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Effects of Cadmium on Protocerebral Neurosecretory Neurons and Fitness Components in *Lymantria dispar* L.*

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Changes in fitness components including larval stage duration, relative growth rate (RGR), and mass of the gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae), were investigated in caterpillars fed a synthetic diet with or without a cadmium supplement (10, 30, 100, 250 μg Cd/g dry food weight). Morphometric changes of large protocerebral dorsomedial A2 neurosecretory neurons, their nuclei and the electrophoresis profiles of brain proteins were analyzed in the 4th instar gypsy moths fed the examined diets. The duration of the fourth larval instars were prolonged and RGR and body mass reduced if the caterpillars were fed diets containing high concentrations of cadmium (100 and 250 μg). The size of large A2 dorsomedial neurosecretory neurons and their nuclei were significantly higher in larvae fed the diets supplemented with 10, 100 and 250 μg Cd. A large amount of neurosecretory material appeared in dorsomedial neurosecretory neurons in larvae fed diets with 100 and 250 μg Cd. Differences in larval brain protein profiles in the region of molecular mass ranges (Mr) of 98kDa, 46kDa and 3.4-6.1 kDa were identified in the experimental groups.

Key words: Growth rate; mass; larval duration; brain proteins; dorsomedial neurosecretory neurons.

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Lymantria dispar Linnaeus (Lepidoptera: Lymantriidae) is a widespread phytophagous forest pest which causes severe tree loss during population outbreaks in Europe, Asia and North America. Heavy-metal accumulation in ecosystems is a topical phenomenon after industrialization and causes changes in the dynamics of animal populations (BILONSKA *et al.* 2002; DAMEK-POPRAWA 2002), including *L. dispar* and other insects (WILLIAMS & LIEBHOLD 1995). Whether insects have or have not adapted to heavy metals, such environmental stress causes changes in physiological responses and affects insect populations. Cadmium from industrial effluents and other sources accumulates in plants (BONNARD *et al.* 2009) that insects, including *L. dispar*, use as food (NIEMINEN *et al.* 2001). Like many other pollutants, cadmium is non-biodegradable and can be accumulated by *L. dispar* at a level that affects its physiological state, susceptibility to parasites, population density and outbreak dynamics (ALSTAD *et al.* 1982).

Various effects of cadmium on insects have been described. Cadmium consumption leads to inhibition of growth, development, reproduction, hatchability (VAN STRAALEN *et al.* 1989; GINTENREITER *et al.* 1993; DUTTA & KAVIRAJ 1996; NIU *et al.* 2002; CERVERA *et al.* 2004) and alters respiratory (ORTEL & VOGEL 1989) and other metabolic processes (BISCHOFF 1995; NIU *et al.* 2002;) etc. In recent years, knowledge of the adverse effects of cadmium on organisms, including insects, has accumulated, especially concerning heat shock proteins important for protein folding, protein transport and cell stabilization during cadmium

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stress (COURGEON *et al.* 1984; SINGER *et al.* 2005; WILCZEK *et al.* 2008).

All stressors, regardless of their nature, and including pollution stress, can cause changes in the activity of the neuroendocrine system (RAABE 1982; IVANOVIĆ & JANKOVIĆ-HLADNI 1991) and morphometric characteristics of protocerebral neurosecretory neurons (PERIĆ-MATARUGA *et al.* 2001, 2004, 2008). Neurohormones are the master regulators of all physiological and metabolic processes, including secretion of the hormones that regulate growth, development, reproduction, metabolic processes, stress responses and homeostasis in insects. They regulate biochemical and physiological processes that take part in the adaptive response and, by various compensatory mechanisms, enable the insects to overcome the harmful effects of environmental stress, RAABE 1982; IVANOVIĆ & JANKOVIĆ-HLADNI 1991; NIJHOUT 1994).

