Original Article

INFLUENCE OF ELECTROMAGNETIC FIELD WITH FREQUENCY OF 50 HZ IN FORM OF DOSES ON SELECTED BIOCHEMICAL MARKERS OF HONEY BEE

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Abstract

The honey bee has a significant environmental and economic impact. While collecting food or water, bees are exposed to negative physical and chemical factors that lead to physiological and behavioral changes and, consequently, even death. Along with the development of technology and communication, electromagnetic fields produced by artificial emitters have begun to have an impact on the environment. The aim of the study was to check whether the electromagnetic field also impacts antioxidant enzymes functioning in the honey bee's organism. The study was conducted under laboratory conditions, and one-day and seven-day-old honey bee workers were used in the experiment. Honey bee workers were exposed to an electromagnetic field with a frequency of 50Hz and variable intensity in the range of 1-10 kV/m. Immediately after the end of the exposure, hemolymph was collected from the bees for biochemical analysis. The results of the research did not show clearly whether changes in the activity of biochemical markers were affected by the time spent in the electromagnetic field or its intensity but did show that there was a difference in physiology between one-day-old and seven-day-old bees.

Keywords: biochemical markers, electromagnetic field, enzymatic markers, honey bee, 50 Hz

INTRODUCTION

With the development of civilization, the artificial electromagnetic field (EMF) has also started to rapidly affect the environment. The Earth is the natural source of EMF, and living organisms in its natural field have adapted and even learned to use it (Rochalska, 2009). Unfortunately, new artificial sources of electromagnetic fields have formed, and the growing energy consumption and communication ranges take advantage of their different frequencies and intensities (Bieńkowski & Wyszkowska, 2015) impacting the environment's fauna and flora (Rochalska, 2009). Currently, all devices and installations that are used in energy, industry, homes, public places and transport artificially generate an EMF (Strzałka-Gołuszka & Syrek, 2008).

Besides development of the entire economy, there is also technological development, which has disturbed the symbiosis between the

environment and the honey bee and these changes that threaten the environment and its proper functioning (Papa et al., 2022). In good weather, the natural strength of the earth's electromagnetic field is 100-150 V/m, but in bad weather,e.g. during a storm, the intensity can reach even 20 kV/m (Strzałka-Gołuszka & Syrek, 2008). The most common frequency found in the environment that comes from artificial sources is 50 Hz. In the environment, the honey bee is most often exposed to an artificial electromagnetic field of 1 to 10 kV/m, present under high voltage lines (220-400 kV); (Strzałka-Gołuszka & Syrek, 2008; Szychta et al., 2022).

Electromagnetic fields have varying effects on living organisms and the environment depending on their frequency and intensity. Despite this issue having been studied for over thirty years, it is still difficult to draw conclusions based on the available data about the potential effects of very low frequency EMFs. Apart from such factors as agrochemicals (pesticides, fertilizers, etc.), diseases and predators, physical factors also influence the daily functioning of the honey bee ,especially the defense systems of worker honey bees ([2]Migdał et al., 2020). In studies on the influence of th, EMF has been shown to have a negative effect on both the honey bee's physiology ([2]Migdał et al., 2021) and behavior ([1]Migdał et al., 2021).

Protease and protein transformation enzymatic markers are an important part of the metabolic system. Proteases and their inhibitors are responsible for activating many metabolic processes and supporting the antioxidant system in the bee hemolymph. Damaged proteins accumulate in cells as a result of antioxidant processes, and proteolytic enzymes are designed to recognize and neutralize them, thus protecting the body against excessive deposition of protein aggregates. Enzymatic markers involved in the protein transformation are alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamylotranspeptydase (GGTP), which are known to be indicators of various physiological and pathological changes. In humans, most proteins are synthesized in the liver and fat bodies, while in bees it occurs in the fat body, which is involved in the body's detoxification processes (Łoś et al., 2019; Migdał et al., 2022).

Research shows that the activity of AST, ALT, ALP and GGTP biochemical markers responsible for the antioxidant and detoxification system of the honey bee's organism decreased after exposure to EMF. This reduction can impair key metabolic cycles in the honey bee body, including the Krebs cycle, ATP synthesis, oxidative phosphorylation and β-oxidation. Exposure to EMF's of varying intensity may change the concentration of creatinine and albumin, which are important non-enzymatic antioxidants ([2]Migdał et al., 2021; Migdał et al., 2022).

