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## Proteomics and Immune Response Differences in *Apis mellifera* and *Apis cerana* Inoculated with Three *Nosema ceranae* Isolates

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1 **Proteomics and immune response differences in *Apis mellifera* and *Apis***  
2 ***cerana* inoculated by three *Nosema ceranae* isolates**

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31

32

33 **Abstract**

34 *Nosema ceranae* infects midgut epithelial cells of *Apis* species and jumped from its  
35 original host *A. cerana* to *A. mellifera* worldwide asking questions on how the new host is  
36 responding. We compared *A. mellifera* and *A. cerana* responses to *N. ceranae* isolates from  
37 *A. cerana*, *A. mellifera* from Thailand and *A. mellifera* from France. Proteomics and  
38 transcriptomics results were merged to deeper understand the impact on immunity of the two  
39 species. This represents the first combination of omics analysis to evaluate the impact of *N.*  
40 *ceranae* spores from different origin and provides new insights into the differential immune  
41 responses in honeybees inoculated by *N. ceranae* from original *A. cerana*. No difference in  
42 the antimicrobial peptides (AMP) was observed in *A. mellifera*, while these peptides were  
43 altered in *A. cerana* when compared to controls. Inoculation of *A. mellifera* or *A. cerana* with  
44 *N. ceranae* caused up-regulation of the AMP genes and cellular mediated immune genes but  
45 did not greatly alter apoptosis-related gene expression. *A. cerana* showed a stronger immune  
46 response than *A. mellifera* after different *N. ceranae* isolate inoculation. *N. ceranae* from *A.*  
47 *cerana* caused a highly negative impact on *A. mellifera* and *A. cerana* health compared to  
48 other *Nosema* isolates.

49 **Keywords:** honeybees, immune response, microsporidia, hemolymph, transcriptomics and  
50 proteomics

51

52 **INTRODUCTION**

53           The Western honeybee (*Apis mellifera*) and the Eastern one (*A. cerana*) have been  
54 promoted as economic pollinators for beekeeping in Asia<sup>1</sup>. Nowadays honeybees face several  
55 harm factors (biotic or abiotic) including pesticides, lacking of food source and pathogens,  
56 resulting in colony losses across the globe<sup>2-5</sup>. Among those factors, nosemosis is considered  
57 as one of the most serious diseases of adult honeybees and has been reported to decrease  
58 honeybee population<sup>6,7</sup>.

59           Microsporidian parasites are obligated unicellular spore-forming fungal parasites  
60 causing nosemosis or *Nosema* disease in honeybee population (*Apis* spp.)<sup>8</sup>. The disease is  
61 caused by *N. apis* and *N. ceranae* originally affecting *A. mellifera* and *A. cerana*,  
62 respectively<sup>9-11</sup>. *N. ceranae* has been reported to infect *A. mellifera* rather recently<sup>12,13</sup> and  
63 now worldwide<sup>14</sup>. These obligate intracellular parasites infect the digestive tract of adult  
64 honeybees where they can develop and multiply before being transmitted horizontally  
65 through spore consumption from contaminated foods, via fecal-oral transmission or ingestion  
66 of contaminated water<sup>11,15,16</sup>. A visual symptom of *Nosema* infection is diarrhea, while  
67 microscopic observations reveal tissue lesions, spore accumulation and colonization by  
68 *Nosema* mycelium in the gut tissue and Malpighian tubule system. In the adult honeybees, a  
69 *Nosema* infection gives rise to several behavioral and molecular alterations such as (i)  
70 reduction of the lifespan of workers<sup>17</sup>; (ii) cognitive changes including acceleration of the  
71 behavior-age polyethism<sup>18</sup>, change of the olfactory learning and memory<sup>17,19,20</sup>; (iii) dysbiosis  
72 of the endogenous microbial communities of the gut and impairment of the defense system of  
73 the host<sup>21,22</sup>; (iv) alteration of the hemolymph carbohydrate homeostasis<sup>23</sup>, lipid loss<sup>24</sup>; and  
74 (v) dysfunction of the gut tissue<sup>25</sup>.

75 Like other insects, honeybees have evolved defense mechanisms against pathogens, helping  
76 them to survive under biological stress, including *Nosema* invasion<sup>26</sup>. The mechanisms of the

77 insect immune system against parasites and pathogens relies on both humoral and cellular  
78 responses<sup>27</sup>. Recognition of foreign pathogens involves specific receptors for sensing  
79 infection. These include  $\beta$ -glucan recognition proteins and peptidoglycan recognition  
80 proteins, the activation of these receptors will turn on signaling pathways that activate  
81 immune genes. Like all other insects, two key signaling pathways, Toll and immune  
82 deficiency (Imd), regulate the induction of honeybee immune effectors that target microbial  
83 pathogens<sup>28</sup>. Amongst these effectors are antimicrobial peptides (AMPs) that are presumed to  
84 be produced by the *nuclear factors kappa B* (NF- $\kappa$ B) Dorsal and *relish* from the Toll and Imd  
85 pathways, respectively<sup>29</sup>. Honeybees have an open circulatory system which contains  
86 transparent or light yellowish hemolymph for molecular transport, offering a readout of the  
87 humoral immune defenses e.g. synthesis by the fat body of antimicrobial peptides (AMPs),  
88 and physiological monitoring<sup>30</sup>. Honeybee hemolymph contains high concentration of  
89 inorganic ions, amino acids, sugars and proteins compared to vertebrate blood<sup>31</sup>. In addition  
90 to the AMPs synthesis by the fat body and hemocytes of honeybees (such as Defensins,  
91 Abaecin, Apidaecins and Hymenoptaecin)<sup>28</sup>, the humoral immune response is also associated  
92 with melanization mediated by the prophenoloxdase pathway<sup>32-34</sup> and vitellogenin<sup>35</sup>.  
93 Vitellogenin (Vg) is a 180 kDa female-specific protein<sup>36</sup> that is synthesized by the fat body,  
94 released into hemolymph and transported to ovaries and other tissues through receptor-  
95 mediated endocytosis<sup>37</sup>. Vg also has functions in immune transport<sup>38</sup>, immune priming<sup>39</sup> and  
96 honeybee lifespan regulation<sup>40</sup>. Nevertheless, several factors are able to alter the *vitellogenin*  
97 levels in honeybees i.e., *N. ceranae*<sup>41</sup> and insecticides<sup>42</sup>. Hemocytes are also participating to  
98 the cellular responses through encapsulation and phagocytosis mechanisms<sup>43</sup>. *Lysozyme*  
99 (*LSY*) is also an important component of the innate immune system which varies depending  
100 on the developmental stage and gender of the honeybees<sup>44,45</sup>. Honeybees have three *lysozyme*  
101 genes, two encoding c-type *lysozymes* and one encoding i-type *lysozyme*. Only the c-type lys-

102 1 gene is up-regulated in infested bees<sup>26,28</sup>. Apoptosis-related genes are also involved in the  
103 fights against viruses and pathogens<sup>46,47</sup> including *relish (REL)*, *nuclear factor of activated T*  
104 *cell (NFAT)* and *apoptosis regulator RI-like (RI)*<sup>48</sup>. Previous study reported that apoptosis-  
105 related genes, *NFAT*, *RI* and *REL*, expressions in *Nosema* inoculated honeybees (*A. mellifera*  
106 and/or *A. cerana*) were significantly down-regulated<sup>48</sup>, showing a common response to  
107 against *Nosema*. Recent results also evidenced that the proteomic profiles of *Nosema*-infected  
108 honeybees (*A. mellifera*) gut revealed that several new proteins were detected to be  
109 implicated with host response mechanisms including metabolic, oxidative, and energetic  
110 pathways, especially the collagen IV NC1 domain-containing protein<sup>49</sup>. This protein has been  
111 considered as an early prognostic marker of *Nosema* spp. infection in honeybees<sup>49</sup>. Energetic  
112 stress of honeybees was exhibited after *Nosema* infection causing behavioral and  
113 physiological changes<sup>50</sup>. The energetic pathway related proteins were up-regulated after *N.*  
114 *ceranae* and *N. apis* infection which may affect honeybee health status<sup>51</sup>.

115 In the present study, we aim to compare the immune response of the original host, *A.*  
116 *cerana* and the new host *A. mellifera* by proteomics and transcriptomics approaches. Both  
117 species were submitted to three different *N. ceranae* isolates inoculations, one coming from  
118 the original host [*A. cerana* from Thailand (NC1)], another coming from the new host but in  
119 the original location [*A. mellifera* from Thailand (NC2)], and the third coming from the new  
120 host in a very distant location [*A. mellifera* from France (NF)]. Proteomic analyses  
121 demonstrate that AMPs were up-regulated after *Nosema* inoculation, especially in *A. cerana*.  
122 Moreover, the differential proteomics reveal that Ferritin may be involved in the defense  
123 mechanisms developed by *A. mellifera* and *A. cerana* in response to the inoculation of  
124 *Nosema* spores, iron sequestration is a recognized innate immune mechanism. In addition,  
125 our transcriptomic results suggest that in *A. cerana*, the immune response pathways were  
126 more altered than in *A. mellifera* after inoculated with the different *Nosema* spore isolates.

127 This will provide new insights into how gene and protein expressions are modulated in *Apis*  
128 host in response to *Nosema* infections.

