

## ACTIVITY OF GYPSY MOTH DORSOLATERAL NEUROSECRETORY NEURONS UNDER INCREASED REARING DENSITY

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**Abstract** - *Lymantria dispar* caterpillars were reared under two different rearing densities for the first three days of the 4<sup>th</sup> larval instar: 5 larvae that were kept in a Petri dish ( $V = 80$  ml) belonged to the intense stress ( $D_1$  group); 5 larvae that were kept in a plastic cup ( $V = 300$ ml) belonged to the group exposed to less intense stress ( $D_2$  group). In the control group, single larvae were reared in a Petri dish. Morphometric changes in L1, L2 and L2' dorsolateral neurosecretory neurons (nsn) were analyzed. After keeping 5 larvae in a Petri dish, the size of L2 neurosecretory neurons (nsn) significantly increased. Rearing 5 larvae in a plastic cup significantly increased the size of L1 nsn nuclei and the number of L2'nsn. A decrease in relative band densities in the region of molecular masses (11-15 kD) that correspond to prothoracicotropic hormones in the gypsy moth was observed in the electrophoretic profiles that were obtained after both treatments in comparison to the control group .

**Key words:** Rearing density, gypsy moth, dorsolateral neurosecretory neurons, Serbia

### INTRODUCTION

Gypsy moth populations show periodic fluctuations in population density, passing through several phases: latency (low density), progradation (increase in density), culmination (high density) and retrogradation (decrease in density).

High larval density leads to a crowding effect in many lepidopteran larvae. Population density affects development and growth, and consequently morphological changes and further behavioral patterns. *Lymantria dispar* is an insect pest that defoliates forest complexes during population outbreaks. In Europe, these events culminate every eight to eleven years and last for three years. During this gradation period, the gypsy moth has a high population density and intensive defoliation capacity, and it defoliates large forest complexes.

Increased density is known to change larval development (Roberts 1998; Tammaru et al., 2000), survival, body weight and fecundity (Hirschberger 1999, Hooper et al., 2003). At high density of population, gypsy moth development was 2-3 weeks shorter, caterpillars and pupae have a lower weight, and fecundity was decreased (Leonard 1981). In addition, the size of the eggs was reduced, but not the quantity of vitellogenin (Diss et al., 1996). Under increased rearing density, the activity of gypsy moth amylase was not significantly affected, while the activity of trypsin was increased (Lazarević et al., 2004).

Density-dependent responses such as increased respiration, mobilization of carbohydrates and lipids, different expression of glycolytic and other enzymes (Applebaum and Heifetz, 1999), are regulated by insect neurohormones (Perić-Mataruga et al., 2006) synthesized in neurosecretory neurons.

Our previous investigation revealed changes in the size and cytological characteristics of protocerebral dorsomedial neurosecretory neurons (nsn) in gypsy moth caterpillars exposed to elevated rearing density (Ilijin et al., 2010). In this paper we have analyzed the changes of L1, L2 and L2' gypsy moth nsn, located in the dorsolateral part of the protocerebrum. The neurohormones synthesized in these nsn (e.g. allatostatins and a large form of the prothoracicotrophic hormone (PTTH)) are regulators of the metabolic and morphogenetic processes in insects. From the obtained results, we discuss the underlying neurosecretory mechanisms in response to elevated population density. In addition, we have examined changes in brain protein profiles in the region of molecular masses (Mr) 11-15kD, known as the Mr range of PTTH in the gypsy moth (Kelly et al. 1991).

## MATERIALS AND METHODS

### *Insect rearing*

We collected gypsy moth egg masses in a poplar forest 30 km from Belgrade (Opovo). From hatching, larvae were reared on a high wheat germ (HWG) diet (O'Dell et al., 1985) in transparent plastic containers ( $V = 300\text{ml}$ ) at a temperature of  $23^{\circ}\text{C}$  and photoperiod of 16L:8D. Larvae were randomly assigned to three experimental groups for histochemistry and three groups for electrophoresis of brain proteins. The following groups were created: **C** – control group in which single larvae were reared in a Petri dish; **D1** – five larvae were kept in a Petri dish ( $V = 80\text{ml}$ ) for the first three days of 4<sup>th</sup> larval instar, and **D2** – five larvae were kept in a plastic cup ( $V = 300\text{ml}$ ) for the first three days of 4<sup>th</sup> larval instar. Larvae were fed *ad libitum* with a basic diet until hatching in the 4<sup>th</sup> instar.

