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► **To cite this version:**

Martin Quque, Claire Villette, François Criscuolo, Cédric Sueur, Fabrice Bertile, et al.. Eusociality is linked to caste-specific differences in metabolism. *Cellular and Molecular Life Sciences*, 2022, 79 (1), pp.29. 10.1007/s00018-021-04024-0 . hal-03538817

HAL Id: hal-03538817

<https://hal.science/hal-03538817v1>

Submitted on 21 Jan 2022

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1 **Eusociality is linked to caste-specific differences in metabolism,**
2 **immune system, and somatic maintenance-related processes in an ant**
3 **species.**

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23 **Abstract**

24 The social organisation of many primate, bird and rodent species and the role of individuals within
25 that organisation are associated with specific individual physiological traits. However, this
26 association is perhaps most pronounced in eusocial insects (e.g., termites, ants). In such species,
27 genetically close individuals show significant differences in behaviour, physiology, and life
28 expectancy. Studies addressing the metabolic changes according to the social role are still lacking.
29 We aimed at understanding how sociality could influence essential molecular processes in a
30 eusocial insect, the black garden ant (*Lasius niger*) where queens can live up to ten times longer
31 than workers. Using mass spectrometry-based analysis, we explored the whole metabolome of
32 queens, nest-workers and foraging workers. A former proteomics study done in the same species
33 allowed us to compare the findings of both approaches. Confirming the former results at the
34 proteome level, we showed that queens had fewer metabolites related to immunity. Contrary to
35 our predictions, we did not find any metabolite linked to reproduction in queens. Among the
36 workers, foragers had a metabolic signature reflecting a more stressful environment and a more
37 highly stimulated immune system. We also found that nest-workers had more digestion-related
38 metabolites. Hence, we showed that specific metabolic signatures match specific social roles.
39 Besides, we identified metabolites differently expressed among behavioural castes and involved
40 in nutrient sensing and longevity pathways (e.g., sirtuins, FOXO). The links between such
41 molecular pathways and ageing being found in an increasing number of taxa, our results confirm
42 and strengthen their potential universality.

43 **Keywords:** social insects, omics, division of labour, task specialisation, metabolic profile, life-history
44 trade-offs

45 **Declarations**

46
47 *Funding*

48 The study was supported by the CNRS and the French Proteomic Infrastructure (ProFi; ANR-10-
49 INSB-08-03). M. Quque PhD was funded by the University of Strasbourg and the French Ministry of
50 Education, Research and Innovation.

51
52 *Competing interests:* The authors declare no conflict of interest.

53
54 *Availability of data and material*

55 All the data analysed are available online as electronic supplementary material (ESM1, Tables S1-S7).

56
57 *Code availability:* Not applicable.

58
59 *Authors' contributions*

60 DH, FB, FC, CS and MQ designed the experimental protocol, MQ performed the behavioural
61 observations and prepared samples before use in omics; DH prepared samples for metabolomics and
62 performed the raw data processing; CV performed the LC-HRMS injections for metabolomics; MQ
63 performed the whole statistical analysis and looked for manual functional annotation of metabolites;
64 FB retrieved KEGG functional information; MQ wrote the first draft; CV and DH wrote
65 methodological parts related to metabolomics of this draft; all authors edited the first draft.

66
67 *Ethics approval:* Not applicable.

68 (Experimental protocols involving ants do not require ethical approval)

69
70 *Consent to participate:* Not applicable.

71 *Consent for publication:* Not applicable.

73 1. Introduction

74 Metabolomics is the science that analyses the metabolome, *i.e.* the set of final or intermediate
75 products of metabolism, called metabolites: *e.g.* vitamins, lipids, nucleic acids, oligosaccharides,
76 amino acids. Since the 2000s, metabolomics has considerably developed in insects, especially social
77 insects [1], and has made it possible to refine our understanding of biological processes such as
78 immunity [2], diapause and resistance to cold [3], exposure to insecticides or pollutants [4–6],
79 mutualism or parasitism interactions with bacteria and fungi [7–9], and venom composition [10]. A
80 large part of the literature using metabolomics in social insects deals with chemical communication
81 through pheromones and cuticular hydrocarbons. Cuticular hydrocarbons serve as individual
82 markers [11–16] and allow the recognition of the colony, sex, age and caste [17–19]. They also reflect
83 the ovarian activity and, therefore, the reproductive status of individuals [20–22]. Pheromones and
84 other glandular secretions have been intensively described and associated with a great variety of
85 functions. For instance, several of them exhibit antibiotic properties (Beattie *et al.* 1986; Ortius-
86 Lechner *et al.* 2000; Fernández-Marín *et al.* 2006; Tragust 2016), while others are territory or route
87 markers [27–29], and various alarm and defensive compounds have been identified [30–33]. These
88 studies have led to a better understanding of the molecular language that governs the complex
89 organisation of social insect colonies. Except some studies focusing on the microbiome and
90 combining genomics and metabolomics [reviewed in 34], there are still few comparative metabolomic
91 studies in social insects actually addressing the interrelationship between the metabolome and a key
92 element of eusocial evolution, the division of labour. The division of labour in eusocial species is
93 characterised by individuals specialising in the performance of certain tasks within the colony (*e.g.*,
94 foraging, breeding, brood care) and sometimes losing the ability to perform other tasks. Such groups
95 with distinct behaviours are usually referred to as 'castes'.
96

97 Individuals of different castes do not only differ from each other in their behaviour, but also in
98 their anatomy and/or age and/or physiology and/or molecular signature [35, 36]. In most ant species,
99 for example, the queen (or queens for polygynous species) is larger and the only one to be fertilised
100 to ensure the sexual reproduction of the colony. The queen also differs from the workers in her
101 lifespan, on average ten times longer [37]. Several studies have tried to understand the mechanisms
102 underlying these differences in development and longevity. For example, a higher concentration of
103 ecdysteroids and vitellogenin in reproductive individuals has been observed [38, 39], as well as
104 higher expression levels for genes related to life extension and immunity [*e.g.* 40–43]. However, inter-
105 individual differences are not restricted to queens versus workers, and some also exist between
106 workers. Workers perform a wide variety of tasks depending on the ecology of their species and
107 specific morphological adaptations can even be observed [44–47]. Although some behaviours are
108 species-specific, workers can be separated into two groups: on the one hand, the nest-workers that
109 perform their tasks in the shelter of the nest (*e.g.* care of the queen and larvae, nest construction); and
110 on the other hand, the foragers that bring food back from outside to the colony. It has been shown
111 that being a forager accelerates the individual ageing rate [48, 49] and, in bees, the transition from
112 foragers to nest-workers can restore immune and cognitive functions [50–52]. Besides, concentrations
113 of vitellogenin, juvenile hormone and insulin/insulin-like growth factor not only reflect the
114 reproductive status but also task specialisation of workers [53–58]. It is highly likely that these
115 differences in anatomy, behaviour, longevity, and physiology of individuals according to their
116 behavioural caste (queens, nest-workers, foragers) will be associated with differences in their
117 metabolome.
118

119 This study aimed to identify the metabolic signature of the division of labour among the castes
120 of a social insect, the black garden ant (*Lasius niger*). Our comparison was not restricted to the usual
121 opposition of queens *vs.* workers, but we also investigated the differences between worker
122 behavioural castes, which have been little studied so far. The use of the black garden ant gave us a
123 point of comparison with a previous proteomics study carried out under similar conditions in the
124

