptic forms or immune system modulation through the action of secreted molecules
71 (Schrank and Vainstein, 2010). *Metarhizium* spp. produce a diverse range of enzymes and
72 secondary metabolites that are active against insects, fungi, bacteria, viruses and cancer cells
73 (Roberts and St Leger, 2004; Gao et al., 2011); most notably the cyclic hexadepsipeptidic
74 destruxins (Schrank and Vainstein, 2010) which display antiviral, antitumor, insecticidal,
75 cytotoxic, immunosuppressive, phytotoxic and anti-proliferate effects (Kershaw et al., 1999;
76 Sowjanya Sree et al., 2008; Liu and Tzeng, 2012). *Beauveria bassiana* is known to produce
77 cyclic peptides that are cytotoxic and immunosuppressive (Hung et al., 1993) and a diverse
78 selection of secondary metabolites including nonpeptide pigments and polyketides (e.g.
79 oosporein), non-ribosomally synthesized peptides (e.g. beauvericin) and secreted metabolites
80 that have roles in pathogenesis and virulence (Xiao et al., 2012). These metabolites have
81 insecticidal properties and can also inhibit growth of other microorganisms (van der Weerden
82 et al., 2013).

83 Here we report a cellular and proteomic analysis of the effect of culture filtrate of three EPF
84 species on the immune system of *H. abietis*. The primary aim of this work was to investigate
85 the immunomodulatory potential of EPF on the insect immune response. This was achieved
86 in part using label free quantitative (LFQ) mass spectrometry to investigate proteomic
87 expression of pine weevils exposed to EPF extracts, a strategy that has been successfully
88 applied to the lepidopteran *Galleria mellonella* (McNamara et al., 2017). To facilitate the
89 proteomic analysis and to compensate the lack of genomic information for *H. abietis*; a *de*
90 *novo* transcriptome for *H. abietis* was produced. The three species of EPF chosen for this
91 work were *M. brunneum* (Petch)(Met52), *B. bassiana* and *B. caledonica*. *Beauveria bassiana*

92 and *Metarhizium* spp. are two of the most commonly employed EPF in biocontrol. Both have
93 a wide host range and global distribution, and are used to control plant pests and vectors of
94 human disease (Shah and Pell, 2003; Glare et al., 2008; Gao et al., 2011; Xiao et al., 2012;
95 Lacey et al., 2015; Butt et al., 2016). *Beauveria caledonica* was found to be a naturally
96 occurring pathogen of pine bark beetles in New Zealand (Glare et al., 2008; Reay et al., 2008)
97 and *H. abietis* in Ireland (Glare et al., 2008; Williams et al., 2013). Morphologically, *B.*
98 *caledonica* is similar to *B. brongniartii*, with cylindrical conidia, but molecular studies (Glare
99 & Inwood 1998; Rehner and Buckley 2005), including the current research, have shown the
100 species to be distinct. In contrast, *B. bassiana* has globose conidia.

101

102 2. Materials and Methods

103 2.1 Origin of EPF strains and preparation of culture filtrate

104 A commercial strain of *M. brunneum* (Met52; previously *M. anisopliae*) produced by
105 Novozymes (Denmark) was used and was purchased on rice grains from National
106 Agrochemical Distributors, Lusk, Dublin. *B. bassiana* experimental strain 1694 was supplied
107 by Becker Underwood (Littlehampton, UK). *B. caledonica* (2c7b) is a native strain isolated
108 from a soil sample from soil close to a pine stump in a felled forest in Hortland, Co. Kildare
109 (Ireland). The soil sample was baited with *G. mellonella* larvae and fungus from the infected
110 cadaver was identified through DNA sequencing of an ITS PCR product (a region of the
111 internal transcribed spacer unit of the ribosomal DNA, ITS4, was amplified by PCR). EPF
112 were cultured in Sabouraud Dextrose liquid medium (Oxoid) for 48 h, 72 h and 96 h in a
113 shaking incubator at 25°C and 250 rpm. After each time point the culture was filtered through
114 0.45 µm syringe filters and then through 0.2 µm syringe filters (Sartstedt). The filtrate was
115 collected and stored at -80 °C.

116

117 2.2 Inoculation of *Hylobius abietis* larvae

118 Late instar *H. abietis* larvae were collected from pine stumps and stored at 4 °C for a
119 maximum of 3 weeks until used in experiments. For each of the laboratory bioassays larvae
120 were injected with fungal culture filtrate through an abdominal spiracle using a Myjector

121 U100 insulin syringe (Terumo Europe, Leuven, Belgium). Larvae were placed in 24 well
122 culture plates (Costar) with filter paper and stored at 20 °C.

123

124 2.3 Enumeration of haemocyte and yeast cell densities, and infection susceptibility assays

125 The density of circulating haemocytes in larvae was assessed as described by Bergin *et al.*,
126 (2003). All experiments were performed with three biological replicates.

127 To test the effect of EPF on the immune response to a subsequent infection, larvae were
128 inoculated with EPF culture filtrate or Sabouraud dextrose (control) and incubated for 24 h at
129 20 °C, after which they received a second inoculation through an abdominal spiracle with
130 *Candida albicans* (10^4 cells in 20 μ l). *Candida albicans* MEN (serotype B, wild-type
131 originally isolated from an eye infection (a gift from Dr. D. Kerridge, Cambridge, UK) was
132 cultured to the stationary phase overnight in yeast extract peptone dextrose (YEPD) at 30 °C
133 and 200 rpm on an orbital shaker (Browne *et al.*, 2015). In each of the three replications, five
134 larvae were injected per treatment and time. Following the second inoculation, larvae were
135 incubated for a further 24 h or 48 h at 20 °C and were homogenized in 3 ml of sterile
136 phosphate buffered saline (PBS). After serial dilution in PBS, 100 μ l of each sample was
137 spread on YEPD plates containing erythromycin (1 mg/ml). The plates were incubated for 48
138 h at 30 °C. Yeast cell density was calculated per larva.

139 To test whether EPF would make larvae more susceptible to a second pathogen, larvae were
140 inoculated through an abdominal spiracle with 20 μ l of culture filtrate or Sabouraud dextrose
141 and incubated at 20 °C. After 24 h, larvae were given a second injection with *C. albicans*
142 ($1 \times 10^4/20$ μ l, culture as above), or PBS. In each of the three replications, ten larvae were
143 injected per treatment and time. Larvae were incubated at 20 °C and mortality was recorded
144 for up to 14 days.

145

146 2.4 RNA extraction of *H. abietis* larvae

147 One larva was crushed to a fine powder in liquid nitrogen using a sterilized pestle and mortar.
148 Trizol was added and the sample was homogenized with a power pestle. The sample was
149 spun at 13,000 x g for 10 minutes at 4 °C. Then, 200 μ l of chloroform was added to the

150 sample, vortexed and left at room temperature for 10 min. The sample was spun at 12,000 x g
151 for 10 min 4 °C and the top clear layer was removed to a fresh centrifuge tube. Isopropanol
152 was added to the clear layer and inverted several times. The sample was left for 10 min at
153 room temperature. The sample was spun again at 12,000 x g for 10 min, and the resulting
154 pellet was washed in 70 % ethanol. The sample was spun to remove ethanol, the pellet was
155 allowed to air-dry. The pellet was resuspended in 100 µl of elution buffer (Sigma GenElute
156 Mammalian Total RNA Miniprep Kit). A Sigma GenElute Mammalian Total RNA Miniprep
157 Kit and protocol was used to do complete extraction of the sample. This method was carried
158 out twice: 1. Untreated *H. abietis* larva, and 2. *H. abietis* larva injected with *M. brunneum*
159 culture filtrate.

160

161 2.5 *H. abietis* transcriptome

162 The *H. abietis* transcriptome *de novo* study was completed by Beijing Genomics Institute
163 (BGI, Hong Kong) using Illumina HiSeq 4000. After extraction of total RNA and treatment
164 with DNase I, Oligo (dT) adapters were used to isolate mRNA. The mRNA was fragmented
165 by mixing with the fragmentation buffer and cDNA was synthesized using the mRNA
166 fragments as templates. Short fragments were purified and resolved with elution buffer for
167 end reparation and single nucleotide A (adenine) addition. The short fragments were
168 connected to adapters and suitable fragments were selected for the PCR amplification. During
169 the QC steps, Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System were
170 used in quantification and qualification of the sample library. Then the library was sequenced
171 using an Illumina HiSeq 4000. After sequencing, the raw reads were filtered for low-quality,
172 adaptor-polluted and high content of unknown base (N) reads to get clean reads. *De novo*
173 assembly was performed using Trinity with clean reads to obtain the Unigene set. After that,
174 simple sequence repeats (SSR) detection, Unigene expression analysis, heterozygous single
175 nucleotide polymorphisms (SNP) detection, and Unigene functional annotation were
176 performed. Unigenes were divided into two classes; clusters with the prefix “CL”
177 (comprising several Unigenes with sequence similarity of 70% and above) and singletons
178 with the prefix “Unigene”. The predicted protein sequences for the *H. abietes* transcriptome
179 was analysed using InterProScan (version 5.18-57.0) to provide functional annotations based
180 on protein family (Pfam) domains (Jones et al., 2014). To assess the completeness of *H.*

181 *abietis* assembled transcriptome a BUSCO (Benchmarking Universal Single-Copy Orthologs)
182 (Simão *et al.* 2015) assessment was carried out on the predicted nucleotide and protein fasta
183 files. Raw sequence reads were deposited in the Sequence Read Archive (SRA) hosted by the
184 National Center for Biotechnology Information under BioProject PRJNA419715
185 (<https://www.ncbi.nlm.nih.gov/bioproject/419715>) and BioSample SAMN08095620
186 (<https://www.ncbi.nlm.nih.gov/biosample/SAMN08095620/>).

