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Effect of heavy metals (Cd, Cu, and Zn) on feeding indices and energy reserves of the cotton boll worm *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae)

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Abstract: Third-instar larvae of *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) were exposed to 12.5, 25, and 50 mg/kg concentrations of cadmium (Cd). The third-instar larvae were also exposed to 25, 50, and 100 mg/kg concentration of copper (Cu), and 25, 50, and 100 mg/kg concentrations of zinc (Zn). The heavy metal concentrations were each introduced separately into the artificial diet of the third-instar larvae. The third-instar larvae were 24 h old at the start of the treatment. The larvae were maintained in controlled conditions (26±1°C, 65±10% RH and 16L : 8D h) for 7 days. The feeding indices and the level of total protein, glycogen, cholesterol, and triglyceride were measured after the treatments. The results showed that high concentrations of Cd significantly increased approximate digestibility (AD). The relative growth rate (RGR) was significantly enhanced with a 25 mg/kg concentration of Cu. Efficiency of conversion of the ingested food (ECI%) increased significantly with lower concentrations of copper (25 and 50 mg/kg). The amount of cholesterol was also enhanced with 12.5 and 25 mg/kg concentrations of cadmium while the amount of triglyceride was significantly reduced in all Cd treatments. Glycogen, protein, and cholesterol were significantly enhanced in all Cu treatments. The amount of triglycerides at 25 and 50 mg/kg of Cu was significantly increased. Glycogen in all Zn treatments was significantly increased. Protein and cholesterol levels showed significant reduction with a 25 and 50 mg/kg concentration of zinc while triglyceride was enhanced with a 50 mg/kg concentration of Zn. It is clear from the present results that the presence of such heavy metals in the environment has an intense impact on *H. armigera* as far as food consumption and biochemical indices are concerned. Therefore, a need is shown for a more comprehensive study on the life table and immunology of this insect, under the presence of heavy metals.

Key words: carbohydrate, environmental pollution, food consumption, growth, lipid, protein

Introduction

Natural activities including volcanic eruptions, erosion, and spring water, and human activities such as exploration, mining, agriculture, and the search for fossil fuels form an accumulation of heavy metals in the soil and implicit toxicity to plants, animals, and humans (Sharma and Agrawal 2005; Boyd 2010). It has been proven by Lindqvist (1992), that there exists an accumulation of heavy metals in insects that feed on plants containing one, two or all three of the heavy metals: cadmium (Cd), copper (Cu), and zinc (Zn). Some heavy metals are essential, such as cooper and zinc (Jensen and Trumble 2003), Sharma and Agrawal (2005), however, mentioned that some heavy metals such as cadmium (Cd), nickel (Ni), arsenic (As), chromium (Cr), and lead (Pb) are increasing to a dangerous levels for humans, plants, and animals. Heavy metals in insects have a clear effect on growth (Warrington 1987), mortality (Mitterbock and Fuhrer 1988), and physiology (Fountain and Hopkins 2001; Ilijin *et al*. 2009). Zinc and copper connect to the cytosol metalothionein in the midgut of many organisms and are essential elements, but at high concentrations can be toxic (Jensen and Trumble 2003). Cadmium is highly toxic, even at a low concentration, and can create mutations in the organism (Emre *et al*. 2013). Feeding indices such as relative growth rate (RGR), relative consumption rate (RCR), and other consumption data are very useful when comparing various influential factors. For example, the effects of environmental factors on feeding intake can take on indicators of nutritional influence (Waldbauer 1968). The effect of heavy metals on an index such as RGR is quite various. For example, the effect of a high concentration of nickel in *Spodoptera litura* Fabricus reduced RGR but a low concentration of nickel increased RGR (Sun *et al*. 2011).

Intermediary metabolism includes multiple pathways in insects and the energy is stored as carbohydrates, lipids, and proteins for energy production through degradation or synthesis (Nation 2008). Carbohydrates, lipids, and proteins act as energy sources and have a great effect on insect populations (Dadd 1985; Lagadic *et al*. 1994; Olson *et al*. 2000; Hogervost *et al*. 2007). Amino acids act as a facilitator of neural transmission, phospholipid synthesis, energy production, and morphogenetic activities (Chen 1985). Lipids affect sexual maturation, egg produc-

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tion, and energy saving (Olson *et al*. 2000; Ryan and Van Der Horst 2000).

