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RESEARCH ARTICLE

Cadmium effects on the fitness-related traits and antioxidative defense of *Lymantria dispar* L. larvae

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Abstract Cadmium, like many other pollutants, is nondegradable and can be accumulated by *Lymantria dispar* at a level that affects fitness components, physiology, and development, which could indicate presence of environment pollution by heavy metals. The cadmium effect on fitness-related traits in the third, fourth, fifth, and sixth instar of *L. dispar* L. was determined. Furthermore, activities of the following antioxidative defense components after the larvae had been fed on the artificial cadmium-supplemented diet (50 μg Cd/g dry food) were assessed: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APOX), total glutathione amount (GSH), glutathione-*S*-transferase (GST), glutathione reductase (GR), and the amount of free sulfhydryl (SH) groups. Statistically significant delay of development in the fourth, fifth, and sixth instar and decrease of the larval mass in the third and fourth instar were estimated after the exposure to cadmium through food in comparison to the control. There were no changes in SOD activity of cadmium-treated larvae. Significantly lower CAT, APOX, and GR activities were recorded in the third, fifth, and in the third instar, respectively. At the same time, higher activity was recorded in the sixth instar, while GST activity was higher in the third. GSH content was significantly lower during all instars after treatment but the amount of SH groups was higher in older larvae. The strategy of antioxidative defense and the adjustment or modulation of fitness-related traits in presence of cadmium was dependent on the age of larvae in *L. dispar*, which might be used in early metal risk assessment in Lepidoptera and other insects.

Keywords Cadmium (Cd) · *Lymantria dispar* L. · Larvae · Development · Larval mass · Antioxidative defense

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Introduction

As a transition liquid metal, cadmium (Cd) enters the environment through natural and anthropogenic sources, and it represents one of the 129 priority pollutants according to the Environmental Protection Agency (USEPA 1991). Toxic effects of Cd are most intensively noticed in organisms living in habitats with direct sources of pollution, such as industrial waste disposal systems, mineral exploitation sites, areas treated with pesticides and phosphate fertilizers, etc. (Chaney et al. 1999). Cd can be absorbed by host plants and accumulated in phytophagous insects through the food chain (Devkota and Schmidt 2000a, b Pedersen et al. 2008). Upon entering an organism, Cd can induce irreversible damage ultimately resulting in death. Cd is a cumulative toxicant and is of concern for phytophagous insects as its concentrations in the organism tend to increase with age (Heliovaara and Vaisanen 1990).

There is serious scientific evidence about the cryptogenic, mutagenic, and carcinogenic effects of Cd to organisms including invertebrates (Filipić et al. 2006; Vlahogianni et

al. 2007). Through inhibition of different metabolic and antioxidative enzymes, and attachment to their thiol groups, Cd increases prooxidative processes responsible for causing oxidative stress. Cd is considered a non-redox metal and can indirectly cause the production of reactive oxygen species (Wang et al. 2004). Furthermore, Cd can interfere with antioxidative defense systems, resulting in the production of free radicals and consequently lipid peroxidation; it can damage the nucleic acids, perturb osmosis–ion balance, affect the calcium and sulfur homeostasis, change the activity of important groups of enzymes, etc. (Badisa et al. 2007).

Antioxidative defense systems in insects consist of functionally connected enzymes and low molecular mass components, which have the biological role of neutralizing and suppressing oxidative damage. The antioxidative defense in insects, including *Lymantria dispar*, mainly consists of the following enzymes: superoxide dismutase (SOD), catalase (CAT), isoforms of glutathione-*S*-transferase (GST) with peroxidase activity, glutathione reductase (GR), ascorbate peroxidase (APOX) and non-enzymatic cellular antioxidants—glutathione (GSH), ascorbic acid, etc. (Ahmad and Pardini 1990; Perić-Mataruga et al. 1997; Blagojević and Grubor-Lajšić 2000). Superoxide radicals generated under oxidative stress are rapidly converted to hydrogen peroxide (H_2O_2) by SOD, which can be decomposed by CAT (Ahmad 1995). A number of related publications have demonstrated that Cd stress can change the activities of these antioxidative enzymes in insects. For example, high concentrations of cadmium caused the induction of superoxide dismutase and catalase activity in *Oxya chinensis* (Li et al. 2006). Glutathione contents and activity of glutathione-dependent enzymes in the body of insects inhabiting polluted areas have been shown to help with maintaining redox homeostasis, and the intensity of enzyme responses can depend on developmental stage and state of nutrition (Migula et al. 2004). GSTs are ubiquitous in nature and have been demonstrated to be involved in detoxification of xenobiotic compounds in vertebrates and invertebrates (Pickett and Lu 1989). GST is the most important enzyme of phase II detoxification and has a central role in defense against various environmental pollutants. Induction of GST during detoxification processes is followed by reactions which expend cellular GSH and generate its oxidized form glutathione disulfide (GSSG). Induction of glutathione reductase activity under the influence of cadmium has been shown in *Oncopeltus fasciatus* (Cervera et al. 2003). In herbivorous insects such as *L. dispar*, ascorbate recycling enzymes which decompose H_2O_2 using GSH as reducing agents are an important part of antioxidative defense. APOX activity, which catalyzes the oxidation of ascorbic acid with the concurrent reduction of H_2O_2, was found in Lepidoptera larvae. Given that most insect groups lack Se-dependent glutathione peroxidase, APOX is important in removing peroxides in insects (Mathews et al. 1997).