187

188 *2.6 Protein sample preparation and mass spectrometry*

189 Larvae were either injected with 20 µl of fungal culture filtrate or Sabouraud dextrose
190 (procedural control) and incubated for 48 h at 20 °C. Five larvae per treatment were bled into
191 a pre-chilled 1.5 ml centrifuge tube and spun at 1,500 x g for 5 min at 4 °C. Samples were
192 diluted in PBS and a Bradford assay was carried out to determine protein quantity. Protein
193 (100 µg) was removed to a pre-chilled 1.5 ml centrifuge tube and ice cold 100 % acetone was
194 added at ratio of 1:3 (sample: acetone) and precipitated at -20 °C. The sample was
195 centrifuged at 13,000 x g for 10 min and the protein pellet was resuspended in 100 µl of
196 resuspension buffer (6 M urea, 2 M thiourea, 5 mM calcium chloride). Protein (75 µg) was
197 reduced with dithiothreitol (200 mM) and alkylated with iodoacetamide (1 M). Samples were
198 digested with sequence grade trypsin (Promega, Ireland) at a trypsin:protein ratio of 1:40,
199 overnight at 37 °C. Three replicate samples were prepared for each treatment.

200 Tryptic peptides were purified for mass spectrometry using C18 spin filters (Medical Supply
201 Company, Ireland) and 1 µg of peptide mix was eluted onto a QExactive (ThermoFisher
202 Scientific, U.S.A) high resolution mass spectrometer connected to a Dionex Ultimate 3000
203 (RSLCnano) chromatography system. Peptides were separated by an increasing acetonitrile
204 gradient (2-40 %) on a Biobasic C18 Picofrit™ column (100 mm length, 75 mm ID), using
205 a 120 min reverse phase gradient at a flow rate of 250 nL /min. All data were acquired with
206 the mass spectrometer operating in automatic data dependent switching mode. A full MS scan
207 at 140,000 resolution and a scan range of 300-1700 m/z was followed by an MS/MS scan, at
208 resolution 17,500, to select the 15 most intense ions prior to MS/MS.

209

210 2.7 Quantitative mass spectrometry data analysis

211 Protein identification from the MS/MS data was performed using the Andromeda search
212 engine in MaxQuant (version 1.2.2.5; <http://maxquant.org/>) to correlate the data against a
213 predicted protein set derived from the *H. abietis de novo* transcriptome generated in this
214 study. To insure that all identified proteins were derived from the insect and not the fungal
215 supernatant an additional search of all MS data was performed against the predicted protein
216 set for *Beauveria bassiana* (Joint Genome Institute, downloaded April 2018) derived from
217 the *B. bassiana* genome initiative (Xiao et al., 2012). The following search parameters were
218 used: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5 ppm with
219 cysteine carbamidomethylation as a fixed modification and N-acetylation of protein and
220 oxidation of methionine as variable modifications and a maximum of 2 missed cleavage sites
221 allowed. The MS proteomics data and MaxQuant search output files have been deposited to
222 the ProteomeXchange Consortium (Côté et al., 2012) via the PRIDE partner repository with
223 the dataset identifier PXD008232. Results processing, statistical analyses and graphics
224 generation were conducted using Perseus v. 1.5.0.31. Label free quantification (LFQ)
225 intensities were \log_2 -transformed and t-tests comparing EPF treated larvae with controls were
226 performed using a p-value of 0.05. Proteins were kept in the analysis if they were found in all
227 3 replicates in at least one group. Principal component analysis (PCA) was used to emphasize
228 variation and visualize strong patterns in the data. Proteins found to be absent (below the
229 level of detection) in one or more treatments and present (above the level of detection) in
230 three or fewer treatments were termed 'uniquely detected proteins'. These proteins were also
231 used in statistical analysis of the total differentially expressed group following imputation of
232 the zero values with values that simulate low abundant proteins. These values were chosen
233 randomly from a distribution specified by a downshift of 1.7 times the mean standard
234 deviation (SD) of all measured values and a width of 0.33 times this SD. Volcano plots were
235 generated in Perseus to visualize differentially abundant proteins between control and
236 treatment groups by plotting negative log p-values from pairwise Student's t-tests on the y-
237 axis and \log_2 fold-change values on the x-axis for each pair-wise comparison. Proteins with a
238 p-value < 0.05 were considered statistically differentially abundant. To reduce the numbers of
239 proteins with minor fold changes proteins with a relative fold change < 1.5 were removed
240 from the analysis. To obtain an overall proteomic profile of abundance for all significantly

241 expressed and exclusive proteins, hierarchical clustering (displayed as a heat map) on Z-score
242 normalised intensity values was performed to resolve clusters of proteins with similar
243 abundance and expression profiles. The Blast2Go suite (Conesa *et al.*, 2005;
244 www.blast2go.com) of software tools was utilized to BlastP search all identified proteins
245 against the NCBI non redundant database with the following search settings: number of blast
246 hits: 5, high-scoring segment pair length cutoff: 33, Blast expect value: 1.0e-5. Blast2Go was
247 used to assign gene ontology (GO) terms for biological processes, molecular function and
248 cellular components in addition to enzyme codes and InterPro identifiers for all proteins.
249 Annotations derived from the InterProScan-based Pfam annotation of the *H. abietis*
250 transcriptome were used to provide annotations in cases where Blast2Go failed to provide
251 one.

252

253 2.8 Statistical analysis

254 Statistical analysis was carried out using Minitab version 16 statistical software and GraphPad
255 Prism version 5. All data were first tested for normality, where data were found not to be
256 normal, the data were either transformed before further analysis was carried out or a suitable
257 non-parametric test was used. For alterations to haemocyte densities and yeast densities, data
258 were analysed using two-way ANOVA with EPF culture time and assessment time (24 or 48
259 h post injection) as the factors. Bonferroni post-hoc tests were used to compare EPF
260 treatments to relevant controls. To determine whether EPF culture filtrate increases
261 susceptibility of *H. abietis* to a subsequent infection, data for yeast-injected and PBS-injected
262 larvae were compared using paired t-tests.

263

264 3. Results

265 3.1 Alterations in haemocyte densities following injection of larvae with EPF culture filtrate

266 Larvae injected with all three EPF culture filtrates showed a significant alteration in
267 haemocyte densities at both 24 h and 48 h (*B. caledonica*; $F_{3,16}=8.21$, $p<0.01$, *B. bassiana*;
268 $F_{3,16}=49.36$, $p<0.001$ and *M. brunneum*; $F_{3,16}=8.89$, $p<0.001$) (Figure 1A). All significant
269 differences in haemocyte densities between treatments and their appropriate controls were in

270 the direction of reduction. All three EPF caused a decrease in the haemocyte densities of
271 larvae following inoculation with 96 h fungal culture filtrate relative to their appropriate
272 controls.

273

274 3.2 Alterations in yeast cell density following injection of larvae with EPF culture filtrate

275 Injection of larvae with *B. caledonica* culture filtrate did not have a significant effect on yeast
276 cell density in larvae (Figure 1Bi). In larvae injected with *M. brunneum* and *B. bassiana*
277 culture filtrate, both treatment (*M. brunneum*; $F_{3,16}=36.85$, $p<0.001$, *B. bassiana*; $F_{3,16}=20.93$,
278 $p<0.001$) and time (*M. brunneum*; ($F_{3,16}=36.43$, $p<0.001$), $p<0.001$, *B. bassiana* $F_{3,16}=77.85$,
279 $p<0.001$) had a significant effect, there was also a significant interaction between treatment
280 and time (*M. brunneum*; ($F_{3,16}=12.96$, $p<0.001$), $p<0.001$, *B. bassiana* $F_{3,16}=6.62$, $p<0.01$).
281 After a 24 h incubation, injection with *M. brunneum* 48 h, 72 h, and 96 h culture filtrate
282 resulted in a significant alteration in yeast density, with a fold increase of 6.2, 2.1 and 16.8
283 respectively, relative to controls. After 48 h incubation, injection with 72 h and 96 h *M.*
284 *brunneum* culture filtrate resulted in a significant alteration in yeast density, with a fold
285 increase of 12.8 and 20.8 respectively, relative to controls (Figure 1Biii). After a 48 h
286 incubation, injection with *B. bassiana* 72 h culture filtrate resulted in a significant alteration
287 in yeast density ($p<0.001$), with a fold increase of 44.4 (Figure 1Bii).

288

289 3.3 Effect of EPF culture filtrate on susceptibility of larvae to subsequent infection

290 Larvae injected with a combination of fungal culture filtrate and *C. albicans* showed higher
291 mortality than those that received fungal culture filtrate only, and the difference was
292 significant for both *B. bassiana* ($T=-17$, $p<0.01$) and *B. caledonica* ($T=-4.91$, $p<0.05$) (Figure
293 1C). Larvae that were injected with EPF culture filtrate before treatment with *C. albicans*
294 also had higher mortality than larvae treated with *C. albicans* alone, where no death occurred.