## 129 **EXPERIMENTAL SECTION**

### 130 **Chemicals and Reagents**

131 Tris-acetate-EDTA (TAE) buffer was prepared as a 50X stock solution for laboratory  
132 use. The Tetro™ cDNA Synthesis kit and the SensiFAST™ SYBR® No-ROX kit were from  
133 Bioline (Meridian Life Science Inc, USA). TRIzol reagent was from Invitrogen, dithiothreitol  
134 (DTT), 4-vinyl-pyridine (4-VP), hexofluoroisopropanol (HFIP), alphacyano-4-  
135 hydroxycinnamic acid (4-HCCA), ammonium bicarbonate (NH<sub>4</sub>HCO<sub>3</sub>, ABC), phenylthiourea  
136 (PTU, Sigma-Aldrich) and phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich) were  
137 reagent grade from Sigma-Aldrich (St. Louis, MO). RapiGest™ SF surfactant was purchased  
138 from Waters (Milford, USA). The LC-MS grade formic acid (FA), trifluoroacetic acid (TFA)  
139 and acetonitrile (ACN) were from Carlo-Erba Reagents (Val de Reuil, France). Sequencing-  
140 grade modified trypsin from Promega (Madison, WI) was used for in solution protein  
141 digestion. Phosphate Buffered Saline (PBS) was from Thermo Fisher Scientific (Waltham,  
142 MA, USA). Protein Calibration Standard I (ProtMix) was purchased from Bruker Daltonics  
143 (Bremen, Germany) while APISCAL (composition detailed in Houdelet et al., 2020) is a  
144 homemade calibration solution. All solutions were prepared in ultrapure MilliQ water (Merck  
145 Millipore, Billerica, MA).

### 146 **Honeybees**

147 The asymptomatic western honeybee (*Apis mellifera*) and eastern honeybee (*A.*  
148 *cerana*) samples were collected from three different apiaries in Chiang Mai, Thailand.  
149 Newly-emerged bees were randomly kept to a cage (three apiaries, three cages per apiary  
150 total of 270 bees with 30 bees per cage and nine cages per experiment; **Figure 1**) and placed

151 in a controlled incubator at 34°C with 60-70% relative humidity<sup>52</sup>. Honeybees were fed with  
152 a 50% sucrose solution (w/v) *ad libitum*.

### 153 **Spore identification**

154 *Nosema* spp. were confirmed using a light microscope (400X) and by using specific  
155 primers for identification of microsporidia by PCR (**Table S1**). The PCR conditions were  
156 95°C for 2 min, followed by 40 cycles of 94°C for 15 sec, 55°C for 30 sec and 68°C 30 sec<sup>53</sup>.  
157 The PCR products were resolved on 1% agarose gel in TAE buffer. The 100 bp DNA ladder  
158 was used as a marker. The sequences have been deposited in NCBI GenBank under accession  
159 numbers MZ671824 (NC1), MZ671825 (NC2), MZ671826 (NF) and MZ671827 (NA).

### 160 **Inoculum preparation**

161 A filtrated microsporidian suspension from each strain (*Nosema* spp.) was  
162 individually fed to honeybees by a 50% (w/v) sucrose solution with a final spore  
163 concentration of 10<sup>5</sup> spores/mL using homemade boxes as reported by Houdelet et al., 2021.  
164 The *A. mellifera* and *A. cerana* experiments were divided into four experimental conditions  
165 including (i) *N. ceranae* obtained from BioPark, France (NF), (ii) *N. ceranae* isolated from *A.*  
166 *cerana* at Chiang Mai University, Thailand (NC1), (iii) *N. ceranae* isolated from *A. mellifera*  
167 at Chiang Mai University, Thailand (NC2) which feed on both bee host species, and (iv) the  
168 control (non-experimentally inoculated). Dead honeybees were enumerated every days in  
169 order to evaluate the survival rate. Honeybees were collected at different time intervals: day  
170 4, 7 and 13 post inoculation (d.p.i.).

### 171 **Hemolymph collection**

172 Four days and thirteen days after *Nosema* spore inoculation, hemolymph from three  
173 (at 4 d.p.i.) and five honeybees (at 13 d.p.i.) per cage were collected (a total of 99  
174 haemolymph samples and bled bees per condition at the end of the experiment; **Figure 1**).  
175 For this part, no individual was collected at 7 d.p.i. to ensure sufficient number for the end of



176 the experiment (13 d.p.i.). Honeybees were individually bled by piercing the abdomen  
177 dorsally between the second and third tergite using pulled glass capillaries (Sutter Instrument  
178 Corp, Novato, California). The hemolymph (~5  $\mu$ L) was transferred into a chilled LoBind  
179 Protein microtube (Eppendorf, Germany) containing dried solutions of PTU at 20  $\mu$ M and  
180 PMSF at 5 mM to minimize melanization and proteolysis, respectively. The hemolymph  
181 samples and the corresponding bled bodies were kept at -20°C until further use. Samples with  
182 contamination from the gut or crop content were removed from the analyses.

### 183 **MALDI-BeeTyping® mass spectrometry analysis**

184 Each hemolymph sample was analyzed by MALDI-mass spectrometry (referred as  
185 MALDI-BeeTyping®) after a 10-fold dilution in 5% TFA. One microliter of the sample was  
186 spotted on a MALDI MTP 384 polished steel plate (Bruker Daltonics), dried for 15 min  
187 under mild vacuum before adding 1 $\mu$ L of a 4-CHCA matrix solution (15 mg/mL in 70%  
188 ACN and 2.5% TFA) on each individual spotted sample and dried again as mentioned above  
189 before analysis. Each individual sample was spotted three times.

190 Spectra were acquired manually in a positive ion linear mode on a MALDI AutoFlex  
191 III Smartbeam (Bruker Daltonics) using the software FlexControl 3.4 (Bruker Daltonics).  
192 Instrument was calibrated using two distinct calibration sets (APISCAL and ProtMix)  
193 covering the dynamic range of interest. The following parameters were applied for spectral  
194 acquisition: laser at 200 Hz with an overall attenuation adjusted so as to obtain a signal  
195 intensity of 104 (arbitrary unit) for the majority of the samples, voltage of source 1 at 20 kV,  
196 voltage of source 2 at 18.7 kV, voltage lens at 9.25 kV, linear detector voltage at 1.906 kV,  
197 pulsed ion extraction times at 120 ns and detector window at 600 Da. The MALDI-MS  
198 spectra were acquired between 600 Da and 18 kDa by accumulating 2,000 laser shots. Each  
199 spot (three spots per sample) was acquired three times, resulting in nine spectra per sample.  
200 Data was visualized using FlexAnalysis 3.4 software.

201 **Off-gel digestion & nano-LC-ESI-MS/MS, data searching and label free quantification**  
202 **(LFQ)**

203 After MALDI acquisition, nine hemolymph samples from the same condition were  
204 combined (resulting in three and five pools of nine samples per condition at 4 and 13 d.p.i,  
205 respectively), treated for off-gel digestion bottom-up proteomics. Each pooled hemolymph  
206 sample was suspended in 0.1% RapiGest™ surfactant in 50 mM ammonium bicarbonate.  
207 Proteins were reduced with DTT at 56°C and alkylated using 55 mM 4-VP at room  
208 temperature, both reactions being performed in the dark. Digestion was proceeded overnight  
209 at 37°C with 0.5 µg of trypsin. Digested samples were acidified with 5 µL of 20% ACN/10%  
210 TFA to stop enzymatic digestion and neutralize the buffer. After a 45-min incubation at  
211 37°C, samples were centrifuged for 10 min at 15,000 g and transferred into inserts for high-  
212 performance liquid chromatography (HPLC) autosampler vials for injection.

213 Digested hemolymph samples were loaded onto a Thermo Fisher Scientific Ultimate  
214 3000 nano-HPLC equipped with an Acclaim C<sub>18</sub> PepMap100 column (3 µm, 75 µm × 250  
215 mm). The tryptic-digested peptides were separated using a biphasic linear liquid  
216 chromatography gradient of ACN in 0.1% formic acid of a 2-32% in 100 min, 32-65% in 5  
217 min followed by a wash and a column equilibration between each injection. The eluted  
218 peptides were online electrosprayed into a Thermo Fisher Scientific Q-Exactive Orbitrap  
219 mass spectrometer and analyzed in a positive and data-dependent acquisition mode, using the  
220 *m/z* range 380-2,000, as previously described in Houdelet et al., 2021<sup>49</sup>. The resulting  
221 MS/MS-derived spectra were matched against a protein database, totaling 111,814 entries  
222 from *A. mellifera*, *Nosema* species, viruses, and bacteria species related to *A. mellifera*,  
223 downloaded from the UniProtKB/TrEMBL UniProt in January 2020. The mass spectrometry  
224 proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE  
225 partner repository with the dataset identifier PXD034645 and 10.6019/PXD034645

226 **RNA extraction and cDNA synthesis**

227 Non-bled live bees from the 4 d.p.i. experiment, and randomly selected bled honeybee  
228 bodies kept after hemolymph collection at 4, 7 and 13 d.p.i., were individually crushed in  
229 sterile mortar with liquid nitrogen (9 individuals per condition; **Figure 1**). Total RNA was  
230 extracted using TRIzol reagent, following the manufacturer's protocol. DNA was removed  
231 using DNase I reaction follows 37°C for 1 h and 75°C for 10 min. First strand cDNA was  
232 synthesized using Tetro™ cDNA Synthesis Kit (Bioline), following manufacturer's protocol.  
233 cDNA synthesis reaction followed: 10 min at 25°C and 30 min at 45°C for incubation,  
234 terminated reaction at 85°C for 5 min and chilled on ice.

