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# Cadmium-induced changes of gypsy moth larval mass and protease activity

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ABSTRACT

Cadmium uptake takes place mainly through food. *Lymantria dispar* larvae were exposed to dietary cadmium in concentrations of 10 and 30 μg Cd/g dry food (NOEC, no-observed-effect and LOEC, lowest-observed-effect concentration, respectively) for acute and chronic treatment and recovery. We established that metal contamination decreased mass only during the chronic treatment at 30 μg Cd/dry food with no recovery on removal of cadmium for 3 days. Significant reduction of protease activity was detected at LOEC after the acute and chronic treatments. Protease showed enhanced plasticity with regard to the fitness trait (mass) during environmental stress and the higher cadmium load, when it changed. The statistically significant higher index of phenotypic plasticity for protease correlated with lower variability. Protease isoforms at the same cadmium treatments differed between genotypes, while some protease isoforms from one egg-mass differed between cadmium treatments. Owing to the low sensitivity and plasticity of mass change during exposure to cadmium, as well as its small influence, we concluded that larval mass is not a good indicator of cadmium presence in food. We suggest that proteases, with further research, might be a suitable indicator of dietary cadmium contamination, as well as nutriment utilization during heavy metal stress.



## 1. Introduction

### 1.1. Insects in pollution research

Evaluation of pollutants at various levels of biological organization (from molecular to population) is widely used in environmental studies. Considering that each type of pollution represents a great threat for individual organisms and populations, it is essential to diagnose causes and predict possible negative effects on living organisms (Tsangaris et al., 2007). Many insects can be used as environmental pollution bioindicators. In Australia, ants are employed for measuring pollutants in borealis forests (Peck et al., 1998), while predatory insects, bees and wasps are used for monitoring heavy metals, pesticides and herbicides in the environment (Balestra et al., 1992; Ghini et al., 2004; Urbini et al., 2006; Nummelin et al., 2007), and chironomidae larvae are bioindicators for toxic sediment stress (Servia et al., 1998). Insects consume large amounts of food and, per se, are food for other predators. Therefore, they are an important link in cycling and transport of metals between trophic levels. Several studies have demonstrated the effect of cadmium on insect physiology and biochemistry, development, growth and reproduction (Van Straalen et al., 1989; Gintenreiter et al., 1993; Ortel, 1995a, 1995b, 1996; Rayms-Keller et al., 1998; Cervera et al., 2004). Changes at the biochemical level in the presence of heavy metals occur more rapidly than variations of fitness related traits.

### 1.2. Midgut enzymes as indicators of contamination

Biochemical and cellular markers are the most sensitive indicators of contamination and may reflect specific toxic mechanisms of xenobiotics (Marigómez et al., 2002; Bolognesi et al., 2004; Tsangaris et al., 2007). Non-specific enzymes are usually associated with tissue damage and in some cases, can be used as stress indicators or general biomarkers of stress (Lagadic et al., 1994; Cajaraville et al., 2000; Hyne and Maher, 2003; Vlahović et al., 2009, 2012). There is a direct relationship between the activity of an animal's digestive enzymes and its access to energy via food. In *Orchesella cincta*, 92% of the total cadmium in the body was found in the gut (Hensbergen et al., 2000). This points out the relevance of digestive enzymes in the exposure and/or effect of single or multiple pollutants in the environment. The sensitivity of digestive enzymes to exposure of an organism can be shown by measurements. Responses connected to this type of enzyme are able to highlight exposure and/or the effect of single or multiple xenobiotics. Considering that digestive enzymes influence disruptions at the individual level (mass, growth, mortality), they can be a very important factor in (eco)toxicological studies.

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Proteases (peptidases, proteinases EC 3.4.x.x) are digestive enzymes that hydrolyze peptide bonds. They are distributed in subclasses depending on the catalytic mechanisms and substrate, as well as pH optimum. Specificity is only used in the identification of individual enzymes within a subclass (Terra et al., 1996). Protein breakdown and recycling depend on the levels of proteolytic enzymes and are an essential part of the insects' response to environmental stress.

In various insects heavy metals are mostly distributed in the midgut (Poulson and Bowen, 1952; Maroni and Watson, 1985; Lauverjat et al., 1989). Decrease of peptidolytic activity in the presence of heavy metals was established in *Musca domestica* (Blahovec et al., 2006) and in the palm weevil (Evans, 2011), while bivalent cations, such as zinc and cobalt, partially inhibited purified aminopeptidases in *Morimus funereus* (Božić et al., 2003).

### 1.3. *Lymantria dispar* larvae as biomonitors

Lepidoptera are very sensitive to environmental changes (heavy metals, magnetic fields, temperature) (Gilbert, 1984; Heliovaara and Vaisanen, 1990; Ilijin et al., 2011, 2012, 2013). *Lymantria dispar* (gypsy moth) is a phytophagous pest insect that feeds on more than 500 plant species. It has a short generation time, precisely defined developmental stages, well-known physiological processes and is easily manipulated. Therefore, this species can be considered a model organism for toxicological studies. The primary objective was to examine how acute and chronic cadmium effects [10 μg Cd/g dry food (NOEC) and 30 μg Cd/g dry food (LOEC)] influence protease activity and larval mass, to calculate the index of phenotypic plasticity of the examined fitness and biochemical traits and to investigate the relationships between heavy metal exposure and protease isozyme variations. NOEC and LOEC values referred to the relative growth rate of gypsy moth larvae 4th larval instar (Vlahović et al., 2001).