124 same species [59]. The cross-use of multi-omics data makes possible the capture of several aspects of
1 125 the same phenomenon, *e.g.* different steps of regulatory mechanisms, and thus to have a better
2 126 comprehension and a more detailed view [60–62]. Besides, metabolites represent the ultimate
3 127 downstream products of the genome. Consequently, their study (*i.e.* metabolomics) depicts the
4 128 observed phenotype very closely. Based on this previous proteomic study and the literature cited
5 129 above, we expected to find larger amounts of metabolites related to reproduction and somatic
6 130 maintenance in queens. Regarding the immune system, genomic studies generally show
7 131 overexpression in queens, however, our previous proteomic study [59] did not support this
8 132 conclusion. It was therefore interesting to evaluate whether metabolomics is more in line with
9 133 genomics or proteomics in such context. Concerning the differences between workers, based on the
10 134 previous proteomics study in *L. niger*, we predicted the foragers to have more metabolites related to
11 135 xenobiotic detoxification and nest-workers to have more metabolites related to food absorption and
12 136 digestion.
13 137

17 138 2. Methods

20 139 2.1. Ant keeping and preparation of samples before metabolomics

21 140 The black garden ant (*Lasius niger*, Linnaeus 1758) is a common species in Western Europe and
22 141 is found widely in urban areas [63]. There is no dimorphism between workers, with individuals of
23 142 about 3 mm long, living for up to 3 years. Queens can reach 7-9 mm and live on average 20 years [64].
24 143 Adult colonies are monogynous (only one queen lays eggs). For our study, wild newly mated queen
25 144 ants were collected in July 2018 and housed in Strasbourg, France (48°36'29.3"N 7°42'50.0"E). After
26 145 collection, queens were placed individually in glass tubes in the dark. Nineteen queens established a
27 146 viable colony. Colonies were kept at a temperature of 21 °C at night and 26 °C during the day, relative
28 147 humidity of 50-60%, and the photoperiod mimicked the natural photoperiod of the capture area. We
29 148 let the colonies grow freely for two months and then we removed the larvae before hatching until
30 149 collecting samples one year after. In this way, we obtained sufficiently large colonies where all ants
31 150 were about one year old, thus minimising a potential effect of age on the proteome and metabolome.
32 151 The ants were fed with a 0.3 M sugar water solution and mealworms (*Tenebrio molitor*; VenteInsecte,
33 152 Courthézon, France) once a week.
34 153

35 154 Since we were interested in the differences between foragers and nest-workers and there is no
36 155 dimorphism between them, we separated them according to their behaviour. To do this, food sources
37 156 were removed for 48 hours, then 1 M sugar water was supplied. Once the first forager discovered the
38 157 food source, we waited five minutes for optimal recruitment of the largest number of foragers. After
39 158 these five minutes, all the ants that came to the food source were collected and marked on the
40 159 abdomen with acrylic ink (Posca ©). On the other hand, nest-workers were identified by the fact they
41 160 form immobile clusters inside the nest and do not move outside. Those individuals were marked
42 161 with a different colour. Although easily recognisable, queens were also marked to ensure all ants
43 162 used in the study were handled the same way. This protocol was carried out three times to make sure
44 163 that all the foragers were recruited, but with a four-day interval to allow the colony to rest.
45 164

46 165 Because of the small amount of biological material represented by one ant, one sample analysed
47 166 by mass spectrometry consisted of a pool of several ants. The queens were pooled in pairs and the
48 167 workers (foragers and nest-workers) in groups of 50. In order to prevent a possible bias owed to
49 168 different origins (different colonies), the pools of workers contained a balanced mixture of ants
50 169 coming from the 19 colonies used in the experiment. In total, 8 samples from each behavioural caste
51 170 were formed, *i.e.* using 16 queens, 400 foragers and 400 nest-workers. Prior to mass spectrometry
52 171 analysis, the ants were snap-frozen in liquid nitrogen. The ink on the foragers' abdomens was
53 172 removed with acetone. The ants were ground under liquid nitrogen for 1 min at 30 Hz with steel
54

173 beads (Mixer Mill MM400, Retsch, Eragny Sur Oise, France). Tubes containing the resulting powder
174 were stored at -80 °C until analysis.

175 2.2. Metabolomic analysis

176 Deionised water was filtered through a Direct-Q UV (Millipore) station. Isopropanol and
177 methanol were purchased from Fisher Chemicals (Optima ® LC/MS grade). Deuterium labelled
178 [²H₆](+)-cis,trans-abscisic acid (²H₆-ABA) was purchased from OIChemIm. NaOH was obtained from
179 Agilent Technologies, acetic acid formic acid from Sigma Aldrich.

180 2.2.1. Sample preparation

181 30 mg of ground ant powder from each behavioural caste were suspended in 1 ml of cold (5°C)
182 methanol spiked with an internal standard of deuterium labelled [²H₆](+)-cis,trans-abscisic acid (²H₆-
183 ABA) at 0.1 µg/ml. After 10 seconds of vortexing, the samples were stored for 16h at -20 °C, then
184 centrifuged at 13 000 rpm for 15 min at 4°C. The supernatant was collected and dried by sublimation
185 using a SpeedVac concentrator (Savant SPD121P, Thermo Fisher). The dry samples were suspended
186 in 200 µl of methanol and analysed using liquid chromatography coupled to high-resolution mass
187 spectrometry (LC-HRMS) described in section 2.2.2.

188 2.2.2. LC-HRMS analysis

189 Samples were analysed using liquid chromatography coupled to high-resolution mass
190 spectrometry on an UltiMate 3000 system (Thermo) coupled to an Impact II (Bruker) quadrupole
191 time-of-flight (Q-TOF) spectrometer. Chromatographic separation was performed on an Acquity
192 UPLC ® BEH C18 column (2.1x100mm, 1.7 µm, Waters) equipped with an Acquity UPLC ® BEH C18
193 pre-column (2.1x5mm, 1.7 µm, Waters) using a gradient of solvents A (Water, 0.1% formic acid) and
194 B (MeOH, 0.1% formic acid). Chromatography was carried out at 35 °C with a flux of 0.3mL.min⁻¹,
195 starting with 5% B for 2 minutes, reaching 100% B at 10 minutes, holding 100% for 3 minutes and
196 coming back to the initial condition of 5% B in 2 minutes, for a total run time of 15 minutes. Samples
197 were kept at 4°C, 10 µL were injected in full loop mode with a washing step after sample injection
198 with 150 µL of wash solution (H₂O/MeOH, 90/10, v/v). The mass spectrometer was operated in
199 positive ion mode on a mass range of 20 to 1000 Da with a spectra acquisition rate of 2 Hz in
200 AutoMS/MS scan mode. The end plate offset was set to 500 V, capillary voltage to 2500 V, nebulizer
201 at 2 Bar, dry gas at 8 L.min⁻¹ and dry temperature at 200 °C. The transfer time was set at 20-70µs and
202 MS/MS collision energy to 80-120% with timing of 50-50% for both parameters. The MS/MS cycle time
203 was set to 3 seconds, absolute threshold to 816 cts and active exclusion was used with an exclusion
204 threshold at 3 spectra, release after 1 min and precursor ion was reconsidered if the ratio current
205 intensity/previous intensity was higher than 5. A calibration segment was included at the beginning
206 of the runs allowing the injection of a calibration solution from 0.05 to 0.25 min. The calibration
207 solution used was a fresh mix of 50mL isopropanol/water (50/50, v/v), 500 µL NaOH 1M, 75 µL acetic
208 acid and 25 µL formic acid. The spectrometer was calibrated in high precision calibration (HPC)
209 mode with a standard deviation below 1 ppm before the injections, and recalibration of each raw data
210 was performed after injection using the calibration segment.

