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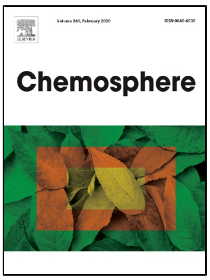

The effect of long term exposure to cadmium on *Ostrinia nubilalis* growth, development, survival rate and oxidative status

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The effect of long term exposure to cadmium on *Ostrinia nubilalis* growth, development, survival rate and oxidative status

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Key words: heavy metals, bioaccumulation, larvae, pupae, gene expression, MDA

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Abstract

In this study the effect of long term exposure to cadmium (Cd) on *Ostrinia nubilalis* larval growth, development, survival rate and oxidative status was analyzed. Newly hatched first instar - L1 larvae were reared on a Cd contaminated diet until the larvae reached the final, fifth instar - L5 or developed into pupae. In total, six experimental groups, five treatments (concentrations of Cd in fresh diet: Cd I: 0.73, Cd II: 3.70, Cd III: 6.85, Cd IV: 41.71 and Cd V:

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77.53 mg kg^{-1}) and a control group (C) were set up. The results of the experiment showed that exposure to higher concentrations of Cd (41.71 and 77.53 mg kg^{-1}) had a significant influence on development and redox status of *O. nubilalis* larvae: (1) the development rate was strongly reduced resulting in a prolonged pupation time; (2) the survival rate of larvae was prominently lower; (3) bioaccumulation factor (measured in pupae) was reduced which indicated that larvae could accumulate Cd to a certain level; (4) the level of the lipid peroxidation was significantly higher, which points to oxidative damage; (5) the expression of *Mtn* was significantly up-regulated while *Cat* and *GPx* genes down-regulated. In conclusion, long term exposure to dietary Cd in a concentration of 41.7 mg kg^{-1} and higher, induced oxidative stress and slowed down growth and development of *O. nubilalis* larvae.

Introduction

Environmental pollution is a consequence of both natural and anthropogenic activities. Atmospheric deposition (e.g. forest fires, volcanic eruptions, soil erosion, air pollution), industrial by-products, the application of phosphate fertilizers in agriculture (mineral, sewage sludge and manure) represent major sources of Cd input in soils (Hooda, 2010; Roberts, 2014). Fertilizers contains Cd, since phosphate rock is used as an essential feedstock for industrially produced fertilizers. According to Van Kauwenbergh (2001) fertilizers can contain from 2 to over 100 mg kg^{-1} of Cd depending on the phosphate rock source. This could have a serious impact on organisms feeding on plants on which these plant products were applied.

Cadmium (Cd), a toxic heavy metal, accumulates in plants to concentrations that could be potentially toxic to most herbivores without having any effect on plant growth (Hooda, 2010; Godinho et al., 2018). Long term exposure to heavy metals in some insects can reduce growth,

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development time, reproduction, and/or hatchability (Schmidt et al., 1991; Gintenreiter et al., 1993; Sildanchandra and Crane, 2000; Niu et al., 2002; Cervera et al., 2004; Gao et al., 2017). According to Vigneron et al. (2019) Cd tolerance inheritance within the field population does not demonstrate genetic adaptation, as the transgenerational plasticity was induced by parental exposure to cadmium.

Cadmium has been a main point of interest for many years due to different aspects of its pro-oxidative nature (Bertin and Averback 2006; Gallego et al., 2007). The detrimental action of cadmium reflects not only on DNA strand breaks induction, chromosome aberration and gene expression alterations, but can also have other severe consequences including tissue damages, morphological deformities and even death of living organisms (Wang et al., 2013). Several reports have shown that oxidative stress is an important mechanism of Cd toxicity (Kim and Sharma, 2006; Filipic et al., 2006). The key defense mechanisms of front line protection, important for the reduction of the toxic effect of metals on a molecular level, include the synthesis of chelating agents, activation of enzymatic and non-enzymatic antioxidative processes and activation of metallothioneins, small cysteine-rich heavy metal-binding proteins, with a very prominent role in metal homeostasis (Stegeman et al., 1992; Augustyniak and Migula, 2000; Chandrana et al., 2005; Augustyniak et al., 2009; Wang et al., 2013). Cells with more metallothioneins are protected against heavy metal toxicity and oxidative stress, whereas under-expression in cell lines lead to elevated sensitivity to cadmium resulting in oxidative stress (Andrews, 2000; Wang et al., 2013). The oxidative stress caused by metals needs an effective antioxidant defense system to meliorate potential tissue damage. Among various mechanisms of antioxidant defense, catalase (CAT), superoxide dismutase (SOD), glutathione transferase (GST) as well as glutathione peroxidase (GPx) play a crucial role, as their gene expression is up-

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regulated as front line defense against Cd toxicity (Andrews, 2000; Wang et al., 2013; Nikolić et al., 2016). Lipid peroxidation, indicated by malondialdehyde (MDA) level, is one of the key parameters for the assessment of oxidative stress, commonly accepted as an effective biomarker of toxic pollutant exposure (Li et al., 2012; Wang et al., 2013).

The European corn borer (*Ostrinia nubilalis*, Hbn.) is a holometabolous polyphagous highly adaptable agricultural pest, widespread through the temperate regions of Eurasia and North America. It has a very significant economic impact, primarily as a pest of maize, and several other crops. In Southeast Europe it has mainly two generations per year: one non-diapausing and one diapausing (overwintering). *O. nubilalis* has highly adaptive characters for external environmental conditions as its larvae are able to survive under very diverse harsh environmental conditions as a result of physiological adaptations (Vukašinović et al., 2018). The 5th instar larvae of diapausing generation of *O. nubilalis* are able to survive highly unfavorable environmental conditions such as harsh winters, as cold-hardy freeze tolerant larvae (Grubor-Lajšić et al., 1991), inside corn stalk debris, left on harvested fields. As the generation of non-diapausing larvae is actively feeding in the corn stalk during summer, causing significant damage to crops, they may be exposed to accumulated cadmium from the field. We were interested in the ability of larvae that originated from field population to cope with high concentrations of cadmium in their diet since according to our knowledge, there are no studies about the influence of heavy metals on this species, nor about concentrations that influence growth, development and survival rate. The aim of this study was to explore the effect of long term exposure to cadmium on *O. nubilalis* larval growth, development, survival rate and oxidative stress.