### 235 **Real-time quantitative PCR**

236 Total cDNA was used to quantify the levels of gene expression using SensiFAST™  
237 SYBR® No-ROX Kit (Bioline) on a qTOWER<sup>3</sup> thermal cycler. According to Sinpoo et al.<sup>48</sup>,  
238 primer sequences used in this study including AMP genes (*defensin*, *abaecin*, *apidaecin* and  
239 *hymenoptaecin*), humoral immunity-related enzyme genes (*eater gene (AmEater)*, *glucose*  
240 *dehydrogenase (GLD)*, *lysozyme (LSY)* and *phenol oxidase (PO)*), *vitellogenin (Vg)* as a  
241 female-specific protein gene, apoptosis-related enzyme genes (*nuclear factor of activated T*  
242 *cell (NFAT)*, *Relish (REL)* and *Apoptosis regulator RI-like (RI)*), and two housekeeping  
243 genes (*Rps5* and  *$\beta$ -actin*) (**Table S1**). The PCR conditions were 95°C for 2 min followed by  
244 40 cycles of 95°C for 20 sec, 60°C for 30 sec and 72°C for 1 min. A negative control (NTC)  
245 was included in each run. The relative quantification of gene expression was calculated using  
246 the  $\Delta\Delta C_t$  method<sup>54</sup>.

### 247 **Statistical analysis**

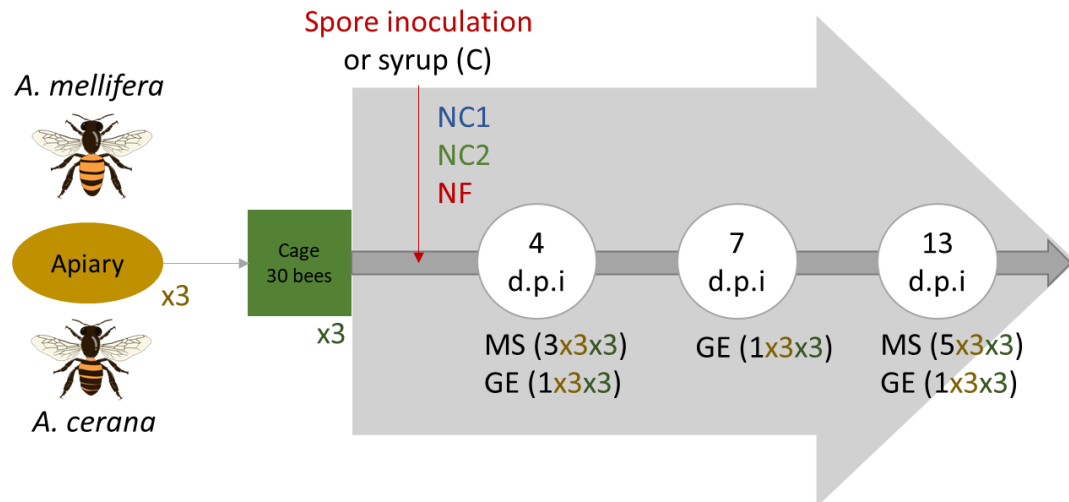
248 Differences in the survival rates and gene expression in both honeybees, *A. mellifera*  
249 and *A. ceranae*, and gene expression levels were analyzed using SPSS program version 17.0  
250 for Window (SPSS, Inc.). Statistical analysis was performed using one-way analysis of  
251 variances (ANOVAs).

252 Datasets obtained by MALDI-MS were analyzed with the ClinProTools 2.2 software  
253 (Bruker Daltonics). Spectra were submitted to a baseline subtraction and smoothing. The  
254 average spectra and the peak picking were fixed at a signal/noise of 5. The post-processing  
255 step involving spectra normalization and peak selection was performed with a 95%  
256 confidence interval. The statistical test for nonparametric data, Wilcoxon-Mann-Whitney,  
257 was applied to assess the differences between the mean intensities of the peaks selected for  
258 principal component analysis (PCA). In the case of data respecting normality (Anderson-  
259 Darling test,  $p > 0.05$ ), we retained the statistical value of the parametric T-test.

260 Datasets obtained by nano-LC-ESI-MS/MS were analysed as described by Houdelet  
261 et al., 2021. We fixed that the protein was up-regulated with a log<sub>2</sub> fold-change up to 2 and  
262 down-regulated when value is under -2. The significance of deregulation is considered with  
263 p-values less than 0.05.

264 Transcriptomic section, the normal distribution and homogeneity of variances data  
265 were assessed to conform to the assumptions to parametric tests or non-parametric tests using  
266 SPSS program version 17.0 for Window (SPSS, Inc.). For parametric tests, one-way  
267 ANOVAs were performed with Tukey-HSD test, comparison with an alpha value ( $p$ ) of 0.05.  
268 For genes that conform to the consumption of non-parametric tests, the statistical  
269 significances were analyzed using Kruskal–Wallis tests with a Bonferroni correction. Overall  
270 mean transcript levels across all treatments are presented.

271



272

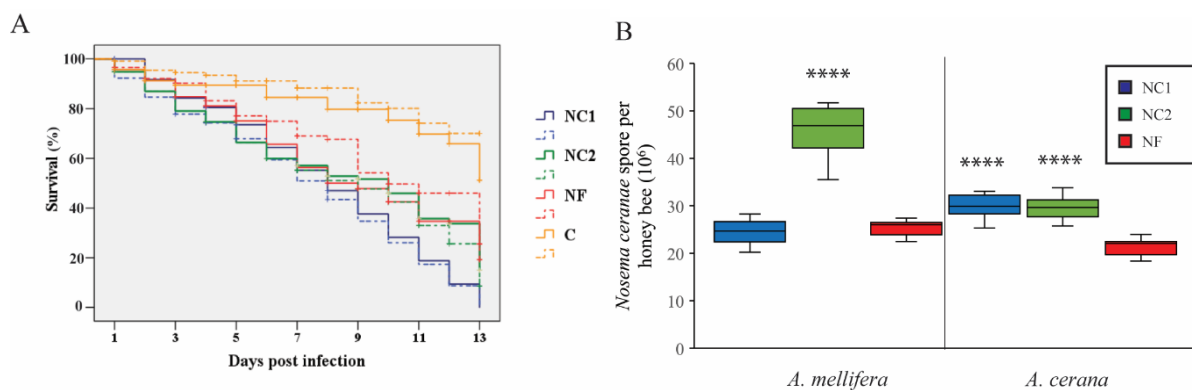
273 **Figure 1: Experimental design.** Three apiaries of *Apis mellifera* and *A. cerana* were used.  
 274 The number in brackets represented the total number of bees collected by days, the numbers  
 275 in yellow and in green corresponded to the three apiaries and the three cages per apiaries,  
 276 respectively. MS: mass spectrometry analyses, GE: gene expression analyses, d.p.i.: days  
 277 post inoculation, NC1: *N. ceranae* from *A. cerana* from Thailand, NC2: *N. ceranae* from *A.*  
 278 *mellifera* from Thailand, NF: *N. ceranae* from *A. mellifera* from France, C: control bees that  
 279 were not inoculated by spores.

280

## 281 RESULTS AND DISCUSSION

### 282 Survival rate of inoculated versus control honeybees

283 The effect of *Nosema* spp. isolates collected from different origins [*N. ceranae* from  
 284 France (NF) and from *A. cerana* from Thailand (NC1), and (*N. ceranae* isolated from *A.*  
 285 *mellifera* in Thailand (NC2)] were tested on the survival of *Apis mellifera* and *A. cerana*  
 286 hosts by individual *per os* inoculation of  $10^5$  spores/mL filtrated microsporidian suspensions  
 287 from each strain (*Nosema* spp.). Non-experimentally infected bees were used as control and  
 288 the absence of spore was confirmed by observation of crushed gut under microscope. The  
 289 survival rate was followed every days during the 13 days of experiment.