Hemolymph sugar and carbohydrate metabolism (which is neurohormonally regulated) play an essential role in the cadmium stress response in insects as a source of energy in many protective physiological mechanisms, such as detoxification, protein synthesis in the fat body and water transport across the rectal wall BISCHOFF 1995). Insulin-like neuropeptides (bombyxin and the small form of prothoracicotropic neurohormones) are involved in the regulation of carbohydrate metabolism and accelerate the breakdown of hemolymph ("insect blood") trehalose. This finding indicates that insect insulin-like neurohormones are also metabolic hormones similar to vertebrate insulin and suggests that the physiological role of bombyxin in insects includes regulation of metabolism, including sugar utilization (SATAKE *et al.* 1997).

Prothoracicotropic hormones (PTTHs) are ecdysiotropic neurohormones which exist in two forms in various insect species: large PTTH (11-29 kDa) and small PTTH (4-7 kDa) (GILBERT 1981). Both large and small PTTH are produced mainly in neurosecretory cells of the brain and, in response to endogenous and/or exogenous cues, are released from neurohemal organs into the hemolymph and transported to the prothoracic glands.

Small PTTH (bombyxin) extracted from *Bombyx mori* was the first insulin-related peptide identified in insects (ISHIZAKI & SUZUKI 1994). In the gypsy moth, *L. dispar*, KELLY and collaborators (1991) estimated the size ranges for large and small PTTH to be 11-15 kDa and 4-6 kDa, respectively. They are included into the vertebrate insulin superfamily on account of amino acid sequence homology, as well as similar DNA and gene structure (NAGASAWA *et al.* 1984; IWAMI 1990). Production and characterization of a monoclonal antibody generated against a synthetic N-terminal decapeptide of the bombyxin a chain have allowed for the identification of the neurosecretory neurons which produce bombyxin or other insulin-like peptides. Using this antibody, bombyxin neurosecreting neurons (average diameter 20 μm) have been found in the large dorsomedial protocerebral part of the brain of *Bombyx mori* (MIZOGUCHI *et al.* 1990; IWAMI 1990), *Manduca sexta* (GRAY *et al.* 1994; DAI *et al.* 1994), *Galleria mellonella* (ZITNAN *et al.* 1990), *Drosophila melanogaster* (ZITNAN *et al.* 1993), *Locusta migratoria* (ZACHARY *et al.* 1988) and *Tenebrio molitor* (LAVERDURE *et al.* 1995).

Our aim was to examine how different cadmium concentrations (10, 30, 100, 250 μg Cd/g diet) affect gypsy moth fitness components, the electrophoretic profiles of their brain proteins and the morphometric traits of protocerebral dorsomedial A2 neurosecretory neurons.

Material and Methods

Insect rearing

Gypsy moth egg masses were collected in a poplar forest (locality Opovo: 20°25'49E, 45°3'8N, altitude 67m, 30 km from Belgrade). The egg masses were kept in a refrigerator at 4°C from October to March when they were set for hatching. After hatching, 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 in transparent plastic 200 ml cups. Larvae were randomly assigned (n=15) to five experimental groups for histochemistry, five experimental groups (same design) for brain electrophoresis and five experimental groups (same design) for duration of larval instar, relative growth rate calculation and larval mass. Larvae were reared on the basic diet until hatching in the 4th instar and then fed diets with cadmium supplements (10, 30, 100, 250 μg Cd/g dry food weight) or without cadmium (control). There were 15-larvae in each experimental group (control, 10, 30, 100, 250 μg Cd) for each data point.

Duration of the 4th larval instar, body mass of the 5th instar and relative growth rate

Relative growth rate was calculated on a mass basis according to the formula (FARRAR *et al.* 1989):

$$RGR = \frac{W_5 - W_4}{D_4 \times W_4}$$

Larval mass at the beginning of the 4th instar (W_4) and after moulting into the 5th instar (W_5), as well as the duration of the 4 th instar (D_4), were recorded.

