Arch. Biol. Sci., Belgrade, 65 (1), 359-367, 2013 DOI:10.2298/ABS1301359I

SENSITIVITY OF GYPSY MOTH NEUROSECRETORY NEURONS TO ACUTE THERMAL STRESS

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Abstract - In gypsy moth caterpillars exposed to a temperature of 35ºC (for 1, 12 and 24 h and caterpillars that were exposed to elevated temperature for 12 h and were allowed to recover for 12 h at 23ºC), changes in the brain protein profiles and morphometric characteristics of A1' medial and L2 lateral protocerebral neurosecretory neurons were analyzed. In all groups, protein bands with a molecular mass corresponding to that of members of heat-shock protein families were detected, indicating that acute exposure to this temperature likely induced the synthesis of HSP. Increased morphometric parameters of A1' neurons and the large amount of neurosecretory material in the neuron body implicate that the temperature of 35˚C is not in the temperature range that exerts stimulatory effects on growth and survival. Changes in the morphometric characteristics of L2 neurosecretory neurons from the lateral part of the protocerebrum, and retention of neurosecretory material in their cytoplasm indicate a low level of secretion.

Key words: Gypsy moth, neurosecretory neurons (nsn), acute thermal stress

INTRODUCTION

Environmental temperature is one of the most important variables that determine the distribution and abundance of species (Cossins and Bowler, 1987), as well as population density and gradation power. Increased temperature increases mortality, decreases development time and the size of insects, which leads to a decreased number of laid eggs (Ochieng-Odero, 1992; Matsuki et al., 1994). In addition, increased oxygen consumption has been reported, along with changed food quantity and speed of food consumption as a consequence of increased temperature (Roe et al., 1985; Reynolds and Nottingham, 1985). An unsuitable external temperature affects insect intermediary metabolism (Ivanović et al., 1992), and Burnell et al. (1991) observed an increased activity of the detoxification system after exposure to high temperature.

Temperature stress can delay or completely inhibit the development of many hemimetabolous and holometabolous insects by disturbing the hormonal equilibrium (Wiggleswort, 1952). Maksimović (1958) observed that variable temperatures are more favorable for the development of *Lymantria dispar* caterpillars.

The insect neuroendocrine system is sensitive to high temperatures, which may disturb the regular synthesis and release of neurosecretory material (Ivanović and Janković-Hladni, 1991). Changes in the morphological parameters and activity of dorsomedial and dorsolateral neurosecretory neurons (nsn)

were detected in different insect species (Gruntenko et al., 2000; Leković et al., 2001; Mrdaković et al., 2005). In *Morimus funereus* larvae, an increased volume of *corpora allata* was detected, while the activity of dorsomedial and dorsolateral protocerebral nsn was inhibited (Leković et al., 2001; Mrdaković et al., 2007). Temperature shock induces supernumerary molting in *Drosophila*, in response to elevated levels of the allatotropin regulatory neurohormone that is synthesized in protocerebral nsn (Gruntenko et al., 2000).

In the present study, we examined the effects of high temperature on *L. dispar* L. larvae. The aim was to analyze the changes in the total brain protein content and changes in brain protein profiles in 4th instar caterpillars of gypsy moth exposed to a temperature of 35°C for 1, 12 and 24 h, as well as caterpillars returned to recovery (12 h at 23 °C). We also examined the morphometric characteristics of A1' medial and L2 lateral nsn in all experimental groups. We analyzed the underlying neurosecretory mechanisms in response to an elevated rearing temperature.

MATERIALS AND METHODS

Insect rearing

Gypsy moth egg masses originated from a poplar forest (locality Opovo, 30 km from Belgrade). Caterpillars were reared on a synthetic HWG (high wheat germ) diet (O'Dell et al., 1985) in transparent plastic containers (V = 200ml) at 23˚C and a 16 h light:8 h dark photoperiod. Caterpillars were randomly assigned to the following experimental groups: five groups for histochemistry (n=15), and five groups for brain sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (n=15).