295

296 3.4 Transcriptome of *H. abietis* larvae

297 Approximately 17.7 Gb bases in total were generated after Illumina Hiseq sequencing. After
298 assembly 49,960 unigenes were generated, with a total length of 59,001,875 bp, an average

299 length of 1,180 bp, N50 of 2,241 bp, and GC content 39.27 %. The unigenes were annotated
300 with 7 functional databases; NR (Non Redundant Protein), NT (Non Redundant Nucleotide),
301 GO (Gene Ontology), COG (Clusters of Orthologous Groups of proteins), KEGG ((Kyoto
302 Encyclopedia of Genes and Genomes), Swissprot and Interpro. With functional annotation,
303 27,653 coding domains (CDS) were detected, and after ESTScan with the remaining
304 unigenes, a further 2,936 CDS were found. 3,121 SSRs were detected on 2,788 unigenes. To
305 determine the comprehensiveness of the assembly and annotation a BUSCO (Benchmarking
306 Universal Single-Copy Orthologs) assessment was carried out on the nucleotide fasta file.
307 The BUSCO analysis was conducted using the single-copy ortholog set of 1,658 conserved
308 genes derived from the class Insecta. The *H. abietis* transcriptome used in this study
309 contained ~97% (1,607/1,658) of the queried BUSCO orthologs (1214 Complete and single-
310 copy, 340 Complete and duplicated, 53 Fragmented and 51 Missing). These conserved genes
311 are expected to be present in the genomes of all Insecta species and given that a transcriptome
312 captures what is constitutively expressed at the time of RNA extraction, the high number of
313 BUSCO orthologs identified here indicates that our pine weevil transcriptome is particularly
314 comprehensive.

315

316 3.5 LFQ analysis of *H. abietis* larval hemolymph following EPF culture filtrate treatment

317 Label free quantification (LFQ) was used to compare the haemolymph proteome of *H. abietis*
318 larvae treated with EPF filtrate relative to control larvae. The groups analyzed were larvae
319 treated with *M. brunneum*, *B. caledonica* and *B. bassiana* culture filtrate grown for 96 h and
320 control larvae (treated with sabouraud dextrose media). In total, 157 proteins were identified,
321 155 proteins having two or more peptides (Table S1). Seventy seven of these proteins were
322 either significantly changed in abundance (Table 1) or uniquely detected across the four
323 treatments analyzed. Across the four sample groups, 43 proteins were deemed to display
324 exclusive distribution either being present in at least one group but undetected in one or more
325 groups. These proteins were termed 'uniquely detected proteins'; notably, several of these
326 were glycosyl hydrolase proteins uniquely detected in *B. caledonica* treated larvae only.
327 These proteins were included in subsequent statistical analysis of the total differentially
328 expressed group following imputation.

329 Proteomic analysis indicates that the larval response to the culture filtrate from *B. caledonica*
330 was the most divergent relative to the control; this is evident in the PCA analysis (Figure 2
331 A). Hierarchical clustering resolved proteins that had similar expression profiles in response
332 to treatment with different fungal culture filtrates (Figure 2B) and a number of protein
333 clusters were identified (Table S2). Cluster A comprises proteins with higher levels of
334 abundance in larvae treated with *M. brunneum* and *B. caledonica* relative to control larvae
335 and includes several proteins involved in sensing and recognition such as odorant-binding 29,
336 chemosensory 6 and β -1,3-glucan-binding. Cluster B comprises proteins with higher levels of
337 abundance in larvae treated with *B. caledonica* relative to all other treatments and control
338 larvae. It consists of a number of proteins involved in metabolic processes including
339 numerous members of glycosyl hydrolase families 1, 2, 20, 28, 31 35, 45, 48 and 79 a
340 member of the carboxylesterase family. Cluster C comprises proteins with lower levels of
341 abundance in larvae treated with *M. brunneum* relative to all other treatments and control
342 larvae. It includes proteins that may be involved in the proPO cascade such as serpin, serine
343 protease easter and serine protease persephone isoform X2. Cluster D comprises proteins
344 with higher levels of abundance in control larvae relative to all EPF treated larvae and
345 includes the antimicrobial peptide (AMP) defensin. Two sample t-tests ($p < 0.05$) identified
346 23, 37 and 33 statistically significant differentially abundant (SSDA) proteins in pine weevils
347 treated with *B. bassiana*, *B. caledonica* and *M. brunneum* culture filtrates in comparison to
348 control treatments, respectively (Table S3).

349 The Blast2GO annotation software was used to group proteins based on their conserved gene
350 ontology (GO) terms in order to identify processes and pathways affected by the different
351 EPF treatments (Figure 4; Table S4). In relation to cellular processes *M. brunneum* appears to
352 affect the largest number of process in the insect haemolymph, with *M. brunneum* and *B.*
353 *caledonica* affecting the most processes in common. In relation to molecular function *M.*
354 *brunneum* appears to affect the largest number of functions in larval haemolymph including
355 ion binding and hydrolase activity. *M. brunneum* and *B. bassiana* affect the most functions in
356 common. While *B. caledonica* affects the least functions it has 25 proteins involved in
357 hydrolase activity alone. In relation to biological processes *M. brunneum* appears to affect in
358 the largest number of processes in the larval haemolymph. While *B. caledonica* affects the

359 least number of processes, it affects 23 proteins involved in organic substance metabolic
360 process and 22 proteins involved in primary metabolic process (Figure 4).

361 A number of the identified haemolymph proteins are typically associated with the insect
362 digestive system, highlighting the potential that the EPF filtrate damaged or altered midgut
363 cells which resulted in their release into the haemolymph. However, seven glycosyl
364 hydrolases were detected in the control samples (Table S5) which suggests that their presence
365 is not directly associated with exposure to EPF filtrate. To account for the possibility that
366 some of the identified proteins were components of the injected EPF filtrate MS data was
367 searched against the predicted proteome for *B. bassiana*. Only seven proteins were identified
368 with two or more peptides (Table S5) many of which are highly conserved eukaryotic
369 proteins. In addition a number of serine proteases annotated as trypsin or chymotrypsin were
370 identified in pine weevil haemolymph. These annotations were based on the presence of the
371 pfam motifs that matched to the trypsin reference (PF00089) or the top hit BLAST result
372 (Table S1). In total 17 proteins with the pfam code PF00089 were identified in pine weevil
373 haemolymph across all treatments and where no additional annotation was available they
374 were annotated as trypsin- or chymotrypsin-like.

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379 4. Discussion

380 The principal objective of most insect biocontrol studies is to identify efficient means of
381 utilising entomopathogenic organisms. In many cases this is achieved through a better
382 understanding of the molecular strategies adopted by the control agent and its interaction with
383 its target and the latter's cellular and immunological systems. However the lack of available
384 genomic resources for both target and biocontrol organism can dramatically inhibit our
385 understanding of and insight into this interaction and therefore reduce the effectiveness of
386 novel control strategies. This bottleneck can now be circumvented through advances in high
387 throughput sequencing of genomes and transcriptomes which enable functional research on
388 'non-model' organisms, including those of great ecological or evolutionary importance
389 (Haas *et al.* 2013). For the purposes of this study we produced an assembled transcriptome
390 that was used to facilitate proteomic analysis of *H. abietis* exposed to culture filtrates of three
391 EPF. A number of cellular immune assays were also conducted which highlighted differences
392 in effects of EPF filtrate on *H. abietis*. Transcriptome characteristics, immune assays and
393 significant altered protein pathways and processes are discussed in detail below.

394 4.1 *H. abietis* transcriptome

395 The comprehensiveness of the *H. abietis* transcriptome was evaluated through a comparative
396 analysis of highly conserved single-copy orthologs using 1,658 BUSCO for the class Insecta.
397 BUSCO assessment allows for informative comparisons of, for instance, newly sequenced
398 draft genome and transcriptome assemblies through comparisons to high quality model
399 genomes (Simão *et al.* 2015). This *H. abietis* transcriptome produced in this study comprised
400 97% of the Insect BUSCO indicating a particularly high degree of comprehensiveness
401 making it a suitable resource not only for the proteomic analysis conducted in this study but
402 to the wider communities of plant-insect interactions, insect immunology, pest control and
403 insect genetics and phylogenetics.

404 4.2 EPF culture filtrates induce variant immune responses in *H. abietis*.

405 Injection with filtrate led to reduction in circulating haemocyte number. One possibility for
406 the reduction in haemocyte number is due to death and disintegration of haemocytes and/or
407 reduced proliferation of haemocytes (Strand, 2008) or due to activated haemocytes becoming
408 adhesive and attaching to inner organs such as the fat body (Browne *et al.*, 2013). Injection of

409 larvae with *M. brunneum* or *B. bassiana* filtrate facilitated a significantly increased yeast cell
410 density suggesting these filtrates are modulating the insect immune system allowing a
411 subsequent pathogen to proliferate. Larvae co-injected with *B. bassiana* or *B. caledonica*
412 filtrate and *C. albicans* showed significantly increased mortality. The proposed
413 immunomodulation of larvae by the EPF that is rendering the host more susceptible to a
414 subsequent pathogen is caused by spore-free culture filtrate which contains a diverse mixture
415 of enzymes, proteases and secondary metabolites (Vey *et al.* 2001; Sánchez-Pérez *et al.*
416 2014). Destruxin, the most abundantly produced secondary metabolite in *Metarhizium* spp.,
417 induced a similar response in *D. melanogaster* (Pal *et al.* 2007).

