Arch. Biol. Sci., Belgrade, 62 (1), 27-37, 2010 DOI:10.2298/ABS1001027I

THE INFLUENCE OF INCREASED REARING DENSITY ON MEDIAL PROTOCEREBRAL NEUROSECRETORY NEURONS OF *LYMANTRIA DISPAR* L. CATERPILLARS

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Abstract – Morphometric changes of A1, A1' and A2 protocerebral dorsomedial neurosecretory neurons, total brain protein content and brain protein profiles were analyzed in 4th instar *Lymantria dispar* larvae under elevated rearing density, i.e. under intense stress when 5 larvae were kept in a petri dish (V = 80 ml), less intense stress when 5 larvae were kept in a plastic cup (V = 300 ml). In the control samples the larvae were reared in isolated conditions. Protein pattern changes in the brain were observed. Proteins with the following molecular masses: 30, 14, 10 and 3.4-2.5 kD were detected in the experimental groups. The size and cytological characteristics of protocerebral dorsomedial neurosecretory neurons were changed under elevated rearing density.

Key words: Rearing density, brain protein content, brain protein pattern, medial neurosecretory neurons

UDC 595.78:591

INTRODUCTION

Many organisms, including insects, experience irregular and unpredictable fluctuations in population density (Begon et al., 1990). The gypsy moth (*Lymantria dispar* L.) has periodic fluctuations in its population density with several phases: latency (low density), progradation (increase in density), culmination (high density) and retrogradation (decrease in density) (Elkinton and Liebhold, 1990). In Europe (as well as in Serbia) culmination lasts for 3 years and appears every 8-11 years. During outbreaks the gypsy moth caterpillars defoliate forests and cause great damage.

Several environmental factors have influence on herbivorous populations, such as food quality, humidity, temperature, competitors, predators and parasites. Food quality depends on the season, weather and density of the population, as well as on other herbivorous populations due to competition for food resources. All organisms from host-plants and insects to hyperparasites are affected by the weather conditions, storms, floods, etc. The influence of each of these environmental factors affects the population physiological status and the physiological and behavioural states of the organism (Elkinton and Liebhold, 1990). The neuroendocrine system of insects quickly reacts to environmental changes (Ivanović and Janković–Hladni, 1991). Neuromodulation of the activity of neurosecretory neurons and the amount of neurohormones effect these changes. They are accompanied by the onset of sexual maturity or reproductive status (Fabre–Nys et al., 1997; Broad et al., 2002), the development and metamorphosis (Lehman et al., 2000a; Mercer and Hildebrand, 2002). Neurohormones synthesized in *pars intercerebralis* neurosecretory neurons represent the major regulatory proteins of all biochemical, physiological and behavioral processes in stress-protective mechanisms.

Increased respiration, mobilization of carbohydrates and lipids, different expression of glycolytic and other enzymes (Applebaum and Heifetz, 1999), increased lipid content (Ferguson et al., 1997) and induction of Hsp70 expression (Sorensen and

Loeschcke, 2001) represent density-dependent responses. Literature data indicate that elevated population density induces a decrease in juvenile hormone (JH) level (Injeyan and Tobe, 1981) and 20-hydroxyecdysone (Tawflik et al., 1996), an increased level of adipokinetic hormone precursors (Ayali et al., 1996) and biogenic amines (Iba et al., 1995).

High population density changes the physiology and behavior of *L. dispar* so that development is 2-3 weeks shorter, larval and pupal masses are reduced and fecundity decreases (Leonard, 1981). Elevated density leads to decreased egg size (Diss et al., 1996), changes in oviposition preference, a broadening of the host-plant range (Barbosa, 1978), a higher proportion of males (Barbosa et al.,1981), disturbed circadian feeding rhythm (Lance et al., 1986), and an increase in dark coloration (Ponomarev,1994). Overcrowding reduces the resistance to parasites and viruses (Martemyanov and Bakhalov, 2007). Overpopulation depends on *L. dispar* quality, i.e. on biochemical, physiological, developmental and behavioral traits. They further influence fitness components, stress resistance and tolerance, dispersal ability etc. (Rossiter, 1994).

