HEALTH AND NUTRITIONAL BIOMARKERS IN HONEYBEES: OPPORTUNITIES AND CHALLENGES UNDER FIELD CONDITIONS

Isani Gloria[1], Rudelli Cecilia[1], Bellei Elisa[2] and Andreani Giulia[1]

[1]Department of Veterinary Medical Sciences, University of Bologna, Bologna, Italy. gloria.isani@unibo.it

[2]Department of Surgery, Medicine, Dentistry and Morphological Sciences, Proteomic lab, University of Modena and Reggio Emilia, Italy

Introduction

The decline of honeybee (*Apis mellifera*) populations has negative consequences not only for agriculture and beekeeping, but also for ecosystems. In human and veterinary medicine, proteomics and metabolomics provide valuable biomarkers to assess the health and nutritional status of organisms. In honeybees, the application of these techniques is still in its infancy and remains underexplored from a clinical perspective.

This study aims to investigate the most abundant proteins of honeybee hemolymph as potential biomarkers of health and nutritional status at the colony level. In addition, an untargeted metabolomics-based approach was applied to honeybee extracts.

Materials and Methods

Samples of hemolymph were collected from honeybees in different apiaries in the province of Bologna in different periods of the year, from April to November. Hemolymph proteins were separated and quantified by 1D SDS-PAGE, as reported by Isani et al. (2023).

Honeybee cytosolic extracts were fractionated using size exclusion chromatography (SEC) and metabolites were analyzed in fractions using mass spectrometry (Orbitrap Exploris 480, Thermo Fisher). Metabolites were detected by Compound Discoverer 3.3 software (Thermo Fisher).

Different statistical tests were used depending on whether sample distribution was normal or not.

Differences between honeybee sub-castes or seasons were considered statistically significant for $p<0.05$.

Results

The five most abundant hemolymph proteins, namely vitellogenin, apolipophorin I and II, transferrin and hexamerin 70a, represent a panel of biomarkers related to key metabolic processes. These proteins show interesting variations depending on many physiological and environmental factors, such as:

- **the age of honeybees:** nurse bees had the highest vitellogenin concentration compared to the other two sub-caste (Figure1);
- **the season:** in autumn, a peak of vitellogenin and transferrin concentration was observed in winter bees;
- **the presence of parasites:** a decrease of vitellogenin, apolipophorin II, transferrin, and hexamerin 70a was detected in bees parasitized by Varroa.

After SEC fractionation of cytosolic extracts of honeybees (Figure 3) and mass analysis, one hundred and ninety-eight different pathways and more than 2000 metabolites were identified. The most abundant metabolites belonged to the flavone pathway (Table 1), followed by the lipoxygenase pathway. Most metabolites are of plant origin and may be related to the environmental availability of nectar and pollen, which in turn are essential for honeybee nutrition, suggesting a possible role as biomarkers of nutritional status.

