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THE RESPONSE OF DORSOMEDIAL A1' AND DORSOLATERAL L2' NEUROSECRETORY NEURONS OF *LYMANTRIA DISPAR* L. CATERPILLARS TO THE ACUTE EFFECTS OF MAGNETIC FIELDS

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Abstract - The morphometric changes (size of neurons and their nuclei) of protocerebral dorsomedial A1' and dorsolateral L2' neurosecretory neurons were analyzed in *Lymantria dispar* larvae after exposure to strong static (SMF, 235 mT) and extremely low frequency magnetic fields (ELF MF, 2 mT). Increase in the size of A1' neurons and their nuclei were observed after acute exposure to SMF. Decrease in the size of these neurons and their nuclei was observed after exposure to ELF MF. The size of L2' neurons and their nuclei tend to decrease after exposure to SMF and ELF MF. The quantification of protein bands within the Mr range corresponding to the large form of the prothoracicotropic neurohormone indicates that the amount of protein decreased after exposure to both types of magnetic fields.

Key words: Magnetic fields, protocerebral neurosecretory neurons, *Lymantria dispar*

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INTRODUCTION

In addition to the well-known natural influence of the the Earth's magnetic field (or geomagnetic field), artificial magnetic fields also affect all organisms. During the last decade the level of artificial magnetic fields has increased as a result of electrification and development of telecommunication systems. The intensity of these magnetic and electromagnetic fields is several-fold greater than the geomagnetic field. They are described as electromagnetic pollution. Up to now, the effects of magnetic fields on insects have been poorly studied in comparison to other environmental stressors (Tenforde 1979; Prolić and Jovanović 1986; Prolić and Nenadović 1995; Prolić et al. 2002; Nenadović et al. 2005).

Sensitivity to geomagnetic fields has been documented in several groups of insects (Wiltschko and Wiltschko 1995). The physiological mechanisms of magnetoreception are still unknown, but there are several hypotheses regarding the detection of magnetic fields in living organisms. Kirschvink et al. (2001) have pointed out the existence of biogenic magnetite that transduces the energy of the magnetic fields into physical forces that the nervous system can detect. Ritz et al. (2000) proposed a second model system which includes the induction of free radical reaction in sensitive photoreceptors by a magnetic field. In all hypotheses concerning magnetoreception and the effects that magnetic fields have on living systems, the neuroendocrine system is viewed as the target site and a key factor (Binhi 2001; Johnsen and Lohman 2005).

The neuroendocrine system of insects, *via* neurohormones, regulates the basic life processes and reacts quickly to environmental changes, including the effects of magnetic fields (Nenadović et al. 2005). Neurohormones determine the level and

interplay of juvenile hormones, ecdysteroids and other hormones which coordinate metabolic and morphogenetic activities in insects. Literature data strongly suggest that ecdysteroids and juvenile hormones (JH) have an important role in the mechanisms of the response to environmental stress in insects (Chernysh 1991; Rauchenbach 1991). The large form of the prothoracicotropic neurohormone (PTTH) synthesized in L2′ neurosecretory neurons (nsn) (Ilijin 2009) has a tropic effect on the prothoracic gland by inducing the synthesis of ecdysteroids. Exogenous and endogenous factors that change the activity of neurosecretory neurons, and their number, also change the neurohormonal balance and thereby the dynamics of insect development and metabolism (Ivanović and Janković-Hladni 1991). Literature data indicate that protocerebral dorsomedial neurosecretory neurons, including A1' type, participate in the regulation of digestive processes, affecting the protease secretion in insect gut (Ivanović et al. 1978; Raab 1989; Leković et al. 2001).

Our goal was to examine the effects of acute exposure to a strong static magnetic field (235 mT) and an extremely low frequency magnetic field (2 mT), on protocerebral neurosecretory neurons of A1' and L2' type, in 4th instar *Lymantria dispar* caterpillars. We have also examined the changes in brain protein profiles after the acute exposure of 4th instar larvae of *L. dispar* L. to magnetic fields (strong static magnetic and extremely low frequency magnetic fields) in the region of a molecular mass of about 14-16 kD, known as the Mr range of the large form of prothoracicotropic neurohormones in insects.

