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# 1 Division of labour in the black garden ant (*Lasius* 2 *niger*) leads to three distinct proteomes

3 Martin Quque\*<sup>1</sup>, Margaux Benhaim-Delarbre<sup>1</sup>, Jean-Louis Deneubourg<sup>2</sup>, Cédric Sueur<sup>1</sup>, François  
4 Criscuolo<sup>1†</sup>, Fabrice Bertile<sup>1†</sup>

5 <sup>1</sup> Université de Strasbourg, CNRS, IPHC UMR 7178, F-67000 Strasbourg, France – 23 rue du Loess, 67037  
6 Strasbourg Cedex 2, France

7 <sup>2</sup> Université Libre de Bruxelles, CPBT, CP231 Av. F. Roosevelt 50 1050 Bruxelles, Belgique

8 † Share co-seniorship of the paper

9 \* Correspondence: [martin.quque@iphc.cnrs.fr](mailto:martin.quque@iphc.cnrs.fr) - +33 388 106 951

10 **Abstract:** Task specialization in social insects leads to striking intra-specific differences in behaviour,  
11 morphology, physiology and longevity, but the underlying mechanisms remain not yet fully  
12 understood. Adult colonies of black garden ants (*Lasius niger*) have a single queen fertilized by one  
13 or a small number of males. The inter-individual genetic variability is thus relatively low, making it  
14 easier to focus on the individual molecular differences linked to the division of labour. Mass  
15 spectrometry-based proteomics enabled us to highlight which biological functions create the  
16 difference between queens, foragers and nest-workers. The proteome of each caste reflected nicely  
17 their social role: e.g., reproduction for queens, pesticide resistance for foragers – that are the most  
18 exposed to environmental risk factors – and, interestingly, digestion for nest-workers, thus  
19 highlighting proteomic profiles differences even among workers. Furthermore, our exploratory  
20 approach suggests energy trade-off mechanisms – in connection with the theory of social immunity  
21 – that might explain the difference in longevity between queens and workers. This study brings  
22 evidence that proteomics is able to highlight the subtle mechanisms of molecular regulation  
23 induced by social organization.

24 **Keywords:** task specialization, social insect, trade-off, social immunity, longevity, mass spectrometry

## 25 1. Introduction

26 Animal species display different schemes of social organization – from solitary to eusocial  
27 species. Eusociality exists in certain mammals (Burda, Honeycutt, Begall, Locker-Grütjen, & Scharff,  
28 2000), crustaceans (Duffy, Morrison, & Ríos, 2000) and insects (Wilson, 1971). The latter include  
29 eusocial Hymenoptera (wasps, bees, ants) and termites, the complex social organization of which is  
30 based on division of labour. Each individual belongs to a caste and displays a caste-specific set of  
31 behaviours. While the queen's main role is to produce offspring, the task specialization among  
32 workers is highly species-dependent and can result in a broad range of sizes and shapes within the  
33 same species (Harvell, 1994; Jeanne, 1986; Morton Wheeler, 1908; Seeley, 1986). Castes also differ in  
34 terms of longevity, queens reaching a dramatically longer lifespan than workers, living up to  
35 ten-fold longer (Keller & Genoud, 1997). The reproductive division of labour leads to a higher  
36 concentration of ecdysteroids and vitellogenin in reproductive individuals (Gospocic et al., 2017;  
37 Robinson, Strambi, Strambi, & Feldlaufer, 1991). Intriguingly, hormone concentrations do not reflect  
38 only the reproductive status but also the task specialization among workers, particularly the trio  
39 constituted by Insulin/Insulin-like growth factor Signalling, vitellogenin and juvenile hormone  
40 (Azevedo, Zanuncio, Delabie, & Serrão, 2011; Corona et al., 2013; Guidugli et al., 2005; Kohlmeier,  
41 Feldmeyer, & Foitzik, 2018; Libbrecht et al., 2013; Nelson, Ihle, Fondrk, Page Jr, & Amdam, 2007).  
42 Thus, resulting from division of labour in eusocial insects, genetically close individuals may  
43 nevertheless greatly differ from each other in terms of behaviour, morphology, physiology and  
44 longevity.

45  
46 Genomics and proteomics picture different levels of gene expression (Gygi, Rochon, Franza, &  
47 Aebersold, 1999; Hunt et al., 2010). Studying differences between individuals at the proteome level  
48 allows consideration of changes which are likely closer to phenotypic variation than, for instance,  
49 gene expression (Baer & Millar, 2016). The study of the molecular basis of social life by genomic tools  
50 (*i.e.* sociogenomics) has already led to the identification of numerous genes (Robinson, Grozinger, &  
51 Whitfield, 2005; Sumner, 2006). By contrast, proteomics studies in social insects are less focused on  
52 behaviour and social interaction, concern mainly the honeybee biology (but (LeBoeuf et al., n.d.)),  
53 and are biased toward larvae rather than adults. In the latter case, the proteome of queen-destined  
54 and worker-destined larvae has been shown to differ in protein quantity for the following processes:  
55 resistance to oxidative stress, energy production, lipid metabolism, amino acid metabolism,  
56 development, protein biosynthesis, protein folding and cytoskeleton (Li et al., 2010). Larval  
57 mitochondrial (Begna, Fang, Feng, & Li, 2011) and larval nuclear (Begna, Han, Feng, Fang, & Li,  
58 2012) proteome studies have shown similar results, as well as the comparison at an adult stage of  
59 antennal proteome between drones (male bees), workers and queens (Fang et al., 2012).

60  
61 Black garden ants (*Lasius niger*) combine low genetic variations and a marked division of  
62 labour. Even though worker's ovaries are functional (Have, Boomsma, & Menken, 1988), the queen  
63 monopolizes the reproduction, she is larger and lives far longer than workers (Parker, 2010). These  
64 elements appoint black garden ants as a wise choice to stimulate the field of "socioproteomics", *i.e.*  
65 to determine the influence of the social role on individual's proteome, and then phenotype. In ants,  
66 proteomics has been hitherto used to specifically analyse spermatheca or venom compositions  
67 (Malta et al., 2014; Wiese et al., 2006), and protein response to desertic conditions (Willot, Gueydan,  
68 & Aron, 2017). With the hypothesis that abundance of proteins specific to given tasks and/or related  
69 to longevity and reproduction physiology should differ between the castes, we compared the  
70 proteome of black garden ant individuals, with the aim to characterize the differences between  
71 queens, foraging workers and non-foraging workers (respectively referred to as foragers and  
72 nest-workers).