The gypsy moth (*L. dispar* L.), a polyphagous herbivore, is dangerous pest insect of forest and fruit trees. Its host range is estimated at more than 500 plant species from 73 families (Lance 1983). Our previous research, as well as studies by other authors, showed that Cd affects the individual performance (life span, development time, etc.) of the gypsy moth (Ginterneiter et al. 1993; Ortel 1995; Vlahović et al. 2001, 2008; Mirčić et al. 2010). In these previous experiments, significant acute effects of cadmium on the relative growth rate in the fourth instar were observed. No-observed-effect concentrations and lowest-observed-effect concentrations were determined to be 10 and 30 μg Cd/g of dry food, respectively (Vlahović et al. 2001). Therefore, *L. dispar* caterpillars were treated with concentration of Cd (50 μg Cd/g of dry food). The aim of this study was to investigate effects of chronic exposure of *L. dispar* to a cadmium-supplemented diet during larval development. Specifically, the effects on fitness-related traits (mortality, larval developmental duration, and larval mass) and the antioxidative system (antioxidative enzyme activities (SOD, CAT, GR, GST, APOX), total glutathione, and sulfhydryl (SH) groups) were studied.

Materials and methods

Insect rearing and cadmium treatments

In our previous experiment, significant effects of cadmium on the components of the adaptive values and digestive enzymes (alkaline phosphatase, glycosidase, leucine aminopeptidase, glucosidase, and esterase) of gypsy moth larvae were recorded using the concentration of 50 μg Cd/g of dry food (Mirčić et al. 2010; Vlahović et al. 2008, 2012). In rearing larvae on food containing this dose of Cd, the chronic effect on larval mass was significant and ubiquitous during the recovery of the larvae (Vlahović et al. 2008). Therefore, the dose of 50 μg Cd/g of dry food was chosen for this experiment.

Gypsy moth egg masses were collected in Carina, 150 km south of Belgrade, in forests mainly populated by oak trees (*Quercus frainetto*, Tenore; *Quercus cerris* L.). Egg masses were kept in a refrigerator at 4 °C until hatching. After hair removal, eggs were disinfected in 0.1 % sodium hypochlorite (bleach) for 5 min and were then rinsed with distilled water and air-dried, which is particularly important in terms of the prevention of fungal contamination. Hatched larvae were reared at a temperature of 23 °C, a photoperiod of 12 light:12 dark, and were fed on the HWG artificial diet (Odell et al. 1984) without cadmium (control group, consisting of 165 individuals). The group exposed to cadmium consisted of individuals (n=165) whose HWG diet was supplemented with 50 μg Cd/g ad libitum dry food

during the third, fourth, fifth, and sixth instar of larval development. The active cadmium ingredient was Cd $(NO_3)_2.4H_2O$. Molting of the larvae was recorded daily. The effects of cadmium on the components of the antioxidative defense system in larval instars were measured on the third day of each instar.

Preparation of homogenates

All individuals were euthanized by freezing in liquid nitrogen. Larvae were euthanized on the second day after molting into the third and fourth instars and on the third day after molting into the fifth and sixth larval instars. Frozen larvae were kept at the temperature of −24 °C until the preparation of the homogenates. Larvae were homogenized on ice in 0.25 M sucrose buffer (pH 7, 100 mg/2 ml), using an Ultra-Turrax homogenizer (IKA-Werke, Staufen, Germany) for 3×10 s at 2,000 rpm followed by 3×15 s sonication steps with a 50-W sonifier (Bandelin sonoplus HD2070, Berlin, Germany). Then, homogenates were centrifuged using a Beckman L7-55 ultracentrifuge (Beckman, L7-55, Ultracentrifuge, Nyon, Switzerland) at 105,000×*g* at 4 °C. The supernatants were extracted and frozen at −24 °C until use. In order to determine the GSH content, a portion of the sonicated homogenates was used to precipitate proteins using 10 % sulfosalicylic acid, and the GSH content was measured after centrifugation for 20 min at 10,000×*g* and 4 °C (Model 5417R, Eppendorf, Hamburg, Germany).

Activity of antioxidative defense

Determining the activity of SOD was conducted according to the methods described by Mistra and Fridovich's (1972). This method is based on the ability of SOD to prevent adrenaline autoxidation in an alkaline medium. The adrenaline conversion into adrenochrome is followed by the release of superoxide anion radicals that lead to the acceleration of the autoxidation reaction. Adrenaline autoxidation rate was determined spectrophotometrically through the absorption change at a wavelength of 480 nm at 25 °C. Superoxide dismutase activity was expressed as the amount of enzyme causing a 50 % inhibition of adrenaline autoxidation milligram protein (in units per milligram protein).

Catalase activity was determined according to the method by Beutler (1982) using spectrophotometric determination of dissolution of the standard concentration of H_2O_2 (10 mM) at 230 nm. Activity was expressed as the amount of dissolved H_2O_2 reduced per minute per milligram protein (in micromolars per minute per milligram protein).

