



## Metabolomic Studies in *Apis mellifera*

Jewon Jung\*

Department of SmartBio, College of Life and Health Science, Kyungsung University, Busan 48434, Republic of Korea

### Abstract

The application of metabolomic analysis has emerged as a potent tool for exploring honeybee physiology. This technique enables researchers to detect thousands of chemical compounds simultaneously in a single sample, which represent a combination of gene expression, enzyme activity, and environmental factors. The utilization of metabolomics has facilitated a broad range of investigations in honeybees, yielding novel insights into gene function and metabolic status that would otherwise be unattainable. The present review outlines the applications of metabolomic analysis since its introduction in the field, while also discussing the main techniques employed for metabolomic studies in honeybees. Furthermore, we identify and highlight the new avenues for future research in this field.

### Keywords

Metabolomics, Honeybees, Pathogens, Pesticides, Diets

## INTRODUCTION

Pollinators are vital for maintaining ecosystem equilibrium, enabling sustainable agriculture, and ensuring food security (Potts *et al.*, 2016). Animal pollination is necessary for the production of roughly 75% of vegetable food crops and seeds, which increases crop quality, shelf life, and commercial value (Klein *et al.*, 2007). Despite being the most critical pollinators worldwide, *Apis mellifera* honeybee colonies have declined globally in recent decades, attracting considerable attention (vanEngelsdorp and Meixner, 2010). Climate change, habitat fragmentation and loss, invasive species, pathogens, and agrochemicals are suspected of negatively impacting their survival (Higes *et al.*, 2007; Genersch, 2010; Henry *et al.*, 2012; Guez, 2013). Agrochemicals are extensively utilized in agriculture and pose a significant threat to populations, as they can easily expose and impact them during foraging and pollination, causing widespread concern.

To elucidate disorders and timely detect alterations in bee physiology, it is crucial to employ molecular instruments for screening the gamut from genome to metab-

olome (Lankadurai *et al.*, 2013). Of these instruments, metabolomics is the most informative, as it focuses on the final stage of the “omics cascade” by examining small metabolites and strongly correlates with the phenotype (Alonso *et al.*, 2015; Liu and Locasale, 2017). After the completion of whole genome sequencing of various insect species such as fruit flies, mosquitoes, and honeybees, research and development in the field of omics, including transcriptomics, have been actively pursued to understand the functions of individual genes. Among them, metabolomics is a research field that tracks quantitative or qualitative changes in metabolites within an organism and interprets various physiological phenomena or mechanisms at the cellular or organismal level in a comprehensive manner based on this information.

Insect metabolomics research, using flies and mosquitoes, is actively ongoing, and research on honeybee metabolism has greatly increased since the 2010s (Table 1). Honeybee metabolomics research, specifically, is a study that comprehensively understands the complex physiological phenomena of honeybees by tracking and investigating the temporal and spatial changes of all