## 73 2. Materials and Methods

### 74 2.1. Animal model

75 The black garden ant (*Lasius niger*, Linnaeus 1758) is a very common species in Western Europe,  
76 in urban and rural areas (Sterry, 1997). They are omnivorous and widely known to farm aphids for  
77 the honeydew they excrete (Domisthorpe, 1927). In adult colonies, only one queen lays eggs  
78 (monogynous species). She is fertilized by one or two males, and very rarely by more (Boomsma &  
79 Have, 2002; Fjerdingstad, Gertsch, & Keller, 2002; Fjerdingstad, Keller, & Tregenza, 2004). The queen  
80 is 7-9 mm long and has an average lifespan of 20 years, whereas workers are 2.5-5 mm long and live  
81 for 3 years on average (Hölldobler & Wilson, 1990). Unlike in other species, black garden ant  
82 foragers and nest-workers do not morphologically differ from each other.

83

84 The colonies used in this study came from wild newly-mated queens captured on the site  
85 "Campus Plaine" at the Université Libre de Bruxelles (50°49'08.4"N 4°23'57.0"E) and tended during  
86 two years in lab. We removed the eggs to control that all individuals were 2 years old. Colonies,  
87 consisting of only females (queen and worker ants), were housed in IPHC-DEPE (Strasbourg,  
88 France) at a temperature of about 25°C with 50-60% relative humidity and were fed with sugar water  
89 (0.3M) and mealworms once a week. Even though no law regulates the care and use of insects, we  
90 applied internal animal welfare policies by minimizing the number of ants used in experiments and  
91 by preventing any form of avoidable suffering.

92

## 93 2.2. Caste identification

94 The worker castes differ from each other by their interaction pattern and spatial segregation  
95 (Mersch, Crespi, & Keller, 2013). Active individuals, spending time in the foraging area were  
96 identified as foragers. On the other hand, the nest-workers were identified by no move outside the  
97 nest and their tendency to form immobile clusters. To stimulate the foraging behaviour, we used a  
98 4-day fast and then placed a high concentration sugar solution (1M) in a plastic tray. To ensure an  
99 optimal recruitment, we waited for 5 minutes after the fifth individual came to the food source, then  
100 picked up all the forager individuals seen at the food source for one hour. No foraging behaviour  
101 was noticed after this period in preliminary tests. We then collected the nest-workers. Ants were  
102 anesthetized by cold (0.5-1 min, -20°C). A pen filled with acrylic ink (Posca®) was used to mark their  
103 abdomen, with a different colour according to the caste. When they woke up, the ants were carefully  
104 watched for a few minutes. None of them exhibited any sign of aftereffects. They were then put back  
105 into their colony where usual food and water were provided. This process was repeated three times,  
106 every 48 hours, to reduce the number of false positive (starved nest-workers exiting from the nest)  
107 and false negative (non-recruited or non-captured forager).

## 108 2.3. Proteomic analysis

### 109 2.3.1. Sample preparation

110 We used 15 colonies, individuals of which were homogeneously distributed among the  
111 samples. One sample is made of three queens or ten workers (nest-workers or foragers). We had five  
112 samples per caste, except in nest-workers, where only four samples had a sufficiently high protein  
113 content to be analysed by mass spectrometry. Frozen ants were ground under liquid nitrogen for 45  
114 seconds at 30Hz using a Mixer Mill MM400 (Retsch, Eragny Sur Oise, France), and total proteins  
115 were extracted from the resulting powder using 200µl of extraction buffer (8 M urea, 2 M thiourea,  
116 0.1 M Ammonium Bicarbonate, 1% DTT, protease inhibitors; Sigma-Aldrich, Lyon, France). After  
117 sonication on ice (2 x 10 seconds, 135 watts) and centrifugation (2000 x g, 2 minutes) to eliminate  
118 cuticle residues, 8 volumes of cold acetone were added to samples that were kept at -20°C overnight.  
119 Precipitated proteins were pelleted by centrifugation (13500 x g, 20 minutes, 4°C), and after  
120 discarding supernatants, dissolved in Laemmli buffer (10 mM Tris pH 6.8, 1 mM EDTA, 5%  
121 β-mercaptoethanol, 5% SDS, 10% glycerol). Samples were centrifuged to eliminate the remaining  
122 cuticles (2,000 g, 2 minutes). Total protein concentrations were determined using the RC-DC Protein  
123 Assay kit (Bio-Rad, Hercules, CA, USA). At this stage, a reference sample comprising equal amounts

124 of all protein extracts was made, to be injected regularly during the whole experiment and thus  
125 allow QC-related measurements.

126 20 µg of proteins from each sample were electrophoresed on SDS-PAGE gels (12%  
127 polyacrylamide) for 60 minutes at 50 V followed by 15 minutes at 100 V. After protein fixation (50%  
128 ethanol, 3% phosphoric acid), gels were stained overnight using colloidal Coomassie Blue. For each  
129 lane, five 2mm bands were excised, and proteins were in-gel digested with trypsin (Promega,  
130 Madison, WI, USA; 120 ng/band) at 37°C overnight after de-staining, reduction (10mM DTT),  
131 alkylation (55mM iodoacetamide), and dehydration using a MassPrep station (Micromass, Waters,  
132 Milford, MA, USA). Tryptic peptides were extracted using 60% acetonitrile, 0.1% Formic acid in  
133 water for one hour at 450 rpm on an orbital shaker. The organic solvent was then eliminated using a  
134 vacuum centrifuge (SpeedVac, Savant, Thermoscientific, Waltham, MA, USA), and peptides were  
135 re-suspended in 90 µl of 1% acetonitrile, 0.1% formic acid in water. A set of reference peptides (iRT  
136 kit; Biognosys AG, Schlieren, Switzerland) was finally added to each sample prior to LC-MS/MS  
137 analyses.