Thermal stress (temperature regimes)

The acute effects of a stressful high temperature of 35°C were tested on gypsy moth caterpillars. The first group was exposed to 35°C for 1 h (**1h**); the second for 12 h (**12h**); the third group of caterpillars was transferred for 12 h to 23°C after 12 h exposure to 35°C (**12/12h**), and the fourth group was exposed to high temperature for 24 h (**24h**). The control group was reared at 23°C (**C**). The larvae were exposed to this temperature in a thermostat, with constant humidity.

SDS-PAGE electrophoresis

Brains were homogenized at 20,000 g (200 mg brain/ml distilled water) and then centrifuged at 10,062 g at 4˚C. The supernatant was collected and SDS-PAGE electrophoresis was performed on 12% gels according to Laemmli (1970). The gels were stained with Coomassie Brilliant Blue R 250 (Serva), followed by destaining in 50% methanol (Lach-Ner) – 10% acetic acid solution (Merck). The molecular weight (Mr) of the proteins was estimated using commercial standards of Mr 4-250 kD (Invitrogen Corporation).

Histological procedures

On the 3rd day of the 4th instar, the caterpillars were killed by decapitation and the head capsules were fixed in Bouin's solution for 24 h (Merck). Brain complexes were dissected, rinsed in 70% ethanol (Hemos), dehydrated in a graded series of ethanol, impregnated in xylol (Hemos) and embedded in paraffin wax (59ºC, Merck). 3.5 μm-serial sections were cut for histochemistry (microtome 820 Spencer) and collected on 0.2% gelatin/0.05% chrome alum (Sigma Aldrich) coated slides. Sections were stained by a modified (Panov, 1980) Ewen paraldehyde fuchsin technique (Ewen, 1962). In all nsn, the neurosecretory material was stained different shades of purple in the cytoplasm, while nucleoli were observed as light pink-colored spheres in the nuclei (Panov, 1980). The activity of all four types of nsn was determined by monitoring the size of the nsn and their nuclei (expressed as means of the shortest and longest diameters in μm). Data were evaluated by one-way analysis of variance (ANOVA) and a *post-hoc* multiple range test (Fisher's least significant difference – LSD). The parameters were analyzed and measurements made using an image processing and analysis system (QWin image analysis tool kit) linked to a Leica DMLB light microscope (Leica).

RESULTS

Differences in the electrophoretic patterns of brain homogenates of 4th instar *L. dispar* caterpillars obtained by 12% SDS-PAGE after exposure to a high temperature of 35˚C for 1, 12 and 24 h and a group returned to 12 h recovery at a control temperature of 23˚C, after 12 h exposure to 35˚C, are presented in Fig. 1A. We examined changes in the Mr region from 4 to 98 kD. Protein profiles were similar to the majority of protein bands located in the Mr area, 45-50 kD. In all experimental groups, two protein bands with close molecular masses are detected in the Mr regions of 10, 16, 30 and 36 kD. In the Mr region of 50-60 kD, two close protein bands were detected, and one with a smaller mass was less intense. In the groups with a prolonged exposure to 35˚C, these protein bands exhibited the same intensity of staining. After 24 h exposure to high temperature, the brain protein profile becomes less intense in comparison to all other experimental groups.

In Fig. 1B, changes in the total brain content after exposure to this acute stressor are presented. A significant decrease in total protein content was detected in caterpillars exposed to 35˚C for 1 and 24 h and in the group returned to 12 h recovery at a control temperature of 23˚C after 12 h exposure to 35˚C in comparison to the control group and the group exposed to high temperature for 12 h (Fisher's LSD test). All groups exposed to high temperature showed a significant decrease in brain total protein content in comparison to the control group (one-way ANOVA $F_{4,15} = 10.348$, $P<0.001$), apart from the group exposed for 12 h to high temperature.

Based on their size and morphological characteristics, we divided the protocerebral nsn of *L. dispar* into groups to facilitate the monitoring of the results. We analyzed changes in the morphometric parameters of dorsomedial A1' nsn of average diameter 25.47 ± 0.71 μm (Fig. 2), and L2 dorsolateral group of nsn with average diameter 17.03 ± 0.25 μm (Fig. 4) after exposure to the temperature of 35˚C.

