FEDERAL STATE BUDGET INSTITUTION OF SCIENCE

PAVLOV INSTITUTE OF PHYSIOLOGY

OF THE RUSSIAN ACADEMY OF SCIENCES

APPROVE

Director of FSBIS

Pavlov Institute of Physiology of RAS

Akad. of RAS L.P Filaretova

«6» May 2020

REPORT

UNDER THE AGREEMENT ON SCIENTIFIC COOPERATION

WITH AIRES HUMAN GENOME RESEARCH FOUNDATION

Subject: Study of electromagnetic radiation 5G impact and Aires resonators 5G influence on behavior, genetic and epigenetic processes in brain : models of rat Rattus norvegicus and honey bee Apis mellifera L.

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«6» May 2020

Saint Petersburg, 2020

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SECTION 1 (March - May 2020): Study of the effects of electromagnetic radiation (WiFi router, 2.4 GHz) and Aires Defender Pro resonators on the behavior of different rat strains in a series of tests to assess motor activity, emotionality, anxiety, and cognitive abilities.

Systems to protect against industrial electromagnetic radiation (EMR) are being developed in various fields, taking into account changes in the characteristics of EMR sources. The Aires Foundation has developed resonators that influence the response of animals and humans to non-ionizing electromagnetic radiation and have a protective effect (Zhabrev et al., 2005; Jasitis et al., 2018), but the biological mechanisms of these influences have not been studied.

The features of the influence of EMR on innate elements of behavior, motor activity, emotionality, anxiety, and cognitive abilities in animals (Karthick et al., 2017), in particular, with a different functional state of the nervous system, as well as the effectiveness of devices designed to prevent the harmful effects of electromagnetic radiation on the body and the independent effect of these devices also have not been studied sufficiently.

Previously, we showed that the nature of the orienting-exploratory reaction of animals to the weakening of external electric and magnetic fields, as well as to a router's EMR under these conditions, depends on the hereditary excitability of the nervous system and affects various components of behavior (Shiryaeva et al., 2020). The action of the Aires Defender Pro resonators changed the behavior of animals and caused their activity to increase when placed in a new environment. Specific behavioral components of the animals' reaction to the action of resonators were identified, indicating their selective positive influence on specific elements of behavior.

For further more detailed study of the effects of EMR and the effects of properties of resonators on behavior, it is necessary to expand the range of tests used in preclinical trials of pharmacological preparations, using the Wi-Fi router EMR exposure schedule of 4 days at 6 hours a day, as we showed earlier, with the most powerful genotoxic effect in bone marrow cells (Dyuzhikova et al., 2019). Along with an assessment of orienting-exploratory activity and emotionality in an open field, it is necessary to study anxiety and other reactions of animals in a light-dark box and an elevated plus maze.

Previously It was shown that animals' exposure in a Faraday cage without additional influences and in combination with the router's action disturbed the retention in memory of the conditioned reflex of passive avoidance in male Wistar rats. Resonators partially prevented a negative effect on memory from animal exposure in a Faraday cage 24 hours after training (Shiryaeva et al., 2020).

It seems important to study the action of the EMR of a WiFi router and Aires Defender Pro resonators on learning processes and spatial memory using an additional test - the Morris water maze, as well as to continue to study the features of the formation and retention of the

conditioned reflex of passive avoidance in rats of strains with contrasting nervous system excitability immediately after the animals' exposure under given experimental conditions.

Purposes of the work

Study the influence of Aires Defender Pro resonators without and with the action of the

EMR of a standard Wi-Fi router for 4 days at 6 hours a day under standard Faraday cage conditions:

1) on behavior in open field, light-dark box, and elevated plus maze tests;

2) on the production and retention of the passive avoidance (PA), as well as on spatial memory in the Morris water maze.

in rats of strains with high-threshold (HT) and low-threshold (LT) contrasting nervous system excitability.

MATERIAL AND METHODS

The work was carried out on 5-month-old male rats of the HT and LT strains, bred at the Laboratory of Higher Nervous Activity Genetics (Vaido, 2000, Vaido, et al., 2018) and included in the Biocollection of Federal State Budget Institution I.P. Pavlov Institute of Physiology of the RAS (No. GZ 0134-2018-0003). Breeding Achievement Patents No. 10769 and 10768, issued by Federal State Budget Institution Russian Federation State Commission for the Testing and Protection of Breeding Achievements, were registered in the state register of protected breeding achievements on January 15, 2020. The males were kept in groups of 6 specimens in standard cages on a standard diet.

We used a Wi-Fi router (LinkSys E1200-EE/RU wireless router) with technical specifications: wireless frequency: 2.4 GHz, number and type of antennas: 2 internal antennas, gain of the standard antenna(s), dBi: 4 dBi. To study the action of a router's EMR, a "home" cage with animals was placed in a Faraday cage. Under the cage's top cover, a router was placed on a removable shelf in the center. The experimental groups were exposed to the router for 4 days for 6 hours a day (8:00 AM - 2:00 PM) without resonators and in combination with the action of resonators, as well as only in the presence of the action of resonators without a router. The control was group of rats placed in a Faraday cage at the same time, but without a router and resonators.

Aires Defender Pro resonators were used in the experiments (Zhabrev et al., 2005; Jasaitis et al., 2018). Six resonators were used to assess the effect of resonators with and without the action of the router's EMR. They were placed in the center of each face of the Faraday cage.

After the end of the animals' exposure in a Faraday cage under the experimental conditions described above, the behavior of the animals was assessed in tests in the following

sequence: 1) Open field; 2) Light-dark box; 3) Elevated plus maze 4) Passive avoidance task 5) Morris water maze.

We used the standard equipment by NPO Open Science (Russian Federation, Moscow Region, Krasnogorsk) and the original by Laboratory of Higher Nervous Activity Genetics, which are included in the list equipments for performing psychopharmacological tests pursuant to Order N 281 of the Russian Ministry of Health of April 30, 2013.

The **"open field"** apparatus used was a circle with a diameter of 160 cm, bounded by a border that was 35 cm high. The floor of the circle was divided into 20 cm squares. Above the center of the floor at a height of 60 cm a 500 W lamp was hung with a mirror reflector, providing illumination at the floor level from 2000 lx in the center to 1500 lx at the edges. The apparatus was placed in a darkened room. During the test, a rat was placed in the central square of the circle and its behavior was monitored for 5 minutes.

The behavior parameters were recorded:

Latency of the first movement (s)

Horizontal motor activity (number of intersected squares)

Vertical motor activity (raising up on the hind legs, number of upright positions)

Emotionality - defecation (number of boluses)

Grooming (number of acts)

Freezing (number of acts)

Turns (number of acts)

Rotations (number of acts)

An "open field" apparatus is designed to study the orienting-exploratory and emotional behavior of rodents under conditions of being transferred to a new environment; it allows a comprehensive assessment of natural controlled behavior and its changes under the influence of various factors (Amiksheeva et al., 2003): assessment of the level of emotion, motor activity (the horizontal is the number of crossed sectors, and the vertical is upright positions reflecting orienting and exploratory activity), and the severity of the anxiety-depressive component (activity in the central sector of the field, the number of turns and rotations), fear level based on a freezing response, and stereotypical behavior (Kaluev, 1998, Buresh et al., 1991).

Light-dark box

The "light-dark box" apparatus is a box divided by a removable partition with a hole into two compartments — light (30x30x30 cm) and dark (30x30x30 cm) (Fig. 1). The dark compartment also has a removable cover. The walls and floor of the chamber are made of white polyvinyl chloride. The dark compartment is covered inside with a black matte film.

The apparatus is designed to study the behavior of rodents under conditions of variable stress and makes it possible to assess: preference for darkness and light; intensity and dynamics of "peeping out" behavior; habituation.

Assessment of the behavior of rats in a light-dark box is included in the list of tests for preclinical research of new pharmacological preparations. http://www.openscience.ru/index.php?page=ts&item=007&lang=en

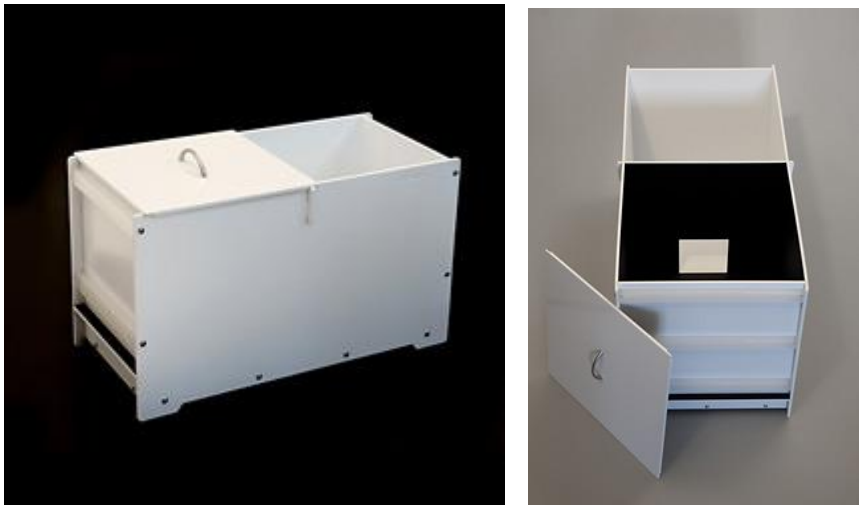

Fig. 1. "Light-dark box" apparatus for studying the behavior of rats.

The rat was placed in the center of the light chamber, with its tail towards the hole into the dark compartment, and its behavior was observed for 5 minutes.

The following parameters were recorded:

Latency of entry into the dark compartment (s)

Time spent in the light chamber (s)

Time spent in the dark chamber (s)

Number of transitions

Number of boluses in the light chamber

Number of boluses in the dark chamber

Elevated plus maze

"Elevated plus maze" (EPM) apparatus (Fig. 2) is designed to study the behavior of rodents in variable stressogenic conditions and makes it possible to evaluate: anxiety level of the animal (according to the preference for darkness/light, fear of heights, intensity and dynamics of "peeping out" behavior). According to the Guidelines for Preclinical Study of New Pharmacological Substances, the assessment of the behavior of rats and mice in an elevated plus maze is included in the list of tests to study tranquilizing (anxiolytic) action (basic tests). Normal

animals prefer to spend most of their time in the closed (dark) arms of the maze. The anxiolytic effect of the influence or drug is assessed based on an increase in the number of entries into the open (light) arms and the time spent in them, without an increase in overall physical activity. http://openscience.ru/index.php?page=ts&item=002&lang=en

Fig. 2. "Elevated plus maze" apparatus for assessing the behavior of rats.

The rat was placed on the central area of the maze with its nose toward one of the open arms and its behavior was observed for 5 minutes.

The following parameters were recorded:

1) number of entries into the closed arms;
2) time spent in the central zone;
3) time spent in the open arms;
4) time spent in the closed arms;
5) number of times the head is lowered;
6) number of boluses;
7) presence/absence of urination;
8) the ratio of animals in the experimental group that entered and did not enter the open arms.

A **passive avoidance task** (PAT) with one-time negative pain reinforcement was produced using an apparatus consisting of two chambers — a light chamber and a dark chamber, which had no lighting — connected with each other by a passage. As is known, rats normally spend most of their time in the dark compartment, which is associated with the animals' instinctive desire to be in a dark and cramped space — a burrow (burrowing reflex). The method is based on producing in rats a conditioned reflex for passive avoidance of the dark chamber in

response to an unconditioned electrocutaneous pain stimulus. The rat was placed in the center of the light chamber, with its tail toward the hole into the dark chamber. The animal was given 2 minutes to explore the compartments; during this time, it found the hole into the dark chamber and entered it. In the dark chamber, the animal received an electrocutaneous pain stimulus of 1 mA for a duration of 1 min. This concluded production of the reflex. If the animal did not enter the dark chamber for 2 minutes, it was excluded from the subsequent experiment. The percentage of animals that did not enter the dark compartment was recorded.

Morris water maze

The water maze is a pool with a diameter of 93 cm and height of 50 cm, filled with 36 cm of water. A hidden 10×10 cm platform was located in the center of one of the pool's quadrants at a depth of 2 cm under water and its position did not change during the experiment. To achieve opacity, powdered milk was added to water at a temperature of 25-30 °C. Elements of the experimental room acted as external visual reference points.

The experiment was carried out for two consecutive days; the rat was placed at the starting point on the periphery of the pool with its nose facing the wall and given 2 minutes to search for the platform hidden under water; after finding it, the rat was left on the platform for 30 seconds, after which it was moved to an individual cage to rest and dry off for 2 minutes; if the rat did not find the platform itself within 2 minutes, then it was brought to the platform; each rat did 12 consecutive attempts during each day;

Recorded parameters:

1) latency of finding the hidden platform (s).

Statistical processing

Averages and medians were calculated. The medians are shown in the figures. The significance of differences between the options was determined using nonparametric criteria, the Mann-Whitney test, the multiple range test, as well as the criterion for the significance of differences in shares (Plokhinsky, 1970). We used the Friedman test for multiple comparisons of related samples, Kruskal-Wallis test for group comparisons based on individual parameters, and Benjamini-Hochberg correction for multiple comparisons based on the number of parameters. We used Statgraphics Centurion XV111 software.

RESULTS AND DISCUSSION

The open field, light-dark box, and elevated plus maze apparatuses were used to study the effects of exposing rats of two HT and LT lines in a Faraday cage without additional influences,

as well as in combination with the EMR of a standard WiFi router (4 days x 6 hours per day), a router and resonators, and only resonators. We also studied the influence of these effects on the cognitive abilities of rats in the conditions of a Morris water maze and in the formation and retention of a conditioned passive avoidance reaction. Let us consider the results obtained in succession.