### *Histological techniques*

Dissected brain complexes were immersed in Bouin's fixative (Merck), rinsed in 70% ethanol (Hemos) and dehydrated in a graded series of ethanol (Hemos) before embedding in paraffin wax. Serial sections of brain complexes were cut at  $3\ \mu\text{m}$  for histochemis-

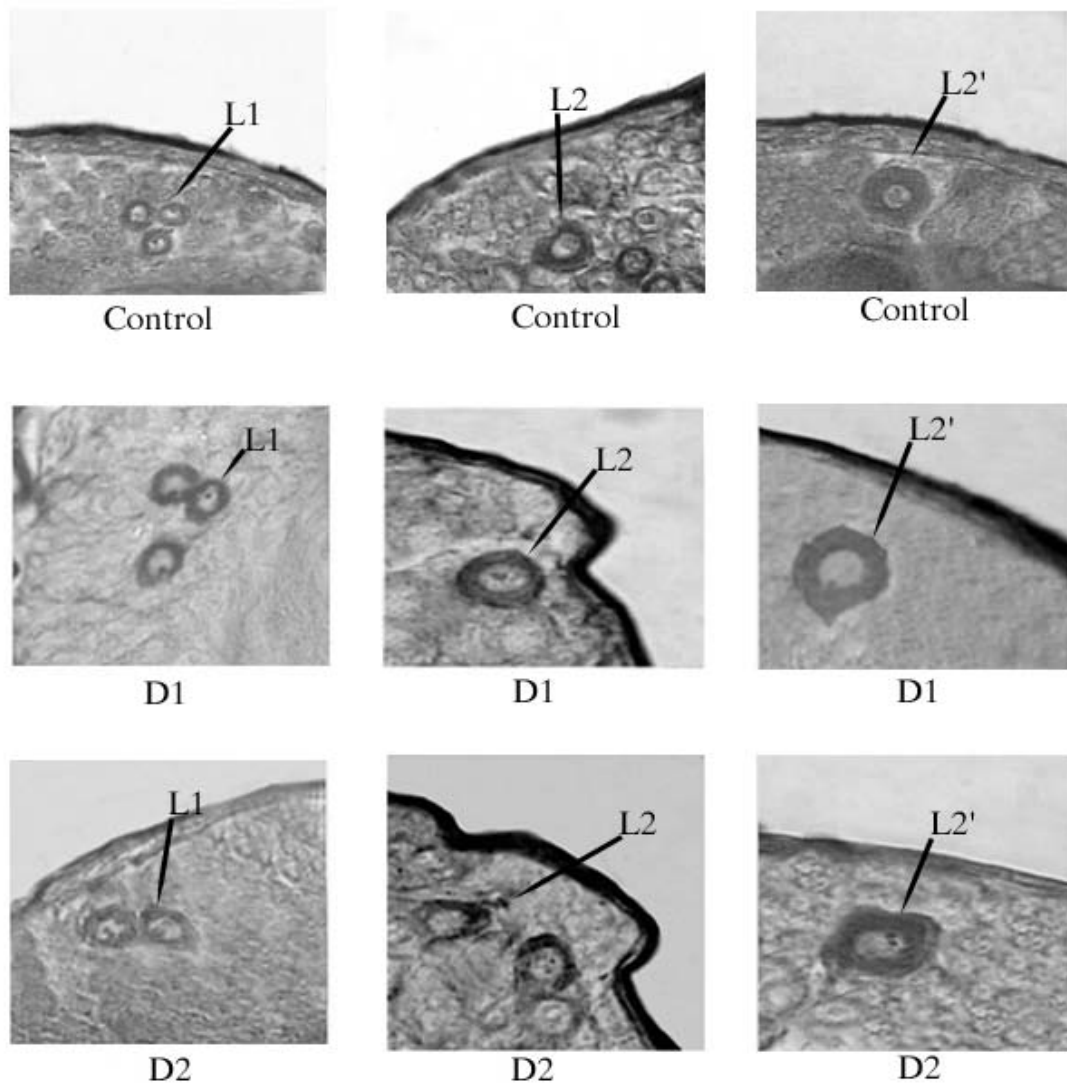
try (microtome- "820" Spencer). After being dried for 48 h at  $37^{\circ}\text{C}$ , the sections were deparaffinized in xylene (Hemos), rehydrated to 10 mM phosphate buffered saline (Sigma Aldrich), and stained by modified (Panov 1980) Ewens paraldehyde fuchsin technique. The size of the protocerebral dorsomedial nsn and nuclei was expressed as the mean value of their smallest and largest diameter (in  $\mu\text{m}^2$ ). The parameters were analyzed and measurements made using the image processing and analysis system (QWin image analysis tool kit) linked to a Leica DMLB light microscope (Leica).