Material and methods

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Insect material

Newly hatched non-diapausing larvae (first instar, L1) of *Ostrinia nubilalis* were obtained from several egg masses laid on maize leaves in June, 2018, from the Institute of Field and Vegetable Crops, located near Novi Sad, Serbia (19°51E; 45°20N; 84 m a.s.l.).

Long term cadmium exposure (experimental design)

The influence of long term exposure to Cd on the development of *O. nubilalis* larvae was followed by rearing newly hatched L1 larvae on Cd contaminated diet and maintained until larvae reached L5 or developed to pupae. In total, six experimental groups, five treatments (Cd I, Cd II, Cd III, Cd IV and Cd V) and a control (C, non-treated diet) were set up. The nominal concentrations of exposure solutions in Cd I, Cd II, Cd III, Cd IV and Cd V were 1, 5, 10, 50 and 100 mg kg^{-1} while the actual concentrations were 0.73, 3.70, 6.85, 41.71, 77.53 mg kg^{-1}, respectively. Each group was comprised of three biological replicates between fifteen and twenty L1 larvae per replicate. Larvae were reared in laboratory under controlled conditions with naturally fluctuating temperatures between 25 and 30ºC and a long-day photoperiod. For each biological replicate, 70 g of artificial diet was weighted and added to 200 ml glass jars with manually perforated lid allowing air to flow. Larvae of the control group (C) were fed on a diet without Cd consisting of a mixture of wheat germs, oat brans, barley flakes, fresh yeast, sucrose, agar, vitamin C, sodium benzoate and distilled water. Treatments consisted of a diet with increasing concentration of Cd, contaminated by addition of $CdCl_2$, thoroughly mixed and left for several hours before newly hatched L1 larvae were fed with it.

Sampling regime

The sampling was performed on the 18th, 22nd and 28th day of development (Table 1). First sampling of larvae (18th day) was performed when the first pupae were noticed in the control (C) and treated groups (Cd I, Cd II and Cd III). Nine larvae were sampled from each experimental group. Second sampling of larvae and pupae (22nd day), was performed when approximately 50% of larvae developed to pupae in the control (C) and Cd I, Cd II and Cd III groups. The following number of L5 larvae and pupae were collected: C: 12 larvae, 16 pupae; Cd I: 9 larvae, 15 pupae; Cd II: 14 larvae, 25 pupae; Cd III: 13 larvae, 25 pupae; Cd IV: 9 larvae. The last sampling of larvae and pupae (28th day), was performed when approximately 50% of larvae developed to pupae in Cd IV group only one pupa in Cd V group appeared, while larvae reached L4-L5 instar. From Cd IV group, 8 larvae of L5 instar and 6 pupae were collected and from Cd V group, 6 L5 larvae and 1 pupa. Larval weight and pupal weight and length were measured at the specified sampling times. Samples of larvae, pupae and artificial diet were immediately frozen and kept at -80°C until further analysis.

Table 1. Larval stage appearance/pupae notification.

	C	**Cd I**	**Cd II**	**Cd III**	**Cd IV**	**Cd V**
1. day	L1 larvae					
18. day	first pupa noticed L4-L5 larvae, #				L3-L4 larvae, #	L2-L3 larvae, #
22. day	~50% pupae, * L5 larvae, #				first pupa noticed L4-L5 larvae, #	L3-L4 larvae
28. day					~50% pupae, * L5 larvae, #	first pupae noticed, * L4-L5 larvae, #

larvae sampling, * pupae sampling

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First, second, third, fourth and fifth instar larvae: L1, L2, L3, L4 and L5, respectively. Names of experimental groups and the concentrations of Cd in artificial diet (mg kg^{-1}): Cd I: 0.73, Cd II: 3.70, Cd III: 6.85, Cd IV: 41.71, Cd V: 77.53 and control C: 0.03.

Metal concentration assessment

Concentration of Cd was measured in pupae and diet. Pupae from Cd I, Cd II, Cd III and control (C) groups were collected during the second sampling (22^{nd} day) while from Cd IV and Cd V during third sampling (28^{th} day). From each of the three biological replicate for each experimental group, 2-9 pupae and about 2 g of diet was intended for Cd concentration analysis. The exception was the Cd V group which was represented with only one pupa. Cd measurements were made in technical triplets.

The moisture of pupae and diet samples was measured by the gravimetric method. The amount of moisture of pupae and diet was about 70% and 3%, respectively. The other part of the original pupae samples was dried at 50ºC during 48 h, before further preparations. The pupae and diet samples were prepared by microwave digestion (1:24, w/v) in concentrated HNO_3 and H_2O_2 (5:1, v/v) by gradually heating up to 180°C using a Milestone Vario EL III for 55 min. The concentration of Cd was determined by ICP-OES (Vista Pro-Axial, Varian) in accordance with US EPA method 200.7:2001. Quality control was periodically carried out with reference materials SRM1515 (Apple leaves) and IRMM BCR 142R (Light sandy soils) and deviations were within ±10% of the certified values. The limit of detection (LOD) was 0.01 mg kg^{-1}, which provided adequate sensitivity to analyze.