Histological techniques

Based on their size and morphological characteristics, we divided protocerebral neurosecretory neurons of *Lymantria dispar* L. (for ease of monitoring the results) into groups; one of these is the A2 group of neurons with average diameter of 20 μm (PERIĆ-MATARUGA *et al*. 2001; PERIĆ--MATARUGA & LAZAREVIĆ 2004).

Brain complexes of gypsy moths were dissected in insect Ringer solution and immediately immersed in BOUIN's fixative (picric acid-saturated solution 75%; formaldehyde 20%; acetic acid 5%). Brain complexes were rinsed in 70% ethanol and fully dehydrated in a graded series of ethanol (from 80% to 100%) before embedding in paraffin wax. Serial sections of brain complexes were cut at 3μm for histochemistry (microtome- "820"Spencer) 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, stained by the modified (PANOV 1980) Ewens paraldehyde fuchsin technique and tested cytologically. Neurosecretory granules in neurosecretory neurons were stained dark purple-paraldehyde fuchsin positive, nuclei border were clear and nucleoli were intensive orange (EWEN 1962).

The sizes of the protocerebral dorsomedial A2 neurosecretory neurons and their nuclei were expressed as the mean values of the smallest and largest diameters (in μm^2). All measurements were performed and parameters analyzed using the image processing and analysis system (QWin image analysis tool kit) linked to a Leica DMLB light microscope (Leica, Cambridge, UK).

SDS PAGE Electrophoresis

After decapitation, caterpillar brains were dissected on ice and weighed. The brains were homogenized (20000xg) in cold distilled water (200 mg brain/ml distilled water) and afterwards centrifuged at 10 000 rpm for 10 min at 4°C. The supernatant was collected and SDS-PAGE electrophoresis performed according to LAEMMLI (1970), on 12% 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 50% methanol and 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) and 2.5-17 kD (Sigma).

Statistical methods

The results were analyzed statistically using the program STATISTICA, version 6.0. LSD tests were used as post-hoc tests after detecting significant effects with the appropriate one way ANOVA (following the examination of normality and assumption of homogeneity of variance), applied on log transformed results (SOKAL & ROHLF 1981).

Results

The duration of the 4th instar, relative growth rate, and 5th larval instar masses in *L. dispar* caterpillars

The duration of the 4th instar in the groups of *L. dispar* caterpillars fed diets supplemented with high cadmium concentrations (100 and 250 μg Cd/g) was significantly prolonged in comparison with the other experimental groups (P<0.001, Fig. 1).

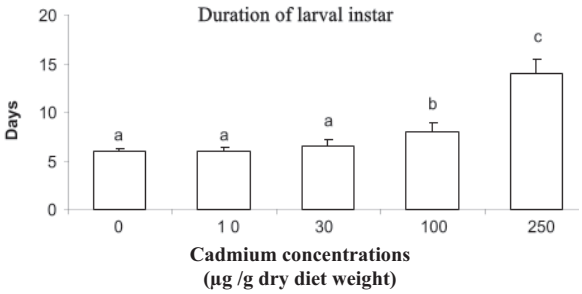

Fig. 1. Duration of 4th larval instar in *Lymantria dispar* caterpillars after administration of different dietary cadmium concentrations (0μg; 10μg 30μg; 100μg; 250μg. Values indicated by different letters (a,b,c) are significantly different (LSD test, P<0.001).

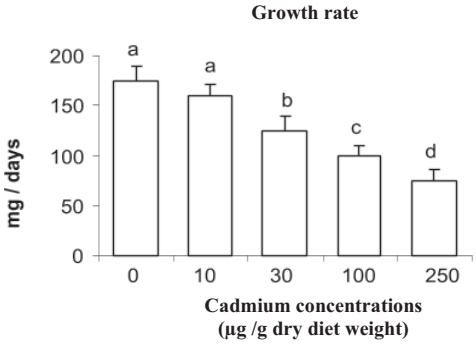

Fig. 2. Relative growth rate (GR) of 4th instar *Lymantria dispar* caterpillars exposed to various cadmium concentrations (0 μg; 10 μg; 30 μg; 100 μg; 250 μg/g of diet). Values indicated by different letters (a,b,c,d) are significantly different (LSD test, P<0.01).