1. Open field

In the highly excitable rats of the **LT** strain, we observed changes (a decrease), which were comparable in magnitude, in the latency of the first movement (**Latency**) after the action of the router and after the action of the resonators relative to the control group (Fig. 3). After the combined action of the router and resonators, the Latency tended to increase, not reaching the level of significance. Rats with low-excitability of the HT strain of the control group had lower Latency values compared to the alternative line. No changes in the studied trait were recorded for them in any of the three experimental exposure options.

As for vertical motor activity (VMA) (number of upright positions), only known genetically-determined interstrain differences in rats of the HT and LT strains were shown (Fig.4). Intensified VDA is a distinctive feature of the highly excited LT strain. The experimental factors did not influence this indicator of the rats' motor activity.

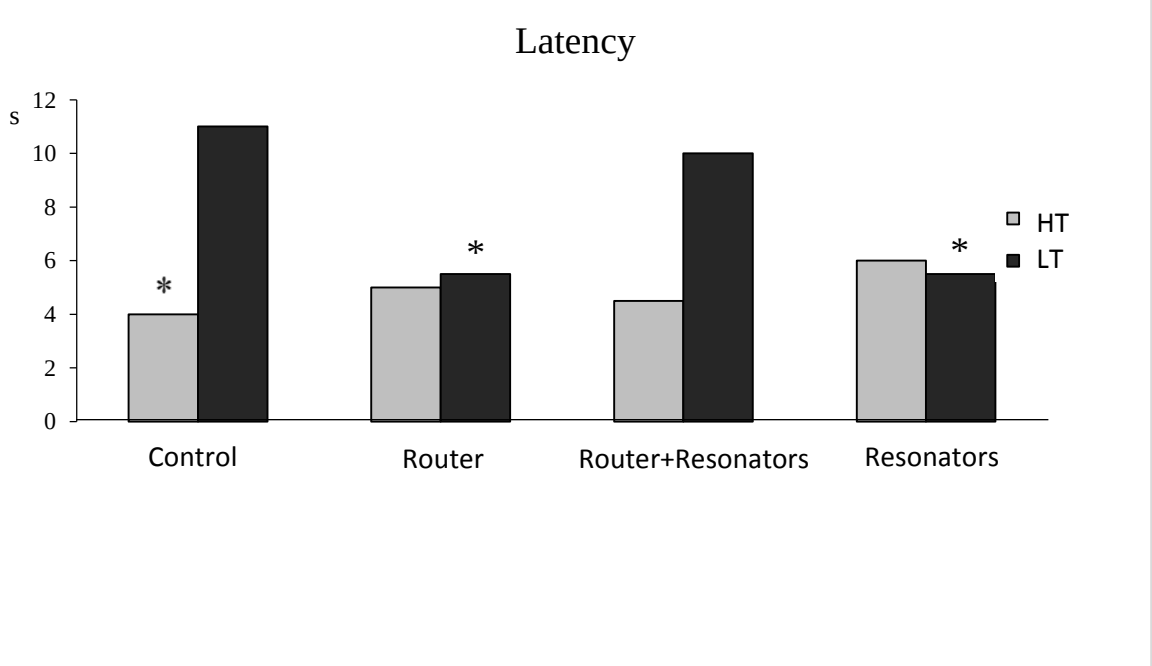

Fig. 3. Latency of reactions in the open field test of male rats of the HT and LT strains in the various experimental groups. Exposure of animals: Control — 4 days (x 6 hours a day) in a Faraday cage, Router — 4 days (x 6 hours a day) in a Faraday cage with a Wi-Fi router, Router+resonators — 4 days (x 6 hours a day) in a Faraday cage with a Wi-Fi router and resonators, Resonators — 4 days (x 6 hours a day) in a Faraday cage with resonators.
Key: *- The LT strain s differences with the control are significant (P<0.009, P<0.009, P<0.04).

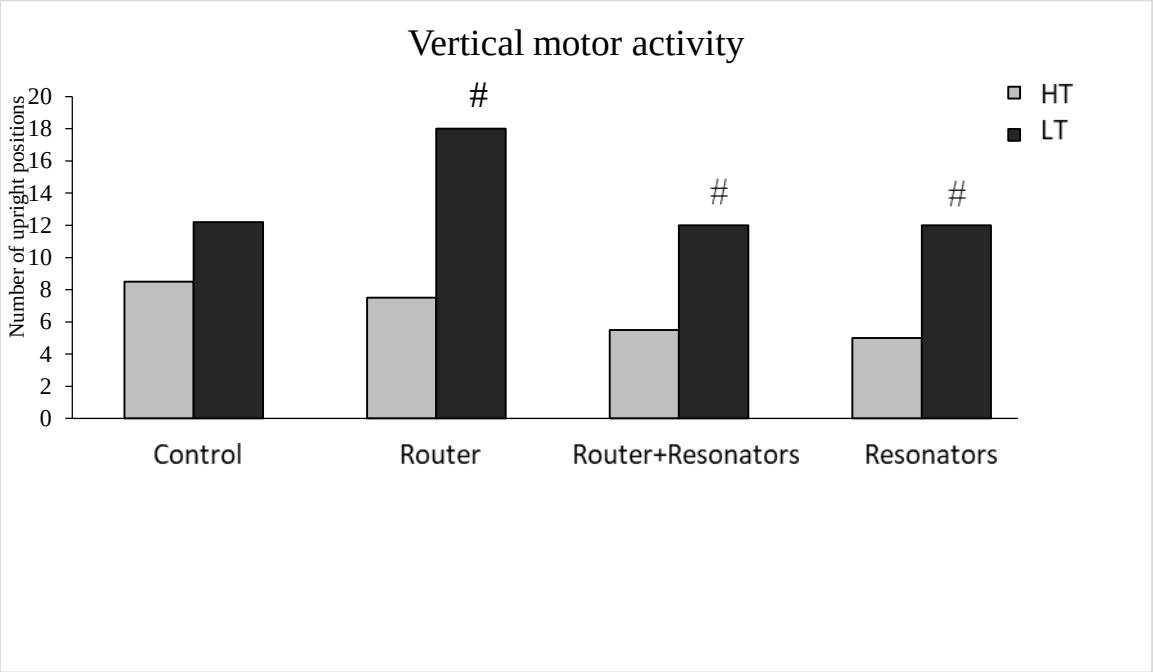

Fig. 4. Vertical motor activity (number of upright positions) in the open field test of male rats of the HT and LT strains in the various experimental groups (same as in Fig. 1).
Key: #- The HT strain's differences with the alternative group are significant ($P<0.03$)

Horizontal motor activity (**HMA**) (number of intersected squares), which characterizes the animals' locomotor activity, was affected, decreasing relative to the control, by the action of both the resonators alone and the resonators together with the router (Fig. 5), but only in the **LT** strain with low excitability. Significantly lower values of the trait in the control group were observed in the LT strain compared with the HT strain, which did not change under the influence of the experimental actions.

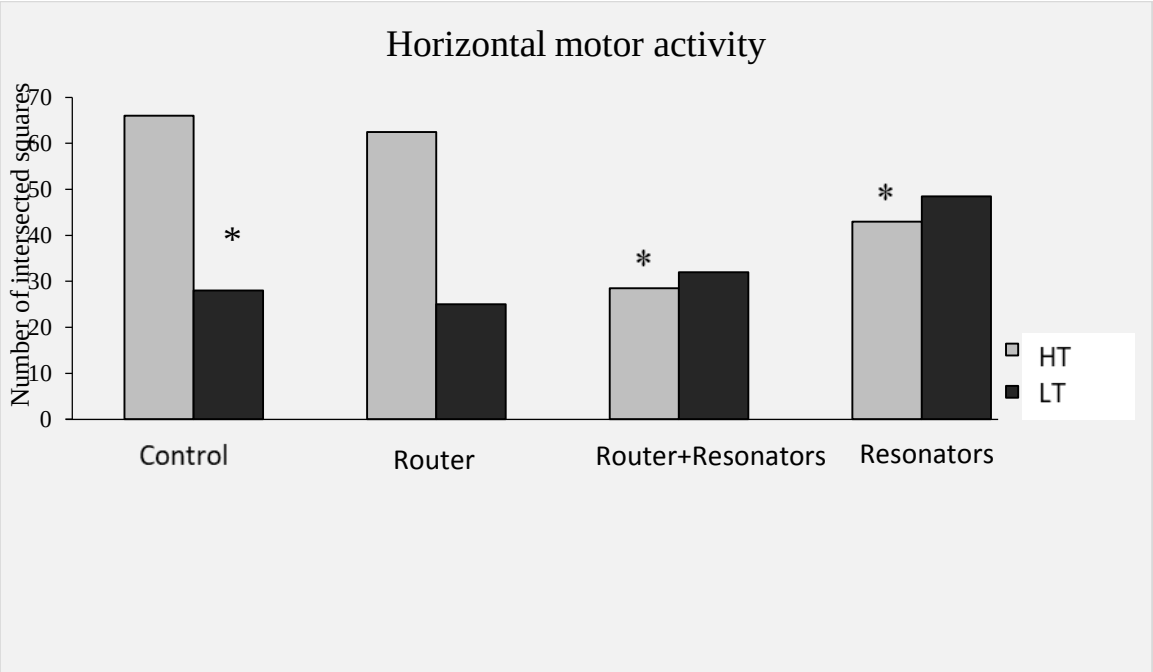

Fig. 5. Horizontal motor activity (number of intersected squares) in the open field test of male rats of the HT and LT lines in the various experimental groups (same as in Fig. 1). Key: *- The HT line's differences with the control are significant ($P<0.05$).

As for **freezing**, ambiguous multidirectional changes were observed in both strains (Fig. 6). Under the conditions of the action of the router together with resonators, freezing increased in rats of the **LT** strain in comparison with the action of the router alone, and the action of the resonators alone. By contrast, in rats of the **HT** strain, exposure with resonators increased freezing behavior compared to the combined action of a router and resonators.

There were no differences in grooming behavior (Fig.7).

The number of **turns** decreased in rats of the **LT** strain under the action of a router together with resonators in comparison with the control group (Fig. 8).

In rats of the **HT strain, rotations** decreased to the same extent both under the action the router alone, and under the action of resonators alone. However, their combined action brought the indicator's values back to the control level (Fig. 9). In rats of the **LT** strain, the action of the resonators reduced the number of rotations relative to the control and the action of the router.

The router together with the resonators enhanced **emotionality** in rats of the **LT** strain relative to the control (Fig. 10). However, exposure with resonators alone reduced this indicator both with respect to the action of the router and with respect to its joint action with resonators.

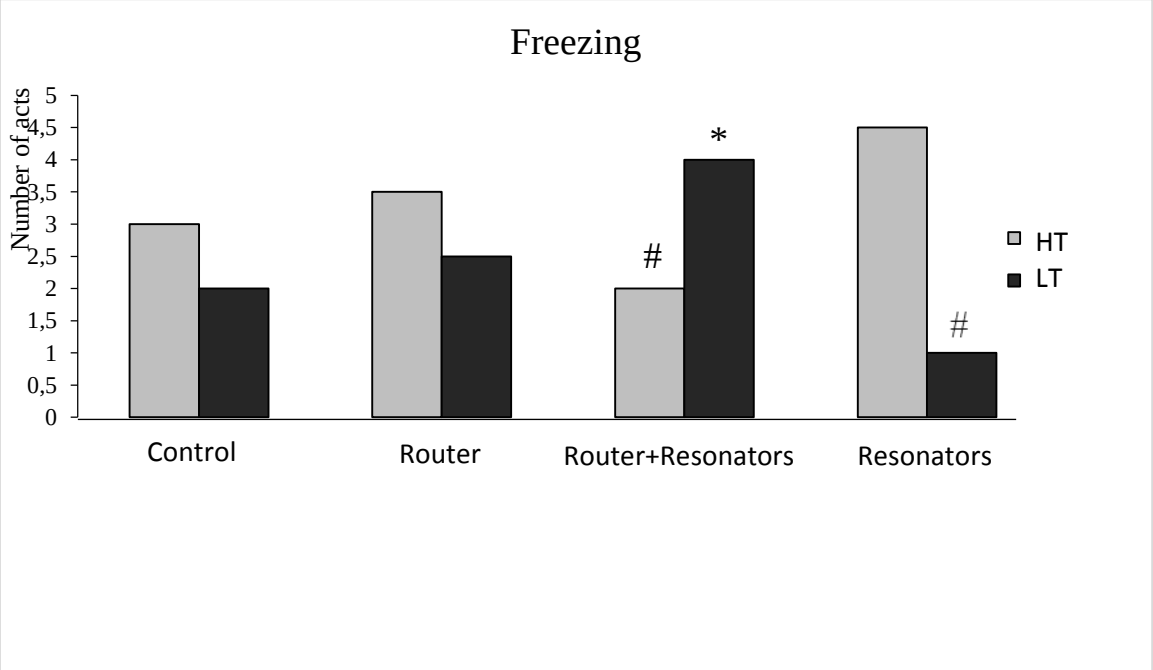

Fig. 6. Number of acts of freezing in the open field test of male rats of the HT and LT strains in the various experimental groups (same as in Fig. 1)

Key: #- The differences with the HT Resonators group are significant ($P<0.04$); *- The LT strain's differences with the Router and Resonator groups are significant ($P<0.02$, $P<0.007$).