Malondialdehyde (MDA) level measurements

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The content of MDA was measured in L5 larvae from Cd I, Cd III, Cd IV and control (C) groups collected during the second sampling (22nd day). Due to high larval mortality in the experimental group Cd V, we did not have enough larvae to analyze this parameter. The larvae of *O. nubilalis* were frozen in liquid nitrogen (three biological replicates, of each experimental group, consisting of 3 larvae per replicate) and ground to a fine powder with a mortar and pestle. Subsequently, the powder was homogenized with ice cold 50 mM phosphate buffer pH 7.0 (20%, w/v). The crude homogenates were centrifuged at 10 000 g (4°C) for 10 min. The supernatants were placed into new tubes and stored at -80ºC until analysis.

MDA levels in homogenate were measured according to the method of Rehoncorna et al. (1980), which is based on formation of colored complex as a result of the reaction of malondialdehyde, the specific product of lipid peroxidation, and thiobarbituric acid (TBA). The maximum absorption is at 532 nm, expressed in nmol per milligram of protein. The protein concentration in homogenates was determined using the Bradford method, with bovine γ-globulin as protein standard (Bradford, 1976).

Total RNA isolation, cDNA synthesis, quantitative PCR

Relative gene expression was measured in larvae from Cd I, Cd III and control (C) groups collected during the first sampling (18th day), and Cd IV and Cd V groups collected during the third sampling (28th day). Total RNA from fifth instar larvae (2 larvae from each of the three biological replicate for each experimental group) was extracted using TRIzol. Integrity of isolated RNA was tested on 1% agarose gel electrophoresis, while the concentration and purity was checked using BioSpec-nano Micro-volume UV-Vis Spectrophotometer (Shimadzu). Synthesis of cDNA was carried out using High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems) according to the manufacturer's protocol, starting with 2

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µg of total RNA. Relative gene expression was measured for metallothionein (*Mtn*), catalase (*Cat*) and glutathione peroxidase (*GPx*), using genes for β-actin (*ActB*) and ribosomal protein S5 (*Rps5*) as endogenous controls. Primers were designed using the NCBI PrimerBlast (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Primer efficacy was calculated using a standard curve with a 10 fold dilution series of cDNA. PCR primer sequences and efficiencies are shown in Table 2.

All analysis were done in a technical duplicate. Reaction included 7µL of 2X SYBR® Green PCR Master Mix (Applied Biosystems), 200 nM of each primer and 50 ng cDNA in total volume of 14µL. Quantitative PCR on the cDNA products was carried out using MasterCycler RealPlex^4 (Eppendorf). The amplification program consisted of a pre-incubation step at 95^{o}C (10 min) and 40 cycles of 95^{o}C (15 s) and 60^{o}C (1 min) with an additional melt-curve analysis to confirm the specificity of amplification.

Table 2. PCR primer sequences.

Amplification target		**Sequence**	**Efficiency**	**Reference**
β-Actin (150 bp)	*ActB.F*	CAGAAGGAAATCACAGCTCTAGCC	89.9%	Popović et al., 2015
	ActB.R	ATCGTACTCCTGTTTCGAGATCCA		
Ribosomal protein S5 (101 bp)	*Rps5.F*	AACTCCGGACCCCGTGAAGA	112.5%	
	Rps5.R	ATGGCCTGGTTCACACGACG		
Metallothionein (144 bp)	*Mtn.F*	CGTCGGCTTGCACCTGTGTAT	90.7%	
	Mtn.R	CAGCGCAGTTGCACGAAGAG		
Catalase (111 bp)	*Cat.F*	GCTTCAAGAGACCCCGCCTC	96.3%	
	Cat.R	TGCAGTCTTCACGCCCACAG		
Glutathione peroxidase (137 bp)	*GPx.F*	TGGCGATACAGCGAGTCCAC	92.7%	
	GPx.R	TTTGTGGGCCATGGCGTTCT		

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Data analysis

Development time of larvae was defined as the number of days between hatching and the day when the first pupa was observed. Survival rate (expressed in percentages) was defined as the sum of the number of larvae and pupae from three samplings, divided by the number of L1 larvae at the beginning of the experiment.

Bioaccumulation factor (BAF) was defined as the ratio of metal concentration in pupae over the metal concentration in diet.

The data on heavy metal and MDA concentrations were analyzed using STATISTICA 13 software with ANOVA followed by Dunnett's post-hoc test and expressed as mean ± SE. Significant difference was estimated with $p<0.05$ confidence intervals.

The difference in the analyzed gene expression was calculated by REST 2009 software (Qiagen), where relative up- or down- regulations were calculated and tested for statistical significance by the integrated Bootstrap randomization test (2000 iterations) between control and treated groups for $p<0.05$ confidence intervals.

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Results

The concentration of Cd in pupae and diet

The average concentration of Cd in diet and pupae, as well as calculated bioaccumulation factor (BAF) are given in Table 3. The results showed that with increasing concentration of Cd in diet the concentration of Cd in pupae has also increased (Table 3). However, the bioaccumulation factor (BAF) decreases with increasing concentration of Cd in diet. In the experimental groups Cd I, Cd II and Cd III the concentration of Cd highly exceeded the

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concentration in diet, BAF was above 3.5. On the other side in the experimental groups Cd IV and Cd V the accumulation was lower and BAF was below 1.

Table 3: The concentration of Cd in pupae and diet [mgkg^{-1}] and the bioaccumulation factor (BAF) in Cd treated groups and control of *O. nubilalis*.

	C	Cd I	Cd II	Cd III	Cd IV	Cd V
Diet [mg kg^{-1}]	0.03	0.73	3.70	6.85	41.71	77.53
Pupae [mg kg^{-1}]	1.10 (n=16)	3.12 (n=15)	14.67 (n=25)	25.05 (n=25)	38.66 (n=6)	57.23 (n=1)
BAF		4.30	3.96	3.66	0.93	0.74

Mean values of Cd (mg kg^{-1}) in pupae and diet. Names of experimental groups and the concentrations of Cd in artificial diet (mg kg^{-1}): Cd I: 0.73, Cd II: 3.70, Cd III: 6.85, Cd IV: 41.71, Cd V: 77.53 and control C: 0.03.