**Table 1.** Applications of metabolomics to *Apis mellifera*

Research topic	Sample type	Techniques utilized	Conclusions	References
Carbendazim exposure	Brain	LC/MS	Metabolites associated with energy and amino acid metabolism showed high abundance.	(Chen <i>et al.</i> , 2021)
Carbaryl and acetamiprid exposure	Body of larvae	LC/MS	L-proline, phenylpropionylglycine, N2-acetyl-L-ornithine, dimethylglycine, L-anserine and DL-tryptophan, were significantly decreased in bee larvae after exposure to carbaryl.	(Gao <i>et al.</i> , 2022)
Clothianidin exposure	Brain	LC/MS	Healthy bee brains have high levels of alkyl-ether linked phospholipids (plasmanyl) (51.42%) and low levels of plasmalogens.	(Morfin <i>et al.</i> , 2022)
Glyphosate exposure	Head part	<sup>1</sup> H NMR	Metabolomic perturbations can occur in response to short-term exposure to other environmental stressors or long-term exposure.	(Wang <i>et al.</i> , 2022)
Thiacloprid exposure	Head part	LC/MS GC/MS	Honeybees activate their detoxification system to resist thiacloprid toxicity, as indicated by abundant metabolites enriched in oxidative stress and detoxification pathways.	(Shi <i>et al.</i> , 2018)
Acetamiprid exposure	Hemolymph	LC/MS	As acetamiprid concentration increased, traumatic acid content increased while tryptophan metabolite 1-kynurenine and indole content decreased, along with a decrease in lipid content.	(Shi <i>et al.</i> , 2023)
Nicotine exposure	Larvae	LC/MS	Honeybee larvae can efficiently break down dietary nicotine through an adaptive detoxification process, significantly impacting their primary metabolism, growth, and carbohydrate and energy metabolism.	(du Rand <i>et al.</i> , 2017)
Nosema infection	Hemolymph	GC/MS	The parasite disrupts honeybee physiology, impacting energy mobilization mechanisms in infected individuals.	(Aliferis <i>et al.</i> , 2012)
Nosema infection	Hemolymph	LC-MS NMR	Found that <i>Nosema ceranae</i> infection in honeybees causes changes in the composition of hemolymph, including alterations in amino acid, carbohydrate, and lipid metabolism.	(Jousse <i>et al.</i> , 2020)
Varroa infection	Brain	LC-MS/ MS	Linoleic acid metabolism; propanoate metabolism; and glycine, serine, and threonine metabolism were acutely perturbed in varroa mite-infected honeybees.	(Wu <i>et al.</i> , 2017)
Varroa infection	Abdomen (Heads, poison sacks, and gastrointestinal tracts were removed)	<sup>1</sup> H NMR	Parasitised worker bees exhibited low levels of saccharides and amino acids.	(Kunc <i>et al.</i> , 2023)
Ascospaera infection	Gut	LC-MS	Ascospaera infection affects the ability of honeybee larvae to handle oxidative stress, revealing new understanding of altered physiological responses during the infection.	(Li <i>et al.</i> , 2020)

**Table 1.** Continued

Research topic	Sample type	Techniques utilized	Conclusions	References
Artificial diets	Head, thorax (excluding legs and wings), abdomens with guts intact.	LC/MS GC/MS	Metabolomics results help understand how microalgae diets improve bee growth, antioxidant activity, and heat shock protein gene expression.	(Ricigliano <i>et al.</i> , 2022)
Pollen composition and diets	Hemolymph	LC/MS	Pear pollen had the highest nutritional value among the three bee pollens in terms of amino acid levels, followed by rape and apricot pollen, and these differences in amino acid composition influenced the lipid and amino acid metabolism pathways in early adult honeybee hemolymph.	(Chang <i>et al.</i> , 2022)
Dietary phytochemicals	Body	LC/MS	Feeding bees pollen/nectar from various plants decreased pesticide residue in bees exposed to imidacloprid, tau-fluvalinate, and tebuconazole.	(Ardalani <i>et al.</i> , 2021)
Gut bacteria	Hemolymph	LC/MS	Different species regulate specific modules of metabolites in the hemolymph; Gilliamella regulates metabolites involved in carbohydrate and glycerophospholipid metabolism pathways, while Lactobacillus Firm4 and Firm5 mainly alter amino acid metabolism pathways.	(Zhang <i>et al.</i> , 2022)
Gut microbiota	Gut hemolymph	GC/MS	The gut community's effects on gut and hemolymph metabolic profiles indicate that gut bacteria break down plant polymers from pollen, providing nutrients to the host.	(Zheng <i>et al.</i> , 2017)
Summer and winter bees	Body	<sup>1</sup> H NMR	Winter bees had a higher concentration of trehalose and decreased levels of all amino acids except proline and alanine compared to summer bees.	(Lee <i>et al.</i> , 2022)