211 2.2.3. Metabolite annotation and quantification

212 Raw data were processed in MetaboScape 4.0 software (Bruker): molecular features were considered
213 and grouped into buckets containing one or several adducts and isotopes from the detected ions with
214 their retention time and MS/MS information when available. The parameters used for bucketing were
215 a minimum intensity threshold of 10,000, a minimum peak length having enabled the acquisition of
216 +4 spectra, a signal-to-noise ratio (S/N) of 3 and a correlation coefficient threshold set to 0.8. The
217 [M+H]⁺, [M+Na]⁺ and [M+K]⁺ ions were authorised as possible primary ions. Replicate samples were
218 grouped together and only the buckets found in 80% of the samples in one group were extracted from
219 the raw data. The obtained list of buckets was annotated using SmartFormula to generate raw

220 formula based on the exact mass of the primary ions and the isotopic pattern. The maximum allowed
1 221 variation on the mass ($\Delta m/z$) was set to 3 ppm, and the maximum mSigma value (assessing the good
2 222 fitting of isotopic patterns) was set to 30. To put a name on the obtained formulae (*i.e.* annotate),
3 223 analyte lists containing raw formulae and names were derived from FooDB (<http://foodb.ca>),
4 224 LipidMaps (<https://www.lipidmaps.org>) and SwissLipids (<https://www.swisslipids.org/>). The
5 225 parameters used for the annotation with the analyte lists were the same as for SmartFormula
6 226 annotation.

9 227 2.3. Data filtering and statistics

11 228 Unless otherwise specified, the analysis and graphical representations were made using R
12 229 software, version 4.0 [65]. A metabolite was considered present for sure in a given group only when
13 230 it was present in at least 80% of the samples from this group, *i.e.* 6 samples out of 8. Conversely, a
14 231 metabolite was considered completely absent from a given group only when none of the samples
15 232 from this group contained the metabolite. Hence, all metabolites found in 1 to 5 out of 8 samples were
16 233 excluded from the analyses. In the resulting dataset, the missing data (values not found in 1 or 2
17 234 samples for a given metabolite) were imputed using an iterative PCA (principal component analysis)
18 235 algorithm [MissMDA package v.1.17, 66]. Missing data represented 2.6% of the data analysed. When
19 236 all three behavioural castes are considered, there is a greater probability that a metabolite is present
20 237 in less than 80% of the samples for at least one of the three castes. Therefore, when comparing
21 238 workers, we did not consider the queen samples to filter the related dataset. This resulted in a
22 239 significant increase in the number of metabolites (see results section) for the comparison between
23 240 nest-workers and foragers, and thus gave further information about the underlying metabolic
24 241 differences.
25 242

26 243 In a first step, we aimed at exploring the different sources of variation among our samples, from
27 244 both an inter- and intra-group perspective. For this purpose, we performed a PCA with the
28 245 FactoMineR package [v.2.3, 67]. The data were standardised (centred and scaled) before running the
29 246 PCA. Only the metabolites correlated to more than 50% with PCA axes and with a \cos^2 greater than
30 247 0.8 were retained. The results of this preliminary PCA are available in electronic supplementary
31 248 materials: table with metabolite identification, classification, and correlation to the axes (**ESM1**, Table
32 249 S5), as well as the PCA plot (**ESM2**, Fig. S1). Those most informative and discriminating metabolites
33 250 were then grouped according to their chemical class. We ran a second PCA with the chemical class
34 251 as an explanatory variable to highlight the metabolites typical to each behavioural caste of the black
35 252 garden ant. Here, we integrated the absent metabolites because their null values were averaged with
36 253 the other metabolites of the same class.
37 254

38 255 The PCA provided a global picture of the inter- and intra-caste variation, based on the chemical
39 256 classes of metabolites. We wanted to refine this global picture by highlighting the metabolites that
40 257 differed most strongly between the behavioural castes compared two-by-two. For this purpose, we
41 258 calculated \log_2 fold-changes (further referred to as Log2FC) for each metabolite using the DESeq2
42 259 package [v.1.28, 68]. In this analysis, we retained only the metabolites with a false discovery rate
43 260 (FDR) lower than 0.05 and a Log2FC higher than 2 (up-regulated) or lower than -2 (down-regulated).
44 261 Absent metabolites could not be used here because of the incompatibility of a null value with the log
45 262 function. We built heat maps with the ComplexHeatmap package [v.2.42, 69]. In order to better
46 263 highlight the metabolic pathways involved, we used the online tool MetaMapp [70]. This online
47 264 software builds the biochemical relationships between all identified metabolites by combining the
48 265 information available in the following databases: KEGG reaction, Tanimoto chemical, and NIST mass
49 266 spectral similarity scores. Then, it provides matrices readable by the free software Cytoscape
50 267 (<https://cytoscape.org/>) which allows the construction of network graphs associated with the
51 268 identified metabolites. We presented in the section 'results' only the networks showing at least one
52 269 metabolite present in a significantly (FDR < 0.05) and strongly (Log2FC > 2 or < -2) different quantity
53 270 in the targeted caste compared to the control one.

271 2.3. Metabolite functional and structural classification

1 272 To interpret metabolomics data, we looked for the biological processes in which the
2 273 differentially expressed metabolites were involved, and we classified them according to their
3 274 chemical class (e.g. sphingolipids, amino acids, fatty acids). We proceeded in two steps. First
4 275 automatically, by retrieving data either from Kyoto Encyclopedia of Genes and Genomes database
5 276 (KEGG; biological processes, <https://www.genome.jp/kegg>) or from the use of ChemRICH
6 277 [metabolite classes, 71], then manually when the automatic method did not work. For biological
7 278 processes, the manual completion method consisted of searching the literature for articles relating to
8 279 the metabolite concerned or metabolites of the same type (references indicated in tables). To complete
9 280 the classes of metabolites not found automatically, we used the sub-class of the 'Chemical Taxonomy'
10 281 section of the HMDB database (<https://hmdb.ca>). If the metabolite was not present in HMDB, the
11 282 subclass from the 'Classification' section of the FooDB database (<https://foodb.ca>) was used.
12 283

13 284 To consolidate this manual procedure and to avoid potential biases in the interpretation of the
14 285 biological functions, we ran an automated metabolite set enrichment analysis (aka. MSEA) through
15 286 the online platform MetaboAnalyst (v. 5.0, www.metaboanalyst.ca). [72] MSEA is a method to detect
16 287 significantly enriched biological patterns in quantitative metabolomic data. Usually, metabolites are
17 288 assessed individually for their significance under the study conditions. In contrast, MSEA directly
18 289 assesses the significance of a set of functionally related metabolites. During the quantitative
19 290 enrichment analysis, the data were normalised with the 'auto-scaling' option and the databases
20 291 (SMPDB and KEGG) were queried considering all molecules in these databases that had at least two
21 292 entries. However, the databases used by MetaboAnalyst are mainly derived from human studies.
22 293 This led to only 14 metabolites recognised by the platform in comparisons involving queens, and 200
23 294 (out of 460 annotated metabolites) for comparisons between workers. These databases therefore do
24 295 not sufficiently cover our dataset to fully depict its diversity, especially in queens where there were
25 296 too few recognised metabolites to be relevant. For workers, we kept the MSEA to be sure we did not
26 297 omit an important biological function when looking at the literature. However, since half of the
27 298 metabolites were not recognised by the platform, we did not use the results from this analysis alone.