Effect of Cd exposure to larval development and survival rate

The first pupae were noticed on the 18th day of the experiment (1st sampling), in the control (C) and experimental groups Cd I, Cd II and Cd III and during this period, the larvae reached L4 or L5. In experimental groups Cd IV and Cd V the larvae reached L3-L4 and L2-L3, respectively (Fig. 1; Table 1).

Approximately 50% of larvae developed to pupae on the 22nd day of the experiment (2nd sampling) in the control (C) and experimental groups Cd I, Cd II and Cd III. At the same time, in the Cd IV group the first pupa was noticed while larvae mainly reached L4-L5 and in Cd V group larvae developed to L3-L4 (Table 1).

Aproximatelly 50% of larvae developed to pupae on the 28th day of the experiment (3rd sampling) in the Cd IV group while in the Cd V group only one pupa emerged at the same time and larvae mainly reached L4-L5 (Table 1).

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In the experimental groups with lower concentrations of Cd (Cd I, Cd II and Cd III) the development time of larvae lasted for 18 days and did not differ from the development time of larvae in the control group (C). The first appearance of pupae was noticed on the same day as in the control (C) (Table 1). In the experimental groups with higher concentrations (Cd IV and Cd V), the development rate of larvae was strongly reduced. The first pupa emerged 4 and 10 days later than in the control group (C) (Table 1), i.e. the overall development time of *O. nubilalis* larvae, from L1 until pupation, lasted 22 and 28 days, respectively.

The inhibiting effect of Cd on the development was also confirmed on the 18th day of the experiment (1st sampling) analyzing the weight of larvae exposed to various Cd treatments and control (Figure 2A). The larval weight was significantly lower in Cd IV and Cd V groups, in comparison to the control (C). Additionally, the body weight of larvae, successfully developed to L5, was significantly lower in the experimental groups exposed to Cd concentration 41.71 (Cd IV) and 77.53 (Cd V) mg kg^{-1} of fresh diet in comparison to the control (Fig. 2B). Consequently the pupal weight and length were significantly lower in Cd IV as well (Fig. 2C and D). Because of high mortality in Cd V group, only one larva developed to pupa, so the statistical parameters could not be calculated for this experimental group.

The survival rate of larvae in experimental groups Cd IV and Cd V was highly reduced to 60% and 40%, respectively, in comparison with C, Cd I, Cd II, and Cd III groups with the survival rate over 85%.

Effect of Cd exposure on the level of MDA

In the experimental group Cd IV, the level of MDA was significantly higher in L5 *O. nubilalis* larvae compared to the control group (C). In experimental groups Cd I and Cd III the level of MDA was not significantly different compared to the control group (C) (Fig. 3).

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Effect of Cd exposure to gene expression

Results for relative gene expression for metallothionenins (*Mtn*), catalase (*Cat*) and glutathione peroxidase (*GPx*) in L5 *O. nubilalis* larvae exposed to different Cd concentrations, are presented on Figure 4A, B, C. The expression of *Mtn* gene was significantly up-regulated in experimental groups Cd I, Cd III, Cd IV in comparison to the control group (C) while in experimental group Cd V did not change (Fig. 4A). The expression of both genes *Cat* and *GPx* was significantly reduced in Cd III, Cd IV and Cd V groups, in comparison to the control group (C) (Fig. 4B and C).

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Discussion

In this study the effect of long term exposure to dietary cadmium (Cd) on larval growth, development, survival rate, gene expression of stress response protein- metallothionein (*Mtn*), two antioxidative enzymes (*Cat* and *GPx*) and oxidative status (MDA) was analyzed. Our results clearly demonstrated that concentrations of Cd of 41.71 and 77.53 mg kg^{-1} in fresh diet, alter the normal growth and development of *O. nubilalis* larvae, as the body weight of larvae successfully developed to L5 and consequently the weight and length of pupae, showed a significant decrease compared to larvae developed on non-contaminated diet. The development time of larvae fed on these two diets (Cd IV and Cd V), from L1 to pupation, was strongly reduced, with a delayed appearance of the first pupa, noticed 4 and 10 days later then in the control group, respectively. The concentrations of Cd selected in our experiments were higher than those occurring naturally within polluted areas thus providing a strong test of the insect ability to deal with this toxic metal. According to Kabata-Pendias and Pendias (1992) the average concentrations of Cd in non-polluted soils are between 0.06 to 1.1 mg kg^{-1}, with a minimum of 0.01 and a maximum of 2.7 mg kg^{-1}. Our findings are in accordance with the findings of other authors about development alterations of Cd exposed insect species, manifesting in a significant reduction or retardation in their growth and development (McCadhon and Pascoe, 1991; Schmidt et al., 1991; Gintenreiter et al., 1993; Ortel, 1996; Sildanchandra and Crane, 2000; Maryaski et al., 2002; Cervera et al., 2004; Al-Momani and Massadeh, 2005; Wu et al., 2006; Mirčić et al., 2010; Gao et al., 2017). The survival rate of treated larvae showed clear reduction to 60% and 40% with two highest concentrations of Cd in diet (41.71 and 77.53 mg kg^{-1}, respectively) in comparison with all other analyzed groups with survival rate over 85%. . Once Cd passes through the cellular membrane, it can bind to different biomolecules, accumulate or deposit in subcellular compartments and it