### *SDS PAGE Electrophoresis*

Brain samples (200 mg/ml of distilled water) were homogenized ( $20\ 000 \times g$ ) and then centrifuged at  $10\ 062 \times g$  for 10 min at  $4^{\circ}\text{C}$ . SDS PAGE electrophoresis was performed on 12% gels (Laemmli, 1970). The gels were then stained for proteins with a Coomassie Brilliant Blue R 250 solution (Serve Electrophoresis) overnight at  $4^{\circ}\text{C}$ , followed by destaining in a 50% methanol, 10% acetic acid solution. The molecular weight of the proteins in SDS PAGE was estimated using commercial standards with Mr of 4-250 kD (Invitrogen Corporation). Protein band intensities in the region of the big form of PTTH Mr (11-15 kD) were analyzed using National Institute of Health (NIH) software ImageJ 1.42q.

## RESULTS

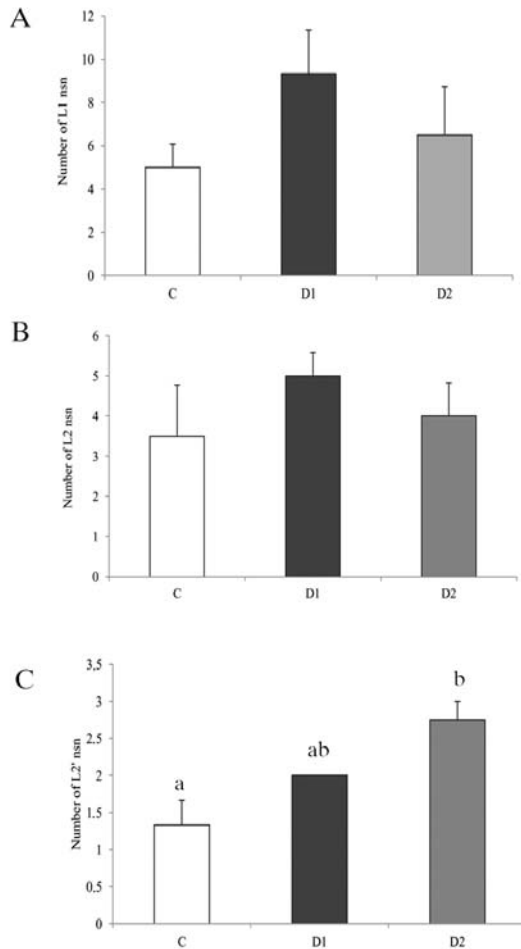
Based on their size and morphological characteristics, we divided the dorsolateral nsn of *L. dispar* L. into three groups (L1, L2 and L2'). Changes in the activity of the lateral neurosecretory neurons in gypsy moth caterpillars reared under two different rearing densities are presented in Fig. 1. Number of L1 nsn as well as the size of these nsn tended to increase, on both rearing densities, in comparison to control group (Figs 2 and 3). The average size of L1 nsn was  $11.83\ \mu\text{m}$ . The size of L1 nuclei increased significantly (one-way ANOVA,  $F_{2,71} = 6.02$ ,  $p < 0.001$ , Fig. 4) in D2 group in comparison to the control group. Majority of these nsn had a large amount of neurosecretory material in cytoplasm and large nuclei (Fig.



**Fig. 1.** – Brain transverse cross-sections of *Lymantria dispar* 4<sup>th</sup> instar caterpillars after rearing in different rearing densities: C – control group in which single larvae were reared in a Petri dish (isolated conditions), D1 – 5 larvae *per* Petri dish (V = 80 ml), for the first three days of 4<sup>th</sup> larval instar, and D2 – 5 larvae *per* plastic cup (V=300 ml). Arrows indicate the dorsolateral L1, L2 and L2' neurosecretory neurons. The bar represents 10  $\mu$ m.