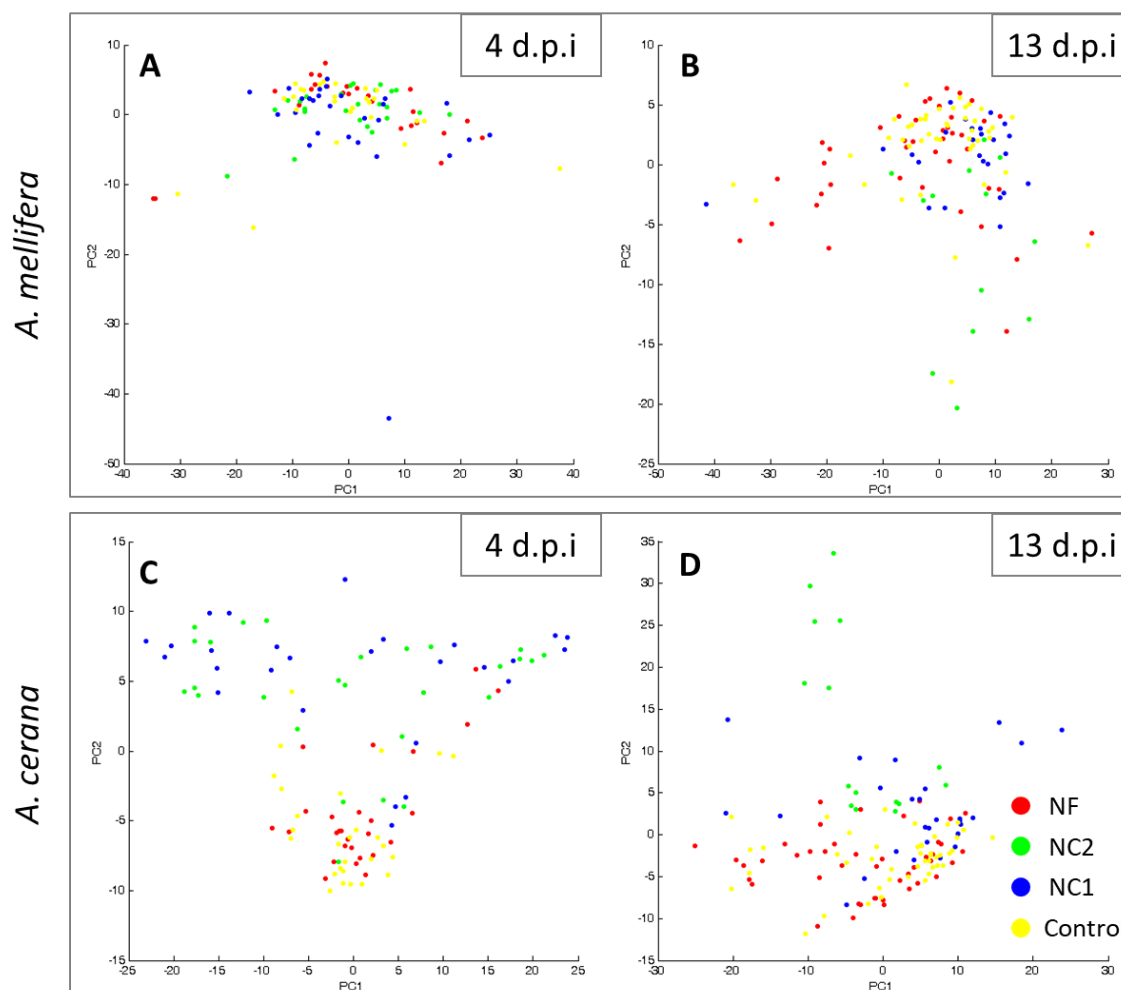
290  
 291 **Figure 2:** Survival rate and spore loads of honeybees exposed to spore populations from  
 292 different origins. (A) Survival of *Apis mellifera* (solid line) and *A. cerana* (dashed line) when  
 293 infected with *N. ceranae*. Honeybees were infected with *N. ceranae* from *A. cerana* from  
 294 Thailand (NC1, blue); *N. ceranae* from *A. mellifera* from Thailand (NC2, green); *N. ceranae*  
 295 from *A. mellifera* from France (NF, red) and non-experimentally infected bees as control (C,  
 296 yellow). (B) Boxplot of the *Nosema* spore loads per honeybee in *A. mellifera* and *A. cerana*.  
 297 NC1, *N. ceranae* from *A. cerana* from Thailand; NC2, *N. ceranae* from *A. mellifera* from  
 298 Thailand; NF, *N. ceranae* from *A. mellifera* from France.

299 The statistical analysis of honeybee global survival rate (**Figure 2A**) showed that the  
 300 different *N. ceranae* inoculums were pathogenic for both *A. mellifera* and *A. cerana* hosts  
 301 (ANOVA factor '*Nosema* species'  $\chi^2$ , 126.262, d.f. 7,  $p < 0.0001$ ). During the 13 days of the  
 302 experiment, three main events were observed. A first distinction of the survival curves was  
 303 observed at 3-4 d.p.i. between non-inoculated and inoculated bees, supporting an early impact  
 304 of *N. ceranae* isolates. A second separation was occurred at 6 d.p.i. where NF inoculated *A.*  
 305 *mellifera* host showed lower survival rate than *A. cerana*. Finally, from 8-9 d.p.i. another  
 306 separation came between the *Nosema* originated from *A. mellifera* (NC2 and NF), and those  
 307 originated from *A. cerana* (NC1) that were more virulent both for *A. mellifera* and *A. cerana*  
 308 (**Figure 2A**). In parallel to the follow-up of the survival rate, *Nosema* spore loads in the  
 309 different sets of *A. mellifera* and *A. cerana* were monitored [NC1:  $25 \times 10^6$  spores/bee, NC2:  
 310  $46 \times 10^6$  spores/bee, and NF:  $24 \times 10^6$  spores/bee for *A. mellifera* and NC1:  $30 \times 10^6$   
 311 spores/bee, NC2:  $30 \times 10^6$  spores/bee, and NF:  $21 \times 10^6$  spores/bee (**Figure 2B**)]. *Nosema*  
 312 spore loads in *A. mellifera* from *A. cerana* from Thailand (NC1) was significantly higher than  
 313 for other isolates ( $F = 104.06$ ,  $p < 0.0001$ ) (**Figure 2B**). For *A. cerana*, results revealed that

314 *N. ceranae* from *A. cerana* from Thailand (NC1) and *N. ceranae* from *A. mellifera* from  
315 Thailand (NC2) were significantly higher than the third isolate (NF) ( $F = 39.86$ ,  $p < 0.0001$ )  
316 (**Figure 2B**). This suggest that *N. ceranae* from its original host *A. cerana* caused negative  
317 impact on both *Apis* hosts compared to *N. ceranae* from *A. mellifera*. At 13 d.p.i. (end point  
318 of our experiment), we observed that all NC2 inoculated *A. mellifera* bees had died. *N.*  
319 *ceranae* infection led to higher prevalence and spore loads than *N. apis* in both *A. mellifera*  
320 and *A. cerana* due to high proliferation<sup>13,48</sup>. Our results support a previous study from Sinpoo  
321 et al. (2018)<sup>48</sup>, which compared the *A. mellifera* and *A. cerana* mortality with *Nosema* spp.  
322 cross-infection, and found that *N. ceranae* could successfully infect both *Apis* hosts and cause  
323 colony mortality.

#### 324 **Potential impact of *Nosema* spp. on *A. mellifera* and *A. cerana* evaluated by MALDI-** 325 **BeeTyping® approaches**

326 To measure the impact of different isolates of *Nosema* spores (NF, NC1 and NC2) on  
327 the Western and Asian honeybees, a total of 27 and 45 individual hemolymphs were  
328 collected at 4 and 13 d.p.i, respectively (**Figure 1**). Each hemolymph sample was analyzed by  
329 MALDI-BeeTyping® to visualize the potential impact of *Nosema* spp. inoculations on the  
330 hemolymph peptidome profile (MFPs) as a read out of the immune status of bees. To this  
331 end, the MFPs of hemolymph collected on non-treated control bees and *per os* infected bees  
332 were compared (i.e., bees kept for 4 and 13 d.p.i.). We focused on the antimicrobial peptides  
333 (AMPs) known to circulate in bee hemolymph in response to an immune challenge, namely  
334 Apidaecin 1A ( $m/z$  2107, accession Q06602 · APD73\_APIME), Abaecin ( $m/z$  3878,  
335 accession P15450 · ABAE\_APIME) and Defensin 1A ( $m/z$  5519, accession P17722 ·  
336 DEFI\_APIME). Hymenoptaecin ( $m/z$  10270, accession Q10416 · HYTA\_APIME) was  
337 hardly detectable in our MALDI-BeeTyping® conditions. All these AMPs are known to be  
338 weapons of the bee immune system to protect honeybees from invading microorganisms.



339  
 340 **Figure 3:** Principal component analyses of the repartition of the molecular mass fingerprints  
 341 generated by MALDI-BeeTyping® of hemolymph samples of *A. mellifera* (A and B) and *A.*  
 342 *cerana* (C and D) at 4 (A and C) and 13 d.p.i. (B and D). In blue, NC1: *N. ceranae* from *A.*  
 343 *cerana* from Thailand, in green NC2: *N. ceranae* from *A. mellifera* from Thailand, in red NF:  
 344 *N. ceranae* from *A. mellifera* from France, in yellow C: not inoculated control bees.