418 Proteomic analysis indicated that the response to *M. brunneum* culture filtrate was the most
419 divergent relative to the control (Figure 2). Injection with *M. brunneum* affects the largest
420 number of processes within the haemolymph. Injection with *B. caledonica* affects 25 proteins
421 involved in hydrolase activity alone and influences 23 proteins involved in organic substance
422 metabolic process and 22 proteins involved in primary metabolic process (Figure 4).

423 4.3 EPF culture filtrate alters abundance of serine proteases and their inhibitors

424 Insect hemolymph contains numerous serine protease/proteinase inhibitors (serpins) that are
425 involved in diverse processes including development and defence (Kanost 1999; Broehan *et*
426 *al.* 2010; Vilcinskas 2010; Butt *et al.* 2016). Serine proteases are major components of the
427 insect immune proPO pathway, which is regulated numerous serpins (Butt *et al.* 2016). In
428 addition serpins directly inhibit fungal and bacterial proteinases, regulate coagulation and
429 activate cytokine signaling processes (Kanost 1999). A number of serine proteases and
430 serpins were differentially abundant in *H. abietis* larvae injected EPF filtrate in a species
431 specific manner. *M. brunneum* filtrate resulted in a significant alteration in abundance of the
432 serine proteases easter, papilin-like protein and stubble in addition to a serpin (annotated as
433 kunitz & bovine pancreatic trypsin inhibitor domain containing protein; Figure 3). Larvae
434 injected with *B. bassiana* filtrate had an alteration in abundance of papilin-like protein and a
435 melanin-inhibiting protein whereas larvae injected with *B. caledonica* filtrate had an
436 alteration in the abundance of serine proteases and their inhibitors including trypsin- and
437 chymotrypsin-like proteins (Figure 3). Although chymotrypsin and trypsin generally have
438 digestive functions in insects, the specific function of these particular proteins in pine weevil

439 haemolymph has yet to be determined. It is most likely that these proteins are poorly
440 annotated and are members of the immune associated serine protease/serpin cascade
441 pathways found in insect haemolymph. The fact that the EPF of the different fungal species
442 alters different members of these cascades may explain the different levels of
443 immunomodulation observed in the cellular bioassays. Interestingly similar observations
444 were made by Mc Namara *et al.* (2017) who showed that the filtrate of *M. brunneum* and *B.*
445 *caledonica* also altered the abundance of serine proteases and serpins in the haemolymph of
446 *G. mellonella* larvae highlight potentially conserved immunomodulatory effects of the
447 excretory/secretory products of entomopathogenic fungi.

448

449 4.4 EPF culture filtrate alters abundance of detoxification enzymes in haemolymph

450 Insects have developed mechanisms to deal with EPF and their secretory products; insects
451 exposed to fungal toxins generally have higher antioxidant enzyme activity (Butt *et al.* 2016).
452 Larvae of the Colorado potato beetle, *Leptinotarsa decemlineata*, demonstrated elevated
453 activity of esterases and glutathione-S-transferase (GST) when infected with *M. brunneum*
454 (Dubovskiy *et al.* 2010). *Hylobius abietis* injected with *B. caledonica* filtrate had increased
455 abundance of carboxylesterases. In *G. mellonella* larvae injected with *B. bassiana* culture
456 filtrate there was a higher level of expression of alpha-esterase and carboxylesterase when
457 compared to *M. brunneum* and *B. caledonica* treated larvae (McNamara *et al.* 2017). Insects
458 have been shown to produce an array of humoral defences to resist fungal infection including
459 lectins, protease inhibitors, PO, AMPs and reactive oxygen and nitrogen radicals (Butt *et al.*
460 2016). However these reactive species can damage both the host and pathogen. Thus, both
461 possess antioxidant systems and detoxifying enzymes, aimed at neutralizing these reactive
462 species. In insects, these enzymes include superoxide dismutase (SOD), catalase, peroxidase
463 and GST (Felton and Summers 1995, Butt *et al.* 2016). *Hylobius abietis* larvae treated with
464 *M. brunneum* or *B. bassiana* filtrate demonstrated alterations in abundance of proteins
465 involved in oxidative stress: copper and zinc SOD and peroxidase isoform X, respectively.

466 4.5 EPF culture filtrate alters the abundance of proteins involved in reception and detection

467 The ability to perceive, discriminate and respond to chemical cues by chemoreception
468 strongly impacts on fitness and survival. This process is necessary for identification of food

469 resources, avoiding intoxication and to communicate with or detect other organisms including
470 fungi (Boucias *et al.* 2012). *Hylobius abietis* larvae injected with *M. brunneum* filtrate had
471 altered abundance of proteins involved in reception and detection: chemosensory 6, odorant-
472 binding 29 and β -1,3-glucan-binding protein (GBP) were increased in abundance, while
473 peptidoglycan-recognition SC2 and an odorant binding protein from the pheromone binding
474 protein & general odorant binding protein family were decreased in abundance (Figure 3). In
475 *G. mellonella* larvae injected with *M. brunneum* and *B. caledonica* there were alterations in
476 abundance of peptidoglycan recognition-like (due to *M. brunneum*) and β -1,3-GBP and two
477 peptidoglycan recognition proteins (due to *B. caledonica*) (Mc Namara *et al.* 2017). Insects
478 can differentiate between major groups of microbes using pattern recognition receptor
479 (PRRs) such as PGRPs, hemolin and β -1,3-GBP. PRRs function by binding to Pathogen-
480 associated molecular patterns (PAMPs) on microbial cells such as β -1,3-glucan from fungi
481 that acts as a signal to activate the antifungal functions of Toll (Stokes *et al.* 2015). These
482 receptors are crucial to recognition of pathogens and activation of an appropriate immune
483 response (e.g. proPO pathway). Two major gene families are involved in the perireceptor
484 events of the chemosensory system: the odorant binding and chemosensory protein families
485 (Vieira and Rozas 2011). Chemosensory 6 was increased in abundance in *H. abietis* larvae
486 injected with *B. caledonica* filtrate. Chemosensory and odorant-binding 29 were increased in
487 abundance following injection with *B. bassiana* filtrate (Figure 3).

488 4.6 EPF culture filtrate alters the abundance of AMP in the insect haemolymph

489 Biologically active peptides exhibiting antibacterial, antifungal and antiviral activity are
490 found abundantly in insects. Most insects have high anti-microbial peptide (AMP) activity
491 against Gram-positive bacteria but less against Gram-negative bacteria, fungi and yeasts
492 (Faruck *et al.* 2016). Anti-microbial peptides (AMP) are expressed in the fat body and
493 secreted into the haemolymph in response to infection. *Hylobius abietis* larvae treated with
494 *M. brunneum* filtrate had an altered abundance of attacin and pathogenesis-related 5
495 (thaumatin). Thaumatin-like peptides were identified in *T. castaneum*, and were found to act
496 as an AMP against filamentous fungi (Altincicek *et al.* 2008), potentially indicative of the
497 insect mounting an immune response to EPF. Attacin is an antibacterial protein, originally
498 isolated from haemolymph of *Hyalophora cecropia*, where it was produced in response to
499 bacterial infection (Carlsson *et al.* 1998). The production of immune effectors is costly for

500 the insect, so production of several in lower concentrations that work together would be very
501 advantageous (Butt *et al.* 2016). One implication of an upregulation in AMP active against
502 bacteria, following treatment with EPF filtrate, is that antibacterial activity can be beneficial
503 to EPF as it might help exclude opportunistic infections that would be disadvantageous to the
504 fungus (Butt *et al.* 2016).

505 *Hylobius abietis* larvae treated with *B. caledonica* filtrate had increased abundance of
506 pathogenesis-related 5 and decreased abundance of a defensin. Larvae treated with *B.*
507 *bassiana* filtrate had increased abundance of attacin C and decreased abundance of a
508 defensin. Defensins are anti-bacterial peptides highly active against Gram-positive bacteria
509 (Faruck *et al.* 2016), they form voltage-dependent channels, leading to rapid leakage of K⁺
510 and other ions (Hoffmann 1995). A coleopteracin was increased in abundance in *M.*
511 *brunneum* and *B. bassiana* treated larvae relative to *B. caledonica* treated larvae (Figure 3).
512 Antibacterial coleopteracins have been identified in the yellow mealworm beetle, *Tenebrio*
513 *molitor*, and were upregulated following bacterial challenge and parasitisation (Zhu *et al.*
514 2014). *Galleria mellonella* larvae treated with EPF filtrate also displayed alterations in
515 abundance of AMP (McNamara *et al.* 2017).

516 4.7 EPF culture filtrate affects the abundance of proteins involved in insect development

517 Susceptibility to infection can depend on insect developmental stage, recently moulted insects
518 being particularly vulnerable as new cuticle is not fully sclerotized (Butt *et al.* 2016).
519 *Hylobius abietis* larvae are more susceptible than adults to both EPF (Ansari and Butt 2012)
520 and EPN (Williams *et al.* 2015), potentially in part due to differences in cuticle thickness.
521 *Hylobius abietis* larvae injected with *M. brunneum* filtrate had an alteration in abundance of
522 proteins involved in development, metamorphosis and structure: JHPB, endocuticle structural
523 glyco ABD-4, tropomyosin 1 and actin 5C were increased in abundance, myosin regulatory
524 light chain 2 and a chitin binding protein Peritrophic matrix 9 precursor were decreased
525 (Figure 3). Although typically associated with muscle and the cytoskeleton many of these
526 proteins are commonly reported as soluble components of insect haemolymph (Handke *et al.*,
527 2013; Li *et al.*, 2012; McNamara *et al.*, 2017). Larvae injected with *B. bassiana* filtrate had a
528 decrease in abundance in proteins involved in development: diapause-associated transcript-2
529 and myosin regulatory light chain 2 (Figure 3).

