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CELLULAR AND MOLECULAR BIOLOGY

Biological effects of chronic exposure of *Blaptica dubia* (Blattodea: Blaberidae) nymphs to static and extremely low frequency magnetic fields

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Abstract: In this paper, we analyzed the effects of chronic exposure (5 months) to static magnetic field (110 mT; SMF) and extremely low frequency magnetic field (ELF MF; 10 mT, 50 Hz) on *Blaptica dubia* nymphs. We have examined acetylcholinesterase (AChE) activity and heat shock protein 70 (HSP70) level, two sensitive biomarkers of stress in terrestrial insects. Relative growth rate (RGR), as a life history trait, was estimated. AChE activity was determined spectrophotometrically and HSP70 levels were quantified using indirect non-competitive ELISA and Western blotting. Calculated RGR was significantly changed upon exposure to both types of ambiental MFs. The effects of chronic exposure of *B. dubia* nymphs to SMF and ELF MF (50 Hz) were observed as decreased activity of AChE. The increased level of HSP70 was present only after exposure to SMF. The strength of ELF MF was most likely below the energy level needed to induce the expression of this stress protein. Different patterns of the expression of two HSP70 isoforms, where isoform 2 was sensitive only to SMF, are most likely a possibly switch – off in the expression of constitutive and/or inducible HSP70 isoforms.

Key words: Acetylcholinesterase, HSP70 isoforms, magnetic field, relative growth rate.

INTRODUCTION

During the last century, the level of ambiental MFs has increased. The intensity of these magnetic and electromagnetic fields is several-fold greater than that of the geomagnetic field and they are described as electromagnetic pollution. Manmade static MFs were found to influence the neurophysiological system, blood cells and hemopoetic system in vertebrates and humans (WHO 2006). In 2002, the international agency for research on cancer (IARC) classified the extremely low frequency magnetic field (ELF MF) generated by electrical devices as potentially carcinogenic to humans (IARC 2002).

Magnetoreception is detected in wide range of organisms, from bacteria to high vertebrates, as the sensory ability to detected the Earth`s magnetic field (geomagnetic field). In insects, received information are than processed by nervous system and have an important role in behavioural processes like homing, foraging and courtship behaviour (Wajnberg et al. 2010). The ability to perceive the MF has been described in several different species of cockroaches (Vácha 2006, Bazalova et al. 2016, Vácha et al. 2010). The American cockroach, *Periplaneta americana*, is characterized by magnetic alignment in the resting position (Vácha et al. 2010), and changes in locomotor activity were detected upon periodic changes in the geomagnetic field (Vácha et al. 2009). Recently, in this cockroach biomagnetism was determined and characterized in vivo (Kong et al. 2018). In our environment numerous

objects we commonly use and encounter are equipped with permanent magnets which generate static magnetic fields of several mT, like: rail transport using traction (around 50 mT) or magnetic levitation (10-100mT); nuclear magnetic resonance in medical imaging centres (from 1.5 even 3T) as well as electrochemical industry using electrolysis technique (Perrin & Souques 2012).

Ambiental MFs influence growth and development (Graham et al. 2000, Todorović et al. 2012) and increase locomotor activity (Zmejkoski et al. 2017). Magnetic fields have a great influence on biochemical processes and chemical reactions and it is necessary to examine the role of MFs in the modification of cellular function. Exposure to a strong MF (up to 2 T) reversibly inhibited the Na+, K+-pump activity in HeLa cells (Park et al. 1999), inhibited acetylcholinesterase (AChE) activity in murine bone marrow (Stegemann et al. 1993), and in Mediterranean flour moth (*Ephestia kuehniella*) larvae it induced DNA damage and oxidative stress (Pandir & Sahingoz 2014).