### 138 2.3.2. Nano LC-MS/MS analyses

139 Samples were analysed on a nanoUPLC-system (nanoAcquity, Waters) coupled to a  
140 quadrupole-Orbitrap hybrid mass spectrometer (Q-Exactive Plus, Thermo Scientific, San Jose, CA,  
141 USA) using a randomized sequence within block injections. Each block consisted of one biological  
142 sample of each group plus the reference sample. To reduce carry-over, two solvent blank injections  
143 were included in between each sample. Briefly, one µl of each sample was concentrated/desalted on  
144 a Symmetry C18 pre-column (0.18 x 20 mm, 5 µm particle size; Waters) using a mobile phase  
145 composed of 99% of solvent A (0.1% formic acid in water) and 1% of solvent B (0.1% formic acid in  
146 acetonitrile) at a flow rate of 5 µl/min for 3 minutes. Afterwards, peptides were eluted using a UPLC  
147 separation column (BEH130 C18, 200 mm x 75 µm, 1.7 µm particle size; Waters) maintained at 60 °C  
148 with the following gradient: from 1% to 6% B in 30 seconds, from 6% to 35% B in 59.5 minutes.

149 Q-Exactive Plus was operated in positive ion mode with source temperature set to 250°C and  
150 spray voltage to 2.0 kV. Spectra were acquired through automatic switching between full MS and  
151 MS/MS scans. Full scan MS spectra (300-1800 m/z) were acquired at a resolution of 70,000 at m/z 200  
152 with an automatic gain control (AGC) value set to  $3 \times 10^6$  ions, a maximum injection time set to 50  
153 ms, and the lock-mass option being enabled (polysiloxane, 445.12002 m/z). Up to 10 of the most  
154 intense precursors (with a minimum of 2 charges) per full MS scan were isolated using a 2 m/z  
155 window and fragmented using higher energy collisional dissociation (HCD), with normalised  
156 collision energy set to 27 eV and dynamic exclusion of already fragmented precursors set to 60  
157 seconds. MS/MS spectra were acquired at a resolution of 17,500 at m/z 200 with an AGC value set to  
158  $1 \times 10^5$  and a maximum injection time set to 100 ms, and the peptide match selection option was  
159 turned on. The system was fully controlled by Xcalibur software (v3.0.63; Thermo Fisher Scientific).  
160 Peak intensities and retention times of reference peptides were monitored in a daily fashion.

### 161 2.3.3 Protein identification and quantification

162 MS raw data processing was performed using MaxQuant (v 1.5.3.30). Peak lists were searched  
163 against a UniProtKB-derived protein database created using the MSDA software suite (Carapito et  
164 al., 2014). The database contained *Lasius niger* (TaxID 67767) protein sequences (February 2017; 18075  
165 sequences) to which sequences of common contaminants were added (247 entries;  
166 contaminants.fasta included in MaxQuant). A minimal number of one peptide (unique or razor) was  
167 required for protein identification. A maximum number of one missed cleavage and a false  
168 discovery rate (FDR) of 1% for both peptide-spectrum matches (minimum length of seven amino  
169 acids) and proteins was accepted during identification. From the use of a *Lasius niger* protein  
170 database, we identified 57 “uncharacterized” proteins (~4% of all identified proteins) for which we  
171 searched known homologous proteins in the Protostomia clade. This was done by using BLAST  
172 searches (FASTA program v36; downloaded from  
173 [http://fasta.bioch.virginia.edu/fasta\\_www2/fasta\\_down.shtml](http://fasta.bioch.virginia.edu/fasta_www2/fasta_down.shtml)), and only the best hits were retained.

174 To validate this procedure, we automatically extracted orthology annotations and sequence domains  
175 of *Lasius niger* uncharacterized proteins and of their homologues from the OrthoDB (Kriventseva et  
176 al., 2019) and InterPRO (Mitchell et al., 2019) resources. The relevance of the match between *Lasius*  
177 *niger* uncharacterized proteins and their homologues was then checked manually.

178 Regarding quantification, data normalisation and protein abundance estimation were  
179 performed using the MaxLFQ (label-free quantification) option implemented in MaxQuant (Cox et  
180 al., 2014) using a “minimal ratio count” of one. “Match between runs” was enabled using a one  
181 minute time window after retention time alignment. Only unmodified peptides were considered for  
182 quantification (except those for which a modified counterpart was detected) while shared peptides  
183 were excluded. All other MaxQuant parameters were set as default.). Only proteins quantified with  
184 at least two unique peptides and detected in at least three samples in a given caste were kept for  
185 further analysis. The mass spectrometry proteomics data have been deposited to the  
186 ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository with the  
187 dataset identifier PXD006779.

188 Regarding quality controls, we found that the median coefficient of variation (CV) of retention  
189 times and raw intensity of iRT peptides when considering all injections was 0.96% and 22%,  
190 respectively. The median CV regarding the raw intensity of all quantified proteins across a repeated  
191 analysis of the reference sample was 16%. These different values support the good stability of the  
192 nanoLC-MS/MS system during the whole duration of analyses, and good reproducibility of protein  
193 abundance determination.

