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Original article

Electromagnetic field of extremely low frequency has an impact on selected chemical components of the honeybee

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Abstract

The electromagnetic field (EMF) is an environmental factor affecting living organisms. The aim of this study was to demonstrate the effect of an extremely low frequency electromagnetic field (ELF-EMF) on selected chemical components of the honeybee (*Apis mellifera L.*) using Fourier Transform Infrared (FTIR) spectroscopy. The FTIR method provides information on the chemical structure of compounds through identification and analysis of functional groups. The honeybees were treated with EMF at a frequency of 50 Hz and magnetic induction of 1.6 mT for 2, 6, 12, 24 and 48 hours. Analysis of FTIR spectra showed that EMF exposure longer than 2 hours induced changes in the structure of chemical compounds, especially in the IR region corresponding to DNA, RNA, phospholipids and protein vibrations, compared to control samples (bees not EMF treated). The results confirm the effect of EMF on bees depending on the duration of exposure.

Key words: *Apis mellifera*, ELF-EMF, FTIR, spectra analysis, body chemical composition

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Introduction

The honeybee (*Apis mellifera L.*), a species widespread throughout the world, is an insect of great importance for the biosphere and economy. It is responsible for pollinating 70% of arable crops, which represents about 35% of world food production (Klein et al. 2007). The profit achieved as a result of pollination by *Apis mellifera* is approximately 25-30% of total crop gain (Giannini et al. 2015).

For decades, the phenomenon of mass disappearance of bees (Colony Collapse Disorder - CCD) has been observed. This phenomenon, sometimes contributing to the extinction of 80-100% of the colonies in a given area, was recorded in Europe including Poland (Moritz et al. 2010, Topolska et al. 2018, Gray et al. 2020) and in North America (Van Engelsdorp et al. 2009). Due to its specific symptoms (mainly leaving the hive by the collectors), its etiology is not fully understood to this day. Researchers point to various causes of this phenomenon: malnutrition of bees (Harpe and Hayden 2009), stress caused by the transport of bees to new habitats (Naug 2009), viral diseases (Bonning 2009), parasites (Paxton 2010), excessive shortening of telomeres in the wintering worker group (Stindl and Stindl 2010), pesticides (Cresswell et al. 2012), general weakness of the colony caused by many factors acting simultaneously (Stindl and Stindl 2010) or electromagnetic field (EMF) of various origins (Kumar 2018), mainly EMF with a frequency of 50 Hz (Wyszkowska et al. 2019). In recent years, there has been a significant overlap of technology that has increased the amount of artificial sources of EMF. The universality of access and the use of the advantages of electricity requires efficient operation and continuous expansion of the equipment for its production, transmission and distribution. Electricity supplied to all recipients is generated in power plants. Transmission of energy from the power plant to the recipient is possible thanks to the extensive network of power lines and substations. This results in living organisms which are surrounded by these devices being continuously exposed to its effects.

EMF is a factor that is particularly dangerous for bees, because apiaries in crops are often located in the immediate vicinity of high-voltage power lines - places which, due to problems with operating heavy agricultural equipment, are excluded from cultivation.

Animals can use the Earth's magnetic field as an indicator of their movement direction. Bees also belong to such animals. Bees sense a permanent magnetic field with a magnetic induction of just 26 nT. Detection of a variable magnetic field is less sensitive and amounts to 100 μT (Etheredge et al. 1999). Bees have clusters of magnetite in the abdominal areas. For millions of years, before the electrification of the Earth by humans, honeybees had not been exposed to varying EMF with frequencies below 10 Hz (Skiles 1985). Currently, they are exposed to the 50 Hz EMF (in Europe) due to the increasing number of sources. Recent studies have shown that ELF-EMF disturbs the functioning of the nervous system in honeybees, which is manifested by a decrease in cognitive abilities and an increase in the level of aggression (Shepherd et al. 2019).