The information on the presence of an external magnetic field most probably is transmitted by the neuroendocrine system. AChE is a primary neurotransmitter in the sensory and neuromuscular system in most animal species. Inhibition of this enzyme leads to the accumulation of acetylcholine, which causes a continuous and excessive stimulation of nerve and muscle fibres, leading to tetany and paralysis (Kirby et al. 2000, Solé et al. 2000). Neuroenzymes such as AChE have been used as biomarkers of the effects of neurotoxic contaminants (Forget et al. 2003).

Heat shock proteins (HSPs) have a key role in cellular defences against external stresses that affect cells and cell systems. Among the different classes of HSPs, HSP70 is the most abundant and most important in stress defence (Kultz 2005). These proteins are useful biomarkers that have already been used to monitor the impact of environmental factors on different invertebrate species (Singh & Lakhotia 2000, Mukhopadhyay et al. 2002). MFs have been shown to induce increased transcription and translation of HSP70 (Malagoli et al. 2006, Pooam et al. 2017). Critical early steps in stress response induced by ambiental magnetic fields are activation of heat shock factors (HSF) and increased heat shock element (HSE) (Lin et al. 1997). This increased expression of HSP70 gene could be a result of the effects of magnetic fields on ions, protein-ions complexes and changes in increased permeability of ionic channels in membranes.

The influence of short- term exposure insects to ambiental MFs was described in numerous studies, while long-term exposure in general has been very poorly investigated. The effects of ambiental MFs can be monitored at different levels of biological organization. AChE inhibition and HSP70 expression have already served as sensitive biomarkers of stress in terrestrial insects (Singh & Lakhotia 2000). Since magnetoreception was observed and described in cockroaches at the behavioural level, our work aimed was to detect the possible influence of long-term (5 months) exposure to MFs on these two biomarkers in *Blaptica dubia* (Blattodea: Blaberidae) cockroaches.

MATERIALS AND METHODS

Insect rearing

B. dubia, also known as the orange-spotted cockroach, is a medium-sized cockroach that grows to around 4.0-4.5 cm. *B. dubia* is found in Central and South America. They are ovoviviparous, giving birth to live young (20-40 nymphs per month in favourable conditions). Individual male and female pairs were taken

from a stock population of *B. dubia* cockroaches reared in the Institute for Biological Research "Siniša Stanković" in Belgrade. Month-old offspring of each pair were randomly assigned to each experimental group (20-25 individuals), i.e. experiment was conducted under blind conditions. Nymphs were reared in Petri dishes (3.5 cm in diameter), 3-5 nymphs per dish, fed once a week with dog chops (dry dog food), ground biscuits and fresh apples. Insects were kept at a constant temperature of 26±0.2 °C, 60-70% humidity and a 12 h dark/12 h-light regime. Experimental groups were as follows: control (C: which was kept clear of the influence of the magnets), SMF and ELF MF (50 Hz) groups. Insects were exposed to the effects of both magnets for 5 months.

The SMF and ELF MF (Figure 1) systems were modified system previously used in our experiment with *Baculum extradentatum* (Todorović et al. 2012). The SMF system was applied with a permanent, double, U-shaped magnet (Model 6002, Raytheon, Waltham, MA). The upper half of the magnet consisted of two north (N) poles positioned at the terminal ends of the magnet and a centrally positioned south (S) pole. Conversely, at the terminal ends of the lower half of the magnet there are two S poles, while the N pole was centrally positioned. The magnet pole area was 25.6 cm^2 and the gap between the poles, where 6 Petri dishes with 3-5 nymphs were placed, was 4.5 cm. A relatively homogenous MF was created between the poles, with an average magnetic induction of 110 mT. The ELF MF exposure was applied with a locally designed system comprising three pairs of coils composed of 84 copper wires (1 mm in diameter) in each coil. The coils were placed around a regular laminated transformer core. The ELF MF system was fed and permanently grounded using an AC power supplier which was permanently switched on. 6 Petri dishes with 3-5 nymphs were placed in the space between the poles (dimensions 95 x 48 x 75 mm). An electric current (0.4 A, 50 Hz) was used to generate an ELF MF (50 Hz) average magnetic induction of 10 mT.