345 For *A. mellifera* (**Figure 3A, 3B**) no significant difference in the MFPs between  
 346 controls and inoculated honeybees were observed at 4 d.p.i. At 13 d.p.i., a part of the bees  
 347 inoculated by NF and NC2 isolates does not show the same MFP profiles than the ones from  
 348 control honeybees (**Table S2**). This suggests that immune related peptide/proteins other than  
 349 the referenced AMPs (Apidaecins, Abaecin, Defensins) are responsible for the observed  
 350 differences in the MFPs recorded in *A. mellifera* after *N. ceranae* inoculation. For example,  
 351 *N. ceranae* is able to induce energy metabolism proteins in *A. mellifera* midguts due to  
 352 energetic stress at high *Nosema* infection levels e.g. cytochrome C oxidase subunit 6A1, and

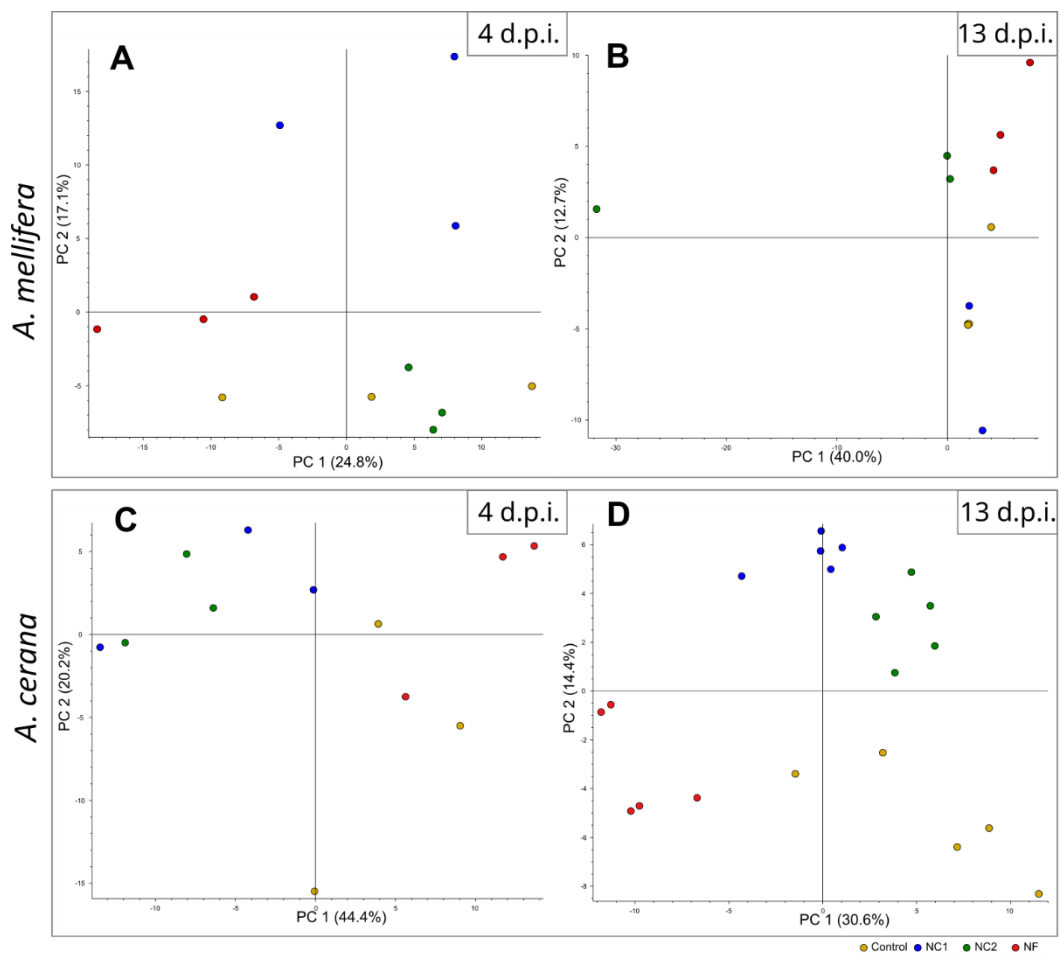


353 ATP synthase subunit beta<sup>51</sup>. For *A. cerana* (**Figure 3C, 3D**), different PCA profiles were  
354 obtained. At 4 d.p.i, the MFPs recorded from bees inoculated by NC1 or NC2 were detached  
355 from the non-infected hemolymph spectra (**Figure 3C**). The statistical analyses revealed 14,  
356 17 and 2 molecular ions with a statistical difference of intensity between controls and the  
357 NC1, NC2 and NF condition, respectively (**Table S2**). The two molecular ions at  $m/z$  2112.59  
358 (Apidaecin) and 5515.07 (Defensin), had a higher intensity on spectra of bees infected by  
359 NC1 and NC2, compared to control bees (**Table S2**). The intensity of the Apidaecin  
360 molecular ion was found also higher in bee hemolymph collected from individuals infected  
361 by NF (**Table S2**). While still observed at 13 d.p.i., the difference was less visible on PCA  
362 (**Figure 3D**). Moreover, the spectra recorded on the hemolymph of bees inoculated by NC1  
363 were separated in two opposite clusters (**Figure 3D**). Among the 12 molecular ions  
364 statistically different between NC1 infected and control bees, the molecular ion of Apidaecin  
365 was at a higher intensity in the infected individuals. The same observation was done after the  
366 NC2 inoculation. Additionally, a higher intensity was observed for the molecular ions of  
367 Defensin and Abaecin at  $m/z$  5515.89 and  $m/z$  3903.45, respectively. At 13 d.p.i., a slight  
368 increase of intensity of the Defensin molecular ion was observed after NF inoculation. The  
369 peptide profiles revealed that Apidaecin, Defensin and Abaecin, were up-regulated after *N.*  
370 *ceranae* inoculation in *A. cerana*, suggesting that turning on the immune response as  
371 “imaged” by the presence of circulating AMPs may prevent microsporidian infection more  
372 efficiently in *A. cerana* than in *A. mellifera*<sup>55</sup>.

### 373 **Bottom-up proteomics analysis of the hemolymph of honeybee in response to different** 374 ***Nosema* inoculums**

375 To further investigate the molecular impact of *Nosema* inoculation on the hemolymph  
376 proteome, the hemolymph samples were pooled according to the MALDI-BeeTyping® MFPs

377 recorded per type of experiment. These pools were subjected to an off-gel digestion in the  
 378 presence of 0.1% RapiGest™ surfactant and the digests were analysed by LC-ESI-MS/MS  
 379 bottom-up proteomics. Label-free quantitative (LFQ) proteomics were conducted on the  
 380 hemolymph pools based on the relative abundance of the proteins. Fold-change ratios were  
 381 calculated as a measurement of the protein regulation in the infectious over the control  
 382 conditions. In *A. mellifera* samples, 382 and 262 proteins at 4 d.p.i. and 13 d.p.i. were  
 383 identified, respectively, while in *A. cerana* only 185 and 159 proteins were identified at 4  
 384 d.p.i. and 13 d.p.i., respectively. Within those protein lists, we highlight differentially  
 385 abundant proteins in the context of *A. mellifera* and *A. cerana* infected by various *N. ceranae*  
 386 isolates and those of non-infected bees (**Figure 4; Table 1**).



387

388 **Figure 4:** PCA based on LC-ESI-MS/MS and clustering of hemolymph of *A. mellifera* (A  
389 and B) and *A. cerana* (C and D) at 4 (A and C) and 13 d.p.i. (B and D). Each dot is  
390 representing a pool of individuals. In blue, NC1: *N. ceranae* from *A. cerana* from Thailand,  
391 in green NC2: *N. ceranae* from *A. mellifera* from Thailand, in red NF: *N. ceranae* from *A.*  
392 *mellifera* from France, in yellow C: control bees that were not inoculated by spores.

393 For *A. mellifera*, at 4 d.p.i. and 13 d.p.i. no important difference were observed between the  
394 four condition (**Figure 4A**). Actually, the LFQ results revealed that two proteins were  
395 statistically down-regulated in the NC2 condition: Ferritin and GMC\_OxRdtase\_N domain-  
396 containing protein (**Table 1**). Ferritin, a protein that plays a pivotal role in the regulation of  
397 iron content, has been reported to be indispensable for iron metabolism and iron homeostasis  
398 through the storage and release of iron ions, especially Fe<sup>+</sup><sup>56,57</sup>. Iron sequestration is a well-  
399 recognized innate immune mechanism referred as nutritional immunity developed by the host  
400 against invading pathogens<sup>58,59</sup>. To sequester iron from pathogens, the host relies on several  
401 iron-binding proteins, among which Transferrin members and Ferritin frequently play a  
402 pivotal role<sup>58</sup>. In insects, Ferritin is found mainly in the ER and nuclear envelope and serve as  
403 an iron storage protein<sup>60</sup>. Then, Ferritin is circulating in the hemolymph and functions in iron  
404 transport<sup>61</sup>. In honeybee, Ferritin was found to be highly detected in newly-emerged  
405 honeybee hemolymph<sup>57</sup> and its gene expression considerably increased after an exposure to  
406 imidaclopride<sup>62</sup>. The modulation of iron sequestration may improve the survival of *N.*  
407 *ceranae*-infected bees as it was demonstrated for *Transferrin 1* in *Drosophila* faced to  
408 pathogen infection<sup>63</sup>. GMC\_OxRdtase\_N domain-containing protein is a part of the glucose  
409 oxidase complex that is involved in catalyzing the oxidation of glucose into hydrogen  
410 peroxide for its antibacterial activity<sup>64</sup>. Our results confirm that the GMC\_OxRdtase\_N  
411 domain-containing protein was found in the hemolymph of non-infected *A. mellifera*. This is  
412 suggesting that Ferritin and GMC\_OxRdtase\_N domain-containing protein down-regulation  
413 is the consequence of the honeybee immune system reaction to the microsporidian infection.  
414 At 13 d.p.i., differences were only observed for the NF infection with a lower abundance of  
415 the IU\_nuc\_hydro domain-containing protein and the ML domain-containing protein. ML

