FEDERAL STATE BUDGET INSTITUTION OF SCIENCE
PAVLOV INSTITUTE OF PHYSIOLOGY OF THE RUSSIAN ACADEMY OF SCIENCES

«APPROVE»
Director
of Pavlov Institute of Physiology
of the RAS
corresponding member of RAS
L.P Filaretova
«12» 07 2019

REPORT
UNDER THE AGREEMENT ON SCIENTIFIC COOPERATION WITH AIRES HUMAN GENOME RESEARCH FOUNDATION

Subject: Study of high-frequency electromagnetic radiation impact and Aires resonators influence on behavior, genetic and epigenetic processes in cells of central and peripheral organs (models organisms: rat (*Rattus norvegicus*) and honeybee (*Apis mellifera L.*)

FIFTH STAGE: Study of the effect of «Aires Defender Pro» resonators on the expression of the stress-reactive *hsp70* gene in the brain of a honeybee.

Principal investigators

Chief Research Scientist
Laboratory of Higher Nervous Activity Genetics
Dr.Sci., Prof. N.G.Lopatina

Senior Research Scientist
Laboratory of Higher Nervous Activity Genetics
Ph.D. T.G.Zachepilo

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REPORT

UNDER AGREEMENT ON COOPERATION IN SCIENCE BETWEEN FEDERAL STATE BUDGET-FUNDED INSTITUTION OF SCIENCE PAVLOV INSTITUTE OF PHYSIOLOGY OF THE RUSSIAN ACADEMY OF SCIENCE AND AIRES HUMAN GENOME RESEARCH FOUNDATION

Subject:

Study of High-Frequency Electromagnetic Radiation Impact and Aires Resonators Influence on Behavior, Genetic and Epigenetic Processes in Cells of Central and Peripheral Organs (*Rattus norvegicus* and *Apis mellifera* L.Honey Bee Models)

STAGE FIVE (December – June, 2019): Study of Aires Resonators Influence on Stress-Responsive *hsp70* Gene Expression in the Honey Bee Brain.

INTRODUCTION

Over millions of years natural electromagnetic fields (geomagnetic field, solar radiation, atmospheric electricity) have been a constant ecological factor, influencing condition of living organisms and ecosystems. Evolving organisms adapted to influence thereof. Today, as a result of progress in science and technology, there is a great number of electric devices, helping a man in various areas of life. Most electric devices around us are sources of electromagnetic radiation of different frequency and power. High level of electromagnetic pollution of the environment can be harmful to living organisms. Degree of EMR impact on living beings depends on field power and strength, oscillations frequency, exposure duration and mode of generation thereof (pulse and continuous fields) (Kudryashov et al., 2008).

Insects (butterflies, ants, cockroaches, flies) are deemed to be the best experimental animals to study EMR impact as they are highly sensitive to magnetic and electric fields (Kumar et al., 2011).

It has already been proved that impact of high-frequency radiation decreases queen bee's fertility and leads to reduction of honey and bee-bread amount in a family (Kumar et al., 2011). Unconditional-reflex food excitability and short-time memory of a honey bee deteriorates (Lopatina et al., 2019). Molecular and cellular mechanisms of this phenomenon are unknown and need careful study. Apparently, change of the natural electromagnetic background is a stressor for bees.

Heat shock proteins (HSP) are universal stress reaction sensors. Heat shock proteins are one of the most conservative and phylogenetically ancient proteins: homology degree of

eucaryotes and procaryotes HSP makes more than 50%, and some domains thereof are totally identical; structural similarity of human and mice HSP is up to 95%. A certain amount of heat shock proteins (HSP) is continuously synthesized in any nuclear cells, in numerous intracellular structures (in the nucleus, cytoplasm, endoplasmic reticulum, chloroplasts and mitochondria) of all multi-cellular organisms, regardless of exposure to stress factors. Heat shock proteins are molecular chaperones, participating in protein folding (tertiary structure formation), HSP prevent nonspecific proteins aggregation and protect them from premature proteolysis. HSP protect the cell from impact of mutant or misfolded proteins, from death of cells caused by stress. Increase of HSP intracellular synthesis is caused not only by heat shock, but also by any exposure to stress: external impact (UV, heavy metals, heat shock, amino acids), pathologic impact (viral, bacterial and parasitic infections, inflammation, malignant transformation, autoimmune response) or even physiological impact (growth factors, cell differentiation, hormonal stimulation, tissue growth) (Nikitin, 2008).