The induction of the MFs was measured using a GM05 gaussmeter with a PT 2837 probe (Hirst Magnetic Instruments, Falmouth, UK). The force lines of the MFs were parallel to the vertical component of the geomagnetic field. During the experiment, the values of the local geomagnetic field (448380N; 208460E) were in the range of 47500-47532 nT for the vertical component, and 22643-22660 nT for the horizontal component (Republic Geodetic Authority, Sector for

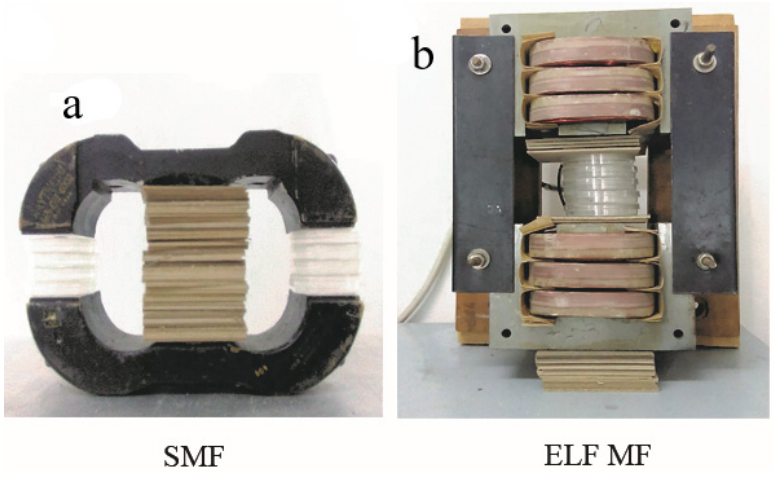

Figure 1. Setup for exposure of *Blaptica dubia* nymphs to a- Static magnetic fields (SMF) with average magnetic induction of 110 mT; b- Extremely low frequency magnetic field (ELF MF, 10 mT, 50 Hz).

Geodetic Works, Department of Geomagnetism and Aeronomy, Belgrade, Serbia).

Ethical standards

All animal procedures were in compliance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade.

Relative growth rate (RGR)

Individuals were weighed every week for five months. In each experimental group, the RGR was monitored and the RGR was calculated on a mass basis according to the formula:

$$RGR = W_5 - W_1 / T_{0-5} \times W_1$$

Larval mass (g) at the beginning of each treatment (W_1) and after five months (W_5), as well as treatment duration (T_{0-5}, 5 months or 140 days), were recorded.

AChE activity

The brain tissues were dissected from head capsules, on ice, using Olympus stereo microscope SZ51 (magnification 3.2). Isolated brain tissues were pooled by experimental group (n=20-25 brain tissues per group) and diluted in distilled water (1:9/w:V). Nymphs brains were homogenized on ice at 5000 rpm, during the three intervals of 10 sec, with 15 sec pauses between them (MHX/E Xenox homogenizer, Germany). Homogenates were centrifuged at 10 000 g for 10 min, at 4°C (Eppendorf centrifuge, 5417R, Germany). Supernatants were used for the enzyme assays. Protein concentration was determined spectrophotometrically using the Bio-Rad DC protein assay kit (Richmond, CA, USA), with bovine serum albumin (BSA) as the standard (Bradford 1976). AChE activity was determined spectrophotometrically (Ellman et al. 1961) and quantified by absorbance at 406 nm. To each cuvette, 100 µl of 0.025 mM acetylcholine iodide, 1 ml of 0.4 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) in 50 mM potassium phosphate buffer (pH 7.9) and 15 µl of brain tissue sample were added in succession. Enzyme kinetics were monitored every 15 s for 3.5 min at 25°C with a UV mc2 spectrophotometer (SAFAS, Monaco City, Monaco). All samples were measured in triplicate (brain tissues pooled for each group), and the linear relationship between the protein content and enzyme activity was tested. The rate of enzyme activity was expressed as 1 µmol of substrate hydrolyzed per minute per mg of protein.