416 domain-containing protein is classified to the group of genes associated with pathogen  
417 recognition, immune response, and lipid metabolism<sup>65,66</sup>. This matches well with previous  
418 tick study demonstrating that ML protein, detected in gut, salivary glands and hemolymph, is  
419 involved in the tick innate immunity<sup>67</sup>. For *A. cerana*, the obtained PCAs shown a  
420 discrimination between the protein content profiles of inoculated bees and control bees  
421 (**Figure 4C, 4D**). At 4 d.p.i. of spores of *N. ceranae* isolated from Thailand (NC1 and NC2),  
422 Apidaecin type 73 was upregulated (**Table 2**). For NC2, Apidaecins and Glyco\_18 domain-  
423 containing protein were upregulated while Ferritin and two uncharacterized proteins were  
424 downregulated (Table 2 and Supplementary **Table S3**). The results are in the same trend with  
425 the hemolymph results obtained with MALDI-BeeTyping®, showing that AMPs, especially  
426 Apidaecin, were upregulated in *A. cerana* hosts after 4 d.p.i. of NC1 and NC2 inoculation.  
427 Ferritin may be involved in the defense mechanisms in honeybees, which downregulated in  
428 both *A. mellifera* and *A. cerana* host after 4 d.p.i. of *N. ceranae* from *A. mellifera* from  
429 Thailand (NC2) inoculation. Ferritin was also downregulated at 13 d.p.i. for conditions NC1  
430 and NF. Moreover, odorant binding proteins (OBPs) were suppressed in *A. cerana* immune  
431 response after NC1 and NF inoculation at 13 d.p.i., especially OBP13 (**Table 2**). OBPs, water  
432 soluble proteins, are in charge of mediation of volatile organic compounds to the odorant  
433 receptors<sup>68,69</sup> but more recently, OBPs were discovered in non-sensory organs and so  
434 associated with other physiological processes<sup>70-72</sup>. In 2022, Zhang and collaborators<sup>71</sup>  
435 demonstrated the relation of OBPs with Toll pathway innate immunity of locusts. In *A.*  
436 *mellifera* and *A. cerana*, 21 and 17 OBPs were identified, respectively that are related with  
437 olfactory system and physiological functions. The OBP13 was previously detected in  
438 different stages of both *A. mellifera* without any threats<sup>57,73,74</sup> and *A. cerana*<sup>73</sup>. Thus, it was  
439 inferred that the odor perception of insect was harm by *N. ceranae*.

440 To sum up, *A. mellifera* shows no or little response at 4 and 13 d.p.i., with the different  
 441 isolates of *Nosema ceranae*. In contrast, *A. ceranae* shows an early response with the up-  
 442 regulation of Apidaecin at 4 d.p.i., for Thai isolates of *Nosema* (NC1 and NC2), and no  
 443 response to the French isolate (NF). At 13 d.p.i., the response is more complex, with multiple  
 444 proteins involves up or down regulated, and always the presence of Ferritin.

445 **Table 1.** List of dysregulated proteins in pools of hemolymph of *A. mellifera* at four- and  
 446 thirteen-days post-inoculation. In red, downregulated proteins. NC1: *N. ceranae* from *A.*  
 447 *cerana* from Thailand, NC2: *N. ceranae* from *A. mellifera* from Thailand, NF: *N. ceranae*  
 448 from *A. mellifera* from France, C: control bees that were not inoculated by spores.

<i>Nosema</i> isolates	Day post inoculation	Description	Accession	Peptides	Coverage [%]	Ratio Exp/Ctrl	<i>p</i> -value	Biological Process
NC1	4	Not significant						
	13	Not significant						
NC2	4	Ferritin	AOA087ZXW2	6	52	0.19	0.042	cellular iron ion homeostasis
		GMC_OxRdtase_N domain-containing protein	AOA087ZVX2	1	4	0.04	0.016	Unknown
	13	Not significant						
NF	4	Not significant						
	13	IU_nuc_hydro domain-containing protein	AOA087ZSJ3	3	17	0.08	0.002	nucleobase-containing compound metabolic process
ML domain-containing protein		AOA088A045	6	47	0.12	0.000	intracellular cholesterol transport	

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451 **Table 2.** List of dysregulated proteins in pools of hemolymph of *A. cerana* at four- and  
 452 thirteen-days post-inoculation. In red, downregulated proteins and in green, upregulated  
 453 proteins. NC1: *N. ceranae* from *A. cerana* from Thailand, NC2: *N. ceranae* from *A. mellifera*  
 454 from Thailand, NF: *N. ceranae* from *A. mellifera* from France, C: control bees that were not  
 455 inoculated by spores. Uncharacterized proteins have been removed from this table, but the  
 456 complete table is available in the supplementary **Table S3**.

<i>Nosema</i> isolates	Day post inoculation	Description	Accession	Peptides	Coverage [%]	Ratio Exp/Ctrl	<i>p</i> -value	Biological Process
NC1	4	Apidaecins type 73	Q06602	3	58	6.00	0.044	innate immune response
		COesterase domain-containing protein	A0A088ABF6	2	6	20.11	0.002	metabolic process
		Defensin	B9UKF1	2	27	3.21	0.034	defense response;response to stimulus
		IRP30	G9F9Z6	6	19	28.84	0.009	
	13	Anarchy 1	Q4PKR8	1	3	0.45	0.006	
		Ferritin	A0A087ZXW2	4	45	0.10	0.010	cellular homeostasis;metabolic process;transport
		GMC_OxRdtase_N domain-containing protein	A0A087ZVX2	1	2	0.12	0.009	metabolic process
		ML domain-containing protein	A0A087ZRW9	1	7	0.12	0.010	transport
		ML domain-containing protein	A0A088A045	4	38	0.14	0.018	transport
		OBP13	Q1W641	5	36	0.21	0.023	
		OBP21	Q1W633	1	7	0.18	0.009	
		PA2c domain-containing protein	A0A088A4R3	1	7	0.29	0.023	metabolic process;transport
		SHSP domain-containing protein	A0A088APK1	1	8	0.34	0.002	
		SVWC domain-containing protein	A0A088APR8	3	19	0.17	0.000	
TIL domain-containing protein	A0A088AF72	1	15	0.07	0.005			
NC2	4	Apidaecin	B9UKF6	7	52	7.60	0.022	Unknown
		Apidaecins type 73	A0A088AIG0	3	58	6.80	0.017	Unknown
		Glyco_18 domain-containing protein	A0A087ZUC1	1	4	10.45	0.017	metabolic process
		Ferritin	A0A087ZXW2	3	20	0.08	0.022	cellular homeostasis;metabolic process;transport
	13	COesterase domain-containing protein	A0A088ABF6	6	2	32.59	0.000	metabolic process
		Defensin	B9UKF1	27	2	5.88	0.022	defense response;response to stimulus
		IRP30	G9F9Z6	19	6	29.86	0.036	
		SVWC domain-containing protein	A0A088APR8	19	3	0.43	0.027	
NF	4	Not significant						
	13	Alpha-glucosidase	Q25BT7	1	2	7.99	0.022	metabolic process
		Apidaecin	B9UN18	6	46	10.26	0.001	
		Apidaecin	B9UN24	6	46	15.36	0.001	
		Apolipoprotein III-like protein	B0LUE8	3	30	0.14	0.009	transport
		Chitin-binding type-2 domain-containing protein	A0A088AG75	1	32	0.02	0.000	metabolic process
		Ferritin	A0A087ZXW2	4	45	0.17	0.044	cellular homeostasis;metabolic process;transport
		Fructose-bisphosphate aldolase	A0A087ZPQ8	11	45	0.18	0.004	metabolic process
		ML domain-containing protein	A0A088A045	4	38	0.02	0.001	transport
		OBP13	Q1W641	5	36	0.02	0.000	
		Omega-conotoxin-like protein 1	H9KQJ7	1	31	0.05	0.020	defense response;response to stimulus
		SHSP domain-containing protein	A0A088APK1	1	8	0.37	0.009	
		SVWC domain-containing protein	A0A088APR8	3	19	0.20	0.000	
		TIL domain-containing protein	A0A088AF72	1	15	0.03	0.001	