1) indicating intensive synthetic activity. Second type of lateral nsn, analyzed in this paper, was L2 type, with average size of 15.54  $\mu$ m. Their number tended to increase on both treatments (Fig. 2). The L2 nsn size was significantly increased in group D1 in comparison to the control group (Fig. 3). From Fig. 4 we can see that the size of L2 nuclei tended to decrease in D2 group, while the D1 group showed an

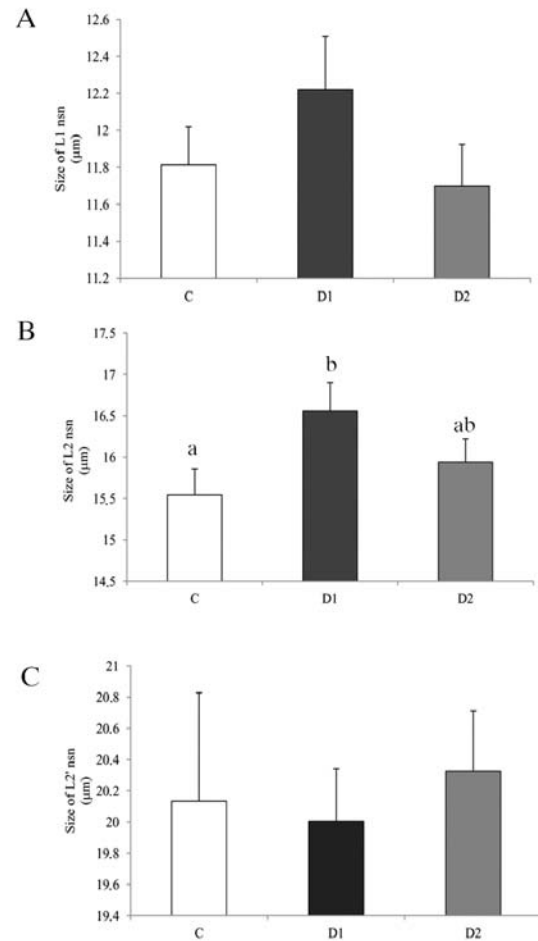
opposite trend. Increased rearing density (D1 group) intensified the activity of L2 nsn, which is indicated by the accumulation of neurosecretory material in neurons and the presence of large nuclei (Fig. 1). Significantly increased number of L2'nsn was detected in group D2 (Fig. 2), while their size was decreased in D1 group and increased in D2 group (Fig. 3) in comparison to control group (average size was 20.13



**Fig. 2.** - The number of dorsolateral L1 nsn (A) L2 nsn (B), L2'nsn (C) in 4th instar *Lymantria dispar* caterpillars rearing in different rearing densities. All abbreviations are same as in Fig. 1. Error bars indicate the standard error of the mean (SEM) Different letters (a,b) indicate significant differences between groups (LSD test,  $P < 0.01$ ).

$\mu\text{m}$ ). The size of their nuclei from both treatments was similar to the control group (Fig. 4). From Fig. 1 and morphometric changes (Figs. 2, 3 and 4), we can see that neither of the rearing densities provoked significant changes in L2'nsn activity.

Differences in the electrophoretic patterns of the 4<sup>th</sup> instar *L. dispar* brain homogenates, after three day exposure to different rearing densities in the region of molecular masses 11-15 kD, known as the Mr range of prothoracicotrophic hormones in *L. dispar*,