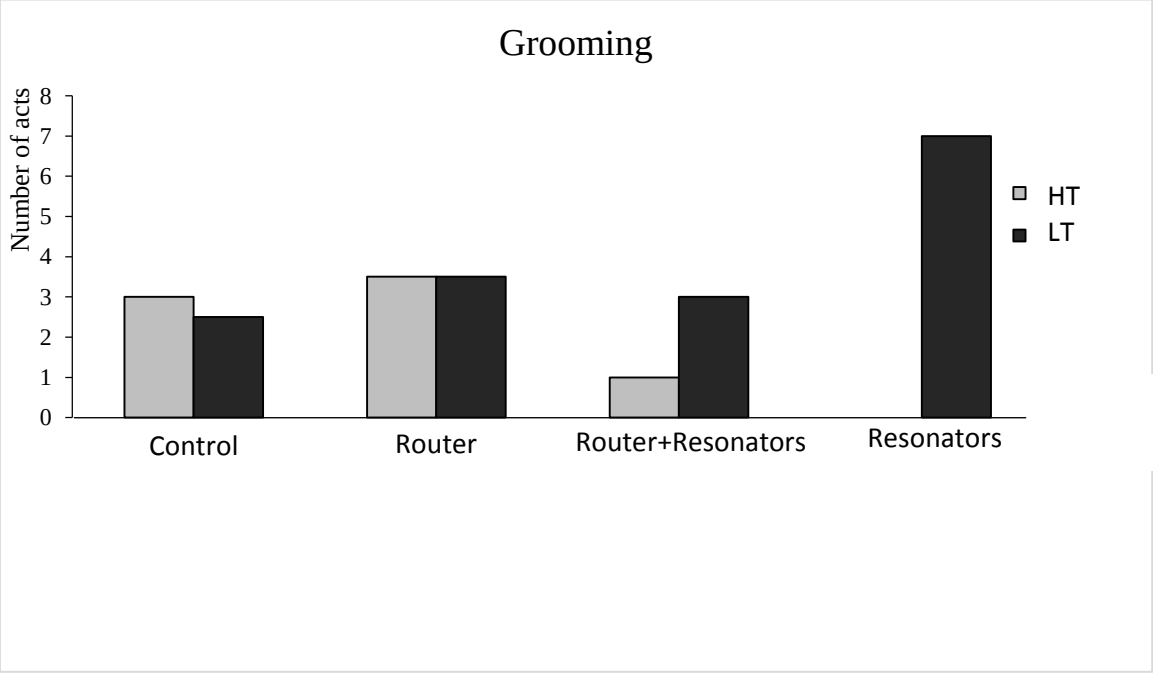

Fig. 7. Number of acts of grooming in the open field test of male rats of the HT and LT strains in the various experimental groups (same as in Fig. 1).

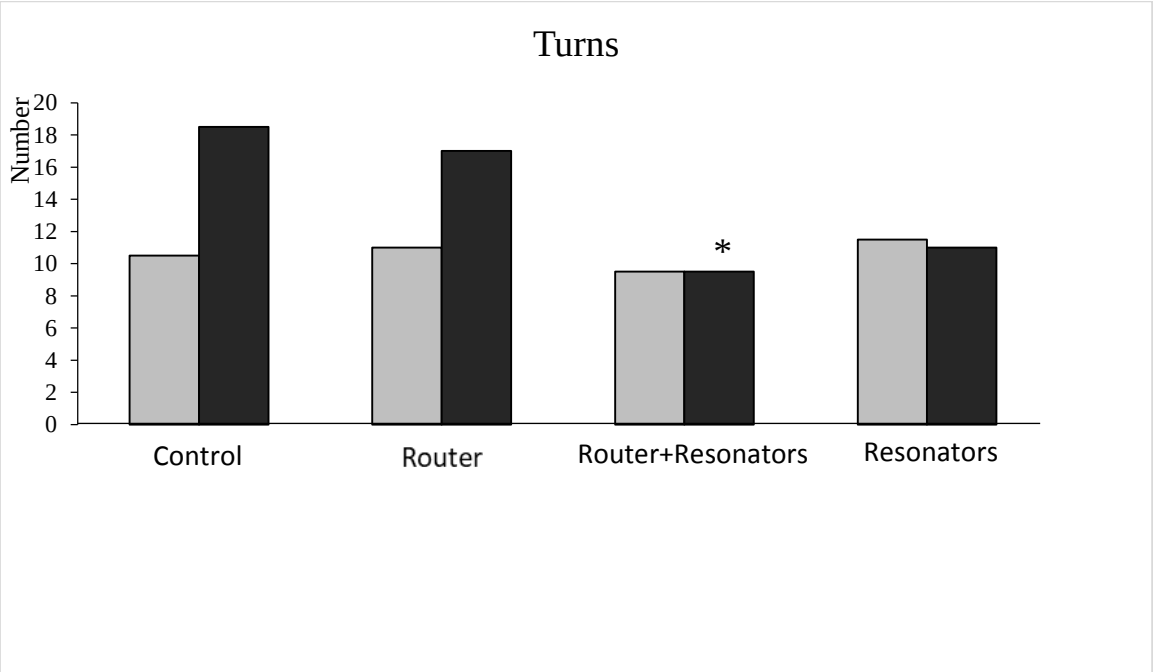

Fig. 8. Number of turns in the open field test of male rats of the HT and LT strains in the various experimental groups (same as in Fig. 1). Key: *- The LT line's differences with the control are significant (P<0.03).

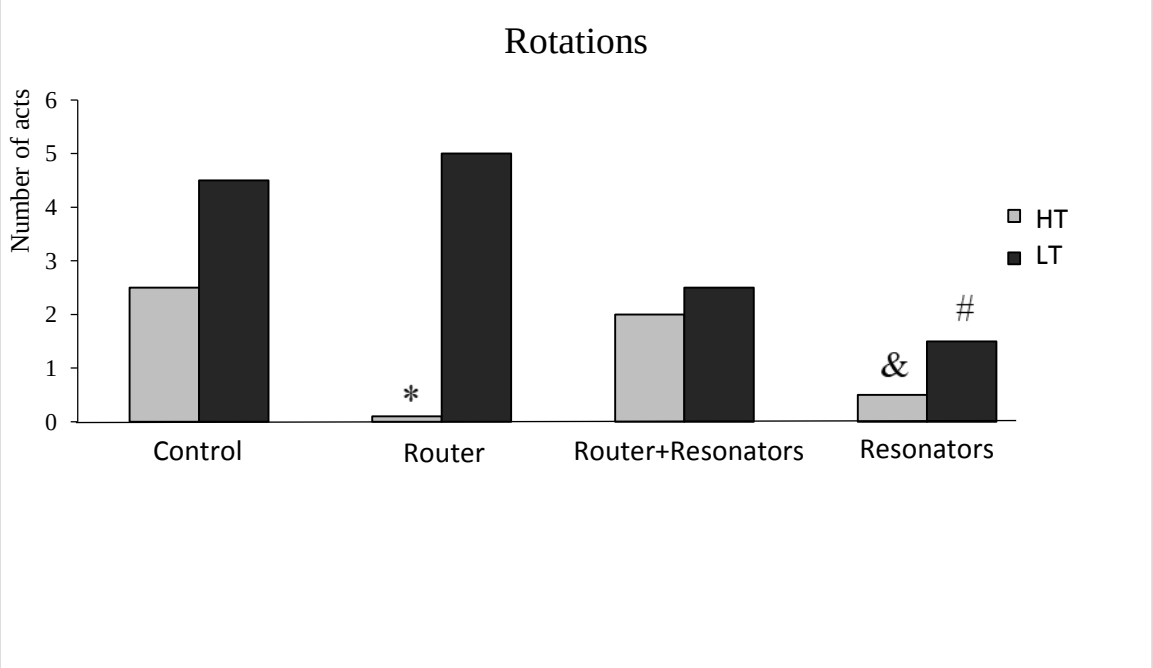

Fig. 9. Number of rotations in the open field test of male rats of the HT and LT strains in the various experimental groups (same as in Fig. 1). Key: *- The differences with the control and the Router+Resonators group of the HT strain and with the Router group of the LT strain are significant (P<0.007, P<0.04, P<0.004); #- The LT strain's differences with the control and the Router group are significant (P<0.04); &- The HT strain's differences with the control are significant (P<0.02).

Fig. 10. Emotionality (number of boluses) in the open field test of male rats of the HT and LT strains in the various experimental groups (same as in Fig. 1).
Key: *- The LT line's differences with the Router and Router+Resonators groups are significant (P<0.04, P<0.007); #- The LT strain's differences with the control are significant (P<0.04).

2. Light-dark box

When testing animals of 8 groups in a light-dark box, we found that the experimental action did not impact the latency of entry into the dark compartment (Fig. 11) nor the time spent by animals in the dark compartment (Fig. 13).

But the decrease in the time spent by rats in in the light chamber was revealed (Fig. 12) : in the LT rats after the router together with the resonators action and only the resonators action relative to the group "Router", and in the HT rats only after the action of resonators relative to both the Router group and the control.

Number of transitions between the light and dark compartments decreased only in rats of the **LT** strain after the resonators with a router action in comparison with the action of the router alone (Fig. 13).

The router action increased **emotionality** in the light chamber compared with the control in rats of both strains, while the action of the resonators reduced emotionality relative to the router action and the router absence to the control level only in rats of the **LT** strain (Fig. 14).

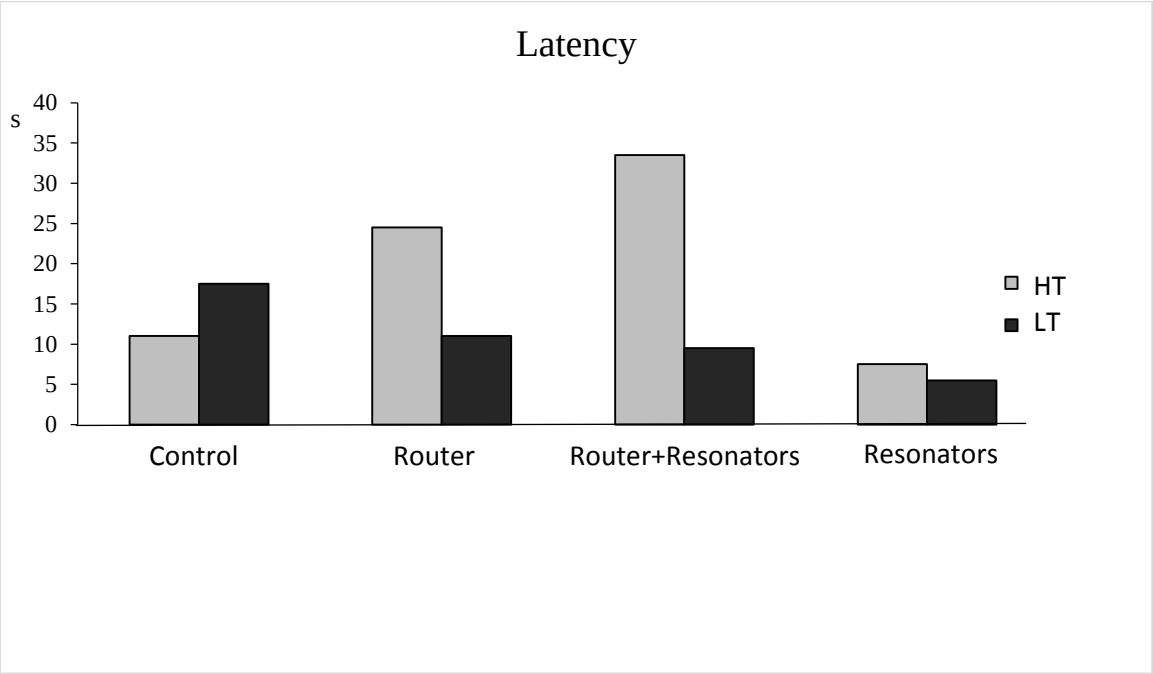

Fig. 11. Latency of entry into the dark chamber in the light-dark box for male rats of the HT and LT strains in the various experimental groups. Exposure of animals: Control — 4 days (x 6 hours a day) in a Faraday cage, Router — 4 days (x 6 hours a day) in a Faraday cage with a Wi-Fi router, Router+resonators — 4 days (x 6 hours a day) in a Faraday cage with a Wi-Fi router and resonators, Resonators — 4 days (x 6 hours a day) in a Faraday cage with resonators.