FTIR spectroscopy is a tool which can be used for chemical analysis of tissues. The IR regions show a series of absorptions that result from all kinds of vibrations bent in the molecule. This indicates that this method can be useful for analyzing changes in cellular structures (at the molecular level) caused by pathogenic and environmental factors. Thus, the FTIR method was chosen to study the biochemical changes in bees caused by EMF.

After analyzing the available publications we decided to conduct a study to evaluate the effect of EMF in the extremely low frequency range at different durations of treatment (2, 6, 12, 24 and 48 hours) on honeybees. We checked the chemical compositions of bees treated with EMF (at a frequency of 50 Hz and magnetic induction of 1.6 mT) and bees not treated with EMF.

Materials and Methods

Animals

Honeybees (*Apis mellifera L.*) were the research model. A total of 400 *A. mellifera carnica* workers were used for the study, taken from a single hive with a population of 40 - 45 thousand individuals, located in an apiary located in the Biotechnology Center of the University of Rzeszów, (South-Eastern Poland). Animals were taken from the hive, so that the collected samples were only minimally loaded with the load of foreign substances (pollen, nectar, gutation water carried to cool the nest). The collection took place each time between 6.30-7.00 in the morning. The research was carried out in the period June-July 2018.

Insect incubation

The bees were placed on eight Petri dishes 10 cm in diameter (10 bees per dish), in which they had unlimited access to an aqueous sugar solution 1:1 (w/w) (sugar solution was topped up every 6 hours). The dishes were placed in a culture chamber type S 711 (Chłodnictwo-Madej, Poland) under constant lighting conditions, at a temperature of 26 ± 0.5ºC and a humidity of 60 ± 5%. Of the eight dishes, four dishes constituted a control group (incubated under the same condi-

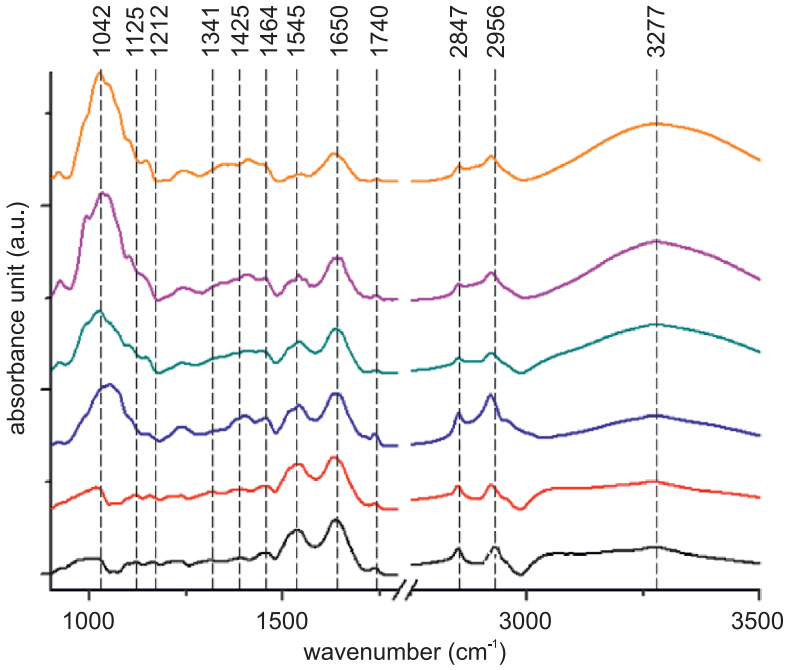

Fig. 1. Average FTIR spectra of honeybees treated with EMF at a frequency of 50 Hz and magnetic induction of 1.6 mT. Control group (black spectrum) and groups treated with EMF for: 2 h (red spectrum), 6 h (blue spectrum), 12 h (green spectrum), 24 h (pink spectrum), 48 h (orange spectrum).

tions as the tested group, but not subjected to EMF action), and the next four were exposed to EMF for the entire study period. This scheme was used for bees incubated in the chamber for 2, 6, 12, 24 and 48 h.