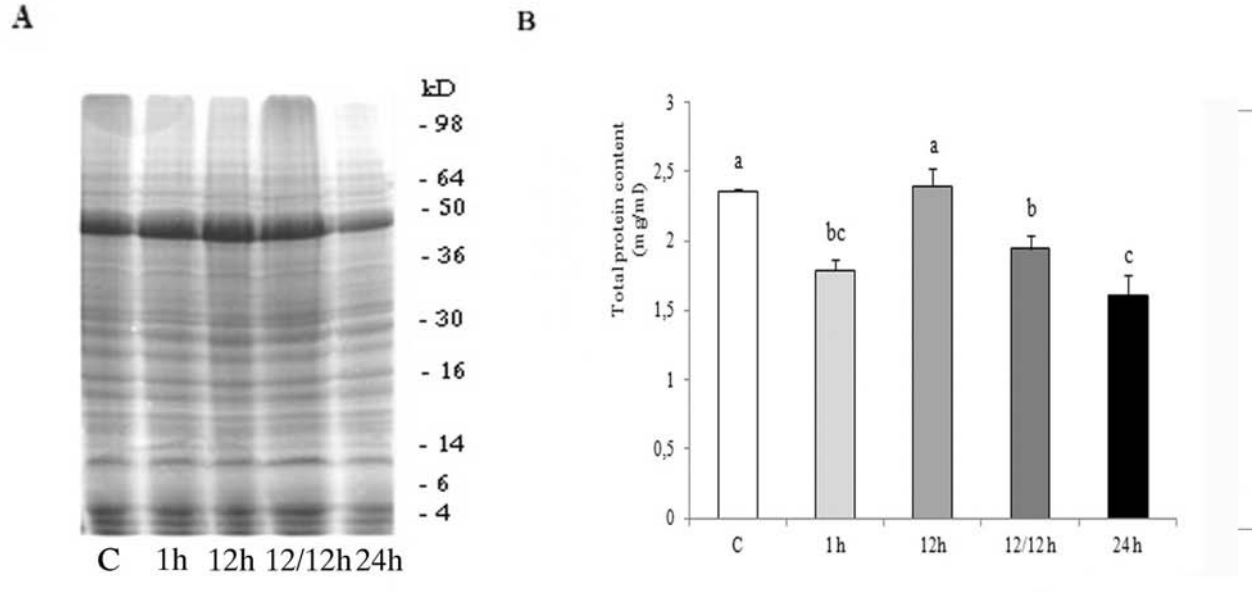

Fig. 1.– (**A**) Electrophoretic patterns of brain homogenates of 4th instar *Lymantria dispar* caterpillars obtained by 12% SDS PAGE, after exposure to high temperature of 35˚C for **1h**, **12h**, **24h** and the group that was allowed to recover for 12 h at the control temperature of 23˚C after a 12 h exposure to 35˚C (**12/12h**). The control group was kept at 23˚C (**C**); (**B**) Total protein content in 4th instar *Lymantria dispar* caterpillars brain homogenates after acute exposure to high temperature of 35˚C. All abbreviations are the same as for Fig. 1A.