Changes in neuropeptides in response to elevated population density in gypsy moth are poorly investigated. In the present study, we examined the effects of population density in *L. dispar L.* larvae by maintaining them in isolated and crowded conditions. The aim was to analyze the changes in the morphometric characteristics of medial neurosecretory neurons (A1, A2 and A1') in 4th instar caterpillars of gypsy moth under elevated rearing density. We also examined the total brain protein content and changes in brain protein profiles. From obtained results, we discussed underlying neurosecretory mechanisms in response to elevated population density.

MATERIALS AND METHODS

Insect rearing

Gypsy moth egg masses were collected in a poplar forest (locality Opovo). The egg masses were kept in a refrigerator at 4°C from October to March when they were set for hatching. After hatching, larvae were reared on a synthetic HWG (high wheat germ) diet (O´Dell et al., 1984) at 23°C with a 16 h light : 8 h dark photoperiod. Larvae were randomly assigned to 3 experimental groups for histochemistry and 3 groups for brain protein electrophoretic analysis. Larvae were fed *ad libitum* with the basic diet until hatching in the 4th instar so they were not starving. The following groups were examined: ***C*** – control group in which single larvae were reared in a petri dish (isolated conditions), ***D1*** – 5 larvae were kept in a petri dish (V = 80 ml), for the first 3 days of 4th larval instar, and ***D2*** – 5 larvae were kept in a plastic cup (V = 300 ml) for the first 3 days of 4th larval instar.

SDS PAGE Electrophoresis

After decapitation, the caterpillar brains were dissected on ice and weighed. The brains were homogenized in cold distilled water (200 mg brain/ml distilled water) and centrifuged at 10 000 rpm for 10 min at 4˚C. The supernatant was collected and SDS-PAGE performed according to Laemmli (1970) on 12% and 16% gels. The gels were then stained for proteins with Coomassie Brilliant Blue R 250 solution overnight at 4˚C, followed by destaining in a 50% methanol, 10% acetic acid solution. The molecular weight of the proteins after SDS-PAGE was estimated using commercial standards with Mr ranging from 4-250 kD (Invitrogen) and 2.5-17 kD (Sigma).

Histological techniques

Based on their size and morphological characteristics, we divided medial protocerebral neurosecretory neurons of *L. dispar* L. (for easy monitoring of the results) into 3 groups (A1, A2 and A1'). Brain complexes were dissected in insect Ringer solution and immersed in Bouin's fixative (picric acid-saturated solution 75%; formaldehyde 20%; acetic acid 5%). The brain complexes were rinsed in 70% ethanol and fully dehydrated in a graded series of ethanol solutions (from 80% to 100%) before embedding in paraffin wax. Serial

sections of the brain complexes were cut at 3 μm for histohemistry (using a "820" Spencer microtome) and collected on 0.2% gelatin/0.05% chrome alum (Sigma, France) 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 the Ewens paraldehyde fuchsine technique and modified by Panov (1980).

The size of the protocerebral dorsomedial neurosecretory neurons was expressed as the mean value of the smallest and largest diameter (in μm^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, Cambridge, UK).

Statistical methods

The results were analyzed statistically using the program STATISTICA, ver. 6.0. Statistical significance of differences was estimated using the LSD test.

RESULTS

In the D1 group (intense stress) where 5 larvae were kept in a petri dish for 3 days the total brain protein content was significantly decreased in comparison to the D2 group (less intense stress) or a group where 5 larvae were kept in a plastic cup for 3 days (Fig. 1). No significant changes in the protein content were detected between the control group and the D1 or D2 groups.

