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1 **Title**

2 A combined LC-MS and NMR approach to reveal metabolic changes in the hemolymph of
3 honeybees infected by the gut parasite *Nosema ceranae*

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19

20 **Highlights**

21 * Honeybee hemolymph metabolome is altered by the gut parasite *Nosema ceranae*

22 * 15 metabolites were identified by LC-MS and NMR as candidate biomarkers of infection

23 * Putative biomarkers are involved in carbohydrate, amino acid and lipid metabolic
24 pathways

25

26 **Abstract**

27 *Nosema ceranae* is an emerging and invasive gut pathogen in *Apis mellifera* and is
28 considered as a factor contributing to the decline of honeybee populations. Here, we used a
29 combined LC-MS and NMR approach to reveal the metabolomics changes in the hemolymph
30 of honeybees infected by this obligate intracellular parasite. For metabolic profiling,
31 hemolymph samples were collected from both uninfected and *N. ceranae*-infected bees at
32 two time points, 2 days and 10 days after the experimental infection of emergent bees.
33 Hemolymph samples were individually analyzed by LC-MS, whereas each NMR spectrum was
34 obtained from a pool of three hemolymphs. Multivariate statistical PLS-DA models clearly
35 showed that the age of bees was the parameter with the strongest effect on the metabolite
36 profiles. Interestingly, a total of 15 biomarkers were accurately identified and were assigned
37 as candidate biomarkers representative of infection alone or combined effect of age and
38 infection. These biomarkers included carbohydrates (α/β glucose, α/β fructose and
39 hexosamine), amino acids (histidine and proline), dipeptides (Glu-Thr, Cys-Cys and γ -Glu-
40 Leu/Ile), metabolites involved in lipid metabolism (choline, glycerophosphocholine and O-
41 phosphorylethanolamine) and a polyamine compound (spermidine). Our study
42 demonstrated that this untargeted metabolomics-based approach may be useful for a better
43 understanding of pathophysiological mechanisms of the honeybee infection by *N. ceranae*.

44 **Keywords:** Honeybee; Metabolomic; *Nosema ceranae*; Stress Biomarkers

45

46

47 **1. Introduction**

48 Honeybees provide essential ecosystem services in agricultural and natural areas by
49 pollinating many crops and native plants. However, these insect pollinators are exposed to
50 multiple abiotic (pollutants, pesticides) and biotic (infectious agents, parasites) stressors
51 which are detrimental to their health and lifespan. Among them, both parasites and
52 pesticides seem to be the most important stressors affecting honeybees and contribute to
53 the decline of their populations (Goulson et al., 2015; vanEngelsdorp et al., 2009;
54 vanEngelsdorp and Meixner, 2010).

55

56 The gut parasite *Nosema ceranae* is among the most common pathogens in *Apis mellifera*,
57 with a worldwide distribution (Goulson et al., 2015) and is now considered to be a major
58 threat to the Western honeybee at both the individual and colony levels (Fries, 2010; Higes
59 et al., 2013; Paris et al., 2018; vanEngelsdorp and Meixner, 2010). Similar to other
60 microsporidian species, *N. ceranae* can alter the bee physiology and behavior in order to
61 maintain a more favorable environment for its reproduction. Several studies have
62 demonstrated that *N. ceranae* infection impairs tissue integrity in the midgut (Dussaubat et
63 al., 2012), alters the energy demand in honey bees (Alaux et al., 2009; Martín-Hernández et
64 al., 2011; Mayack and Naug, 2009; Naug and Gibbs, 2009) and decreases hemolymph sugar
65 level (Mayack and Naug, 2010). The infection also significantly suppresses the bee immune
66 response (Alaux et al., 2009; Antúnez et al., 2009; Aufauvre et al., 2014; Chaimanee et al.,
67 2012; Dussaubat et al., 2012) and alters pheromone production in worker and queen honey
68 bees (Alaux et al., 2011; Dussaubat et al., 2010; Holt et al., 2013). Some studies also revealed
69 that *N. ceranae*-infected honeybees have shorter lifespans than uninfected honeybees
70 (Alaux et al., 2009; Goblirsch et al., 2013; Higes et al., 2006; Vidau et al., 2011). However, the
71 presence of *N. ceranae* is not systematically associated with honeybee weakening and

72 mortality (Cox-Foster et al., 2007; Gisder et al., 2010; Invernizzi et al., 2009), suggesting
73 modulations in the parasite virulence. Possible explanations for this variation include
74 parasite or host genetics (Chaimanee et al., 2010; Dussaubat et al., 2013; Medici et al., 2012;
75 Williams et al., 2008), climate (Chen et al., 2012; Gisder et al., 2010), nutrition (Alaux et al.,
76 2010b; Fleming et al., 2015), or interactions with other stressors such as environmental
77 contaminants or other parasites. Indeed, some recent studies demonstrated that *N. ceranae*
78 can sensitize the honeybees to chemical stressors (Alaux et al., 2010a; Aufauvre et al., 2014,
79 2012; Pettis et al., 2012; Retschnig et al., 2014; Vidau et al., 2011; Wu et al., 2012).

80 In order to explain disorders and detect (early) modifications on bee physiology, we must
81 acquire molecular tools to screen from genome to metabolome (Lankadurai et al., 2013).
82 Among these tools, metabolomics is the most informative, looking at the end of the “omic
83 cascade” for the small metabolites, and strongly linked to the phenotype (Alonso et al.,
84 2015; Bundy et al., 2008; Fiehn, 2002; Liu and Locasale, 2017). Most of the time, to visualize
85 a wide range of metabolites, several analytical techniques are required. Mass spectrometry
86 (MS) is one of the most sensitive and precise technique, depending on mass spectrometer
87 accuracy, but is limited by chromatographic coupling and in-source ionization. Nuclear
88 magnetic resonance (NMR) spectroscopy provides the widest coverage of all techniques.
89 Nevertheless, sensitivity and resolution are regular drawbacks.