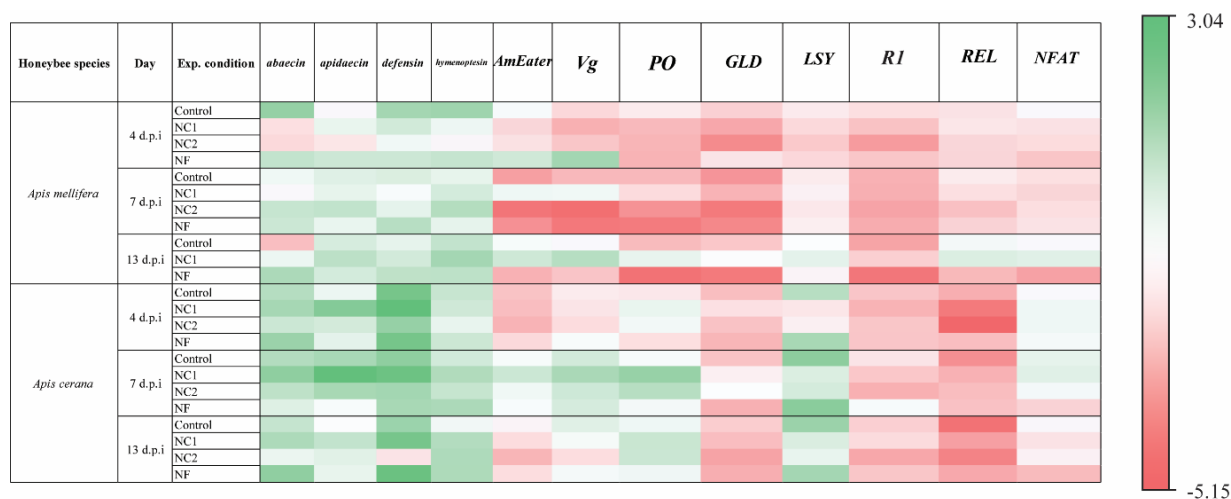
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460 **Transcriptomics analysis of the hemolymph of honeybee in response to different *Nosema***  
 461 **inoculums**

462 The body of *A. mellifera* and *A. cerana* honeybees used for proteomic analyses were sampled  
 463 in order to study the modulation of expression of immune related genes after *Nosema*  
 464 inoculation. For this part, another time point at 7 d.p.i. was added to complete the dynamic of  
 465 genes expression during infection. We focused on the genes coding AMPs (*abaecin*,  
 466 *apidaecins*, *defensin* and *hymenoptaecin*) and on the genes involved in cellular defense  
 467 reactions (**Figure 5**). The level of expression of the 12 targeted immune genes was examined  
 468 in response to the different *N. ceranae* isolates inoculated to *A. mellifera* and *A. cerana* at 4, 7



469 and 13 days post inoculation (d.p.i.).

470 **Figure 5:** Heat map of immune regulation between both *A. mellifera* and *A. cerana* during  
 471 *Nosema* inoculation at 4 days post-inoculation (4 d.p.i.), 7 d.p.i. and 13 d.p.i. In green,  
 472 upregulated gene expression and in red, downregulated gene expression. *AmEater*: *eater*  
 473 *gene*, *Vg*: *vitellogenin*, *PO*: *phenol oxidase*, *GLD*: *glucose dehydrogenase*, *LSY*: *lysozyme*,  
 474 *R1*: *apoptosis regulator R1-like*, *REL*: *relish*, *NFAT*: *nuclear factor of activated T cell*.

475 **Antimicrobial peptide genes**

476 For *Apis mellifera*, the number of *abaecin* and *hymenoptaecin* transcripts were lower  
 477 at 4 d.p.i. with NC1 and NC2 isolates than in non-experimentally challenged bees with  
 478 microsporidian spores (ANOVA,  $p < 0.005$  for *abaecin* and  $p < 0.001$  *hymenoptaecin*)  
 479 (**Figure 5, Figure S1**). The number of transcripts of the *defensin* gene was decreased after

480 NC2 inoculation at 4 and 7 d.p.i. (ANOVA,  $p < 0.001$ ; **Figure 5, Figure S1**). Chaimanee and  
481 colleagues<sup>75</sup> also observed a significant decrease of *apidaecin* and *defensin* gene expression  
482 three days after an inoculation of  $10^6$  spores/ml of *N. ceranae*. From 7 d.p.i., all genes coding  
483 for the AMPs from *Nosema*-inoculated *A. mellifera* were up regulated which is similar to  
484 previous studies on *Nosema*-infected *A. mellifera*<sup>48,55</sup>. After 13 d.p.i. of NF inoculation, the  
485 mRNA level of *abaecin* significantly increased (ANOVA,  $p < 0.005$ ; **Figure 5, Figure S1**).  
486 In *A. cerana*, the *apidaecin* gene expression was upregulated after NC1 inoculation at 4 d.p.i.  
487 (ANOVA,  $p < 0.05$ ; **Figure 5, Figure S2**). The *abaecin*, *defensin* and *hymenoptaecin* gene  
488 expressions were significantly increased after NF inoculation rather than NC2 inoculation at  
489 13 d.p.i. (ANOVA,  $p < 0.005$ ,  $p < 0.001$  and  $p < 0.001$  for the *defensin* and *hymenoptaecin*  
490 genes, respectively; **Figure 5, Figure S2**). Interestingly, the *apidaecins* responses to *N.*  
491 *ceranae* inoculated *A. cerana* was marked by the upregulation of the gene which is the same  
492 trend with our proteomic results of Apidaecins. We further discovered that *defensin* of *A.*  
493 *cerana* at 13 days post NC2 inoculation was suppressed which is similar response to that  
494 proteomic study. In *A. cerana*, AMPs were increased after *N. ceranae* from *A. cerana* (NC1)  
495 inoculation whereas *abaecin* and *defensin* gene expressions were down regulated after *N.*  
496 *ceranae* from *A. mellifera* (NC2) inoculation, suggesting that *N. ceranae* from non-original  
497 host has less of an impact on *A. cerana*. The *abaecin* and *defensin* genes are correlated with  
498 MyD88 an adaptor protein in the *Toll* signaling pathway<sup>76</sup>. This result demonstrated that *Toll*  
499 pathway regulation was inhibited during NC2 inoculation in *A. cerana*.

#### 500 Cellular response genes

501 In *A. mellifera*, the mRNA levels of the genes coding for *AmEater*, *PO* and *GLD* were  
502 significantly decreased after NF inoculation compared to NC1 inoculation at 13 d.p.i.  
503 (ANOVA,  $p < 0.01$ ,  $p < 0.001$  and  $p < 0.001$  respectively; **Figure 5, Figure S3**). This showed  
504 that *N. ceranae* from *A. mellifera* reduced cellular immune responses in *A. mellifera* host



505 while cellular immune genes were induced after 7 d.p.i. of *N. ceranae* from *A. cerana*. This  
506 pattern suggested that humoral and cellular defense mechanisms were suppressed in *A.*  
507 *mellifera* after *N. ceranae* from *A. mellifera* inoculation. Seven days after NC1 inoculation,  
508 there was a significant increase in the gene expression coding for *PO*) compared to control *A.*  
509 *cerana* (ANOVA,  $p < 0.001$ ; **Figure 5, Figure S4**). *PO* is responsible for melanization which  
510 is an important defense mechanism against fungi pathogens<sup>77</sup>. *LSY* mRNA level was  
511 significantly showed down-regulated after NC1 and NC2 compared to the control experiment  
512 in all *A. cerana* treatments (4, 7 and 13 d.p.i.) (ANOVA,  $p < 0.001$ ,  $p < 0.001$  and  $p < 0.001$   
513 respectively; **Figure 5, Figure S4**).

#### 514 ***Apoptosis related and vitellogenin genes***

515 The expression of the Apoptosis-related genes (*NFAT*, *RI* and *REL*) significantly  
516 decreased at 13 d.p.i. in *A. mellifera* after NF inoculation compared to NC1 (ANOVA,  $p <$   
517  $0.01$ ,  $p < 0.01$  and  $p < 0.001$  respectively; **Figure 5, Figure S5**). *Vg* gene expression of *A.*  
518 *mellifera* significantly increased after NF inoculation at 4 d.p.i. (ANOVA,  $p < 0.01$ ; **Figure**  
519 **5, Figure S5**). The vitellogenin protein is a metabolic regulator of stress responses and cell  
520 damage recognition<sup>78,79</sup>. At 7 and 13 d.p.i., *vitellogenin* gene expression was significantly  
521 down regulated after NF inoculation compared to NC1 inoculation (ANOVA,  $p < 0.01$  and  $p$   
522  $< 0.01$  respectively; **Figure 5, Figure S5**). A decrease in *Vg* expression was found in all *A.*  
523 *mellifera* and *A. cerana* treatments, except NC1 inoculated *A. mellifera*, in which *Vg*  
524 expression increased after 7 days. This finding is consistent with previous studies showing  
525 that *N. ceranae* inoculation lead to reduced honeybee host lifespan with *Vg* gene  
526 suppression<sup>80,81</sup>. In *A. cerana*, NF inoculation showed significant suppression of *NFAT* at 7  
527 d.p.i. compared to control (ANOVA,  $p < 0.01$ ; **Figure 5, Figure S6**). *Apoptosis regulator RI*  
528 mRNA showed significant down-regulation after NC2 inoculation in both *A. mellifera* and *A.*  
529 *cerana* at 4 d.p.i. and 7 d.p.i., respectively (ANOVA,  $p < 0.01$  and  $p < 0.001$  respectively;

