

## Temperature-induced stress response in *Lymantria dispar* neurosecretory neurons

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**Abstract:** The release of neurosecretory material from A2 neurosecretory neurons (NSNs) was stimulated in *Lymantria dispar* fourth instar caterpillars exposed to a temperature of 35 °C for 1, 12, and 24 h, as well as those allowed recover after exposure (12 h at 35 °C, then 12 h at 23 °C). The levels of 2 protein forms with the same molecular mass as bombyxin (3–4 and 4–5 kDa) increased with prolonged exposure to 35 °C. The second band was present only in the groups exposed to this stressor. There was intensified synthetic activity and a low level of secretion in L2' NSNs after exposure to 35 °C. We previously found these NSNs to be immunopositive for prothoracicotropic neurohormone. After this stress, densitometric analysis revealed a decreased amount of the 11–12 kDa isoform (present in the control group). The new isoform (13–15 kDa), expressed after exposure of the insects to a high temperature, increased in amount with prolonged exposure and after recovery at 23 °C. Short-term exposure of caterpillars to high temperatures (35 °C) is a stressor and activates carbohydrate metabolism, while PTTH immunopositive NSNs are secretory-inactive during acute thermal stress regimes.

**Key words:** High temperature, gypsy moth, medial and lateral neurosecretory neurons

### 1. Introduction

Insects are the largest group of invertebrates and very diverse; however, only a few neurohormones regulate all of their living processes. Neurohormones are synthesized mainly in neurosecretory neurons (NSNs) of the insect brain, and several peripheral neurons make up less than 1% of all neurons in the nervous system.

The type of NSNs can be distinguished by greatest diameter, staining affinities, neurosecretory granule size, and protocerebral location (Raab, 1982). In the *Lymantria dispar* brain the majority of NSNs are located in the medial and dorsolateral part of the protocerebrum. Based on morphological characteristics and protocerebral location, we divided the medial group of NSNs into groups A1, A1', and A2 and the dorsolateral group into L1, L2, and L2' (Perić Mataruga et al., 2001; Perić Mataruga and Lazarević, 2003).

Neurohormones play an important role in regulating insect development (McBrayer et al., 2007), physiology (Kim and Rulifson, 2004), and behavior (Renn et al., 1999). The synthesis of ecdysone, a major morphogenetic hormone, is regulated by prothoracicotropic

neurohormones (PTTHs), which play a central role in postembryonic development. The structure of PTTHs is known for several insect species (Kelly et al., 1991; Kim et al., 1997; Ishizaki, 2004). Ishizaki and Suzuki (1988) revealed that PTTHs are peptides present in several isoforms in the silkworm (*Bombyx mori*).

In Lepidoptera, lateral NSNs are known to produce a large form of PTTHs (Mizoguchi and Gilbert, 1994), while a pair of medial NSNs produces a small, insulin-like form (Dai et al., 1994). In *Lymantria dispar* caterpillars we detected PTTH-immunoreactive molecules in L2' type dorsolateral NSNs (Ilijin et al., 2012), while bombyxin-like material was found in the A2 type medial protocerebral NSNs (Ilijin et al., 2011). Axons from both pairs of NSNs terminate in the corpus allatum, a specialized secretory gland of the neuroendocrine system (Mizouchi et al., 1990; Dai et al., 1994).

Once released from the corpora allata into the hemolymph, PTTHs target the prothoracic gland and regulate the production and release of ecdysone (Rybczynski, 2005). The small form of PTTH is released from the corpus allatum into the hemolymph (Ishizaki

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and Suzuki, 1992). This insulin-like protein (bombyxin) is active in carbohydrate mobilization and provides the energy necessary for metamorphosis and other developmental processes by releasing it from glycogen energy depots.

Neurosecretory neurons in insect brains known to synthesize PTTHs (small and large form) receive stressogenic stimuli or environmental stimuli (photoperiod, high temperature, plant allelochemicals, etc.) through receptor systems (Perić Mataruga et al., 1999; Mizoguchi et al., 2001, 2002; Gäde and Goldsworthy, 2003). Changes in the titer of PTTHs correlate with alterations in the ecdysteroid titer in circulation as well as morphological and behavioral changes typical for metamorphosis or the metabolic response to stress (Gu et al., 2000). Under some environmental conditions decreased levels of PTTHs and ecdysone are an adjustment. Environmental signals inform the organism of unfavorable conditions and postpone ecdysis to the next larval stage until favorable conditions return.

Insects are very sensitive to high temperature. Heat from the sun or an artificial source increases the body temperature of small poikilotherms to a lethal level very quickly. In addition, there is the problem of maintaining water balance in the organism (Denlinger et al., 1991). Temperature also affects phytophagous insect species directly or indirectly by influencing host plant metabolism. This can lead to disturbed development and qualitative and quantitative changes in the chemical composition of the host plant. Moreover, elevated temperature as a stressor raised the level of allatotropins and altered the juvenile hormone endocrine system in *Drosophila* (Gruntenko et al., 2000). In some lepidopteran species high temperature increased mortality, shortened development time, and reduced the size of individuals, consequently decreasing the number of eggs laid (Reynolds and Nottingham, 1985; Ochieng-Odero, 1992). Thermal sensitivity of the metabolic rate is a significant characteristic of different insect species (Berrigan, 1997).