Glutathione reductase activity was determined according to Glatzle et al. (1974). In brief, changes in the amount of NADPH consumed by the reduction of standard amount of oxidized glutathione (GSSG) were measured spectrophotometrically at a wavelength of 340 nm. The activity was expressed as nanomoles NADPH per minute per milligram protein.

Glutathione-*S*-transferase activity was determined according to the method by Habig et al. (1974). Glutathione-*S*-transferase catalyses the conjugation of 1-chloro-2.4-dinitrobenzene (CDNB) with the SH groups of glutathione. The amount of derived CDNB–glutathione complex was measured spectrophotometrically at 340 nm and expressed in nanomoles GHS per minute per milligram protein.

Determination of the total concentrations of glutathione (reduced GSH and oxidized GSSG) was conducted according to the method described by Griffith (1980). Sample proteins were precipitated in the homogenates by sulphosalicylic acid. The method is based on the recycling procedure where the oxidation of GSH with DTNB (producing 5.5-dithiobis(2-nitrobenzoic acid)) and its reduction by glutathione reductase with NADPH are conducted reciprocally. The rate of formation of 2-nitro-5-thiobenzoic acid was monitored spectrophotometrically at 412 nm, and the concentration of total glutathione was calculated in accordance to the standard and was expressed per grams of wet mass.

Determination of APOX activity was conducted according to Hossain et al. (1984). In brief, catalase activity of each sample was inhibited by addition of aminotriazole, and the reaction was then started by adding hydrogen peroxide. APOX was monitored spectrophotometrically at 290 nm and was expressed as nanomolar per minute per milligram protein.

Determination of the amount of free SH groups was conducted according to Ellman (1959), using dithiobis(2-nitrobenzoic acid)−DTNB as substrate. The product of the reaction was monitored at 412 nm. The amount of free SH groups in the sample were expressed in millimolars per milliliter and calculated from standard curve using GSH as a standard. Protein concentration was determined according to Bradford (1976) using bovine serum albumin as the standard.

Statistical analysis

The results were processed using the statistical package SAS 9.1.3 (2003). Prerequisite for the analysis of variance was the normality of distribution within a group which was achieved through logarithmic transformations of the traits (Sokal and Rohlf 1981). Differences in the average values between the control and treatment experimental groups within each larval instar were assessed by one-way ANOVA and Tukey's HSD test. Two-way ANOVA was applied in order to test the significance of the main effects, cadmium (Cd) and larval instar (I), and their interaction ("Cd×*I*").

Results

Cadmium effects on larval fitness-related traits

Development of *L. dispar* larvae fed on cadmium-supplemented artificial diet was significantly delayed in the fourth, fifth, and sixth instar, while the duration during the third instar was not significantly changed in response to cadmium intoxication (Table 1). There was a decreasing trend in the mass of cadmium-treated larvae in all investigated instars, but statistically significant differences were found only in the fourth and fifth larval instar in comparison to the control group (Table 2).

Cadmium effects on antioxidative defense

Antioxidative defense strategies by *L. dispar* were dependent of larval stage as shown by the patterns of antioxidative enzyme activities across all larval stages tested. No significant differences were observed for SOD activities between controls and Cd treatment groups (Fig. 1).

In the third larval instar, CAT activity was significantly decreased in response to the presence of Cd in the diet, while the sixth instar showed significantly higher activities of CAT in larvae reared on the cadmium-supplemented diet, compared to the control groups (Fig. 2). Two-way ANOVA revealed a significant effect of larval stage and a significant "Cd×I" interaction, i.e., differences of the CAT activities among larval instars in response to the stressor (Fig. 2).

APOX was dependent on *L. dispar* larval stage (Fig. 3). Statistically significant decreases in APOX activity were found in cadmium-treated experimental groups of the fifth larval instar when compared to untreated larvae (Fig. 3). Similar to CAT activity, the activity of GR in gypsy moth larvae significantly decreased in the third instar but significantly increased in the sixth instar larvae after the exposure to Cd treatment when compared to untreated controls. GR activity significantly decreased during development of control group larvae, while GR activity of cadmium-treated larvae did not change throughout development (significant "I×Cd" interaction) (Fig. 4).

A significant increase of GST activity was observed in the third larval instar in cadmium-treated larvae relative to controls (Fig. 5). There was a weak but significant decrease in total glutathione concentrations after exposure of all instar stages to Cd (Fig. 6). A significant increase in SH groups was observed after exposure of instars II, III, and IV to Cd. While SH group concentrations in control animals decreased as a function of increasing larval stage, an increasing trend occurred for Cd-treated moths (Fig. 7). These results also indicate that total glutathione amount and SH groups were dependent both on cadmium treatment and larval instar. Developmental changes differed significantly between control and cadmium-treated groups (I×Cd). While the amount of SH groups decreased through the development of control larvae, the opposite trend can be noticed in larvae reared on cadmium-supplemented diet (Fig. 7).

Discussion

Insects are frequently exposed to heavy metals in the environment and are therefore dependent on efficient detoxification systems for their survival under such conditions. Biochemical defense mechanisms against heavy metal stress (changes in many enzyme activities, low molecular mass molecules, metallothionein, Hsp synthesis, etc.) protect insects against adverse effects of metal intoxication (Korsloot et al. 2004). However, these mechanisms are energetically expensive and can consequently affect fitness-related traits in insects (Mouneyrac et al. 2011). The results of our study showed significant effects of cadmium-supplemented diet on fitness-related traits in *L. dispar* larvae. The duration of larval stages was prolonged and larval body mass was reduced after the exposure to Cd (Tables 1 and 2) which is all in agreement with literature data on the gypsy moth and other insects (Schmidt et al. 1991; McChaton and Pascoe 1991; Vlahović et al. 2001; Niu et al. 2002; Cervera et al. 2004; Mirčić et al. 2010).