#### 194 2.4. Protein selection procedure and PCA

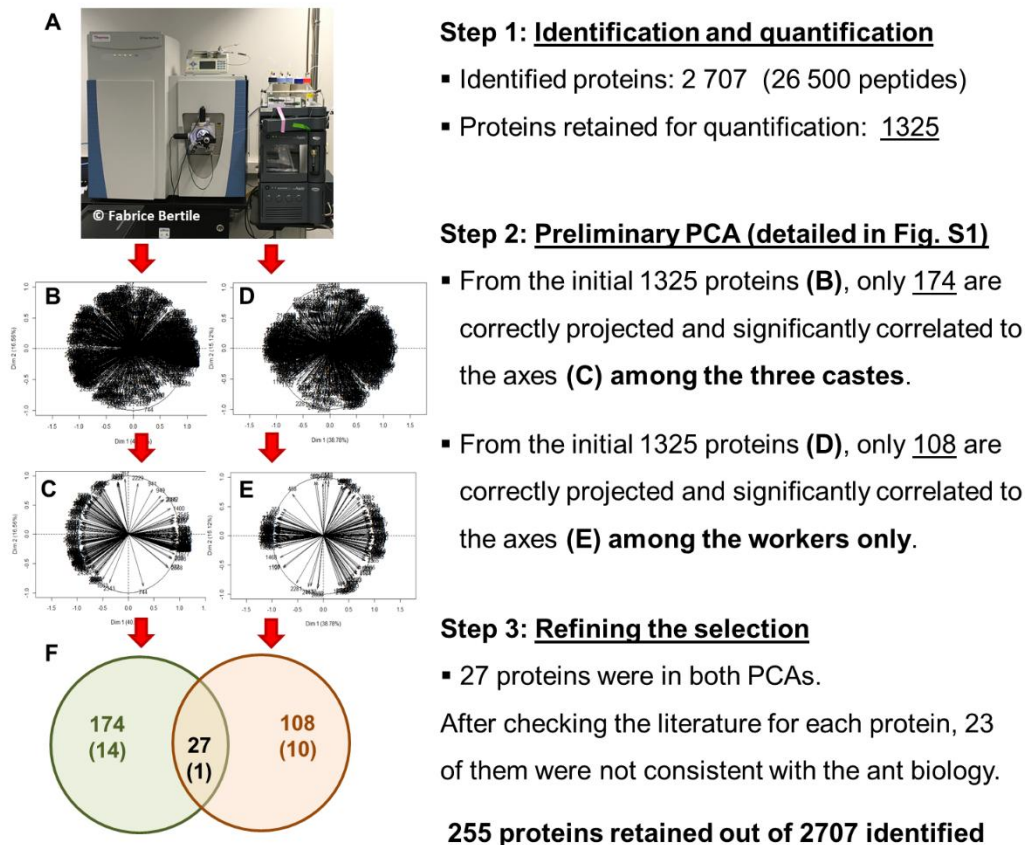
195 In total, 2707 proteins were identified, of which 1325 fulfilled the criteria for accurate  
196 quantification (see above). This original dataset is available online in supplementary material (Table  
197 S1-S3). To properly run the PCA (principal component analysis); missing data were inferred by  
198 regularized iterative PCA algorithm (missMDA package; (Josse & Husson, 2016). The PCA was  
199 performed (FactoMineR, v.1.34; Lê et al., 2008) on all three castes, and also only the two workers  
200 castes separate from the queen caste, in order to have a more precise insight into potential  
201 differences. First, we used PCA as a filter to shrink the number of variables and only retain proteins  
202 giving rise to differences between the castes. Only 174 proteins for the three castes and 108 for the  
203 workers were strongly correlated to the axes ( $p < 0.05$ ) and faithfully projected ( $\cos^2 > 0.8$ ; Fig. S1 C  
204 and D). Most of the time, protein databases in insects are unfortunately patchy and based on  
205 predicted annotations. This makes an automatic annotation highly prone to misassignment. The  
206 protein functions were therefore “manually” attributed by using several database resources (Ortho  
207 DB, InterPro, UniProt) that contain annotations for proteins from the Protostomia clade that are  
208 homologous to *Lasius niger* proteins, as well as results from previous studies in insects (social insects  
209 whenever possible) to avoid misleading functional annotations. In the case of pleiotropic proteins,  
210 we kept all the different functions (proteins indicated in blue in Table S2-S3) and did not arbitrarily  
211 choose one among the others. Finally, 255 proteins were kept for further analyses (**Fig. 1**), and they  
212 were clustered according to their functions (categories in **Table 1**). These functional groups were  
213 used as variables to build the axes of the second set of PCAs (**Fig. 2**), in order to determine which  
214 biological functions allow discriminating queens from nest-workers from foragers."

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218 protein functions were therefore “manually” attributed by crossing several databases (Ortho DB,  
219 InterPro, UniProt) and actual studies in insects (social insects whenever possible) to avoid  
220 misleading functional annotations. In the case of pleiotropic proteins, we kept all the different  
221 functions (proteins indicated in blue in Table S2-S3) and did not arbitrarily choose one among  
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225 allow discriminating queens from nest-workers from foragers.

## 226 2.5. Statistical tests

227 Statistical tests were performed using R software (R Core Team, v3.4, 2017) at the significance  
228 threshold  $\alpha = 5\%$ . To statistically test whether any biological function isolates one caste from another,  
229 we used the PCA's coordinates as variables. With them, we performed Kruskal-Wallis (KW) tests (>2  
230 modalities, heteroscedasticity) and Wilcoxon rank sum test (2 modalities, homoscedasticity). If the  
231 KW tests were significant, they were followed by Conover-Iman posthoc test with Bonferroni  
232 correction (conover.test v.1.1.5; Alexis Dinno). Homoscedasticity was assessed by Bartlett test.



233  
234

235 **Fig. 1. Protein selection procedure.** The underlined numbers are the subset in the step n used in the  
236 step n+1. **A:** First, the mass spectrometer and the MaxQuant Software were used to analyse the  
237 proteins. Amongst the 2707 identified proteins, 1325 could be quantified. One PCA regarding the  
238 three castes (**B**) and one regarding the workers only (**D**) were performed with these proteins. Only  
239 the proteins properly projected ( $\cos^2 > 0,8$ ) and significantly correlated to the axes ( $p < 0,05$ ) were kept  
240 for the three castes (**C**) and the workers only (**E**). **F:** The number in brackets refers to the number of  
241 inconsistent proteins among the total number. A total of 23 proteins did not show strong evidence to  
242 be involved in insect's processes or in evolutionarily conserved mechanisms. 27 proteins were  
243 redundant between the two PCAs. The 255 proteins remaining at the end of this procedure were  
244 clustered according to their biological function in the further analysis. *Photo credits: Fabrice Bertile,*  
245 *co-author of the publication.*

246  
247  
248  
249  
250  
251

**Table 1. Functional groups retained to build the axes of the PCA.** Functional category's names are adapted from GO term annotations ([www.geneontology.org](http://www.geneontology.org)) of proteins homologous to *Lasius niger* proteins, identified using several database resources (Ortho DB, InterPro, UniProt), as well as literature examination. The IDs corresponds to the numbers found on the PCA plots (figures 2 and S1). The attribution of proteins to functional groups is detailed in the 'Materials and Methods' section.