530 Insect growth, development and reproduction are regulated by juvenile hormone (JH). Its
531 presence during larval moulting prevents metamorphosis, and it reappears in the adult to
532 regulate female reproductive maturation (Jindra *et al.* 2013). Insecticides have been
533 developed that mimic the action of insect growth and developmental hormones; the steroidal
534 20-hydroxyecdysone and the sesquiterpenoid JH (Dhadialla *et al.* 1998). The cuticle is the
535 first and most important barrier to EPF and chitin is a major component of the cuticle. EPF
536 produce an extensive array of enzymes such as lipases, proteases and chitinases, with some of
537 these cuticle-degrading enzymes being considered virulence determinants (Butt *et al.* 2016).
538 Insect growth and morphogenesis are dependent on the capability to remodel chitin-
539 containing structures. Thus, insects repeatedly produce chitin synthases and chitin-lytic
540 enzymes. These alterations in abundance of developmental proteins following injection with
541 EPF filtrate may be indicative of the insect trying to regenerate and protect itself from
542 pathogens or it could be a reflection of EPF natural products (e.g. enzymes or secondary
543 metabolites) within the culture filtrate having an impact on the insect.

544 *4.8 EPF filtrate has a significant effect on proteins involved in cellulolytic and other* 545 *metabolic processes in H. abietis*

546 Larvae injected with *B. caledonica* filtrate had a higher abundance of proteins involved in
547 metabolic processes (Figure 4) with GO term mapping indicating that a considerable portion
548 were involved in cellulolytic processes. It was surprising to identify the large number of
549 cellulases and carbohydrases (annotated as glycoside or glycosyl hydrolases (GHs) in *H.*
550 *abietis* haemolymph, although GHs have been identified previously in insect haemolymph
551 (Zhang *et al.*, 2014; Rocha *et al.*, 2016). In total 24 GHs were identified across all treatments
552 and a number of these were present in the haemolymph of non-exposed larvae indicating that
553 they are endogenous to haemolymph and not artefacts of damage to the gut by the filtrate
554 contents. We also explored the potential that these proteins were fungal in origin, with
555 homology to insect GHs and were delivered into the insect via the filtrate. However no GH
556 proteins were identified when the mass spectrometry data was searched against a reference
557 proteome for *Beauvaria*.

558

559 The largest effect on GH abundance was observed in larvae injected with *B. caledonica*
560 filtrate, which had higher abundance of proteins from GH families 1, 2, 31, 35, 38, 48 and 79

561 with proteins annotated as members of GH families 28 and 45 having relative fold changes of
562 over 100 in comparison to the non EPF exposed controls. A smaller number of GHs were
563 altered in abundance in *B. bassiana* (GH family 28 and 45) and *M. brunneum* (GH family 1)
564 treated *H. abietis* larvae, but not to the same extent as in *B. caledonica* treated larvae (Figure
565 3). Although typically associated with cellulytic activity many of these protein families
566 display considerably diverse functions in insects. GH family 1 consists of β -glucosidase, 6-
567 phospho- β -glucosidase and β -galactosidase that are involved in carbohydrate transport and
568 metabolism whereas GH family 2 contains β -galactosidase, β -mannosidase and β -
569 glucuronidase activities involved in chlorophyll, carbohydrate and starch metabolic
570 processes. GH family 28 includes polygalacturonase and rhamnogalacturonase, enzymes that
571 are important in cell wall metabolism. GH family 31 is comprised of key enzymes of
572 carbohydrate metabolism whereas members of GH family 45 are endoglucanases which
573 function in the hydrolysis of soluble β -1, 4 glucans. GH48 are in most cases components of
574 complex proteins that include additional functional domains. APAP I, from family GH48
575 from the leaf beetle *Gastrophysa atrocyanea* has chitinase activity but is also involved in
576 diapause termination by JH (Fujita *et al.*, 2006).

577
578 A number of these proteins belonging to GH families are potential cell wall degrading
579 enzymes (PCWDEs). Xylophagous insects, such as *H. abietis*, are well adapted to feeding on
580 wood and possess efficient systems to convert cellulosic biomass in their bodies (Watanabe
581 and Tokuda 2010). PCWDEs degrade cellulose, hemicellulose, or pectin in plant cell walls,
582 liberating sugars, minerals, and other nutrients from woody plant tissues. Although many of
583 these GHs have been well characterized in other insects considerable analysis has now to be
584 performed to determine the correct annotation, source and functional assignment of the GH
585 proteins identified here. Although initially thought to be absent in insects (through the
586 analysis of the genomes of model insect organisms including *D. melanogaster* and *B. mori*),
587 recent work has shown that PCWDEs are in fact both present and diverse in insects (Pauchet
588 *et al.*, 2010; Watanabe and Tokuda, 2010), particularly in the Coleoptera. The PCWDEs
589 present in the *H. abietis* transcriptomes were found in other beetle species previously;
590 mountain pine beetle (Keeling *et al.*, 2013), asian longhorned beetle (McKenna *et al.*, 2013),
591 coffee berry borer (Vega *et al.*, 2015), Colorado potato beetle (Schoville *et al.*, 2018). A

592 pectinesterase was also increased in abundance following injection with *B. caledonica* culture
593 filtrate. Given the presence of PCWDEs including pectinesterases are carbohydrate esterases
594 that belong to family 8 (CE8) (Markovic and Janecek, 2004; Kirsch *et al.*, 2016) and
595 demethylate galacturonic acid residues of homogalacturonan to facilitate the action of
596 polygalacturonases (GH28). These enzymes are commonly found in species of Curculionidae
597 and have important biotechnological applications for the processing of pectin (Habrylo *et al.*,
598 2018). Thus the determination of a treatment of pine weevil larvae that results in the
599 considerable over expression of potential PCWDEs and other enzymes of potential
600 importance highlights the potential biotechnological significance of our work. However
601 considerable analysis of the GH proteins is warranted to determine the specific function and
602 origin of these diverse and abundant group of proteins not typically associated with insect
603 haemolymph.

604

605 *Conclusion*

606 Elucidating how EPF modulate the immune response leaving insects more susceptible to
607 subsequent pathogens may have application in improving biocontrol in the field in a number
608 of ways: selecting superior strains with immune modulating characteristics to overcome
609 problems with EPF killing target pests inefficiently compared to their chemical counterparts,
610 selecting strains that could be used in combination with other plant protection products to
611 enhance control (achieve synergy). Additionally, EPF isolates could be screened for their
612 ability to produce particular secreted products that induce immunomodulation in target
613 insects. This aim of this work was to investigate the effect of culture filtrates from three EPF
614 species on the insect immune response using larvae of the economically important forestry
615 pest *H. abietis* larvae.

616 The immune responses induced in *H. abietis* larvae were in response to injection with spore
617 free culture filtrate, so it is a reflection of the immune response induced by EPF secreted
618 products. A number of fungal secreted products are known to be important virulence
619 determinants that can induce changes to immune response of insects affecting AMP and the
620 proPO cascade as well as the cellular immune response. These findings aid in understanding

621 how the desired synergism between biocontrol agents could mechanistically occur e.g.
622 interfering with the proPO cascade and the production of AMP.

623 Bioassays allowed assessment of the immunomodulation of different treatments and
624 proteomic analysis aided in understanding mechanistically how these variations may have
625 occurred e.g. alterations to proteins/pathways that may render the insect more susceptible to
626 subsequent pathogens. Injection with *M. brunneum* or *B. bassiana* culture filtrate facilitated a
627 significantly increased yeast cell density in larvae. Larvae co-injected with either *B.*
628 *caledonica* or *B. bassiana* culture filtrate and *C. albicans* showed significantly increased
629 mortality. Injection with EPF culture filtrate was shown to alter the abundance of protease
630 inhibitors, detoxifying enzymes, antimicrobial peptides and proteins involved in
631 reception/detection and development in *H. abietis* larvae. Larvae injected with *B. caledonica*
632 culture filtrate displayed significant alterations in abundance of proteins involved in
633 cellulolytic and other metabolic processes in their haemolymph proteome. Together these
634 results suggest that EPF culture filtrate has the potential to modulate the insect immune
635 system which may allow subsequent pathogens to proliferate.