299 3. Results

300 The mass spectrometry analysis (LC-MS/MS) revealed 1991 metabolites in the three studied
301 behavioural castes of the *Lasius niger* ant, i.e. queens, nest workers, and foragers. When considering
302 the three castes, 121 metabolites were present in at least 6/8 samples for each group, and 97
303 metabolites were completely absent from at least one group. When considering only the workers,
304 1807 metabolites were present in at least 6/8 samples for each group, whereas 52 were completely
305 absent from foragers or nest-workers (see Fig. S2 in ESM3, for a visual overview). Out of the 1991
306 detected metabolites, 486 were automatically annotated. These annotations mainly came from
307 FoodDB and LipidMAPS metabolomics databases. Original datasets are available in the electronic
308 supplementary material (ESM1, Table S1-S3). The references for the biological processes to which we
309 refer below can be found in ESM1 (Table S4-S7). For a better understanding of the results below, we
310 draw the reader's attention to two points of terminology. First, when the term 'workers' is used alone,
311 it refers to both nest workers and foragers, as opposed to queens. The second point concerns the
312 naming of metabolites. In metabolomics, the definitive identification of a given molecule is ensured
313 by comparison with a reference standard. However, we did not use a standard for the 1991
314 metabolites in this study. The comparison of the signal obtained with an online database is called
315 annotation: in this study, the presented annotations are at level 3 of Schymanski's classification [73].
316 Consequently, when a metabolite is referred to as annotated, it means named and not functionally
317 annotated, contrary to the terminology used in other fields.

318 3.1. Absent metabolites

319 Table S4 (ESM1) lists all the annotated metabolites absent from at least one group both for the
320 analysis that considered the three behavioural castes (Table S4 A) and the analysis of workers only
321 (Table S4 B). Queens, when compared to workers, lack metabolites related to oxidative damage,
322 immune system, mandibular secretion, and nutrient availability signalling. Foragers are the only
323 ones, when compared to the two other castes, to have 2S-amino-3R,4R,5S-trihydroxy- 2-
324 (hydroxymethyl)-14-oxo-eicos-6E-enoic acid (a sphingolipid) and the F-2-alpha prostaglandin. When
325 comparing workers solely, only the foragers had metabolites completely absent from nest-workers.
326 Among these forager-specific metabolites, some were related to oxidative damages (methyl 5-
327 hydroperoxy-6,8,9,11-bisepidioxy-12,14-eicosadienoate) and associated buffering pathways (1-
328 nonadecene-2,3R-dicarboxylic acid, Prostaglandin F-2-alpha), and others were terpenoids and
329 sphingolipids, involved in numerous metabolic pathways (see Table S4 B). The 4-Ethyl-7,11-
330 dimethyldodeca-trans-2-trans-6-1-o-trien-1-al might be a defensive secretion against predators.

331 3.2. Inter- and intra-caste variation assessed through a classification-based PCA

332 When considering the three behavioural castes of black garden ants, the first, second and third
333 principal components (PC1, PC2 and PC3) respectively explained 59.1%, 14.5% and 10.4% of the total
334 variation (84.07% of total variance). Workers, and especially foragers, were positively correlated with
335 PC1, while queens were negatively correlated with it (Fig. 1 A). Hydroxy fatty acids, isoquinolines,
336 oxo fatty acids, gangliosides, dioxepanes, ergosterols derivatives, glutarates, phytofurans,
337 arachidonic acid derivatives, urea derivatives, saturated fatty acids, guanidine and derivatives,
338 ketones, phosphatidylcholines, sphingolipids, gamma butyrolactones, prostaglandins were found in
339 larger amounts in workers, especially in foragers (positively correlated to PC1). On the contrary,
340 glutamine and derivatives, dicarboxylic acids, alkenes, tertiary alcohols, serine and derivatives, and
341 cyclohexenones were negatively correlated to PC1 and thus typical of queens (Fig. 1 C). The intra-
342 caste variation among queens appeared to depend mainly on metabolite classes along PC3: proline
343 and tryptophan derivatives (Fig. 1 B and D). We do not discuss PC2 because it separated the workers
344 and this difference is studied more precisely in the dedicated PCA below. However, the complete list
345 of all metabolites, including PC2, is available in Table S6 (ESM1).

346
347 When considering the metabolic differences among the workers only, PC1, PC2 and PC3
348 respectively explained 50.3%, 22.6% and 7.8% of the variation (80.7% of total variance). The list of
349 metabolites correlated with the different axes being too long to be quoted, we invite the reader to see
350 Fig. 2 C, D and E, as well as the online supplementary Table S6 (ESM1). While PC1 clearly segregated
351 the foragers (negative values) from the nest-workers (positive values), PC2 and PC3 highlighted
352 metabolic differences among workers, with F1 and NW1 being markedly distinguished from the
353 other worker samples (Fig. 2 A and B).

354 3.3. Pairwise comparison of ant behavioural castes' metabolomes

355 For each pairwise comparison (foragers vs. queens, foragers vs. nest-workers and nest-workers
356 vs. queens), Table S7 (ESM1) identifies the metabolites significantly (FDR < 0.05) and strongly down-
357 regulated ($\log_2FC < -2$) or up-regulated ($\log_2FC > 2$) and provides the related molecular formula,
358 chemical class, and biological processes.

359
360 Like the PCAs, the heat maps (Fig. 3) constructed from the 50 metabolites with the highest
361 \log_2FC confirm that the metabolic differences allow the three behavioural castes of black garden ants
362 to be clearly separated using blind hierarchical classification. There were, however, intra-group
363 variations similar to those highlighted by the PCA, e.g. the queen sample Q3 was separated from the
364 other queen samples.

365
366 Regarding the metabolic differences in workers, the metabolites less abundant in foragers
367 compared to nest-workers mainly belonged to the classes of phosphatidylcholines, carnitine and

368 derivatives, and alkenes. There were metabolites with antifungal activity (pipericine) or linked to
369 membrane and lipid transport: e.g. (3-[(4Z)-dec-4-enoyloxy]-4-(trimethylazaniumyl) butanoate,
370 2,3,4,5 - tetranor-9S,11R,15S-trihydroxy-13E-prostenoic acid). The metabolites more abundant in
371 foragers compared to nest-workers belonged to several kinds of fatty acids, naphthofurans
372 dicarboxylic acids, cyclohexanones, prostaglandins, sesquiterpenoids, arachidonic acid derivatives,
373 pentanones, and ketones. Those metabolites are known to be mostly embedded in the cell membrane
374 and involved in cell signalling, notably through inhibition of protein kinase C.
375