90 Untargeted metabolomics has the advantage to highlight both known and unknown
91 metabolites resulting from phenotypic evolution. Based on statistical filters, all signals from
92 analytical devices (MS and/or NMR) are examined for possible correlation with the observed
93 biological effect (Wishart et al., 2008). Those with high correlation will be submitted to
94 deeper analyses as data/base mining, MS-MS and/or 2-dimensional NMR experiments if
95 needed, to reveal the identity of the associated biomarker.

96 One of the most established protocols in metabolomics is the metabolic profiling of plasma
97 (Simón-Manso et al., 2013; Zhao et al., 2010) and urine (Zhang et al., 2012) on “higher
98 animals”. Biological fluids are easy to collect and store and contain a wide range of
99 metabolites that can be impacted by many physiological disturbances including medication,
100 nutrition or disease (Zhang et al., 2020). In this way, researchers aim to find host biomarkers
101 for early diagnosis of infectious diseases, collect evidence of metabolic disorders along time-
102 periods or improve nutrition benefits. Hemolymph is the sole biofluid in the insect,
103 analogous to the blood in invertebrates, and is composed of fluid plasma in which
104 hemolymph cells (hemocytes) are suspended. It circulates in the interior of the insect body
105 and remains in direct contact with the animal's tissues. Analyzing this fluid is an opportunity
106 to access to metabolic pool impacted by stressors (Aliferis et al., 2012; Wang et al., 2019).
107 Aliferis et al. (2012) previously reported by GC/MS the metabolite profiling of hemolymph in
108 bees naturally infected by *N. ceranae*. They revealed that a gut parasite can induce a general
109 disturbance of the honeybee physiology.

110 In our study, we have developed a methodology to perform metabolomics on hemolymph
111 samples, using combined LC/MS and NMR approaches. The aim was to investigate the
112 metabolic response of honeybees following experimental infection of emergent bees by *N.*
113 *ceranae*. Metabolome of the hemolymph was examined in both infected and uninfected
114 honeybees at two post-infection times.

115

116 **2. Materials and methods**

117

118 ***2.1. Biological experiments***

119

120 **2.1.1. Honeybee artificial rearing**

121 All experiments were performed with a mixture of honeybees taken from three Buckfast
122 colonies of the same apiary at the Laboratoire Microorganismes : Génome et Environnement
123 (UMR 6023, Université Clermont Auvergne, France). We confirmed that the three colonies
124 were free of *Nosema* (sampling of 30 foragers for each colony) by PCR using specific primers
125 as previously described (Higes et al., 2006). Two frames of sealed brood were placed in an
126 incubator in the dark at 33°C with 60% relative humidity. Emerging honeybees (100 per
127 colony) were collected, confined to laboratory Pain-type cages in two groups (infected vs
128 uninfected, see below for the infection procedure) of 50 individuals, and maintained in the
129 incubator. During this time, honeybees were fed *ad libitum* with candy (Apifonda®)
130 supplemented with fresh pollen (Naturapi). In order to mimic the colony environment, a
131 small piece of wax and a 5-mm piece of Beeboost® (Pherotech, Delta, BC, Canada) releasing
132 five queen mandibular pheromones, were placed in each cage. Each day, feeders were
133 replaced; dead bees were counted and removed.

134

135 **2.1.2. Experimental infection with *Nosema ceranae***

136 Spores of *N. ceranae* were obtained from bees experimentally-infected in the laboratory and
137 the infection process was conducted as previously described (Vidau et al., 2011). Briefly, the
138 intestinal tract of infected bees was dissected and homogenized in PBS and the resulting
139 suspension was filtered through Whatman No 1 filter paper, cleaned by centrifugation and
140 resuspended in PBS. At 5 d post-emergence, caged honeybees were starved for 3 h, CO₂-
141 anaesthetized and individually transferred in “infection boxes” consisting of 40 ventilated
142 compartments (3.5 cm³). Each compartment was supplied with a tip containing 125,000
143 spores of *N. ceranae* diluted in 3 µL of water. “Infection boxes” were placed in the incubator

144 and 1 h later, bees that had consumed the total spore solution were again caged (50 bees per
145 cage). Uninfected bees were similarly treated without *N. ceranae* spores in the water. At the
146 end of the experiment, we checked that the control remained uninfected.

147

148 **2.1.3. Hemolymph sampling**

149 Hemolymph samples were collected at day 2 (D2) and day 10 (D10) post-inoculation from
150 both infected and control (uninfected) honeybees using the method described by (Mayack
151 and Naug, 2010). Bees were first CO₂-anaesthetized and placed on ice, before antenna
152 cutting. Immediately, honeybees were placed into PCR tube and centrifuged at 16,000 x *g*
153 during 30 s. Hemolymph was collected in a new tube and stored at -80°C until analysis.

154

155 **2.2. Chemical analyses**

156

157 **2.2.1. Chemicals and reagents**

158 Creatinine (Fluka), phenylalanine and tryptophan (Sigma-Aldrich) were used as external
159 standards for LC/MS quality control. For NMR experiments, Deuterium oxide (D₂O) was
160 purchased from Eurisotop and 3-(trimethylsilyl) propionic-2,2,3,3- tetra-d₄ acid sodium salt
161 (TSP-d₄) was purchased from Sigma-Aldrich. Acetonitrile (Optima LC-MS), water (Optima LC-
162 MS) and ammonium acetate (Optima) were purchased from Fisher Scientific for LC/MS
163 analyses. MS external calibration was performed using lithium hydroxide monohydrate and
164 formic acid (Fluka).