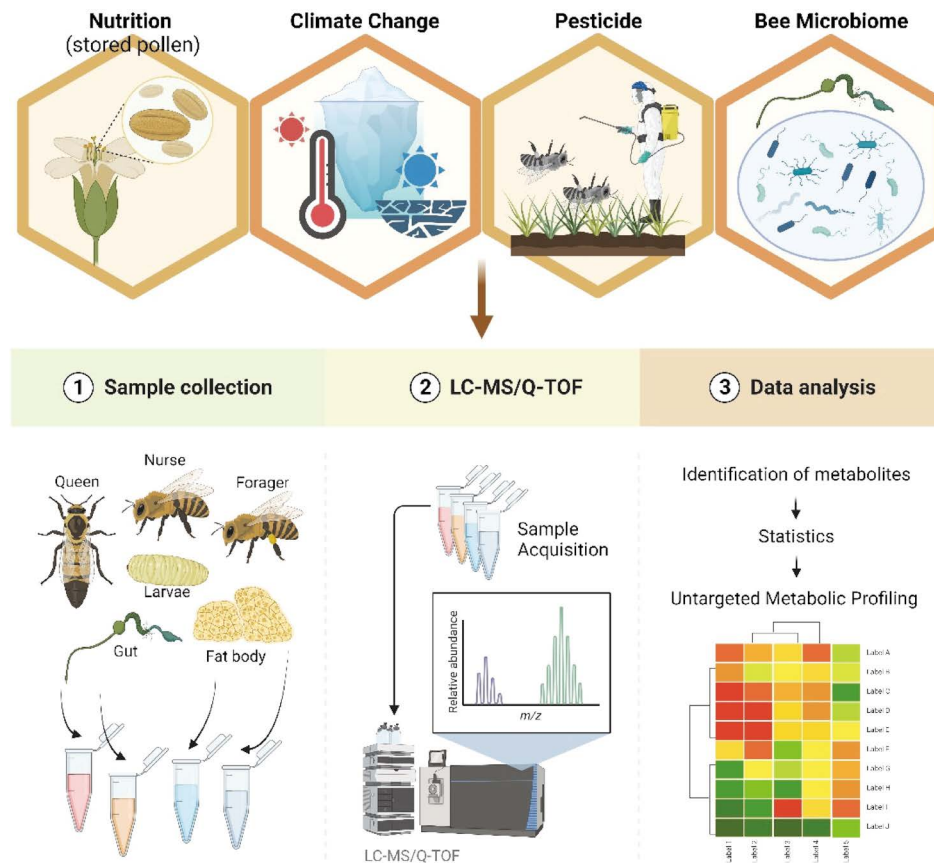
metabolites present in honeybee tissues in response to various internal and external environmental conditions, such as stress responses to behavior, infection, and temperature changes, as well as symbiosis with bacteria (Chakrabarti *et al.*, 2019; Lee *et al.*, 2022; Ricigliano *et al.*, 2022; Kunc *et al.*, 2023). Currently, the field of honeybee metabolomics research is widely used as a research tool for functional genomics, including the elucidation of metabolic regulatory mechanisms and the understanding of physiological responses, due to the biological information provided by previously known individual honeybee genomics and the development of analytical instruments.

The goal of metabolism research is to understand and interpret the metabolic processes and pathways within

cells and tissues. Therefore, honeybee metabolism research is also evolving in the direction of understanding metabolic pathways from a systems biology perspective for the purpose of identifying various unknown metabolic pathways (Fig. 1). In this paper, we aim to introduce the current status of honeybee metabolism research.

## METHODS USED FOR METABOLOMIC ANALYSIS IN HONEYBEES

The metabolome refers to any small molecule (< 1,500 Da) that an organism ingests, produces, catabolizes, or encounters in its surroundings (Wishart *et al.*, 2007). While the specific composition of the honeybee metabo-



**Fig. 1.** A descriptive overview is given regarding the procedure of extracting and studying metabolites. Honeybee metabolomic studies were conducted under four different conditions, including nutrition, climate change, pesticide exposure, and microbiome analysis. Samples were collected from bee larvae, adult bees, and specific tissues, and their metabolic compositions were examined using untargeted and targeted liquid chromatography-mass spectrometry (LC-MS) or gas chromatography-mass spectrometry (GC-MS) techniques. The data obtained from LC-MS or GC-MS were processed using specialized metabolomics software.

lome has not yet been defined, the Human Metabolome Database currently contains information about more than 42,000 small molecules (Wishart *et al.*, 2013). This number is expected to increase as technology advances. Given the high conservation of metabolic pathways between flies and humans (Rajan and Perrimon, 2013), it is likely that the honeybees metabolome also encompasses a similar range of compounds. However, due to the diverse nature of these molecules, no single method can effectively measure all of them. Consequently, each study must focus on measuring a specific subset of the metabolome.