376 When comparing foragers to queens, the following classes of metabolites were found up-
377 regulated: glutarates, guanidine and derivatives, oleic acids, unsaturated fatty acids, ureas, and
378 phosphatidylcholines. It was worth noting the presence of isobutyridene, a compound of pesticides
379 or soil fertilizers. We also found a worker Dufour's gland secretion, the 9Z-octadecenamide. Other
380 metabolites found are known to be embedded in the cell membrane or involved in amino acid
381 metabolism (valine, leucine, isoleucine, arginine, proline). We found few metabolites in greater
382 abundance in nest-workers than in queens. They belonged to metabolite classes already highlighted
383 in the comparison of foragers *vs.* queens: glutarate derivatives, oleic acid and derivatives, and urea
384 derivatives. Among them, we found one annotated metabolite also up-regulated in foragers *vs.*
385 queens: the isobutyridene. No metabolite was significantly found in lower quantity in any worker
386 group compared to queens.
387

388 The metabolites that are significantly different between these three groups can be seen in a
389 metabolic network (**Fig. 4**). It is thus possible to visualise both the metabolites with significantly
390 different amounts between the behavioural castes, but also to see the interactions between these
391 metabolites and thus to get an idea of the metabolic cascades involved.
392

392 3.4. Metabolite set enrichment analysis (MSEA)

393 The 50 metabolites the most enriched in worker comparisons, according to the MSEA performed
394 with Metabo Analyst 5.0 can be found in **Table 1**. MSEA highlighted that the nest-worker
395 metabolome, compared to forager metabolome, was enriched in molecules involved in the
396 metabolism of many amino acids (*e.g.* glycine, arginine, alanine, histidine, leucine, glutamate),
397 vitamins, carnitines, nicotinamide, glutathione, glycerophospholipids and other fatty acids.
398

398 4. Discussion

399 Our metabolomic analysis combining principal component analysis (PCA) and log₂ fold change
400 calculation (Log₂FC) highlighted distinct metabolic profiles between the behavioural castes of the
401 black garden ant (*Lasius niger*). In addition to inter-caste differences, we also underlined intra-caste
402 variation in the metabolomes of queens, foragers, and nest-workers. The exhaustive lists of
403 metabolites are available in Electronic Supplementary Material (**ESM1**). In the discussion below, we
404 first stress metabolites involved in functions or life-history traits underlining the physiological and
405 behavioural specificities of each group. Then, we address the more general question of hormone and
406 pheromone synthesis. Finally, we discuss the intra-caste variation and the metabolites related to
407 reproduction in queens in the context of the low proportion of metabolites that were annotated and
408 linked to biological processes. All along with the discussion, we compare the present metabolomics
409 results with data from a previous proteomics study in *L. niger*, sharing the same experimental design
410 [59].
411

411 4.1. Queens are protected from stress, pathogens and pollutants

412 Log₂FC showed that isobutyridene, a soil fertiliser and insecticide compound [74, 75], was
413 present in larger amounts in workers than in queens. This reflects the capacity of workers to serve as
414 a buffer against detrimental incomes from outside the anthill. The social network structure of an ant
415 colony probably prevents the queen from being in contact with high doses of noxious xenobiotics.
416

416 Such a benefit to the queen has been described under the name of *social immunity* in the context of
1 417 pathogenic infections [76–80]. If queens are less exposed to outside threats, we could therefore
2 418 understand why metabolites related to the immune system are less present and that we found some
3 419 missing metabolites in queens potentially related to immunity. Indeed, the classification-based PCA
4 420 revealed that arachidonic acid derivatives were found in larger amounts in workers than in queens.
5 421 For example, N-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoyl amine was completely absent from queens
6 422 but present in both foragers and nest-workers. Arachidonic acid and derivatives are major
7 423 components of the insect cuticle and are thought to be involved in the formation of nodules during
8 424 melanisation [81, 82], the main insect immune response to bacterial infections [83, 84]. Moreover, 3-
9 425 Ethyl-5-methyl-1,2-cyclopentanedione was also lacking in queens. This ketone is produced by
10 426 bacteria and has antifungal and antibacterial activity [85]. The presence of communities of bacteria or
11 427 fungi on ant cuticle has already been described [see 86 for a review of ant-microbiome interactions]
12 428 and contributes to their immune defences [87, 88], as well as the caste recognition, reflecting the
13 429 division of labour [34, 89, 90]. Here metabolomics brings supporting pieces of evidence to our
14 430 previous proteomic study [59], where we found the queens to be characterised by lower amounts of
15 431 proteins linked to the immune system (*e.g.* T-cell immunomodulatory protein, ferritin) when
16 432 compared to workers. We hypothesise that the immune protection granted by social immunity to
17 433 social insect queens allows them to reduce the energetic investment in their own immune system,
18 434 likely for the benefit of reproduction and somatic maintenance. At the species level, phylogenetic
19 435 studies support the idea that the social structure may incur a reduced immune system with the
20 436 evolution of eusociality: a larger colony size is associated with a weaker melanisation response [91],
21 437 social insects have fewer genes involved in immune functions than less social insects [57], and a
22 438 decrease in immune gene activity and diversity during the evolution of termites has also been
23 439 evidenced [92]. On the other hand, at the individual level, the results in social insects are equivocal,
24 440 finding up-regulation of gene expressions involved in immunity in workers [93, 94] or queens [40,
25 441 95]. As our results are obtained through proteomics and metabolomics, the divergent conclusions
26 442 might exemplify how gene expression can be modulated downstream. This hypothesis would need
27 443 an experimental design with concomitant analyses of the transcriptome, proteome and metabolome
28 444 to be properly tested. Genes, proteins and metabolites may be involved in several processes. For
29 445 example, as stated by Graeff and collaborators [40], the up-regulation of a histone 2A homologue in
30 446 *L. niger* queens may be related to immunity but also a high rate of cell division. Vitellogenin is
31 447 strongly associated with caste differentiation in social insects [*e.g.* 53, 54, 58] and its up-regulation in
32 448 queens found in some studies [*e.g.* 40, 95] may also be due to their reproductive status. Similarly in
33 449 our study, arachidonic acid derivatives are involved in many processes (*e.g.* cell membrane
34 450 components, cellular signalling, pheromone synthesis) [96] and their up-regulation in workers (more
35 451 especially the foragers) might not be linked only to the immune system. In future studies, directly
36 452 challenging the immune system of queens and workers with controlled pathogens would provide
37 453 more definite answers.

454
455 Out of six metabolites completely absent from queens, two of them are known to be related to
46 456 oxidative damages. Xi-salsolinol has been shown to be a neurotoxin [97, 98] and to cause oxidative
47 457 damage to cytochrome C [99]. (E)-12-(5-ethyl-4-hydroxytetrahydrofuran-2-yl)-9,12-dihydroxydodec
48 458 -10-enoic acid belongs to the class of phytofurans that are known biomarkers of peroxidation of
49 459 polyunsaturated fatty acids (PUFA) in plants [100–102]. As we have seen above, the social structure
50 460 of the colony means that the queens are less exposed to pollutants and pathogens which are two
51 461 sources of oxidative stress [103–107]. This could explain why the queens show fewer metabolites
52 462 associated with oxidative damage. Moreover, less oxidative stress in queens would require a less
53 463 active antioxidant system. This assumption agrees with the lower enzymatic antioxidant activity
54 464 previously found in *L. niger* [108].