Stress activates numerous physiological processes and mechanisms necessary to overcome negative effects. The insect neuroendocrine system is a primary activator of stress response mechanisms in cases of environmental stress. Our goal was to analyze underlying neurosecretory mechanisms that react to short-term exposure to high temperature in order to better understand the mechanisms of neurosecretory stress responses in insects. We examined the morphometric characteristics of NSNs, which synthesize the large and small form of PTTH, and differences in the intensity of brain protein bands in the region of their molecular masses in fourth instar caterpillars of gypsy moths exposed to 35 °C for 1, 12,

or 24 h, as well as in caterpillars allowed to recover after exposure (12 h at 35 °C and then 1 h at 23 °C).

## 2. Materials and methods

### 2.1. Insect rearing

*Lymantria dispar* is a widespread polyphagous herbivorous insect with a host range of more than 500 plant species (Lance, 1983). Optimal habitats are oak forests (Janković, 1958), but larvae were also collected from different hazelnut trees in the Black Sea region of Turkey (Demir et al., 2012). This pest defoliates forest complexes in North Africa, Asia, North Europe, and America during population outbreaks. Scientists are currently trying to supplement hazardous chemical pesticides with biological control agents (Demir et al., 2009).

Gypsy moth egg masses were collected in a poplar forest (locality: Opovo; 20°25'49"E, 45°3'8"N; altitude, 67 m; 30 km from Belgrade) and kept in a refrigerator at 4 °C from October to March, when they were set for hatching at 23 °C with a 16 h light:8 h dark photoperiod. After hatching, *L. dispar* caterpillars were reared on a synthetic, high wheat germ (HWG) diet (O'Dell et al., 1985) in transparent plastic containers (V = 200 mL) at 23 °C and a 16 h light:8 h dark photoperiod. Caterpillars were randomly assigned to 5 experimental groups for histochemistry (n = 15) and 5 groups for brain sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) (n = 15).

### 2.2. Thermal stress

Caterpillars were reared at 23 °C until entry into the fourth instar when they were exposed to high temperature stress in a thermostat with constant humidity. The first group was kept at 35 °C for 1 h (1h), the second for 12 h (12h), the third group for 12 h followed by transfer to 23 °C (12/12h), and the fourth group was exposed to the high temperature for 24 h (24h). The control group was reared at 23 °C (C).

### 2.3 Histological techniques

Caterpillars were sacrificed on the third day of the fourth instar by decapitation, and head capsules were fixed for 24 h in Bouin's solution (Merck, Darmstadt, KgaA, 64271, Germany). Brain complexes were dissected, dehydrated in a graded series of ethanol, impregnated in xylol (Hemos, Belgrade, Serbia), and embedded in paraffin wax (59 °C; Merck, Darmstadt, KgaA, 64271, Germany). For histochemistry 3.5-µm serial sections were cut on an 820 Spencer microtome and collected on 0.2% gelatin/0.05% chrome alum- (Sigma Aldrich, GmbH, Taufkirchen, Germany) coated slides. Sections were stained by a paraldehyde fuchsin technique (Ewen, 1962) modified by Panov (1980). In all NSNs, neurosecretory material was stained different shades of purple in the cytoplasm, while nucleoli were observed as light pink spheres in the nuclei (Panov, 1980). The activity of both types of NSNs was

determined by monitoring the size of NSNs and their nuclei (in micrometers), calculated as means of the shortest and longest diameters of each measured NSN or nucleus. Data were evaluated by one-way analysis of variance (ANOVA) and a post-hoc multiple range test [Fisher's least significant difference (LSD)] using STATISTICA version 6.0. 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, Wetzlar, Germany).

#### 2.4. SDS PAGE electrophoresis

Caterpillar brains were dissected on ice, homogenized in cold distilled water (200 mg brain/mL distilled water, i.e. 3–4 brains/sample), and then centrifuged at  $10,062 \times g$  for 10 min at 4 °C. In each experimental group 15 samples were analyzed. The supernatant was collected, and SDS PAGE electrophoresis was performed on 16.5% gels according to Laemmli (1970) using a MINI PROTEAN II system (Bio-Rad, USA). The gels were then stained with Coomassie Brilliant Blue R 250 (Serva Electrophoresis GmbH, Heidelberg, Germany), followed by destaining in a 50% methanol (Lach-Ner, Neratovice, Czech Republic)/10% acetic acid solution (Merck, Darmstadt, KgaA, 64271, Germany). The molecular weight (Mr) of the proteins was estimated using commercial standards (Mr: 2.51–16.95 kDa) (Sigma Aldrich). Protein band intensities in the molecular mass regions of bombyxin Mr (4–6 kDa) and PTTH (11–15 kDa) were analyzed densitometrically using Photo-Capt software version 12.4 (Vilber Lourmat, France).