The majority of metabolomic investigations rely on three main types of equipment: Gas Chromatography/Mass Spectrometry (GC-MS), Liquid Chromatography/Mass Spectrometry (LC-MS), and Nuclear Magnetic Resonance (NMR). Each of these methods has its own

advantages and disadvantages, and although the availability of equipment often determines the choice of analysis, comprehending the capabilities of each instrument can greatly enhance the efficiency of study design.

## APPLICATIONS OF METABOLOMICS IN *APIS MELLIFERA*

Despite the fact that the technology needed for metabolomic research has only been accessible for about 15 years, more than 20 studies in honeybees have taken advantage of this technology, encompassing a broad spectrum of topics as shown in Table 1. These applications have demonstrated their significance in the identification of metabolites that are specific to certain tissues, and in examining the impacts of pathogens, diets, envi-

ronmental conditions, or pesticide stresses on the organism's overall physiology, also demonstrated in Table 1. This type of analysis has frequently provided unique insights into metabolism that would not have been possible using any other methods.

### **1. Comparative analysis of metabolites among honeybee groups according to season (summer and winter)**

Due to global warming, climate change has occurred, leading to a decrease in the habitat of honeybees and a subsequent decrease in the number of honeybee populations (Le Conte and Navajas, 2008). The gradual and consistent reduction in the capacity of honeybee populations to survive through winter in different parts of the world, such as North America and Europe, is a concerning occurrence that has been reported in various studies (vanEngelsdorp *et al.*, 2015; Gray *et al.*, 2019). In the last few decades, an increase in mortality rates during overwintering has been observed (vanEngelsdorp *et al.*, 2009), which is likely attributable to a combination of biotic and abiotic stressors. These stressors include the *Varroa destructor* mite (van Dooremalen *et al.*, 2012), *Nosema* sp. Microsporidia (Higes *et al.*, 2008), viral infections (Highfield *et al.*, 2009), exposure to pesticides (Simon-Delso *et al.*, 2014), dietary and nutritional deficiencies (Requier *et al.*, 2017), unfavorable winter conditions (Steinhauer *et al.*, 2015), and climate change (Le Conte and Navajas, 2008). Depending on the temperature change, the honeybee population can be divided into two groups: summer bees with a short lifespan and winter honeybees with a long lifespan. Summer bees mainly engage in brood rearing, nectar and pollen collection, and have a lifespan of about 15–38 days. On the other hand, as brood rearing begins to decline in the fall, winter bees begin to emerge, and their main task is to keep the colony warm throughout the winter and to raise the first new generation in late winter or early spring, with a lifespan of about 140–320 days (Page and Peng, 2001).

Although many studies have revealed the biochemical parameters of the difference in lifespan between summer honeybees and winter honeybees, relatively few studies have been conducted on metabolic changes within honeybees. Recently, differences in metabolic profiling between summer honeybees and winter honey-

bees were revealed through  $^1\text{H}$  NMR analysis (Lee *et al.*, 2022). According to the results, there were 28 metabolites that showed statistically significant differences between the two groups, which can be divided into carbohydrates, amino acids, choline-containing compounds, and unknown compounds based on the metabolite groups. Compared to summer bees, winter bees had higher concentrations of fructose, sucrose, and trehalose, while their levels of amino acids, except for proline and alanine, were lower than those of summer bees. Carbohydrates such as sucrose and trehalose are essential energy sources for bees and are stored as glycogen in the fat body (Blatt and Roces, 2001). Therefore, high levels of carbohydrates can be converted to high levels of glycogen, which can be used as a survival energy source during the winter. Additionally, trehalose is known to protect bees from various stresses such as dryness, low temperature, and hibernation (Watanabe *et al.*, 2002; Kostal *et al.*, 2007; Overgaard *et al.*, 2007). Therefore, the high trehalose levels in winter bees are considered an adaptive response to withstand cold temperatures and other environmental stresses.