55 465 Finally, N-(5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl)-ethanolamine, a.k.a. EPEA, is another metabolite
56 466 we did not detect in queens. EPEA has been found with an omega 3 fatty acid (DHA) to induce
57 467 apoptosis and autophagy through PPAR γ activation in cancer cells [109, 110]. According to the longer

468 lifespan of queens and the positive association between autophagy and longevity described in diverse
469 taxa [111–114], we would not expect this metabolite to be absent from queens. Yet, EPEA has also
470 been shown to decrease thermal resistance and lifespan in *C. elegans* [115], where it probably acts as
471 a sensor of nutrient availability and energy state [116]. Together these studies appear to point out
472 nutrient-sensitive but autophagy-independent anti-ageing mechanisms in ant queens.

4.2. Among workers, nest-workers up-regulate pathways linked to nutrition and longevity

474 Compared to foragers, protein biosynthesis appeared to be more active in nest-workers, since
475 the classification-based PCA found several amino acids and derivatives to be up-regulated, including
476 serine, alanine, glutamine, glutamate, tryptophan, lysine, arginine, and proline. Besides, the average
477 enrichment ratio in nest-workers regarding these amino acids was 10.6. Only methionine and its
478 derivatives were found in larger amounts in foragers. Serine, glutamate, tryptophan, tyrosine, alanine
479 and derivatives appeared to share common metabolic maps, all related to digestion and absorption
480 of nutrients. In fact, they belong to one or more of the following KEGG maps: protein digestion and
481 absorption (map04974), bile secretion:digestive system (map04976), vitamin digestion and absorption
482 (map04977), mineral absorption (map04978). Similarly, Log2FC showed larger amounts of piperidine
483 and carnitine in nest-workers, both metabolites notably involved in nutrient absorption (map04974
484 and map04976). Similarly, carnitine and vitamin (*e.g.* A1, B1, B2) metabolism were also highlighted
485 in the MSEA showing that such pathways were enriched in nest-workers (**Table 1**). These findings
486 echoed those from our previous proteomic study [59], in which nest-workers overexpressed proteins
487 linked to digestion (*e.g.* alpha amylase) when compared to foragers. As far as we know, the
488 importance of digestion in ant nest-workers had not yet been reported and should be further
489 investigated to specify the meaning and depth of greater amounts of digestion-related metabolites
490 and proteins. We may assume that the storage of food excess by nest-workers [117] followed by pre-
491 digestion would make it quickly available for further use, thus enhancing the fitness of the whole
492 colony by buffering environmental unpredictability in food resources, but also by increasing the
493 efficiency in food processing by conspecifics. Nest-workers may also pre-digest food for castes that
494 do not perform this task very well. According to several studies, ant larvae do not require help to
495 digest food [118–120]. The pre-digested food could be more valuable to queens, allowing them to
496 invest less in digestive metabolism and save energy for other costly life-history traits such as
497 reproduction and a long lifespan.

499 Among workers, nest-workers also exhibited up-regulated glutamic acid (aka glutamate) and
500 nicotinamide when compared to foragers, and related pathways were enriched in nest-workers
501 (respectively around 10.7 and 12.6 of enrichment ratio, **Table 1**). Glutamic acid is involved in
502 numerous pathways, notably the metabolic pathways of other amino acids (see KEGG maps in **ESM1**,
503 *e.g.* Table S7), but it is worth noting also its implication in nutrient sensing and metabolic activity
504 through the activation of the FOXO transcription factor pathway (map04068). This activation is
505 mediated by N-methyl-D-aspartic acid (NMDA) receptors of glutamate [121]. When activated, FOXO
506 transcription factors inhibit tumour development and increase lifespan through antioxidant activity
507 and DNA repair mechanisms [122–124]. FOXO transcription factors are linked to the mechanistic
508 target of the rapamycin pathway (mTOR), inhibited by insulin or insulin-like growth factor, but
509 activated under dietary restriction [122, 123]. Regarding nicotinamide, in addition to being involved
510 in the absorption of vitamins (map04977), it is also part of the metabolic map 04212: longevity
511 regulating pathway - worm. Accordingly, the oxidised form of nicotinamide dinucleotide (NAD⁺)
512 activates histone deacetylases called sirtuins [125]. Sirtuins have been shown to have a positive effect
513 on longevity, in particular by activating autophagy and slowing down mitochondrial activity [113,
514 126–128]. Here, the comparison between the most and the least senescent workers, respectively
515 foragers and nest-workers, reinforces the link between metabolic activity, somatic maintenance and
516 longevity. This link was already found in the comparison between queens and workers in our study
517 (section 4.1), as well as in different contexts across several taxa in other studies [*e.g.* 113, 129–131].

518 4.3. Foragers appear stressed, exposed to pathogens, and with larger amounts of saturated fatty acids

519 We found 1,8-octanedioic to be in larger amounts in foragers than in nest-workers. This
520 metabolite has been detected in the honey bee royal jelly and associated with metabolism activation
521 [132, 133]. A higher metabolic rate is associated with more oxidative stress, notably through the
522 enhanced production of reactive oxygen species by the mitochondria [134, but see 135]. Supporting
523 the possibility that metabolic rates and oxidative stress are higher in foragers than nest-workers, up-
524 regulated potential markers of oxidative stress were found in foragers. Prostaglandin F-2-alpha was
525 detected only in foragers and another prostaglandin (5-Hexyltetrahydro-2-furanoctanoic acid) was
526 up-regulated in foragers compared to nest-workers. In marine invertebrates, prostaglandins have
527 been shown to raise after exposure to stressful environmental conditions [136 and references therein].
528 More prostaglandins in foragers might thus be expressed concomitantly or as a response to
529 accumulating oxidative damages caused by higher metabolic activity or higher stress levels.
530 Moreover, in support of higher oxidative stress levels in foragers, foragers were also the only one
531 where we detected methyl5-hydroperoxy-6,8,9,11-bisepidioxy-12,14-eicosadienoate, a hydroperoxy
532 fatty acid resulting from lipid peroxidation and involved in oxidative stress-related cell death in
533 plants [137]. Besides, the glutathione pathway, a well-known antioxidant, was found enriched in
534 nest-workers (see **Table 1**).

535
536 When compared to nest-workers, foragers had a greater amount of two arachidonic acid
537 derivatives (N - 5Z,8Z,11Z,15Z - eicosatetraenoyl - alanine and N - hydroxy - 5Z,8Z,11Z,14Z -
538 eicosatetraenoyl amine). As previously stated, arachidonic acid derivatives are precursors in the
539 melanisation mechanism in response to bacterial infection. Moreover, 2S - amino - 3R,4R,5S -
540 trihydroxy - 2 - (hydroxymethyl) - 14 - oxo - eicos - 6E - enoic acid, a.k.a sphingofungin E, was also
541 only detected in foragers. Like other sphingofungins, sphingofungin E has antifungal activity [138].
542 Finding more potential immune metabolites in foragers agrees with the fact that they are the most
543 exposed to pathogens since they go outside to bring food back to the colony. Such an antifungal
544 activity would ensure that foragers do not bring pathogens back into the anthill, thus contributing to
545 the social immunity mentioned above.