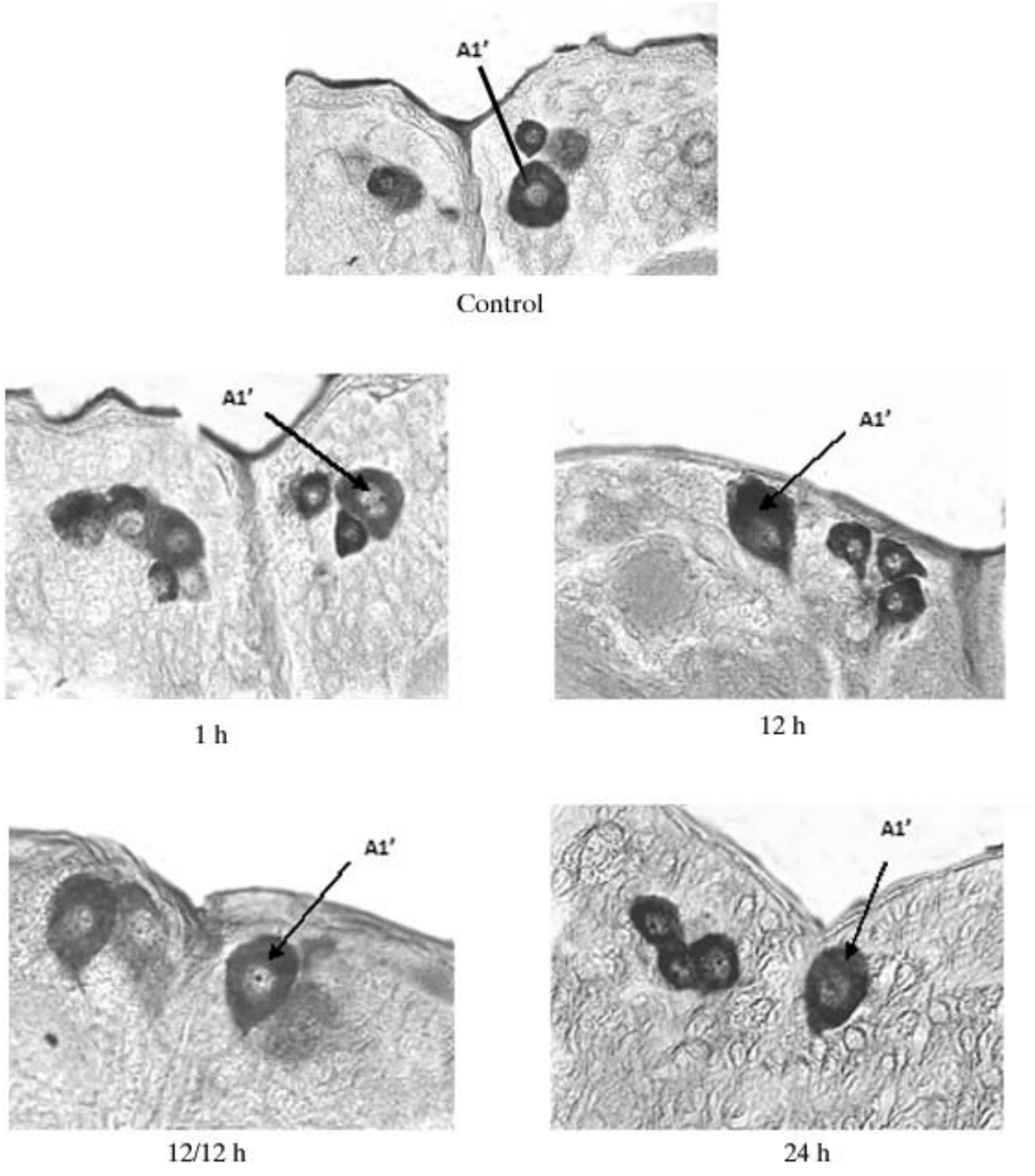

Fig. 2.- Brain transverse cross-sections of *Lymantria dispar* 4th instar caterpillars after acute exposure to high temperature of 35˚C. All abbreviations are the same as for Fig. 1A. Arrows indicate the dorsomedial A1' neurosecretory neurons (nsn).The bar represents 10 µm.

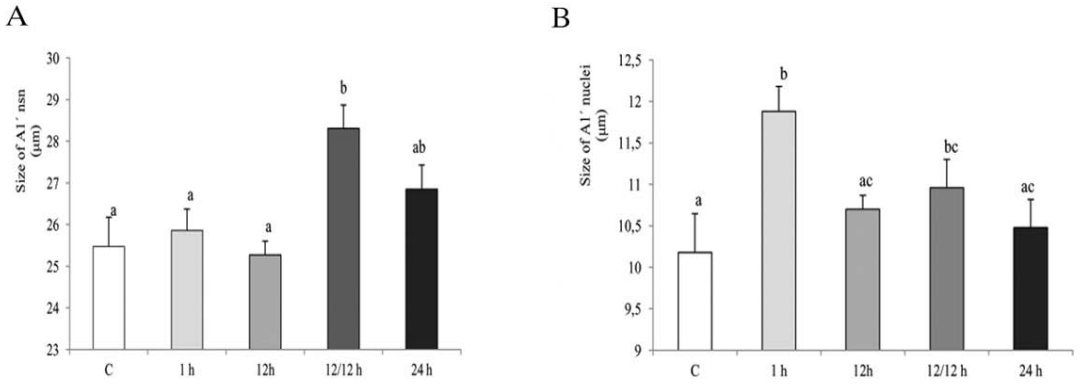

Fig. 3.- The size of A1' nsn (**A**), and size of A1' nsn nuclei (**B**) in 4th instar *Lymantria dispar* caterpillars after acute exposure to high temperature of 35˚C. All abbreviations are the same as for Fig. 1A. Error bars indicate the standard error of the mean (SEM) Different letters (a,b,c) indicate significant differences between groups (LSD test, $P < 0.01$).