The results also showed that higher cadmium intake (30, 100, 250 μg) led to a significant decrease in relative growth rate of *L. dispar* 4th instar larvae, compared to that of larvae fed the control diet (P<0.01, Fig. 2). This was especially obvious for caterpillars given diets containing 100 and 250 μg of cadmium.

The higher cadmium concentrations also led to a reduction of the 5th instar larval mass. Thus, 5th instar caterpillar mass was significantly lower in groups reared on diets with high cadmium contents (100 and 250 μg Cd/g; P<0.01; Fig. 3).

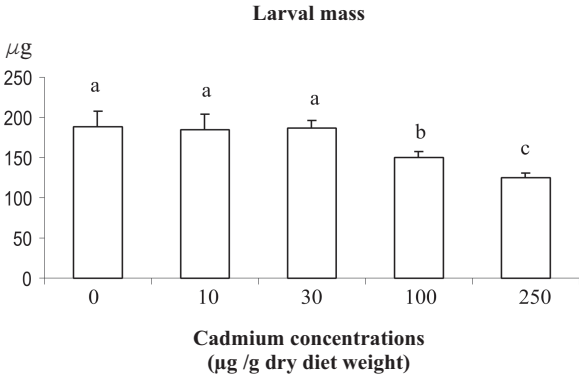

Fig. 3. Larval mass of 5th instar *Lymantria dispar* after administration of various cadmium concentrations (0 μg; 10 μg; 30 μg; 100 μg; 250 μg/g of diet). Values indicated by different letters (a,b,c) are significantly different (LSD test, P<0.01).

Large protocerebral dorsomedial A2 neurosecretory neurons and their morphometric parameters

A2 neurosecretory neurons were localised in the anterior dorsomedial region of the pars intercerebralis (Fig. 6). The size of these neurons was approximately 20 μm (depending on the physiological state).We detected a significant increase in size of the A2 neurosecretory neurons with elevation of dietary cadmium content in relation to the control (P<0.01, Fig. 4) with the exception of caterpillars fed a diet containing 30 The nucleus size in dorsomedial protocerebral A2 neurosecretory neurons was greater in the groups fed a cadmium supplemented diet in comparison with the control group (P<0.001, Fig. 5). A large amount of neurosecretory material was observed in A2 neurosecretory neurons in caterpillars given the largest amounts of cadmium (100 and 250 μg, Fig. 6). Fine granulation of neurosecretory material in insects is an indication of secretion activity of the neurosecretory neurons, involving the breakup of large neurosecretory granules into microvesicles and the microvesicles then releasing their contents (HIRUMA & AGUI 1977).

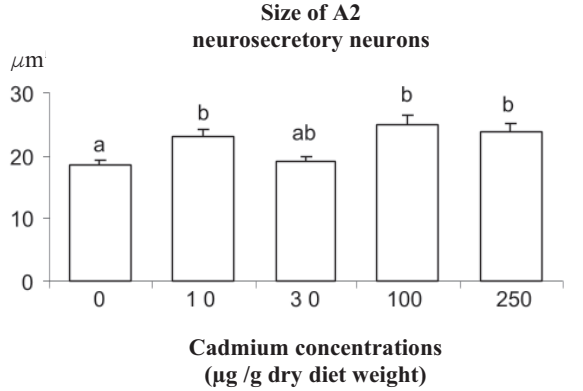

Fig. 4. The size of protocerebral dorsomedial A2 neurosecretory neurons in 4th instar *Lymantria dispar* caterpillars after exposure to various cadmium concentrations (0 μg; 10 μg; 30 μg; 100 μg; 250 μg/g of diet). Values indicated by different letters (a,b) are significantly different (LSD test, P<0.01).