165

166 **2.2.2. Standard solutions and sample preparations for LC/MS and NMR**

167 Stock solutions were prepared for each standard compound (creatinine, phenylalanine and

168 tryptophan) at a concentration of 0.5 mg/ml in water/acetonitrile 1:1 containing 0.1% formic
169 acid and were stored at -20°C. A mix of all standard solutions was extemporaneously
170 prepared, for a final concentration of 5 µg/mL. Repeated analyses of the mixed standard
171 solution ensure system stability before analysis of biological samples. Forty-eight hemolymph
172 samples (10 µL each, 12 per modality) were diluted in water (LC/MS grade) 1:4 to decrease
173 viscosity and provide a sufficient volume, considering LC sampling capabilities. Finally, 5 µL
174 was injected in LC/MS for each analysis. For ¹H-NMR analyses, 51 hemolymph samples (12
175 bees per modality, but 15 for infected ones at D10) were centrifuged at 14000 x g for 10 min
176 at 4°C. Three hemolymph samples (from the same experimental condition) were pooled (to
177 reach the minimum volume required for NMR experiment) and the final volume was
178 adjusted to 50 µl with D₂O. The Metabolic Profiler[®] platform robot (Bruker) prepared all
179 analytical samples by mixing 50 µl of pooled samples with 150 µl of phosphate buffer (1.5 M
180 phosphate in D₂O at pH 7.06). D₂O was used for shimming and locking, whereas TSP-d₄
181 constituted a reference for chemical shifts (0 ppm) for NMR. Finally, 180 µl of the solution
182 was injected, through a capillary, for the online ¹H-NMR profiling. Quality control samples
183 were also prepared using aliquots from each analytical sample. They ensure system stability
184 and permit to improve spectra processing.

185

186 ***2.2.3. Metabolic profiler[®] platform (Bruker Biospin, France)***

187 Ultraperformance liquid chromatography-mass spectrometry (UPLC-MS) used was a
188 MicroToF system (Bruker) equipped with an electrospray ionization probe (ESI) and a 1200
189 series chromatographic system (Agilent). Chromatographic separation was performed with a
190 kinetex HILIC column (100 x 2.1mm, 2.6 µm, Phenomenex) with pre-column. The mobile
191 phase was composed with acetonitrile (A) and water (B) solvents, both containing 10 mM

192 ammonium acetate. The flow rate was 0.4 mL/min and the gradient elution was carried out
193 as follows: 0-2 min at 100% A; 2-4 min linear gradient to 80% A; 4-13 min linear gradient to
194 20% A and back to 100% A into 10 sec (initial conditions); 1 min 50 sec equilibration wash
195 with 100% A, for a total run of 15 min. The injection volumes for both samples and standards
196 were 5 μ L and the column temperature was set at 25°C. Blanks of pure water and pure
197 acetonitrile were injected after every 10 hemolymph samples, for cleaning the
198 chromatographic system. The mass spectrometer was calibrated with lithium formate
199 clusters (5 mM into water) and operated in positive ion mode for full scan (50-1000 m/z)
200 detection. Nitrogen was used as the nebulizer and the drying gas. The nebulizer pressure was
201 2 bars, the desolvation gas flow rate was 8 L/min and desolvation temperature was
202 maintained at 200°C. Capillary tension was 4000 V.

203 On flow 1-dimensional ^1H -NMR experiments were done on an Avance III 500 MHz NMR
204 spectrometer, using an inflow 3-mm FISEI z-gradient (^1H - ^{13}C) probe with a 60- μ l cell.
205 A standard one dimensional noe spectroscopy sequence (noesygppr1d with water
206 presaturation and gradients) was used with low power irradiation of the water resonance
207 during the recycle delay of 4 s and the mixing time of 10 ms. 256 scans were collected with
208 an 90° impulsion time of 9.29 μ s, an acquisition time of 3.28 s, a spectral window of 10000
209 Hz and 64K data points zero-filled to 128K before Fourier transformation with 0.3 Hz line
210 broadening. All NMR spectra were recorded at 300K and processed with Topspin version 2.1.

211

212 ***2.2.4. Ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS-MS)***

213 For identification purposes, a few samples from each condition, and that were representative
214 for the features in the model, were analysed in full scan (80-1000 m/z) for positive ion mode
215 on an LTQ Orbitrap Velos MS with same chromatographic conditions (gradient, column) as for

216 UPLC-MS profiling. The m/z of each feature was searched in the resulting chromatograms to
217 obtain a better mass accuracy of the features, which was used to determine the most
218 probable molecular formulae. The parent ion was identified from in-source fragments and
219 adducts, and additional structural information on the features was obtained by performing
220 MS/MS fragmentation on relevant ions using MS-MS in product ion scan mode with collision
221 energies of 10, 20, and 30 eV. All information on the features was used to search into
222 chemicals and metabolites databases (cf. § 2.5.3).

223

224 **2.2.5. 1D and 2D NMR experiments for metabolite identification**

225 For the metabolite identification step, the sample corresponded to a pool of 25 hemolymphs
226 (mix of both infected and uninfected bees). The total volume of hemolymph (300 µl) was
227 mixed with 300 µl of phosphate buffer in D₂O. The NMR spectrometer used (TGIR - CNRS de
228 Gif/Yvette) was a Bruker Avance III 950 MHz equipped with a cryoprobe (1.7, 3 and 5 mm
229 tube) TCI (¹H/¹³C/¹⁵N/²H) with z-gradient coil probe (Bruker Biospin Wissenbourg, France).