The differences in electrophoretic protein patterns of crude *L. dispar* larvae brain homogenates obtained by 12% SDS-PAGE are presented in photo 1. The protein profiles from all 3 experimental groups are very similar, with the most intense band at 43 kD. In the D2 group, i.e. the group where 5 larvae were kept in a plastic cup and in the control group, 4 protein bands were detected in the Mr region from 30-16 kD. When 5 larvae were kept in a petri dish only one band with the smallest Mr was present. One band of lower

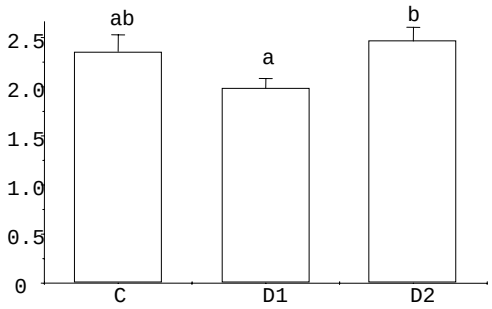

Figure 1. Total protein content in 4th instar Lymantria dispar caterpillars brain homogenates after exposure to different rearing densities. C – control group in which single larvae were reared in a petri dish, D1 –5 larvae were kept in a petri dish (V=80ml) for 3 days and D2 –5 larvae were kept in a plastic cup (V=300ml) for 3 days. Values indicated by different letters (a,b) are significantly different (LSD test, P<0.01).

intensity with a Mr of about 10 kD was observed in the control group. In the D1 group where caterpillars were induced to intense stress, two protein bands were observed: the one with a lower Mr was less intense. Under more intense stress (D2 group) the opposite situation was observed: the band of higher mass was not so intense while and the one with the smaller Mr was identical to the band from the control group. The protein patterns obtained from the control group and the D2 group are very similar.

The differences in protein profiles from the crude brain homogentes of the 4th instar *L. dispar* larvae exposed to high density contitions and obtained by 16% SDS-PAGE are presented in photo 2. A great similarity between all 3 experimental groups was observed in the region of small molecular masses. Only one protein band was detected in the region of about 14 kD in the control group, while in the D1 and D2 groups 2 bands with close Mr were present. Two protein bands of Mr 6-3.4 kD were detected in all experimental groups, but in the brain protein profiles of the caterpillars exposed to these acute stressors bands were more intense. The region of molecular masses from 3.4-2.5 kD was characterised by 2 bands in the control group, while after exposure to both crowding

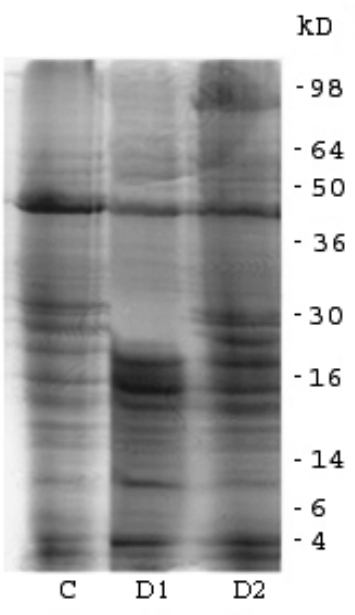

Photo 1. Electrophoretic patterns (12% SDS-PAGE) of 4th instar Lymantria dispar caterpillars brain homogenates after exposure to different rearing densities. C – control group in which single larvae were reared in a petri dish, D1 –5 larvae were kept in a petri dish (V=80ml) for 3 days and D2 –5 larvae were kept in a plastic cup (V=300ml) for 3 days.

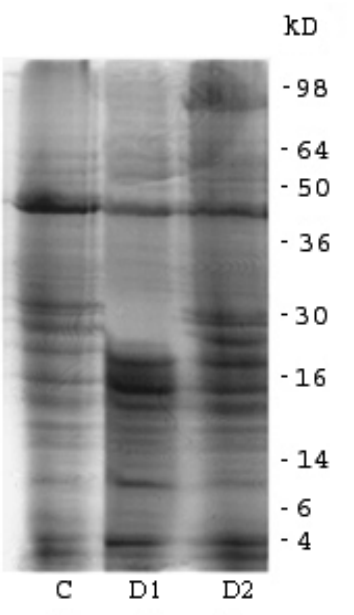

Photo 2. Electrophoretic patterns (16% SDS-PAGE) of 4th instar Lymantria dispar caterpillars brain homogenates after exposure to different rearing densities. C – control group in which single larvae were reared in a petri dish, D1 –5 larvae were kept in a petri dish (V=80ml) for 3 days and D2 –5 larvae were kept in a plastic cup (V=300ml) for 3 days.

conditions one new protein band with a smaller mass appeared.