Previous authors' work indicated that biological effects depend not only on field strength, but also on exposure time and questioned whether biological effects were affected by the interchangeability between field strength and exposure time. The dose was defined as $D_{LF}=E*t$ [(V/m)*h], a value of such interchangeability for the low-frequency range. Such a definition of the dose results from the analysis of the mechanisms of EMF effects on organisms; in the frequency range up to 100 kHz, the dominant effects are related to eddy currents and excitation of the nervous system, for which field strength is an important parameter (ICNIRP, 2010). For high frequencies, the primary biological effects are related to thermal effects (ICNIRP, 1998) - where the EMF energy related to the square of the field strength is important - for this frequency range, a better approximation of the dose would be $D_{HF}=E^2*t$ [$(V/m)^2$*h]. Therefore, the results of the studies presented by Migdał et al., (2020), [1]Migdał et al. (2021), [2]Migdał et al. (2021) and Migdał et al. (2022) were analyzed to compare the effects at similar D_{LF} doses, and the measurement scenario of the presented studies was planned to obtain the same doses at different field strengths and exposure times. The aim of this study was to investigate how EMF with a frequency of 50 Hz and of varying intensities in the range of 1-10 kV influenced selected biochemical markers of the honey bee.

MATERIAL AND METHODS

Research material

One-day and seven-day-old honey bee workers were used in the experiment because investment in immune functioning in bees older than seven days varies extremely as individuals change their physiological state and behavioral task and begin to immunosenesce (Amdam et al., 2005; Simone et al., 2009). For the research, colonies of *Apis mellifera carnica* were chosen as a brood source. Queens originating from the same mother-queen colony were inseminated with the semen of drones from the same father-queen colony. Five randomly picked queens were placed in isolators with empty Dadant combs (435×300 mm) for egg-laying. Each queen was kept in a separate colony.

On day 20 of development, brood combs were transported to a laboratory and placed in an incubator with conditions similar to those in the hive, temperature 34.5±0.5°C and humidity 70±5% according to Migdał et al. (2020). In the incubator, honey bee workers had access to protein (bee bread) and carbohydrates (sucrose syrup 1 mol/dm^3) *ad libitum*. One-day-old honey bee workers were then placed in wooden cages (200x150x70 mm) with two feed dispensers (5 ml each); each cage contained ninety workers. Each of the experimental and control groups consisted of six cages. The bees were fed with a 1 mol/dm^3 sucrose solution administered *ad libitum* until exposition started. The honey bee workers were kept in experimental cages with access to sucrose syrup ad libitum and electromagnetic field conditions <2 kV/m for up to seven days.

To maintain the same dose, the experimental groups were exposed to different E-field intensity levels and different exposure times. The combination of used field strength and exposure time to the 50 Hz E-field are presented in Tab. 1. The control group was exposed to a E-field <0.2 kV/m. During the experiment, the group was kept in experimental cages with access to sucrose food *ad libitum*.

Generation of an E-field

A homogeneous E-field with a frequency of 50 Hz was generated in the exposure system using a plate capacitor according to [2]Migdał et al. (2020). The field intensity was set at 1, 2, 4, or 8 kV/m. The exposure time was according to each intensity 2, 1, 0.5, and 0.25 h, so each of the experimental groups was exposed to the same dose energy but at different times (Tab. 1). The changes in the homogeneity and stability of the E-field did not exceed ±5% in the whole time and space to which the worker bees were exposed throughout the experiment. The field intensity and homogeneity in the research area were verified by measurements made by a research laboratory accredited by LWiMP (certificate AB 361 of the Polish Center for Accreditation) using ESM-100 meter No. 972153 with a calibration certificate LWiMP/W/056/2021 of February 15, 2021, issued by the accredited PCA AP-078 calibrating laboratory.

Analysis of biochemical markers

Immediately after the end of the workers exposure to doses of E-field, hemolymph was collected. To do this the bees' antennae was removed with sterile tweezers and bee's abdomen was gently pressed according to the method by Borsuk et al. (2017) and [1]Migdał et al. (2020). The hemolymph was collected in 20 µl end-to-end glass

Table 1.