EMF exposure system

The use of electric energy, its generation, transmission and distribution are associated with the generation of EMF, delivered at a frequency of 50 Hz in Europe and New Zealand, and 60 Hz in the USA. This range is within the extremely low frequency of the electromagnetic spectrum (frequency <300 Hz).

In this study we used a Magneris EMF generator (Astar, Poland) with innovative flat applicators. The device generated EMF with a frequency range from 2 to 120 Hz and magnetic induction up to 10 mT. On the basis of the field distribution measurements, the area of exposure and the value of the magnetic induction in the area of bee movement were determined. The applicators were placed directly above the dishes. The bees were subjected to EMF at a frequency of 50 Hz, and magnetic induction of 1.6 mT for the entire measurement period, i.e. 2, 6, 12, 24 and 48 hours for particular groups of bees. After exposure bees were frozen (-18°C) for further analysis. The EMF generation system was described in our previous papers (Koziorowska et al. 2017, 2018).

Preparation of samples for analysis

Frozen bees were transferred to a lyophilizer (Labconco Freezone 2.5 freeze dryer; Labconco, USA) for 168 hours at a pressure of 0.050 mbar; temperature -50°C. After this period, the lyophilisate was triturated under a liquid nitrogen atmosphere. Samples prepared in this way were stored at - 80ºC until the FTIR analysis was performed.

FTIR Spectroscopy

We used a Vertex 70 (Bruker) spectrometer with the Attenuated Total Reflectance (ATR) technique and a diamond crystal was used in this experiment to measure the FTIR spectra of bees. The range of selected infrared radiation was the average IR (400 - 4000 cm^{-1}). The spectral resolution was 2 cm^{-1} and in each measurement 64 scans were done. Normalization and baseline correction of obtained spectra were performed. The second derivative spectra were calculated from the ATR–FTIR spectra to determine the secondary structure of proteins. All spectra were analyzed using OPUS software.

Statistical analysis

Principal component analysis (PCA) was used to obtain information about the bee spectra variation depending on EMF treatment time. PCA is a non-parametric method for extracting relevant information from

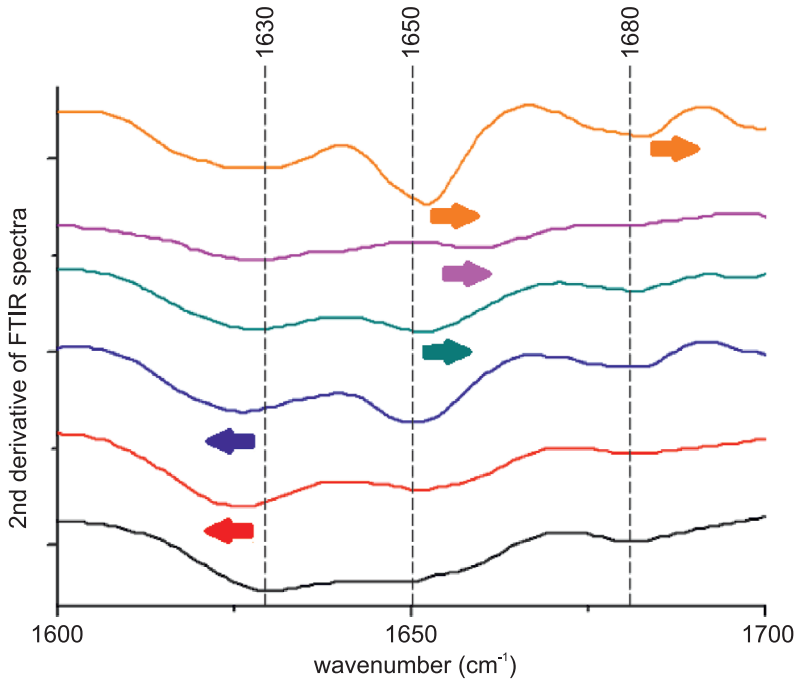

Fig. 2. Second derivative of average FTIR spectra of honeybees treated with EMF at a frequency of 50 Hz and magnetic induction of 1.6 mT. Control group (black spectrum) and groups treated with EMF for: 2 h (red spectrum), 6 h (blue spectrum), 12 h (green spectrum), 24 h (pink spectrum), 48 h (orange spectrum).

