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Gas chromatography–mass spectrometry metabolite profiling of worker honey bee (*Apis mellifera* L.) hemolymph for the study of *Nosema ceranae* infection

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ABSTRACT

Here, we are presenting a gas chromatography–mass spectrometry (GC/MS) approach for the study of infection of the worker honey bee (*Apis mellifera* L.) by the newly emerged obligate intracellular parasite *Nosema ceranae* based on metabolite profiling of hemolymph. Because of the severity of the disease, early detection is crucial for its efficient control. Results revealed that the parasite causes a general disturbance of the physiology of the honey bee affecting the mechanisms controlling the mobilization of energy reserves in infected individuals. The imposed nutritional and energetic stress to the host was depicted mainly in the decreased levels of the majority of carbohydrates and amino acids, including metabolites such as fructose, L-proline, and the cryoprotectants sorbitol and glycerol, which are implicated in various biochemical pathways. Interestingly, the level of glucose was detected at significantly higher levels in infected honey bees. Metabolomics analyses were in agreement with those of multiplex quantitative PCR analyses, indicating that it can be used as a complementary tool for the detection and the study of the physiology of the disease.

1. Introduction

Each organism is a complex functional entity in which the quantitative and qualitative metabolite composition or metabolomes of its different compartments/organs fluctuate within "normal" values. The latter depends on several factors such as, genotypic composition, developmental stage, nutrition, and environmental factors. Exposure of an organism to stress results in behavioral and physiological alterations, leading to a new metabolic equilibrium. Therefore, the monitoring of metabolic regulatory networks could provide valuable insights of the factors that cause their disturbance and furthermore, could be used to define the status of an organism as normal/healthy or not (Fiehn, 2002). Although metabolomes are complex, the development of metabolomics applying advanced analyzers have facilitated the monitoring of global metabolic networks of organisms and their fluctuations, thus making metabolomics a robust bioanalytical tool for diagnostic and monitoring purposes (Aliferis and Jabaji, 2011; Hunter, 2009; Kaddurah-Daouk and Krishnan, 2008; Simpson et al., 2011; Vinayavekhin et al., 2009).

Biological fluids of vertebrates such as urine and blood, are fundamental components of their physiology, which among others, contain metabolic signatures of various biochemical pathways that could be used for the characterization of their physiological status. Based on this principal, metabolomics approaches have been successfully developed for the discovery of disease biomarkers in blood and urine, as well as early detection and monitoring of disease progress in humans (Bogdanov et al., 2008; Kim et al., 2010; Madsen et al., 2011; Slupsky et al., 2009; Zhou et al., 2010). In contrast, the application of metabolomics in the study of the hemolymph, which is strictly speaking analogous to blood in invertebrates, is largely unexploited and has lagged behind mammalian serum studies. Only a handful of reports had focused on hemolymph metabolome-based toxicity studies conducted on the Daphnid *Daphniae magna* (Poynton et al., 2011; Taylor et al., 2010) and disease studies of hemolymph of the Atlantic Blue Crab (Schock et al., 2010).

The European honey bee (*Apis mellifera* L., Hymenoptera) is the world's most important pollinator accounting for an estimated gross world annual income of $217 billion USD (Gallai et al., 2009). Crop systems benefit from pollination by having an increased yield, fruit size, seed set, and better quality of fruits. Absence of pollination from *A. mellifera* is estimated to cause significant yield losses (Klein et al., 2007).

Among the reported bee parasites, *Nosema ceranae* represents a recently emerged threat for bee colonies worldwide which can result in decreased pollination and honey yield, hive depopulation, or hive death (Fries, 2010; Higes et al., 2008; Martín-Hernández et al., 2007; Vanengelsdorp and Meixner, 2010; Vinayavekhin et al.,

Abbreviations: HCA, hierarchical cluster analysis; OPLS-DA, orthogonal partial least squares-discriminant analysis; RT-PCR, real-time polymerase chain reaction.

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2009). Because of the severity of the disease, early detection is crucial for efficient control. Seasonal patterns of *N. ceranae* infections, however, are inconclusive (Copley and Jabaji, 2012; Gisder et al., 2010; Martín-Hernández et al., 2007; Traver and Fell, 2011) making early detection of the parasite difficult. Microsporidian fungi often alter their host's physiology, suppress their immune system and alter behavior in order to maintain a more favorable environment thus prolonging their ability to reproduce (Antúnez et al., 2009; Fisher, 1963; Fisher and Sanborn, 1964; Keeling and Fast, 2002; Kelly et al., 2002; Mathis, 2000). Recent studies have demonstrated that *N. ceranae* infections can alter the energy demands of honey bees (Martín-Hernández et al., 2011; Mayack and Naug, 2009; Naug and Gibbs, 2009), suppress their immune system (Antúnez et al., 2009) and alter pheromone production in worker and queen honey bees (Alaux et al., 2011; Dussaubat et al., 2010). The fungal parasite enters the adult bee orally and multiplies in its gut and spreads to different glands (Copley and Jabaji, 2012), imposing a metabolic demand on its host (Naug and Gibbs, 2009) and proliferates until the nutrients in the host-cells are exhausted, which triggers sporulation.