### 3. Results

#### 3.1. A2 NSNs after acute thermal stress

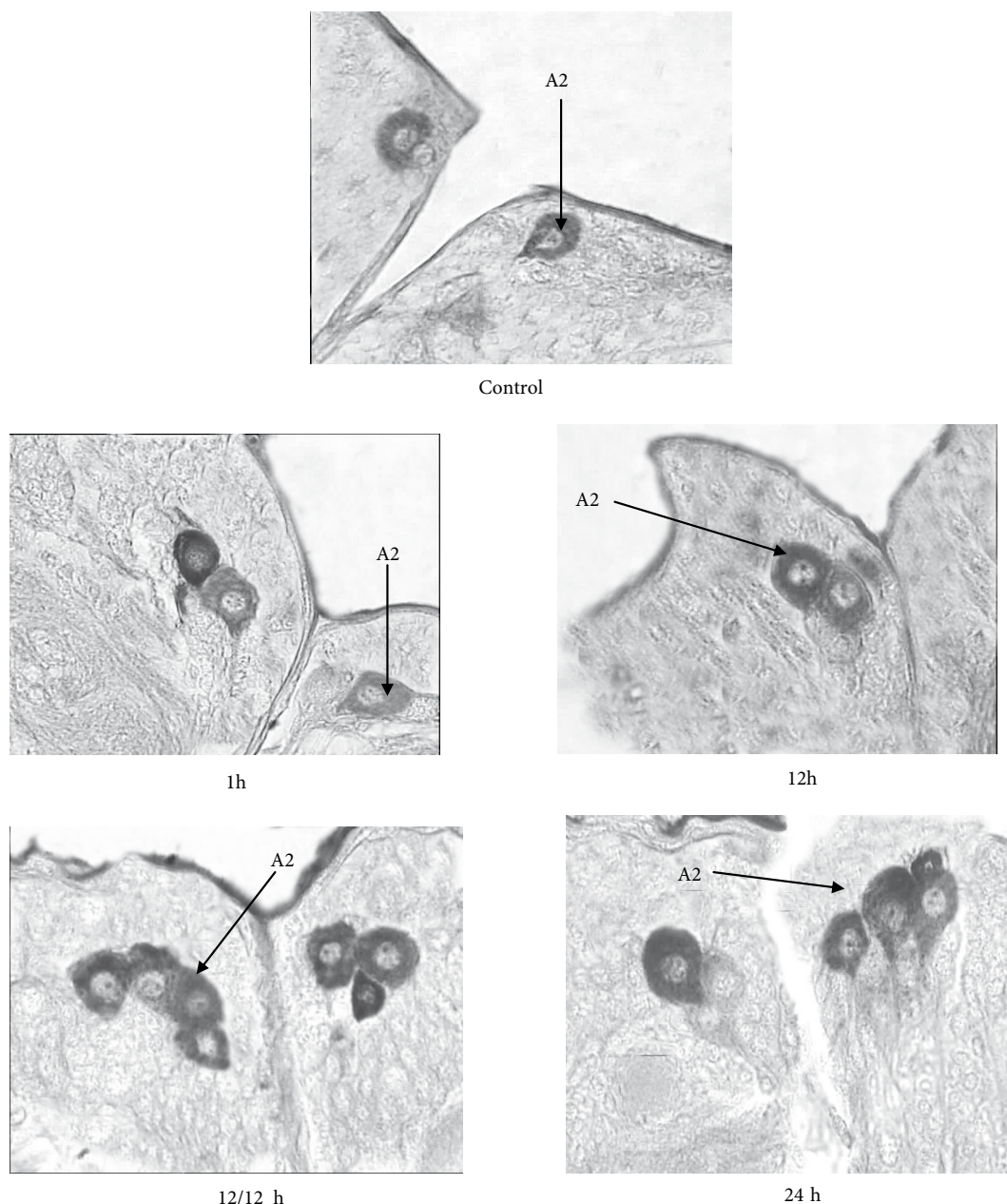
A2 NSNs from all experimental groups are presented in Figure 1. After acute exposure to a temperature of 35 °C for 1, 12, and 24 h, the cytoplasm of A2 NSNs was filled with a considerable amount of large granulated neurosecretory material. In the group allowed to recover at 23 °C after exposure to 35 °C, the same type and amount of neurosecretory material was observed. The number of NSNs increased after exposure to high temperature (Figure 2a), but this was statistically significant only in the group allowed to recover, in comparison with the control group and all other experimental treatments (LSD). The size of A2 NSNs did not change significantly after exposure to acute thermal stress (Figure 2b). However, there was a decrease in the size of A2 nuclei (Figure 2c) in caterpillars returned to 23 °C in comparison to all other experimental treatments except the control group (LSD). Acute exposure to high temperature induced an increase in A2 nuclei size in comparison to the control group (one-way ANOVA  $F_{4,168} = 2.794$ ;  $P < 0.01$ ).

#### 3.2. L2' NSNs after acute thermal stress

After acute exposure to high temperatures, large-grained neurosecretory material was visible in the cytoplasm of L2' NSNs (Figure 3). The number of these neurons and the size of their nuclei were similar in all groups (Figures 4a and c). On the other hand, significant changes in the size of L2' NSNs (Figure 4b) were observed after exposure to high temperature, in comparison with the control group (one-way ANOVA  $F_{4,86} = 4.804$ ;  $P < 0.01$ ). Fisher's LSD test revealed significant increases in the size of these NSNs both after 1 h of exposure to 35 °C and in the group of caterpillars allowed to recover for 12 h after 12 h of stress, in comparison to the other experimental groups.