MATERIALS AND METHODS

Insect rearing

Gypsy moth egg masses were collected in a poplar forest (locality Opovo: 20˚25'49E, 45˚3'8N, altitude 67 m, 30 km from Belgrade). The egg masses were kept in a refrigerator at 4˚C from October to March, when they were set for hatching. After hatching, the *L. dispar* caterpillars were reared in transparent plastic containers (V=200 ml) at 23˚C. They were fed with standard artificial diet for the gypsy moth (O'Dell et al., 1984) in a 16 h light:8 h dark photoperiod. The larvae were randomly assigned to three experimental groups for histochemistry (n=15) and for brain SDS PAGE (n=15).

Magnetic fields

On the first day of 4th instar, one group of caterpillars were transferred to a strong static magnetic field (SMF). The plastic containers (V=80 ml) were placed between the poles (dimension was 26 cm^2) of a permanent double U-shaped magnet (Raytheon 6002, USA) with a maximum magnetic induction B at the poles of approximately 235 mT (for details see Prolić and Nenadović 1995). The upper half of the magnet has two North (N) poles at the terminal end of magnet, and a centrally positioned South (S) pole. The lower half of the magnet has two S poles at the terminal end of the magnet and a centrally positioned N pole. S poles were facing N poles and between them a magnetic field with a relatively homogenous strength was created.

The second group of caterpillars was transferred to plastic vials (same volume as the Petri dishes) and placed in an extremely low frequency magnetic field (ELF MF) obtained by an electromagnet of three pairs of coils placed around a regular laminated transformer core. The dimensions of the poles were 44.6 cm^2 and the space between them was 3.2 cm. The electromagnet was supplied by a current of 1 A, producing an alternating magnetic field with an average magnetic induction of B=2 mT, at a frequency of 50 Hz. The magnetic fields were measured by a gaussmeter (HIRST' GAUSSMETER GM 05, with probe PT 2837- Hirst Magnetic Instruments LTD, Tesla House, Tregoniggie, and Cornwall, UK). The force lines of the magnetic field were parallel to the vertical component of the geomagnetic field. The magnitude of the geomagnetic field was characterized as "quiet" and "very quiet" for the days of the experiments, as obtained from the Geomagnetic Institution in Belgrade, measured by a GSM 10 proton magne-

tometer. A control group (C) was kept 5 m from the magnets, in the same Petri dishes (V=80 ml), exposed only to a natural magnetic field.

Histological techniques

Based on their size and morphological characteristics, we divided the protocerebral neurosecretory neurons of *L. dispar* L. (for easy monitoring of the results) into groups (dorsomedial - A1, A2, A1' and dorsolateral - L1, L2, L2'). The brain complexes were dissected in insect Ringer solution, immersed in Bouin's fixative (picric acid-saturated solution 75%; formaldehyde 20%; acetic acid 5%) and fixated for 24 h. After that, the brain complexes were rinsed in 70% ethanol and fully dehydrated in a graded series of ethanol (from 80% to 100%) and then embedded in paraffin wax (Merck 59°C). Serial sections of the brain complexes were cut at 3.5 μm for histochemistry (microtome - "820" Spencer) and collected on 0.2% gelatin/0.05% chrome alum (Sigma) coated slides. After drying for 48 h at 37°C the sections were deparaffinized in xylene, rehydrated to 10 mM phosphate buffered saline and stained by Ewen's paraldehyde fuchsine technique (Ewen 1962 modified by Panov 1980). On the basis of morphological characteristics, the A1' and L2' type nsn were easily selected, neurosecretory granules in nsn cytoplasm were stained different shades of dark purple and the nucleoli were light pink (Panov 1980).

The activity of protocerebral A1' and L2' neurosecretory neurons was determined by combined monitoring of the following cytological parameters: the size of the nsn and their nuclei (expressed as the mean values of the smallest and largest diameters in μm). Data analysis was carried out by one-way analysis of variance (ANOVA) and *post hoc* multiple range test (Fisher's least significant difference-LSD), using the program STATISTICA, version 6.0.