252

Functional group	ID	Description
Cell Activity	1	Protein involved in several mechanisms highlighting general cell activity: transcription/translation, ATP synthesis...
Ageing +	2a	Direct and/or strong association with the individual ageing status.
Ageing -	2b	Direct and/or strong association with a slower ageing rate or extended lifespan.
Apoptosis +	3a	Inducing or fostering apoptosis.
Apoptosis -	3b	Inhibiting or delaying apoptosis.
Tissue Growth	4	Tissue growth especially during embryonic development.
Chaperone	5	Protecting the cell against harmful conditions (oxidative stress, pH variation...). Ensuring a proper protein folding.
Cell Cycle	6	Controlling cell cycle (mitosis/meiosis, blocking cell cycle...).
Cytoskeleton	7	Part of the cytoskeleton or associated with (actin, dynein, kinesin...).
Detoxification	8	Soma repair after a stressful event.
Digestion	9	Protein involved in the digestion metabolism.
Cell Dynamics	10	Non-focused action proteins involved in structural cell mechanisms: controlling cell shape, adhesion...
GnExpression+	11a	Activating gene expression (transcription and/or translation).
GnExpression-	11b	Inhibiting gene expression (transcription and/or translation).
Calcium Homeostasis	12	Regulating the calcium level.
Human Pathologies	13	Human diseases – degenerative most of the time (e.g. Alzheimer).
Xenobiotics Detox	14	Resistance to chemicals, especially pesticides.
Immunity	15	Resistance to pathogens.
Larvae	16	Proteins related to larval development.
NclAcid Metabolism	17	Nucleic acids synthesis or modification.
Energy Metabolism	18	Protein involved at least in one of the following pathways: glycolysis, Krebs cycle, gluconeogenesis, ATP synthesis.
Glucid Metabolism	19	Glucid modification, not for direct use in glycolysis or Krebs cycle
Lipid Metabolism	20	Lipid modification, not in an energetic purpose.
Protein Metabolism	21	Protein synthesis or modification.
Muscles	22	Protein required for muscle contraction.
IR	23	Irrelevant: unknown function or inconsistent in <i>L. niger</i>
Cell Proliferation	24	Protein directly involved in cell proliferation.
Glucid Recycling	25	Breakdown of glucids to provide the cell with new fatty materials.
Protein Recycling	26	Breakdown of proteins to provide the cell with new amino acids.
Redox	27	Promoting redox reactions in physiological conditions, ensuring the redox balance within the cell.
Reproduction	28	Proteins related to the gametes.
Secretion	29	Secreted proteins: hormones, pheromones, in saliva.
Nervous System (NS)	30	Growth, maintenance and repair of the nervous system.
Sensitive NS	31	Protein involved in the sensitive nervous system.
Membrane Trafficking	32	Protein involved in membrane trafficking between RE and Golgi or



### 253 3. Results

#### 254 3.1. Biological functions splitting the castes according to the PCAs

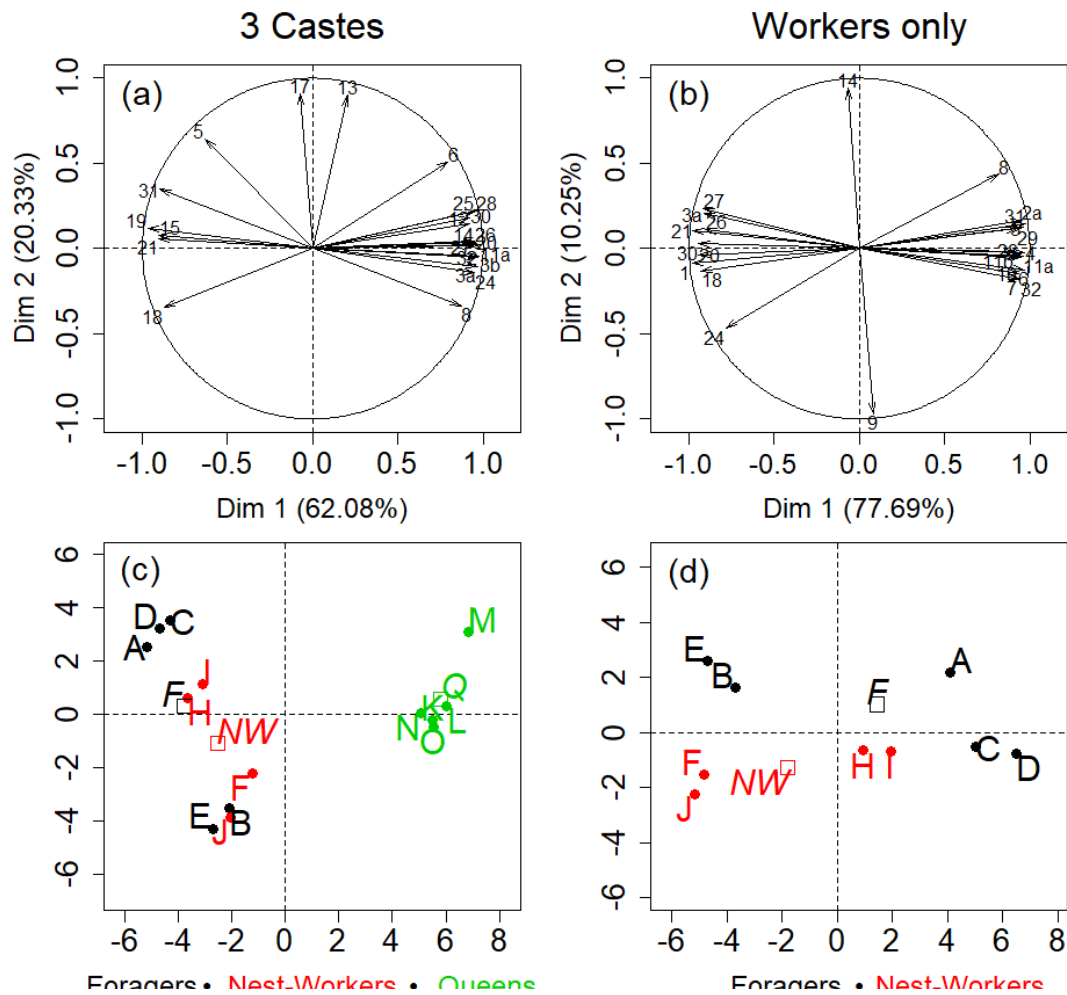
255 In the supplementary material section, we supply accession numbers, names and relative  
256 amounts of the 1325 quantified proteins (Table S1 B). Table S2 and S3 aggregate the 255 retained  
257 proteins and their functional category. We identified 35 functional groups (**Table 1**) according to our  
258 selection procedure.

259 Regarding the three-caste analyses, the first two dimensions (**Fig. 2 A**) of the PCA explained  
260 82.41% of the variance, which is considered significant (Table S4). Only the first axis statistically  
261 separated all castes from each other ( $\chi^2KW_{\text{axe1}} = 9.926$ ,  $p_{\text{axe1}} = 0.01$  and  $\chi^2KW_{\text{axe2}} = 0.591$ ,  $p_{\text{axe2}} = 0.744$ ):  
262 forager-nest-worker ( $t = 1.82$ ,  $p = 0.048$ ), queen-nest-worker ( $t = -3.71$ ,  $p = 0.002$ ), forager-queen ( $t = -5.86$ ,  
263  $p < 0.001$ ). Queens were mainly characterised by proteins involved in somatic maintenance –  
264 mechanisms aiming to avoid or repair damages to macromolecules – cell division, gene expression  
265 regulation and trafficking. While workers expressed more proteins related to metabolic pathways  
266 (except lipid metabolism) and sensitive nervous system or immunity.