230 For 1D ¹H-Spectra, a standard one dimensional noe spectroscopy sequence (noesygppr1d
231 with water presaturation and gradients) was used with low power irradiation (31 µW) of the
232 water resonance during the recycle delay of 10 s and the mixing time of 10 ms. 128 scans
233 were collected with a 90° impulsion time of 8.1 µs, an acquisition time of 3.3 s, a spectral
234 window of 10000 Hz and 64K data points zero-filled to 128K before Fourier transformation
235 with 0.3 Hz line broadening. For 2D homonuclear (COSY TOCSY, JRES) and heteronuclear
236 (¹H/¹³C HSQC and HMBC) experiments were performed with quadrature phase detection in
237 dimensions, using state-TPPI or QF detection mode in the indirect one. For each 512 (80 for
238 JRES) increments in the indirect dimension, 2K data points (8K for JRES) were collected and
239 16 transients were accumulated in the direct dimension. ¹³C decoupling (GARP) was

240 performed during acquisition time for heteronuclear experiments. A $\pi/2$ shifted square sine-
241 bell function was applied in the two dimensions before Fourier transformation. Spectra were
242 treated with Topspin version 3.1. All NMR spectra were recorded at 300K.

243

244 **2.3. Data management**

245

246 **2.3.1. Data extraction**

247 MS data were converted into NetCDF format and extracted to data matrix using XCMS
248 package under R environment (v. 2.15.3). Briefly, after ion extraction (m/z), a retention time
249 (RT) correction was performed before production of the matrix including for each ion
250 (m/z@RT) the relative intensity (area) detected into each analytical sample. Finally, CAMERA
251 package (Kuhl et al., 2012) proposed a first annotation according to common adducts and
252 natural isotopes. NMR data were binned using AMIX software (v. 3.9.10, Bruker) after
253 alignment and baseline correction. Extraction process was chosen as follows: fixed bucketing
254 of 0.01 ppm, from 0.13 to 10 ppm; solvent signal exclusion between 4.7 and 4.9 ppm; signals
255 integration on the sum of intensities; normalization on total intensity. The resulting matrix
256 was used for statistics.

257

258 **2.3.2. Statistical analyses**

259 Multivariate analyses were performed using SIMCA-P+ (v. 12.0.1.0, Umetrics) for non-
260 supervised (PCA) and supervised ((O)PLS-DA) methods. Two normalization methods were
261 tested, univariate (UV) and pareto (PAR), in order to obtain the most robust statistical
262 models. All statistical models were validated according to explanatory (R^2) and predictive
263 (Q^2) values as well as with random permutations. Lists of signals (ions or buckets) were

264 selected for each model according to VIP (variable importance in projection) scores (>1). For
265 all valid models, a list of ions considered to be the most implicated was selected, according
266 to VIP score. Univariate analyses were performed on Excel software (Windows) for ANOVA
267 filtering on MS data. ANOVA multiway, with interaction, was performed in order to separate
268 analytical effects from biological ones and to distinguish, when possible, age and infection
269 metabolic effects.

270

271 **2.3.3. Metabolite identification**

272 Identification of VIP ions was performed manually or using R script for data mining on
273 several public metabolic databases (KEGG, MetaCyc, HMDB). MS-MS fragmentation features
274 were compared to MassFrontier (v. 7; ThermoScientific) theoretical schemes and databases
275 (MassBank, METLIN, HMDB, PRIME). NMR VIP signals (buckets) were attributed manually
276 using previously published data (Fan, 1996; Nicholson et al., 1995; Willker et al., 1996) and
277 the human metabolome public database (HMDB).

278

279 **3. Results**

280 For metabolic profiling, hemolymph samples from both uninfected (UI) and *N. ceranae*-
281 infected (I) bees were collected at two time points considered as early (D2) and late (D10)
282 times of infection, respectively. Forty eight samples (12 for each modality) were individually
283 analyzed by LC-MS in two batches (23 samples for batch 1 and 25 samples for batch 2). For
284 ¹H-NMR experiments, hemolymph from 51 bees were pooled in 17 groups of three.

285

286 **3.1. LC-MS profiles and metabolite identification**

287 All hemolymph samples analyzed by LC-MS showed similar “total ion chromatogram”
288 profiles (**Suppl. Fig. 1**). Multivariate models were then applied to measure age (D2 vs D10)
289 and treatment (I vs UI) effects from a data matrix of 1294 ions. Among each analytical batch,
290 PLS-DA models clearly showed that the age of bees remained the parameter with the
291 strongest effect (**Figure 1**) and no statistical model was valid considering the infection effect
292 only. ANOVA filtering permitted discrimination between batch effect and combined effect of
293 age and infection, and all ions with a p-value above 0.05 were eliminated. A final list of 15
294 ions according to VIP scores, with levels significantly up- or down-regulated (at D2 and/or
295 D10), was then used for metabolite identification. Eight metabolites, corresponding to
296 potential biomarkers representative of infection alone or combined effect of age and
297 infection, were accurately identified according to spectral data and/or standard comparisons
298 (**Table 1**). These biomarkers included 3 dipeptides (Glu-Thr, Cys-Cys and γ -Glu-Leu/Ile), one
299 amino acid (histidine), one amino sugar (hexosamine), two metabolites involved in lipid
300 metabolism (glycerophosphocholine and O-Phosphorylethanolamine) and ethyl aconitate, a
301 tricarboxylic acid derivative, listed as flavoring agent. For each selected marker,
302 infected/uninfected (I/UI) ratios were mentioned at both D2 and D10 post-infection times.
303 The most interesting metabolite was hexosamine. In hemolymph of infected bees, the
304 circulating amount of hexosamine was 3x lower at D2 and 7-8x higher at D10 compared to
305 the control (**Table 1**). Similarly, in infected bees compared to control, levels of circulating
306 histidine and another “amino acid-like” putative compound were lower at D2 and higher at
307 D10. Three other metabolites (glycerophosphocholine and two dipeptides, Cys-Cys and Glu-
308 Thr) exhibited an opposite pattern. These circulating metabolites were more abundant at D2
309 and lower at D10. O-phosphorylethanolamine, a “sulfur-containing” putative metabolite and
310 peptides (γ -Glu-Leu/Ile and another putative “short peptide”) were detected at higher levels