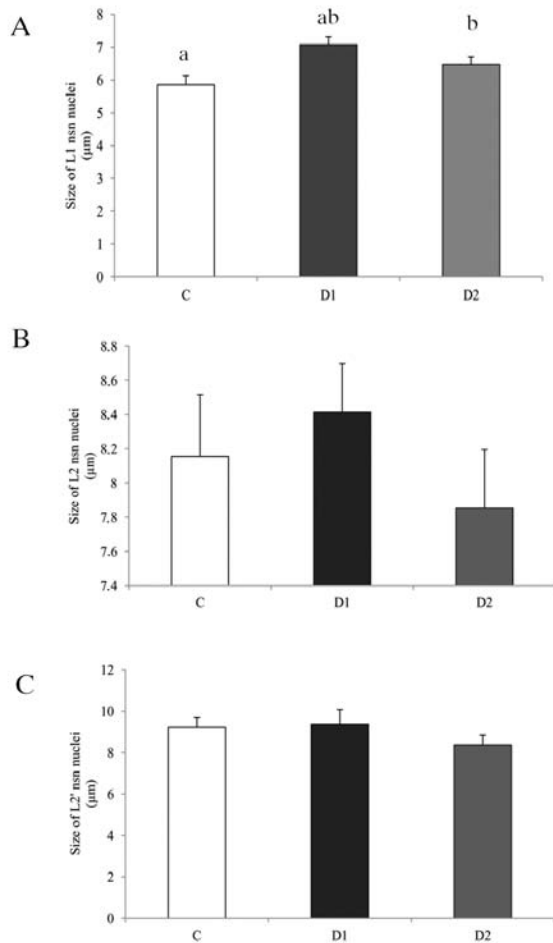


**Fig. 3.** - The size of dorsolateral L1 nsn (A) L2 nsn (B), L2'nsn (C) in 4th instar *Lymantria dispar* caterpillars rearing in different rearing densities. All abbreviations are as in Fig. 1. Error bars indicate the standard error of the mean (SEM) Different letters (a,b) indicate significant differences between groups (LSD test,  $P < 0.01$ ).

are shown in Figure 5. A decrease in relative band density was present in both treatments in comparison to the control group. When the caterpillars were reared in D2 density, the relative band density was increased in comparison to the D1 density.

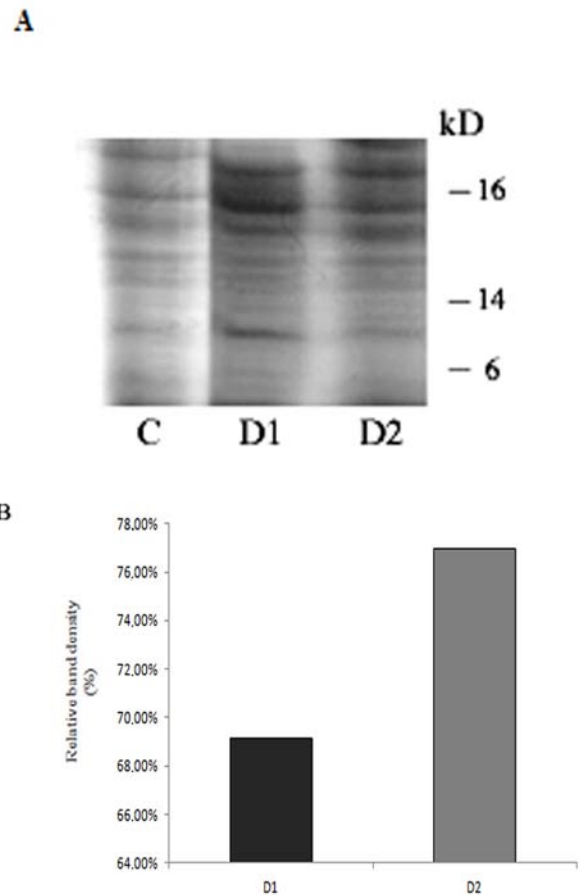
## DISCUSSION

Insects are often exposed to unpredictable and irregular fluctuations in population density (Begon et al., 1990). These fluctuations can induce the appearance



**Fig. 4.** - The size of dorsolateral L1 nsn (A) L2 nsn (B), L2'nsn (C) in the 4<sup>th</sup> instar *Lymantria dispar* caterpillars rearing on different rearing densities. All abbreviations are as in Fig. 1. 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 different phenotypes characterized by significant changes in physiological, morphological and behavioral traits (Iba et al., 1995). Intraspecific competition, resulting from increased population density, influence insect development (Applebaum and Heifetz, 1999), mostly due to the changes in population density related to food availability (Putman, 1977). During *L. dispar* population outbreaks compensatory reactions are present. At increased rearing density of gypsy moth caterpillars, survival was reduced and pupal mass decreased (Lazarević et al., 2000).