Proteomics

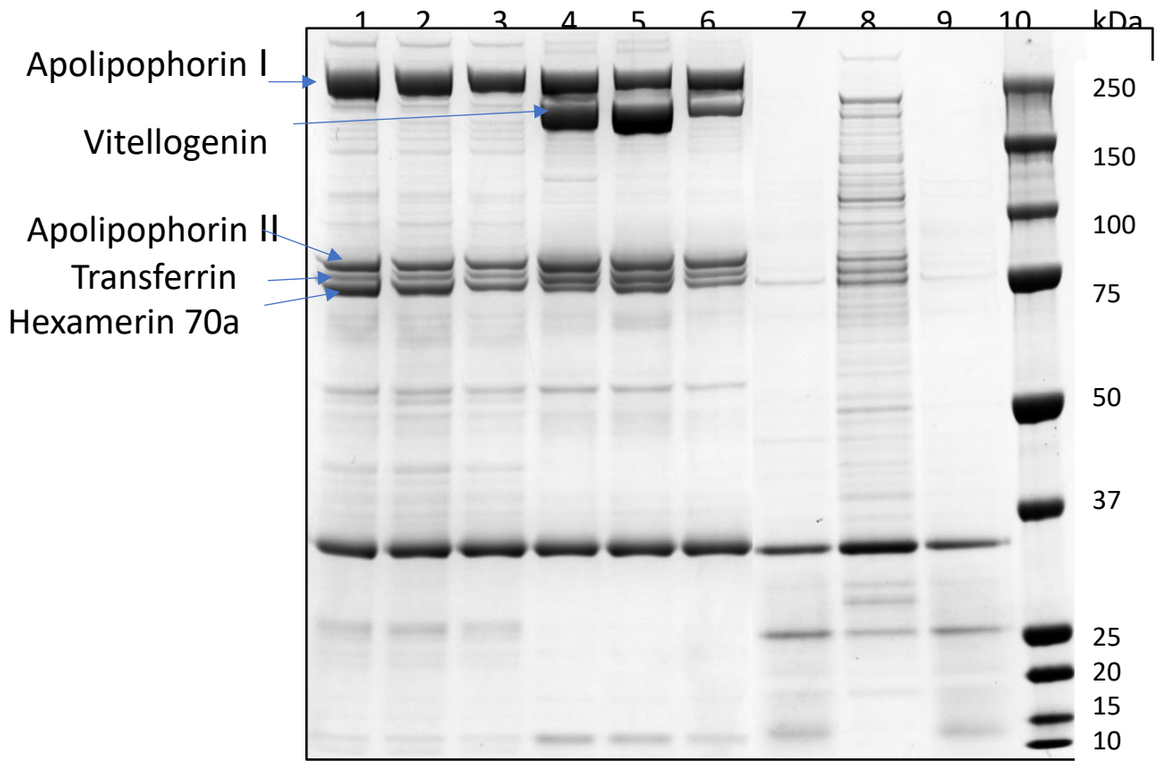

Figure 1. Representative SDS-PAGE of honeybee hemolymph: lanes 1, 2, and 3 represent the protein profiles of newborn bees; lanes 4, 5, and 6 represent the protein profiles of nurse bees; lanes 7, 8, and9 represent protein profiles of foraging bees; lane 10 represents the molecular mass marker.