636

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640

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- 854

855

856 **Fig. 1.** Haemocyte and yeast cell densities and mortality in *H. abietis* larvae pre-treated with EPF
 857 culture filtrate. (A) Haemocyte density (mean \pm SE) in *H. abietis* larvae treated with EPF culture
 858 filtrate. Following inoculation with fungal culture filtrate, larvae were incubated for 24 h or 48 h at 20
 859 °C before bleeding and enumeration. Sab Dex: Control medium, (i) Bc: *B. caledonica*, (ii) Bb: *B.*
 860 *bassiana* and (iii) Met: *M. brunneum*. X-axis represents length of time EPF was cultured for: 48 h, 72
 861 h, 96 h. Asterisks indicate significant difference to relevant control * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
 862 (B) Yeast cell density in *H. abietis* larvae pre-treated with EPF culture filtrate. Number (mean \pm SE)
 863 of *C. albicans* cells per larva after incubation for 24 h and 48 h at 20 °C. Larvae were treated with
 864 fungal culture filtrate 24h prior to inoculation with *C. albicans*. Sab Dex: Control medium, (i) Bc: *B.*
 865 *caledonica*, (ii) Bb: *B. bassiana* and (iii) Met: *M. brunneum*. X-axis represents length of time EPF
 866 was cultured for: 48 h, 72 h and 96 h. Asterisks indicate significant difference to relevant control *
 867 $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (C) Mortality of *H. abietis* larvae treated with EPF culture filtrate
 868 alone and in combination with *C. albicans*. + *C. albicans* indicates larvae that received a dose of *C.*
 869 *albicans* after 24 h, - *C. albicans* indicates larvae that did not. Sab Dex: Control media, Bc: *B.*
 870 *caledonica*, Bb: *B. bassiana* and Met: *M. brunneum*. All EPF were cultured for 96 h. Mortality one
 871 week after infection with *C. albicans*. Data were tested for significance using paired T-tests. *
 872 $p < 0.05$, ** $p < 0.01$.

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874

875 **Fig. 2.** Principal component analysis (PCA) and hierarchical clustering of haemolymph proteomic
 876 profiles of larvae treated with EPF culture filtrate versus control. (A) PCA of three replicates of each
 877 treatment included in LFQ analysis. Dashed circles denote sample groups. The two axes account for
 878 74.1 % of total variation within the dataset. (B) Heat map based on hierarchical clustering of the
 879 median protein expression values of all statistically significant differentially abundant and uniquely
 880 detected proteins. Hierarchical clustering (columns) resolved four distinct clusters comprising the
 881 replicates from their original sample groups and four protein clusters (rows) based on expression
 882 profile similarities.

883

884 **Fig. 3.** Volcano plots of post imputed data highlighting proteins altered in abundance in haemolymph
 885 of *H. abietis* larvae following injection with EPF culture filtrate. Proteins above the dashed line are
 886 considered statistically significant (p -value < 0.05) and those to the right and left of the vertical lines
 887 indicate relative fold changes of ≥ 1.5 . Volcano plots are annotated with the most differentially

888 abundant proteins identified in larvae inoculated with (A) *B. caledonica*, (B) *B. bassiana* and (C) *M.*
889 *brunneum* culture filtrate versus control larvae (inoculated with sabouraud dextrose).

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891

892 **Fig. 4.** Alterations in biological processes at level 3 gene ontology following injection of *H. abietis*
893 larvae with fungal culture filtrate in comparison to control larvae. Only processes identified in all
894 three EPF treatments are given.

Table 1 Number of statistically significant differentially abundant (SSDA) proteins between EPF filtrate injected and control larvae. Annotations, mass spectrometry information and sequence characteristics for SSDA haemolymph proteins (two sample t-tests, $p < 0.05$) between treated and control larvae. Relative fold changes are given for EPF injected SSDA proteins with \uparrow and \downarrow representing higher or lower abundance relative to the controls

Protein ID	Protein Annotation	Relative fold change			Intensity	MS/MS	Peptides	Mol. Weight	Sequence length
		BB	BC	MET					
Unigene8018	Chemosensory 6	10.71 \uparrow	7.41 \uparrow	130.5 \uparrow	1.0x10 ¹⁰	98	13	14.9	129
Unigene10957	Glycosyl hydrolase family 79	2.347 \uparrow	17.19 \uparrow	3.46 \uparrow	4.8x10 ⁸	35	9	52.46	468
Unigene3665	FKBP-type peptidyl-prolyl cis-trans isomerase	2.56 \uparrow	2.28 \uparrow	3.09 \uparrow	1.7X10 ⁹	81	12	24.13	220
CL416.Contig1	Heat shock 70 kDa cognate 4	1.5 \uparrow	2.1 \uparrow	2.7 \uparrow	2.8x10 ⁸	53	11	71.8	655
CL2534.Contig1	Lectin C-type domain	2.14 \downarrow	2.74 \downarrow	2.17 \downarrow	2.7x10 ⁸	33	6	16.02	143
CL1928.Contig2	Odorant-binding 29	1.6 \uparrow	-	3.6 \uparrow	1.1x10 ¹¹	313	17	14.8	136
CL943.Contig7	Papilin-like Protein	2.01 \uparrow	-	1.73 \uparrow	4.8x10 ⁸	89	19	278.9	2546
Unigene7330	Attacin C	1.66 \uparrow	-	1.51 \uparrow	4.1x10 ¹⁰	128	11	13.8	133
Unigene5426	Myosin regulatory light chain 2	6.35 \downarrow	-	18.56 \downarrow	7.0x10 ⁸	30	6	22.1	206

CL4537.Contig3	No annotation	9.4↑	144.2↑	-	1.4×10^9	20	2	12.7	120
Unigene3946	Glycosyl hydrolase family 28	2.52↑	130.12↑	-	2.5×10^9	53	8	38	364
Unigene9634	Glycosyl hydrolase family 45	3.88↑	105.8↑	-	2.4×10^9	18	2	23.8	225
CL2640.Contig1	Pathogenesis-related 5	1.52↑	48.4↑	-	3.2×10^9	46	10	27.0	253
Unigene2311	Alpha-L-fucosidase	2.45↑	25.2↑	-	3.9×10^8	28	8	49.2	429
Unigene3970	Glycosyl hydrolase family 28	1.76↑	22.81↑	-	7.0×10^8	39	11	35.8	335
Unigene5303	Defensin	1.54↓	1.76↓	-	1.2×10^{10}	72	3	9.1	85
Unigene28106	Glycosyl hydrolase family 1	-	26.88↑	2.6↑	1.3×10^9	18	7	25.8	227
Unigene11176	Peritrophic matrix 9 precursor	-	1.67↓	2.17↓	1.2×10^9	60	7	29.7	268
CL1224.Contig1	Lectin C-type domain	-	4.16↓	2.12↓	1.9×10^9	73	6	13.588	123
CL515.Contig1	Diapause-associated transcript-2	6.18↑	-	-	3.2×10^8	22	5	18.8	162
Unigene9585	No annotation	1.57↑	-	-	3.3×10^{10}	183	10	17.4	160
Unigene2445	Regulatory CLIP domain of proteinases	1.55↑	-	-	1.8×10^9	43	3	8.1	71
CL2420.Contig2	Melanin-inhibiting protein	1.53↑	-	-	1.1×10^{10}	182	14	32.4	286
CL2563.Contig1	Peroxidase isoform X1	1.51↑	-	-	2.9×10^9	301	35	80.4	716