Indirect non-competitive enzyme-linked immunosorbent assay (ELISA)

The concentrations of HSP70 in *B. dubia* brain tissue were quantified using an indirect non-competitive ELISA. Homogenized samples (15 µg of tissue/well) were coated on a microplate (Multiwell immunoplate, NAXISORP, Thermo Scientific, Denmark) overnight at 4 °C in the dark. Indirect non-competitive ELISA was performed using monoclonal anti-HSP70 mouse immunoglobulin G, subclass 1(IgG1) (1:5000 dilution, clone BRM-22, Sigma) and mouse anti-mouse HSP70 horseradish peroxidase conjugate antiserum (1:10000 dilution, Sigma Aldrich); absorption was measured on a microplate reader (LKB 5060-006) at 450 nm. To ensure statistically valid comparisons of the experimental groups across multiple microplates, each microplate contained an HSP70 standard curve. Homogenized brain tissues were pooled after each treatment and loaded on microplates in a matched design to ensure that equal replicates

from each experimental group were used per microplate.

Western blot

Western blotting was used to detect the presence of HSP70 isoforms. *B. dubia* brain tissue homogenates were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) on 12% gels at 4 °C. Monoclonal anti-HSP70 mouse IgG1 (1:4000 dilution, clone BRM-22, Sigma Aldrich) and secondary mouse anti-mouse HSP70 horseradish peroxidase conjugate antiserum (1:5000 dilution, Sigma Aldrich) were used for detection of the *B. dubia* HSP70. Bands were visualized by chemiluminescence (ECL Plus, Amersham). The images of both gels were matched to determine the molecular masses of the HSP70 bands. HSP70 isoform intensities were analyzed densitometrically using Photo-Capt software, ver. 12.4 (Vilber Lourmat, France).

The obtained data are expressed as means±standard error. The normality of data distribution was estimated by the Kolmogorov-Smirnov and Lilliefors tests for normality. Changes in RGR upon experimental treatments were estimated using Kruskal Wallis ANOVA and Median test for continuous variable. Differences in AChE activity between the control group and groups exposed to SMF and ELF MF (50 Hz) were analyzed by one-way analysis of variance (ANOVA) and post hoc multiple range test (Fisher's least significant difference (LSD) test). In all cases the probability $p<0.05$ was considered as statistically significant.

RESULTS

Relative growth rate (RGR)

Changes in calculated RGR for both magnetic fields (20-25 individuals in each magnetic field) was significantly changed (H (2, N) =98; *p=0.0508*) in comparison to the control group (20-25 individuals), but RGR was not significantly different between SMF and ELF MF (50 Hz). RGRs are presented in Figure 2.

AChE activity

The changes in AChE activity in *B. dubia* brain tissue (n=20-25 brain tissues per group) upon exposure to SMF and ELF MF (50 Hz) are presented in Figure 3. There was a significant decrease in AChE activity in nymphs exposed to both ambiental MFs in comparison to control group (F (2, 21)=7,06448; p=0.0029).

Effects of MFs on HSP70 in *B. dubia*

Changes in HSP70 concentrations were detected using an indirect non-competitive ELISA, and the obtained results are presented in Figure 3. Cockroaches exposed to the SMF had significantly increased relative levels of brain tissue HSP70 (n=20-25 brain tissues per group) in comparison to the control group (one-way ANOVA, F(2,21)=11,54; p=0,0004). The LSD post hoc test showed significant differences between HSP70 concentrations in cockroaches exposed to SMF and ELF MF (50 Hz). Using Western blot analysis, we detected 2 HSP70 isoforms in the control group of cockroaches, as well as in groups exposed to SMF and ELF MF (50 Hz) (Figure 4a). The monoclonal anti-HSP70 used in our analysis localized with both the constitutive (HSP73) and inducible (HSP72) forms. We observed changes in the relative concentration of two HSP70 isoforms (Figure 5). Densitometric analysis revealed an increased concentration of the more abundant isoform 1 upon exposure to both ambiental MFs, while isoform 2 decreased in concentration when the nymphs were exposed to the SMF in comparison to the control and ELF MF (50 Hz) groups (Figure 4b).