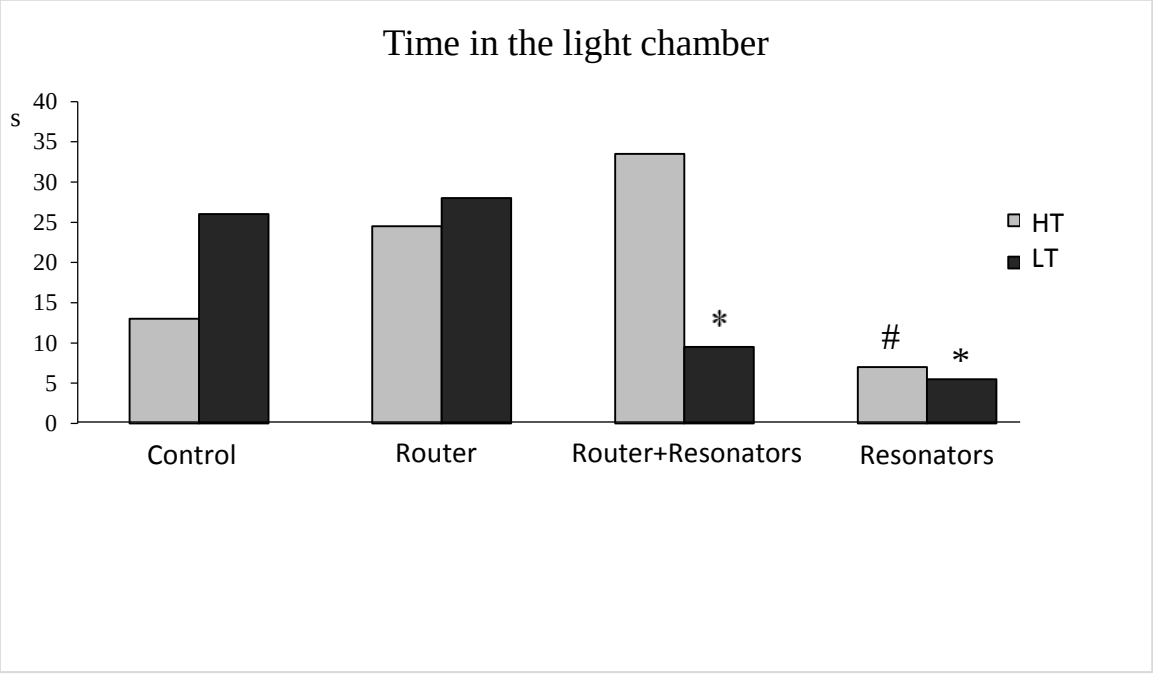

Fig. 12. Time spent in the light chamber of the light-dark box for male rats of the HT and LT strains in the various experimental groups. Key: *- The differences with the Router group of the LT strain are significant ($P<0.05$); #- The HT strain's differences with the control and the Router group are significant ($P<0.01$).

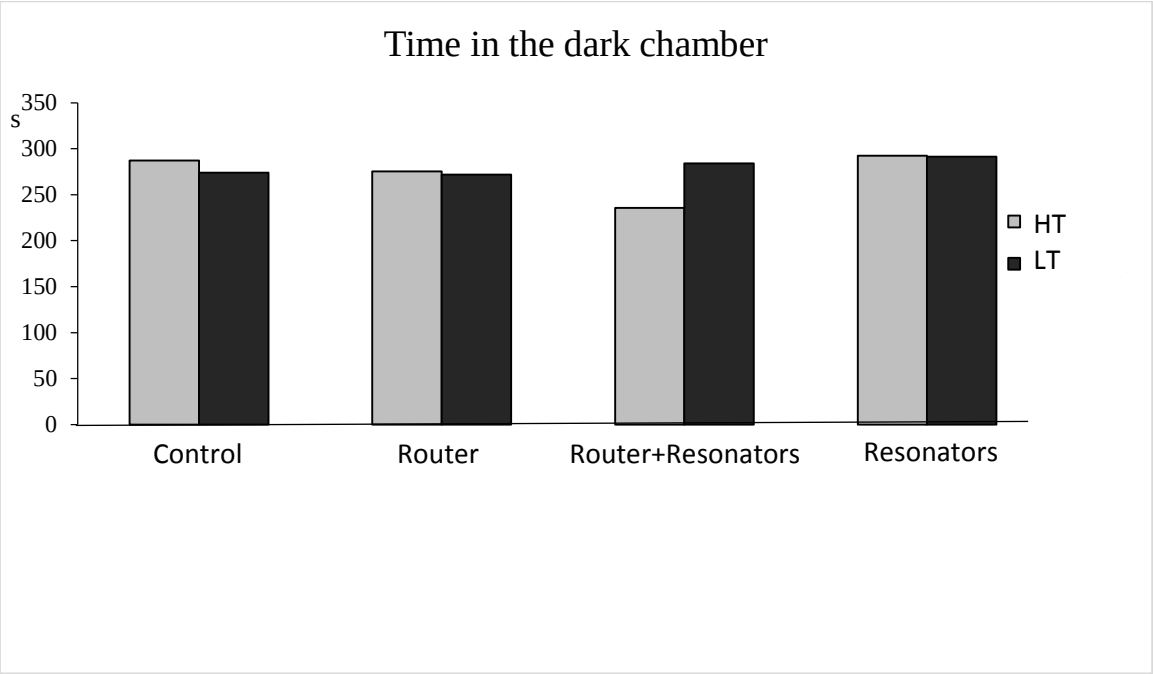

Fig. 13. Time spent in the dark chamber of the light-dark box for male rats of the HT and LT strains in the various experimental groups.

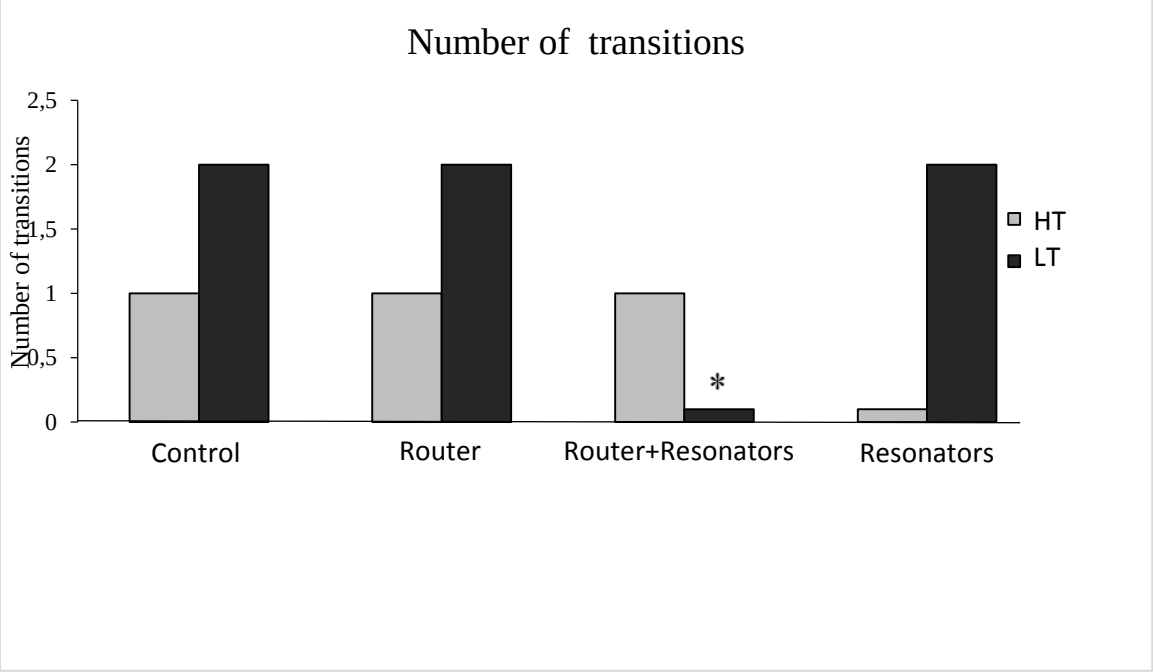

Fig. 14. Number of transitions between the dark and light chambers in the light-dark box for male rats of the HT and LT strains in the various experimental groups. Key: *- The differences with the Router group of the LT strain are significant ($P<0.05$)

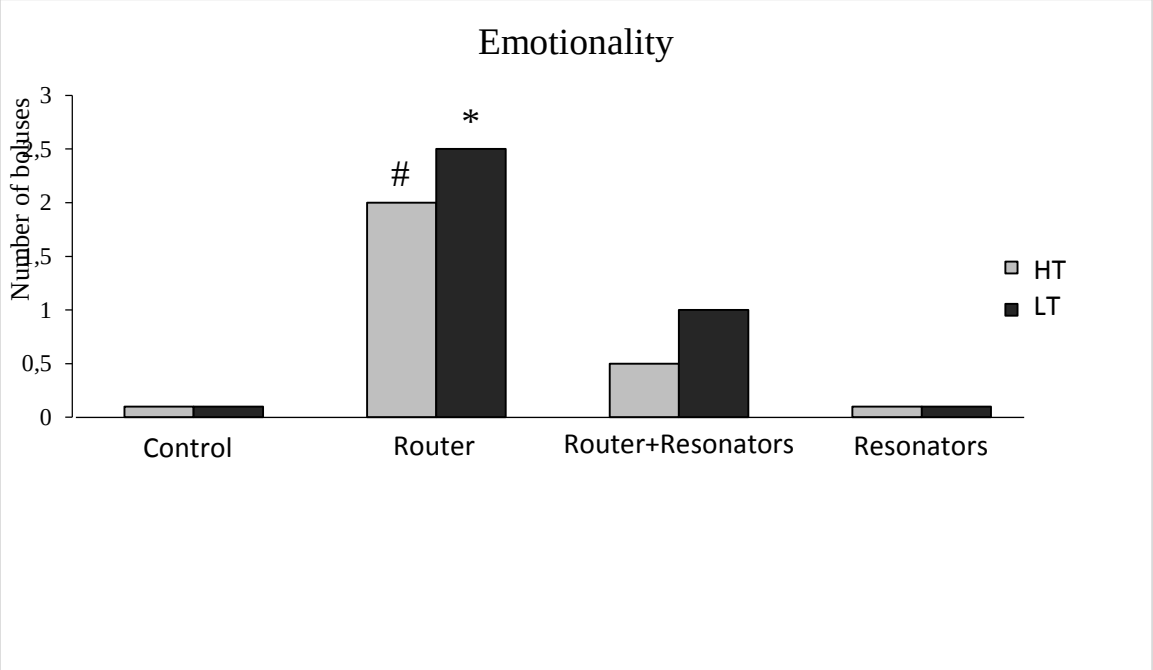

Fig. 15. Bowel movements in the light chamber of the light-dark box for male rats of the HT and LT strains in the various experimental groups. Key: *- The LT strain's differences with all the remaining groups are significant (P<0.03); #- The HT strain's differences with the control are significant (P<0.009).

3. Elevated plus maze

Testing of animals of the experimental groups in an elevated plus maze (EPM) revealed changes in all recorded parameters. Several of indicators of the EPM test are traditionally used to assess the level of anxiety in animals. Here we are mainly referring to the time spent in the open, most illuminated arms of the maze, and the number of entries into them — the longer the stay, the lower the anxiety level.

Number of entries into the open arms in the **HT** rats decreased relative to the router action in groups after exposure with a router and resonators, and with resonators alone (Fig. 16).

Time spent in the open arms decreased only in the **HT** rats after exposure with a router and resonators, and with resonators alone both in comparison with the action of the router and with the control conditions of the Faraday cage (Fig. 17).

The **number of entries into the closed arms** were observed to decrease under the action of resonators relative to the group with the router in the **HT** rats (Fig. 18). **Time in the closed arms** increased relative to the router action after exposing rats of the **HT** strain to the router and resonators, and the resonators alone (Fig. 19). Moreover, the greatest increase in time in the closed arms occurred under the action of resonators without being combined with other influences.

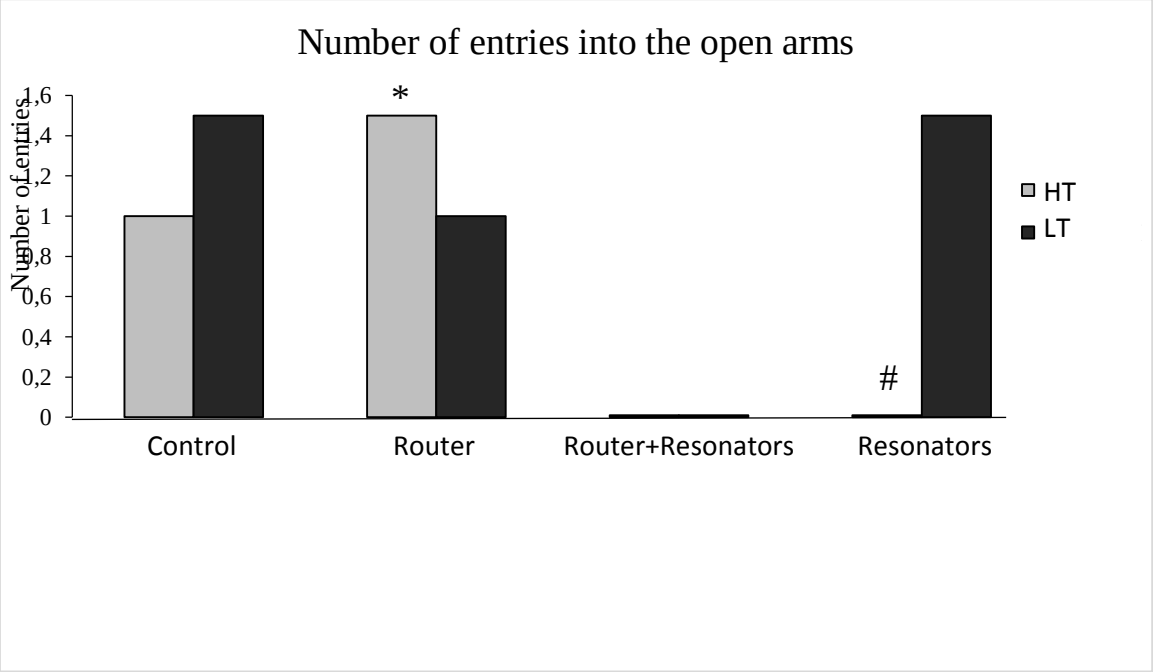

Fig. 16. Number of entries into the open arms of the EPM by male rats of the HT and LT strains in the various experimental groups. Key: *- The HT strain's differences with the Router+Resonators and Resonators groups are significant (P<0.01; P<0.005); #- The differences with the control group of the HT strain and Resonators group of the LT strain are significant (P<0.01; P<0.04).