Dose level in experimental group

Field strength	Time [h]	Dose level [(kV/m)*h]
1 kV/m	2	1*2=2
2 kV/m	1	2*1=2
4 kV/m	0.5	4*0.5=2
8 kV/m	0.25	8*0.25=2

* it is understood as the exposure of bees to the same amount of energy

capillary without anti-coagulant which was placed in 1.5 ml Eppendorf tube with 200 µl Mil-Q water. After the appropriate amount of material was collected, the tubes were transferred to cryo-boxes and placed in a -80°C freezer. From each group, three Eppendorf tubes (3 replicates) were collected with ten glass capillaries each. Four bees were used to fill each 20 µl end-to-end capillary, and 120 bees from each group were analyzed. The following parameters were selected to assess the state of the organism: enzymatic biochemical marker activity : aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma-glutamylotranspeptydase (GGTP) in bee hemolymph. Indicators were measured with the kinetic method with the use Cormay monotests (Lublin, Poland) in accordance with the manufacturer's procedure.

Aspartate aminotransferase - AST

An AST reagent composition at pH 8.1 was prepared from the following compounds:
2-Ketoglutarate (13 mmol/L), L-Aspartate (220 mmol/L), LDH (1200 U/L), MDH (90 U/L), NADH (10 mmol/L), Tris buffer (88 mmol/L) and EDTA (5.0 mmol/L). The AST reagent composition was then heated to 37°C. 100 µL of the AST reagent composition was poured into 10 µL of sample/hemolymph, vortexed for 3-5 seconds, heated for 30 seconds. at 37°C and the absorbance at 340 nm was measured during T0 followed by 1, 2 and 3 minutes after incubation.

Alanine aminotransferase - ALT

ALT reagent composition at pH 7.8 was prepared from the following compounds:
2-Ketoglutarate (13 mmol/L), L-alanine (440 mmol/L), NADH (0.10 mmol/L), LDH (1800 U/L), Tris buffer (97 mmol/L) and EDTA (5.0 mmol/L). The ALT reagent composition was then heated to 37°C. 100 µL of the ALT reagent composition was poured into 10 µL of sample/hemolymph, vortexed for 3-5 seconds and heated for 30 seconds at 37°C, and the absorbance at 340 nm was measured during T0 followed by 1, 2 and 3 minutes after incubation.

Alkaline phosphatase - ALP

The ALP reagent composition was prepared from the following compounds: 2-amino-2-methyl-1-propanol (900 mmol/L), magnesium acetate (1.6 mmol/L), zinc sulfate (0.4 mmol/L) and HEDTA (2.0 mmol/L). The ALP reagent composition was then heated to 37°C. 100 µl of the ALP reagent composition was poured into 2 µl of sample/hemolymph, vortexed for 3-5 seconds, heated for 60 seconds at 37°C. Then 20 µL of 4-NPP (16.0 mmol/L) was added to the sample/solution, vortexed for 5 seconds and heated for 60 seconds at 37°C. Absorbance was measured at 405 nm at T0 followed by 1, 2 and 3 minutes after incubation.
The activity of AST, ALT and ALP was calculated according to the formula:

$$\text{Activity}_{ALT\,/\,AST\,/\,ALP} = \Delta Abs\,/\,min \times F$$

$$F_{ALT\,/\,AST} = (TV \times 1000) / (6.3 \times SV \times P)$$

$$F_{ALP} = (TV \times 1000) / (18.8 \times SV \times P)\ \Delta Abs\,/\,min = ((A2-A1) + (A3-A2) + (A4-A3)) / 3$$

Where:
A1, A2, A3, A4 - individual absorbance readings for samples
TV - total volume of the reaction mixture
SV - sample volume used for the reaction
P - length of the optical path of the cuvette
6.3 - the absorption coefficient of dihydronicotinamide adenine dinucleotide (NADH; at a wavelength of 340 nm)
18.8 - absorption coefficient for 2,4-dinitrophenol (2,4-DNP).
Enzyme activity is expressed in U/L units.

Gamma-glutamylotranspeptydase- GGTP

A kinetic photometric test was used to determine GGTP. The product concentration was determined colorimetrically by measuring the absorbance at a wavelength equal to λ = 405-410 nm. Analysis was conducted with the ABX Pentra GGT CP assay kit (HORIBA ABX Diagnostics, France) according to the manufacturer's recommendation. Enzyme activity is expressed in U/L units.

Statistical analyzes

The normality of the data distribution was analyzed with the Shapiro-Wilk test. With the use of the non-parametric Kruskal Wallis test, data for the global hypothesis within a group and between groups were determined to be statistically significant. Statistical tests were performed with the use of the RStudio program at the significance level $\alpha = 0.05$. In order to check the two age groups of bees, each was analyzed separately using Welch ANOVA and then the differences were tested with the Games-Howell test.