HSP70 heat shock protein (which belongs to the family of proteins with molecular mass more than 70kDa) is the most studied one. HSP70 acts as a chaperone in the cell, besides it participates in stress-related processes, such as aggregation, deaggregation, degeneration and restoration of three-dimensional folding (Nikitin, 2008).

According to the data base NCBI GenBank (LOC408706 heat shock protein 70Cb ortholog), gene length of a honey bee makes 8361 bps. Length of mRNA: XM_623196.5 - 4605 bps, XM_006561162 – 4497 bps. Proteins length: XP_006561225.1 - 831 a.a., XP_623199.2 - 861 a.a.

Purpose of this work is to study stress-responsive *hsp70* gene expression in the honey bee Brain upon exposure to electromagnetic radiation, emitted by WiFi router and simultaneous exposure to WiFi router and Aires Defender Pro resonators.

STUDY MATERIALS AND METHODS

The work was performed using 10-30 day *Apis mellifera carnica* worker honey bees. Bees were bred at the bee house of the Pavlov Institute of Physiology of the Russian Academy of Science. The bees meant for the experiment were kept in the observation queen-bee cell in the special premise at a room temperature and automatic lighting from 8 a.m. to 8 p.m. .

5 groups of bees participated in the experiment (10-16 animal units per each group): intact group, control group (faraday's cage, isolation from external EMR), control group (6 Aires Defender Pro resonators in the center of each faraday's cage face), experimental group (WiFi

router operating in the faraday's cage in the 24h mode + 6 Aires Defender Pro resonators resonators).

The following algorithm was used to study impact of electromagnetic waves on *hsp70* gene expression: exposure to EMR router (2 groups of bees), control without exposure (3 groups of bees), extraction of RNA from brains of bees of all groups, RT-PCR with electrophoretic detection (Fig. 1, 2).