confusing data sets, allowing patterns in data to be identified and to highlight their similarities and differences. PCA reduces the dimensionality, and the number of variables of the data, by maintaining as much variance as possible. Moreover, to determine similarity between analyzed groups we used hierarchical cluster analysis (HAC). These methods were applied for two IR ranges: fingerprint region (800-1800 cm^{-1}) and amide I region (1600-1700 cm^{-1}). Statistical and multidimensional analysis were done using PAST 3.0. software.

Because the results of the control groups for different times did not differ significantly, they have been averaged and the graphs show the relationships between study exposed groups and one control.

Results

We used FTIR spectroscopy combined with PCA analysis to determine the influence of EMF for different durations of treatment. To determine changes in the secondary protein structure we performed a second derivative of FTIR spectra.

FTIR spectra of bees for different durations of EMF treatment (Fig. 1) showed that the chemical compositions of bees were different compared to the control group (Fig. 1 black spectrum). The smallest changes were observed in the FTIR spectrum of bees treated with EMF for 2 hours (Fig. 1 red spectrum). EMF in this case caused a decrease in the maximum absorbance of peaks corresponding to amide II and amide III vibrations. Moreover, in the FTIR spectrum of bees treated with EMF for 6 hours (Fig. 1 blue spectrum), we observe an increase in peaks corresponding to DNA, RNA, phospholipid and lipid vibrations. Bees treated with EMF for 12 hours (Fig. 1 green spectrum) showed an increase in the IR region between 900 cm^{-1} and 1200 cm^{-1}, compared to the FTIR spectrum of the control group. Moreover, in the green spectrum we also noticed a decrease in the peaks originating from protein and lipids vibrations. The same observation as in bees treated with EMF for 12 hours was noted in the case of bees treated by EMF for 24 (Fig. 1 pink spectrum) and 48 hours (Fig. 1 orange spectrum). However, with increasing time, both increases and decreases were greater. Moreover, treatment with EMF (at a frequency of 50 Hz), longer than 2 hours, caused structural changes especially in the IR region corresponding to DNA, RNA, phospholipid and protein vibrations. In the FTIR spectra, peaks attributed to the different chemical compounds of bees were identified (Table 1).

The IR region between 1700-1600 cm^{-1} (amide I region) corresponds to vibration of the C=O group which participates in the formation of hydrogen bonds. In this case, the C = O stretching bands in the IR spectra are very sensitive to the formation and changes in the hydrogen bonds between the peptide groups (Skornyakov et al. 2008, 2009). The hydrogen bonds of the C=O···H-N group form β-forms or β-sheet struc-

Table 1. The peaks attributed to the different chemical compounds of honeybees treated with EMF at a frequency of 50 Hz and magnetic induction of 1.6 mT. Wavenumbers with corresponding vibrations.

FTIR spectroscopy Wavenumber (cm^{-1})	Vibrations
3277	Amide A and OH group from water (Barth 2007)
2956	CH_3 asymmetric stretching (Wrobel et al. 2011)
2847	Symmetric stretching vibrations of CH_2 (Wrobel et al. 2011)
1740	C-O vibrations from lipids (Wrobel et al. 2011)
1650	Amide I (Barth and Zscherp 2002)
1545	Amide II (Barth and Zscherp 2002)
1464	CH_2 group from cholesterol (Yano et al. 2000)
1425	CH_2 deformations and C–O–H bending vibration (Liu and Kim 2017)
1341	CH_2 group from colagen (Elmi et al. 2017)
1212	Amide III (Movasaghi et al. 2008)
1125	Symmetric PO_3^{-2} group from DNA, RNA and phospholipids (Maziak et al. 2007)
1042	Asymmetric PO_3^{-2} group from DNA, RNA and phospholipids (Maziak et al. 2007)