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may undergo metabolic transformation or elimination (Roesijadi and Robinson, 1994; Di Giulio et al., 1995; Wang et al., 2013). In experimental groups distinguished with high survival rate (concentrations in fresh diet: 0.73, 3.70 and 6.85 mg kg^{-1}), cadmium was strongly absorbed and accumulated by *O. nubilalis* larvae and successfully transmitted to the next developmental stage, pupae, as the concentration of Cd in pupae significantly exceeded those in their diet. As the survival rate was high in these experimental groups we suppose that *O. nubilalis* larvae have well developed mechanisms to tolerate elevated concentrations of Cd in their diet. In accordance with obtained results we could conclude that the highest concentration of Cd in pupae that can be effectively regulated without a negative impact on larval development and survival was 25.05 mg kg^{-1}. Larvae exposed to concentrations which cause alterations in development (concentrations in fresh diet: 41.71 and 77.53 mg kg^{-1}) did not show accumulation of Cd to that extent, with a bioaccumulation factor below 1. However, the concentration in pupae continued to increase to 38.66 and 57.23 mg kg^{-1}, and these values showed a negative effect on fitness components. It is well known that the bioaccumulation of metal depends on dosage, food availability and the age of animals and it is species specific. Cd generally has a greater tendency to accumulate than nutritional metals (Janssens et al., 1991; Gintenreiter et al., 1993, Crawford et al., 1996; Augustyniak and Migula, 2000; Maryański et al., 2002; Wang et al., 2013). Larvae of *O. nubilalis* carefully balance heavy metals uptake, storage and excretion probably using mechanisms which limit metal contents in animal body, most likely by defecation, molting or during pupation. *O. nubilalis* does molt four times during its development and once during pupation but so far there is no information about Cd concentration left behind after molting. According to Wu et al. (2006) Cd exposure induced a prominent impact on fitness components of *Boettcherisca peregrina* larvae. The accumulation of Cd increased with the exposure dose and

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time resulting in a decrease in the body weight and length of larvae, especially at the higher Cd concentration. The total duration of larval stage was also extremely affected as the average duration was prolonged significantly by 14 h at the lower Cd concentration (100 μg g^{-1} of fresh weight), while it was increased by 33.7 h over controls at the higher Cd concentration (400 μg g^{-1} of fresh weight). In an experiment with exposure of *Drosophila melanogaster* to extreme concentrations of Cd, results showed that only a high concentration of 500 ppm of Cd in larval diet caused a significant reduction in the survival rate of pupae and adults of the first generation, 65% and 25% respectively. Extreme concentration of Cd, 1000 ppm, decreased the survival rate of pupae and adults to 15 and 0%, respectively (Al-Momani and Massadeh, 2005). The effects of multigenerational Cd exposure of *Spodoptera exigua* larvae showed elevated Cd concentration in the whole body, higher mortality and longer duration of the larval stage in one-generation exposed insects in comparison with those exposed for many generations, suggesting that tolerance to heavy metals builds over time (Kafel et al., 2012).

The degree of cell damage under heavy metal stress depends on the rate of ROS formation and the efficiency and capacity of detoxification and repair mechanism. The production of metallothioneins has evolved as a mechanism to regulate metal levels and distribution within cells and organism, which is induced by and binds Cd to be stored as nontoxic complex (Andrews, 2000; Wang et al., 2013). Cd disrupts the equilibrium between ROS generation and detoxification capacity of the cell, metallothionein and antioxidant defense system, which result in ROS accumulation (Pathak and Khandelwal 2006; Liu et al., 2009; Patra et al., 2011; Wang et al., 2013). The loss in antioxidant capacities results in an intrinsic accumulation of MDA, which can be used as a reliable marker of free radical generation that indicates the risk of membrane damage (Li et al., 2012; Wang et al., 2013). In our study, the MDA level indicated that

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significant oxidative stress was induced in larvae exposed to 41.71 mg kg^{-1} of Cd in fresh diet. A lack of MDA level increase in larvae of experimental groups Cd I, and Cd III indicated negligible oxidative damages caused by oxidative radicals which might be due to the protective effects of the antioxidant system and metallothioneins. These results were in accordance with our findings about growth, development, survival rate and Cd accumulation by larvae of *O. nubilalis*, as the higher concentrations of Cd (41.71 and 77.53 mg kg^{-1}) alter the normal growth, development and caused higher mortality. According to the results, long term exposure to Cd significantly induced the expression of *Mtn* gene in L5 larvae of experimental groups Cd I, Cd III and Cd IV, but not in Cd V. Induction of metallothionein gene indicate its role in cadmium detoxification as shown in other insect and invertebrate species (Nakamori et al., 2010; Tang et al., 2011; Purać et al., 2018). The absence of up-regulation of *Mtn* gene in *O. nubilalis* at the highest concentration of cadmium (77.53 mg kg^{-1}) might indicate that long term exposure to such high concentration inhibit defense mechanisms. In contrast, the *Cat* and *GPx* genes expression was significantly down-regulated in experimental groups Cd III, Cd IV and Cd V in comparison to the control. In different insect species metal exposure variously results in either enhancement or inhibition of antioxidant enzymes, so general conclusion can not be drawn (Migula et al., 2004; Merritt and Bewick, 2017). Changes in ROS-related gene expression during chronic exposures are also less significant compared to acute Cd poisoning in assessing the level of oxidative stress. The reason might be induced adaptation mechanisms such as overexpression of metallothionein following chronic Cd exposures, which in turn diminish Cd-induced oxidative stress (Patra et al., 2011). In future, in order to establish the role of the detoxification mechanisms in the initial state of Cd exposure the acute short term exposure should be provided on *O. nubilalis* larvae.

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O. nubilalis exhibited a well-developed ability to tolerate a high concentration of Cd in its diet. Higher concentrations of Cd (41.71 and 77.53 mg kg^{-1}) cause instability in the development of *O. nubilalis* larvae, delay it, induce oxidative stress. Our results also indicate the existence of effective mechanisms such as induction of metellothionenin gene included in defense against the toxic effects of Cd. It is important to study the mechanisms of toxicity of Cd to obtain a better understanding of its dynamics and mode of action. Considering the wide geographical distribution and the huge economic impact of this pest, a better understanding of the mechanisms behind its heavy metal tolerance could give a better insights into the adaptation mechanisms of this species and could help predicting its distribution.