Metabolomics holds great promise for identifying indicator metabolites that would allow the easy diagnosis of a disease state. To date, such efforts towards identifying such biomarkers in mammalian systems have succeeded (Issaq et al., 2011; Mamas et al., 2011). Therefore, there is no reason to expect that metabolite biomarkers might not be found in other species such as honey bees. Towards this end, we tested the applicability of gas chromatography–electron impact–mass spectrometry (GC/EI/MS) metabolomics for the discovery of changes in the hemolymph of *A. mellifera* L. infected by *N. ceranae* and the detection of corresponding biomarkers. Additionally, analysis is expected to provide insights into the effects of the disease on bee physiology. To our knowledge, this is the first metabolomics study on the effects of *N. ceranae* on the physiology of the honey bee based on analysis of hemolymph. For the validity of metabolomics analyses, detection of *N. ceranae* in the analyzed honey bee samples was confirmed by quantitative real-time polymerase chain reaction (RT-PCR) assay.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents used in the extraction of hemolymph and GC/MS analyses were of the highest available purity. Ethyl acetate and methanol were purchased from Fisher Scientific Company (Ottawa, Ontario, Canada). Pyridine, methoxylamine hydrochloride, and *N*-methyl-*N*-(trimethyl-silyl)trifluoroacetamide (MSTFA) for sample derivatization and analytical standards were purchased from Sigma-Aldrich Ltd. (Oakville, ON, Canada).

2.2. Colony establishment and sampling

As part of a larger three-year study conducted on the seasonality and prevalence of *Nosema* species in Québec honey bees (Copley and Jabaji, 2012), eight honey bee colonies were started in 2008 using freely-mated sister *A. mellifera* hybrid Italian queens from a known established genetic line. The bees were obtained from a local queen breeder (Les reines Moreau®, Saint-Liboire, Québec) along with 1.5 kg of worker bees. Each colony was comprised of a 9-frame Langstroth hive body and placed at an apiary at the Centre de Recherche en Sciences Animales de Deschambault (CRSAD) located in Deschambault (46.6734°N, 71.9169°W), Quebec, Canada. All colonies were treated identically and were placed 1 m apart from each other. For this study, approximately 100 worker bees were collected from the side frames of the top super from each colony in May 2009, 1 year after colony establishment. Bees were euthanized by freezing them in liquid nitrogen and stored at −80 °C until further use.

2.3. Colony screening for Nosema infections

For the development and standardization of metabolomics models and in order to confirm the presence and infection levels of *N. ceranae* in the analyzed samples, spore counts from bee guts and qPCR analyses were performed prior to GC/MS analysis (Copley and Jabaji, 2012). Results confirmed that May of 2009 was the only month in which one out of the eight honey bee colonies was *Nosema* free, another colony was heavily infected only with *N. ceranae* containing an average of 2.58×10^6 spores/bee, while the remaining colonies contained both *N. ceranae* and *Nosema apis* infections and were not used in this study. Therefore, these two colonies were retained for hemolymph metabolomics study and prompted us to collect new samples to further conduct qPCR analysis on the same two hives prior to metabolomics analyses, as described in Section 2.4.

2.4. Tissue dissection and hemolymph collection

For each collection of 100 bees, the abdomens of 70 bees were dissected and prepared for DNA extraction as previously described (Copley and Jabaji, 2012). Following intestinal dissection, the hemolymph of the dissected bees was collected according to the method of Bozic et al. (2007). Briefly, the hemolymph of each bee was removed by making a small incision in the thorax and collected using a 10 μL pipette for a total of 2–3 μL per bee. This procedure has been successfully used in previous honey bee hemolymph studies and ensures that neither the honey bee stomach nor the intestine is punctured during sampling. Specific care was given in order to avoid any contamination of the hemolymph from intestines. In case of contamination of the hemolymph samples were discarded. From the same collection of 70 bees per colony, the intestines were dissected and pooled, into identical groups of 10 for a total of seven pooled samples. DNA was extracted according to Copley and Jabaji (2012).

2.5. Triplex quantitative real-time PCR

Already published species-specific primers (Chen et al., 2009) and probes for *N. ceranae* (Nceranae; GenBank accession #DQ486027), *N. apis* (Napis; GenBank accession #DQ486027) and *A. mellifera* β-actin (GenBank accession #AB023025) as the normalizer gene, resulting in amplified products of 250 bp, 269 bp, and 181 bp, respectively, were synthesized by Integrated DNA Technologies (San Diego, CA) for triplex qPCR. Fluorophore probe labeling for all primers and standard curve preparation was performed according to Copley and Jabaji (2012) to allow for the amplification of all amplicons simultaneously in a triplex qPCR reaction using the Stratagene MxPro 3005. Probes were labeled using the following fluorophores: 6FAM for *A. mellifera* β-actin, TYE665 for *N. ceranae*, and HEX for *N. apis*.

Each triplex amplification mixture contained: 1× PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM each dNTP, 0.3 μM each primer, 0.15 μM each probe, 2 μM ROX, 1.5 U recombinant Taq DNA polymerase (Fermentas, Burlington, ON), and 50 ng DNA in a total volume of 25 μL. All qPCR reactions were run using the conditions described by Copley and Jabaji (2012). Standard curves ranging from 10^2 to 10^9 copies for each amplicon and no-template controls were run with each plate. Analyses were performed in triplicate. The specificity of triplex qPCR was confirmed by gel electrophoresis and sequence analysis to verify the correct size and specificity of qPCR products.