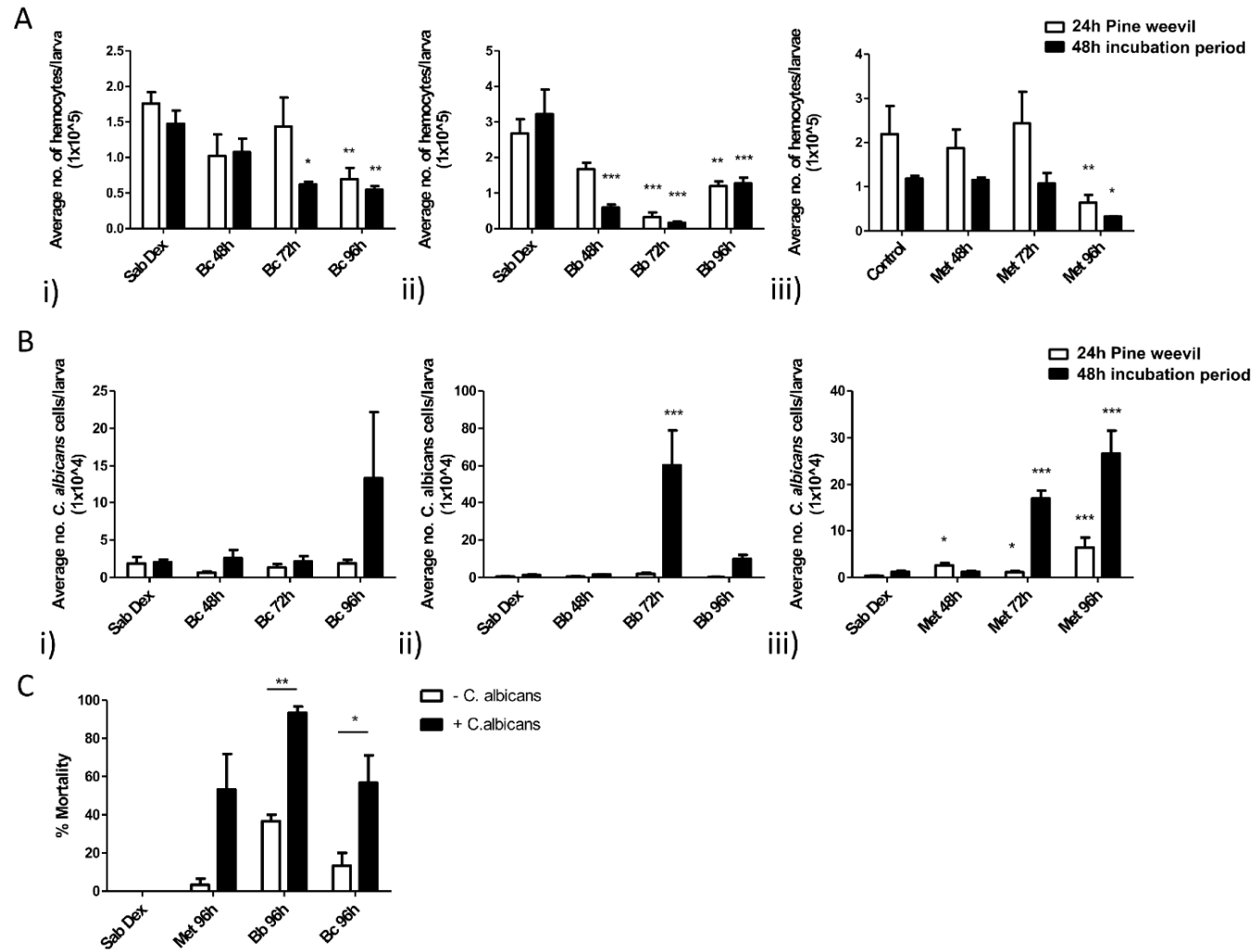
CL5881.Contig1	Actin	6.7↓	-	-	1.4×10^9	27	12	39.8	360
CL61.Contig2	Arylphorin	26.22↓	-	-	1.0×10^{10}	33	73	85.7	717
Unigene11986	Glycoside hydrolase family 48	-	887.1↑	-	2.7×10^{10}	230	26	70.52	633
CL5500.Contig2	Glycoside hydrolase family 48	-	884.3↑	-	4.5×10^{10}	264	29	70.9	638
Unigene3825	Glycosyl hydrolase family 45	-	624.9↑	-	1.5×10^{10}	52	3	23.9	227
Unigene7925	Neutral alpha-glucosidase C	-	266.3↑	-	5.5×10^9	175	31	96.6	844
Unigene9562	No annotation	-	162.2↑	-	5.6×10^9	98	15	40.6	366
Unigene12087	Glycosyl hydrolase family 2	-	143.1↑	-	3.5×10^9	133	32	101.79	894
Unigene13818	Glycosyl hydrolase family 28	-	96.9↑	-	4.7×10^9	67	10	36.4	350
Unigene19514	Pectinesterase	-	74.1↑	-	1.9×10^9	63	12	39.8	380
Unigene6962	Glycosyl hydrolase family 1	-	73.9↑	-	2.6×10^9	40	11	56.1	498
CL921.Contig2	Glycoside hydrolase family 31	-	41.65↑	-	1.4×10^9	69	16	70.7	626
Unigene12565	Glycosyl hydrolase family 35	-	35.95↑	-	1.7×10^9	70	17	71.9	640
Unigene8511	Glycosyl hydrolase family 38	-	30.85↑	-	6.8×10^8	55	18	11.9	988
Unigene13343	Carboxylesterase family	-	29.3↑	-	1.5×10^9	32	10	60.4	545
Unigene3841	Glycosyl hydrolase family 45	-	28.67↑	-	4.4×10^8	12	3	25.8	236

CL2700.Contig4	Carboxylesterase family	-	14.1↑	-	5.6×10^8	32	12	58.5	527	
Unigene3953	Prostatic acid phosphatase	-	9.89↑	-	3.0×10^8	25	7	28.9	381	
CL1864.Contig4	Glycosyl hydrolase family 2	-	7.81↑	-	4.2×10^8	31	8	71.7	631	
Unigene3489	Serpin	-	2↑	-	1.6×10^9	52	3	6.99	62	
Unigene1589	Trypsin-like protein	-	1.74↑	-	9.4×10^8	64	11	42.1	379	
CL797.Contig4	Chymotrypsin--like protein	-	1.67↑	-	2.6×10^8	45	8	49.7	448	
CL5549.Contig2	Sodium channel 60E	-	1.54↓	-	6.7×10^8	50	9	43.8	380	
Unigene13338	Trypsin-like protein	-	2.1↓	-	4.6×10^9	200	22	55.7	488	
Unigene12317	JHBP	-	-	23.78↑	8.5×10^8	36	7	36.7	248	
Unigene17410	JHBP	-	-	22.17↑	1.3×10^9	48	15	26.9	242	
Unigene8077	Endocuticle glycoprotein ABD-4	structural	-	-	11.51↑	2.1×10^8	15	3	11.04	102
Unigene10626	No annotation	-	-	6.67↑	5.7×10^8	33	9	41.4	384	
CL3921.Contig1	Tropomyosin 1	-	-	5.2↑	6.9×10^8	38	17	32.7	283	
CL466.Contig4	Aerine protease easter	-	-	3.24↑	6.1×10^8	57	20	41.2	374	
Unigene2302	Major royal jelly protein	-	-	2.35↑	1.4×10^{10}	323	26	46.1	411	

Unigene27113	28 kDa desiccation stress	-	-	2.22↑	9.8X10 ⁹	265	14	26.2	229
CL1928.Contig3	Odorant-binding 29	-	-	1.88↑	2.0x10 ¹¹	387	15	14.96	136
CL3504.Contig2	Beta-1,3-glucan-binding protein	-	-	1.69↑	1.7x10 ⁹	132	15	53.6	479
CL492.Contig1	Attacin	-	-	1.69↑	2.4x10 ¹⁰	169	10	15.1	140
CL5881.Contig6	Actin-5C	-	-	1.58↑	9.3x10 ⁸	43	21	41.8	376
Unigene8093	Cu ²⁺ ,Zn ²⁺ superoxide dismutase	-	-	1.67↓	6.6x10 ⁹	52	2	16.8	168
CL3832.Contig2	Peptidoglycan-recognition SC2	-	-	1.7↓	3.9x10 ⁹	75	7	20.3	186
Unigene3995	Serine protease easter-like	-	-	1.96↓	8.9x10 ⁹	208	16	40.3	365
CL1617.Contig2	Serine proteinase stubble-like	-	-	2.13↓	5.7x10 ⁹	47	21	48.52	447
CL2247.Contig3	PBP&GOBP family	-	-	2.19↓	9.8x10 ¹¹	1217	13	13.56	124
CL3607.Contig1	Kunitz trypsin inhibitor	-	-	2.32↓	1.8x10 ¹¹	261	6	11.5	102
Unigene4030	Thaumatococcus	-	-	2.63↓	5.6x10 ¹⁰	421	14	26	236
Unigene6368	No annotation	-	-	2.86↓	2.9x10 ⁸	55	17	71.8	615
Unigene417	Aspartyl protease	-	-	6.84↓	2.3x10 ⁸	25	5	47.7	417

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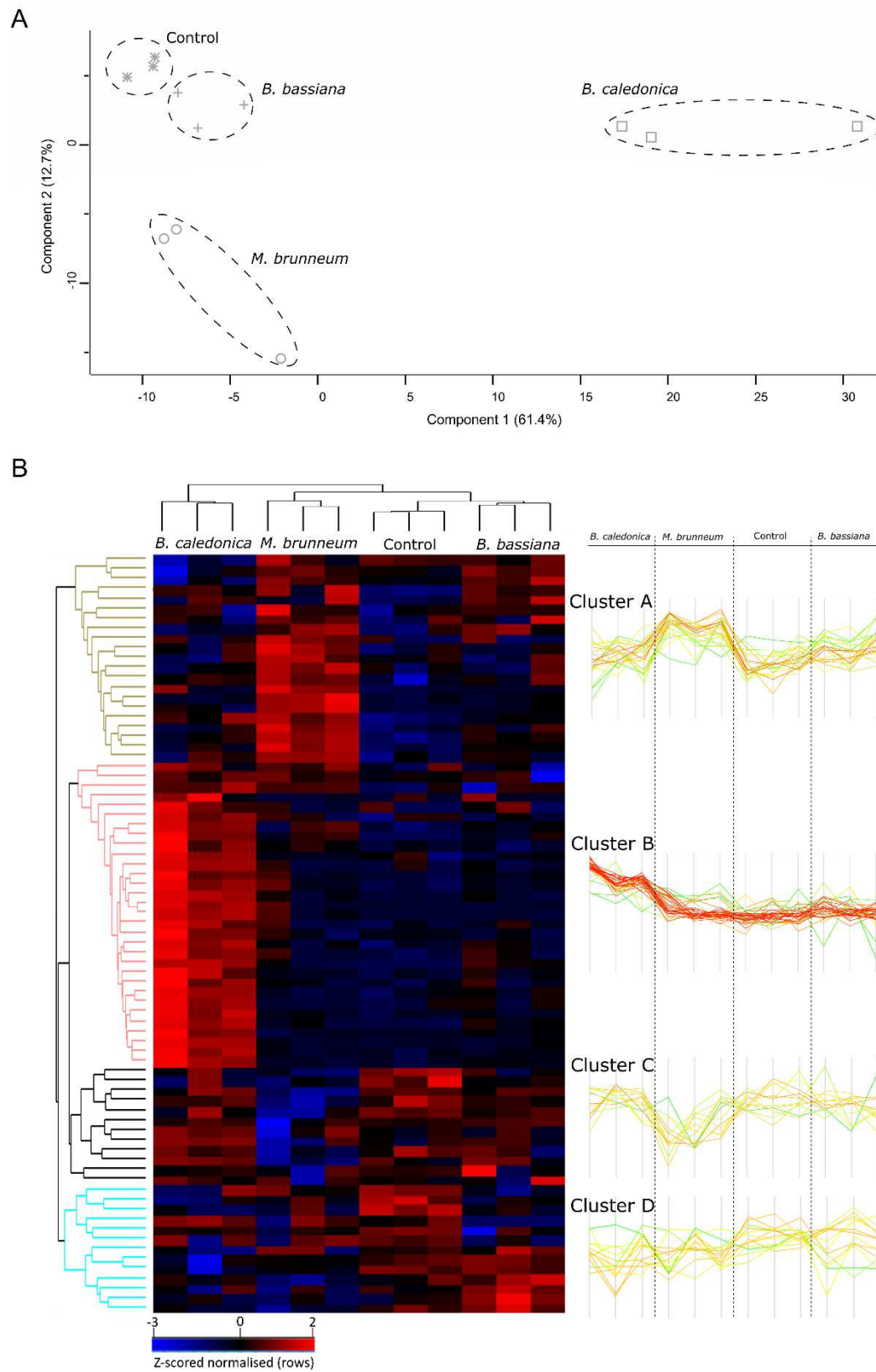
897