Proline is the primary amino acid found in the hemolymph of honeybees (Hrassnigg *et al.*, 2003) and is recognized as an insect cryoprotectant, similar to trehalose (Kostal *et al.*, 2012). Studies on freeze tolerance in fruit flies (*Drosophila melanogaster*) have shown that proline and trehalose are the most abundant metabolites accumulated during cold adaptation (Kostal *et al.*, 2012; Williams *et al.*, 2016). Therefore, the elevated levels of trehalose and proline in winter bees function as cryoprotectants, safeguarding the integrity of cellular membranes during the cold winter period, maintaining optimal water levels, and serving as an energy reservoir.

Meanwhile, Lee *et al.* (2022) showed results that adenosine monophosphate (AMP) and oxidized nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ), which are nucleotide metabolites, were also produced more highly in winter bees than in summer bees. The changes in the concentrations of AMP and  $\text{NAD}^+$  are closely related to the cellular energy metabolism, and it is known that they regulate the expression of AMP-activated protein kinase (AMPK) and sirtuin genes (Hardie, 2007; Bonkowski and Sinclair, 2016), thereby regulating lifespan. In other words, activated AMP and  $\text{NAD}^+$  have been shown in studies using *C. elegans* and *D. melanogaster* to activate

the expression of AMPK and sirtuin, thereby extending lifespan. Therefore, the increased expression of AMP and NAD<sup>+</sup> in winter bees can be considered as one of the switches that promote the transition from a short-lived generation (summer bees) to a long-lived generation (winter bees).

## 2. Metabolic profiles of honeybees in response to changes in artificial flower composition

When nutrient sources are limited, the productivity of honeybees is negatively impacted. Inadequate nutrition causes a decrease in the supply of replacement bees, leading to a decline in the number and vitality of bees within colonies, as well as a reduction in their resistance to prevalent diseases (Paray *et al.*, 2021). Therefore, managed honeybee colonies are commonly provided with artificial diets known as “pollen substitutes” due to insufficient pollen availability in the environment and the consequent risk of malnutrition. Protein-rich components such as soy, corn gluten are typically used in various diet formulations to supply essential amino acids (Brodschneider and Crailsheim, 2010). However, despite the widespread use of these diets, their effectiveness compared to natural pollen remains uncertain. Recent studies have explored the potential of microalgae as a dietary supplement for managed honeybees (Madeira *et al.*, 2017). Notably, *Chlorella*, a eukaryotic microalga, and *Arthrospira*, a prokaryotic cyanobacterium commonly known as spirulina, have been identified as rich sources of protein, fatty acids, sterols, and other bioactive compounds with nutraceutical properties. These microalgae are easily digestible and have been found to mimic the growth characteristics of a natural pollen diet. However, despite promising results, the underlying metabolic mechanisms responsible for the positive effects of microalgae on bee health remain poorly understood.

Ricigliano *et al.* (2022) have found that microalgae can be used as a feed additive, which can be utilized as a natural product to improve honeybee health. They added *Chlorella vulgaris* and *Arthrospira platensis* to the feed and provided it to the honeybees to study the changes in feed consumption and metabolite profiles. Untargeted metabolite analysis was performed using LC-MS and GC-MS to measure the metabolite profiles of honeybees that consumed the natural feed and those that consumed the feed containing microalgae. Two groups of honey-

bees that consumed different diets shared 248 metabolites in LC-MS and 87 metabolites in GC-MS. Twenty-five specific metabolites were identified according to the diet, which were found to contain lipids, essential fatty acids, vitamins, and plant compounds. The honeybees that consumed pollen diet with algae showed higher levels of linoleic acid, zeaxanthin, lutein,  $\alpha$ -tocopherol, and  $\beta$ -carotene. These metabolite analysis results confirmed that algae such as *Chlorella* included in artificial pollen diet can regulate honeybee growth, antioxidant activity, and heat shock protein gene expression. Therefore, algae like *Chlorella* can be used as sustainable artificial supplementary and can be utilized as a natural product to improve honeybee health. This metabolite analysis provides important information for the development of precise nutritional management of honeybee diet.