The A1 type of *L. dispar* larvae medial neurosecretory neurons are localized in the dorsomedial part of protocerebrum. The average size is 15.25 μm. The size of these neurons decreased after exposure of the caterpillars to more intense stress (D1 group) in comparison to the control group of larvae (photo 3, Fig. 2). A1 neurosecretory neurons in caterpillars from the D1 group contained fine-grained neurosecretory granules, large nuclei and centrally positioned large nucleoli, which indicate the induction of neurosecretory neurons activity (Hiruma and Agui, 1977; Raab, 1982).

In the same region of protocerebrum in gypsy moth caterpillars A1' neurosecretory neurons are also present. They are large neurons, round or elliptical in shape. The average size is 25.96 μm. A1' neurons and their sizes are presented in **photo 3** and **Fig. 3**. It can be observed that the A1' neurons from larvae reared

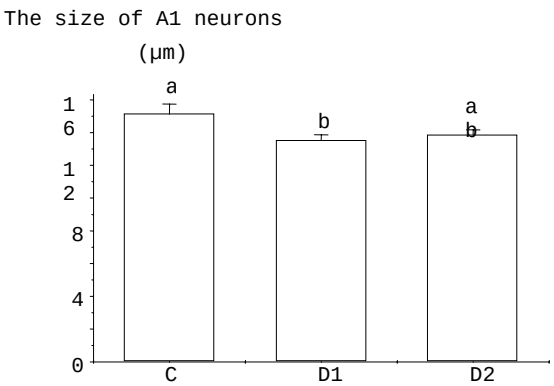

Figure 2. The size of protocerebral dorsomedial A1 neurosecretory neurons in 4th instar Lymantria dispar caterpillars after exposure to different rearing densities. C – control group in which single larvae were reared in a petri dish, D1 –5 larvae were kept in a petri dish (V=80ml) for 3 days and D2 –5 larvae were kept in a plastic cup (V=300ml) for 3 days. Values indicated by different letters (a,b) are significantly different (LSD test, P<0.01).

for 3 days in a plastic cup (less intense stress) were significantly smaller than the A1' neurons from the other experimental groups, but large nucleoli were clearly visible in the nuclei of the neurosecretory neurons of these group which indicate initiation of synthesis of neurosecretory material.

The third type of gypsy moth dorsomedial neurosecretory neurons are of the A2 type. These cells have an average size of 19.58 μm. Obtained changes in A2 neurosecretory size and neurosecretory content are presented in **Fig. 4** and **photo 3**. A significant decrease in the size of these neurosecretory neurons and the presence of agglomerated neurosecretory products was oberved in caterpillars exposed to less intense stress, i.e. the D2 group, in comparison to the control group. The cytoplasm of A2 neurosecretory neurons in caterpillars under intense stress have large nuclei with clearly visible centrally positioned nucleoli (**photo 3**).

DISCUSSION

In nature, *Lymantria dispar* L. populations show periodic fluctuations in population density. At first food quality was considered to be a key factor regulating the gypsy moth population density (Valentin et al., 1983), then it was a maternal effect (Rossiter, 1992; 1994), and recently that role was attributed to pathogens (Berryman, 1996). Crowding triggers behavioral, morphological and physiological responses in the gypsy moth.