The cotton boll worm, *H. armigera* is a highly polyphagous pest of many economically significant crops in parts of Asia, Africa, Australia (including Oceania), and Europe (King 1994). This pest is a polyphagous insect attacking a wide range of crops including legumes, sorghum, cotton, tomato, pepper, sunflower, safflower, and flax (Matthews 1999).

The purpose of this study was to determine the effects of different concentrations of heavy metals such as Cd, Cu, and Zn on feeding indices and energy reserves (carbohydrates, proteins, and lipids) using *H. armigera* as the model insect.

Materials and Methods

The larvae of *H. armigera* were collected from the tomato farms in the city of Astaneh-ye Ashrafiyeh (37°15′35″N 49°56′40″E) in the north of Iran. The larvae were reared on an artificial diet of powdered cowpea, wheat germ powder, yeast, sorbic acid, ascorbic acid, sunflower oil, formaldehyde, and water (Shorey and Hale 1965). The insects were kept in transparent plastic containers (10 × 5 × 5 cm) at 26±1°C, and the relative humidity (RH) was 65±5%. The photoperiod was 16L : 8D h (Hemati *et al*. 2012). The salts of heavy metals used in this work were; cadmium chloride ($CdCl_2$), copper chloride ($CuCl_2$), and zinc chloride ($ZnCl_2$). The different salts were individually dissolved in distilled water to make a stock solution of 2,000 ppm. Each stock solution was then diluted to make a series of different concentrations. The selected concentration of heavy metals was based on the toxicity amount reported in plants, from various reports (Deng *et al*. 2004; Liu *et al*. 2007; Parizanganeh *et al*. 2010; Nazir *et al*. 2011). For copper and zinc: 25, 50, and 100 mg/kg were used, and for cadmium: 12.5, 25, and 50 mg/kg were used. Newly hatched larvae were reared in a plastic container (15 × 10 × × 5 cm) on the artificial diet for the first and second-instar. After that, the third-instar larvae were separated due to high cannibalism. The treatment using heavy metals was initiated for the third-instar larvae when they were 24 h old and continued for 7 days.

Feeding indices

To measure feeding indices, a pre-weighed amount of food was provided to 24 h old third-instar larvae that had been previously starved for 12 h. Food was replaced every two days and feeding data such as weight of the larvae before feeding, weight of wet food before feeding, dry food after feeding, and dry feces production, were measured. The treatment continued for 7 days after which the larvae were dried and weighed. Food, larvae, and feces were dried at 60°C for 48 h. All weights were measured using a monopan balance accurate to 0.1 mg (Sartorius GMBH, Type: A 120 S).

Feeding indices were calculated using the formula described by Waldbauer (1968):

$$\text{Relative growth rate (RGR)} = P/A \times T,$$

$$\text{Relative consumption rate (RCR)} = E/A \times T,$$

$$\text{Efficiency of conversion of the digested food (ECD\%)} = P/E - F,$$

$$\text{Efficiency of conversion of the ingested food (ECI\%)} = P/E, \text{ and}$$

$$\text{Approximate digestibility (AD\%)} = E - F/E.$$

where:
A – dry weight of insect over unit time, E – dry weight of food consumed, F – dry weight of feces produced, P – dry weight gain of insect, and T – duration of feeding period.

Energy reserves

Preparation of whole body homogenates for analysis of energy reserves third-instar larvae which were 24 h old began treatment with heavy metals at the above-mentioned concentrations. After 7 days of treatment, the third-instar larvae were collected and used for biochemical analysis. The whole body was homogenised in the distilled water and samples from each treatment were centrifuged for 10 min at 13.000 rpm at 4°C. The supernatants were transferred to new micro tubes and stored at –18°C until used for measurements of biochemical parameters. Protein was measured based on the method of Lowry *et al*. (1951). A total protein assay kit was used (Ziestchem Co., Iran). To measure the whole body cholesterol, Richmond's method (1973) was used. The principle of this method is based on hydrolysis of cholesterol esterase by cholesterol oxidase, cholesterol esterase, and peroxidase, and measured by a kit (Pars Azmun Co., Iran). For evaluating triglyceride, the method of Rifai *et al*. (1999) and an assay kit (Pars Azmun Co., Iran) were used.