Caterpillars that were allowed to recover for 12 h at the control temperature of 23˚C after 12 h exposure to 35˚C had significantly larger A1' nsn in comparison to the control group and all other experimental groups with exception of the 12/12 group (Fisher's LSD test, Fig. 3A). High temperature induced significant changes in A1' nsn size in all experimental groups in comparison to the control (one-way ANOVA $F_{4,105} = 5.664$, $P<0.001$). High temperature induced increased A1' nuclear size in comparison to the control group (one-way ANOVA $F_{4,105} = 3.802$, $P<0.01$ and Fisher's LSD test, Fig. 3B). In all treated caterpillars, A1' nsn were filled with a high amount of fine-grained neurosecretory material (Fig. 2).

A significant decrease in L2 nsn size was detected only in the caterpillars exposed to high temperature for 1 h (Fisher's LSD test, Fig. 5A). On the other side, the size of their nuclei increased in all group exposed to high temperature, however, this increase was significant only in the group exposed to this acute stressor for 12 h (Fisher's LSD test, Fig. 5B). After all treatments, L2 nsn were filled with fine-grained neurosecretory material (Fig. 4). A decrease in the amount of neurosecretory material was detected in caterpillars that were allowed to recover for 12 h at the control temperature of 23˚C after 12 h of exposure to 35˚C.

DISCUSSION

Insects are exposed to daily fluctuations in temperature. This could be very stressful, even lethal. Insects have thus developed the physiological, biochemical and behavioral mechanisms to overcome these stressful conditions.

In response to extremely high temperatures, insects synthesize heat shock proteins (HSP). These have an important role in the general response to stress. HSP protect newly synthesized proteins ready for transport, and reduce to a minimum the aggregation of nonnative proteins, but if this aggregation happens, they "mark" such complexes for degradation and excretion from the cell (Federe and Hofmann, 1999).

Electrophoretic brain protein profiles (Fig. 1) indicated the presence of intensive protein bands in the molecular mass region of 22 to 29 kD, which suggests the presence of small HSP after the exposure of caterpillars to high temperature. Protein bands of Mr 60 kD were not detected after this acute high temperature of 35ºC. Two bands with close molecular masses, in the Mr region 70-75 kD, are present in all experimental groups. We presume that they could be members of the HSP70 family. The possible presence