Electrophoretic profiles of 4th instar caterpillar brain homogenates

The cadmium induced differences in electrophoretic patterns of the 4th instar *L. dispar* brain homogenates obtained by using 12% SDS-PAGE are shown in Fig. 7A. Changes were observed in the region of 4 to 98 kD. The region around 98 kD was characterized by two close protein bands in the control group and in the experimental groups given 10 and 30g of cadmium per g diet. If caterpillars were fed diets with more cadmium (100 and 250 μg Cd/g), the protein band of higher Mr was absent and the one of lower weight was more intense. The very strong protein band of about 46 kD, detected in all groups, decreased in intensity with higher cadmium concentration in the diet.

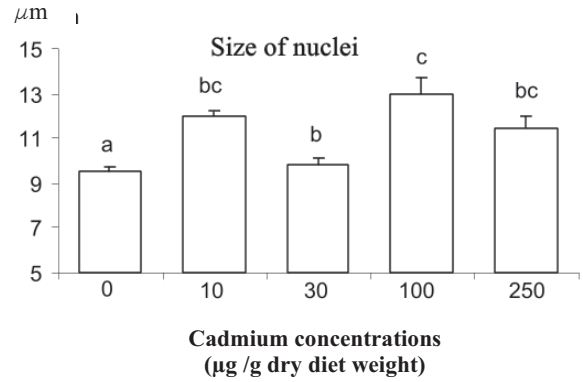

Fig. 5. The size of nuclei in A2 neurosecretory neurons in 4th instar *Lymantria dispar* caterpillars after administration of various dietary cadmium concentrations (0 μg; 10 μg; 30 μg; 100 μg; 250 μg/g). Values indicated by different letters (a,b,c) are significantly different (LSD test, P<0.001).

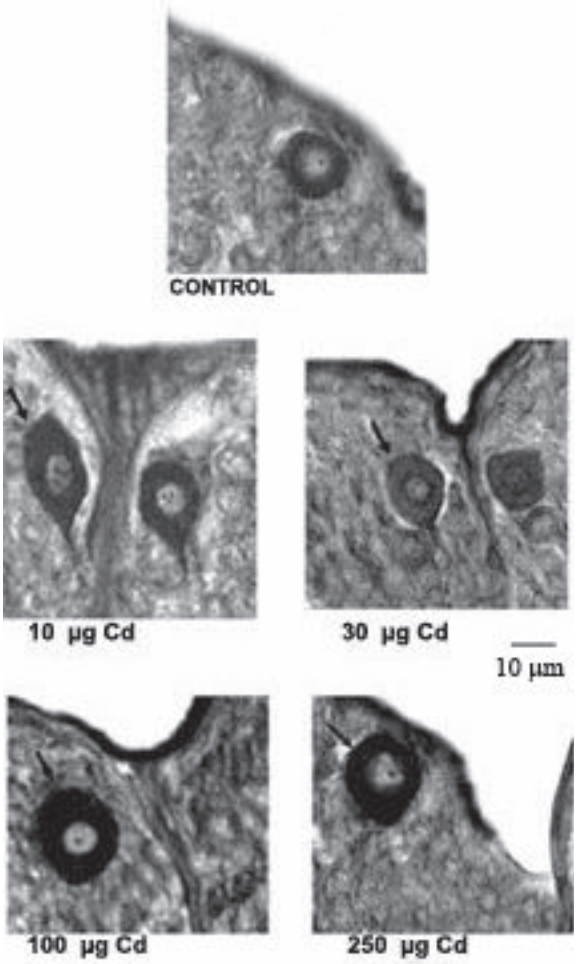

Fig. 6. Brain transverse cross-section of *Lymantria dispar* 4th instar caterpillars fed diets supplemented with various concentrations of cadmium (0 μg; 10 μg; 30 μg; 100 μg; 250 μg/g). Protocerebral dorsomedial A2 neurosecretory neurons are marked (arrows).