Table 1 Effects of Cd on gypsy moth larvae developmental duration

Larval instar	Number	Control (days)	Cd treated (days)	Tukey's HSD test
III	165	12.14±0.17	12.38±0.13	ns
IV	115	16.98±0.12	17.49±0.16	*
V	90	22.45±0.17	23.20±0.20	**
VI	45	28.04±0.19	29.00±0.31	*

Results are expressed as means ± SE. Results are compared by Tukey's honest significance difference (HSD) test

ns nonsignificant

*$P<0.05$; **$P<0.01$ (significant differences to the control)

Table 2 Effects of Cd on gypsy moth larvae body weight during development

Larval instar	Number	Control (g)	Cd treated (g)	Tukey's HSD test
III	50	0.060±0.002	0.055±0.002	ns
IV	25	0.225±0.010	0.182±0.013	**
V	15	0.608±0.051	0.437±0.035	*
VI	15	1.842±0.067	1.886±0.073	ns

Results are expressed as means ± SE. Results are analyzed by Tukey's honest significance difference (HSD) test

ns nonsignificant

*$P<0.05$; **$P<0.01$ (significant differences to the control)

Furthermore, the concentration of cadmium used in our experiment was approximate to the concentration in polluted areas (Knežević 2004).

Chemical properties of Cd and its structure determine the uptake by cells and interaction with biological macromolecules. Literature data demonstrated that the insect gut, as the largest system besides integument in insects, is the main cadmium-accumulating organ. The gut epithelium and peritrophic matrix of insects are targets for environmental pollutants (Lauverjat et al. 1989). High concentrations of cadmium in food can destroy the midgut structure and impact fundamental processes (digestion and absorption of food) causing starvation effects and reduction in larval growth and survival (Rayms-Keller et al. 1998, 2000; Vlahović et al. 2001; Chinni and Yallapragda 2000; Maryanski et al. 2002; Nursita et al. 2005; Wu et al. 2006). Our results showed that chronic Cd treatment by food ingestion reduced body mass of Gypsy moth caterpillars, which is in agreement with general Cd effects.

Together with the reducing effect of the larval mass, exposure to heavy metals can lead to neuroendocrine/hormonal disruption. This is of major concern as these processes regulate development of insects (Ivanović and Janković-Hladni 1991; Ilijin et al. 2009). Our previous results showed that cadmium intoxication was correlate with changes in synthetic and secretory activity of protocerebral neurosecretory cells (Ilijin et al. 2009) which regulate the level of morphogenetic hormones in hemolymph (juvenile hormones and ecdysone). These changes may explain the prolongation of larval instars noted in our experiment (Table 1).

As a non-redox metal, Cd is not directly responsible for the production of free radicals (Dallinger and Rainbow 1991). However, the increasing profile of catalase activity from younger to older larval (the fourth to sixth) instar after treatment of *L. dispar* with Cd indicated the presence of peroxides and other damaging molecular species (Fig. 2). Since there are no changes in SOD activity after Cd treatment, elevated peroxydes levels could be mainly derived from the metabolic oxidoreduction processes performed by various oxidases in peroxisomes such as xanthine oxidase, cytochrome P 450 monooxygenases, etc. (Fornazier et al. 2002; Halliwell and Gutteridge 2007). Catalase activity was