SDS PAGE Electrophoresis

After decapitation, the caterpillar brains were dissected on ice and weighed. The brains were homogenized (20 000 x g) in cold distilled water (200 mg brain/ml distilled water) and after that centrifuged at 10 000 rpm for 10 min at 4°C. The supernatant was collected and SDS PAGE electrophoresis performed according to Laemmli (1970) on 12% gels. The gels were then stained for proteins with Coomassie Brilliant Blue R 250 solution overnight at 4°C, and then destained in 50% methanol-10% acetic acid solution. The molecular weight of the proteins in SDS-PAGE was estimated using commercial standards with an Mr of 4-250 kD (Invitrogen). The intensity of the protein bands in the region of molecular mass of approximately 14-16 kD (Mr of the large form of prothoracicotropic neurohormones, Kelly et al. 1995) were analyzed using NIH software Image J 1.42q (NIH, USA).

RESULTS

Changes in morphometric parameters of A1' and L2' neurosecretory neurons after exposure to both magnetic fields

Fourth instar gypsy moth caterpillars were exposed to a strong static magnetic field (SMF, B=235 mT) and an extremely low frequency magnetic field (ELF MF, B=2 mT) and the observed changes in the morphometric parameters of the A1' and L2' neurosecretory neurons and their nuclei are presented in Fig. 1. The A1' and L2' neurosecretory neurons are located in the medial and lateral parts of the protocerebrum with an average size of 26 μm and 20 μm, respectively.

The trend of the increasing size of A1′ neurosecretory neurons was observed after a three-day exposure to the strong static magnetic field (Fig. 1A). The size of their nuclei was significantly higher in comparison to the control and the group exposed to an extremely low frequency magnetic field (one-way ANOVA $F_{2,52} = 14.938$, $P<0,001$, Fig. 1C). The somata of A1′ nsn in the group of larvae exposed to a strong static magnetic field were filled with a fine-grained neurosecretory product and the axons are clearly visible (Fig. 2), which indicates secretory activity (Laufer 1984, Raab 1982).