#### 3.3. Densitometry and quantitative assessment of PAGE profiles

Analysis of SDS PAGE electropherograms (Figure 5a) and densitograms (Figure 5b) showed quantitative and qualitative differences in protein band intensity among the treatments. The number of protein peaks increased after exposure of caterpillars to 35 °C in all cases, compared to the control group. In the densitograms (Figure 5b) we shaded the bands in the regions of PTTH (11–15 kDa) and bombyxin (4–6 kDa) molecular masses (Kelly et al., 1991). Using Photo-Capt software these protein bands were quantified, and the results are presented in Figure 6. A protein band of Mr 11–12 kDa was detected in all experimental groups (Figure 6a), and the volume (the sum of all intensities included in the defined area) of this band decreased in the groups exposed to the high temperature for 12 h and 24 h and the group returned to 23 °C for 12 h, compared to the control group and the group exposed to 35 °C for 1 h. The second band, with Mr around 13–15 kDa (Figure 6a), was detected only in the control group and groups 12h and 12/12h. High environmental temperature and recovery from this acute stressor increased the size of this protein band in comparison to the control group. The observed changes suggest that prolonged exposure to this temperature may have induced a decrease in the amount of PTTH together with the appearance of increased amounts of the second PTTH isoform. Quantification of protein bands from the Mr region of bombyxin are presented in Figure 6b. In the control group and the groups of caterpillars exposed to a high temperature for 1 h and 12 h, a band was detected at Mr 3–4 kDa. This band increased in size with duration of exposure to the high temperature. A second band (Mr 4–5 kDa) was present only in the groups exposed to the acute stressor for 1 h or 24 h and then allowed to recover for 12 h. In the group exposed for 1 h this band was faint, but in the other 2 groups (12 h and 24 h) it was several times more intense.

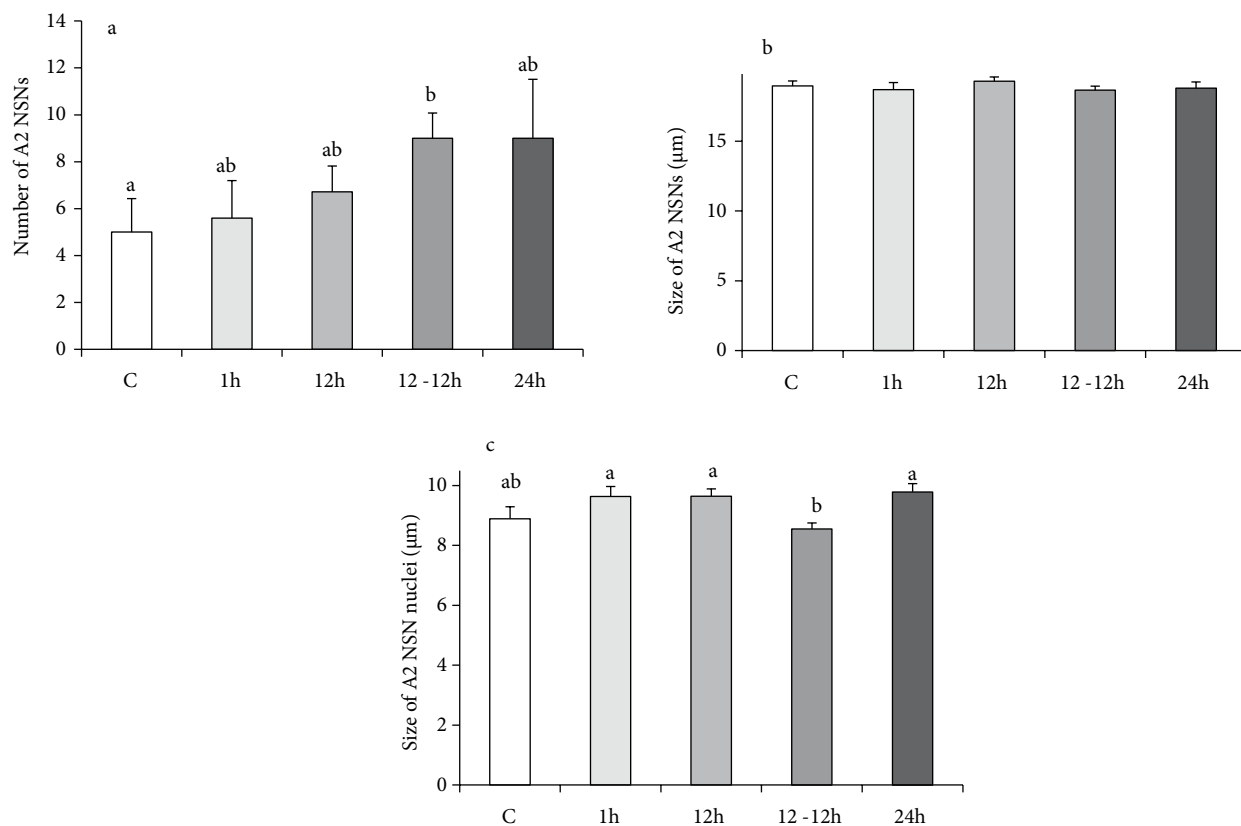


**Figure 1.** Brain transverse cross-sections of *Lymantria dispar* fourth instar caterpillars after exposure to 35 °C for 1 h (1h), 12 h (12h), and 24 h (24h). The 12/12h group of caterpillars was transferred for 12 h recovery at 23 °C after 12 h exposure to 35 °C. The control group was reared at 23 °C (C). Arrows indicate the protocerebral A2 neurosecretory neurons (NSNs). The bar represents 10 μm.