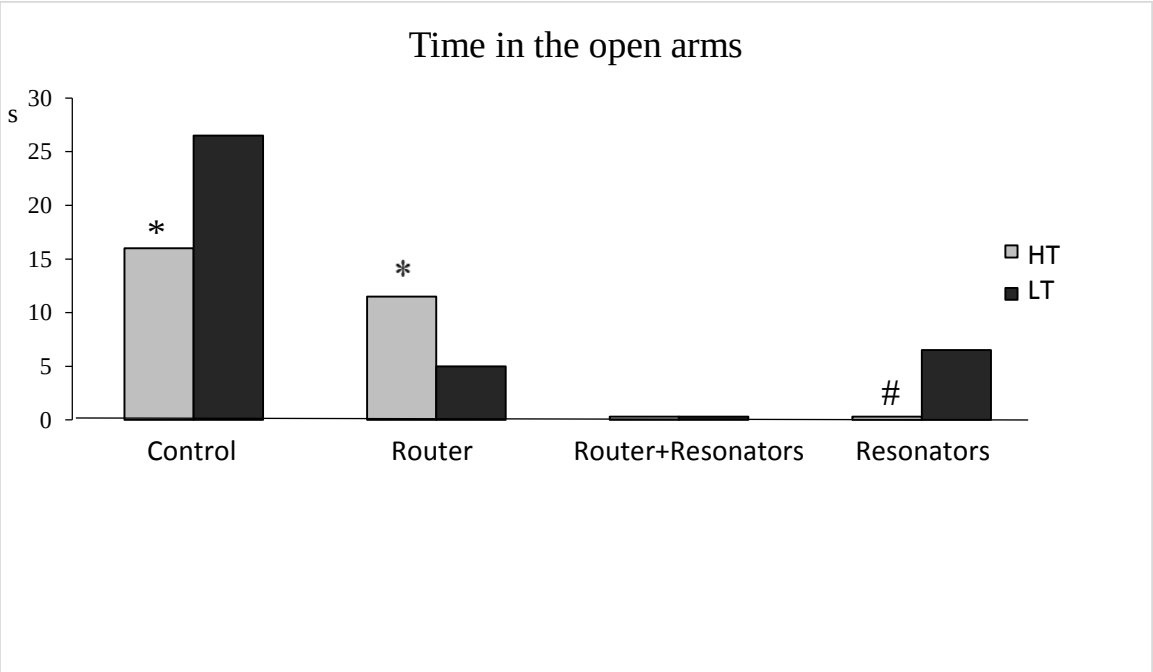

Fig. 17. Time spent in the open arms of the EPM by male rats of the HT and LT strains in the various experimental groups. Key: *- The HT strain's differences with the Router+Resonators and Resonators groups are significant (P<0.02; P<0.01); #- The LT strain's differences with the Resonators group are significant (P<0.04).

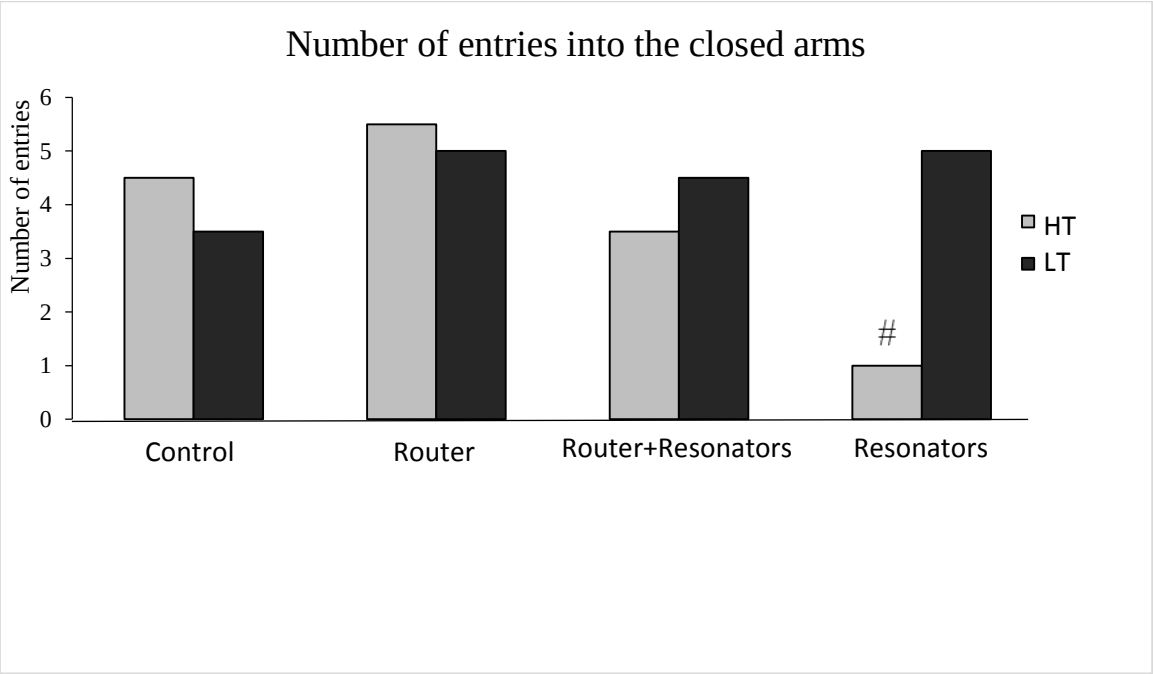

Fig. 18. Number of entries into the closed arms of the EPM by male rats of the HT and LT strains in the various experimental groups. Key: #- The differences with the Router group of the HT strain and with the alternative Resonators group of the LT strain are significant (P<0.01; P<0.02).

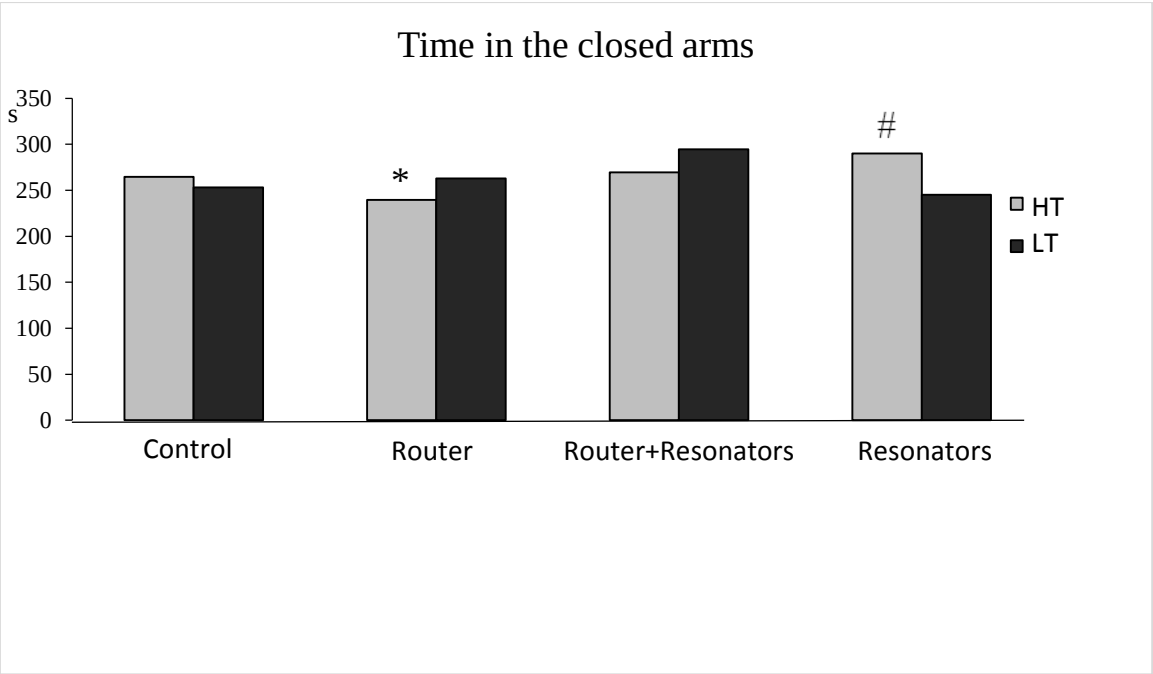

Fig. 19. Time spent in the closed arms of the EPM by male rats of the HT and LT strains in the various experimental groups. Key: *- The HT strain's differences with the Router+Resonators and Resonators groups are significant (P<0.03; P<0.007); #- The differences with the Router+Resonators group of the HT strain and Resonators group of the LT strain are significant (P<0.03; P<0.01).

It should be noted that after the action of the router, all animals of the HT strain entered the open arms of the EPM. However, in the group of rats of the same line that was exposed to resonators, not a single rat entered the open arms. (Fig. 22).

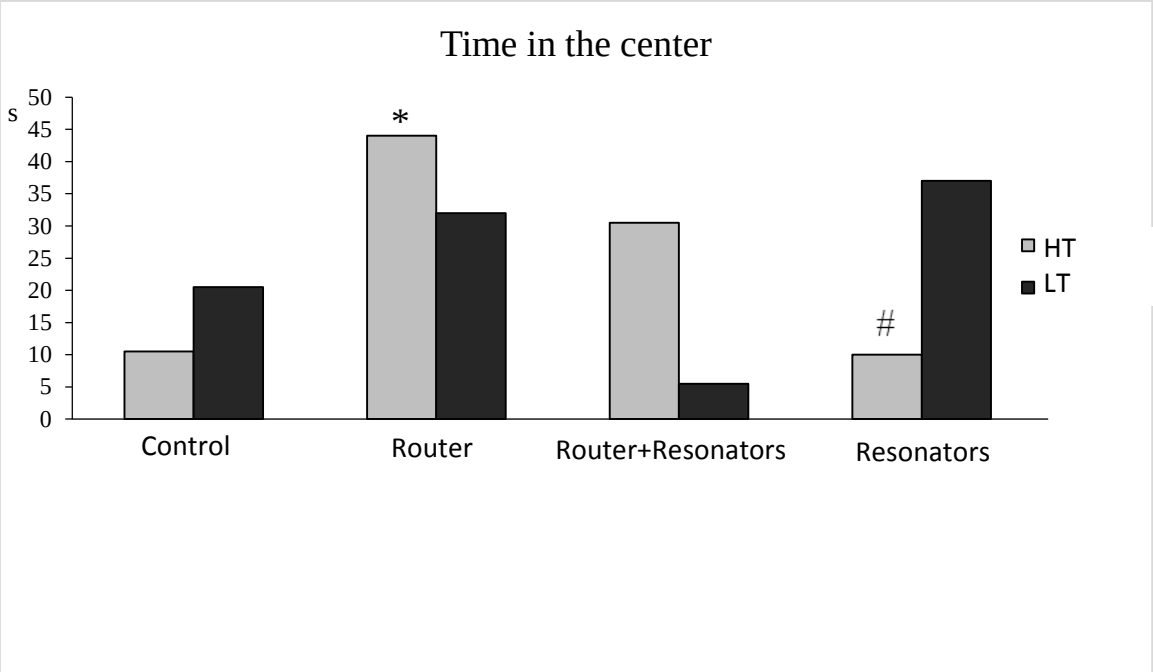

Fig. 20. Time spent in the center of the EPM by male rats of the HT and LT strains in the various experimental groups. Key: *- The HT strain's differences with the control and the Resonators group are significant (P<0.03), #- The LT strain's differences with the Resonators group are significant (P< 0.02).

The action of the router led to an increase in the time spent in the **center** of the EPM by animals of the **HT** strain relative to the control. This time decreased to the control level under the action of resonators (Fig. 20).

In **LT** rats , no changes were seen in any of the five parameters considered above.

It is important to note that the interline differences both in the number of entries and in the time spent in the center, in the open and closed arms, were manifest only under the action of resonators (Fig. 16-20).

The action of the router led to an increase in the **number of times the head is lowered** in rats of the **HT** strain, which decreased when combined with the resonators action, and when under the action of resonators alone (Fig. 21). In rats of the **LT** strain, there was a decrease in this parameter in the Router+Resonators group relative to the control.

Emotionality (number of bowel movements) in the EPM increased both under the influence of a router and a router in combination with resonators relative to the control only in highly excitable animals of the **LT** strain. In fact, the resonators significantly reduced the emotionality in HT rats compared with the the router action (Fig. 23).