## 2. Material and methods

### 2.1. Rearing conditions

Egg-masses of *Lymantria dispar* (Lepidoptera: Erebidae) analyzed in the experiment were collected from a poplar forest in Opovo, Serbia (45° 03′ 49″ N and 20° 27′ 26″ E). Gypsy moth females lay a single egg-mass that is fertilized in a single mating. After collection, the egg-masses were refrigerated at 4 °C until hatching. Larvae were grown in plastic cups (vol = 300 mL). During the first and second larval instar, the density was fifteen larvae per cup, reduced to five larvae per cup during the third instar and one larva per cup at the fourth instar. Rearing temperature was 23 °C, with a photoperiod of 12 h dark:12 h light.

### 2.2. Experimental groups

Larvae were reared on an artificial high wheat germ diet (Bell et al., 1981; O'dell et al., 1984). We used two cadmium concentrations: 10 μg Cd/g dry food and 30 μg Cd/g dry food. As previously described (Vlahović et al., 2009, 2012), twenty egg-masses (genotypes) were collected from the forest. From each egg-mass, ten randomly hatched larvae were taken (200 larvae totally) and distributed into seven experimental groups. Thus, each experimental group consisted of 200 larvae (20 egg-masses × 10 larvae) × 7 groups, so 1400 were reared in total.

#### 2.2.1. Treatments

Control (C) — larvae fed on uncontaminated food throughout;

Acute treatments

$Ac_1$ and $Ac_2$ — larvae were reared on a cadmium-free diet from hatching until entering the 4th instar when they were offered food containing added cadmium (10 and 30 μg Cd/g dry food, respectively) for 3 days before sacrifice.

#### 2.2.2. Chronic exposure

$Chr_1$ and $Chr_2$ — larvae were reared on contaminated food (10 and 30 μg Cd/g dry food, respectively) from hatching until sacrifice on the 3rd day of the 4th larval instar.

Recoveries

$Rec_1$ and $Rec_2$ – larvae were reared on contaminated food (10 and 30 μg Cd/g dry food, respectively) until molting into the 4th instar when they were transferred to an uncontaminated diet for 3 days.

Larvae were treated with $Cd(NO_3)_2 \cdot 4H_2O$. The applied concentrations, 10 and 30 μg Cd/g dry food, were based on the active ingredient (Cd) in the nitrate salt.

Pooled homogenates were made from five larval midguts per twenty egg-masses per each experimental group.

### 2.3. Preparation of homogenates

Before sacrifice larvae were chilled on ice for 60 s to decrease metabolism. Thereafter, they were decapitated, integuments were cut from the abdominal side and midguts were removed and kept at −20 °C. This was conducted on ice the whole time. Considering that insect fluids have buffer values similar to vertebrates (Harrison, 2001), homogenates were made in ice-cold saline (0.15 M NaCl), and the final concentration was 100 mg/mL. The homogenates were centrifuged at 10,000 *g* for 10 min. The pH value of 0.15 M NaCl was 5.5, identically as commercial, midgut homogenate pH of larvae not exposed to cadmium was 7.7 and that of homogenate from chronically exposed larvae was 7.5. Supernatants were used for enzyme assays. This involved 700 midguts (larvae) i.e. 7 experimental groups × 20 egg-masses × 5 larval midguts.

### 2.4. Biochemical analysis of protease activity

Total protease activity was determined according to Kunitz (1947). Casein (1%) dissolved in 0.2 M Gly/NaOH (pH 10) was used as the substrate. The same amount of homogenate and substrate (250 μL) was taken and, after incubation for 1 h at 40 °C, the reaction was stopped by adding 500 μL 10% TCA. Precipitation was conducted in a refrigerator for 30 min followed by centrifugation at 10,000 *g* for 10 min. Activity was measured spectrophotometrically at 280 nm. One unit of protease was defined as the change of optical density at 280 nm per mg of midgut during 1 h. For calculation of specific enzyme activity, the protein concentration was determined according to Bradford (1976), using bovine albumin as the standard.

### 2.5. Electrophoresis

For detection of protease isoforms we used PAGE with 12% gel and a constant current of 20 mA. The sample buffer contained SDS but no β-mercaptoethanol. The method, modified according to Muhlia-Almazan and Garcia-Carreno (2002), is composed of several stages:

- After electrophoresis, the gels were soaked in 3% casein previously dissolved in 50 mM glycine buffer (pH 10) for 30 min at 4 °C, to allow substrate diffusion into the gel at low enzyme activity.
- For maximum protease activity incubation of the gel at 40 °C for 60 min in the same solution is necessary.
- Rinsing with deionized water.
- Staining in 0.1% Coomassie brilliant blue R-250 for 5 min.
- For gel destaining we used 40% methanol and 10% acetic acid overnight.

### 2.6. Statistical methods

Mean values (MV) and standard error of the mean value (±SE) were calculated for relative larval mass and specific activity of total