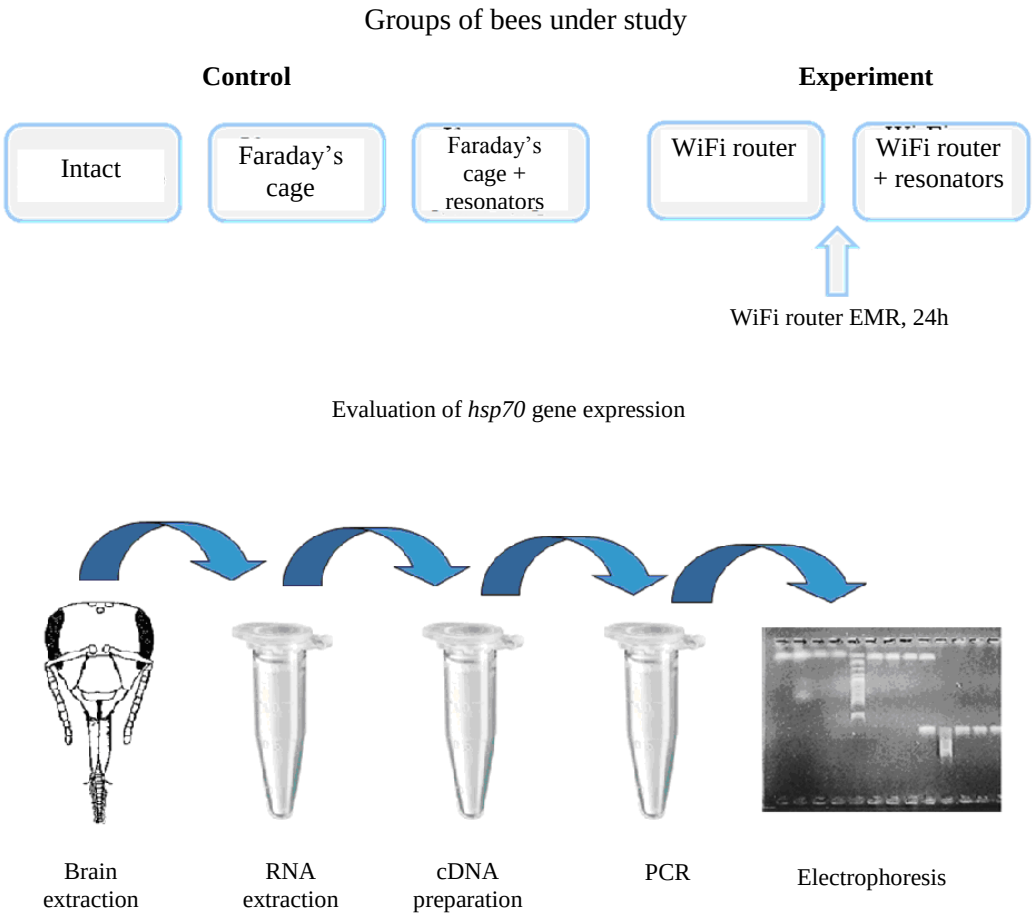

Fig. 1. Design of the experiment

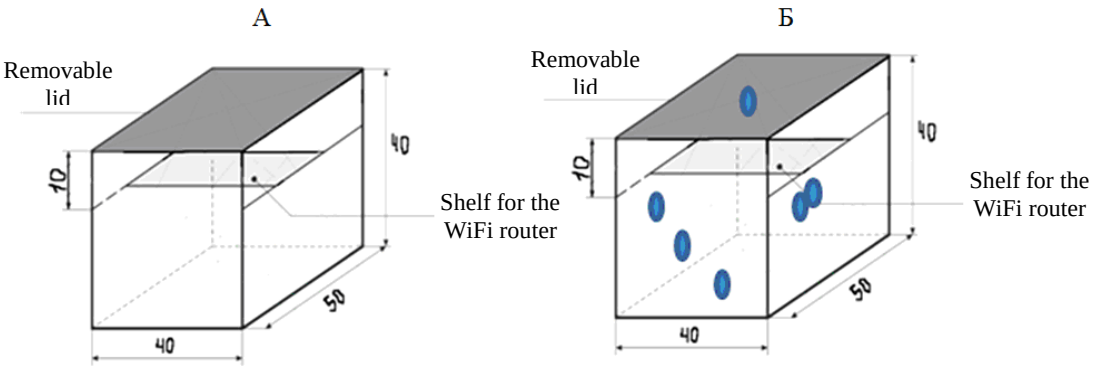

Fig. 2. А – Faraday's cage, Б – Faraday's cage + Aires Defender Pro resonators.

Extraction of total RNA from the brain of the honey bee. The bees were taken from the hive and immediately placed into the freezer for 10 minutes. Afterwards, the head capsule