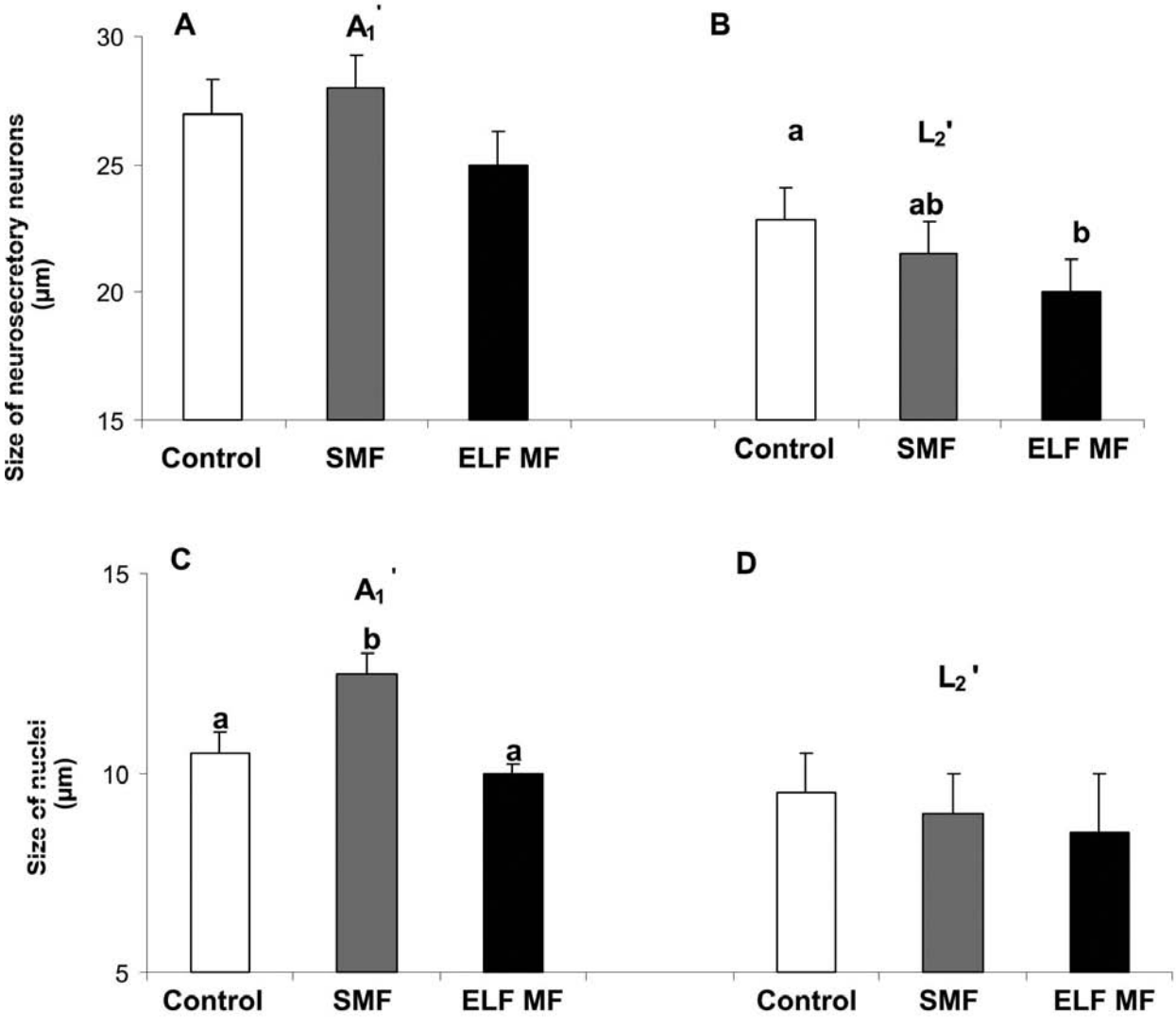
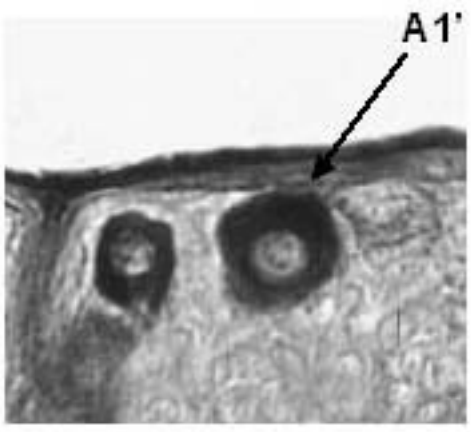
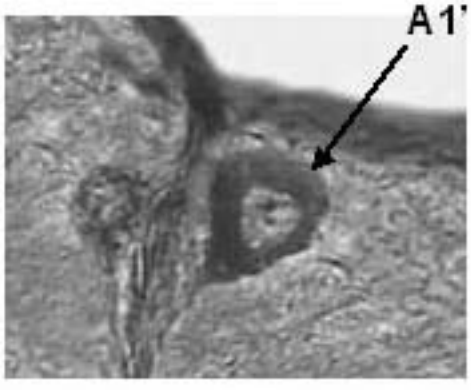


Fig. 1. Means and standard errors (±SEM) of the size of A1' neurosecretory neurons (nsn) (**A**), the size of L2' nsn (**B**), the size of A1' nuclei (**C**) and the size of L2' nuclei (**D**) in protocerebrum of control 4th instar caterpillars and those exposed to a strong static magnetic field (SMF) and an extremely low frequency magnetic field (ELF MF) for 3 days. Different letters (a,b) indicate significant differences between experimental groups (LSD test, P<0.05).;

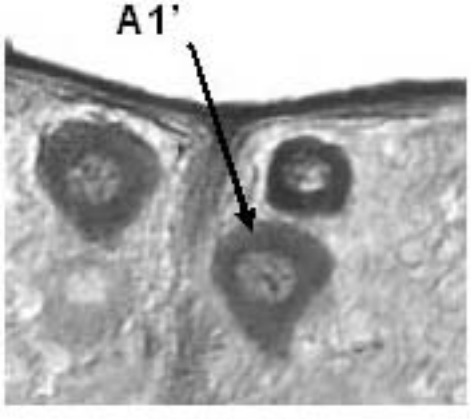
We observed a trend of decreasing size in the L2′ neurosecretory neurons and their nuclei after the exposure of *Lymantria dispar* larvae to both magnetic fields (Fig. 1B). A statistically significant decrease of the L2′ nsn size was detected in the group treated with an extremely low frequency magnetic field in comparison to the control (one-way ANOVA, $F_{2,38}$=3.2805, P<0,05; Figure 1B). The L2′ neurosecretory neurons in the protocerebrum of the larvae exposed to the extremely low frequency magnetic field contained a high amount of large-grained neurosecretory material which forms agglomerations (Fig. 3), indicating a retention of neurosecretory material in the neurons (Laufer 1984, Raab 1982).