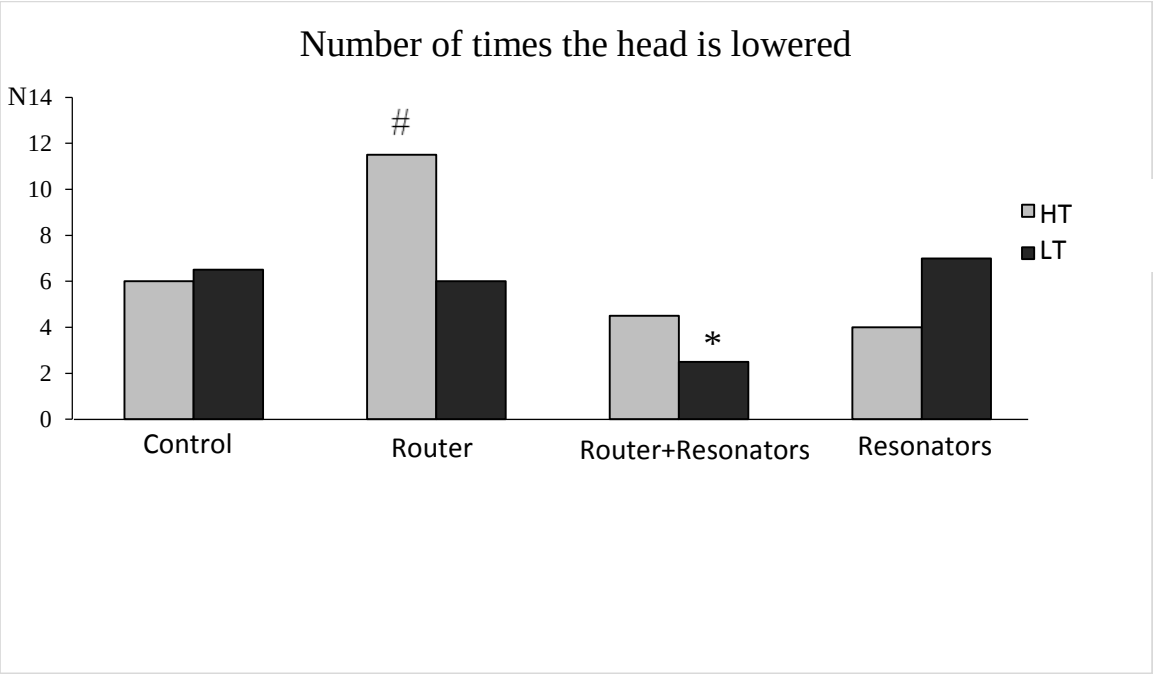

Fig. 21. Number of times the head is lowered in the EPM by male rats of the HT and LT strains in the various experimental groups. Key: *- The LT strain's differences with the control group are significant (P<0.05); # - The HT strain's differences with the Router+Resonators and Resonators groups are significant (P<0.04).

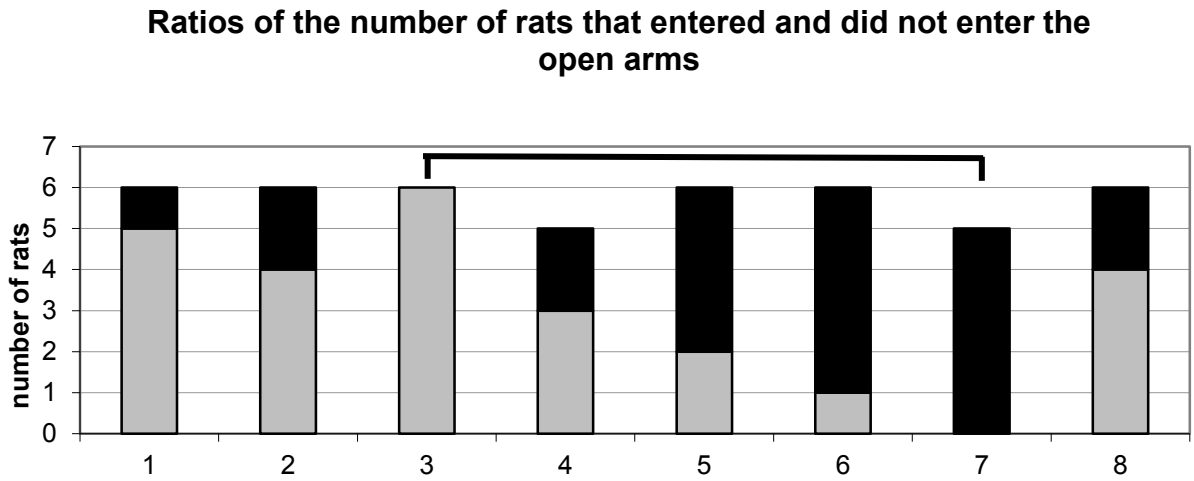

Fig. 22. The ratio of the number of rats from different experimental groups that entered (indicated in black) and did not enter (indicated in gray) into the open arms of the EPM: HT control (1), LT control (2), HT Router (3), LT Router (4), HT Router+Resonators (5), LT Router+Resonators (6), HT Resonators (7), LT Resonators (8). Significantly different values are indicated by the line (P<0.01, chi-squared test).

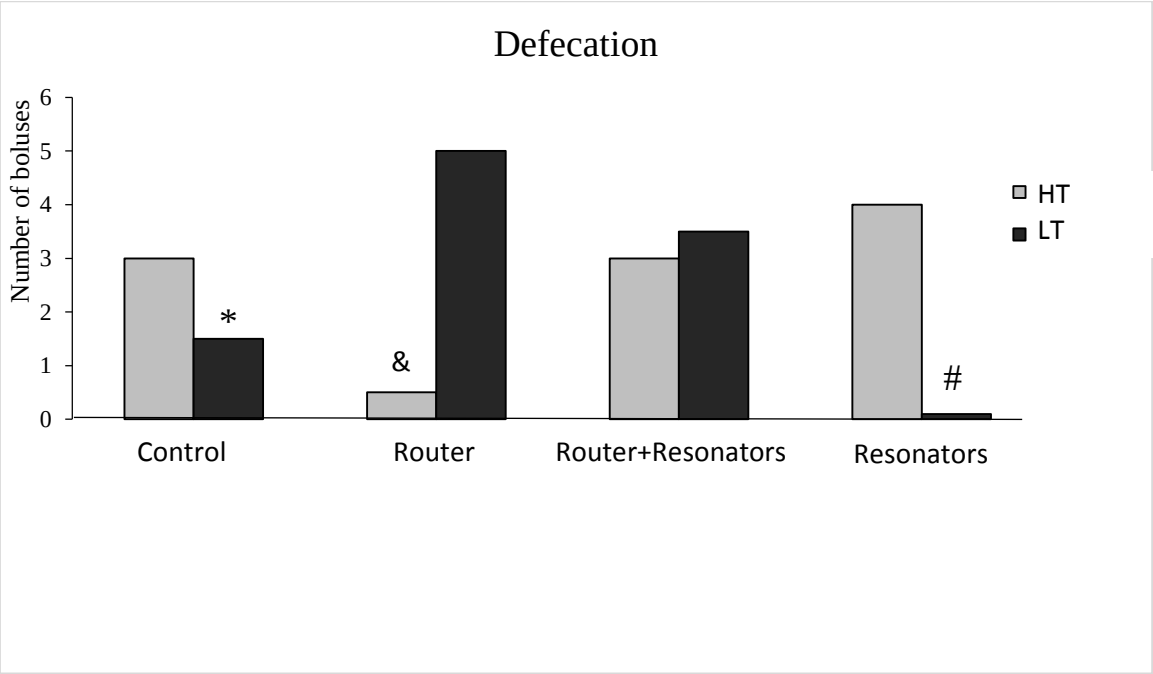

Fig. 23. Bowel movements in the EPM by male rats of the HT and LT strains in the various experimental groups. Key: *- The LT strain's differences with the Router and Router+Resonators groups are significant (P<0.03; P<0.05); #- The differences with the Router group of the LT strain are significant (P<0.02); &- The differences with the Router group of the LT strain are significant (P<0.01).

After applying statistical corrections for multiple comparisons, a highly reliable difference was found between the Router group and the Resonators group in rats of the HT strain in regards to the number of entries into the open arms. Resonators reduce this indicator relative to the router action.

Production and retention of a passive avoidance

Study of the influence of exposure of rats of two HT and LT strains in a Faraday cage, without additional influences, to a router, a router and Aires Defender Pro resonators, and resonators alone on the production and retention of a conditioned passive avoidance reaction showed that the EMR of the router disturbs retention of the **PA** in the **HT** rats with low excitability (Fig. 24). Introducing resonators into the exposure scheme with and without the router caused the PA to be retained in all animals, which may indicate that the resonators have a protective effect in terms of the normalization of cognitive abilities of the HT rats in this test.

To assess the influence of the studied experimental actions on the production and retention of the PA in rats of the LT strain, additional experiments on larger samples are required, because in this experiment the conditioned reflex was developed in less than half of the animals in the control and experimental groups. As a result, it was not possible to analyze the influence of the studied factors on the retention of the reflex in these animals.

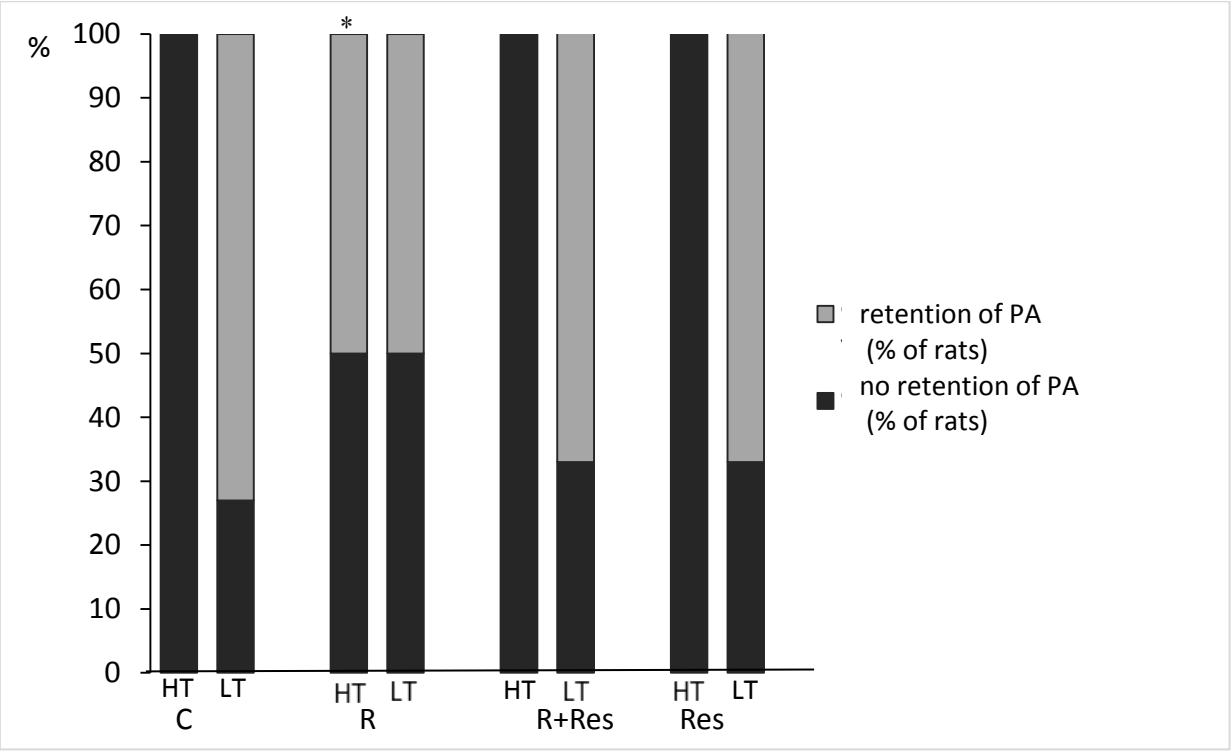

Fig. 24. Retention of the passive avoidance in male rats of the HT and LT strains in the various experimental groups. Exposure of animals: Control — 4 days (x 6 hours a day) in a Faraday cage (C), Router — 4 days (x 6 hours a day) in a Faraday cage with a Wi-Fi router (R), Router+resonators — 4 days (x 6 hours a day) in a Faraday cage with a Wi-Fi router and resonators (R+Res), Resonators — 4 days (x 6 hours a day) in a Faraday cage with resonators (Res). Key: *- The HT strain's differences with all the remaining groups are significant ($P<0.05$).

Previously, in other experimental conditions (exposure with the limitation of external fields, a router, and resonators was performed after the PA was learned), it was shown that the router's own UHF-EMR weakened cognitive functions in Wistar rats, while no changes were found in rats of the HT strain (Shiryaeva et al., 2020). The experiments need to be repeated on larger samples in order to establish the specific nature of the studied actions' influence on the production and retention of the PA and the dependence of the animals' reflexes on the functional state of the nervous system.

Morris water maze

Assessment of the **cognitive** abilities of rats under the influence of the studied experimental actions in the Morris water maze (2-day trials with 12 attempts per day) revealed changes mainly in rats of the **HT** strain with low excitability. On the first day of the experiment, in HT rats, in 5 of 12 attempts, there was a decrease in the platform search time relative to the control group after exposure to a router, router and resonators, and resonators alone (Fig. 26); on the second day, similar changes were seen in 7 of the 12 attempts (Fig. 27). In this case, there were no differences in the platform search time between the last three groups.

In rats of the LT strain, on the first day, in only one attempt out of 12 was the platform search time in the group exposed to a router and resonators seen to decrease relative to the control (Fig.26). On the second day, the Resonators group was found to differ from and the Router group (three attempts), and from the Router+Resonators group (two attempts) and the control (one attempt) (Fig. 27).