#### 4. Discussion

High temperature disrupts the normal synthesis and release of neurosecretory material from NSNs (Ivanović et al., 1975), thereby disturbing the hormonal balance and normal development and metamorphosis (Ivanović and Janković-Hladni, 1991). Highnam (1958) reported increased activity of type A lepidopteran NSNs in *Mimas tiliae* after exposure to high temperature, while Clark

(1966) observed an intensive influx of neurosecretory material in *Locusta migratoria* corpus cardiacum upon exposure to the same stressor. Using the same temperature treatments as in the present study, the increased cytological parameters of dorsomedial A1' NSNs and large amounts of observed neurosecretory material in the neuron body, led us to conclude that in A1' NSN synthetic activity increased (Ilijin et al., 2013). Analyzing some cytological



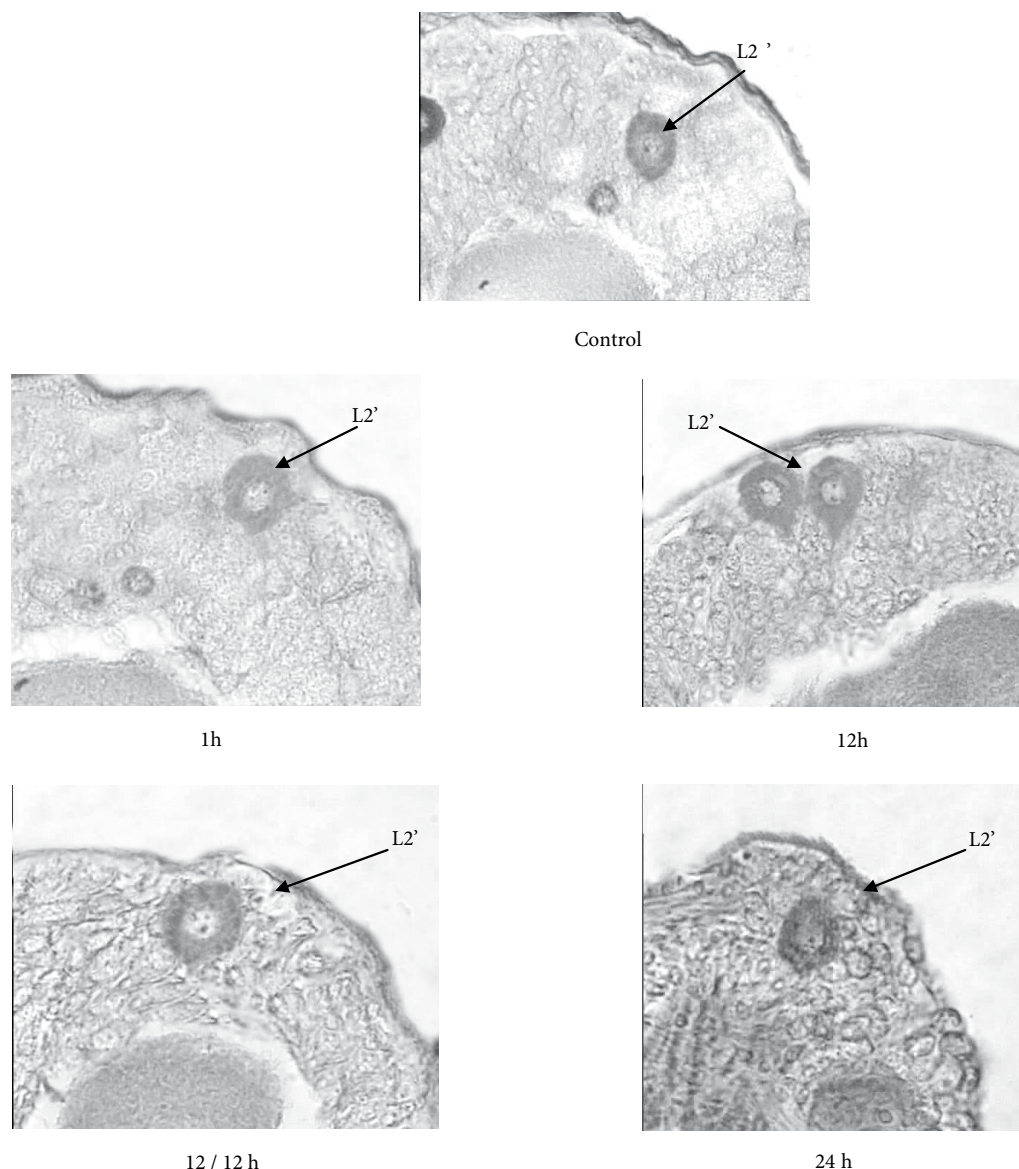
**Figure 2.** Changes in the number of A2 NSNs in *Lymantria dispar* fourth instar caterpillars after exposure to 35 °C (a), their size (b), and the size of their nuclei (c). Caterpillars were exposed to 35 °C for 1 h (1h), 12 h (12h), and 24 h (24h). The 12/12h group of caterpillars was transferred for 12 h recovery at 23 °C after 12 h exposure to 35 °C. The control group was reared at 23 °C (C). Error bars indicate the standard error of the mean (SEM) (n = 15). Different letters (a,b) indicate significant differences between groups (LSD test, P < 0.05).

parameters of A2 NSNs, located in the medial part of the *L. dispar* protocerebrum and previously found to be bombyxin immunopositive (Ilijin et al., 2011), we detected increased activity after exposure to thermal stress (Figures 1 and 2). An increased ambient temperature of 35 °C for a short time (1 h, 12 h, or 24 h) stimulated the release of bombyxin immunopositive material from A2 NSNs. In most insect species carbohydrate metabolism provides energy for activation of the compensatory mechanisms that enable survival under stress, including increased ambient temperature (Ivanović et al., 1992; Đorđević et al., 1995). Trehalose is the principal energy source in the hemolymph, and energy is released by enzymatic hydrolysis to glucose. Insect carbohydrate metabolism is regulated by neurohormones including bombyxin, which increases the level of trehalose utilization in hemolymph. In stressful conditions, bombyxin synthesis is intensive and followed by increased trehalase activity that accelerates trehalose hydrolysis to glucose. Trehalase also improves glucose transport to target tissues and cells to provide the

energy necessary for activation and maintenance of stress defense mechanisms (Satake et al., 1997).