311 in the hemolymph of infected bees at D2 and D10. Finally, ethyl aconitate, levels of
312 circulating “carbohydrate-like” and “amine-like” putative compounds were lower in infected
313 bees than in control bees, at the two time points post-infection.

314

315 **3.2. NMR profiles and metabolite identification**

316 Each NMR spectrum was obtained from a pool of hemolymphs from three honeybees.
317 Spectra analysis showed signal richness of both aliphatic and sugar zones, below 5 ppm
318 (**Suppl. Fig. 2**). Using multivariate statistical analysis tools, we observed a strong effect of
319 ageing with PCA and PLS-DA models (**Figure 2**). Similar to MS results, age had the strongest
320 effect and no valid model was obtained when considering the infection parameter alone.
321 Buckets were selected according to VIP score (above 0.99). Eight biomarkers were clearly
322 identified, according to spectral data and databases comparisons, and I/NI ratios were
323 calculated at both D2 and D10 (**Table 2**). These biomarkers included four carbohydrates
324 (fructose and glucose, both α and β forms), one amino acid (proline), a polyamine compound
325 (spermidine) and two metabolites involved in lipid metabolism (choline and
326 glycerophosphocholine). Signals for carbohydrates (fructose and glucose) shared similar
327 behaviors. These compounds were more abundant in the hemolymph of infected bees
328 particularly at the early infection time (D2). In contrast, proline was always less abundant in
329 infected hemolymph. As for the MS data, levels of circulating glycerophosphocholine were
330 higher in the hemolymph of infected bees at D2, then decreased at D10. Choline showed a
331 completely opposite pattern. Although the amount of spermidine decreased at D2 for
332 infected bees, no significant difference was observed at D10 between I and UI samples.

333

334 **4. Discussion**

335

336 **4.1. Biological sampling**

337 This proof of concept study was performed to develop transposable methodologies for
338 metabolic profiling on honeybee hemolymph from bees in hives and was linked to a study of
339 experimental infection of caged honeybees by the gut parasite *N. ceranae* conducted to
340 reveal metabolic shifts during the infectious process. We also raised several questions about
341 the quality and variability of biological samples.

342 Experimental results have suggested difficulties in assessing the health status of honeybees
343 from hives in field conditions. Both biotic and abiotic stressors may produce effects on the
344 physiology of the bees and modify the metabolic pool. Even using molecular biology (RNA
345 seq and/or qPCR) and analytical (multiresidue detection) tools, it is too time consuming and
346 costly to evaluate unstressed bees (*i.e.* healthy bees). Only visual symptoms and mortality
347 were evaluated during the experiment. In our opinion, difficulty in obtaining clear signatures
348 of *Nosema* infection in natural populations are partly due to honeybee genetic variability.
349 For example, Kurze et al. (2015) demonstrated that *Nosema*-tolerant honeybees were able
350 to escape the manipulation of apoptosis by the parasite. In addition, when a study follows
351 mortality across time, the major risk in sampling surviving bees is to artificially select
352 resistant insects with low-level infections or those less sensitive to infection (*e.g.* genetic
353 tendencies). However, even if honeybees are generally stressed by various factors, our study
354 should reveal the metabolic impact of the infection.

355 Finally, other limitations for biological sampling could be the drastic treatments for bee
356 infection, CO₂ anesthesia and hemolymph harvesting. In order to prevent any
357 artificial/technical disruptions, following a rigorous protocol is mandatory (“The

358 Metabolomics Standards Initiative (MSI) and Core Information for Metabolomics Reporting
359 (CIMR),” n.d.).

360

361 **4.2. Analytical experiments**

362 Discriminating metabolic disturbances is an important issue in metabolomics. Firstly,
363 metabolic coverage, in term of quality and quantity, is strongly linked to analytical tools.

364 Mass spectrometry did not permit easy distinction of isobaric metabolites with similar
365 fragmentation schemes. For this reason, based on our data, it was not possible to distinguish
366 Ile/Leu containing compounds or reveal which hexosamine(s) is (are) impacted. Some NMR
367 signals for ratio calculation were mixed in the same bucket and, thus, polluted. For that
368 reason, they were discarded from our list (e.g. other fructose signals; data not shown). For
369 these reasons, hyphenation using LC-MS, GC-MS and/or NMR is one of the best ways to
370 harvest information, as precisely as possible (Wishart et al., 2008).