## 3. Comparative analysis of honeybee metabolites in response to insecticide exposure

Pesticides have a prevalent application in agriculture, albeit posing significant threats to the well-being of numerous non-target organisms (Desneux *et al.*, 2007; Schafer *et al.*, 2012). In recent times, there has been a significant focus on the impact of neonicotinoid pesticides and their potential to harm the health of honeybee colonies worldwide (Blacquiere *et al.*, 2012). Neonicotinoid insecticides act as systemic insecticides, causing abnormal excitement and convulsive paralysis in insects, and are widely used in agriculture to control various types of pests (Brown *et al.*, 2006; Simon-Delso *et al.*, 2015). The exposure to insecticides is not limited to direct contact; rather, it can also occur through the ingestion of contaminated food sources, such as nectar, pollen, and water. Extensive research has reported the presence of neonicotinoid insecticides such as imidacloprid, thiamethoxam, and thiacloprid residues in various bee-derived products, including honey, beeswax, and pollen, as documented in several scientific studies (Codling *et al.*, 2016; Sanchez-Hernandez *et al.*, 2016; Tosi *et al.*, 2018). Most neonicotinoid insecticides are highly toxic to bees and can induce excessive toxicity reactions, but it has been found that outdoor insecticide doses do not cause acute toxicity (Blacquiere *et al.*, 2012). However, research reports have been published indicating that exposure to sublethal doses of neonicotinoid insecticides can affect brain and thorax development (Oliveira *et al.*,

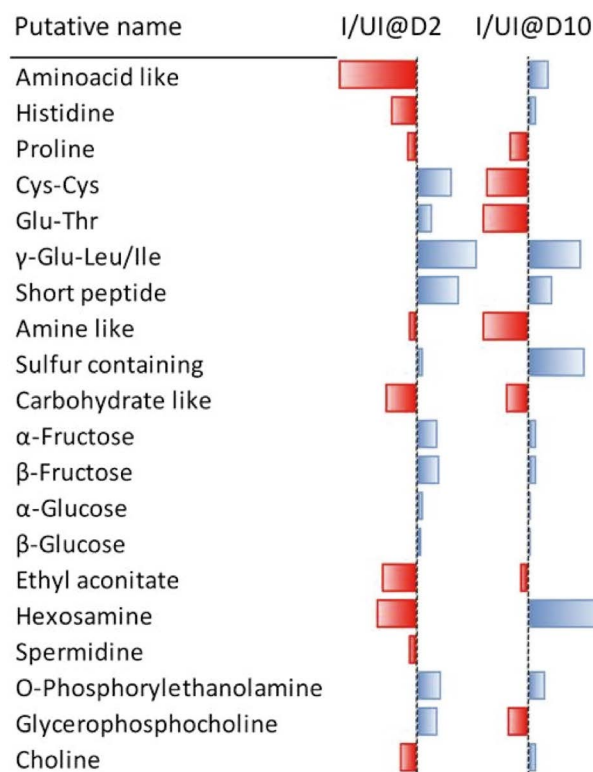
2014), impair learning and memory (Aliouane *et al.*, 2009), and destroy the immune system in bees (Brandt *et al.*, 2016). Furthermore, many outdoor experiments have shown that neonicotinoid insecticides can reduce the foraging and cognitive abilities of bees returning to their hives (Fischer *et al.*, 2014). In a recent study, an investigation was conducted using LC-MS and GC-MS-based metabolomic techniques to explore the metabolic profile variations between honeybees treated with thiacloprid and those in the control group (Shi *et al.*, 2018). The aim of this investigation was to gain a better understanding of the intricate interactions between neonicotinoids and honeybees at the molecular level. Researchers investigated the metabolite profiles of bee heads exposed to 2 mg/l of thiacloprid continuously for three days using both LC-MS and GC-MS. The analysis showed that 115 metabolites showed differences in expression compared to the control group, with 80 metabolites appearing in the LC-MS analysis and 35 metabolites appearing in the GC-MS analysis. The 115 metabolites included various amino acids, fatty acids, phospholipids, and other compounds, with over 70% of these metabolites showing higher expression than in the control group. Interestingly, among the metabolites that showed differences in expression, thiacloprid itself or its metabolites were not detected. This suggests that thiacloprid is rapidly metabolized in the thorax after being absorbed by bees, so only minimal amounts of thiacloprid can reach the bee head. According to previously published research results, when detoxification of alkaloid nicotine occurs in bees, metabolic activity, antioxidant and heat shock responses are increased (Rand *et al.*, 2015). Genes induced by the neonicotinoid thiamethoxam that show differences in expression are primarily abundant in biosynthetic and metabolic pathways related to ribosomes, tyrosine metabolism, and drug metabolism pathways.