The electrophoretic profiles of 4th instar *L. dispar* brain homogenates for the different groups obtained by 16.5% SDS-PAGE are shown in Fig. 7B. In the region of small molecular weight (3.4-16.9 kD) two new bands with close molecular masses from 3.4 to 6.1 kD appeared in larvae exposed to cadmium concentrations of 30, 100 and 250 μg Cd/g.

Discussion

In insects, the control of growth rate is intimately linked to nutritional conditions and hormones. Body mass in insects is predominantly affected by a balance between gut growth rate regulators and neurohormones, such as insulin-like factors and a form of prothoracicotropic neurohormone that sets the feeding interval and moulting (NIJHOUT 1994, 2003; MC BRAYER *et al.* 2007).

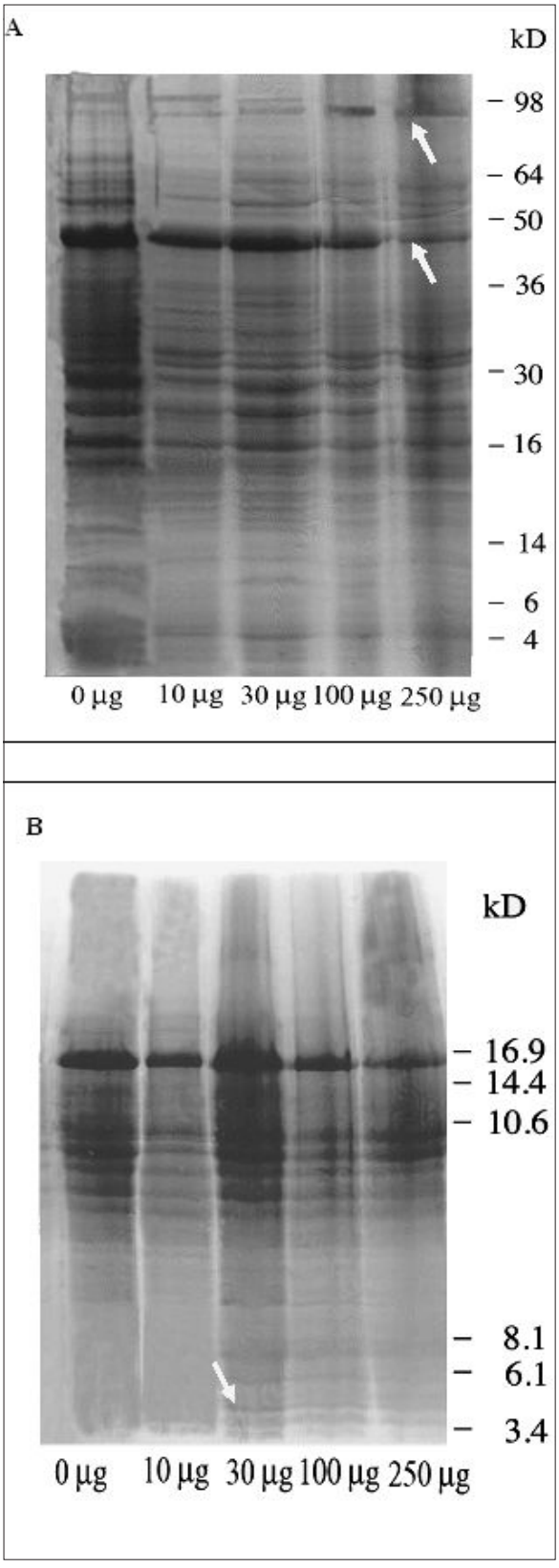

Fig. 7A. Electrophoretic patterns of brain homogenates of 4th instar *Lymantria dispar* caterpillars obtained by 12% SDS-PAGE, after cadmium administartion at 0 μg; 10 μg; 30 μg; 100 μg; 250 μg/g of the diet.

Fig. 7B. Electrophoretic patterns of brain homogenates of 4th instar *Lymantria dispar* caterpillars obtained by 16% SDS-PAGE, after being fed cadmium at 0 μg; 10 μg; 30 μg; 100 μg; 250 μg/ g of the diet.