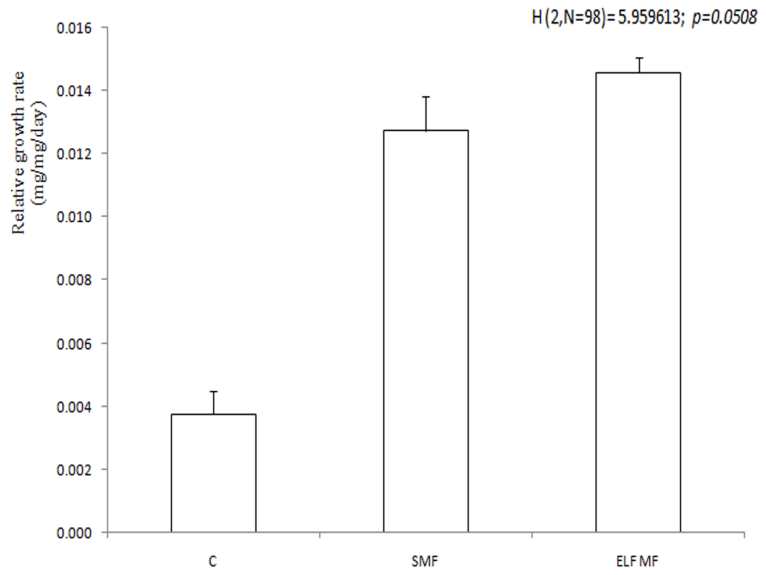

Figure 2. RGR (relative growth rate) of *Blaptica dubia* nymphs after 5 months exposure to ambiental magnetic fields. C – control group kept clear of the influence of the magnets; SMF – static magnetic field (110 mT); ELF MF – extremely low frequency magnetic field (10 mT, 50 Hz). Error bars indicate the standard error of the mean (SEM) for n=20-25 nymphs in each experimental group.

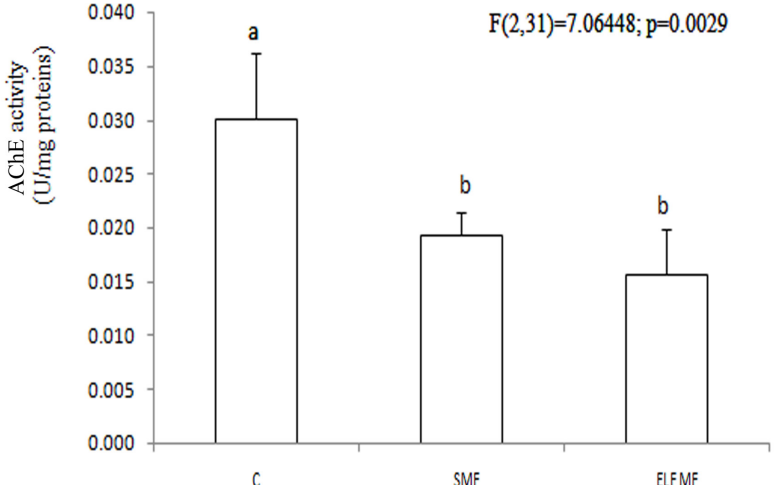

Figure 3. Acetylcholinesterase (AChE) activity in brain tissue of *Blaptica dubia* nymphs after 5 months exposure to ambiental magnetic fields. All abbreviations are as in RGR figure. Data expressed as mean ± standard error, U/mg protein for n=20-25 brain tissues per each experimental group. Different letters (a,b) indicate significant differences between groups (LSD test, $p<0.01$).