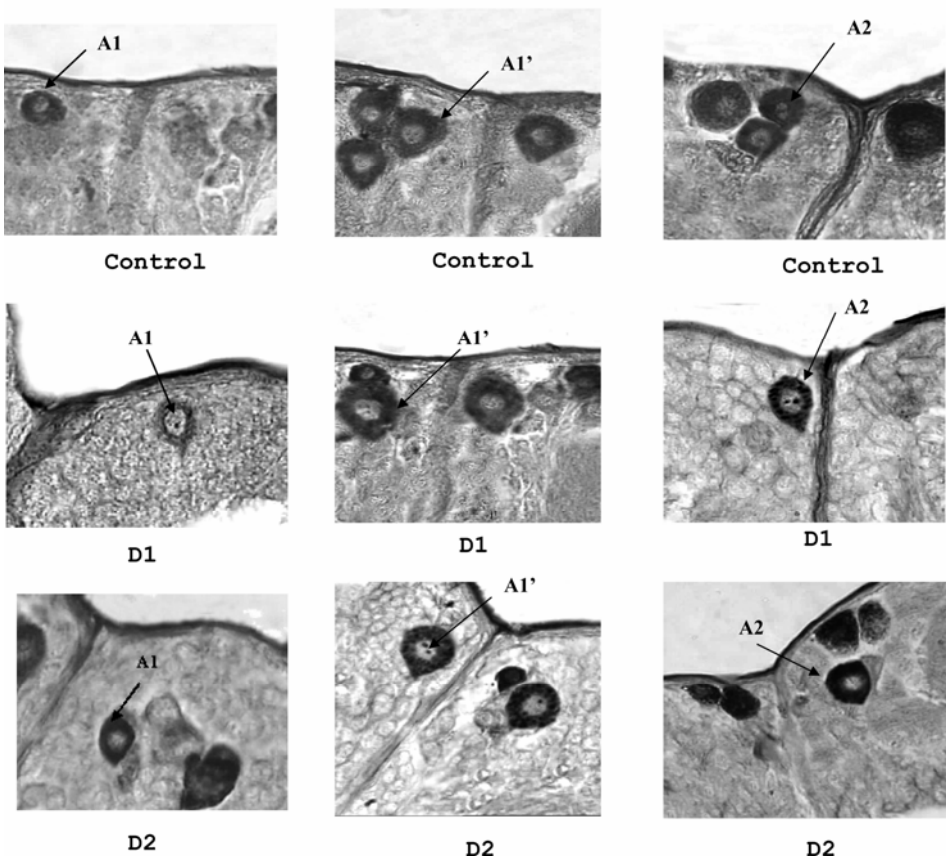

Photo 3. Brain transverse cross-section of *Lymantria dispar* 4th instar caterpillars after exposure to different rearing densities. ***C*** – control group in which single larvae were reared in a petri dish, ***D1*** –5 larvae were kept in a petri dish (V=80ml) for 3 days and ***D2*** –5 larvae were kept in a plastic cup (V=300ml) for 3 days. Protocerebral dorsomedial A1, A1' and A2 neurosecretory neurons are marked.

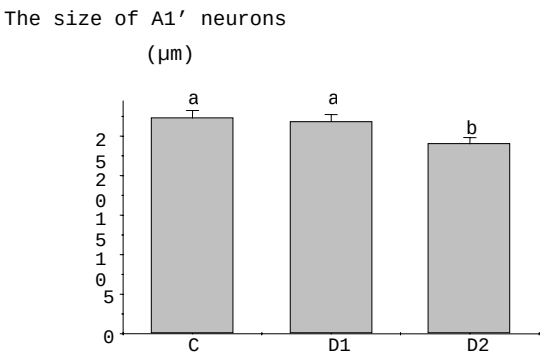

Figure 3. The size of protocerebral dorsomedial A1' neurosecretory neurons in 4th instar Lymantria dispar caterpillars after exposure to different rearing densities. C – control group in which single larvae were reared in a petri dish, D1 –5 larvae were kept in a petri dish (V=80ml) for 3 days and D2 –5 larvae were kept in a plastic cup (V=300ml) for 3 days. Values indicated by different letters (a,b) are significantly different (LSD test, P<0.01).