RESULTS

The activity of the ALT, AST, ALP, and GGTP biochemical markers of bee hemolymph in each experimental group (1 kV/m, 2 kV/m, 4 kV/m, 8 kV/m) at the same age did not differ significantly in comparison with the control group (C) and between them. However, highly significant statistical differences in the activity of the same biochemical markers were found among the experimental bee groups (C, 1 kV/m, 2 kV/m, 4 kV/m, 8 kV/m) of different ages (Tab. 2).

Alanine aminotransferase activity (ALT), Aspartate aminotransferase activity (AST), Alkaline phosphatase activity (ALP)

The highest ALT activity was recorded in one-day-old bees exposed to an electromagnetic field of 2 kV/m (Tab. 2), and this difference compared to the control group (C) of one-day-old bees was not statistically significant. The lowest activity was recorded in the group of seven-day-old bees, also exposed to the field intensity of 2 kV/m, and this difference compared to the control group (C) of seven-day-old bees was not statistically significant. The difference between the highest and lowest activity was 25.6%. Highly significant statistical differences were found between the corresponding groups; the control group (C) of one-day-old bees differed from the control group (C) of seven-day-old bees, and the group of 2 kV/m one-day-old bees differed from the group of 2 kV/m seven-day-old bees. Similarly to the control group, the group exposed to the 2kV/m electromagnetic field had decreased ALT activity with age.

The highest AST activity was recorded in one-day-old bees exposed to a 4 kV/m electromagnetic field (Tab. 2). This difference compared to the control group (C) of day-old bees was not statistically significant. The lowest activity was recorded in seven-day-old bees in the control group (C). The difference between the highest and lowest activity was 33.5%. Highly significant statistical differences were found between the corresponding groups: the control group (C) of one-day-old bees from the control group (C) of seven-day-old bees and the group of 2 kV/m one-day-old bees from the group of 2 kV/m seven-day-old bees. Similarly to the control group, the group exposed to the 2 kV/m electromagnetic field had decreased AST activity with age.

The highest ALP activity was noted in day-old bees in the control group (C) (Tab. 2), and the lowest activity was recorded in the group of seven-day-old bees exposed to a 8 kV/m field. This difference was not statistically significant compared to the control group (C) of seven-day-old bees. The difference between the highest and lowest activity was 8.8%. ALP activity, similar to the activity of other biochemical markers included in the study, also decreased with age, but in this case the difference was not statistically significant.

Gamma-glutamylotranspeptydase activity (GGTP)

The highest GGTP activity was noted in one-day-old bees in the control group (C) (Tab. 2), and the lowest activity was recorded in the group of seven-day-old bees exposed to a 2 kV/m field. This difference was not statistically significant compared to the control group (C) of seven-day-old bees. The difference between the highest and lowest activity was 8.15%. Highly significant statistical differences were found between the control group (C) of one-day-old bees and the control group (C) of seven-day-old bees. GGTP activity decreased with age.

Table 2.

Average value of selected biochemical markers in the hemolymph of worker bees after the first and seventh day after extermination

Group	Time [h]	Age [days]	Selected biochemical markers (±SD)			
			ALT [U/L]	AST [U/L]	ALP [U/L]	GGTP [U/L]
Control	-	1	71.64^{A} (±6.27)	105.96^{A} (±12.66)	5.67 (±0.31)	5.77^{A} (±2.55)
1 kV/m	2		57.35 (±0.05)	112.72 (±0.23)	2.62 (±0.35)	2.57 (±0.26)
2 kV/m	1		74.53^{A} (±0.03)	133.3^{A} (±0.05)	4.68 (±0.28)	2.69 (±0.28)
4 kV/m	0.5		69.24 (±0.01)	144.23 (±0.11)	4.45 (±0.15)	1.14 (±0.06)
8 kV/m	0.25		57.6 (±0.02)	102.07 (±0.04)	3.62 (±0.07)	1.5 (±0.18)
Control	-	7	21.17^{B} (±0.85)	48.37^{B} (±3.73)	3.44 (±1.4)	0.56^{B} (±0.39)
1 kV/m	2		24 (±0.39)	62.18 (±0.89)	1.52 (±0.65)	0.71 (±0.33)
2 kV/m	1		19.06^{B} (±1.28)	49.63^{B} (±3.15)	1.31 (±0.57)	0.47 (±0.51)
4 kV/m	0.5		21.3 (±1.35)	60.48 (±10.65)	1.32 (±0.64)	0.68 (±0.62)
8 kV/m	0.25		21.8 (±2.55)	54.15 (±6.65)	0.5 (±0.01)	1.34 (±0.94)
	p-value		2.315e-06	2.623e-06	3.576e-06	8.433e-06