The increased activity of A2 NSNs was obvious from the band densities in regions of bombyxin molecular mass. After exposure of the caterpillars to 35 °C, a new protein band with a slightly higher molecular mass was detected (4–5 kD instead of 3–4 kD). The band density of both protein isoforms increased upon exposure to this acute stressor (Figure 6b). All analyzed parameters, cytological and densitometric, indicated that short-term exposure of fourth instar gypsy moth caterpillars to a temperature of 35 °C stresses bombyxin immunopositive neurons. Activation of carbohydrate metabolism could be the end result of this short-time exposure to high temperature. After the exposure of caterpillars to the same temperature regimes, protein bands with molecular masses corresponding to those of members of heat-shock protein (HSP) families were detected, indicating that short-time exposure to this temperature probably induces synthesis of HSP (Ilijin et al., 2013).



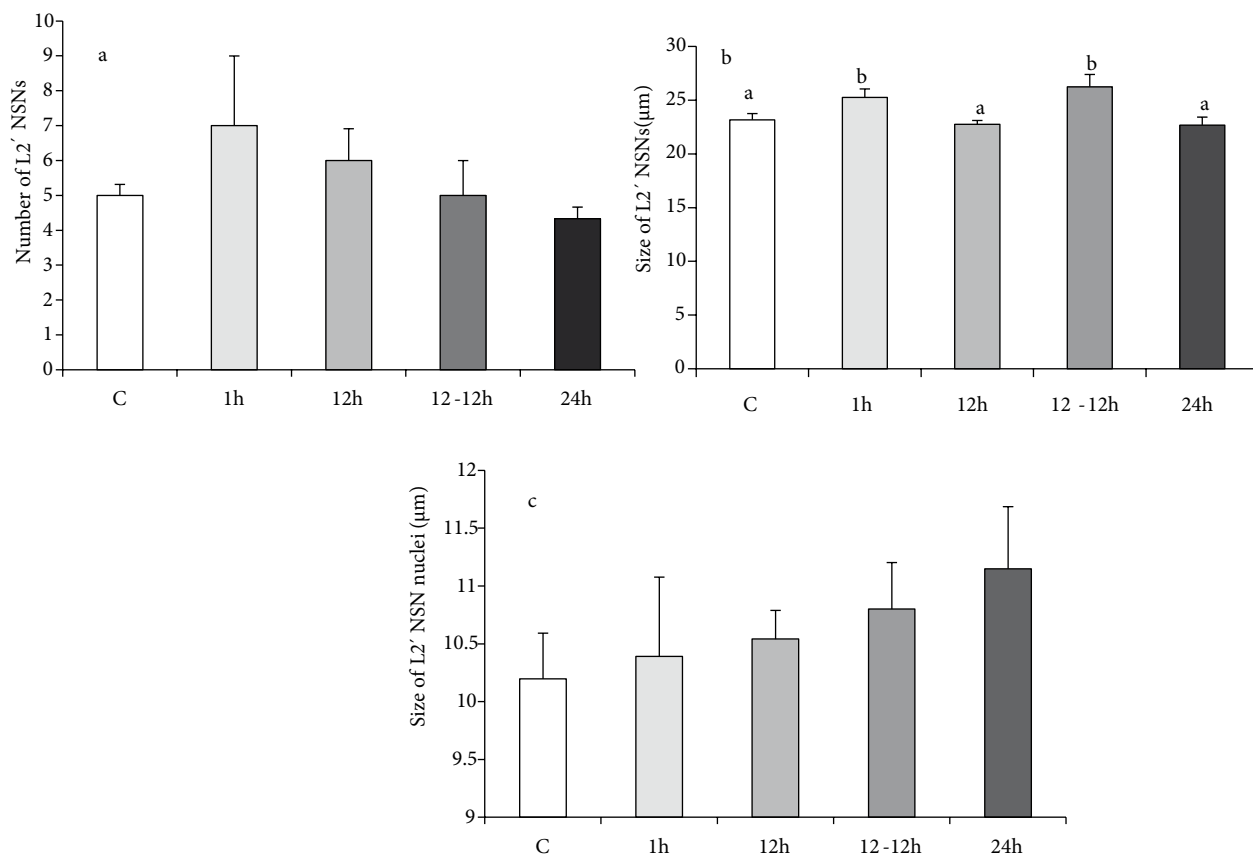


**Figure 3.** Brain transverse cross-sections of *Lymantria dispar* fourth instar caterpillars after exposure to 35 °C. All abbreviations are the same as in Figure 1. Arrows indicate the protocerebral L2' NSNs. The bar represents 10 µm.