**Fig. 5.** - Electrophoretic patterns of brain homogenates of the 4<sup>th</sup> instar *Lymantria dispar* caterpillars obtained by 12% SDS PAGE in the region of 11-15kD (Mr of the big form of PTTH). Using Image J program differences in band density (11-15 kD) after exposure to different rearing densities were estimated and presented as the % of the analyzed bands in comparison to control group (100%).

Neurohormones synthesized in the *pars intercerebralis* of nsn are the major regulatory proteins of all biochemical, physiological and behavioral processes in stress-protective mechanisms. Different types of environmental stressors change the activity of dorsolateral nsn in gypsy moth caterpillars (Ilijin et al., 2011a, 2011b). Changes in neuropeptides as a response to elevated population density are poorly investigated. The size and cytological characteristics of protocerebral dorsomedial nsn were changed under elevated rearing density (Ilijin et al., 2010). Neuro-



hormones from the lateral part of the protocerebrum include prothoracicotropic hormones (PTTH), a tropic factor regulating the synthesis of ecdysteroids (Dai et al., 1994), and allatostatins (Stay and Tobe, 2007). Allatostatins, synthesized in small dorsolateral nsns of the protocerebrum (L1 type), are neuropeptides capable of fast and reversible inhibition of juvenile hormone synthesis in *corpora allata* (Stay and Tobe, 2007). From the results obtained in this experiment it can be assumed that crowding conditions increase the synthetic activity, and decrease the rate of secretion in L1 nsns, and probably small amounts of allatostatins are present in the hemolymph. This was expected, because in gypsy moth caterpillars exposed to crowded conditions, an increased level of release of allatotropins, an antagonist of allatostatins, from A1 nsns, was already detected (Ilijin et al., 2010).

The results obtained here indicate that L2 nsns have increased in size, as well as in the size of their nuclei, i.e. their synthetic activity was high. On the other hand, retention of neurosecretory material in their cytoplasm was obvious and a low level of secretion was detected.

Prothoracicotropic neurohormones (PTTH) stimulate the synthesis and release of ecdysone which regulates molting and metamorphosis (Rybczynski, 2005). In *Lymantria dispar* caterpillars, this neurohormone was identified by Kelly et al. (1991) and its molecular mass was determined as 11-15 kD. L2 nsns were found to be PTTH producing in *L. dispar* caterpillars (Ilijin, 2009). We found that neither of the rearing densities led to significant changes in the activity of L2 nsns. The main mechanism responsible for the delay of larval molting is an increased titer of JH in the hemolymph and retention of PTTH in the brain depots (Ivanović and Janković Hladni, 1991). Our results indicate an elevated activity of L1 nsns, i.e. increased titer of allatostatins that reduced the level of JH in the hemolymph. However, we did not find an increased level of PTTH synthesis. The concentration of the large form of PTTH in the hemolymph peaks before molting to the next stage, which was not the case in this experiment; the increased synthetic activity of L2 nsns was expected later in the 4<sup>th</sup> larval

instar. Therefore, we could presume that the rearing densities we used in our experiment did not disturb the normal larval development of gypsy moth caterpillars.

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## REFERENCES

- Applebaum, S.W. and Heifetz, Y. (1999). Density-dependent physiological phase in insects. *Annual Review of Entomology* **44**, 317-341.
- Begon, M., Harper, J.L., and C.R. Townsend (1990). *Ecology: individuals, populations and communities*, 2<sup>nd</sup> edn. Blackwell Science, Boston.
- Bogus, M.I., and Scheller, K. (1994). Identification of allatotropin secreting cells in the brain of insect larva. *Naturwissenschaften* **8**, 87-89.
- Dai, J.D., Costello, M.J., and Gilbert, L.I. (1994) The prothoracic glands of *Manduca sexta*: a microscopic analysis of gap junctions and intercellular bridges. *Invertebrate Reproduction and Development* **25**, 93-110.
- Diss, A.L., Kunkel, J.G., Montgomery, M.E., and M.E. Leonard (1996). Effects of maternal nutrition and egg provisioning on parameters of larval hatch, survival and dispersal in the gypsy moth, *Lymantria dispar* L. *Oecologia* **106**, 470-477.
- Hirschberger, P. (1999). Larval density affects female weight and fecundity in the dung beetle *Aphodius ater*. *Ecological Entomology* **24**, 316-322.
- Hooper, H.L., Sibly, R.M., Hutchinson, T.H., and S.J. Maud (2003). The influence of larval density, food availability and habitat longevity on the life history and population growth rate of the midge *Chironomus riparius*. *Oikos* **102**, 515-524.
- Iba, M., Nagao, T., and A. Urano (1995). Effects of population density on growth, behavior and levels of biogenic amines in the cricket *Gryllus bimaculatus*. *Zoological Science* **12**, 695-702.
- Ilijin, L. (2009) Protocerebral neurosecretory neurons in acute stress in gypsy moth caterpillars (*Lymantria dispar* L.), PhD Thesis. Biology Faculty, University of Belgrade.
- Ilijin, L., Perić-Mataruga, V., Radojčić, R., Vlahović, M., Mrdaković, M., Mirčić, D., and J. Lazarević (2010). The influence of increased rearing density on medial protocerebral neurosecretory neurons of *Lymantria dispar* L. caterpillars. *Archives of Biological Sciences* **62** (1), 27-37.
- Ilijin, L., Vlahović M., Mrdaković M., Mirčić D., Todorović D., Lazarević J. and V. Perić- Mataruga (2011a). The response