371 Secondly, with a very large amount of metabolic signals, data treatments and statistics are
372 required to filter and reveal significant elements before any identification efforts (Monnerie
373 et al., 2019). In our study, combining LC-MS and NMR metabolomics datasets improved
374 coverage of the metabolome. Processing workflows (R packages; (Giacomoni et al., 2015)
375 are time consuming but ensure data reproducibility and comparability. The differences in the
376 metabolome profiles were deciphered using multivariate statistics (PLS-DA). Many statistical
377 tools are able to manage such datasets, but multidimensional representations permit
378 maintenance of data integrity and complexity. Such approach reveals, at the same time, the
379 variability of the complete dataset and the group of signals that are most relevant
380 considering the scientific hypothesis (Boccard and Rudaz, 2014). Analytical redundancy (e.g.

381 hexosamine in MS; Choline and spermidine in NMR) and metabolic interaction are keys for
382 the explanation of such statistical effects and reinforce the identification of biomarkers.
383 Finally, LC-MS-MS as well as 2D-NMR enhanced the identification of biomarkers. They are
384 low throughput experiments, mostly targeted on the few signals of interest, but increase the
385 quality of the identification. Data mining should be time consuming depending on what kind
386 of metabolite is highlighted. The well-known primary metabolite is quickly identified; those
387 less documented will take time to be identified with certainty. But guessing for “*de novo*”
388 identification (no information in databases) requires high resolution analytical experiments
389 to succeed.

390

391 ***4.3. Biomarkers of the infection and impacts on honeybee metabolism***

392 Because hemolymph is the sole biofluid which circulates in the interior of arthropod body,
393 we can infer that the metabolome of a hemolymph sample reflects the exchanges occurring
394 between organs (digestive, neural, reproductive, etc.) during the infectious process. As
395 expected, the age of bees was the parameter with the strongest effect on the hemolymph
396 metabolome. However, we succeeded in identifying 15 metabolites that were assigned as
397 candidate biomarkers representative of infection alone or the combined effect of age and
398 infection. The levels of these metabolites could be interpreted as either a deleterious impact
399 of infectious process or a defensive response against the pathogen.

400 The same pattern was observed for O-phosphorylethanolamine (PE), the dipeptide Glu-
401 Leu/Ile, glucose and fructose (α and β forms). These metabolites were more abundant in the
402 hemolymph of infected bees, particularly at D2 post-infection. Glycerophosphocholine (GPC;
403 one major form of choline storage) identified by both LC-MS and NMR, as well as two other
404 dipeptides (Cys-Cys and Glu-Thr), were also increased in infected samples but only at early

405 time of infection (D2). These observations could be related to impacts on proteins as
406 building blocks and glycerophospholipid used in nervous and parasympathic systems.
407 Infection is known to increase energetic demand and modify behavior in *N. ceranae*-infected
408 bees (Alaux et al., 2010a; Martín-Hernández et al., 2011; Mayack and Naug, 2009; Naug and
409 Gibbs, 2009). Some dipeptides also are known to have physiological or cell-signaling effects,
410 although most are simply short-lived intermediates on their way to specific amino acid
411 degradation pathways following further proteolysis. Some recent studies showed that lipid
412 depletion is a phenomenon strongly linked to pathogen development (Franchet et al., 2019;
413 Li et al., 2018). The level of amino acids and spermidine was lower in *Nosema*-exposed
414 samples at D2 post-infection, whereas an antagonistic pattern occurred at D10 between
415 proline and histidine. Decreased levels of amino acids, including proline, were also observed
416 by (Aliferis et al., 2012) in the hemolymph of bees naturally infected by *N. ceranae*.
417 Spermidine seems to be unmodified at D10. Proline and spermidine are involved in a key
418 pathway, the arginine and proline pathway, at the boundary of important metabolisms
419 (amino acids, glutathione, polyamine, etc.). Proline, the dipeptide Glu-Thr and GPC
420 decreased at D10. The level of proline decreased as well in infected bees at D2 while the
421 others increased. Hexosamine, the amino acid histidine and the amino alcohol choline
422 showed a different pattern. The levels of these three metabolites were lower in infected
423 bees at D2 and higher at D10 when compared to uninfected bees. Hexosamine may lead to
424 the biosynthesis of chitin, one of the essential components of insect cuticle and peritrophic
425 matrix in the gut. The peritrophic matrix lines the midgut of most insects and is a protective
426 barrier against microbial infections (Kelkenberg et al., 2015). Chitin is also a major
427 component of the cell wall of microsporidian spores (Bigliardi et al., 1996). Some studies
428 revealed a degeneration of the peritrophic membrane in bees infected by *N. ceranae*

429 (Dussaubat et al., 2012). Thus, the higher level of hexosamine detected in the hemolymph of
430 infected bees at D10 could be the result of degradation of the peritrophic matrix during the
431 infectious process.

432

433 **CONCLUSION**

434 This study is the first presenting a complete methodology to analyze bee hemolymph using
435 the up-to-date technologies for metabolomics, MS and NMR. The aim of our study was to
436 detect signatures of the pathological processes during the infection of bees by the gut
437 parasite *N. ceranae*. As a proof of concept, we identified biomarkers that could be useful for
438 a better understanding of pathophysiological mechanisms of the honeybee infection by *N.*
439 *ceranae*.

440 For further experiments, we recommend evaluation of the identified biomarkers, through
441 precise quantification, on workers from hives under different field conditions. This
442 untargeted metabolomic approach could also be used to identify biomarkers in the gut
443 during the progression of the infection. In order to reduce disturbances related to other
444 biotic or abiotic factors, we propose to use hives under insect mesh tunnels and follow
445 health status of colonies through random testing.

446

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451

452 **Figure captions**

453

454 **Figure 1. PLS-DA on LC/MS profiles.** Batch 1 with 23 analyses (1), Batch 2 with 25 analyses
455 (2). Box for Day 2, dot for Day 10, blue figure for uninfected samples and red for infected
456 ones. A strong age effect is shown on the two batches (dashed black line). (1) PC1 (25,7%)
457 and PC2 (18,3%), (2) PC1 (20,9%) and PC2 (12,4%); Hotelling T2 (ellipse) = 95%.