SD - standard deviation

A,B - different letters indicate statistical differences between day-old and seven-day-old bees within each group at $p \leq 0.05$

DISCUSSION

Compared to the control group, there were no statistically significant differences in the activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma-glutamylotranspeptydase (GGTP) in honey bee hemolymph after exposure to the 50 Hz E-field and the intensity of 1 kV/m for 2 h, 2 kV/m for 1 h, 4 kV/m for 30 min and 8 kV/m for 15 min. In contrast, other studies using higher intensities and longer exposure times found that EMFs significantly affected various biochemical markers of insects. [2]Migdał et al. (2021) investigated the effect of a 50 Hz electromagnetic field of variable intensity on biochemical markers in bee hemolymph by using the field strength at the level of 5 kV/m, 11.5 kV/m, 23 kV/m and 34.5 kV/m. Each group was affected by the field for 1, 3, 6 and 12 h. The activity of biochemical markers in the research groups as compared to the control group turned out to be significantly lower. In our own research, each honey bee groups was exposed to an electromagnetic field with a specific energy dose, while [2]Migdał et al. (2021) exposed each group to a different dose of energy, hence the difference in results.

[2]Migdał et al. (2020) investigated electromagnetic fields at 5 kV/m, 11.5 kV/m, 23 kV/m and 34.5 kV/m and their influence on antioxidant

power reducing iron ions (FRAP) and the activity of superoxide dismutase (SOD) and catalase (CAT) antioxidant enzymes. The FRAP level did not differ significantly between the groups, except for exposure to the 50 Hz electromagnetic field and the intensity of 11.5 kV/m and 34.5 kV/m for twelve hours. SOD and CAT activity increased or decreased in the experimental groups. The results indicated that the electromagnetic field could affect the honey bee antioxidant system by altering the concentration of non-enzymatic antioxidants in the hemolymph. This proves a disorder of protein metabolism and increased muscle activity.

Koziorowska et al. (2020) conducted studies in which honey bees were exposed to a human-induced electromagnetic field of 1.6 mT with a frequency of 50 Hz for 2, 6, 16, 24 and 48 hours. An increase in metabolic changes that may have resulted from environmental stress was observed in them. The presented results confirm the influence of the 50 Hz electromagnetic field of as an environmental factor on the activity of selected honey bee biochemical markers and its dependence on the exposure time.

From the presented research, statistically significant differences in the activity of biochemical markers were observed between bees of different ages. The activity of ALT, AST and GGTP decreased in the following days of the experiment (Tab. 2). The decreased activity of these markers concerns both the control groups and the group of bees exposed to the electromagnetic field. ALP activity, as shown by the results (Tab. 2), also decreased in the following days of experiment, but this difference was not statistically significant. Other results had been obtained by Łoś and Strachecka (2018), who noted that the activity of AST, ALT, ALP and GGTP in the hemolymph increased during the experiment. However, in the case of exposure to harmful factors, the activity of these markers decreases.

AST and ALT are transaminases involved in the metabolism of carbohydrates and proteins. The activity of these enzymes varies under various physiological or pathological states (Martin et al., 1981). AST is involved in the conversion of aspartate and α-ketoglutarate to oxaloacetate, and vice versa, during the citric acid cycle and transamination. ALT catalyzes the transfer of an amino group from L-alanine to α-ketoglutarate, and the products of this reaction are pyruvate and L-glutamate. ALP, through dephosphorylation, hydrolyzes phosphate groups in such molecules as nucleotides, proteins and alkaloids under alkaline or acidic conditions. This enzyme may be an indicator of the digestive efficiency and transport of nutrients between the middle intestine, hemolymph and fat bodies (Campos et al., 2011; Jafari et al., 2014). GTTP is an indicator of liver and biliary diseases and in the process of hydrolysis transfers γ-glutamyl residues across membranes to amino acids and small peptides (Mościński et al., 2012).

From the conducted research, it could be concluded that the honey bee is not harmed when subjected to doses of the 50 Hz electromagnetic field and an intensity in the range of 1-10 kV/m. However, considering the information included in the discussion, we could repeat the research by extending the exposure time, because based on the results obtained, we are still unable to determine whether the EMF threatens the proper functioning of the honey bee or not. However, this study demonstrated that the physiological changes that occurred in the body of the honey bee were affected by age. The activity of the ALT, AST, ALP and GGTP biochemical markers decreases with the age of the honey bee, and hence worker bees must be equal age in the experiment.

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