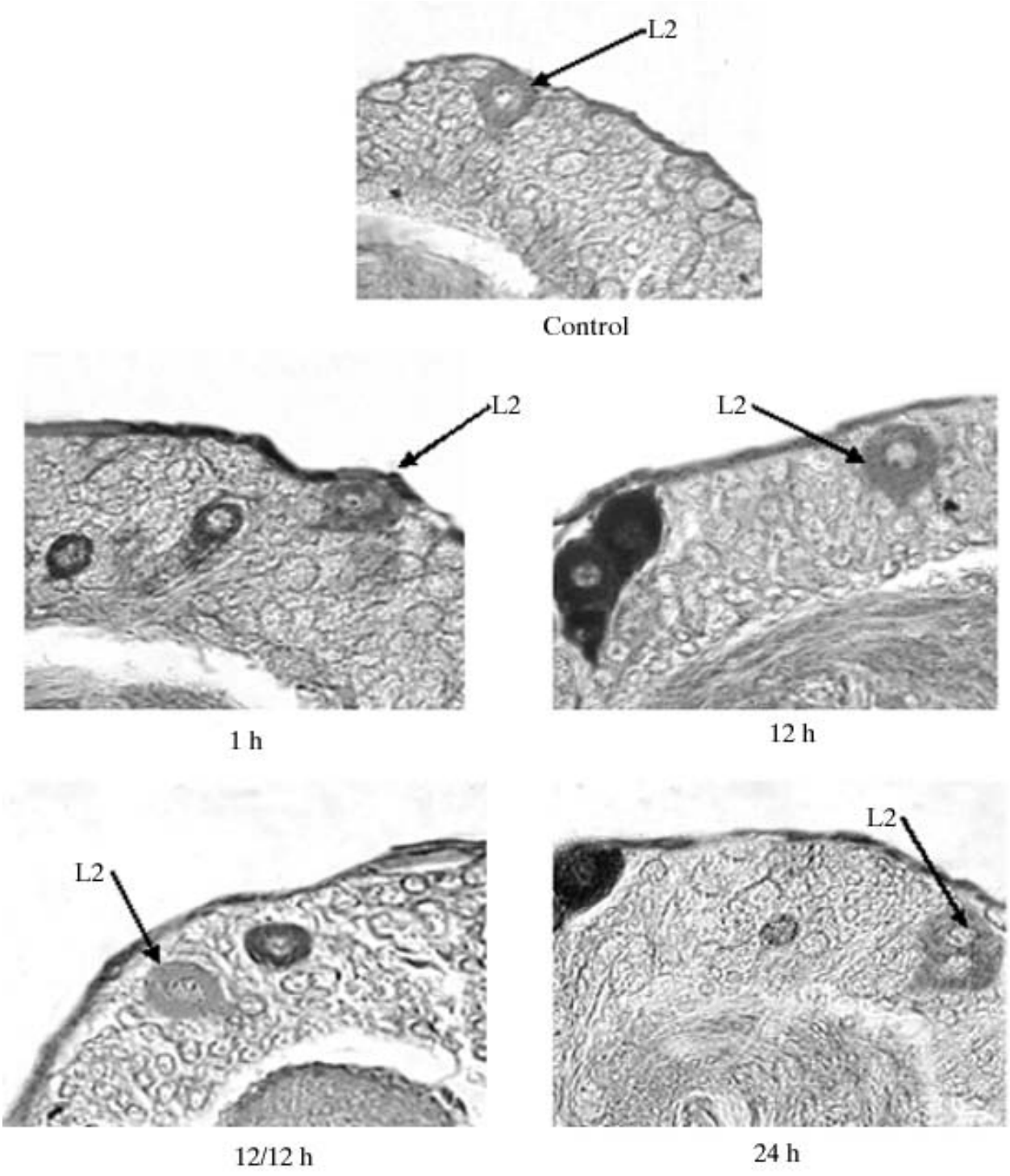

Fig. 4.-Brain transverse cross-sections of *Lymantria dispar* 4th instar caterpillars after acute exposure to high temperature of 35˚C. All abbreviations are the same as for Fig. 1A. Arrows indicate the dorsolateral L2 neurosecretory neurons. The bar represents 10 µm.

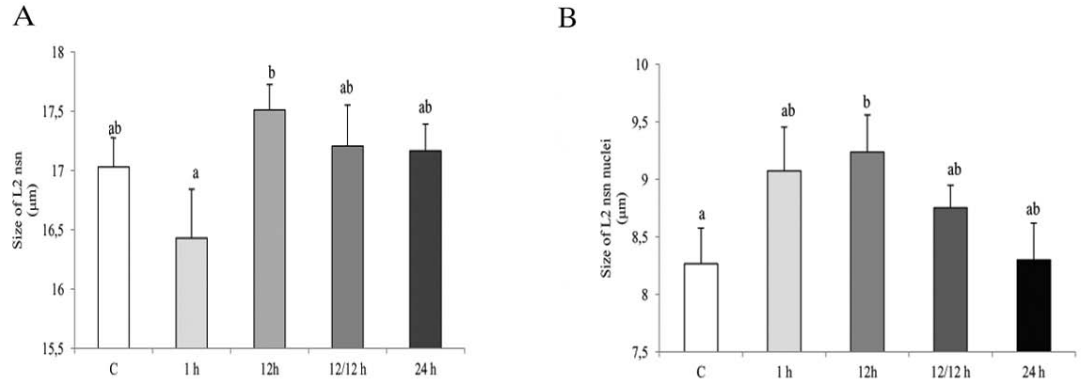

Fig. 5.- The size of L2 nsn (**A**), and size of L2 nsn nuclei (**B**) in 4th instar *Lymantria dispar* caterpillars after acute exposure to high temperature of 35˚C. All abbreviations are the same as for Fig. 1A. Error bars indicate the standard error of the mean (SEM). Different letters (a,b) indicate significant differences between groups (LSD test, $P < 0.01$).