899 **Fig.**

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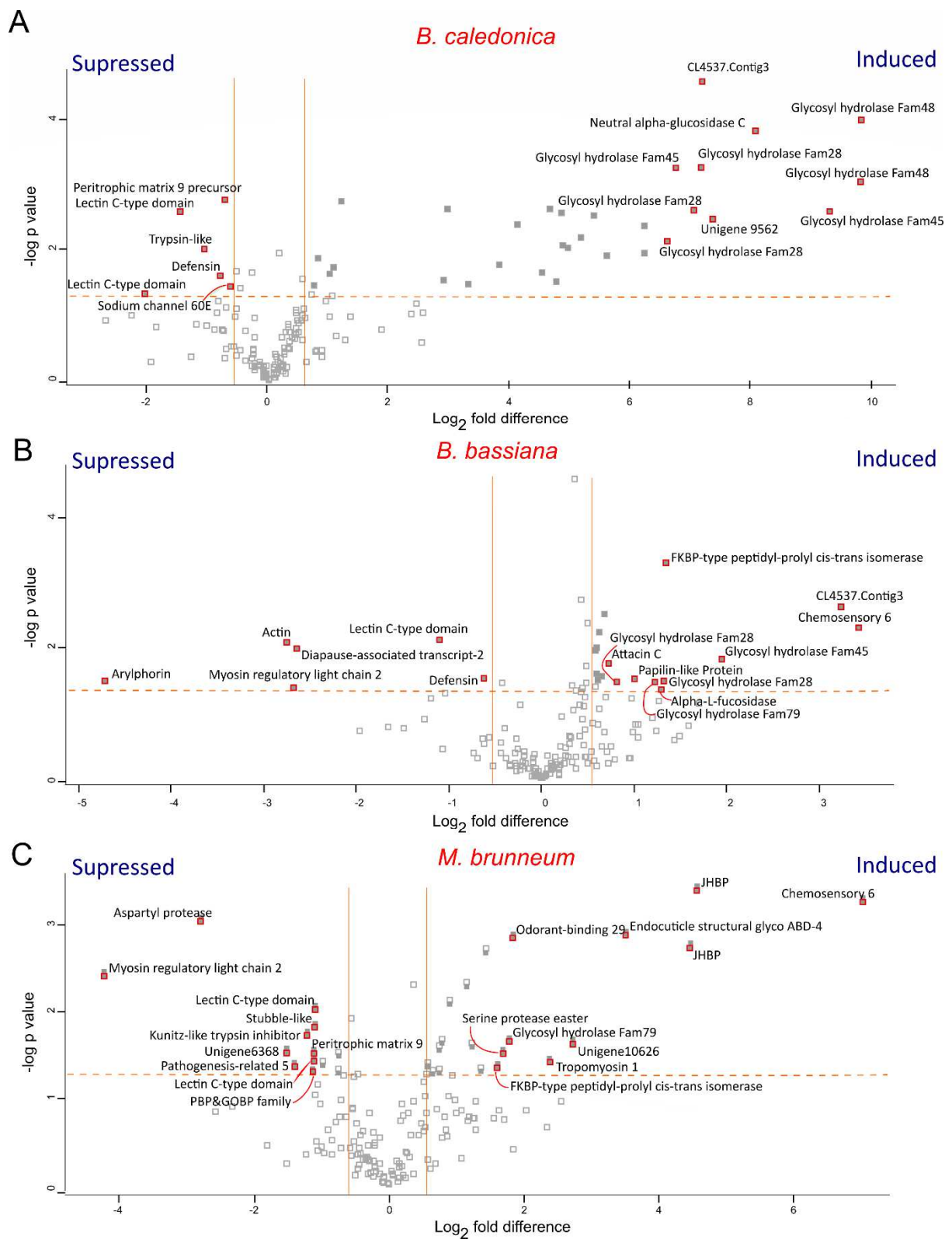
ACCEPTED MANUSCRIPT



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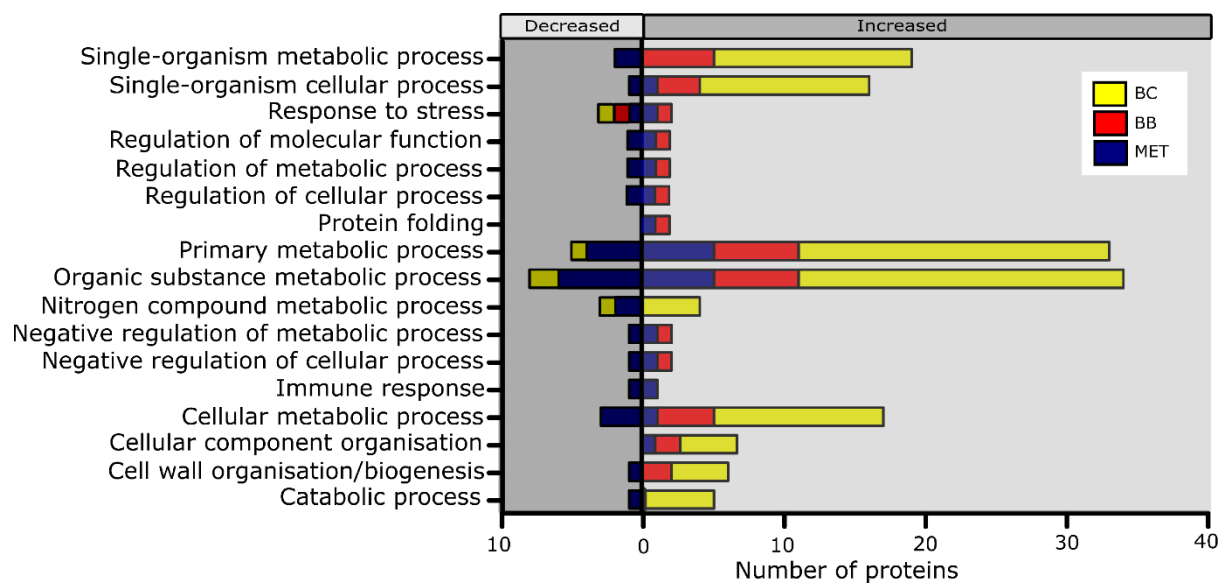
Fig. 2

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903 **Fig. 3**



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905 **Fig. 4.** Bar chart showing number of proteins changed in biological processes at level 3 ontology
 906 following injection of *H. abietis* larvae with fungal culture filtrate. Number of proteins changed in
 907 biological processes common to larvae treated with all three EPF.

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SUPPORTING INFORMATION

916 **Table S1** MS identified proteins from the haemolymph of the large pine weevil, *Hylobius*
 917 *abietis* after treatment with the culture filtrate of *B. bassiana*, *B. caledonica* and *M. brunneum*
 918 and Sabouraud Dextrose liquid medium. (XSLX)

919 **Table S2** Proteins groups identified after hierarchical clustering of SSDA and exclusive
 920 proteins. Four clusters of proteins with similar expression and abundance profiles were
 921 identified.

922 **Table S3** Statistically significantly differentially abundant *Hylobius abietis* haemolymph
923 proteins (2 sample t-tests ; $p < 0.05$) and relative fold change differences for comparisons of *B.*
924 *bassiana* culture filtrate to control; *B. caledonica* culture filtrate to control and *M. brunneum*
925 to control. (XSLX)

926 **Table S4** Blast2Go results for all identified proteins with assigned InterPro ids, enzyme code
927 and gene ontology (GO) terms for biological processes, molecular function and cellular
928 components.

929 **Table S5** The glucosyl hydrolases identified from pine weevil haemolymph. Seven of the 20
930 proteins annotated as glycosyl hydrolases were present in control (not exposed to fungal
931 supernatant filtrate). The results for all MS/MS data searched against the predicted protein set
932 for *Beauveria bassiana* are provided. Of the 15 proteins seven were supported by more than
933 one peptide and only a single protein was annotated as a glucosidase, highlighting that the 20
934 glycosyl hydrolases identified here are more likely insect in origin. (XSLX)

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Highlights

- Injection with EPF filtrate facilitated an increased yeast cell density in larvae
- Co-injection with EPF and *Candida albicans* caused significantly increased mortality
- EPF can modulate insect immune system allowing proliferation of subsequent pathogens
- Injection with EPF culture filtrate significantly altered the haemolymph proteome
- *Beauveria caledonica* altered abundance of proteins involved in cellulolytic/metabolic processes