was opened up and the brain was extracted. The brain was homogenized with a pestle in 1.5mcl centrifugal tube with 200mcl homogenization buffer (Evrogen). The homogenate was incubated at a room temperature during 10 minutes. 300mcl of chloroforme was added for deproteinization, samples were thoroughly vortexed. The samples were incubated at a room temperature during 5 minutes. Then they were centrifugated at 13,000rpm during 3 minutes. The supernatant with nucleic acids was transferred to a clean test tube. 500mcl of isopropyl alcohol was added, the sample was mixed. 100mcl of 3 M Sodium acetate was added, the sample was carefully mixed. The samples were placed into the refrigerator at -20 ^{o}C for 1 hour for nucleic acids to precipitate. Then the samples were centrifugated at 13,000rpm during 3 minutes. The supernatant was removed. 500mcl of 96% ethyl alcohol was added, the samples were mixed. Then they were centrifugated at 13,000rpm during 3 minutes. The supernatant was withdrawn. 500mcl of 70% ethyl alcohol was added, the samples were mixed. Then they were centrifugated at 13,000rpm during 3 minutes. The supernatant was withdrawn. 500mcl of 70% ethyl alcohol was added, the samples were mixed. Then they were centrifugated at 13,000rpm during 3 minutes. The supernatant was withdrawn. The wash-out was repeated (steps 16-18). The sediment was diluted in TE buffer, pH 8.0 (1 mM EDTA, 10mM tris hydrochloride, pH 8.0) at a room temperature during 10 minutes. The samples of the total RNA, prepared in such a way, were stored at -20 ^{o}C.

Reverse Transcription. The reaction of reverse transcription was performed using the obtained samples and the reverse transcription set (Evrogen) with a random primer (Evrogen) according to the manufacturer's recommendations (2 hours at 38 ^{o}C). The obtained cDNA was stored at -20^{o}C.

Polymerase Chain Reaction. cDNA, obtained upon reverse transcription was used as a matrix. PCR was performed according to the manufacturer's recommendations (Evrogen), using Veriti 96-Well Thermal Cycler (Applied Biosystems). Primers annealing temperature was 61^{o} C. Cycles number - 40. Primers (10pmol/mcl, Evrogen): they were selected by T.G. Zachepilo in the GenBank by means of PrimerBLAST online package.

Table 1. Primers

Direct/reverse	Sequence	Product	Primer
Direct	*Apis mellifera* heat shock protein 70Cb ortholog (LOC408706), XM_623196.5	92bps	AAGCACAAGCAAATGAACCACCG
Reverse			CCTCGCACCTCTTCCACCAT
Direct	*Apis mellifera* ribosomal protein L32 (RpL32), NM_001011587.1	109bps	TGTGCTGAAATTGCTCATGGT
Reverse			AGAACGTAACCTTGCACTGG

Electrophoresis in Agarose Gel. PCR-products were mixed with the loading buffer (Evrogen, 1:1). DNA fragments were separated in 10x15cm 1.5% agarose gel (with admixture of ethidium bromide) with TAE buffer in the horizontal electrophoresis chamber (Helicon) at 150V during 40 minutes. Do determine the size of the amplificated fragments, DNA-markers were

applied on the gel: 100bps (Evrogen). Electrophoresis results were detected in the transmitted ultraviolet, using the transilluminator (Vilber Lourmat). The gel was shot, using a digital camera. The results were saved on the computer in JPEG format.

Data Processing. Photos of electrophoregrams were analyzed in ImageJ (NCBI). First, paths were identified in the image, then the area of stained bands was evaluated. Normalization was performed: ratio of area of *hsp70* samples bands to the area of *rp49* reference gene was determined.

Then pairwise comparison of normalized values was performed in all groups (total 10), using non-parametric Mann-Whitney test. Statistical analysis was performed in Statistica 10.

FINDINGS

Expression of *hsp70* stress-responsive gene upon exposure of the honey bee to electromagnetic radiation was studied by the method of RT-PCR and electrophoretic detection.

As a result of performed experiments electrophoregrams were obtained (Fig. 3-5). Expression was compared by matching bands in electrophoregrams. Thick and intensely colored bands refer to strong expression, while thin and weakly colored ones refer to weak expression.

Hsp70 gene expression in the intact group of bees was found in 3 samples (each sample contains the material, consisting of 2 bees brains) of 4, i.e. the animals differ by functional state of the CNS.

In the resonator control group (6 resonators on all faces of the faraday's cage) *hsp70* stress-responsive gene expression was more uniform than in the intact bees control group. It might be related to influence of the faraday's cage and resonators, equalizing functional state of the CNS.

Intact Resonators

Fig. 3. Electrophoregram of PCR-products in the intact and resonator groups. Henceforth, figures are samples numbers, gene under study – *hsp70*, reference gene – *rp49*.