Estimation of glycogen was based on a photometric method using the anthrone reagent as described by Van Handel (1965). The anthrone reagent was prepared by dissolving 0.15 g anthrone in 100 ml of diluted sulphuric acid (76 ml sulphuric acid, d = 1.84, poured into 30 ml water while stirring and cooling). A sample of 1 ml was measured into a centrifuge tube and stirred with 0.05 ml of a saturated solution of Na_2SO_4, followed by 3 ml of ethanol. The tube was placed in a boiling water-bath for 3 min, then cooled in an ice-bath for at least 1 h, and then centrifuged. The ethanol was carefully decanted and the glycogen pellet (after drying) was dissolved in 0.05 ml water. Three ml of freshly prepared anthrone reagent was added and the tube was heated at 90°C for 20 min, cooled in ice water, and measured at 620 nm using a microplate reader (Stat Fax 3200, Awareness Technology, USA)

Statistical analysis

All data are presented as means ±SE. Data were subjected to analysis of variance (ANOVA) using SAS software (SAS Institute 1997). The least significant among treatments were compared using Tukey's multiple range tests. Differences among means were considered to be significant at $p \leq 0.05$.

Results

Effect of heavy metals on feeding indices of *H. armigera*

Cadmium

In Cd treatments, there were no significant differences in the RCR when compared with the control (F = 0.33; df = 3; p = 0.803). The RGR decreased at the highest cadmium concentration (50 mg/kg) (2.24±0.15 mg/mg/day) compared with the control (3.07±0.22 mg/mg/day) (F = 4.26; df = 3; p = 0.045). However, this difference was not significant at the 5% level. The lowest concentration of Cd compared with the highest concentration showed significant differences in RGR. In the efficiency of the ECI% (F = = 0.9; df = 3; p = 0.48) and the ECD% (F = 6.45; df = 3; p = = 0.015), significant differences were not observed between the treatments and the control. The AD% was reduced with the use of the 12.5 and 25 mg/kg concentrations of cadmium (33.1±3.6 and 34.1±2.4%, respectively) decreasing significantly compared with the control (50.2±2.5%) (F = 20.00; df = 3; p = 0.0004) (Table 1).

Copper

Low concentrations of Cu had an intense effect on growth. The RGR demonstrated a significant increase at 25 mg/kg of copper (7.17±0.24 mg/mg/day) compared with the control (3.07±0.22 mg/mg/day) (F = 10.57; df = 3; p = 0.0037). The ECI% increased when there were lower concentrations of copper (25 and 50 mg/kg, 11.9±0.3 and 12.4±1.16%, respectively) (F = 12.43; df = 3; p = 0.002). However, there were no differences concerning the ECD% when compared with the control (F = 5.18; df = = 3; p = 0.028). Treatments and the control showed no differences in the percentage of AD% (F = 10.57; df = 3; p = = 0.0037) (Table 1).

Zinc

Our result demonstrated no significant differences in any of the Zn treatment indices (Table 1).

Effect of heavy metals on energy reserves

Cadmium

The treatments and the control did not show significant differences in glycogen and protein levels, however, the cholesterol level was enhanced at 12.5 and 25 mg/kg concentrations of cadmium compared with the control (F = = 23.98; df = 3; p = 0.0002). Triglyceride was significantly reduced in all treatments which used Cd, compared with the control. The lowest triglyceride amount was observed when the highest concentration of Cd was used (F = 42.16; df = 3; p < 0.0001) (Table 2).