Control

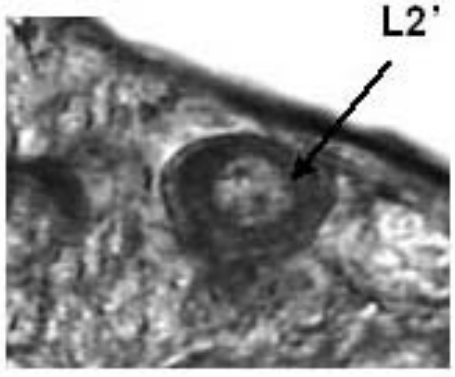


SMF



ELF MF

Fig. 2. - Brain transverse cross-sections (dorsomedial region of the protocerebrum) of *Lymantria dispar* 4th instar caterpillars. Protocerebral A1' neurosecretory neurons are marked by arrow. The bar represents 10 μm. Abbreviations are explained in the caption to Fig.1.



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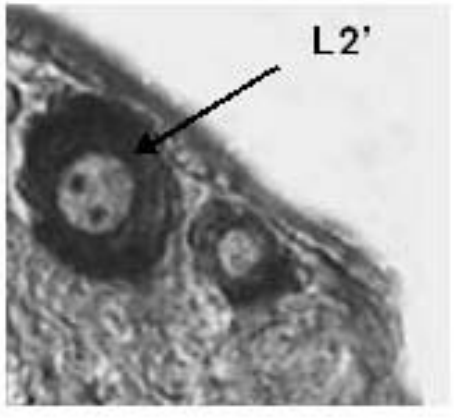
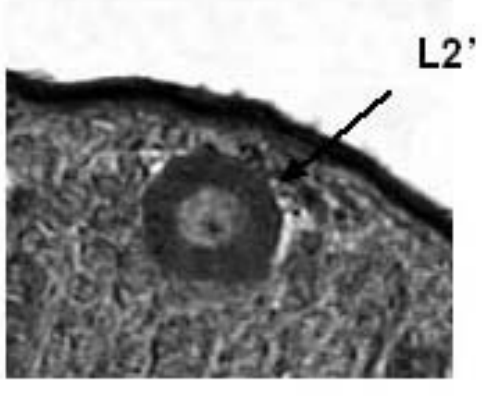


SMF



ELF MF

Fig. 3. - Brain transverse cross-sections (dorsolateral region of the protocerebrum) of *Lymantria dispar* 4th instar caterpillars. Protocerebral L2' neurosecretory neurons are marked by arrow. The bar represents 10 μm. Abbreviations are explained in the caption to Fig.1.