267 In the second PCA (workers only), the first two dimensions (**Fig. 2 B**) explained 87.94% of the  
268 variance, which is considered significant (Table S4). The second axis isolated nest-workers from  
269 foragers, but the first axis did not ( $W_{\text{axe1}} = 16$ ,  $p_{\text{axe1}} = 0.191$  and  $W_{\text{axe2}} = 20$ ,  $p_{\text{axe2}} = 0.016$ ). Foragers  
270 presented more proteins associated with xenobiotic detoxification mechanisms, whereas  
271 nest-workers had more proteins involved in digestion metabolism.

#### 272 3.2. Proteins related to the ToR pathway

273 The Target of Rapamycin (ToR) protein belongs to the serine/threonine kinase family (Helliwell  
274 et al., 1994). A growing literature shows the implication of the ToR pathway in ageing-related  
275 diseases (Skike & Galvan, 2018) and its evolutionarily conserved ability to shorten lifespan in  
276 various taxa (Powers, Kaeberlein, Caldwell, Kennedy, & Fields, 2006). We were thus interested in  
277 knowing whether the amount of ToR-related proteins would differ between the castes. Four proteins  
278 involved in the ToR pathway were significantly different between castes: striatin-3 isoform x2 ( $\chi^2_{\text{KW}} = 10.73$ ,  $p < 0.001$ ),  
279 peptidyl-prolyl cis-trans isomerase ( $\chi^2_{\text{KW}} = 9.23$ ,  $p = 0.01$ ), ubiquilin-1-like isoform  
280 2 protein ( $\chi^2_{\text{KW}} = 6.17$ ,  $p = 0.04$ ) and eukaryotic translation initiation factor 4e ( $\chi^2_{\text{KW}} = 6.94$ ,  $p = 0.03$ ).  
281 Only the cAMP-dependent protein kinase catalytic subunit was not significant ( $\chi^2_{\text{KW}} = 4.06$ ,  $p = 0.13$ ).  
282 However, they are only marginally related to the ToR pathway and could be involved in other  
283 signalling pathways. Hence, we did not perform further analyses.



284

285 **Fig. 2 PCA amongst the castes of *L. niger*.** Left: charts regarding the three castes. 14 ant pools and 33  
 286 variables (biological functions). / Right: charts regarding the workers only. 9 ant pools and 25  
 287 variables (biological functions). (a) and (b): functional groups correlating with at least one of the axes  
 288 ( $p < 0.05$ ) and with a  $\cos^2 > 0.8$ , identified by a number (names and functions in Table 1). (c) and (d):  
 289 representation of the individuals. Similar coordinates mean the individuals show a similar level of  
 290 protein expression in the same biological functions. The empty squares with a capital letter  
 291 (Q=queens, NW=nest-workers, F=foragers) indicate the average coordinates for each caste.

292 **4. Discussion**

293 Our proteomic analysis combined with a PCA-based protein selection highlighted biological  
 294 functions specific to each caste. The exhaustive list of selected proteins and related functions is  
 295 available in supplementary data (Table S2 and S3). Below, we focus the discussion on some  
 296 functions (Table 1) or life-history traits that were either different between queens and workers, or,  
 297 within the workers, between foragers and nest-workers. First, we focus on identified caste-specific  
 298 functions that define the worker castes. Second, we propose other sources of individual variation  
 299 amongst the workers. Third, based on observed differences in queens and workers profiles, we  
 300 discuss possible mechanisms involved in the large difference in longevity. This leads us to question  
 301 the usual fecundity/lifespan trade-off by considering the energetic cost of an active immune system  
 302 in a so particular social context.

303 **4.1. Division of labour has multiple proteomic consequences in workers**

304 **4.1.1. Sensory system**

305 We found a homologue protein (apd-3-like protein) to the bee's apd3 protein which is,  
 306 according to a genomic study, related to the olfactory/gustative function in the antennae (Antony et

307 al., 2016). SAP47 was also part of this functional group and is involved in the learning process of  
308 smells and pictures in *Drosophila*'s larvae (Saumweber et al., 2011). Ant communication is (almost)  
309 all about pheromones. Nest building, foraging, social identification: all rely on those signals  
310 (Beckers, Deneubourg, & Goss, 1993; Khuong et al., 2016; Yan et al., 2017). Moreover, worker ants  
311 must also decipher the environmental cues. For instance, foragers must find the appropriate food  
312 sources according to colony needs within an environment full of non-specific odorants. Hence, it is  
313 not surprising to see in workers high levels of proteins related to the sensitive nervous system (Fig 2  
314 C), allowing them to detect and analyse those specific and non-specific olfactory cues. This  
315 hypothesis is supported by a genomics study in *L. niger*, where workers up-regulate the Ln385\_5  
316 gene, involved in odorant binding (Graeff, Jemielity, Parker, Parker, & Keller, 2007).

#### 317 4.1.2. Immunity

318 Workers had on average a higher amount of proteins associated with the immune system (e.g.  
319 arginine kinase, T-cell immunomodulatory protein). Some studies have opposing results regarding  
320 the expression of immunity-related genes in ants (Graeff et al., 2007) or bees (Grozinger, Fan,  
321 Hoover, & Winston, 2007). Moreover, ferritin, known to withhold iron from invading pathogens  
322 (Ong, Wang, Zhu, Ho, & Ding, 2005) has only been found in queens in our analysis. On the other  
323 hand, a study in *Melipona quadrifasciata* (C. C. Judice et al., 2006) has found an up-regulation in  
324 workers of a gene coding for a scavenger receptor involved in the immune response. The  
325 relationship between the caste and the immune system seems hence to be equivocal.