Copper

Glycogen was significantly enhanced in all treatments (F = = 129.98; df = 3; p < 0.0001) and this increase was also observed in the protein levels (F = 129.98; df = 3; p < 0.0001). The amount of triglycerides at the 25 and 50 mg/kg concentrations of copper was significantly increased (F = = 129.98; df = 3; p < 0.0001). However, no significant differences were observed at the highest concentration used. The amount of cholesterol in all the treatments was significantly increased, compared to the control (F = 12.58; df = 3; p = 0.0021) (Table 2).

Zinc

The present result illustrated enhanced levels of glycogen in all the treatments, when compared with the control (F = = 40.82; df = 3; p < 0.0001). The protein (F = 10.00; df = 3; p = 0.0044) and cholesterol (F = 10.15; df = 3; p = 0.0042)

Table 1. Effect of cadmium (Cd), copper (Cu), and zinc (Zn) on feeding indices of third-instar larvae of *H. armigera**

Heavy metals	Concentrations [mg/kg diet]	RCR [mg/mg/day]	RGR [mg/mg/day]	ECI%	ECD%	AD%
Cd	The control	1.54±0.19 a	3.07±0.22 ab	9.4±1.1 a	18.7±1.3 ab	50.2±2.5 a
	12.5	1.58±0.23 a	3.81±0.36 a	9.18±1.3 a	28.3±6.8 a	33.1±3.6 b
	25	1.44±0.24 a	2.96±0.11 ab	10.1±1.5 a	29.7±5.4 a	34.1±2.4 b
	50	1.77±0.13 a	2.24±0.15 b	7.9±0.5 a	12.9±1.2 b	64 ±1.6 a
Cu	The control	1.54±0.19 a	3.07±0.22 b	9.4±1.1 b	18.7±1.3 a	50.2±2.5 a
	25	1.18±0.05 b	7.17±0.24 a	11.9±0.3 a	29.8±7.9 a	41.7±6 a
	50	1.15±0.11 b	4.64±0.34 ab	12.4±1.2 a	31.2±6 a	40±4.4 a
	100	1.58±0.08 a	2.86±0.15 b	9.1±0.44 b	18.9±0.6 a	48.2±2.9 a
Zn	The control	1.54±0.19 a	3.07±0.22 a	9.4±1.08 a	18.7±1.3 a	50.2±2.5 a
	25	1.32±0.13 a	3.49±0.5 a	10.9±1.04 a	26.8±8.1 a	42.2±5.2 a
	50	1.32±0.13 a	4.45±.041 a	9.9±1.66 a	24±3.7 a	41.2±3.2 a
	100	1.48±0.22 a	3.58±0.61 a	9.8±1.62 a	12±5.2 a	49.9±4.3 a

*the larvae were allowed to feed on the treated diet for 7 days

Within columns, means followed by the same letter do not differ significantly at p < 0.05

RCR – relative consumption rate; RGR – relative growth rate; ECI – efficiency of conversion of the ingested food; ECD – efficiency of conversion of the digested food; AD – approximate digestibility

Table 2. Effect of cadmium (Cd), copper (Cu), and zinc (Zn) on glycogen, protein, cholesterol, and triglyceride of third-instar larvae of *H. armigera**

Heavy metals	Concentrations [mg/kg diet]	Glycogen [mg/l]	Protein [mg/l]	Cholesterol [µg/dl]	Triglyceride [µmol/l]
Cd	The control	16.1±0.5 a	10.1±0.8 a	0.2±0.08 b	5.37±0.89 a
	12.5	17.9±2.1 a	11.8±0.1 a	0.85±0.14 a	2.44±0.5 b
	25	14±2.3 a	11.4±0.6 a	0.73±0.11 a	1.36±0.22 b
	50	18±1 a	10.8±0.6 a	0.28±0.12 b	1.08±0.06 b
Cu	The control	16.1±0.5 c	10.1±0.8 b	0.2±0.08 b	5.37±0.89 c
	25	32.5±0.8 a	12.5±0.2 a	2±0.45 a	7.96±0.5 a
	50	20.9±1.5 b	12.4±0.3 a	1.72±0.13 a	6.92±0.46 ab
	100	22.2±1.1 b	12.3±0.8 a	1.62±0.2 a	5.78±0.24 bc
Zn	The control	16.1±0.5 c	10.1±0.8 b	0.2±0.08 b	5.37±0.89 ab
	25	28.9±1 ab	11.9±0.2 a	2.1±0.71 a	5.05±0.64 b
	50	27±2.2 b	12.8±0.7 a	2.46±0.64 a	7.5±1.34 a
	100	32.1±2.8 a	11.5±0.7 ab	1.12±0.53 ab	5.29±0.66 ab