The midgut, particularly its midway section, is an accumulative organ for cadmium and pathological changes in the epithelium can affect absorption and digestion of food (LAUVERJAT *et al.* 1989).

Molecular biological studies have identified two midgut metal responsive genes (MRGs) in arthropods induced by heavy metals in the midgut epithelium. One of these MRGs codes for an intestinal mucin (RAYMS-KELLER 2000). The integrity of the peritrophic membrane and mucus layer are critical for protection of the arthropod midgut from toxins, microbial trauma, digestive enzymes and physical trauma (WANG & GRANADOS 1997). Another MRG codes for a tubulin gene that is critical for structure and function of the midgut epithelial cells involved in digestion and absorption of food. Concentrations of heavy metals that quantitatively or qualitatively alter alpha-tubulin or alpha-tubulin gene expression in insects could reduce resistance to microbial and toxicological insult (RAYMS-KELLER 1998).

Cadmium also shows chemical similarities to zinc and may affect the properties of zinc containing enzymes. The production of reactive oxygen by cadmium is in part due to inhibition of Zn/Cu superoxide dismutase (SOD) by replacing zinc with cadmium. Low SOD activity increases the chance of reactive oxygen species damaging the midgut peritrophic matrix and epithelium (LIJUN *et al.* 2005). High dose cadmium toxicity in invertebrates causes similar effects to starvation (MORLEY *et al.* 2003).

The response of an organism to nutrient deprivation is complex and involves bichemical, physiological and behavioral changes. The background of these alterations are endocrine events. As shown in Figs 1, 2 and 3, our results demonstrate that diets supplemented with high concentrations of cadmium (100 and 250 μg) incurred considerable negative effects on *L. dispar* larvae, resulting in significant reduction of both larval body mass and relative growth rate (Fig. 2 & 3). Growth rate is a fundamental measure of physiological performance and provides one of the most sensitive measures of stress in organisms and therefore is a valuable biomarker used to monitor the impact of environmental pollution (VLAHOVIĆ *et al.* 2001).

Morphometric characteristics of A2 neurosecretory neurons, insulin-related bombyxin immunopositive neurons in Lepidoptera, (IWAMI *et al*. 1990; KAWAKAMI *et al.* 1990; DAI *et al*. 1994) were modified to a different degree depending on the amount of cadmium in the caterpillar diet (Figs 4 & 6).

Homeostatic regulation of blood/hemolymph sugar levels during stress (including heavy metal stress) is a fundamental physiological process in both invertebrates and vertebrates (NIJHOUT 2003). There are striking metabolic changes associated with nutritive stress including the presence of bombyxin in neurosecretory cells of the pars intercerebralis (MASUMURA 2000). The A2 neurosecretory neurons of gypsy moth caterpillars fed a diet with high concentrations of cadmium (100 and 250 μg) were larger with a larger nucleus than in the control (Figs 4, 5, 6). This cytological characteristic indicates intense synthesis of a probably insulin-like neurosecretory product. Bombyxin stimulates proliferation of cultured midgut stem cells in the lepidopterans *Heliothis virescens* and *Mamestra brassicae.* The number of midgut epithelial cells increased after the addition of bombyxin (GOTO *et al.* 2005). In *Drosophila melanogaster* insulin-like peptide promoted development (NASONKIN *et al.* 1992) and controlled body mass (JOHNSTON & GALLANT 2002). There is a possibility that bombyxin, which regulates carbohydrate metabolism, also has an influence on revitalizing midgut epithelial cells damaged by high cadmium doses.