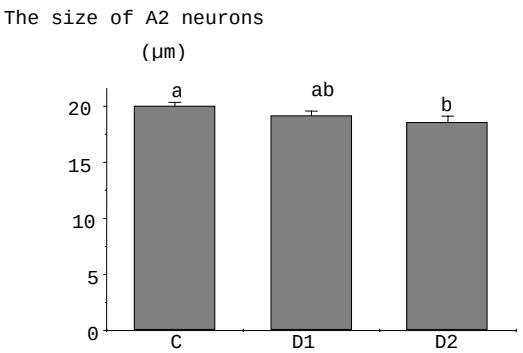

Figure 4. The size of protocerebral dorsomedial A2 neurosecretory neurons in 4th instar Lymantria dispar caterpillars after exposure to different rearing densities. C – control group in which single larvae were reared in a petri dish, D1 –5 larvae were kept in a petri dish (V=80ml) for 3 days, D2 –5 larvae were kept in a plastic cup (V=300ml) for 3 days. Values indicated by different letters (a,b) are significantly different (LSD test, P<0.01).

In gypsy moth populations a compensatory reaction to crowding was detected. In offspring reared in laboratory conditions female pupal mass increased if defoliation in the parental generation was high (Rossiter, 1991a). On the contrary, Lazarević et al. (2004) found a decrease in *L. dispar* pupal mass at high population density. The authors assumed that this could be due to differences in egg quality, changes in factors related to population life history and/or host-plant quality. At high population density survival is reduced, development prolonged, pupal mass reduced and female and male life longevity is shortened (Lazarević, 2000).

Numerous literature data indicate a dominant role of the neuroendocrine system in insect stress response (Cymborowski et al., 1982; Chernysh, 1991; Ivanović and Janković- Hladni, 1991; Harris and Woodring, 1992).

Neurohormones from the medial part of the protocerebrum include prothoracicotropic hormones (PTTH), a trophic factor regulating the synthesis of ecdysteroids (Kawakami et al., 1990; Iwami et al., 1990; Dai et al.,1994), and allatotropic factors which regulate the synthesis of juvenile hormones (Bogus and Scheller, 1994).

Various stressors modulate the activity of neurosecretory neurons, their synthetic activity and release of neurohormones. The response of these neurons is selective and depends on the type of stressor and its duration.

Neurohormones influence processes of lipid (adipokinetic hormone) and carbohydrate (hypertrehalosemic hormone, small form of PTTH) metabolism, stimulate the adoption of carbohydrates (hypoglycemic hormone), water secretion (diuretic hormone) or inhibition of this process (antidiuretic hormone) (Gäde, 1990b; Gäde and Goldsworthy, 2003; Perić- Mataruga et al., 2006; Keeley, 1978). These neurohormones take part in the first phase of stress response, when the insect provides the energy necessary for the physiological processes included in stress response. During the second, later level of stress response, ecdysteroides accompanied these neurohormones. They are responsible for stimulations of macromolecule synthesis and reparative processes. It is well known that ecdysteroides and

juvenile hormones represent growth and development hormones, whose activity is regulated by PTTH and allatoregulatory hormones. Normal insect development depends on the concentration of these neurohormones in the hemolymph.

Juvenile hormones, synthesized in *corpora allata,* regulate the growth, development and reproduction in insects. Allatoregulatory neurohormones (allatotropins and allatoinhibins) are regulators of JH synthesis and release (Bogus and Scheller, 1994; Stay and Tobe, 2007). Allatotropins are neurohormones synthesized in the medial neurosecretory neurons (A1 type) of insect protocerebrum (Zitnan et al., 1995; Bogus and Scheller, 1994; Veelaert et al., 1995; Jeon and Lee, 1999). They have a molecular mass of 20 kD (Bogus and Scheller, 1994) and their role it is to keep the JH titer in the hemolymph high (Bogus and Schaeller, 1994).

In this paper we have analyzed also gypsy moths' A1 medial neurosecretory neurons (synthesize allatotropins, Bogus and Scheller, 1996). These neurons from both crowding conditions showed cytological changes which could indicate increased synthesis of the allatotropic neurohormone, especially under more intense stress where 5 larvae were kept for 3 days in a petri dish. The elevated level of this neurohormone could stimulate the synthesis and release of juvenile hormones. Juvenile hormones are responsible for the insect body tanning. It was determined that implantation of *corpora allata* in young *Schistocerca gregaria* adults reared in crowded conditions induced a light yellow color of insect body, while the allactomized were dark colored (Pener, 1967). Elevated population density induces a high titer of JH and prolonged larval instar and reduces dark body coloration, due to the influence of JH, so it could be concluded that synthesis of these hormones is elevated by crowding conditions (Iba et al., 1995). In crowding conditions *Tribolium freemani* larvae delay JH esterase production (regulators of JH level), and ecdysteroides in comparison to the isolated larvae (Hirashima et al., 1995), so the level of JH is elevated.