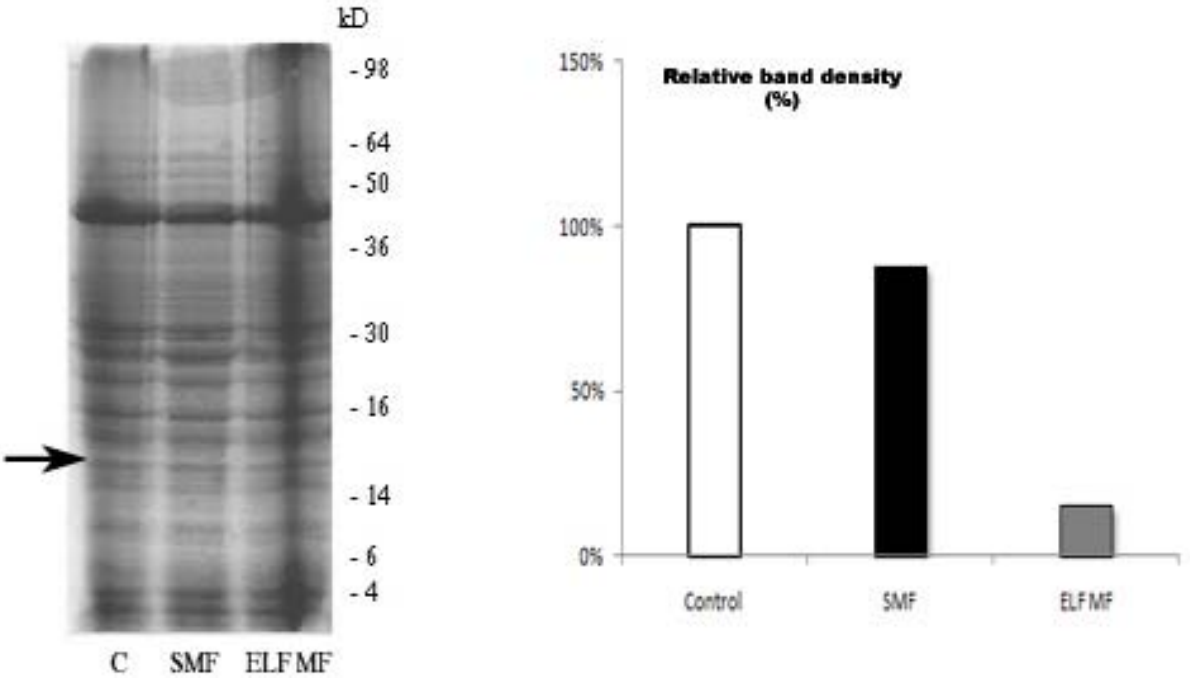


Fig. 4. Means and standard errors (±SEM) of the size of A1' neurosecretory neurons (nsn) (**A**), the size of L2' nsn (**B**), the size of A1' nuclei (**C**) and the size of L2' nuclei (**D**) in protocerebrum of control 4th instar caterpillars and those exposed to a strong static magnetic field (SMF) and an extremely low frequency magnetic field (ELF MF) for 3 days. Different letters (a,b) indicate significant differences between experimental groups (LSD test, $P<0.05$).

Differences in electrophoretic patterns

The differences in the electrophoretic patterns of 4th instar *L. dispar* brain homogenates obtained by 12% SDS PAGE, and induced by the strong magnetic field and extremely low frequency magnetic field are shown in Fig. 4A. The arrow indicates the region of molecular mass of about 14-16 kD, the Mr range of the large PTTH form in insects. Quantification of the bands in this region in all experimental groups revealed a decrease in the amount of protein after exposure to a strong static magnetic field and especially after exposure to an extremely low frequency magnetic field in comparison to the control group (Fig. 4B).

DISCUSSION

Acute or chronic exposure to external magnetic fields is stressful for an organism. The generation, transmission and use of electric energy are associated with the production of alternating artificial magnetic fields. The European Community has established a magnetic flux intensity of 500 μT for occupational exposure, while the IARC (International Agency for Research on Cancer) has proposed that extremely low frequency magnetic fields could be considered as a possible human carcinogen (IARC 2002).

The effects of magnetic fields have been detected in various insect species (Prolić and Nenadović 1995; Kirschvicnk et al.. 1997; Prolić et al. 2002; Nenadović et al. 2005; Perić-Mataruga et al. 2006; Todorović et al. 2007; Perić-Mataruga et al. 2008). Magnetic fields affect growth and development (Prolić and Nenadović 1995; Prolić et al. 1996), enzyme activities (Nossol et al. 1993), DNA replication and transcription (Herada et al. 2001; Nindi et al. 2002), and may even generate mutations (Prolić and Anđelković 1992; Ding et al. 2001).

A strong static magnetic field (375 mT) exhibited a stressogenic influence on the development and survival of the honeybee and *T. molitor* pupae (Prolić and Jovanović 1986; Prolić and Nenadović 1995; Prolić et al. 1996). It decreased the duration of the pupal stage in *T. molitor*, and adult longevity was shortened (Prolić et al. 1996). Additionally, an increase in mortality during the embryonal, larval and pupal development was observed in *D. melanogaster* (Ramirez et al. 1983).