*the larvae were allowed to feed on the treated diet for 7 days

Within columns, means followed by the same letter do not differ significantly at $p < 0.05$

levels at a 25 and 50 mg/kg concentration of zinc were significantly decreased. But it was just at the 50 mg/kg concentration point of this heavy metal that the triglyceride level increased ($F = 4.69$; $df = 3$; $p = 0.0357$) (Table 2).

Discussion

The feeding efficiency is the insect's ability to use the food ingested to the best of its potential (Waldbauer 1968). Unfortunately, most of the previous experiments deal with the effect of heavy metals on the growth of insects. These previous studies neglected the feeding indices that affect growth. A feeding index, like RGR, is a function of body weight gain (Srinivasan and Uthamasamy 2005). The present results showed that the effect of heavy metals does not always negatively affect the organism, as far RGR is concerned. The results indicated an increased RGR in low concentrations of Cu (Table 1). At lower concentrations, metal elements like Cu, Fe, and Zn are considered as important and essential elements of nutrition for the organisms. However, they can be very toxic at higher concentration (Warrington 1987; Tian and Lu 2009; Lu *et al*. 2011; Zan *et al*. 2011). Chang *et al*. (2000) mentioned that Cu stimulates plant growth and 5 to 30 mg/kg of Cu in plant leaves is considered normal. Huang *et al*. (2012) demonstrated that lower concentrations of Cu in the diet (25 and 50 mg/kg) shorten the generation time by 4–5 days, while higher concentrations (100 and 200 mg/kg) increase the duration by 1–2 days. Our result showed that at a 25 mg/kg concentration of Cu, the RGR was significantly increased. In the present experiment, enhanced RGR could be due to the increased efficiency of food eaten. This is well indicated in the percent of ECI which was significantly increased at a 25 mg/kg concentration of Cu. The RCR was reduced and we found that lower concentrations of Cu in the diet could reduce the consumption rate. Energy reserves in this treatment, showed that increased rate and growth was faster than in the control. Therefore, in Cu treatments, with increasing concentrations, negative impacts were observed in the insect. Huang *et al*. (2012) demonstrated that higher concentrations of Cu may cause denaturation in protein leading to dysfunction in *S. litura* at an excessive level. They also mentioned, that if feeding continued at a low concentration, a negative effect on growth was observed due to the accumulation of this heavy metal.

Our results showed that Cd was toxic even at lower concentrations. Our results were similar to the results reported by Hare (1992) who mentioned that "non-essential" elements such as Cd, Pb, and Hg, even at low concentrations, are toxic for organisms. In Cd treatment, the percent AD was enhanced. The increased AD is used to compensate for the lower digestion rate of food while simultaneously trying to achieve steady growth rates by more food consumption (Yazdani *et al*. 2013). Van Ooik *et al*. (2007) reported that polluted birch leaves had low digestibility and this is why *Epirrita autumnata* Borkhausen larvae could not process them as efficiently as in the case of control leaves. They indicated that larvae on polluted trees grew less and produced more frass than larvae on control trees. Our results indicated that a decrease in RGR at higher concentrations could be due to the metal toxicity which impaired food absorption (Yazdani *et al*. 2014). At lower Cd concentrations, the percent of ECI increased. It is probable, that the insect requires a lot of energy to deal with cadmium toxicity (Emre *et al*. 2013). At a higher concentration of Cd, a probable damage to the epithelium of the gut may hinder observation, thus causing an increase in percent AD. Our results are consistent with previous results of reduced growth rate by Cd; growth reduction has been observed in *Aiolopus thalassinus* Fabricius, *Lymantria dispar* Linnaeus, *Chironomus riparius* Meigen, *Oncopeltus fasciatus* Dallas, *Poecilus cupreus* Linnaeus, and *Boettcherisca peregrine* Robineau-Desvoidy (Schmidt *et al*. 1992; Ortel 1996; Sildanchandra and Crane 2000; Maryanski *et al*. 2002; Cervera *et al*. 2004; Wu *et al*. 2006).