546
547 The classification-based PCA has shown that the class of saturated fatty acids was
548 underrepresented in queens, and Log2FC allowed to point out that almost all the saturated fatty acids
549 were found in greater amounts in foragers, whereas nest-workers had larger amounts of unsaturated
550 fatty acids and unsaturated lysophosphatidylcholines. According to our data, it seems that saturated
551 fatty acids characterise the behavioural caste with a faster ageing rate (workers and especially
552 foragers) and unsaturated lipids the behavioural caste with a slower ageing rate (nest-workers *vs.*
553 foragers). This opposes the usual positive association between lipid saturation level of cell
554 membranes and longevity, found in several birds and mammals including humans [139], but also the
555 honey bee [140]. This positive association is usually explained by the fact that saturated lipids are less
556 prone to oxidative damage compared to unsaturated lipids [140–143]. Such a contradiction might be
557 linked to the lack of correlation between ageing and protein, lipid or DNA damages in bee workers
558 [reviewed in 144]. A lipid-specific analysis assessing the precise proportion of i) each fatty acid would
559 allow for more detailed conclusions, and ii) each kind of lipid would help to conclude about the exact
560 composition of the cell membrane in ant queens, foragers, and nest-workers.

561 4.4. Communication, pheromones and other hormones

562 In workers compared to queens, 9Z-octadecenamide was up-regulated. This compound is found
563 in wasps, bees and ants to be a glandular secretion [145–148]. Because of its secretion in ant Dufour's
564 gland, it might be an alarm or trail pheromone [145]. Because of their social role, foragers and nest-
565 workers use more often alarm or trail pheromones. Finding a higher quantity of such a pheromone
566 underlines the link between behaviour and metabolome.

568 Foragers were the only group in which we detected 2-alpha-Ethoxydihydrophytuberin and,
1 569 compared to nest-workers, they had larger amounts of glucosyl (2E,6E,10x)-10,11-dihydroxy- 2,6-
2 570 farnesadienoate. These two sesqui-terpenoids are precursors of two juvenile hormones that are
3 571 crucial insect hormones involved in caste development [149, 150]. In social insect workers, the
4 572 juvenile hormone concentration increases during the caste transition from nest-workers to foragers
5 573 [151–153], potentially under the control of vitellogenin [53], explaining why we found more
6 574 precursors of the juvenile hormone in foragers.

9 575 4.5. Intra-caste variations and non-identified functions

11 576 Our analyses have shown metabolic differences not only between behavioural castes but also
12 577 within them. These differences could be seen on the PCA as well as on the clusters of the heat maps
13 578 (Fig. 1-3). The PCA-based classification allowed us to highlight the classes responsible for these
14 579 differences for both queens and workers (ESM1 Table S5). Unfortunately, very little information in
15 580 databases or the literature was available for these metabolites. We were therefore able to describe
16 581 them, but without providing a functional explanation. Although we were able to detect these intra-
17 582 caste variations, it should be stressed that most of the variation is due to behavioural caste differences
18 583 (Fig. 1 and 2), for which we were able to provide functional explanations.

21 585 Albeit several metabolites or classes of metabolites could not be linked to a particular function,
22 586 it is worth noting that the overwhelming majority of the metabolites we detected are lipid derivatives,
23 587 which may be inherent to sample preparation and composition. The multiple biological roles lipid
24 588 compounds fulfil could hence be highlighted: *e.g.* lipids involved in the protection against desiccation
25 589 and pollutants, mate recognition, energy fuel metabolism, and key components of pheromones and
26 590 sexual hormones [96, 147, 154]. Surprisingly, we did not find any metabolite that appeared to be
27 591 directly related to reproduction in queens, whereas proteins related to sperm motility (*e.g.* dynein
28 592 beta ciliary, radial spoke head protein 3-like protein) were found in our previous proteomics study
29 593 [59]. The metabolites possibly related to such proteins should be mainly involved in energy supply
30 594 to support the flagellum activity (ATP, Krebs cycle molecules, mitochondrial function). These are
31 595 general molecules that are involved in many processes and are therefore unlikely to be found in
32 596 significantly different quantities between two groups and difficult to attribute to only one process,
33 597 such as reproduction. In addition to sperm motility, sperm storage is a key feature for ant queens that
34 598 lay eggs for decades but mate only when founding their colony [64, 155]. Glyceraldehyde-3-
35 599 phosphate metabolism and lipids or lipid-like molecules such as sterols, fatty acyls, prenols
36 600 glycerolphospholipids, have especially been proved to contribute to preserving sperm in the queens'
37 601 spermatheca [156, 157]. Besides, despite the relatively large initial data set (1991 metabolites), the
38 602 proportion of metabolites that matched an annotation remained low (486/1991). In addition, among
39 603 these annotated metabolites, not all could be associated with a specific function. It is therefore highly
40 604 likely that some of these uncharacterised metabolites participate in the queen's reproductive function
41 605 (*e.g.* triglycerides, sterols) [156, 158]. Regarding the mass spectrometry protocol, when working in
42 606 high resolution in a non-targeted way, sensitivity is lower than when applying a targeted approach.
43 607 Reproduction-related molecules might have been in too low concentrations, relative to other
44 608 compounds, to be detected by the instrument used. Moreover, the protocol run did not foster the
45 609 extraction of volatile (*e.g.* monoterpenes) or strongly hydrophilic compounds (*e.g.* carbohydrates,
46 610 amino acids). So if some molecules related to reproduction fall into these categories, they may have
47 611 been missed by our analysis. This should encourage further studies to continue to describe the
48 612 metabolites involved in the different functions of living organisms to reveal the explanatory power
49 613 of metabolomics which, as our study shows, is capable of detecting fine differences between
50 614 genetically related individuals.

58 615 5. Conclusion

616 This metabolomic analysis broadly confirmed the molecular signatures established in our previous
1 617 proteomics study [59], with fewer metabolites related to immune defence and oxidative damage in
2 618 queens, but more of them in foragers, and more metabolites associated with digestion and nutrient
3 619 assimilation in nest-workers. However, this study also shows that proteomics and metabolomics
4 620 approaches are complementary. Indeed, we did not find reproduction-related metabolites in queens,
5 621 while we found proteins related to it. Conversely, nutrient-sensing pathways linked to somatic
6 622 maintenance and longevity were highlighted by the present metabolomics study but not by
7 623 proteomics. The metabolomic analysis also underlined the prevalence of lipids in insect biological
8 624 processes. We propose two possible improvements to this pioneering study. The first is to conduct
9 625 the proteomic and metabolomic analysis jointly to guarantee a comparison of results 100% free of any
10 626 possible sample and temporal bias. The second way of improvement would be to consider other
11 627 factors than only behavioural caste in shaping molecular differences. For instance, some recent
12 628 studies have raised the possibility of a slight difference in worker size depending on the task
13 629 performed [159, 160]. We should also consider that division of labour in *L. niger* is mainly due to age.
14 630 An experimental design studying different behavioural castes at controlled ages would thus make it
15 631 possible to differentiate the age-related changes in the metabolome and/or proteome from those
16 632 purely related to behaviour.
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Tables

Table 1 The 50 most enriched metabolic pathways (nest-workers vs foragers). After running a metabolite set enrichment analysis with MetaboAnalyst 5.0, we kept the 25 most enriched metabolites in both KEGG and SMPDB databases that encompass different molecules. The enrichment ratio is calculated as the number of observed hits in nest-workers / number of observed hits in foragers. It gives an idea of whether a pathway is more (high ratio) or less (low ratio) represented in nest-workers than in foragers. The false-discovery rate (FDR) gives a significance value corrected for the very large number of data (threshold used here: FDR < 0.05). The last column indicates the database where the pathway was found: Kyoto Encyclopedia of Genes and Genomes (KEGG) or Small Molecule Pathway DataBase (SMPDB). Data are sorted according to the enrichment ratio. The dark red colour indicates a high enrichment ratio or a low FDR.