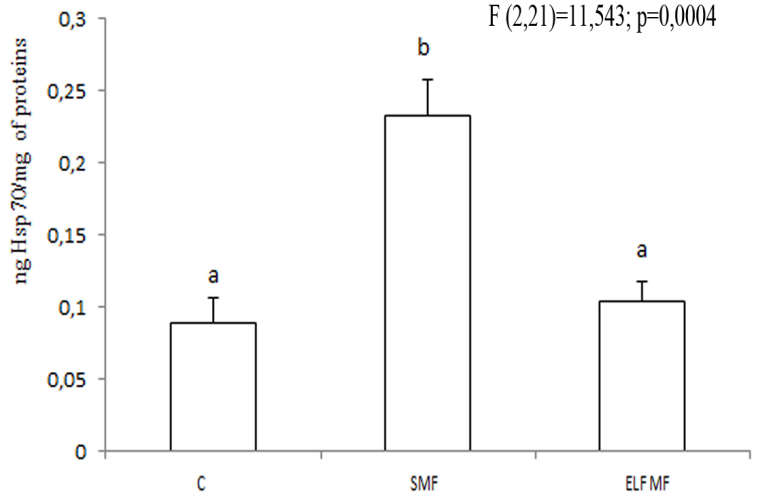

Figure 4. The total HSP70 content from brain tissue of *Blaptica dubia* nymphs after 5 months exposure to ambiental magnetic fields, quantified with indirect ELISA. All abbreviations are as in RGR figure. Data expressed as mean ± standard error, ng/mg of proteins for n=20-25 brain tissues per each experimental group. Different letters (a,b) indicate significant differences between groups (LSD test, $p<0.01$).