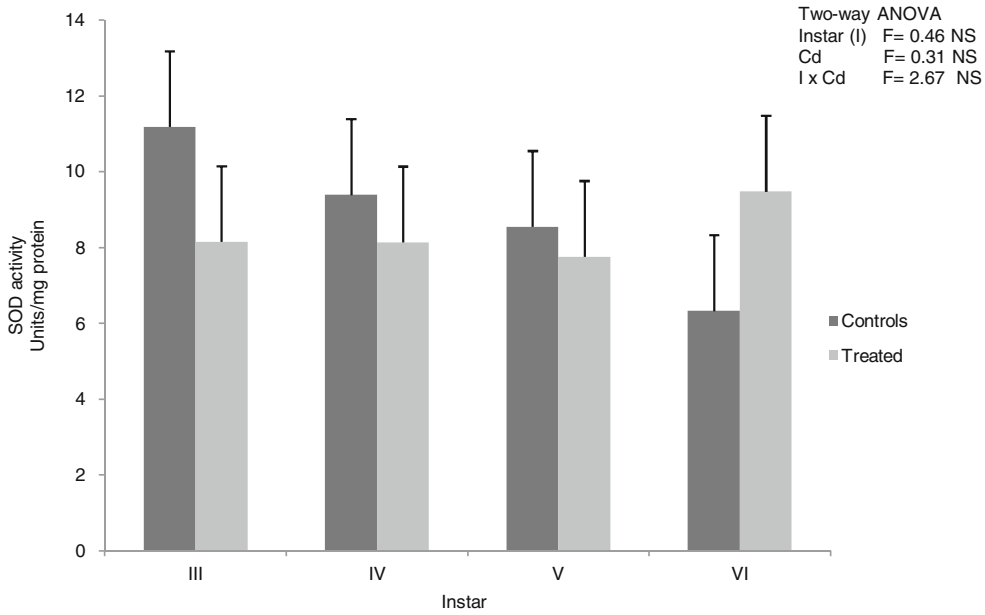

Fig. 1 Effects of cadmium on superoxide dismutase activity in *L. dispar* during larval development (third to sixth instar). *Bars* represent the means (± S.E.), $n=165$. Significance of the effects of instar (*I*), cadmium (*Cd*), and their interaction (*I*× *Cd*) was tested by two-way ANOVA (*F* values are presented; *NS* nonsignificant) and post hoc compared by Tukey's honest significant difference (HSD) test. (*$P<0.05$; **$P<0.01$; ***$P<0.001$)

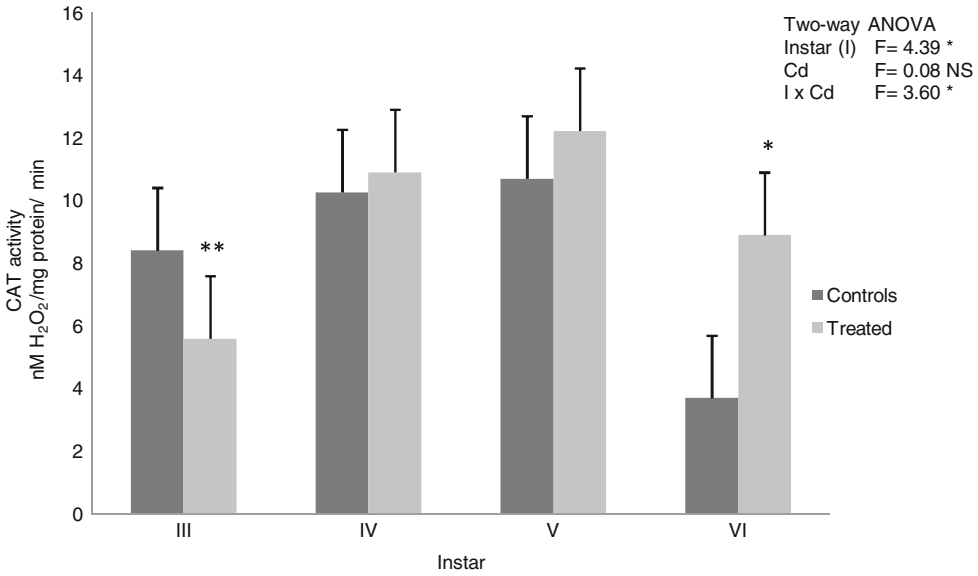

Fig. 2 Effects of cadmium on catalase activity in *L. dispar* during larval development (third to sixth instar). *Bars* represent the means (± S.E.), n=165. Significance of the effects of instar (*I*), cadmium (*Cd*), and their interaction (*I*× *Cd*) was tested by two-way ANOVA (*F* values are presented; *NS* nonsignificant) and post hoc compared by Tukey's honest significant difference (HSD) test. (*P<0.05; **P<0.01; ***P<0.001)

directly induced by the presence of certain concentrations of peroxide in the body (Halliwell and Gutteridge 2007). Two-way ANOVA showed a statistically significant effect for developmental stage and Cd, as well as a statistically significant interaction with catalase activity, suggesting that cadmium effects on CAT activities were dependent on developmental stage (Fig. 2). This also suggests that the influence of Cd-mediated prooxidative pressure depends on the larval instar.

The response of antioxidant enzymes to external stressors can vary among species, different tissues (Wilczek et al. 2004), and development stage (Perić-Mataruga et al. 1997; Jovanović-Galović et al. 2004). Since insects apparently lack Se-dependent glutathione peroxidase, APOX is important for removing H_2O_2 in insects, along CAT (Smith and Shrift 1979; Sohal et al. 1990; Ahmad 1992; Felton and Summers 1995; Jovanović-Galović et al. 2004). APOX uses ascorbic acid as the reducing agent in the first step of the ascorbate–glutathione cycle. Our results showed differences in activity profiles between young (the third and fourth—trend of increase) larvae that are less resistant to intoxication and older larval stages (the fifth and sixth—trend of decrease). Two-way ANOVA showed a statistically significant impact of the developmental stage on APOX activity (Fig. 3). However, decreased APOX activity was compensated by the increased activity of other peroxide-degrading mechanisms like CAT (Fig. 2) or non-enzymatic cellular antioxidants (Fig. 7). In summary, Cd exposure could be