of a member from the HSP 90 family was observed as a protein band of Mr 90 kD. In gypsy moth caterpillars exposed to 35ºC, members of all three HSP families (22, 29, 75, 73, 90 kD) were detected (Yocum et al., 1991). This experiment indicates that a short time exposure to this temperature (1, 12 and 24 h) probably induces the synthesis of HSP.

High temperatures reorganize the synthesis and release of neurosecretory material from nsn, and consequently they disturb the hormonal balance, as well as development and metamorphosis (Ivanović and Janković-Hladni, 1991). Metabolic reactions are faster, and the creation of a receptor-hormone complex is disturbed. The repair of these mechanisms and maintaining of homeostasis is energetically a very expensive process, which needs metabolic reorganization with numerous compensatory reactions.

Located in the medial part of *pars intercerebralis,* A1' nsn are known to synthesize regulatory neuropeptides. They regulate the synthesis and release of adipokinetic neurohormone, secretion of midgut digestive enzymes (proteases and amylases), diuresis, water balance, etc. (Gäde and Goldsworthy, 2003) (Raabe, 1989). The activation of these neurons is necessary for the digestive and metabolic reorganization that compensates for disturbed homeostasis under stressful conditions. In this paper we have observed the increased morphometric parameters of A1' neurons and a large amount of neurosecretory material in the neuron body (Figs. 1, 2). In *L. dispar* caterpillars, acute high temperature stress (t=35˚C) induced a reduction in larval mass and relative growth rate, as well as larval development (Ilijin, 2009). The obtained results implicate that the temperature of 35˚C is beyond the temperature range which exerts stimulatory effects on growth and survival.

In arthropods, high temperatures modulate the level of K^+ and Ca^{2+} ions and change the activity of cerebral nervous system, neurosecretion and muscular activity. Ca^{2+} is a universal intracellular messenger that controls secretion and metabolism. Changes in the morphometric characteristics of L2 nsn from the lateral part of the protocerebrum (Fig. 5), and the retention of neurosecretory material in their cytoplasm (Fig. 3) indicate a low level of secretion. This could be the consequence of inhibited Ca^{2+} influx caused by the acute effect of the high temperature of 35˚C. We observed that after 12 h of recovery from this stressor, almost all measured pa-

rameters reached a level close to control conditions, which is in concordance with the theory that after removing gypsy moth caterpillars from this stressful conditions, normal protein synthesis occurs after 1-3 h (Yocum et al., 1991).

Using temperature as an environmental stressor deriving from global warming, we have analyzed the response of two different categories of neurosecretory neuron (A1' medial and L2 lateral nsn). Nowadays, in ecotoxicology, the focus has moved from the effects of exposure on survival, development and reproductive anatomy to insect behavioral endpoints, sensitive even to small changes in hormone levels. Our results can contribute to a better understanding of the endocrine response and may assist in the development of more sensitive bioindicators.

Acknowledgments - This study was supported by the Serbian Ministry of Education, Science and Technological Development (grant No.173027).

REFERENCES

Burnell, A. M., Reaper, C. and *J. Doherty* (1991). The effect of acclimation temperature on enzyme activity in *Drosophila melanogaster. Comparative Biochemistry and Physiology* **98B,** 609-614.

Cossins, A.R. and *K. Bowler* (1987). Temperature biology of animals. Chapman and Hall, New York.

Ewen, A.B. (1962). An improved aldehyde fuchsin staining technique for neurosecretory products in insects. *Transactions of the American Microscopical Society* **81**, 94-96.

Federer, M.E. and *G.E. Hofmann* (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology* **61**, 243-282.

Gäde, G. and *G.J. Goldsworthy* (2003). Insect peptide hormones: a selective review of their physiology and potential application for pest control. *Pest Management Science* **59**, 1063-1075.