It is interesting that no significant changes were observed in the larval body mass of *L. dispar* reared on low concentrations of cadmium (10, 30 μg/g), although there was a tendency for decreased relative growth rate in the latter case in comparison to the control group. It is possible that small amounts of dietary cadmium do not have a significant negative effect on gypsy moth larvae because they have the capacity for neutralizing the hazardous influences of cadmium by induced defence mechanisms. When fed diets contaminated with low cadmium concentrations, insects and other animals are able to trap the cadmium as spherocrystals in the periphery leaving the cytoplasm unaltered. Cell lysosomes are able to retain cadmium within metallothionein-like proteins in insects (JOHNSON & FOULKES 1980; LAUVERJAT *et al.* 1989). Our results showed that both relative growth rate and larval mass were significantly lower in larvae fed on diets supplemented with high concentrations of Cd (100 and 250 μg) as compared to control larvae (Figs 2 & 3), which is in accordance with earlier results (VLAHOVIĆ 2001, 2008).

In general, low concentrations of pollutants do not have fatal effects on insects, while high concentrations of pollutants are harmful (WU *et al.* 2006). Caterpillars exposed to the highest cadmium concentration were probably not able to counteract its toxic effects, so their nutritional needs could not be satisfied and they starved. In these larvae, body mass and relative growth rate decreased and the duration of the larval instar was prolonged (Figs 1, 2 & 3). Instar prolongation al-

lowed the larvae to reach the critical body mass needed for the next moult.

A2 neurosecretory neurons in caterpillars offered diets with high doses of cadmium (100 and 250 μm/g) were filled with a large amount of retained and agglomerated neurosecretory material and were greater in size than the control group (Figs 4 & 6). In addition, cadmium can act as a substitute for calcium in the protein kinase C (KISS & OSIPENKO 1994). Calcium is of crucial importance for a variety of cellular metabolic functions (VOGEL *et al.* 1999), including involvement in the regulation of release of neurohormones from insect neurons (TAKEDA 1976), but has to compete with cadmium for transport from cells (FOTAKIS *et al.* 2005). The high toxicity of cadmium is tissue-specific but generally includes changes in protein structure (STOHS *et al.* 2001). Our results also indicate qualitative and quantitative differences in protein content of brain homogenates from caterpillars fed diets with different concentrations of cadmium (Fig. 7A, B). In animals, including insects, the brain is an important target for cadmium (LINDQVIST 1992).

The electrophoretic protein profiles of gypsy moth caterpillar brains receiving high cadmium doses exhibited a massive protein band of molecular size around 98 kD (Fig. 7A). This could indicate the presence of some form of stress induced protein. Elevated Hsp 98 has been linked to starvation (carbohydrate metabolism) in several *in vitro* experiments involving isolated cells (LEE *et al*. 1999; WILLMER 2000; SNYDER & MULDER 2001). The expression of Hsp 98 was high during starvation and coincided with periods of high cadmium intoxication and starvation (BRICKMAN *et al*. 1997). These data suggest that heat shock proteins may play an important role in the protection of insect cells against cadmium insult (OVELGONE *et al.* 1995). However, the precise way in which cadmium induces heat shock proteins remains unknown. Several mechanisms have been considered: direct activation by denaturized proteins; increased levels of reactive oxygen species; and/or modulated levels of secondary messengers (JUNGMANN *et al*. 1993).

Our results revealed a decreased intensity of a protein band of about 46 kDa in the brains of caterpillars fed diets supplemented with 100 and 250 μg of cadmium. The Mr of basic components of the cytoskeleton is about 46 kD (intermediate filaments). We previously mentioned that cytoskeletal protein genes in insects are heavy metal responsive (RAYMS-KELLER *et al*. 2000), which may explain this result.

Our electrophoretic protein profiles of gypsy moth brains revealed an increased number of protein bands in the region of 3.4-6.1 kDa with elevation of cadmium concentration in the diet (Fig. 7B). This may indicate an increase of insulin-like isoforms in brain tissue (the small PTTH Mr in the gypsy moth is in the 4-6 kDa range), which may be associated with the cytological characteristics of the neurons which synthesise them. Morphometric variation of A2 neurons associated with the presence of dietary cadmium could be used to monitor the intensity of pollution stress.

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