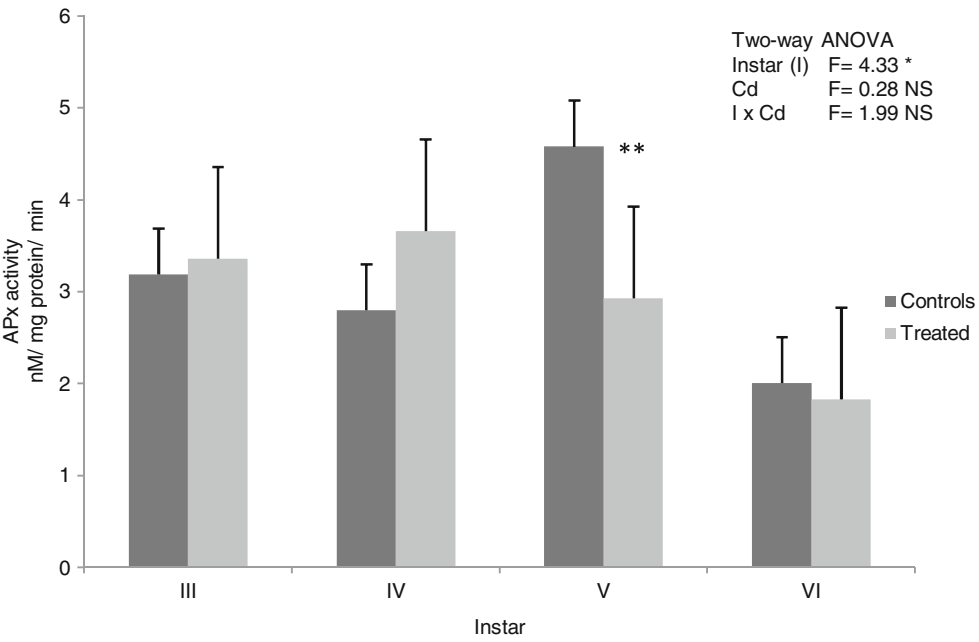

Fig. 3 Effects of cadmium on ascorbate peroxidase activity in *L. dispar* during larval development (third to sixth instar). *Bars* represent the means (± S.E.), n=165. Significance of the effects of instar (*I*), cadmium (*Cd*), and their interaction (*I*× *Cd*) was tested by two-way ANOVA (*F* values are presented; *NS* nonsignificant) and post hoc compared by Tukey's honest significant difference (HSD) test. (*P<0.05; **P<0.01; ***P<0.001)

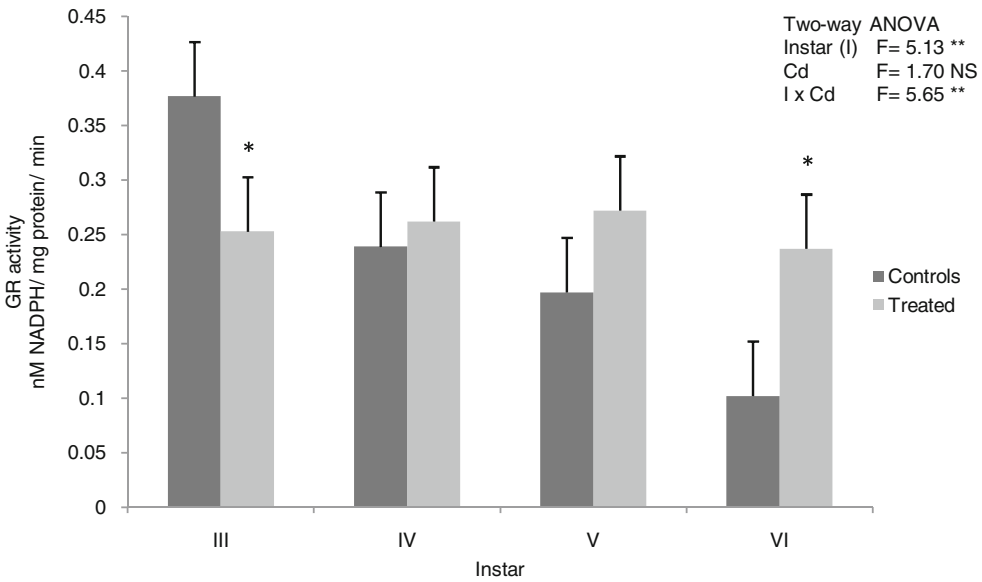

Fig. 4 Effects of cadmium on glutathione reductase activity in *L. dispar* during larval development (third to sixth instar). *Bars* represent the means (± S.E.), $n=165$. Significance of the effects of instar (*I*), cadmium (*Cd*), and their interaction (*I*× *Cd*) was tested by two-way ANOVA (*F* values are presented; *NS* nonsignificant) and post hoc compared by Tukey's honest significant difference (HSD) test. (*$P<0.05$; **$P<0.01$; ***$P<0.001$)

related to either activation or inhibition of components of the antioxidative defense in insects but strongly depends on larval development stage and various morphological changes (Figs. 2, 3, 4, 5, 6, and 7).

GST in insects represents an important line of defense against free radicals and may reflect the adaptation of insects to exposure with high concentrations of toxic chemicals (Perić-Mataruga et al. 1997). Interestingly, GST activity was higher in the youngest larval stage than in the control group after exposure to Cd through food (Fig. 5). This could indicate that at later larval stages (i.e., when the toxic effects of cadmium accumulated), other detoxification mechanisms such as intensive synthesis of metal binding proteins are present. There is evidence that the cumulative effect of Cd can inhibit the activity of glutathione-dependent enzymes including GST, through the inhibition γ-glutamilcysteine synthetase and glutathione biosynthesis (Canesi et al. 1999). This is in accordance with our results that showed less total glutathione in treated animals than in the controls (Fig. 6).