Gruntenko, N.E., Moastirioti, M., Wilson, T.G.; and *I.Y. Rauschenbach* (2000). Stress reactivity and juvenile hormone degradation in *Drosophila melanogaster* strains having stress related mutations. *Insect Biochemistry and Molecular Biology* **30**, 775-783.

Ilijin, L. (2009). Protocerebral neurosecretory neurons in acute stress in gypsy moth caterpillars (*Lymantria dispar* L.), PhD Thesis. Biology Faculty, University of Belgrade.

Ivanović,J., and *M. Janković-Hladni* (1991). Hormones and metabolism in insect stress. Florida, Boca Raton: CRC Press.

Ivanović J.; Janković-Hladni M.; Đorđević S.; Stamenović S.; and *J. Lazarević* (1992). The effect of high temperature on metabolism of *Morimus funereus* larvae during intermoult period. *Journal of Insect Physiology* **38**, 877-883.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Leković, S.; Lazarević, J; Nenadović, V. and *J. Ivanović* (2001). The effect of heat stress on the activity of A1 and A2 neurosecretory neurons of *Morimus funereus* (Coleoptera, Cerambycidae) larvae. *European Journal of Entomology* **98**, 13-18.

Maksimović, M (1958). Experimental research on the effect of temperature on individual development and population dynamics of gypsy moth (*Lymantria dispar* L.). *Biological Institute of* P.R. Srbia, Book **3**.

Matsuki M., Ayres M.P. and *S.F. McLean Jr.* (1994). Temperature effects on growth and molt of *Nematus calais* (Hymenoptera: Tenthredinidae), *Physiological and Chemical Ecology* **23**, 719-725.

Mrdaković, M., Ilijin, L., Janković-Tomanić, M., Vlahović, M., Prolić, Z., Perić-Mataruga, V., Lazarević, J., and *V. Nanadović* (2005). Effects of thermal stress on activity of *corpora allata* and dorsolateral neurosecretory neurons in *Morimus funereus* larvae. *Arch. Biol. Sci.* **57**, 83-92.

Mrdaković, M.; Lazarević, J., Perić-Mataruga, V., Janković-Tomanić, M., Ilijin L.; Vlahović, M., and *D. Mirčić* (2007). Morphometric changes of corpora allata in *Morimus funereus* Müls. (Cerambycidae) larvae during thermal stress. *Archives of Biological Sciences* **59** (3), 47P-48P.

Ochieng-Odero, J.R.P. (1992). The effect of three constant temperatures on larval critical weight, latent feeding period, larval maximal weight and fecundity of *Cnephasia jactatana* (Walker) (Lepidoptera: Tortricidae), *Journal of Insect Physiology* **38**, 127-130.

O'Dell, T.M., Butt, C.A., and *A. W., Bridgeforth* (1984). Lymantria dispar. In: *Handbook of Insect Rearing,* vol.2. (Eds. P. Singht and R. Moore), 355-367. Elsevier, New York, NY.

Panov, A.A. (1980). Demonstration of neurosecretory cells in the insect central nervous system. In: *Neuroanatomical Techniques. Insect Neurosystem*. Springer-Verlag, New York, Heidelberg, Berlin.

Raabe, M. (1989). *Recent developments in insects neurohormones*. Plenum Press, New York, NY.

Reynolds, S.E. and *S.F. Nottingham* (1985) Effects of temperature on growth and efficiency of food utilization in 5th instar caterpillars of tobacco hornworm, *Manduca sexta*. *Journal of Insect Physiology* **31**, 129-134.

Roe, R.M., Clifford, C.W. and *J.P. Woodring* (1985). The effect of temperature on energy distribution during the last larval stadium of the female house cricket, *Acheta domesticus, Journal of Insect Physiology* **31**, 371-378.

Wigglesworth, V.B. (1952). Hormone balance and the control of metamorphosis in *Rhodnius prolixus* (Hemiptera). *Journal of Experimental Biology* **29**, 620-631.

Yocum, G.D., Joplin, K.H., and *D.L. Denlinger* (1991). Expression of heat shock proteins in response to high and low temperature extremes in diapausing pharate larvae of the gypsy moth, *Lymantria dispar. Archives of Insect Biochemistry and Physiology* **18**, 239-249.