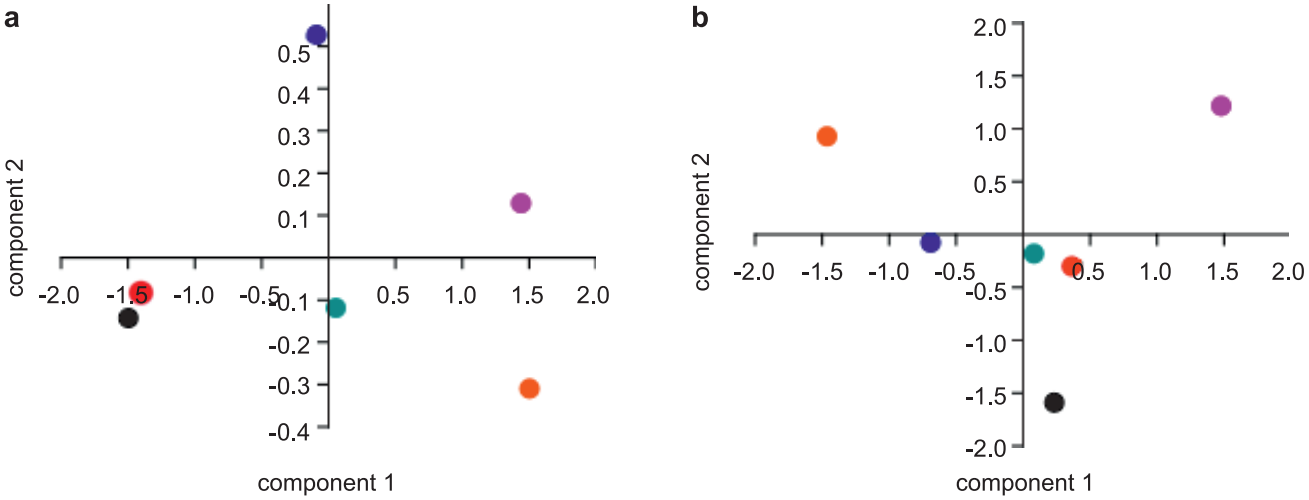

Fig. 3. PCA analysis of Two-dimensional (2D) scores plot of honeybees, due to differences in chemical compositions shown for: IR spectral region between 800-1800 cm^{-1} (a) and second derivative of FTIR spectra in the spectral region between 1600-1700 cm^{-1} (b). Control group (black) and groups treated with EMF for: 2 h (red), 6 h (blue), 12 h (green), 24 h (pink), 48 h (orange).

tures. For α-helix structures, the frequency of vibrational C=O bands are in the range between 1660-1650 cm^{-1}, for the structures of β-forms, frequencies are in the range of 1640-1620 cm^{-1}; while for β-sheet structures the range is 1670-1690 cm^{-1} (Skornyakov et al. 2008). To determine a secondary protein structure a second derivative of FTIR spectra was performed.

The second derivative of FTIR spectra of bees treated with EMF for 2 hours (Fig. 2 red spectrum) and 6 hours (Fig. 2 blue spectrum) caused a shift in the peaks corresponding to β-forms in the lower wavenumber values, in comparison with the control group (Fig. 2 black spectrum). Moreover, in the second derivative of FTIR spectra of bees which were treated by EMF for 12 hours (Fig. 2 green spectrum), 24 hours (Fig. 2 pink spectrum) and 48 hours (Fig. 2 orange spectrum), a shift in the peaks originating from α-helix structures in the higher wavenumber values, was observed, when compared with the control group. Furthermore, in the case of EMF treatment for 48 hours of bees (Fig. 2 orange spectrum), a shift in the peaks corresponding to β-sheet structures was visible.