- of dorsomedial A1' and dorsolateral L2' neurosecretory neurons of *Lymantria dispar* L. caterpillars to the acute effects of magnetic fields. *Archives of Biological Sciences* **63** (1), 167-176.
- Ilijin, L., Vlahović, M., Mrdaković, M., Matić, D., Lazarević, J., Todorović, D., and V. Perić-Mataruga (2011b). Changes in *Lymantria dispar* protocerebral neurosecretory neurons after exposure to cadmium. *Archives of Biological Sciences* **63** (4), 1287-1292.
- Ivanović, J., and M. Janković-Hladni (1991). Hormones and Metabolism in Insect Stress. Florida, Boca Raton: CRC Press
- Kelly, J.T., Masler, E.P., Bell, R.A., Thyagaraje, B.S., Davis, R.E., Fescemyer, H.W., and A.B. Borkovec (1991). Gypsy moth prothoracicotropic hormone. In: *Insect neuropeptides: chemistry, biology and action*, (Eds. Menn J.J., Kelly T.J., Masler P.E.), 27-37, USA: American Chemical Society.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Lazarević, J. (2000). Physiological and genetical mechanisms of adaptation to unsuitable nutrition in *Lymantria dispar* L. PhD thesis, Fac. Nat. Math. Sci, University of Belgrade, Belgrade.
- Lazarević, J., Perić-Mataruga, V., Vlahović, M., Mrdaković, M., and Cvetanović, D. (2004). Effects of rearing density on larval growth and activity of digestive enzymes in *Lymantria dispar* L. (Lepidoptera: Lymantriidae). *Folia Biologica* (Kraków) **52**, 105-112.
- O'Dell, T.M., Butt, C.A., and Bridgeforth, A. W. (1984). *Lymantria dispar*, In: *Handbook of Insect Rearing, vol.2*, (Eds. P. Singh and R. Moore), 355-367. Elsevier, New York.
- 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.
- Perić-Mataruga, V., Nenadović, V., and Ivanović, J. (2006). Neurohormones in insect stress: A review, *Archives of Biological Sciences* **58**(1), 1-12.
- Putman, R.J. (1977). The dynamics of the blowfly *Calliphora erythrocephala* within carrion. *J. Animal Ecology* **46**, 853-866.
- Roberts, D. (1998). Overcrowding of *Culex sitiens* (Diptera: Culicidae) larvae: population regulation by chemical factors or mechanical interference. *Journal of Medical Entomology* **35**, 665-669.
- Rybczynski, R. (2005). Prothoracicotropic hormone. In (Eds L.I. Gilbert, K. Latrou, S.Gill), *Comprehensive Molecular Insect Science*, Volume 3, 61-123, Oxford: Elsevier.
- Stay, B. and S.S. Tobe (2007). The role of allatostatins in juvenile hormone synthesis in insects and crustaceans. *Annual Review of Entomology* **52**, 277-99.
- Tammaru, T., Ruohomäki K., and Montola M (2000). Crowding-induced plasticity in *Epirrita autumnata* (Lepidoptera: Geometridae): weak evidence of specific modifications in reaction norms. *Oikos* **90**, 7-8.