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

Martin Quque^{*1}, Claire Villette², François Criscuolo¹, Cédric Sueur^{1,3}, Fabrice Bertile^{1,4,†}, Dimitri Heintz^{2,†}

¹ Université de Strasbourg, CNRS, IPHC UMR 7178, F-67000 Strasbourg, France – 23 rue du Loess, 67037 Strasbourg Cedex 2, France

² Plant Imaging & Mass Spectrometry (PIMS), Institut de biologie moléculaire des plantes, CNRS, Université de Strasbourg, 12 rue du Général Zimmer, 67084 Strasbourg, France.

³ Institut Universitaire de France, 75005 Paris, France

⁴ Infrastructure Nationale de Protéomique ProFI – FR2048, Strasbourg, France

† Share co-seniorship of the paper

* Correspondence: martin.quque@iphc.cnrs.fr - +33 388 106 951

Orcid IDs:

- MQ: 0000-0001-7386-1416
- CV: 0000-0003-2564-5529
- FC: 0000-0001-8997-8184
- CS: 0000-0001-8206-2739
- FB: 0000-0001-5510-4868
- DH: 0000-0001-7368-1394

Abstract

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

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

Declarations

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Availability of data and material

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

Code availability: Not applicable.

Authors' contributions

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

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(Experimental protocols involving ants do not require ethical approval)

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1. Introduction

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

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

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

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

2. Methods

2.1. Ant keeping and preparation of samples before metabolomics

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

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

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

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

2.2. Metabolomic analysis

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

2.2.1. Sample preparation

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

2.2.2. LC-HRMS analysis

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

2.2.3. Metabolite annotation and quantification

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

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

2.3. Data filtering and statistics

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

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

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

2.3. Metabolite functional and structural classification

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

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

3. Results

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

3.1. Absent metabolites

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

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

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

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

3.3. Pairwise comparison of ant behavioural castes' metabolomes

For each pairwise comparison (foragers *vs.* queens, foragers *vs.* nest-workers and nest-workers *vs.* queens), Table S7 (ESM1) identifies the metabolites significantly (FDR < 0.05) and strongly down-regulated ($\log_2\text{FC} < -2$) or up-regulated ($\log_2\text{FC} > 2$) and provides the related molecular formula, chemical class, and biological processes.

Like the PCAs, the heat maps (Fig. 3) constructed from the 50 metabolites with the highest $\log_2\text{FC}$ confirm that the metabolic differences allow the three behavioural castes of black garden ants to be clearly separated using blind hierarchical classification. There were, however, intra-group variations similar to those highlighted by the PCA, *e.g.* the queen sample Q3 was separated from the other queen samples.

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

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

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

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

3.4. Metabolite set enrichment analysis (MSEA)

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

4. Discussion

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

4.1. Queens are protected from stress, pathogens and pollutants

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

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

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

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

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

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

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

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

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

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

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

4.4. Communication, pheromones and other hormones

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

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

4.5. Intra-caste variations and non-identified functions

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

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

5. Conclusion

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

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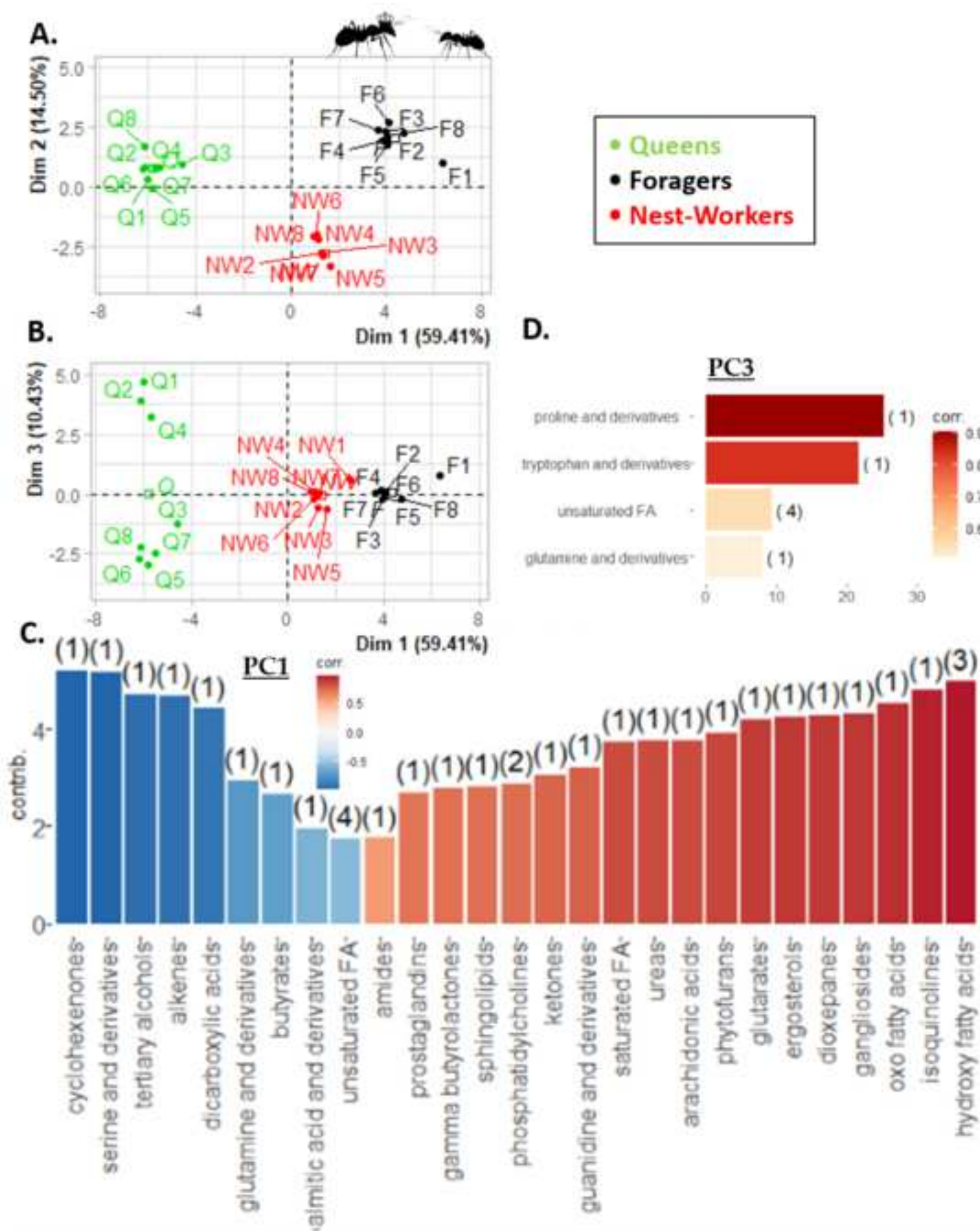


Figure 2

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