Exposure to low frequency magnetic fields (<300 Hz) induced changes in the behavior of social insects (Becker 1976), while Vachá (1997) observed changes in extracardial pulsation in *T. molitor* pupae. Extremely low frequency magnetic fields also induce a decrease in locomotor activity and an increase of stereotypic activity in *Morimus funereus* adults (Prolić et al. 2002). Low frequency magnetic fields (50-60 Hz) appear to disrupt numerous biochemical processes, including protein synthesis, and cause single and double-strand DNA breaks (Lai and Singh 1997) as well as a decrease in cell function and integrity (Tenforde 1991).

However, investigations into changes at the level of the neuroendocrine system (which participates in the regulation of all above-mentioned life processes) provoked by magnetic fields have just begun. The endocrine system is a part of the nervous system and specialized neurosecretory neurons (mainly positioned in the protocerebral region of the brain) show characteristics of 'conventional neurons' in the sense of generating and conducting action potentials. It is interesting that a calcium component in the action potential is a characteristic of insect neurosecretory neurons (Orchard 1984). The data obtained by Blackman (1994) indicate that the magnetic fields' 'information' most probably starts with the Ca^{2+} ions at the neuronal membranes. External magnetic fields influence the Ca^{2+} concentration inside the neurons (Blackman 1994, Karabakhtsian et al. 1994). The exposure of crayfish and marine molluscs to a static magnetic field (4.74 – 43.45 mT) increased the amplitude of the action potential in the neurons (Popescu and Willows 1999, Ye et al. 2004). Changes in the action potential by magnetic field exposure are likely to be mediated by the increasing level of intracellular Ca^{2+} in the neurons, because the chelating of intracellular Ca^{2+} would block the effects induced by exposure to a static magnetic field, while the injection of Ca^{2+} into the neurons could mimic the effects of static magnetic field exposure (Ye et al. 2004). Ca^{2+} is of critical importance for a variety of cellular metabolic functions (Santella 1998) and calcium components of the action potential in the cell bodies of neurosecretory neurons is involved in the regulation of synthesis and release of insect neurohormones (Takeda 1976).

The cytological and morphometric parameters of A1' neurosecretory neurons after exposure of 4th instar *L. dispar* larvae to a strong static magnetic field have showed high synthetic and secretory activity (Fig.1A, 1C, 2). Literature data indicate that medial neurosecretory neurons from the *pars intercerebralis* (where A1'neurosecretory neurons are located) synthesize the neurohormones which regulate the release of adipokinetic neurohormones, regulators of digestive enzyme activity and neurohormones which regulate the diuresis and water balance in insects (Ivanović et al. 1978; Raab 1989; Leković et al. 2001). The stressogenic effects of magnetic fields on organisms manifest as increased cell and tissue metabolism and a more rapid development (Prolić and Nenadović 1995; Prolić et al. 1996; Dini and Abbro 2005). This elevated metabolic activity includes the reorganization of the carbohydrate metabolism regulated by the neurohormones produced in medial neurosecretory neurons. We presume, on the basis of obtained results, that the static magnetic field has a stronger influence on the synthetic and secretory activity of A1' neurons compared to the extremely low frequency magnetic field.

The effects of both magnetic fields, SMF and ELF MF, correlate with the decrease in size of L2' neurosecretory neurons. This effect is stronger after the exposure of *L. dispar* larvae to an extremely low frequency magnetic field (Fig.1B). Literature data point to prolonged insect larval development after exposure to

an extremely low frequency magnetic field (Ramirez *et al.* 1983). The concentration of the large form of prothoracicotropic neurohormones in hemolymph reaches a peak before molting to the next stage. If the larval development was prolonged after exposure to magnetic fields, the synthesis/secretion of prothoracicotropic neurohormones was delayed i.e. the activity of the L2' neurosecretory neurons was also delayed. The decrease in quantity of these neurohormones in the brain of larvae treated with an extremely low frequency magnetic field was confirmed after analysis of the electrophoresis gels (Fig. 4).

The differences in the response of the neurosecretory neurons to different types of magnetic field could derive from changes in the insect's sensitivity at different periods of development to the magnetic fields. The results obtained in this study contribute to a further understanding of the biological effects of static magnetic fields and extremely low frequency magnetic fields on the polyphagous phytophagous forest pest *L. dispar* L. (Lepidoptera: Lymantridae).

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