326 At first glance, the fact that queens are more susceptible to pathogens because of weak immune  
327 defences does not sound evolutionary stable. High productivity in laying eggs is pointless if ant  
328 queens do not survive the first encountered pathogen. Moreover, we know that group living makes  
329 individuals more prone to infection (Godfrey, Bull, Murray, & Gardner, 2006; Schmid-Hempel,  
330 1998). To overcome these issues, eusocial insects have evolved a combination of behavioural  
331 responses, called social immunity (details in Cremer et al., 2007). For instance, infected individuals  
332 are less involved in interactions and they can sometimes even be killed by their own colony. Cremer  
333 et al. also suggest that the structure of the interaction network might be shaped in a prophylactic  
334 way to prevent pathogens from reaching the queen. The queen would be thus "socially" protected  
335 from pathogens. In this context, a weaker immune system would not be an inevitably fatal issue.  
336 Supporting this assumption, a phylogenetic study in five insect species has shown that the larger the  
337 colony size is, the weaker the melanization response (López-Uribe, Sconiers, Frank, Dunn, & Tarpy,  
338 2016). In addition, ants – similarly to other eusocial insect species – have fewer genes involved in  
339 immune functions than less social insects (Libbrecht et al., 2013), what brings evidence that proper  
340 social structure can allow for a reduced immune system.

341

#### 342 4.1.3 Differences within the worker caste

343 Foragers differ from nest-workers by their higher amount of proteins involved in insecticide  
344 resistance – mostly cytochrome P450 (CYP). The role of CYP in insecticide detoxification is well  
345 documented (Oppert et al., 2015; Werck-Reichhart & Feyereisen, 2000). Because of their supplying  
346 role, foragers are directly exposed to pesticides. This larger amount of pesticide-degrading proteins  
347 may help them to face environmental toxic chemicals. Once detoxified by foragers, the food can be  
348 safely distributed to the whole colony. Overexpression of the CYP gene has also been found in the  
349 worker caste of *Melipona quadrifasciata* (C. Judice, Hartfelder, & Pereira, 2004). This could indicate a  
350 common response to insecticides among social insects. A similar response has been observed in  
351 honey bee, where the activity of two detoxification enzymes increases when workers begin to forage  
352 (Smirle & Winston, 2011). Yet, CYP is also involved in metabolic functions in insects such as  
353 hormone degradation (Feyereisen, 1999) and we cannot rule out at that stage other metabolic  
354 implications.

355 We found that a larger amount of digestive proteins (essentially alpha-amylase) are expressed  
356 in nest-workers. By storing food excess (Lenoir, 1979) and pre-digesting it, nest-workers can make it

357 quickly available for further use, thus enhancing the fitness of the whole colony by buffering  
358 environmental unpredictability in food resources, but also by increasing food processing by  
359 conspecifics. Nest-workers may also pre-digest the food for castes that do not perform this task very  
360 well. According to several studies, ant larvae do not require help to digest food (Cassill, Butler,  
361 Vinson, & Wheeler, 2005; Erthal, Peres Silva, & Ian Samuels, 2007; Went, Wheeler, & Wheeler, 1972).  
362 The pre-digested food could be more useful for the queen, allowing her synthesizing fewer digestive  
363 proteins and saving energy for other costly life history traits (*i.e.* reproduction). Whether this may be  
364 of any advantage for other adult castes must be tested by accurately measuring digestive proteins  
365 levels of expression in nest-workers and foragers.

#### 366 4.2. Other sources of variation within the workers

367 Social castes appeared to be of major importance to explain the proteome variability among  
368 adult individuals in the black garden ant. Nevertheless, unexplained variance remained (Fig. 2 C, D)  
369 and nest-workers and foragers were not all perfectly collocated, suggesting other sources of  
370 individual variation. Tan et al. (2017) highlighted that diapause can affect the proteome of the  
371 cabbage beetle (*Colaphellus bowringi*). All the colonies were nonetheless under the same day light and  
372 temperature conditions, it is hence unlikely that some individuals enter diapause and others did not.

373 Usually in ants, there is a temporal division of labour: workers specialize with their age (Jeanne,  
374 1986). However, the colony needs (*e.g.* more brood to feed, galleries to dig) can induce individuals to  
375 change caste – regardless of the age (Robinson, 1992). For instance, if most of the foragers die from  
376 predation, some of the nest-worker workers become foragers to maintain food supply of the colony.  
377 They become foragers earlier than expected. Therefore, we might find individuals of different age  
378 within the same caste. Age-related phenotype including physiological traits would consequently not  
379 be homogenous and thus explain part of the individual variability within a caste. However, we  
380 expect this age-independent caste switching effect to be minimal, since ants were reared under  
381 constant laboratory conditions for more than two years. Furthermore, all the workers used in this  
382 study were at least two years old, damping the potential impact on the proteome of a big age  
383 difference between worker castes. Although the effect of age is mitigated, we cannot be 100% sure  
384 that it does not influence our results, at least in part. Under this assumption, the age effect can be  
385 either independent or confounded with the effect of the caste. If it is independent, then it could  
386 explain the remaining variation not attributable to the caste (axis 2 of the first PCA and axis 1 of the  
387 second one). If age and caste effects are confounded, then this remaining variation would be due to a  
388 third factor, which remains unknown so far.

#### 389 4.3. A possibly multifactorial gap in lifespan

##### 390 4.3.1. Energy metabolism

391 Proteins associated with energy metabolism were more abundant in worker ants. Most of the  
392 proteins forming this group are involved in the Krebs Cycle (*e.g.* NADH dehydrogenase, succinyl  
393 ligase, citrate synthase), ATP synthesis (ATP synthase) or lipid beta-oxidation  
394 (long-chain-fatty-acids ligase 3). This suggests a higher metabolic activity (potentially associated  
395 with higher metabolic rates) in workers than in queen ants. Oxygen consumption at the colony level  
396 was found to be higher in workers than in queens of fire ants (Vogt & Appel, 1999). Oxygen  
397 consumption was even higher in workers moving the most intensively (Ferral, Holloway, Li, Yin, &  
398 Hou, 2017) or the smaller ones (Calabi & Porter, 1989), raising the question whether metabolic rate is  
399 also an important determinant of lifespan in workers.