In the faraday's cage control group expression of *hsp70* was similar to that of the resonator group. Thus, control groups of bees were the same, apart from the intact one.

Faraday's cage

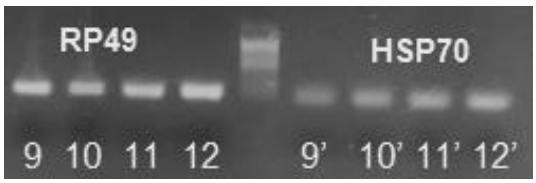

Fig. 4. Electrophoregram of PCR-products in the faraday's cage group

In the router group *hsp70* gene was expressed positively weaker, than in the faraday's cage and router+resonator groups. I.e. 24h impact of EMR, emitted by the router, caused weakening of *hsp70* expression.

Router+resonators Router

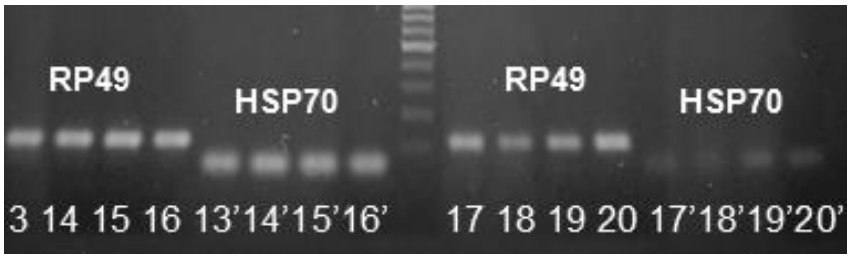

Fig.5. Electrophoregram of PCR-products in the router+resonator and router groups

It is to be noted that any change of electromagnetic background, i.e. weakening of EMF in the faraday's cage, change of EMR parameters in the faraday's cage due to Aires Defender Pro resonators, EMR increase, caused by the router, result in change of *hsp70* expression in comparison with the intact group.

Results of data processing are summarized in Fig. 6.

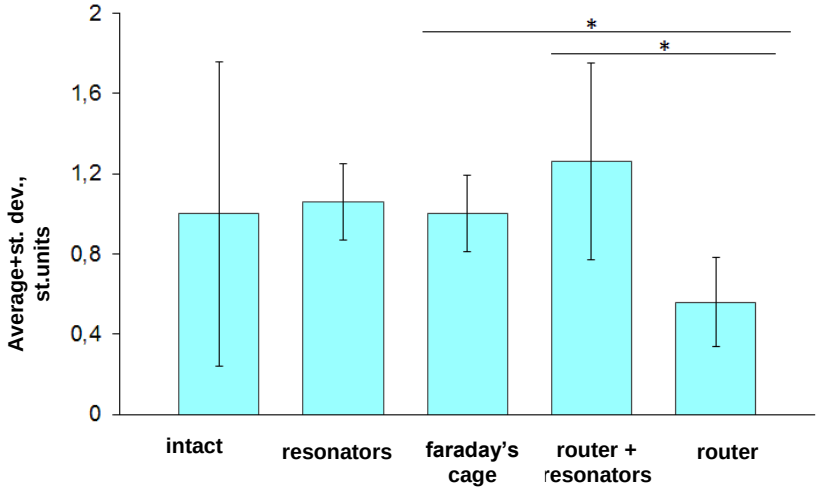

Fig. 6. Expression of *hsp70* gene in the bee's brain (normalized values _ standard deviation). * - differences are significant, $p<0,05$, Mann-Whitney test.

Thus it can be concluded as follows:

1. Level of *hsp70* gene expression in the honey bee's brain is similar in control groups. Intact bees show greater variability of this parameter.

2. After 24-h exposure to high-frequency electromagnetic radiation of a WiFi router, expression of *hsp70* gene in the honey bee's brain weakens which can have negative impact on functioning of the bee's CNS.