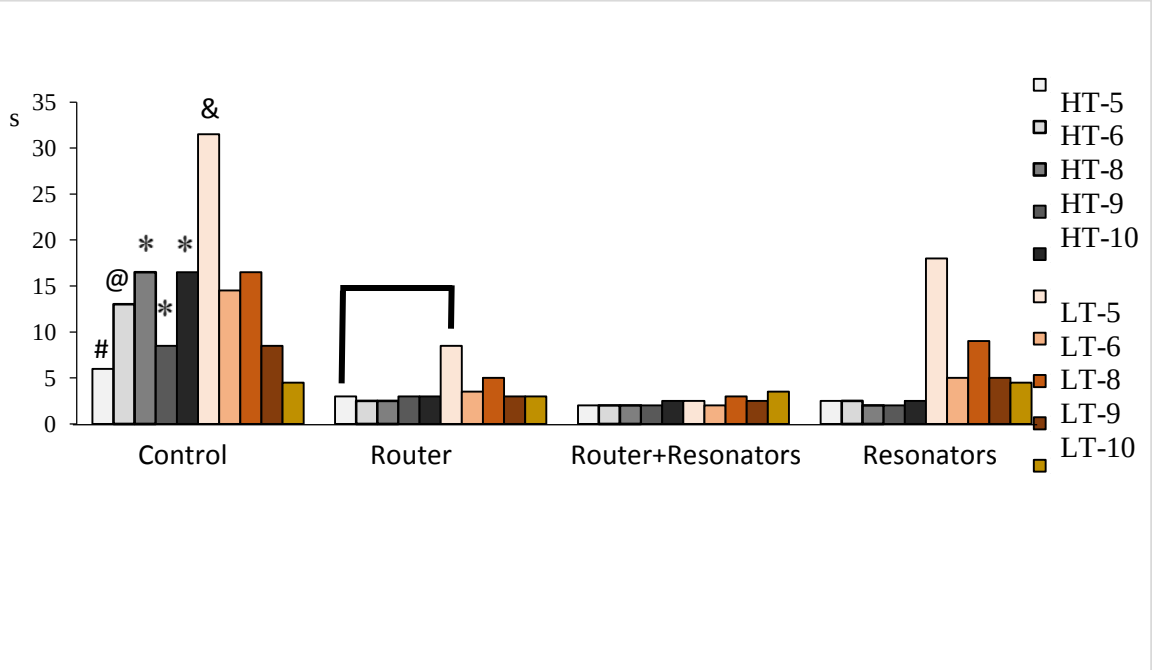

Fig. 26. Latency in the search for the platform in the Morris water maze on the first day of testing (attempts 5, 6, 8, 9, 10) of male of the HT and LT rats in the various experimental groups. Exposure of animals: Control — 4 days (x 6 hours a day) in a Faraday cage, Router — 4 days (x 6 hours a day) in a Faraday cage with a Wi-Fi router, Router+resonators — 4 days (x 6 hours a day) in a Faraday cage with a Wi-Fi router and resonators, Resonators — 4 days (x 6 hours a day) in a Faraday cage with resonators. Key: *- The HT strain's differences with the Router,Router+Resonators, and Resonators groups in attempts 8, 9, and 10 are significant (P<0.03, P<0.006, P<0.01; P<0.007, P<0.005, P<0.007; P<0.03, P<0.03, P<0.008); #- The HT strain's differences with the Router+Resonators and Resonators groups in the 5th attempt are significant (P<0.009, P<0.03); @- The HT strain's differences with the Router and Resonators in the 6th attempt are significant (P<0.01, P<0.02); &- The differences of the LT strain's 5th attempt with the Router+Resonator group are significant (P<0.04). The interstrain differences are indicated by the strain (P<0.04).

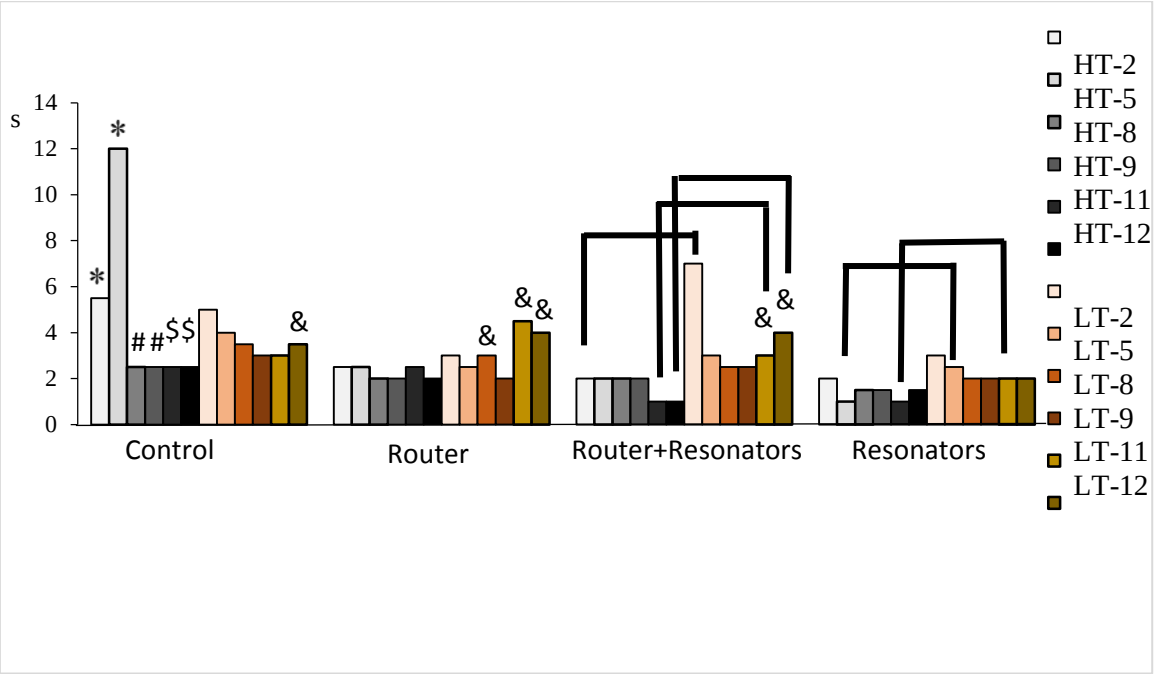

Fig. 27. Latency in the search for the platform in the Morris water maze on the second day of testing (attempts 2, 5, 8, 9, 11, 12) of male rats of the HT and LT strains in the various experimental groups (the same as in Fig. 25). Key: *- The HT strain's differences with the Router,Router+Resonators, and Resonators groups in attempts 2 and 5 are significant (P<0.02, P<0.01; P<0.007, P<0.02; P<0.007, P<0.004); #- The HT strain's differences with the Resonators group in attempts 8 and 9 are significant (P<0.03, P<0.03); $- The HT strain's differences with the Router+Resonators and Resonators groups in attempts 11 and 12 are significant (P<0.01, P<0.04; P<0.01, P<0.02) &- The LT strain's differences with the Resonators group in attempts 8, 11, and 12 are significant (P<0.03). The interstrain differences are indicated by the strain (P<0.01-0.03).

The interstrain differences seen in some attempts when comparing groups of different experimental action options (Fig. 26, 27) indicate that in highly excitable rats, the platform search period is on average longer than in animals with low excitability.

After applying statistical corrections for multiple comparisons, a highly significant difference in the learning dynamics in rats of the HT strain was found between the control group and the Resonators group (in the latter, the speed of finding the platform was higher and the animals showed better spatial memory).

CONCLUSION

In general, according to the results of a behavior analysis in five standard tests of rats of two strains with high and low nervous system excitability after exposure in a Faraday cage, without and with additional exposure factors, such as the electromagnetic radiation of a standard Wi-Fi router and Aires Defender Pro resonators, we can conclude

1. The experimental conditions influenced the behavior of rats of two lines with contrasting nervous system excitability in a study of exploratory and locomotor activity, emotionality, anxiety, and cognitive abilities.

2. The hereditary level of nervous system excitability mediated the nature of the influence of the studied experimental factors on various components of behavior of the rates in the tests used.
3. To the point, Aires Defender Pro resonators had a significant influence on rat behavior. In rats of the highly excitable LT strain, the resonators action compared with the router's EMR under exposure conditions in the Faraday cage mainly affected the emotionality parameters, reducing it to control values, and also caused a decrease in the values of some components of general activity.
4. In rats of the HT strain with low excitability, introducing Aires Defender Pro resonators into the experimental design mainly led to a decrease in locomotor activity with a subsequent increase in anxiety and caused some improvement of cognitive abilities.

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SECTION 2. Continuation of the study of the stress-reactive Hsp70 gene expression in the honeybee brain under the action of EMR (WiFi router, 2.4 GHz) and Aires resonators in early period after exposure.

The ubiquity of wireless communications leads to electromagnetic pollution. The high level electromagnetic pollution can harm living organisms. The EMR's degree of influence on living things depends on the power and strength of the field, oscillation frequency, duration of exposure, and the conditions of generation of the field (pulsed and continuous) (Kudryashov et al., 2008).

The influence of an electromagnetic field, even at a non-thermal level, leads to reversible changes in the regulation of physiological processes: in animals the intensity of metabolic processes, immune activity, and behavior are changed. Insects (butterflies, ants, cockroaches, flies) are among the best experimental animals to study the effects of electromagnetic radiation, as they are highly sensitive to magnetic and electric fields (Kumar et al., 2011). The negative effect of EMR on the honeybees has been shown in a number of studies (Harst et al., 2006). Under the influence of EMR, locomotor activity decreased, the ability to navigate spatially was impaired, the time taken by individual honeybees to return to the hive increased, there was a sharp decrease in honeybees' ability to return to their family, and there was decreased building activity and increased aggression. It has been shown that honeybees perceive EMR as a signal of danger (Favre, 2011). Under the influence of EMR, the queen produces fewer eggs, drone brood appears (Halabi et al., 2013), and there is a sharp decrease in the amount of honey and bee bread in hives (Kumar et al., 2011). Russian researchers have demonstrated behavioral disturbances in the honeybee in response to the action of EMR of various frequencies (Yeskov, Bragin, 1986; Yeskov, Toboev, 2008; Lopatina et al., 2019).

Similar environmentally significant changes in the behavior and reproduction of other insects (locusts, flies, ants) under the influence of electromagnetic radiation have been displayed by Cucurachi et al. (2013). It has been shown that after powerful EMR exposition on *Drosophila*, oxidative stress and gene expression changes were observed (Manta et al., 2017).

Previously, it was proved that the effects of high-frequency radiation reduce a queen fertility and also lead to a decrease in the amount of honey and bee bread in the hive (Kumar et al., 2011). The unconditioned food excitability and short-term memory of the honeybee declined (Lopatina et al., 2019). The molecular-cellular mechanisms of this phenomenon are not well understood and need to be carefully studied. Apparently, a change in the natural electromagnetic background is a stressor for honeybees.

Heat shock proteins (HSP) are universal sensors for stress reactions. A certain amount of heat shock proteins (HSPs) are continuously synthesized in any cells and in many intracellular

structures in all multicellular organisms, independent of stress factors influence. Heat shock proteins also participate in the folding of proteins, preventing non-specific protein aggregation, and protect them from premature proteolysis. Intracellular synthesis of HSP increases in response to not only a heat shock, but also any stressful action: external (UV, heavy metals, heat shock, amino acids), pathological (viral, bacterial and parasitic infections, inflammation, malignant transformation, autoimmune reactions) or even physiological (growth factors, cell differentiation, hormonal stimulation, tissue growth) (Nikitin, 2008).

The heat shock protein HSP70 is the most studied. According to the NCBI GenBank database (LOC408706 heat shock protein 70Cb ortholog), a honeybee has a gene length of 8361 bp. mRNA length: XM_623196.5 - 4605 bp, XM_006561162 - 4497 bp. Protein length: XP_006561225.1 - 831 amino acids, XP_623199.2 - 861 amino acids.

In the report prepared under a scientific and technical cooperation agreement of 2019, we showed that after a 24-hour exposure to the high frequency electromagnetic radiation of a Wi-Fi router *hsp70* expression in the honeybee brain decreases (which may adversely affect the functioning of the honeybee central nervous system). We also showed that 24-hour use of Aires Defender Pro resonators alone "has no effect on the *hsp70* gene expression in the honeybee brain, but when the Aires Defender Pro resonators are used together with the Wi-Fi router for 24 hours, *hsp70* expression in the honeybee brain increases to the control level.

In this report, we present data obtained during 1-hour action of electromagnetic radiation of a Wi-Fi router, combined action of a Wi-Fi router and Aires Defender Pro resonators, and isolated action of Aires Defender Pro resonators.

The purpose of this work is to study the expression of the stress reactive gene *hsp70* in the brain of a honeybee given a 1-hour exposure to the electromagnetic radiation of a Wi-Fi router and the combined action of a Wi-Fi router and Aires Defender Pro resonators.

MATERIAL AND RESEARCH METHODS

The research was carried out on 10–30-day-old worker of honeybees (*Apis mellifera carnica).* The honeybees were bred in the apiary of the Pavlov Institute of Physiology of RAS. For the experiments, the bees kept in an observation hive in a special room at room temperature and with automatic lighting from 8 a.m. to 8 p.m.

The experiment involved 5 groups of the honeybees: intact, control (Faraday cage — isolation from external EMR), control (6 Aires Defender Pro resonators, one in the center of each face of the Faraday cage), experimental (1-hour exposure to the EMR of a Wi-Fi router in the Faraday cage), experimental (1-hour exposure to the EMR of a Wi-Fi router in the Faraday cage + 6 Aires Defender Pro resonators).

To study the effect of electromagnetic waves on the expression of the *Hsp70* gene, we used the following algorithm: exposure to the EMR of a router (2 groups of the honeybees), control without exposure (3 groups of bees), extraction of RNA from the brain of the bees in all groups, RT-PCR with electrophoretic detection (Fig. 1, 2).