458

459 **Figure 2. PLS-DA on NMR data.** Box for Day 2, dot for Day 10, blue figure for uninfected
460 samples and red for infected ones. An age effect is shown on the model (dashed black line).
461 PC1 (44,8%) and PC2 (14,1%); Hotelling T2 (ellipse) = 95%.

462

463 **Table 1: Putative biomarkers identified from LC-MS and MS-MS experiments;** ID was
 464 obtained from XCMS package (p for positive ionization; first number as mass on charge
 465 measurement; T for retention time; second number as the retention time of the ion in
 466 minutes). Infected/Uninfected (I/UI) ratios, at two time points (Day 2 and Day 10), were
 467 calculated on data matrix (relative intensities). Candidate names were proposed according
 468 to data obtained by LC-MS and MS-MS analyses and compared with databases and/or
 469 standards. ANOVA filtering distinguished between biomarkers linked to infection only (I) or
 470 to both infection and age (II). Signals were identified according to a mass precision less than
 471 1 ppm, except for * 5 ppm far and ** 20 ppm far.

ID	I/UI @ D2	I/UI @ D10	Putative name	ANOVA
p133,058T6,3	0,14	1,55	Aminoacid like	I
p142,03T8,2	1,81	1,40	O-Phosphorylethanolamine	I
p179,054T6,4	0,45	0,63	Carbohydrate like	I
p203,059T5,7	0,42	0,86	Ethyl aconitate **	I
p246,249T5,8	0,84	0,36	Amine like	I
p251.044T6.3	1,17	3,40	Sulfur containing	I
p261,045T7,1	4,75	3,09	γ -Glu-Leu, γ -Glu-Ile	I
p399,134T8,2	2,84	1,67	Short (tri or tetra-) peptide	I
p145,052T5,6	0,36	8,57	Fragment of hexosamine	II
p156,078T8,7	0,52	1,18	Histidine	II
p180,093T5,6	0,38	6,64	Hexosamine	II
p181,092T5,6	0,34	7,26	13C of hexosamine	II
p225,035T6,3	2,38	0,41	Cys-Cys *	II
p249,113T6,9	1,47	0,36	Glu-Thr	II
p280,102T8,3	1,21	0,45	Glycerophosphocholine	II

472
 473

474

475 **Table 2: Putative metabolite biomarkers identified from NMR experiments;** ID was
476 obtained with AMIX software (the center of each bucket), and Infected/Uninfected (I/UI)
477 ratios, at the two time points (Day 2 and Day 10), were calculated on extracted matrix
478 (relative intensities). Candidate names were proposed according to Identification realized
479 using previously published data and web databases.

ID	I/UI @ D2	I/UI @ D10	Putative name
3.205	0,60	1,16	Choline
3.225	2,04	0,74	GPC
4.005	1,68	1,19	α -Fructose
4.015	1,75	1,17	β -Fructose
4.135	0,81	0,67	Proline
4.115	1,63	1,12	α -Fructose
3.215	1,81	0,82	GPC
3.055	0,84	1,00	Spermidine
1.775	0,85	0,98	Spermidine
1.765	0,87	1,02	Spermidine
3.045	0,84	0,99	Spermidine
5.235	1,15	1,05	α -Glucose
3.195	0,59	1,19	Choline
1.785	0,79	0,95	Spermidine
3.515	0,78	1,10	Choline
3.065	0,85	0,99	Spermidine
4.645	1,12	1,04	β -Glucose