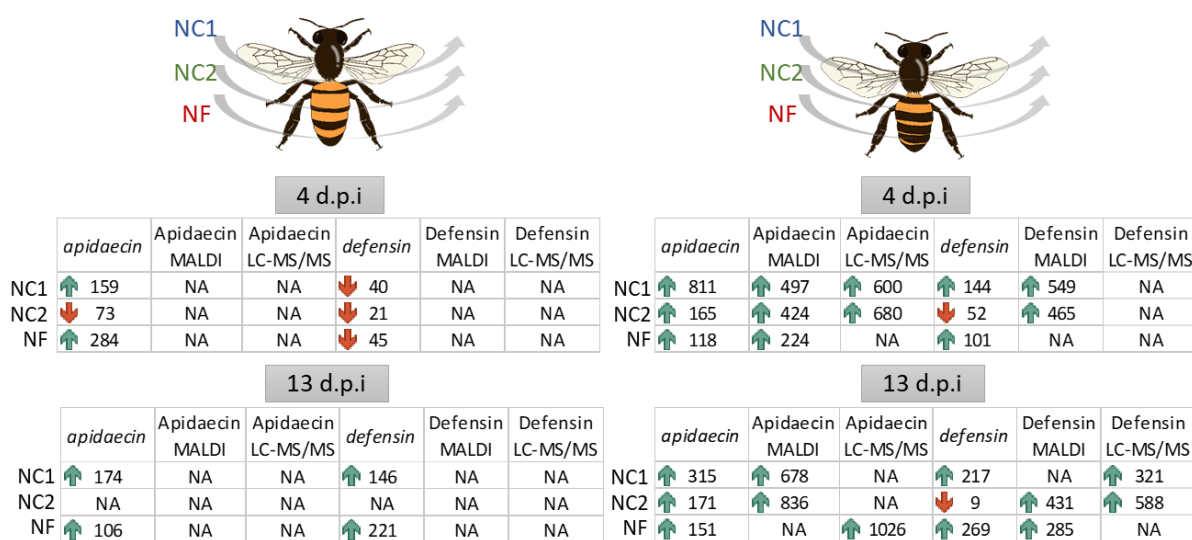
530 **Figure 5, Figure S6**). We found an increase in the *REL* transcript in *A. cerana* after *N.*  
531 *ceranae* (NC1, NC2 and NF) inoculation, which is contrast with Sinpoo et al.<sup>48</sup>. The  
532 apoptosis-related immune up-regulation supports the hypothesis that host cell defense  
533 mechanism leads the programmed cell death pathway, provide a means for infected cell to  
534 limit the spread of pathogens by protecting the integrity cells<sup>82</sup>. Moreover, *NFAT* gene  
535 expressions was reduced in all *A. cerana* treatments. The *NFAT* and *REL* belong to the REL  
536 family, which was involved in the NF- $\kappa$ B pathway, that will allow the host cell to survive  
537 under biological stress<sup>83</sup>. The NF- $\kappa$ B pathway is involved in numerous biological processes  
538 including cell apoptosis<sup>84</sup>. These results imply that this pathway was suppressed following  
539 different *N. ceranae* isolate inoculations in both *A. mellifera* and *A. cerana*.

#### 540 **Contributions of the proteo-transcriptomics to the understanding of nosemosis in *A.*** 541 ***mellifera* and *A. cerana***

542 The results of our multiomics approach that combined MALDI-BeeTyping®, LC-ESI-  
543 MS/MS and qPCR were compared focusing on the dynamics of the two major AMPs  
544 (Apidaecin and Defensin) observed in our study. Both methods are not reflecting the same  
545 phenomenon. First, MALDI-BeeTyping® reflected the presence of mature peptides  
546 circulating in honeybee's hemolymph, through a molecular mass fingerprint without absolute  
547 quantitation of them<sup>85</sup>. Second, LC-ESI-MS/MS allowed the characterization of proteins and  
548 peptides contained in the hemolymph samples and their quantification by LFQ. Third, qPCR  
549 is used to estimated mRNA of specific genes in all body individuals. For *A. mellifera*, the  
550 comparison was impossible because of no difference observed with proteomics tools.  
551 However, for *A. cerana* the results from the three approaches were in the same trend except  
552 for the *defensin* which was downregulated after inoculation of NC2 (at 4 and 13 d.p.i.; **Figure**  
553 **6**), reflecting an earlier stage which does not pretend to measure the effective quantity of

554 peptide. Nevertheless, this may highlight that NC2, non-original isolate, has a lower ability to  
555 alter *defensin* in *A. mellifera*.

556 These synthetic results showed that for *A. mellifera*, there is no or little reaction at the early  
557 stages of the infection, only *apidaecin* was up-regulated for NC1 and NF (**Figure 6**). At day  
558 13 d.p.i. both *apidaecin* and *defensin* were up-regulated, whereas the proteins were not  
559 significantly deregulated (MS analyses). For *A. ceranae*, on the contrary an early response  
560 was observed at 4 d.p.i., for both genes and proteins expression, excepted for NC2. At 13  
561 d.p.i., genes and proteins were still up-regulated, with differences among MALDI-  
562 BeeTyping® and LC-ESI-MS/MS technologies (**Figure 6**).



563

564 **Figure 6:** Comparison of the results of proteomics and transcriptomics approaches in the case  
565 of Apidaecin and Defensin. For qPCR, the value is the ratio between the mean  $2^{\Delta\Delta CT}$  of the  
566 experimental condition by the mean  $2^{\Delta\Delta CT}$  of the control. For MALDI-BeeTyping®, the  
567 result, is the ratio between the mean intensity of the experimental condition by the mean  
568 intensity of the control. For LC-ESI-MS/MS, the value is the ratio of the abundance of  
569 experimental condition by the abundance of the control measures by LFQ. A red or a green  
570 arrow reveals a decrease or an increase of the molecules, respectively.

## 571 CONCLUSIONS

572 *Nosema*-inoculated honeybees revealed different survival rates according to the *N.*  
573 *ceranae* isolate. Especially, *N. ceranae* from *A. cerana* from Thailand (NC1) induced the

574 highest mortality rate in both *A. mellifera* and *A. cerana* hosts. This study suggests that *N.*  
575 *ceranae*, an isolate from *A. cerana* from Thailand (NC1) is the most virulent isolated tested in  
576 our experimental conditions. Our study does not only confirm that AMPs are engaged in the  
577 host response to *N. ceranae* inoculation<sup>75</sup>, it also indicates that GMC\_OxRdtase\_N domain-  
578 containing, Ferritin and Odorant Binding Protein 13 are likely involved as a defense  
579 mechanism in *A. mellifera* and *A. cerana* hosts. Immune-related genes that were studied here  
580 displayed varied expression levels in both *A. mellifera* and *A. cerana* host after different *N.*  
581 *ceranae* isolates inoculation, with some immune pathways shown to be down-regulated,  
582 likely suppressed by the inoculated *Nosema*. Nevertheless, *A. cerana* showed a stronger  
583 immune response than *A. mellifera* after different *N. ceranae* isolate inoculation. This may  
584 also explain why *A. cerana* appears to be less affected by *N. ceranae* inoculation than *A.*  
585 *mellifera*. One hypothesis could be that *A. cerana*, as in contact with *N. ceranae* for long time  
586 acquired a good ability to fight against this pathogen, but for this reason is selecting *Nosema*  
587 of high virulence for him and *A. mellifera*. On the contrary, *A. mellifera*, and even more in  
588 distant locations from the origin, has less performant response, and may not select the most  
589 virulent strains, so when in contact with *A. cerana*, gives little damages to it. This can explain  
590 differential responses among the two bee species in the places where they coexist, but also  
591 gives perspectives in territorial approach of *Nosema* isolates with different variants more or  
592 less virulent. This may orient further research on the factors of virulence in *Nosema* itself  
593 what stays difficult while we have difficulties to cultivate *N. ceranae* in vitro. The differential  
594 microbiota of both bee species may play a role in this game.

## 595 **Supporting information**

596 List and sequences of the primers (**Table S1**); Relative mean intensities of molecular ions  
597 (**Table S2**); Complete list of dysregulated proteins in pools of hemolymph of *A. cerana* and  
598 *A. mellifera* at four- and thirteen-days post-inoculation (**Table S3**); Antimicrobial peptide  
599 gene expression in *Nosema* spp. infected *A. mellifera* (**Figure S1**); Antimicrobial peptide  
600 gene expression in *Nosema* spp. infected *A. cerana* (**Figure S2**); The cellular gene expression

601 in *Nosema* spp. infected *A. mellifera* (**Figure S3**); The cellular gene expression in *Nosema*  
602 spp. infected *A. cerana* (**Figure S4**); Apoptosis and vitellogenin gene expression in *Nosema*  
603 spp. infected *A. mellifera* (**Figure S5**); Apoptosis and vitellogenin gene expression in  
604 *Nosema* spp. infected *A. cerana* (**Figure S6**)

#### 605 **Accession codes**

606 The mass spectrometry proteomics data generated in this study have been deposited in the  
607 ProteomeXchange Consortium via the PRIDE<sup>86,87</sup> partner repository with the dataset  
608 identifiers PXD034645. The MALDI-MS data is available at  
609 <https://doi.org/10.6084/m9.figshare.20122220>.

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#### 616 **Authors Contribution**

617 P.C., C.H., P.B., and M.B., conceived the studies. T.C., C.H., S.V., and C.S. performed the  
618 formal analysis and the methodology. T.C., P.C., P.B., C.H., C.S., and T.D. drafted the  
619 manuscript and all authors contributed to the writing and review of the manuscript. All  
620 authors commented the manuscript at different stages and accepted the final version of the  
621 manuscript.

#### 622 **Competing interests**

623 The authors declare that they have no conflict of interests.

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