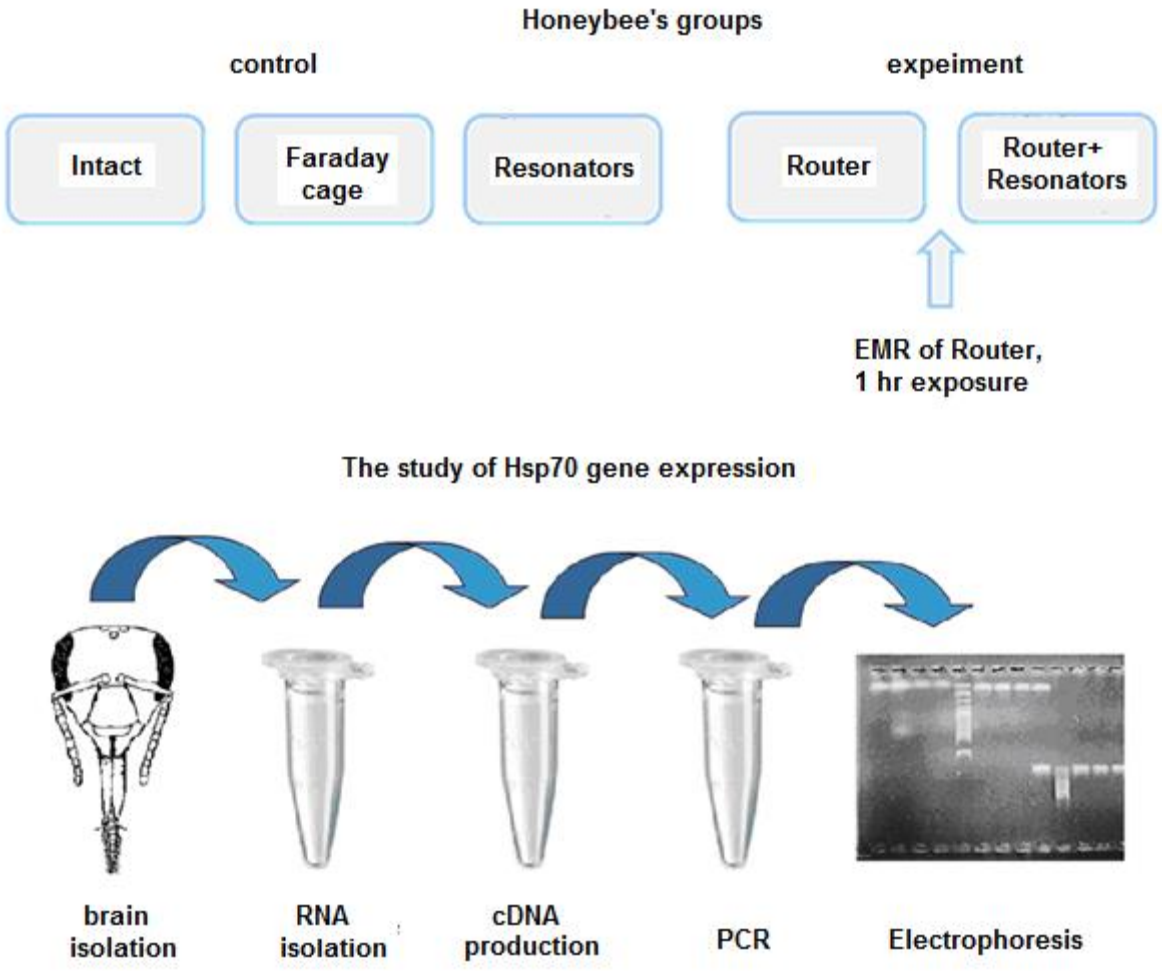

Fig. 1. Experiment design.

A total of 12 samples were analyzed (2 brains in each tube) in the group with the router, 7 samples in the group with the Faraday cage, 6 samples in the intact group, 10 samples in the router+resonators group, 7 samples in the group with resonators alone.

A B

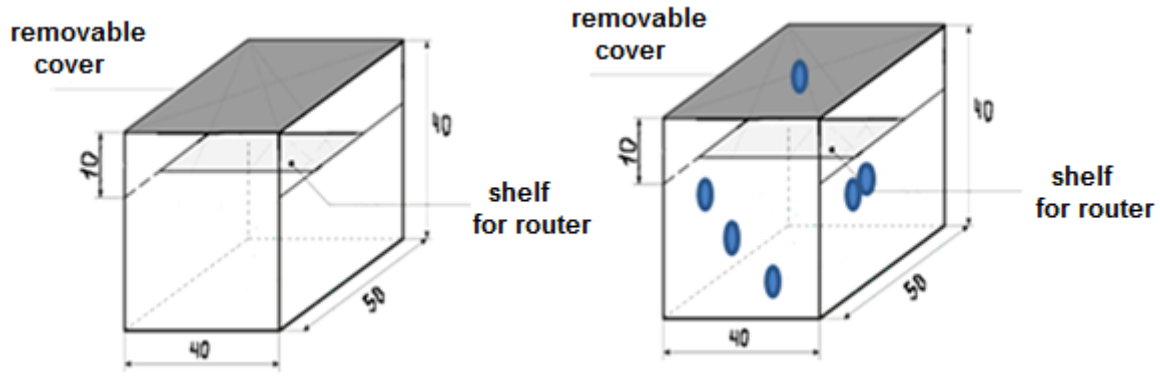

Fig. 2. A – Faraday cage, B – Faraday cage + Aires Defender Pro resonator.

Extraction of total RNA from the honeybee brain. The honeybees were taken from the hive and immediately placed in a freezer for 10 minutes. Then the head capsule was opened and the brain was removed. 2 brains were placed in each test tube. The tissue was homogenized with a pestle in a 1.5 µl centrifuge tube in a 200 µl homogenization buffer (Evrogen). The homogenate was incubated at room temperature for 10 minutes. 300 µl of chloroform was added for deproteinization, and the samples were thoroughly mixed in a vortex. The samples were incubated at room temperature for 5 minutes. They were centrifuged at 13,000 rpm for 3 minutes. The supernatant containing the nucleic acids was transferred to a clean tube. 500 µl of isopropanol was added, and the sample was mixed. 100 µl 3M sodium acetate was added, and the sample was gently mixed. The samples were placed in the refrigerator at -20 °C for 1 hour to precipitate the nucleic acids. They were centrifuged at 13,000 rpm for 3 minutes. The supernatant was removed. 500 µl of 96% ethanol was added, and the samples were mixed. They were centrifuged at 13,000 rpm for 3 minutes. The supernatant was taken out. 500 µl of 70% ethanol was added, and the samples were mixed. They were centrifuged at 13,000 rpm for 3 minutes. The supernatant was taken out. The washing was repeated. The precipitate was dissolved in a TE buffer, pH 8.0 (1 mM EDTA, 10 mM Tris hydrochloride, pH 8.0) at room temperature for 10 minutes. Thus, the prepared samples of total RNA were stored at -20 °C.

Reverse transcription. A reverse transcription reaction was performed with the obtained samples and a reverse transcription kit (Evrogen) with a random primer (Evrogen) according to the manufacturer's recommendations (2 hours at 38 °C). The resulting cDNA was stored at -20°C.

Polymerase chain reaction. The cDNA obtained during reverse transcription served as the matrix. A PCR was performed according to the recommendations of the manufacturer (Evrogen) on a Veriti 96-Well Thermal Cycler (Applied Biosystems). Temperature for annealing primers - 58°C. Number of cycles - 40. Primers (10 pmol/µl, Evrogen): they were selected by T.G. Zachepilo at GenBank with the help of the PrimerBLAST online suite.

Table 1. Primers.

Forward/ reverse	Sequence	Product	Primer
Forward	heat shock protein 70Cb ortholog (LOC408706), XM_623196.5	107 bp.	GCCGTGATTTGACTGACTACC
Reverse			CCAAGCTTGAGGATGATGCAG
Forward	actin related protein 1 NP_001172074.1	165 bp.	TGTGCTGAAATTGCTCATGGT
Reverse			AGAACGTAACCTTGCACTGG

Agarose gel electrophoresis. The PCR products were mixed with a loading buffer (Evrogen, 1:1). The DNA fragments were separated in a 1.5% agarose gel (with the addition of ethidium bromide), 10x15 cm in size, with TAE buffer in a horizontal electrophoresis chamber (Helicon), at a voltage of 150 volts for 40 min. The DNA markers were applied to determine the size of the amplified fragments: 100 bp. (Evrogen). The results of electrophoresis in the passing ultraviolet light on a transilluminator (Vilber Lourmat) were detected. The gel was photographed using a digital camera. The results were saved on a computer in JPEG format.

Data processing. Electropherograms were analyzed using ImageJ (NCBI). The area of the stained bands was evaluated. Normalization was carried out: the area of the bands of *hsp70* samples was attributed to the area of bands of the reference gene a*rp1*.

Then we performed a pairwise comparison of the normalized values in all groups (10 in all) using the nonparametric Mann-Whitney test. Statistical processing was performed in the Past3 program.

RESULTS

The *hsp70* gene expression under the action of weak electromagnetic radiation in a honeybee was studied using the RT-PCR method with electrophoretic detection.

Electropherograms were obtained as a result of the experiments. The expression comparison was performed by comparing the bands on the electropherograms. Thick and intensely stained bands correspond to strong expression, while thin and slightly stained bands correspond to weak.

The expression of the *hsp70 gene* in all groups did not significantly differ. The measurement data is summarized in Fig. 3.

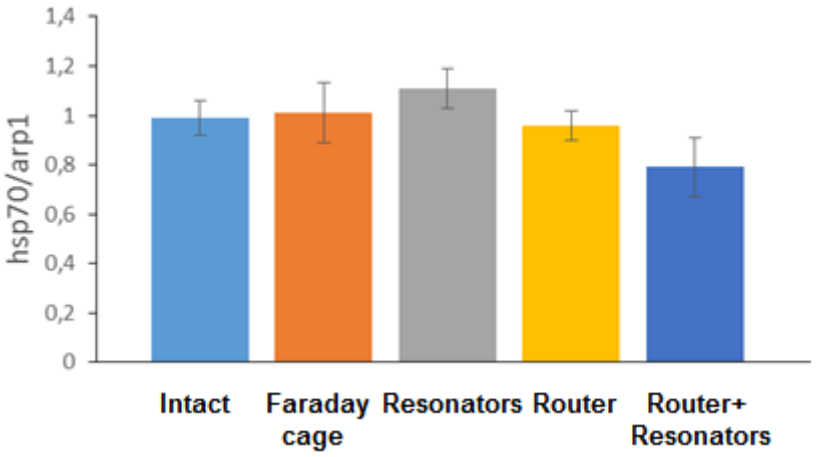

Fig. 3. Assessment of expression of the *Hsp70* gene in all the studied groups.

It should be noted that in the Router+Resonators group (and also in the Router group), additional bands larger than the target product can be seen in the gel. In the Resonators group, such bands are absent (Fig. 4). The appearance of this band may be due to a change in mRNA splicing by *hsp70*, which requires additional research. Additionally, the presence of this band may indicate an increase in the activity of the *hsp70* gene, which is usually associated with cells' stress response.

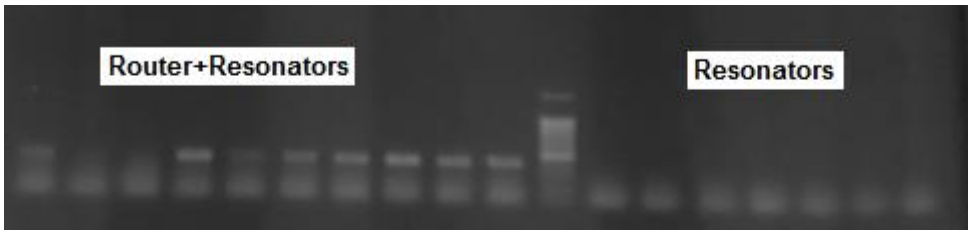

Fig. 4. Electrophoresis of PCR products in Router+Resonators and Resonators groups.

Thus, we can conclude that:

1. The *hsp70* gene expression level in the honeybee brain is similar in all groups.

2. After 1 hour of action of the high frequency electromagnetic radiation of a Wi-Fi router and the combined action of a router and Aires Defender Pro resonators, the content of *hsp70* migh be change.

DISCUSSION

Heat shock proteins play an important role in the life of the cell and the whole organism. HSPs are involved in protein quality control, protecting cells from aggregation of proteins with an irregular three-dimensional structure, or directing them to proteasomes for proteolysis. Almost all cellular proteins at least temporarily interact with HSP70. Under the influence of stress factors, the need for chaperones increases significantly. The target protein's interaction

with HSP70 stabilizes it and then the protein is folded correctly, or another type of chaperone is recruited, after which subsequent restoration of native conformation is possible (Nikitin, 2008).

As we wrote in a previous report, an increase in heat shock protein synthesis may be necessary in some cellular reactions, but not in all (Agustiño et al., 2012) and it is not critical in early adaptation. However, late regulation, including an increase in the amount of heat shock proteins, suggests that stress proteins play a role in promoting long-term tolerance.

According to the literature (Bettencourt et al., 2008), in *Drosophila*, after a 2-hour low-frequency EMR exposure the *hsp70* expression increased (Zhang et al., 2016). Our own data display that development of a stress reaction in the honeybee brain after a 1-hour exposure of high-frequency electromagnetic radiation is not yet observed. Probably, a distinct stress reaction will probably develop after a longer exposure of high-frequency electromagnetic radiation.

The data obtained in this series of experiments is partially consistent with previously obtained data on food excitability and short-term memory in bees under the combined action of a Wi-Fi router and resonators.

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