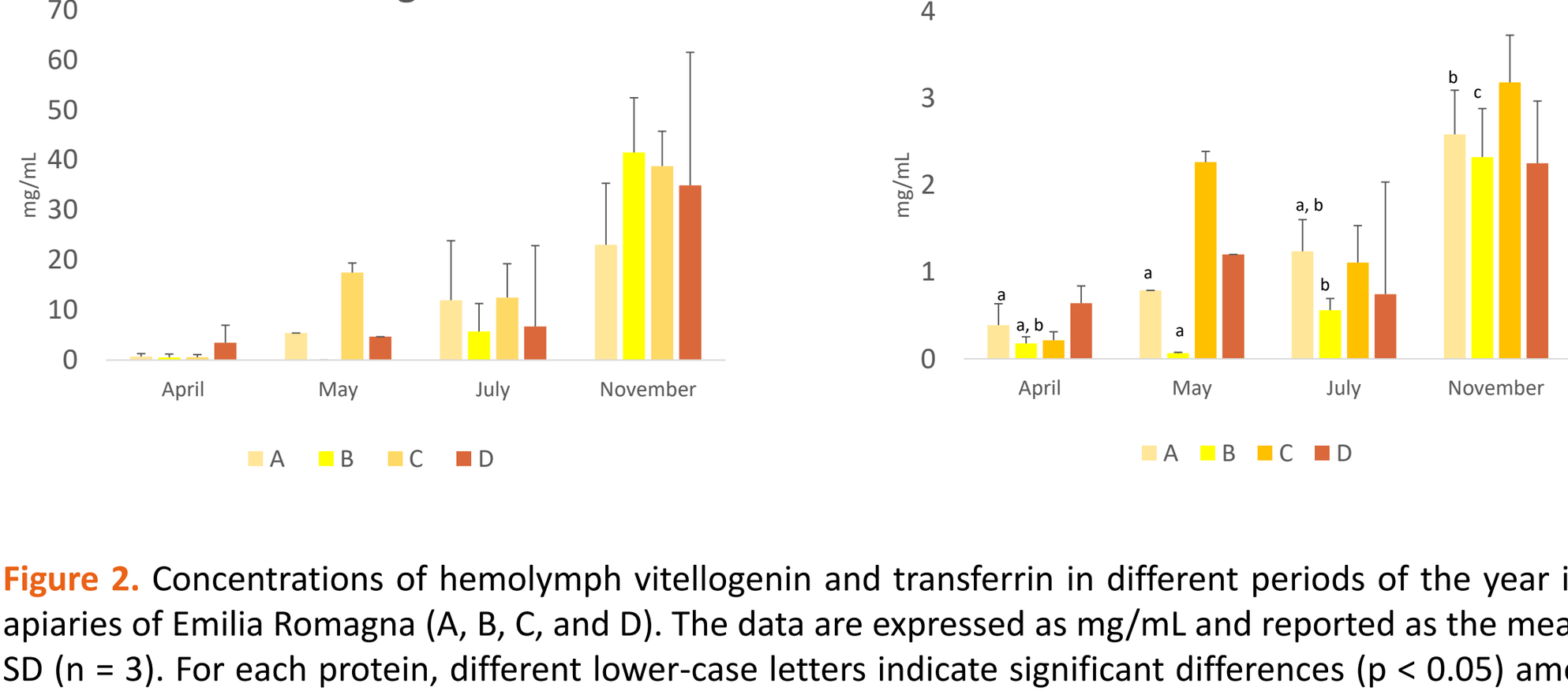

Figure 2. Concentrations of hemolymph vitellogenin and transferrin in different periods of the year in 4 apiaries of Emilia Romagna (A, B, C, and D). The data are expressed as mg/mL and reported as the mean ± SD (n = 3). For each protein, different lower-case letters indicate significant differences ($p < 0.05$) among time points (months) within the same apiary.