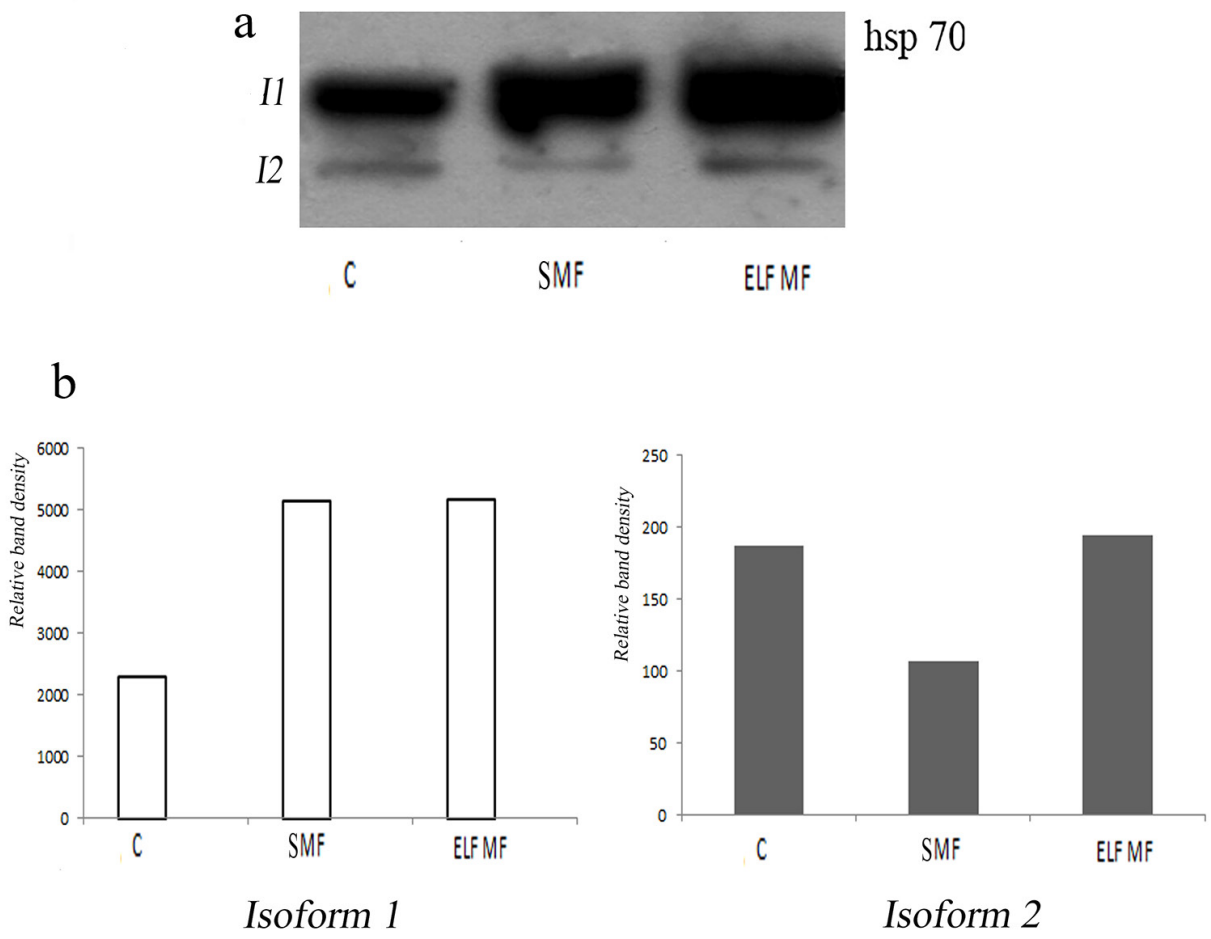

Figure 5. Western blot of HSP70 from brain tissue (n=20-25 brain tissues per each experimental group) of *Blaptica dubia* nymphs after 5 months exposure to ambiental magnetic fields (a). All abbreviations are as in RGR figure. Densitometric analysis of detected isoform 1 and isoform 2 using Photo-Capt software version 12.4 (Vilber Lourmat,France) (b).

DISCUSSION

The effects of environmental stressors can be measured at different levels of biological organization, from the molecular to the community level. Since each type of stressor has different modes of action on the organism, it is of interest to compare their effects on developmental homeostasis. A key factor in responses to magnetic fields is neuroendocrine system and hormone regulated carbohydrate metabolism (Ilijin et al. 2011a, b). A SMF had stress effects on development and survival. Thus, pupal stage duration was decreased and adult life span was shortened in several insect species (see in Todorović et al. 2012). In *Nilaparvata luggens*, egg and nymph development were delayed by exposure to MFs (Wan et al. 2014). In our experiment, chronic exposure of *B. dubia* nymphs to a SMF with a field strength of 110 mT and to an extremely low MF (50 Hz) with a field strength of 10 mT changed significantly the relative growth rate ($p=0.0508$) (Figure 2). Ambiental magnetic fields, used in this experiment, are different types of magnetic fields and changes physiological and biochemical processes in organism differently.