3. Isolated 24-h exposure to Aires Defender Pro resonators has no impact on expression of hsp70 gene in the honey bee's brain.

4. Upon simultaneous 24-h exposure to Aires Defender Pro resonators and WiFi router, expression of *hsp70* gene in the honey bee's brain increases to the control level.

DISCUSSION

Heat shock proteins play an important part in the life of a cell and the organism as a whole. HSP participate in control of proteins quality, in protection of cells from aggregation of misfolded proteins or in forwarding of misfolded proteins to proteasomes for proteolysis thereof. Virtually all cell proteins at least temporarily interact with HSP70. Need in chaperones upon exposure to stress factors increases significantly. Interaction between the protein target and HSP70 result in stabilization of the former, then the protein folds correctly, or chaperons of a different type are recruited, which is followed by further restoration of the intact conformation (Nikitin, 2008). Thus decrease of transcriptional activity of *hsp70* gene causes deficiency of HSP70 protein and increase of misfolded proteins and aggregates thereof. Accumulation of misfolded proteins and aggregates thereof in the neural tissue may result in derangement of learning processes and memory formation.

Despite the fact that development of stress reactions is generally related to increase of HSP70 level, there are also evidences that such conditions as hyperthermia, ageing or disease may decrease reaction of heat shock proteins in the brain (Pardue et al., 2007). This demonstrates that increase of heat shock proteins synthesis can be necessary in some cell reactions, however, not in all of them (Agustiño et al., 2012). The literature also describes decrease of HSP70 in case of cerebral ischaemia (Yang et al., 2005). These data show that increased expression of HSP70 is not critical upon early adaptation. However, regulation at later stages, including increase of heat shock proteins number, suggests that stress proteins are of importance in facilitation of long-term tolerance.

Decrease in number of heat shock proteins is, apparently, indicative of existence of non-thermal physical stimuli, acting through unidentified mechanisms via low-intensity electric fields

without direct connection between power and effect size. Since the animals were exposed to non-ionizing radiation in their entirety, their organism could react to the stress in multiple ways (Agustiño et al., 2012).

A number of works demonstrate negative impact of EMR on honey bees (Harst et al., 2006). Under impact of EMR, their locomotor activity decreased, their ability to orient in space was impaired, it took them more time to get to the hive, their ability to return to their family degraded abruptly, they built less and became more aggressive. It is shown that bees perceive EMR as danger signal (Favre, 2011). When exposed to EMR, egg-laying capacity of the queen bee decreased, drone brood was observed (Halabi et al., 2013), amount of honey and bee-bread in hives decreased abruptly (Kumar et al., 2011). Behavior deviations of a honey bee as a result of exposure to different-frequency EMR are also shown in works of Russian researchers (Yeskov, Bragin, 1986; Yeskov, Toboev, 2008; Lopatina et al., 2019).

Similar ecologically significant changes in behavior and reproduction of other insects (locust, flies, ants) under EMR impact are demonstrated in the work of Cucurachi et al. (2013). It is shown that upon exposure to high-power EMR oxidative stress and change of genes expression are observed in *Drosophila* (Manta et al., 2017). CNS of insects is quite sensitive to EMR: it is shown that it causes decrease of ability to form conditioned food reflex to olfactic and visual stimuli in *Myrmica sabuleti* ants (Cammaerts et al., 2011).

Thus distortions of cognitive activity of honey bees upon long-term exposure to high-frequency electromagnetic radiation may be related to accumulation of misfolded neuronal proteins, caused by decrease of transcriptional activity of *hsp70* gene and deficiency of HSP70 protein.

* * * * *

Results of the performed experiments expressly testify *hsp70* expression normalization under influence of Aires Defender Pro resonators. The data, obtained during the series of experiments is partly in compliance with previously obtained data on food excitability and short-term memory in bees upon simultaneous exposure to WiFi router and resonators. It is possible that *hsp70* normalizing action of resonators has a deferred effect on bees' behavior.

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