However, there is a lack of information on the in vivo effect of the heavy metals on glutathione circle protection system of *L. dispar*. GSH is involved in both enzymatic and non-enzymatic antioxidative processes. Most studies on the relationship between cellular glutathione level and metal toxicity infer that glutathione has a protective function against metal-induced toxicity (Saint-Denis et al. 2001). As a result, GSH becomes depleted and GSSG accumulates, thus indicating oxidative stress. The oxidation of sulfhydryl groups is also critically dependent on pH (Irons and

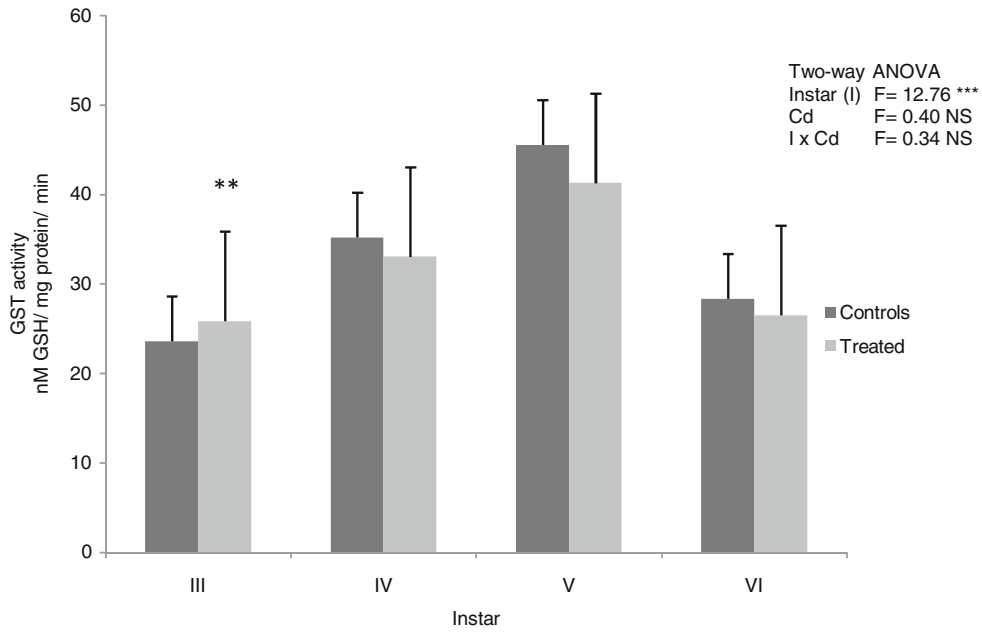

Fig. 5 Effects of cadmium on glutathione-*S*-transferase activity in *L. dispar* during larval development (third to sixth instar). *Bars* represent the means (± S.E.), $n=165$. Significance of the effects of instar (*I*), cadmium (*Cd*), and their interaction (*I*× *Cd*) was tested by two-way ANOVA (*F* values are presented; *NS* nonsignificant) and post hoc compared by Tukey's honest significant difference (HSD) test. (*$P<0.05$; **$P<0.01$; ***$P<0.001$)

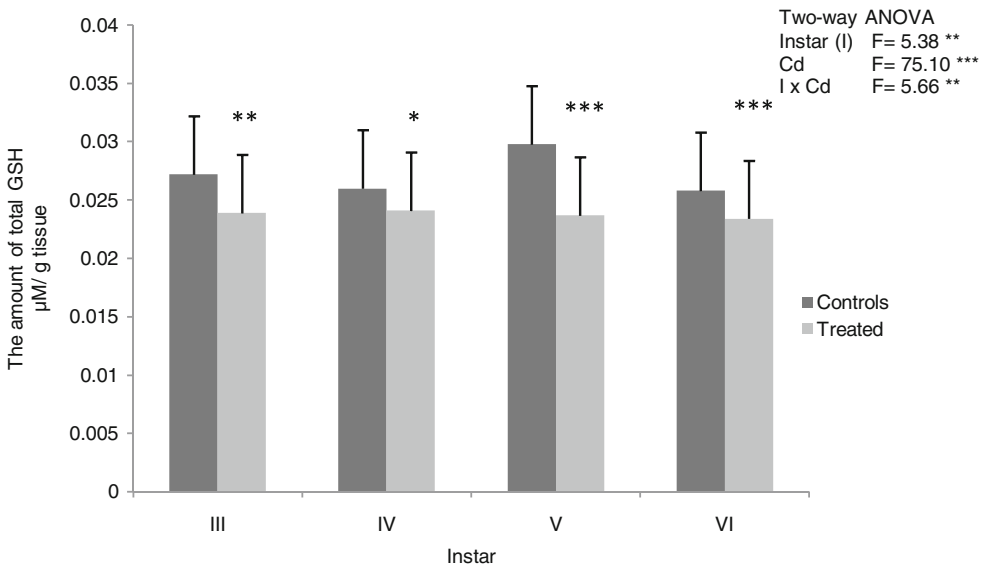

Fig. 6 Effects of cadmium on the amount of GSH in *L. dispar* during larval development (third o sixth instar). *Bars* represent the means (± S.E.), n=165. Significance of the effects of instar (*I*), cadmium (*Cd*), and their interaction (*I*× *Cd*) was tested by two-way ANOVA (*F* values are presented; *NS* nonsignificant) and post hoc compared by Tukey's honest significant difference (HSD) test. (*P<0.05; **P<0.01; ***P<0.001)