#### 4. Effects of pathogen infection with *Nosema ceranae*

*Nosema ceranae*, a gut parasite prevalent in *A. mellifera*, is widely distributed worldwide and poses a significant threat to the Western honeybee, both at the individual and colony levels (Goulson *et al.*, 2015). As with other microsporidian species, *N. ceranae* is capable of manipulating bee physiology and behavior to create a more favorable environment for its reproduction. *N. cer-*

*anae* infection has been shown to weaken the integrity of the midgut tissue (Dussaubat *et al.*, 2012), alter energy demand (Alaux *et al.*, 2009) and hemolymph sugar levels (Mayack and Naug, 2010), and suppress the immune response of honeybees (Alaux *et al.*, 2009). Infected honeybees have been found to have shorter lifespans than uninfected honeybees in some studies, but the presence of *N. ceranae* is not always associated with honeybee mortality, indicating variations in the parasite's virulence (Vidau *et al.*, 2011). These variations may be influenced by host or parasite genetics, climate, nutrition, or interactions with other stressors such as environmental contaminants or other parasites.

Jousse *et al.* (2002) have developed a novel approach for conducting metabolomics analysis on hemolymph samples through a combination of LC/MS and NMR techniques. Their objective was to investigate the meta-



**Fig. 2.** Fifteen biomarkers were successfully detected and categorized as potential biomarkers that indicate infection alone or the combined effect of age and infection. The identified biomarkers include various carbohydrates ( $\alpha/\beta$  glucose,  $\alpha/\beta$  fructose, and hexosamine), amino acids (histidine and proline), dipeptides (Glu-Thr, Cys-Cys, and  $\gamma$ -Glu-Leu/Ile), metabolites related to lipid metabolism (choline, lycerophosphocholine, and O-phosphorylethanolamine), as well as a polyamine compound (spermidine) (Jousse *et al.*, 2020).

bolic changes occurring in honeybees after experimental infection with *N. ceranae*. Hemolymph samples were collected at two time points, 2 and 10 day post-infection, from both uninfected and infected bees. LC-MS was used to analyze individual hemolymph samples, while NMR spectra were obtained from pooled samples. The statistical analysis revealed that the age of bees had the most significant effect on the metabolite profiles. The study identified 15 biomarkers that could distinguish between infected and uninfected bees, as well as the combined effect of age and infection. These biomarkers included carbohydrates ( $\alpha/\beta$  glucose,  $\alpha/\beta$  fructose, and hexosamine), amino acids (histidine and proline), dipeptides (Glu-Thr, Cys-Cys, and  $\gamma$ -Glu-Leu/Ile), metabolites involved in lipid metabolism (choline, glycerophosphocholine, and O-phosphorylethanolamine), and a polyamine compound (spermidine) (Fig. 2). Overall, the untargeted metabolomics-based approach used in this study provides valuable insights into the pathophysiological mechanisms underlying honeybee infection by *N. ceranae*.

## CONCLUSION

Metabolomics has proven to be effectively applied in various honeybee research areas, providing new insights into biology aspects such as behavior, infection, temperature stress responses, and bacteria-insect symbiosis. We are confident that this list of subjects will continue to expand in the near future. In general, it is evident that utilizing a metabolomics approach enables the identification of correlations between a phenotypic state and the underlying cellular metabolism, which older, more specific methods are unable to measure. This unique combination of comprehensive, global analysis and precise quantitative assessment presents an appealing instrument for future honeybee studies.

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