Taking into account the chemical changes, as well as changes in the secondary protein structure induced by EMF, the significance of these differences were investigated using PCA analysis (Fig. 3), while the similarity between analyzed groups were checked by HCA analysis (Fig. 4).

PCA analysis of differences in chemical compounds derived from the fingerprint FTIR region of all analyzed groups (Fig. 3a) showed that the EMF effect caused by 2 (red dot) hours of exposure caused very small differences in the chemical compounds, while when the bees were subjected to EMF for a longer time, the dif-

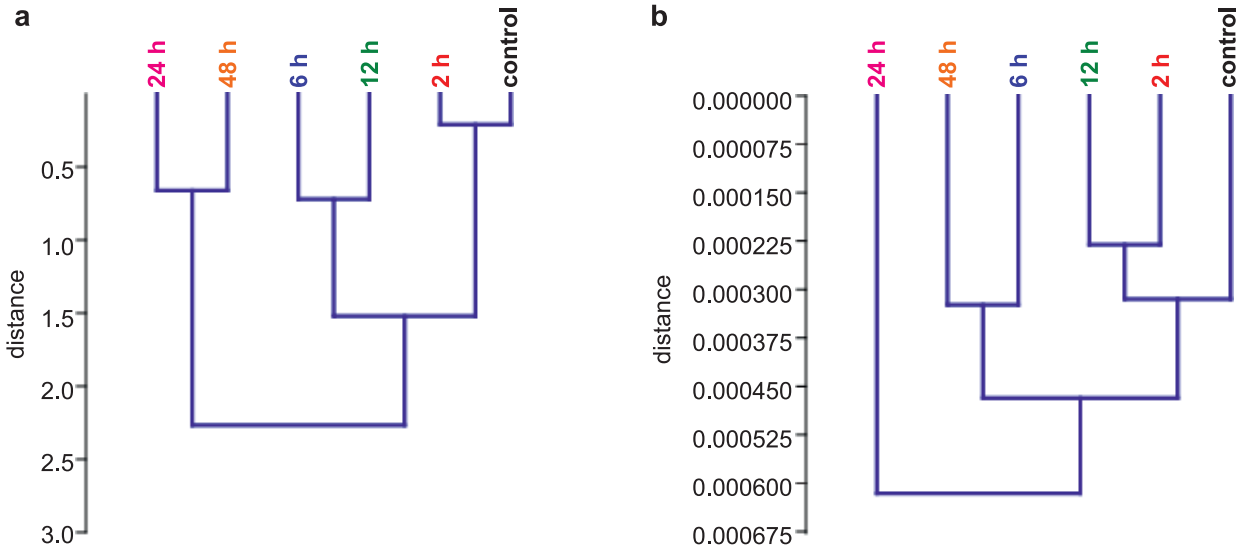

Fig. 4. HCA analysis of differences between IR spectral region between 800-1800 cm^{-1} (a) and second derivative of average FTIR spectra in the spectral region between 1600-1700 cm^{-1} (b). Control group and groups treated with EMF for: 2 h, 6 h, 12 h, 24 h, 48 h.

ferences in the chemical compositions were significant when compared with the control group (black dot). However, when we analyzed the PCA (Fig. 3b) obtained for the amide I region (1600-1700 cm^{-1}), we noticed a similarity in the secondary protein structure in the control group and bees treated with EMF for 2 (red dot) and 12 hours (green dot). In the other groups, we observed significant differences.

HCA analysis of the IR spectral region between 800-1800 cm^{-1} (Fig. 4a) indicates three groups, where the FTIR spectra of the control bees and those subjected to EMF for 2 hours were similar. Moreover, EMF exposure for 6 and 12 hours created a further similarity group. A find similarity group was noted in the FTIR spectra of bees treated by EMF for 12 and 24 hours. However, when we analyzed HCA analysis for FTIR spectra in the spectral region between 1600-1700 cm^{-1} (Fig. 4b), we observed two main similarity groups: between the FTIR spectra of bees exposed to EMF for 2 and 12 hours and between the FTIR spectra obtained for bees exposed for 6 and 48 hours.