Sawahata 1985). At pH 10 in the *L. dispar* larvae midgut, this oxidation can readily occur. At normal levels of oxidative stress, glutathione reductase activity and NADPH availability are adequate to maintain the proper GSSG/GSH ratio in cellular tissues (Halliwell and Gutteridge 2007). Our results showed increasing glutathione reductase activities in cadmium-treated larvae except in the third instar when larvae are most sensitive to intoxication (Perić Mataruga et al. 1988, 1997). In the oldest stage, when the cumulative effect of cadmium was the strongest, GR was significantly increased (Fig. 4.) In our experiment, there was a decreased amount of GSH in cadmium-treated *L. dispar* L. larvae (Fig. 6). Higher GR activity could speed up the reduction of oxidized glutathione and compensate the general lack of GSH to maintain a thiol/disulfide redox potential after cadmium intoxication of *L. dispar* larvae.

Cd tends to bind with any molecule that has sulfur or sulfur–hydrogen combination in its structure and which can reduce the functionality of protein molecules and reduce redox homeostasis. Therefore, the amount of total SH groups is not just the measure of the level of prooxidative pressure but the indice of functional redox state of cells. The presence of large amounts of total sulfhydryl groups in *L. dispar* larvae treated with Cd suggested elevated concentrations of essential thiols in the test organism.

Our results showed that the amount of free SH groups was significantly higher in cadmium-treated larvae in the fourth, fifth, and sixth instar in comparison to the control. The amount of free SH groups depended on larval age and the presence of cadmium in the diet. The level of free SH groups decreased during development of larvae fed on a cadmium-free diet while opposite trends of SH group

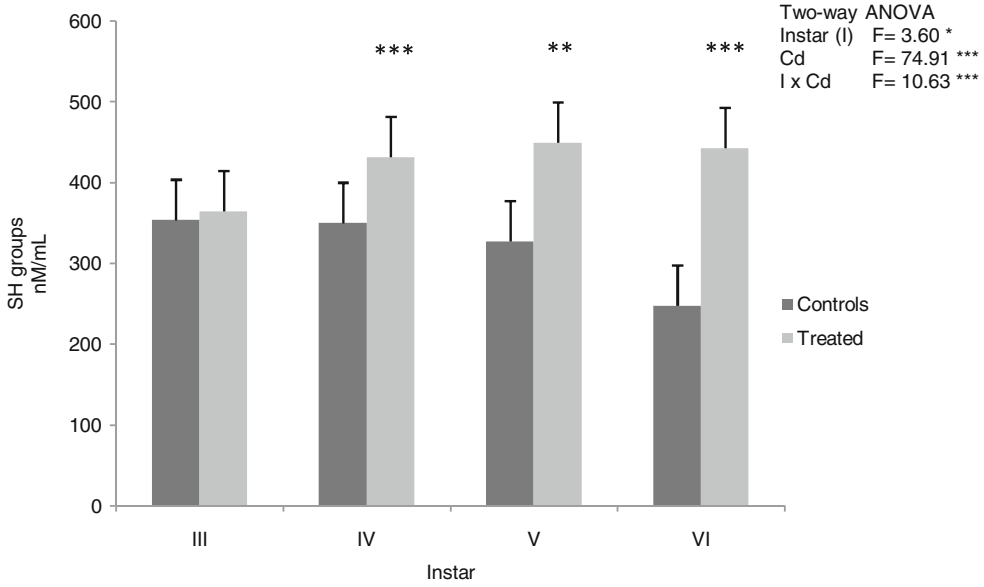

Fig. 7 Effects of cadmium on the amount of total free SH groups in *L. dispar* during larval development (third to sixth instar). *Bars* represent the means (± S.E.), n=165. Significance of the effects of instar (*I*), cadmium (*Cd*), and their interaction (*I*× *Cd*) was tested by two-way ANOVA (*F* values are presented; *NS* nonsignificant) and post hoc compared by Tukey's honest significant difference (HSD) test. (*P<0.05; **P<0.01; ***P<0.001)

concentrations were noticed in cadmium-treated larvae (a significant "I×Cd" interaction). Studying the contribution of protein SH groups in oxidative defense proved that the reactivity of SH groups in relation to GSH varies in different species of animals (Halliwell and Gutteridge 2007). In several studies, it was concluded that exposure to heavy metals such as Cd causes an increase in thiol compounds in living organisms, and it was proposed as an effective indicator of pollution with Cd. Another important feature of the sulfhydryl groups is that their quantity is a marker of protein oxidation after intoxication (Shacter 2000).

Conclusions

Exposure to a Cd-supplemented diet affected fitness-related traits in *L. dispar* larvae. The duration of the larval stages was prolonged and body mass of larvae was reduced after the exposure to cadmium treatment. The interrelationship, activity, and amount of components of antioxidative defense during *L. dispar* development suggested that the regulation of prooxidant/antioxidant equilibrium depends on the susceptibility of particular larval developmental stages to metal toxicity. The antioxidative defense reorganization/detoxification has elevated metabolic cost for processing cadmium-supplemented food and allocation of resources towards defense mechanisms and induction of antioxidative system components which consequently influence on life history traits (development, growth, etc.).

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