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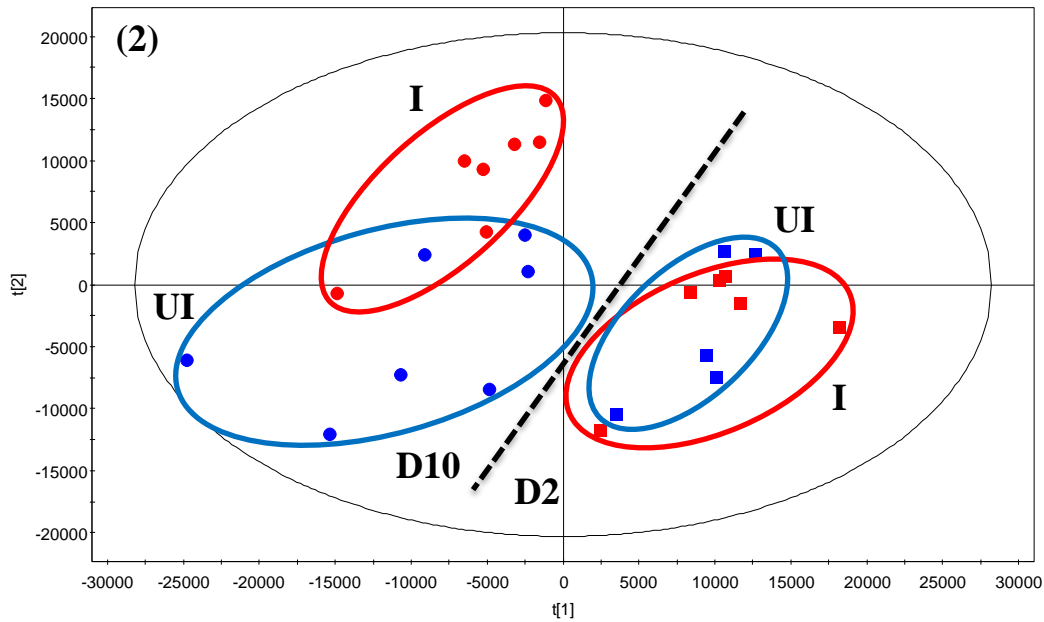
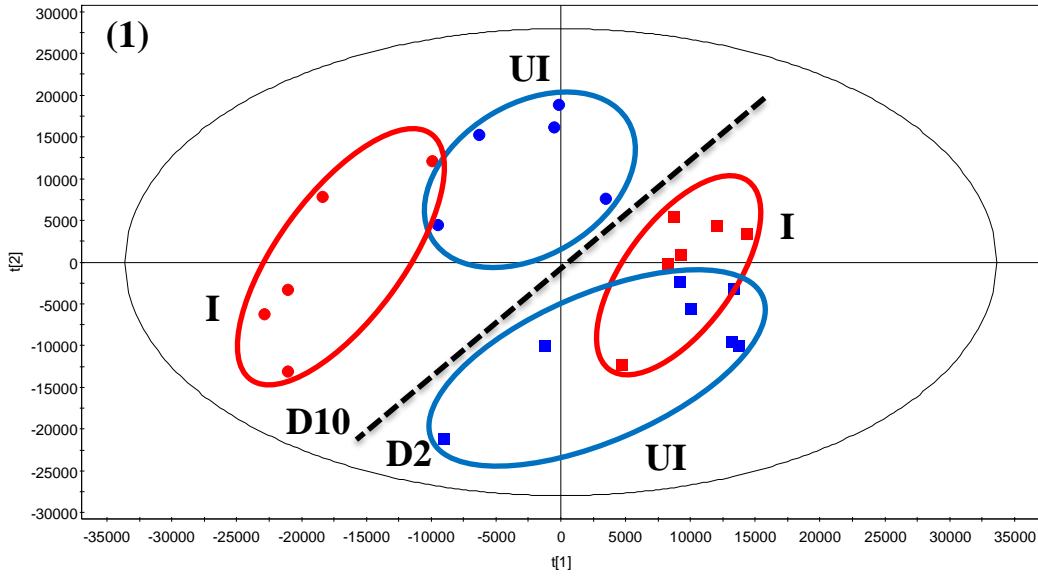
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Fig.1: PLS-DA on LC/MS profiles.



R2X[1] = 0,209422

R2X[2] = 0,124118

Ellipse: Hotelling T2 (0,95)

SIMCA-P+ 12.0.1 - 2019-11-06 16:52:52 (UTC+1)

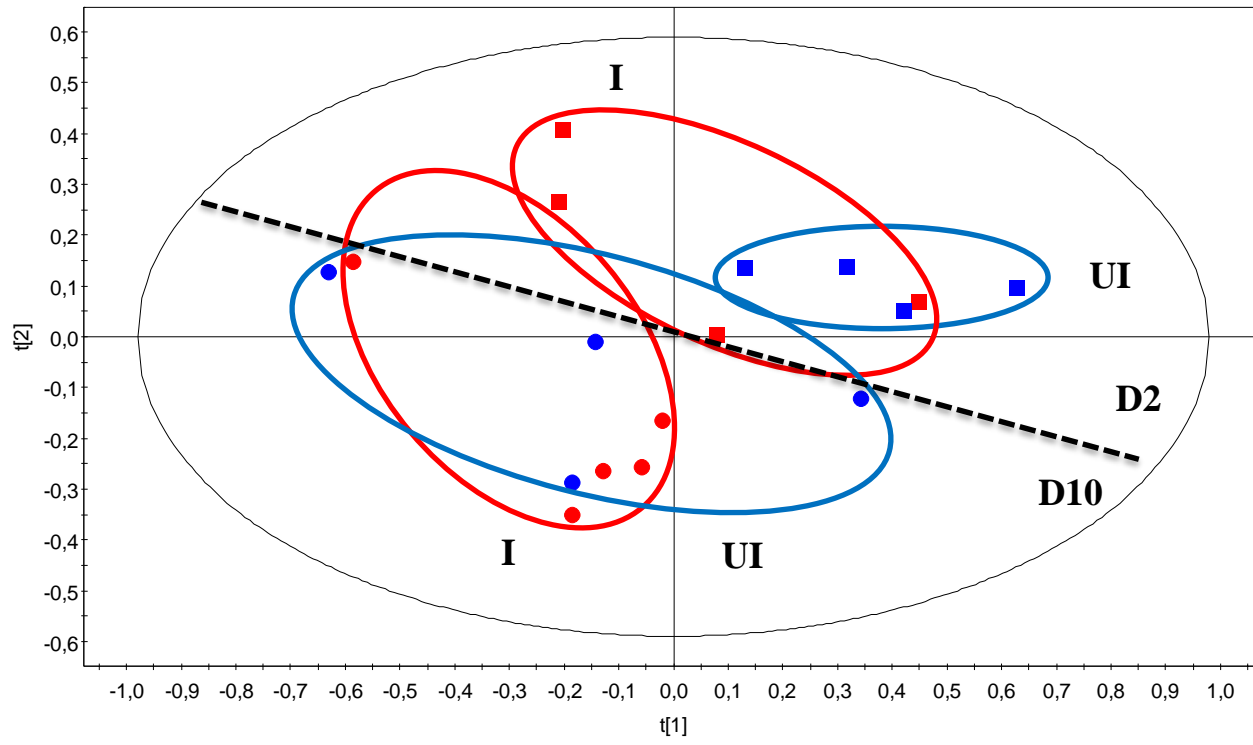


Fig.2: PLS-DA on NMR data.