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Figure 1: Larvae of *O. nubilalis* on the day of the first sampling (18th day) in Cd treated groups and control. Names of experimental groups and the concentrations of Cd in artificial diet (mg kg^{-1}): Cd I: 0.73, Cd II: 3.70, Cd III: 6.85, Cd IV: 41.71, Cd V: 77.53 and control C: 0.03.

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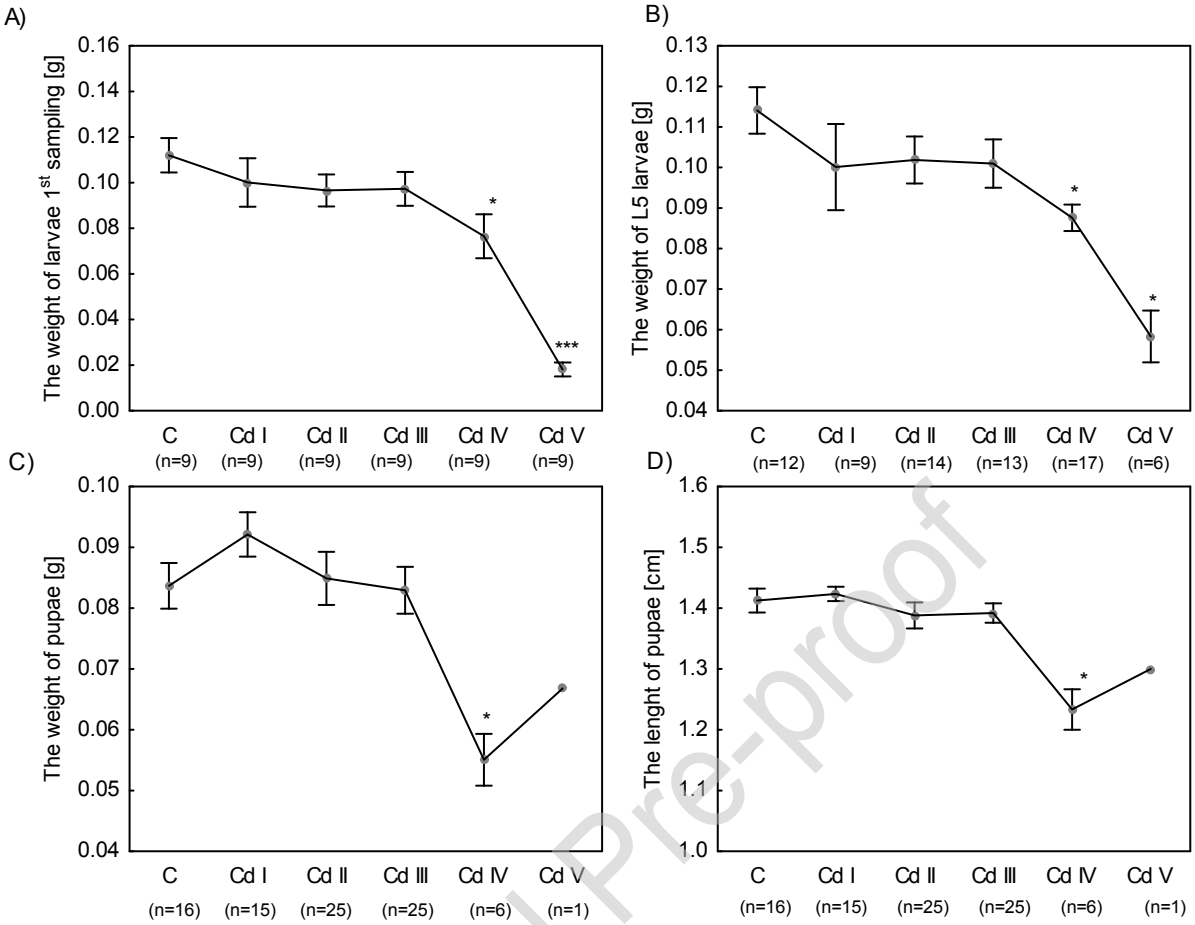

Figure 2. The weight [g] of *O. nubilalis* larvae during 1st sampling (A), the weight [g] of L5 larvae during 2nd and 3rd sampling (B), the weight [g] (C) and length [cm] (D) of pupae in Cd treated groups and control. Names of experimental groups and the concentrations of Cd in artificial diet (mg kg^{-1}): Cd I: 0.73, Cd II: 3.70, Cd III: 6.85, Cd IV: 41.71, Cd V: 77.53 and control C: 0.03. Results are expressed as mean ± SE and analyzed by ANOVA followed by Dunnett's post-hoc test. Statistically significant difference ($p<0.05$) compared to the control group (C) is indicated with an asterisk (*).