Metabolomics

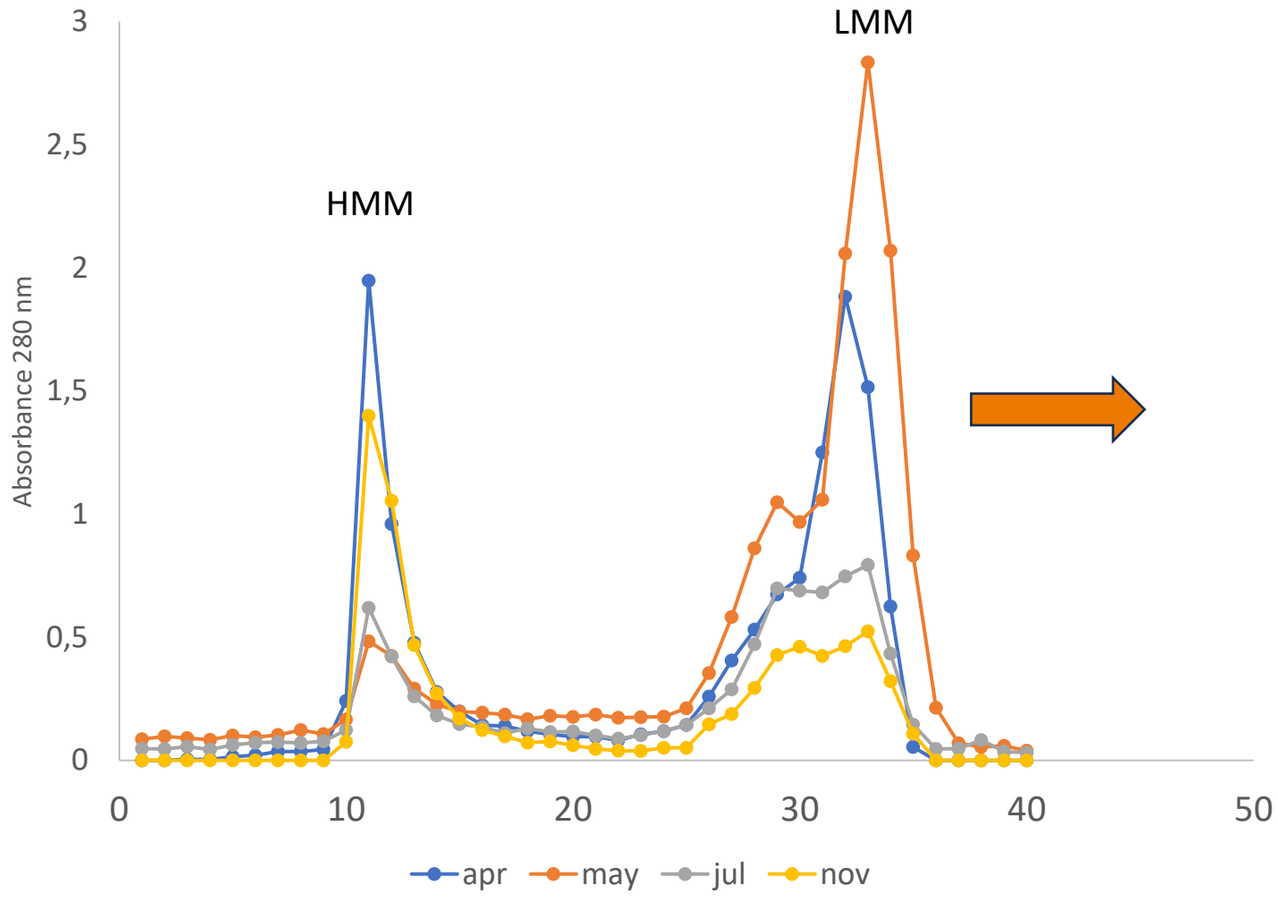

Figure 3. Chromatographic pattern after size exclusion chromatography (SEC) of extracts of honeybees sampled from the same apiary in different periods of the year. Proteins (HMM), peptides and low molecular mass molecules (LMM) were measured at 280 nm.

Superpathway of flavones and derivatives biosynthesis	#1 Apr Site1	#2 May Site1	#3 Jul Site1	#4 Nov Site1	#5 Apr Site2	#6 May Site2	#7 Jul Site2	#8 Nov Site2
Kaempferol-3-glucoside-7-rhamnoside Kaempferol 3-O-rhamnoside-7-O-glucoside	5.8	4.7	2.7	3.6	5.8	3.9	2.7	
Quercetin 3-O-rhamnoside-7-O-glucoside Kaempferol 3,7-O-diglucoside Kaempferol 3-O-β-D-glucosyl-(1->2)-glucoside Kaempferol-3-gentiobioside Rutin	6.2	4.3	6.2	3.6	5.5	2.0	3.7	
Quercetin 3,7-O-diglucoside Quercetin 3-O-sophoroside Quercetin 3,5-O-diglucoside Quercetin-3-gentiobioside	3.0	1.7	5.1	6.6	3.2	5.8	4.7	
Kaempferol-3-rhamnoside-7-rhamnoside		5.2	2.6		6.2	5.5	4.8	4.5
(+)-Dihydromyricetin	3.9	5.8	3.5	2.7	5.1	5.5		
Kaempferol 3-O-rhamnosyl(1->2)glucoside-7-O-rhamnoside Kaempferol-3-O-gentiobioside-7-O-rhamnoside	5.8	2.4	3.1	6.2	6.6	2.1	5.1	
Kaempferol 3-O-β-D-glucosyl-(1->2)-glucosyl-(1->2)-β-D-glucoside		6.2	5.5	5.2	4.7	5.5	6.6	
(+)-Taxifolin	3.5			5.8	3.1	5.4	6.2	5.2
Kaempfenol-3-rhamnoside		1.8	5.0	5.6		5.9	5.8	6.1

Table 1. Major metabolites of the flavone super-pathway detected in LMM fractions of cytosolic extracts from honeybees sampled from hives of two apiaries in the province of Bologna (Site 1 and Site 2).
Colored boxes: peak amount of each compound identified.
Grey boxes: absence of detection.

CONCLUSIONS

These are the first data on the concentration of a panel of hemolymph proteins in different phases of the life cycle and seasons in Italian honeybees. They can be considered as a starting point to establish in the future physiological intervals useful in clinical practice to evaluate the health and nutritional status of honeybees. In addition, many of the metabolites identified in honeybees are of plant origin and can also help to assess the availability of environmental resources in different seasons and locations.

REFERENCES - Isani G, Bellei E, Rudelli C, Cabbri R, Ferlizza E, Andreani G. SDS-PAGE-Based Quantitative Assay of Hemolymph Proteins in Honeybees: Progress and Prospects for Field Application. Int J Mol Sci. 2023 Jun 16;24(12):10216. doi: 10.3390/ijms241210216. PMID: 37373362; PMCID: PMC10299212.

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