In the dorsolateral part of the gypsy moth protocerebrum, L2' type NSNs were immunopositive to the large form of PTTH (Ilijin et al., 2012). This form of PTTH has a multifunctional role in stress-protective mechanisms, stimulating ecdysone synthesis through increased  $Ca^{2+}$  influx in the protothoracic gland cells (Dedos et al., 2005). Under some environmental conditions a decrease in PTTH level is adjustable, i.e. normal development is postponed in an unfavorable environment. The components of this signal transduction cascade are not fixed but vary depending on the stressogenic conditions to which insects are exposed

during development (Rybczynski and Gilbert, 2003).

In our experiment acute exposure to a high environmental temperature led to intensified synthetic activity but a low level of secretion (much of the neurosecretory material was present in the cytoplasm) of L2' NSNs in gypsy moth caterpillars (Figures 3 and 4). Besides L2' NSNs in the dorsolateral part of the protocerebrum, L2 NSNs are found too. Upon exposure to the same short-term temperature regimes used in this experiment, changes in morphometric characteristics and retention of neurosecretory material in the cytoplasm



**Figure 4.** Changes in the number of L2' NSNs in *Lymantria dispar* fourth instar caterpillars after exposure to 35 °C (a), their size (b), and the size of their nuclei (c). All abbreviations are the same as in Figure 2. Error bars indicate the standard error of the mean (SEM) (n = 15). Different letters (a,b) indicate significant differences between groups (LSD test, P < 0.05).

pointed to decreased secretory activity in gypsy moth caterpillars (Ilijin et al., 2013).

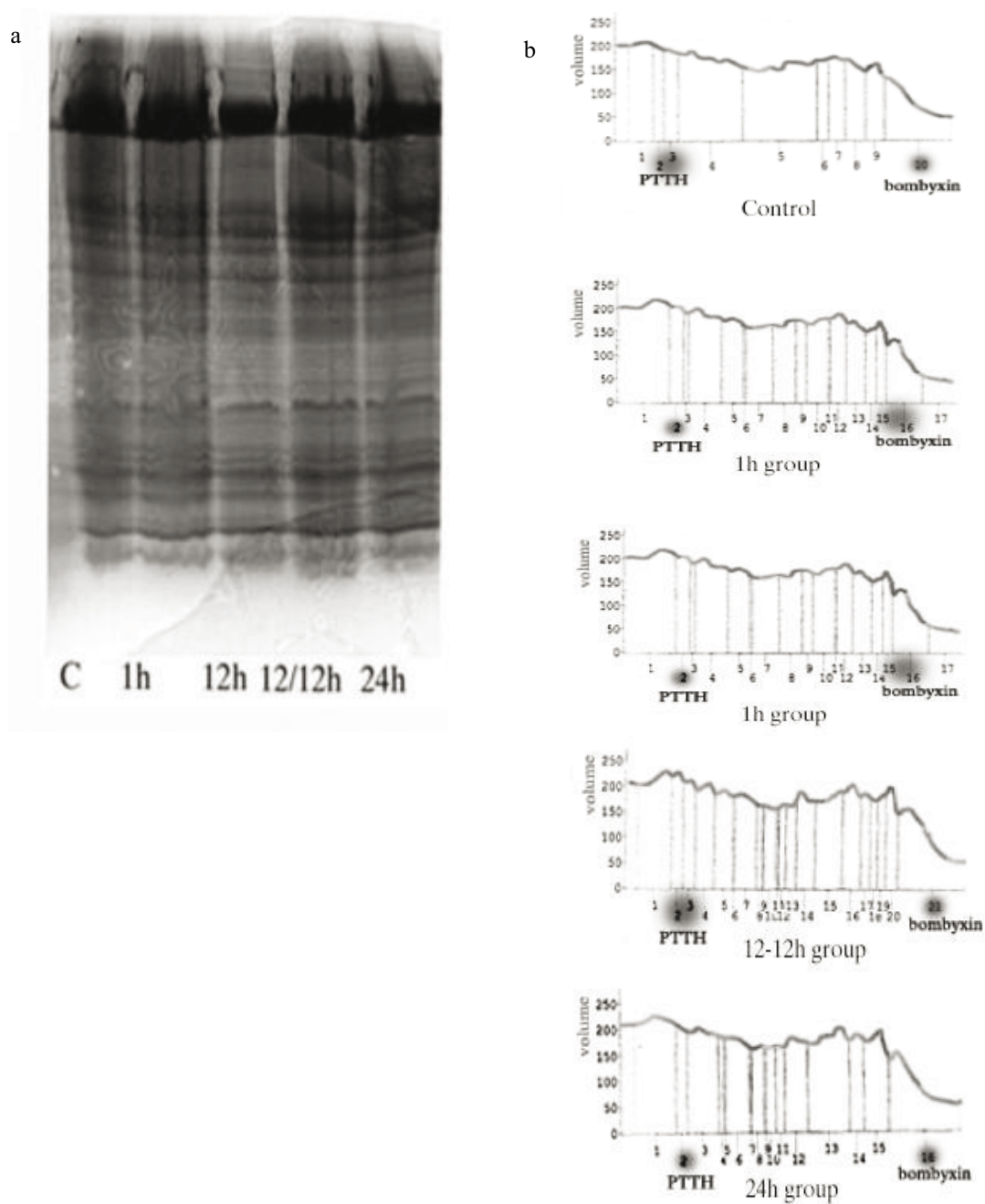
Densitometric analysis (Figure 5b) revealed that high temperatures probably induced a switch-off of PTTH isoforms synthesized in L2' NSNs. The new isoform was detected only upon exposure of caterpillars to this stressor. Moreover, the amount of this isoform increased with prolonged exposure to high temperatures and remained after a 12 h recovery at the control temperature (Figure 6). The isoform detected in the control group was also present in the groups exposed to 35 °C for 1 h and 12 h.