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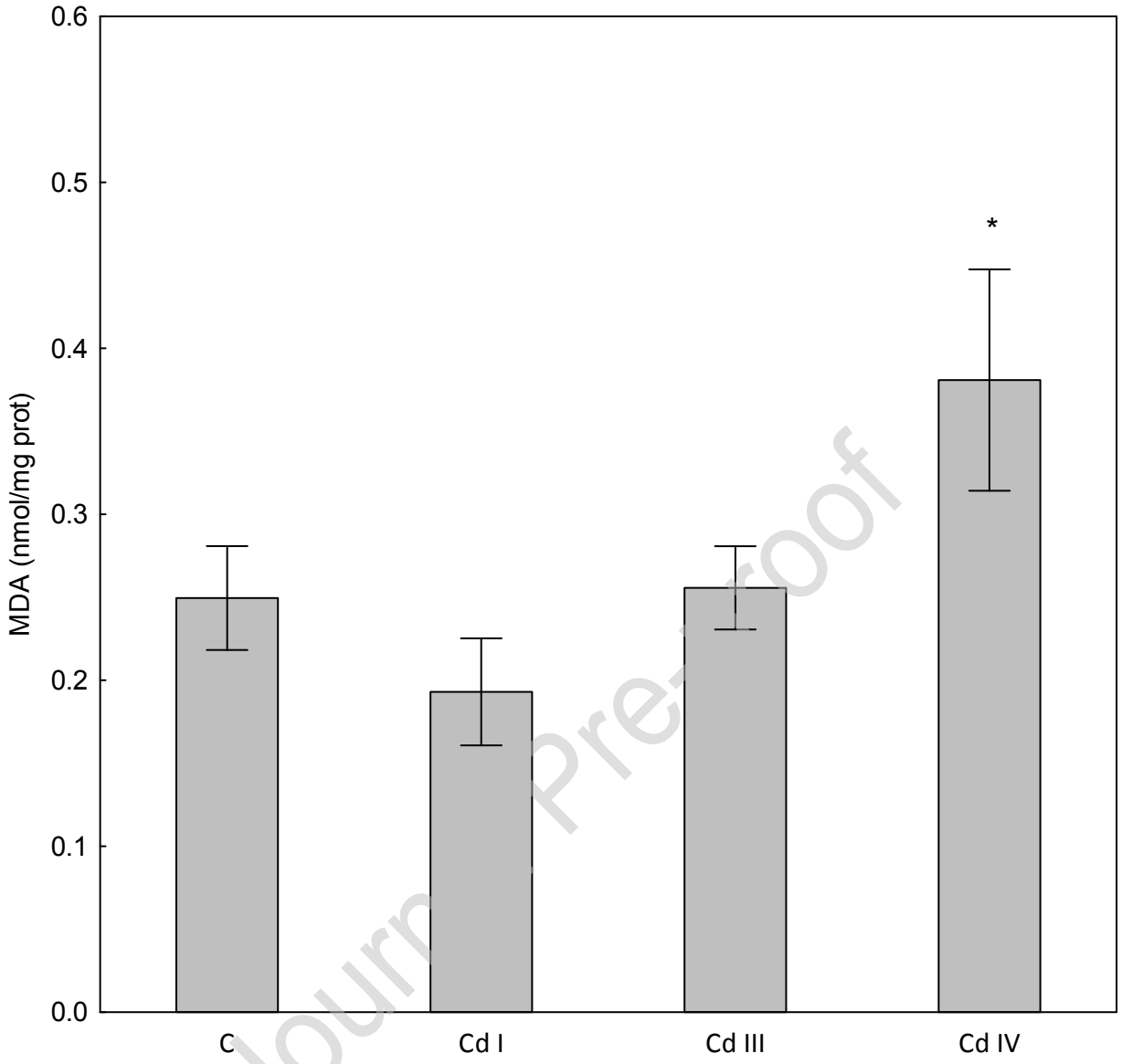

Figure 3. The level of MDA [nmol mg prot^{-1}] in L5 *O. nubilalis* larvae in Cd treated groups and control. The names of experimental groups and the concentrations of Cd in artificial diet (mg kg^{-1}): Cd I: 0.73, Cd III: 6.85, Cd IV: 41.71 and control C: 0.03. Results are expressed as mean ± SE and analyzed by ANOVA followed by Dunnett's post-hoc test. Statistically significant difference ($p<0.05$) compared to the control group (C) is indicated with an asterisk (*).