Among the physiological aspects of organisms, energy metabolism plays a key role in overcoming heavy metal exposure. Energy metabolism will alter survival, growth, and development as well as reproduction (Wu *et al*. 2006). Explaining how Cu and Zn enhanced lipid and glycogen is very complicated. Copper and zinc are metals of great importance in biological processes and metabolism, where they are enzymatic factors (Cass and Hill 1980). Copper is a constituent of several insect enzymes including phenol oxidase and tyrosinae (Hackman 1974; McFarlane 1974; Bagatto and Shorthouse 1996; Tarek *et al*. 2010). Marshall (1983) reported that electron probe X-ray microanalysis of cells in body fat of the *Chaetophyes compacta* Walker and *Pectiniariophyes* sp. contains electron dense granules which are rich in copper and sulphur. He conjectured that copper may be bound to a sulphur containing metallothionein and that granules represent either the end products of copper detoxification or serve as copper stores for synthesis of enzymes and macromolecules by the mycetomal symbionts. This reason could probably be true for Cu, since Cu has a strong ability for binding with metallothioneins (Funk *et al*. 1987; Depledge and Rainbow 1990). The metals Cu and Zn have bioaccumulation (Cheruiyot *et al*. 2013); they are readily taken up and stored by the organism. Metallothioneins are short, cysteine-rich proteins for heavy-metal homeostasis and detoxification; they bind a variety of heavy metals and also act as radical scavengers (Kagi and Kojima 1987; De Moor and Koropatnick 2000). Metallothioneins reduce ion availability but can release them back into the system when needed to replenish low ion levels (Marino *et al*. 1998). Our result showed protein in all the concentrations of Cu and protein was significantly increased in the lower concentrations of Zn. It was shown by Maroni *et al*. (1986), that Cu has the ability to induce proteins to bind with heavy metals in *Drosophila melanogaster* Meigen. The reduction in the protein level at the higher concentrations compared with the low concentrations, could be due to protein denaturation leading to dysfunction in excessive levels (Huang *et al*. 2012).

In our experiment, the reduction in lipid levels when the treatment with Cd was used, is supported by the findings in *Galleria mellonella* Linnaeus, *Pimpla turionellae* Linnaeus, *L. dispar*, and *B. peregrina* (Ortel 1991; Bischof 1995; Shin and Lee 2001; Wu *et al*. 2006; Emre *et al*. 2013), and there was a correlation with the concentrations and exposure time. Our results in Cd treatments, confirm that lipids can complement other energy sources to satisfy the enhanced energy need under stress caused by Cd. Protein is needed by larvae to synthesize metallothionein and the sequestration of cadmium induced in the larvae after Cd exposure; and microorganism satisfy protein levels by degradation of lipids. As we said later, larvae cannot absorb food with high concentrations of Cd (50 mg/kg), and we also stated that the AD% significantly enhanced due to injury in epithelium of midgut. Maybe, both the immune response and a decreased food absorption, lower energy in the larvae since there is a degradation of stored lipids meant to compensate for energy.

The present study clearly confirms the earlier findings about the accumulation of heavy metals in insects. Such an accumulation may hinder the growth by way of suppressing the feeding indices or accumulation of heavy metals may enhance growth. However, the actual effect of heavy metals should be considered as various physiological processes working together. For example, if growth is promoted, is the insect still able to overcome the attack of parasites or predators? If yes, then that means the immunological processes are at same time active and potent as in non-treated insects or even more pronounced. If the insect growth is retarded, what are the circumstances involved in other physiological processes? Our further study on the effect of these heavy metals on growth and immunology of this insect could be a possible answer to these questions.

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