##### 400 4.3.2. Somatic maintenance

401 Queen ants were, among others, characterized by higher amount of apoptosis-regulating  
402 proteins. As recently highlighted (Deursen, 2014), preventing the accumulation of senescent cells  
403 within tissues is a key determinant of an organism's lifespan and health. Killing dysfunctional cells  
404 seems also to be one of the keys to longevity (Berger et al., 2006; Ravikumar, Berger, Vacher, O'Kane,

405 & Rubinsztein, 2006; Tchkonina, Zhu, Deursen, Campisi, & Kirkland, 2013). The negative impact of  
406 senescent cells is mostly mediated through the Senescent Associated Secretory Pathway (Matjusaitis,  
407 Chin, Sarnoski, & Stolzing, 2016; Tchkonina et al., 2013). Contrarily, promoting senescence may also  
408 be beneficial through its implication in tissue repair (Jun & Lau, 2010) and tumour suppression  
409 (Collado, Blasco, & Serrano, 2007). Focusing on the dynamics of senescence markers over life in the  
410 different castes and in different species may be of interest in the near future to estimate how  
411 senescence control has co-evolved with both longevity and sociality in ants. Queens also had higher  
412 amount of proteins belonging to the two functional groups 'Detoxification' and 'Chaperone'. In  
413 these groups are found proteins involved in macromolecules restoration after stress (e.g. aldehyde  
414 dehydrogenase, selenium-binding protein 1-a), ensuring proper folding of proteins (GrpE protein  
415 homolog, T-complex protein 1) or regulating cell energy production during stress (mitochondrial  
416 UCP2). Higher protein quantities from these groups characterized the queens. A previous study in *L.*  
417 *niger* has also found that the expression of somatic maintenance genes is up-regulated in queens  
418 (Graeff et al., 2007). This suggests that the queen's longevity might, at least in part, result from a  
419 higher energy investment in preventing cell damages.

#### 420 4.3.3. Reproduction and refinement of longevity trade-off in queens

421 As expected, the queen's reproductive role was confirmed by the analysis, since proteins related  
422 to reproduction were solely found in queen ants. The Reproduction functional group was only made  
423 of sperm-related proteins (e.g. sperm-associated antigen). This protein abundance can be explained  
424 by the spermathecal storage of sperm in queens. In our study and contrary to genomic studies  
425 (Graeff et al., 2007; Grozinger et al., 2007), vitellogenin was not overexpressed in the reproductive  
426 caste. As highlighted by Amdam et al. (2003), vitellogenin is also found in workers. The difference in  
427 protein quantity might not be sufficient to isolate workers from queens. During oogenesis, lipids are  
428 required for the biosynthesis of the egg cell membrane or lipoproteins (Engelmann, 1979), and a  
429 functional reproductive system synthesizes steroid hormones, which require lipid precursors  
430 (Hoffmann, 1980). Consistently, queens had on average a higher quantity of proteins involved in  
431 lipid transport (e.g. phospholipid-transporting ATPase, apolipoprotein D) or lipid synthesis (fatty  
432 acid synthase). Since energy is limited, investment in reproduction is done at the expense of other  
433 functions. Consequently, when a species or an individual is long-lived, we usually expect a lower  
434 energy investment in reproduction according to the fecundity/lifespan trade-off (Stearns, 1977).  
435 Queens of social insects do not seem to undergo this trade-off, as they are both long-lived and the  
436 only reproductive individual in the colony (Blacher, Huggins, & Bourke, 2017). This is notably the  
437 case in black garden ant queens characterized by intense reproduction combined with extreme  
438 lifespan – up to 28 years in *L. niger* (Parker, 2010). The solution could be not to consider a  
439 lifespan-vs-fecundity trade-off, but a lifespan-fecundity-vs-immunity trade-off. An active immune  
440 system is energy-consuming both at the individual (Moret & Schmid-Hempel, 2000) and colony  
441 level (Evans, Pettis, & Mueller, 2005). The investment in immunity has been shown to impair  
442 reproduction and/or growth (Kopp & Medzhitov, 2009). For instance, up-regulation of immune  
443 genes decreases reproductive success in urban blue tits (Capilla-Lasheras et al., 2017). Consequently,  
444 if the queen invests less in the immune system – as suggested by our data – she might save energy  
445 for reproduction or/and mechanisms aiming to avoid or repair damages to macromolecules.

#### 446 4.3.4. ToR pathway in social insects

447 Proteins whose quantity differed between castes were downstream component and/or weakly  
448 related to the Tor pathway. We cannot therefore highlight a clear involvement of the ToR pathway in  
449 caste differentiation in black garden ants. Whereas, it is unequivocal in honey bee, where  
450 queen-destined larvae upregulate this signalling pathway relatively to worker-destined ones (Page  
451 & Amdam, 2007; Patel et al., 2007). As the queen-destined larvae are overfed, such a finding  
452 confirms the nutrient-sensitive role of ToR pathway to control growth according to the food  
453 availability. On the other hand, activation of the ToR pathway is strongly associated with a shorter  
454 lifespan among diverse taxa (Kapahi et al., 2010). The longevity secret of queen social insects might

455 be a ToR expression modulation depending on their age. When queens are still larvae, an active ToR  
456 pathway (stimulated by overfeeding) would allow somatic growth and ovaries maturation. Then,  
457 ToR expression would decrease with age, protecting the queens from senescence. The opposite  
458 scheme would take place in workers.

## 459 5. Conclusions

460 We showed that proteomics allows assessing fine molecular differences induced by task  
461 specialization in a social insect species. The non-targeted screen of the whole proteome highlighted a  
462 wide diversity of caste-dependent functions from immunity to reproduction to digestion to  
463 insecticide resistance. Our study also raises evolutionary questions about longevity and energy  
464 trade-offs in eusocial species, beyond the classical free-radical theory of ageing. Thanks to our  
465 exploratory approach, we now have a more global insight into all the functions that can be affected  
466 by the division of labour in a eusocial species. Some are well studied (*e.g.* social immunity), others  
467 less, especially in the adult stage (*e.g.* the ToR pathway, difference in the metabolism of digestion).  
468 We therefore hope to pave the way for future experiments to accurately test the numerous and  
469 diverse molecular mechanisms induced by a eusocial lifestyle.

470 **Supplementary Materials:** The following charts and tables are available online. Figure S1: PCAs used for  
471 variable selection (before clustering by biological function). Table S1: Raw data from the mass  
472 spectrometry-based proteome analysis, Table S2: Proteins used for the PCAs with the biological functions  
473 amongst the three castes, Table S3: Proteins used for the PCAs with the biological functions amongst workers  
474 only, Table S4: Inertia's 95th percentile for the first two dimensions of 10,000 PCAs. Tables S1-S3 present raw data and are  
475 available in a separated online repository (doi: [10.17632/xk4rpddxx6.1](https://doi.org/10.17632/xk4rpddxx6.1)).

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