Metabolite Set	Enrichment Ratio	FDR	Database
Fatty acid degradation	14.2968785	1.10E-09	KEGG
Ubiquinone and other terpenoid-quinone biosynthesis	14.1687792	1.78E-09	KEGG
Phenylalanine metabolism	14.1687792	1.78E-09	KEGG
Phenylalanine, tyrosine and tryptophan biosynthesis	14.1687792	1.78E-09	KEGG
Tyrosine metabolism	13.594582	7.82E-11	KEGG
Lysine degradation	13.5731321	5.31E-08	KEGG
Carnitine Synthesis	13.5731321	4.71E-08	SMPDB
Nicotinate and nicotinamide metabolism	13.3373333	1.18E-07	KEGG
Riboflavin metabolism	13.2509337	1.55E-07	KEGG
Riboflavin Metabolism	13.2371338	3.05E-09	SMPDB
Thiamine Metabolism	13.2234839	1.14E-07	SMPDB
Tryptophan metabolism	12.7743861	6.94E-07	KEGG
Taurine and hypotaurine metabolism	12.7718361	6.94E-07	KEGG
Taurine and Hypotaurine Metabolism	12.7718361	5.33E-07	SMPDB
Spermidine and Spermine Biosynthesis	12.5592872	4.98E-08	SMPDB
Glutamate Metabolism	12.3152384	6.60E-10	SMPDB
Betaine Metabolism	12.3138884	1.90E-06	SMPDB
Phenylalanine and Tyrosine Metabolism	12.194189	4.76E-09	SMPDB
Aminoacyl-tRNA biosynthesis	12.1118394	1.02E-09	KEGG
Nicotinate and Nicotinamide Metabolism	11.9169904	3.88E-08	SMPDB
Retinol Metabolism	11.5890921	9.87E-06	SMPDB
Glycine and Serine Metabolism	11.5758921	1.15E-09	SMPDB
Arginine and proline metabolism	11.4878426	6.80E-09	KEGG
Cysteine Metabolism	11.206894	1.14E-07	SMPDB
Propanoate Metabolism	11.206894	1.14E-07	SMPDB
Arginine and Proline Metabolism	11.206894	1.14E-07	SMPDB
Histidine Metabolism	11.206894	1.14E-07	SMPDB
Alanine Metabolism	11.206894	1.14E-07	SMPDB
Urea Cycle	11.206894	1.14E-07	SMPDB
Aspartate Metabolism	11.206894	1.14E-07	SMPDB
Tryptophan Metabolism	10.9823451	5.36E-07	SMPDB
Tyrosine Metabolism	10.4936475	1.15E-09	SMPDB

1	Glutathione metabolism	10.2714986	1.18E-07	KEGG
2	Purine metabolism	9.74245129	1.33E-08	KEGG
3	Purine Metabolism	9.63190184	1.15E-09	SMPDB
4	Alanine, aspartate and glutamate metabolism	9.19030405	3.87E-04	KEGG
5	Histidine metabolism	9.19030405	3.87E-04	KEGG
6	D-Glutamine and D-glutamate metabolism	9.19030405	3.87E-04	KEGG
7	Glyoxylate and dicarboxylate metabolism	9.19030405	3.87E-04	KEGG
8	Butanoate metabolism	9.19030405	3.87E-04	KEGG
9	Porphyrin and chlorophyll metabolism	9.19030405	3.87E-04	KEGG
10	Nitrogen metabolism	9.19030405	3.87E-04	KEGG
11	Lysine Degradation	9.19030405	3.71E-04	SMPDB
12	Folate Metabolism	9.19030405	3.71E-04	SMPDB
13	Glutathione Metabolism	8.83615582	5.51E-06	SMPDB
14	Linoleic acid metabolism	8.34670827	9.37E-04	KEGG
15	Glycerophospholipid metabolism	8.03140984	2.56E-04	KEGG
16	Valine, Leucine and Isoleucine Degradation	7.01711491	8.43E-04	SMPDB
17	Sphingolipid metabolism	6.5395173	9.83E-05	KEGG
18	Arginine biosynthesis	5.88792056	9.58E-05	KEGG

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Figure legends

Fig. 1 Classification-based PCA among the three behavioural castes of *L. niger* (121 metabolites).

We kept the metabolites from the preliminary PCA (Fig. S1 Left), grouped them according to their class and ran a PCA with the classes as variables. **A:** PC1 and PC2. **B:** PC1 and PC3. The bar plots represent the classes of metabolites and their correlation with PCs from blue (negatively correlated) to red (positively correlated), as well as their contribution (bar length) and the number of metabolites in a given class (in brackets). Information is given for each principal component: PC1 (**C**) and PC3 (**D**). All metabolites showed are correlated more than 50%. The list of metabolites is available in Table S6 (ESM1). FA stands for fatty acids.

Fig. 2 Classification-based PCA within workers (1807 metabolites). We kept the metabolites from the preliminary PCA (Fig. S1 Right), grouped them according to their class and ran a PCA with the classes as variables. **A:** PC1 and PC2 among workers only. **B:** PC1 and PC3 among workers only. The bar plots represent the classes of metabolites and their correlation with PCs from blue (negatively correlated) to red (positively correlated), as well as their contribution (bar length) and the number of metabolites in a given class (in brackets). Information is given for each principal component: PC1 (**C**), PC2 (**D**) and PC3 (**E**). All metabolites showed are correlated more than 50%, except for PC1, with which the correlation is at least 80%. The list of metabolites is available in Table S6 (ESM1).

Fig. 3 Metabolite expression profiles among the behavioural caste of *L. niger*. The **upper panel** represents the heat map of the 50 most expressed metabolites among queens (Q), foragers (F), and nest-workers (NW). The **lower panel** represents the heat map of the 50 most expressed metabolites among workers only (F and NW). The left columns indicate the metabolite class. Grey is a NA value. At the bottom are the sample IDs. The right columns indicate the ID rather than the full name for legibility reasons (correspondence is indicated in every table provided). All metabolites presented here have an FDR < 0.05 and Log₂FC < -2 or Log₂FC > 2.

Fig. 4 Metabolomic networks for pairwise comparison of behavioural castes. The caste compared is always the first to appear in the title and the reference caste, the second one. For example, isobutylidene is found in a larger amount in foragers than in queens. We mapped the identified metabolites according to biochemical reactions with MetaMapp and visualised the resulting networks through Cytoscape. An arrow indicates a chemical transformation into the metabolite at the end of the arrow, based on known enzymatic reactions (KEGG reactant pair database) or chemical similarity (Tanimoto chemical and NIST mass spectral similarity scores). The networks without significant and strong metabolomic differences between castes are not shown. As the comparison between workers involved too many metabolites (360) for a suitable display, we show only the metabolites with FDR < 0.05 and Log₂FC > 2 or < -2, and not all the interactions in which they are involved. Dark blue (dark red) indicates a smaller (larger) quantity of a given metabolite with a Log₂FC < -2 (Log₂FC > 2). Light blue (orange) codes for a decreased (increased) quantity of metabolite but with -2 < Log₂FC < 0 (0 < Log₂FC < 2). Grey indicates an absence of significative change (FDR > 0.05). The larger the dot size, the higher the absolute value of Log₂FC is (min = -3.09, max = 6.64).