All our results revealed that PTTH immunopositive L2' NSNs increase synthetic activity, but decrease secretory activity after acute exposure to thermal stress. This is probably due to a low level of PTTH and ecdysone in hemolymph. In a previous work we found that short-term acute temperature stress did not disturb normal synthesis and release of the neurohormones responsible for the synthesis of allatotrophic neurohormones (Jeon and Lee, 1999) in *L. dispar* A1 NSNs (Ilijin, 2009). Allatotropins directly stimulate the synthesis and release of juvenile

hormones in the corpora allata, which represses PTTH and ecdysone secretion. When the juvenile hormone disappears, a PTTH/ecdysonic endocrine cascade is initiated, and the insect can reach its final body size (Nijhout, 2003).

Several stimuli are known to stimulate the synthesis of PTTH and cue from stretch receptors and photoperiod. In Lepidopteran species PTTH synthesis is controlled by critical weight gain and photoperiod. Nijhout (1981) showed that achieving critical weight is a major stimulation factor for PTTH release. However, there are several check points for caterpillars before they attain critical weight. After the minimal viable weight, they continue feeding until starvation no longer affects the time of pupation (Nijhout, 2003). In feeding insects, the nervous system synthesizes and releases bombyxin (Satake et al., 1997; Masumura et al., 2000).

A second stimulus for PTTH secretion is photoperiod. PTTH can be released only during a specific 8-h window each day (Truman and Riddiford, 1974). If caterpillars do not achieve critical weight before this time, feeding



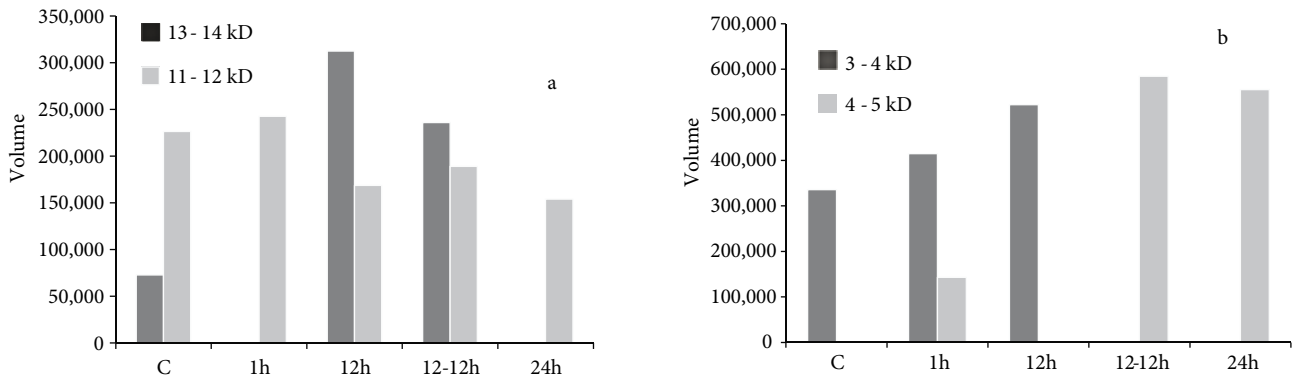
**Figure 5.** SDS PAGE profiles of brain homogenates of the fourth instar *Lymantria dispar* caterpillars obtained by 16.5% SDS PAGE (a) and their densitograms (b). The volume is the sum of all intensities included in the defined area, and it is displayed for each line. Patches show the differences in volume in the region of PTTH and bombyxin molecular masses. All abbreviations are the same as in Figure 1.

continues until the next photoperiod gate. Feeding stimulates the synthesis and release of neurosecretory material (Dogra and Gillott, 1971). McBrayer et al. (2007) indicate that the final insect body size is determined by a balance between insulin-like hormones, which are growth

regulators, and PTTH-like neurohormones, the role of which is to set the duration of the feeding interval.

Our results led us to conclude that brief, 1-h to 24-h exposure of *L. dispar* caterpillars to a temperature of 35 °C represents a stressor. The synthesis and release of bombyxin





**Figure 6.** Differences in volume of bands (the sum of all intensities included in the defined area) in the region of molecular masses of PTTH (Mr 11–15 kD) (a) and bombyxin (Mr 3– 5 kD) (b) obtained from 16.5% SDS PAGE (Figure 5) after exposure to high temperature. All abbreviations are the same as in Figure 1.

is increased, which usually means that trehalose hydrolysis is augmented, and larvae are in a continuous feeding phase. This indicates that environmental conditions are not favorable and that the caterpillars have not reached their critical weight. Therefore, 2 important signal gates for the synthesis and release of prothoracicotrophic neurohormones are not reached, and PTTH immunopositive NSNs are secretorily inactive. Our findings may contribute to the

body of knowledge regarding coherency between these 2 insect neurohormones and their function in normal and stressogenic environments.

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