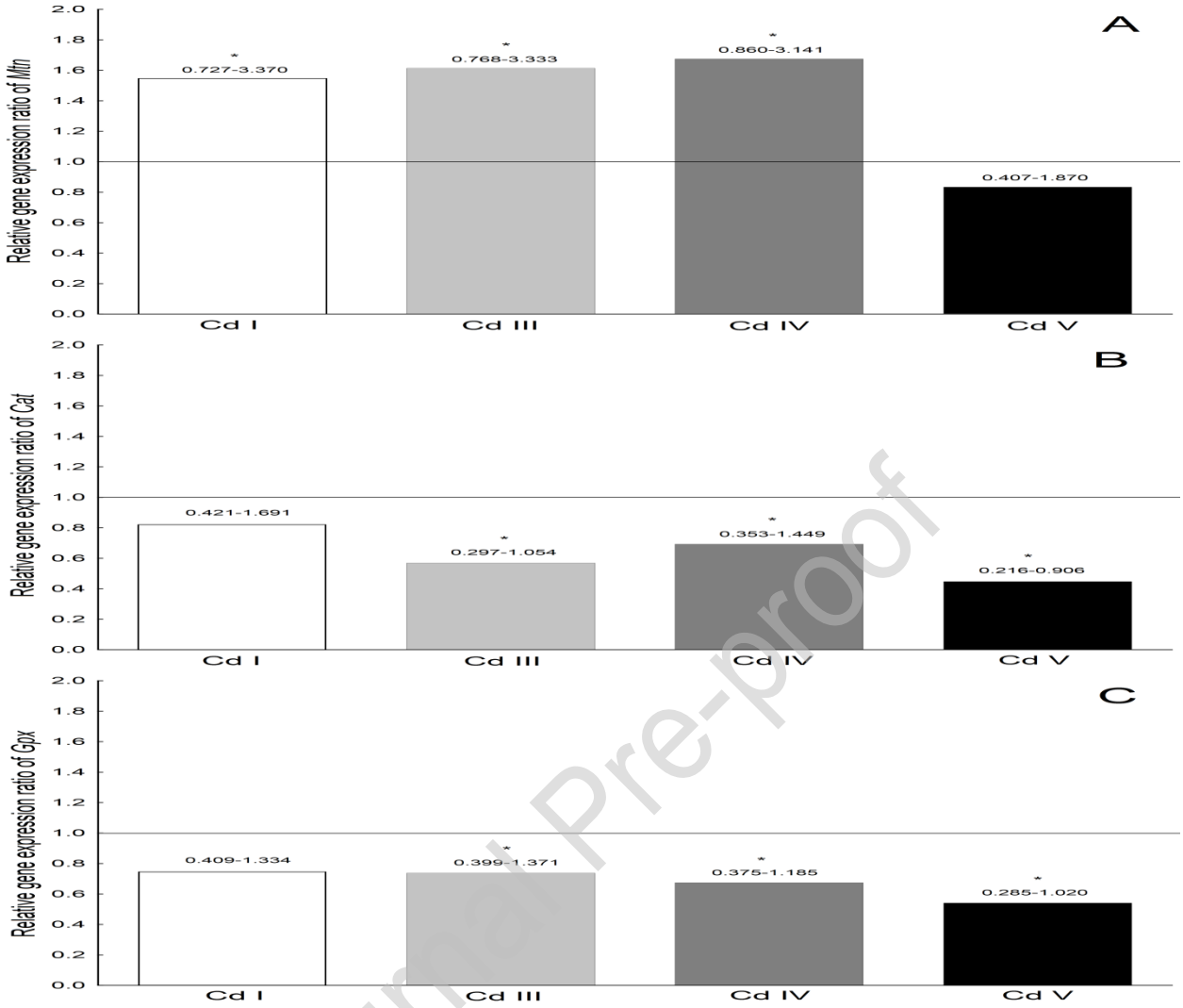

Figure 4. The mean fold changes of gene expression for A) *Mtn* B) *Cat* and C) *GPx* genes in L5 *O. nubilalis* larvae of Cd treated groups and control. Names of experimental groups and the concentrations of Cd in artificial diet (mg kg^{-1}): Cd I: 0.73, Cd III: 6.85, Cd IV: 41.71, Cd V: 77.53 and control C: 0.03. The difference in the analyzed gene expression was calculated by REST 2009 and tested for statistical significance by the integrated Bootstrap randomization test between the control and target groups. The range of values for a confidence interval of 68% is presented above the columns. Statistically significant difference compared to the control group (=1) is indicated with an asterisk (*$p < 0.05$)

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E.L.V.and J.P. designed the study; F.F. contributed to study design; D.K., T.V.Č., S.M. and J.N. performed analyses; D.B. contributed to analyses; E.L.V. and J.P. wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

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Conflict of Interest and Authorship Conformation Form

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- *O. nubilalis* larvae accumulates Cd from diet during long term exposure.
- Cd in diet (≥ 41.71 mg/kg) alters *O. nubilalis* larvae growth and development.
- Cd in diet (41.71 mg/kg) increases MDA level in *O. nubilalis* larvae.
- Cd in diet (0.73; 6.85; 41.71 mg/kg) up-regulates *Mtn* gene expression.
- Cd in diet (≥6.85 mg/kg) down-regulates *Cat* and *GPx* gene expression.

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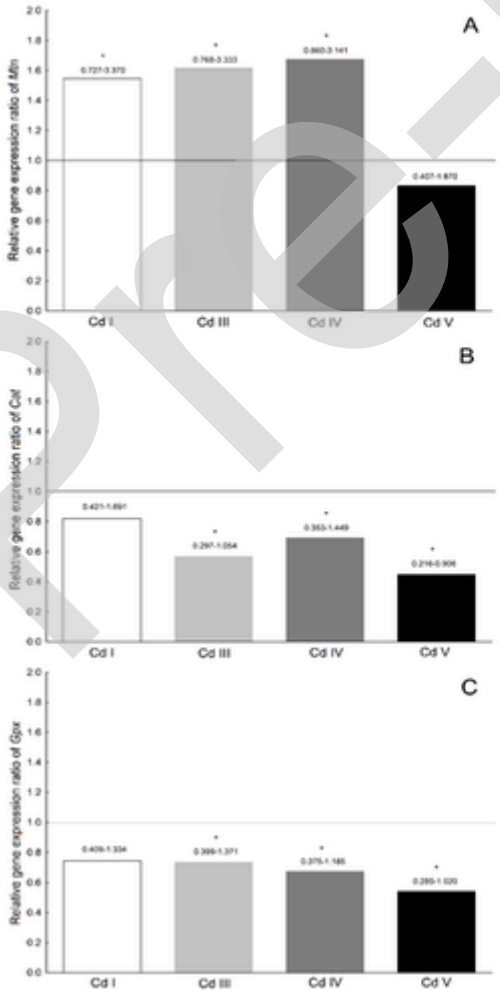
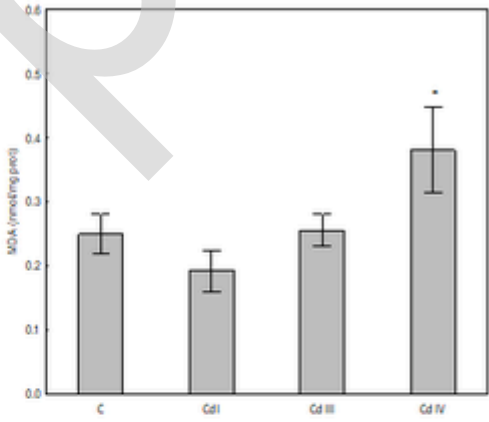
The effect of long term exposure to Cd on *Ostrinia nubilalis* larvae

Groups	C	Cd I	Cd II	Cd III	Cd IV	Cd V
Cd in diet [mg/kg]	0.03	0.73	3.70	6.85	41.71	77.53
Cd in pupae [mg/kg]	1.10	3.12	14.67	25.05	38.66	57.23
BAF		4.30	3.96	3.66	0.93	0.74

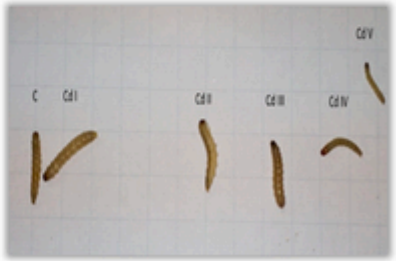
Changed
Mtn, *Cat* and *GPx* genes expression



Induced
oxidative stress



Reduced growth and development



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