Discussion

Electromagnetic radiation from various sources and at different frequencies, circulate together in space in the form of electromagnetic energy waves, permeating each other. The widespread use of electricity in household appliances, the workplace and public transport results in a significant increase in the amount of ELF-EMF in the environment. Exposure to ELF-EMF is ubiquitous and inevitable for both people and animals. The question is whether, and to what extent, these environmental conditions affect natural populations.

In our previous paper we showed that EMF affects animal tissues and infrared spectroscopy can be a method for detecting the changes (Koziorowska et al. 2020). In the present study we showed that exposure to EMF of extremely low frequency affects the chemical composition of honeybees. In our studies honeybees were exposed to a man-made 1.6 mT EMF with a frequency of 50 Hz for different times (2, 6, 12, 24 and 48 hours). We used a modern method of infrared spectroscopy (FTIR) to detect changes in the chemical composition of honeybees.

It is known that bees, in addition to the sense of sight, use the phenomenon of magnetoreception during navigation. Kirschvink et al. (1997) showed that bees are able to detect variable fields with a magnetic induction of 430 μT and frequencies of 10 and 60 Hz (Kirschvink et al. 1997). The sensitivity of the honeybee magnetoreception system depends on the frequency and decreases rapidly as the frequency increases. Bees have been exposed to EMF above 10 Hz only since the introduction of artificial sources of EMF by humans, and it is difficult to determine how efficient the neural mechanisms filtering this type of radiation are (Kirschvink et al. 1997). The magnetoreception system of honeybees is based on the formation iron granules (mainly in the form of Fe_3O_4 and FeOOH with a diameter of 0.5±0.1 μm, deposited in the iron vesicles) in the cytoplasm of trophocytes. The trophocytes encircle the abdomen under the cuticle and are located mainly at the ventral abdomen. The iron granules possess superparamagnetic magnetite and are magnetic granules, which plays a crucial role as a transducer

of the magnetic field. The magnetic field induces the size of the magnetic granules and the release of calcium ions associated with the cytoskeleton. The increase of calcium ions can initiate the neuronal response due to the EMF (Hsu et al. 2007).

Taking into account results obtained for PCA and HCA analyses, in can be concluded that EMF caused greater chemical changes in the DNA, RNA and phospholipid functional groups than in the protein secondary structure, whereas changes in DNA can cause changes in the structure of the protein. Other authors also showed the influence of EMF on model lipid membranes (Koronkiewicz et al. 2001), protein structures (Ikehara et al. 2003) and the distribution of intra-membrane proteins (Balcavage et al. 1996). However, it is difficult to assess whether this is the effect of direct field influence on organic structures or indirect action. The EMF affecting bee magnetoreceptors causes changes such as an increase in the frequency of wing beats, reduction of olfactory perception and disturbances in social behavior (Shepherd et al. 2018). Increased motor activity under the influence of natural EMF was also observed in locusts (Bergh 1979). Also, in our research we observed a significant stimulation of bees exposed to EMF in relation to the control group, manifests as, among others, a change in metabolic rate, including detoxification of active substances of plant protection products (Piechowicz et al. 2020). The observed chemical changes in bees may therefore result from the intensification of metabolic changes related to environmental stress: increased metabolites in the body or accelerated transcription of genes (e.g., responsible for heat shock protein synthesis). An increase in the amount of heat shock proteins under the influence of EMF was observed in the research of Wyszkowska et al. (2016).

The results shown in this paper confirm the influence of EMF with a frequency of 50 Hz, as an environmental factor, on honeybee chemical composition and its dependence on the duration of exposure. Our preliminary results could form the basis for further studies on the detailed molecular mechanism of action of the nervous system and magnetism in bees.

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