In this paper A1' medial neurosecretory neurons from 4th instar *L. dispar* larvae reared for 3 days under slightly elevated density (less intense stress), showed a decrease in size, while these neurons are unchanged in the group of caterpillars under intense stress (Fig. 2, photo 3). Crowding induces behavioral physiological responses in many insects. Adaptive responses to crowding are increased food consumption which increases the activity of the digestive enzymes involved in the adaptive response to stress (Stockhoff, 1991; Lazarević, 2004). Neurohormones from *pars intercerebralis* (from the A1 group) affect the activity of insect digestive enzymes as a consequence of changing food consumption (Muraleedharen and Prabhy, 1981; Leković et al., 2001). It is possible that the caterpillars of gypsy moth in the intense density stress conditions need neurosecretory reorganization as a factor of regulating digestive activity.

PTTH influences adaptation to these conditions by regulating the development and morphological changes. The large form of this hormone stimulates the secretion and release of ecdysteroids from the prothoracic gland (Bollenbacher et al., 1979; Agui et al., 1980), and also has a stress protective role (Gilbert et al., 2000). Changes in the ecdysteroid level lead to ecdysis. The other, small form of PTTH, also known as bombyxin, is a neurohormone with a molecular mass of 4-6 kD in gyspy moth larvae (Kelly et al., 1995). Medial neurosecretory neurons (A2 type) are known to be a place where bombyxin is synthesized (Mizoguchi and Gilbert, 1994). Bombyxin has a large degree of homology with insulin (Nagasawa et al., 1984), and shows hypertrehalosemic activity (Satake et al., 1997). It is also known that this small PTTH form has an important role in metabolic stress responses (Perić-Mataruga et al., 2006).

In our experiment both elevated rearing densities induced more intense protein bands Mr 4 kD. Also, A2 medial neurosecretory neurons showed an increased activity on stress population densities. This group was also characterized by elevated 5th instar larval mass and relative growth rate (unpublished results). All obtained results

indicate intensive carbohydrate metabolism as one of the possible stress response mechanisms. We could presume that the release of allatotropins, synthesis of PTTH in neurosecretory neurons and an increase in juvenile hormone titer could be induced by this acute stressor in gypsy moth caterpillars.

Acknowledgment – This study was supported by the Serbian Ministry of Science and Technological Development (grant No. 143033).

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УТИЦАЈ ПОВЕЋАНЕ ГУСТИНЕ ГАЈЕЊА НА МЕДИЈАЛНЕ НЕУРОСЕКРЕТНЕ НЕУРОНЕ ПРОТОЦЕРЕБРУМА ГУСЕНИЦА *LYMANTRIA DISPAR* Л.

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У овом раду је испитиван утицај повећане густине гајења на гусенице губара *Lymantria dispar* L. Морфометријске промене A1, A1' и A2 дорзомедијалних неуросекретних неурона протоцеребрума, укупна количина протеина у хомогенатима мозга и протеински профили мозга гусеница губара 4. ларвеног ступња су испитивани под следећим експерименталним условима: интензиван стрес – 5 ларви је гајено у петри шољи (V = 80 ml), мање интензиван стрес – 5 ларви је гајено у пластичним чашама (V = 300 ml) и контрола – ларве су гајене појединачно, у изолованим условима. У протеинским профилима промене су уочене у следећим регионима молекулских маса: 30, 14, 10, 3.4-2.5 kD. Величина и цитолошке особине протоцеребралних дорзомедијалних неуросекретних неурона се мењају под деловањем повећане густине гајења.