It has been postulated that MFs interfere primarily with the cell membrane (see in WHO 2006). The cell membrane regulates the intracellular environment, maintains the so-called "resting" potential between the interior and exterior of the cell and regulates molecular flow. Changes in Na^+/K^+ pump activity disturbs the gradient of Na^+ and K^+ ions across the plasma membrane. Shen et al. (2007) showed that 125 mT MF changes the properties of K^+ channels in rat neurons, while Nikolić et al. (2012) observed differences in Na^+/K^+ pump activity upon exposure of nervous system of garden snail to MF of 10 mT. Magnetic fields change the concentration of Ca^{2+} ions in neurons (Gobba et al. 2003). These free ions on both side of cell membrane are included in control of cell volume, metabolic processes and signal transduction. The release of the neurotransmitter acetylcholine is also a consequence of ionic changes in the nerve cell membrane. The accumulation of acetylcholine, as the consequence of AChE inhibition, blocks nerve conduction, causing paralysis and death of the insect. When Morelli et al. (Morelli et al. 2005) exposed mussels to ELF MF (73-151 μT; 75 Hz), they recorded decreases in the activities of membrane-associated enzymes, alkaline phosphatase, AChE and phosphoglycerate kinase, of about 50%. In contrast, Silkstone & Wilson (2016) detected no significant effects of applied MFs (2.5 mT, 75 Hz) on alkaline phosphatase and AChE in human erythrocyte membranes. After exposure of *B. dubia* nymphs to SMF (110 mT) and ELF MF (10 mT, 50 Hz), we measured AChE activity. A significant decrease of activity was detected in nymphs exposed to both ambiental MFs in comparison to control (Figure 3). Esterases have been implicated as insecticide detoxifying enzymes in many species. We presume that in our experiment the chronic exposure (5 months) of *B. dubia* nymphs to SMF and ELF (50 Hz) MFs manifest neurotoxic effects. We analyzed the expression of HSP70, a well-known stress indicator in insects. If ambiental MFs act as general stress factors on *B. dubia*, we would expect significantly higher levels of HSP70 in the brain tissue of nymphs exposed to both investigated field strength densities. A significant increase in HSP70 expression was detected only in nymphs exposed to the SMF (110 mT), while the ELF MF (10 mT, 50 Hz) did not influence the expression of HSP70 (Figure 4). In this experiment an increase in HSP70 protein expression was observed after chronic exposure to strong MFs. Exposure of Dipterans' cells to MFs was shown to increase the synthesis of the stress protein HSP70 (Goodman & Blank 1998). Malagoli et al. (2004) reported that in *Mytilus galloprovincialis* HSP70 expression in immunocytes was unaltered at the transcriptional and translational levels upon exposure to ELF MF (400 μT; 50 Hz). Magnetic fields probably change the expression of HSP70, through their effects on ions, protein-ions complexes and permeability of ionic channels. Although it is known that ELF MFs induce the stress response at low field strengths (Noval et al. 1976), our results indicate that in *B. dubia* nymphs, the ELF MF strength of 10 mT was below the low energy level needed for the induction of a biological response. Western blot analysis revealed that two HSP70 isoforms were present in brain tissue homogenates of *B. dubia* nymphs exposed to both types of MF (Figure 5), as well as in the control group, showing a greater abundance of isoform 1 in all experimental groups. The relative density of the protein band corresponding to isoform 1 increased upon exposure to both types of ambiental MFs, whereas isoform 2 decreased only after exposure to the SMF in comparison to control group of nymphs. The monoclonal anti-HSP70 used in our Western blot analysis localizes with both the constitutive (HSP73) and inducible (HSP72) forms. Our results show the different

patterns of expression of both HSP70 isoforms, which could indicate a possible switch-off in the expression of constitutive and/or inducible HSP70. Isoform 1 appeared to be sensitive to both types of ambiental MFs, while isoform 2 was sensitive only to the SMF. Since HSP70 has a very important role in cellular protection against stressors, e.g. protection of the neuromuscular junctions in stress conditions (Dehghani et al. 2011), further investigations of the response of HSP70 to ambiental MFs are necessary. Our results provide an insight into the biological effects that chronic exposure to a strong SMF (110 mT) and ELF MF (10 mT, 50 Hz) have on the relative growth rate, AChE activity and HSP70 concentration in brain tissue of *B. dubia* nymph. These changes were probably a consequence of the different strengths of the ambiental MFs.

CONCLUSION

Chronic exposure of *B. dubia* nymphs to a SMF (110 mT) and ELF MF (50 Hz;10 mT) changed significantly the relative growth rate. We observed a decrease in AChE activity after chronic exposure of nymphs to both types of MFs. In our experiment, effects were observed as an increased level of HSP70 in *B. dubia* brain issue after chronic exposure to SMF, and as changes in the expression of two HSP70 isoforms. Results indicate that analyzed AChE activity and HSP70 expression change upon long term exposure to different magnetic fields. Both magnetic fields used in this experiment expressed particularities and different properties towards modifying the activity of AChE or expression of HSP70 in *B. dubia* nymphs. Our future research will focus on the sensitivity of other well-known biochemical systems which are a part of important physiological processes and their response to chronic exposure to static and extremely low frequency MFs.

Acknowledgments

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