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Research Article

Effect of copper, zinc, and iron salts on total hemocyte count, nutritional indices, and biological characteristics of *Ephestia kuehniella* (Lepidoptera: Pyralidae)

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Abstract: The accumulation heavy metals in the soil poses a significant risk to the health of plants, animals, and human communities. This study investigated the effect of small amounts of iron, zinc, and copper salts on hemocyte abundance, nutritional indices, and biological characteristics of the *Ephestia kuehniella* Zeller *in-vitro*. Fourth instar larvae were treated with iron, zinc, and copper salts at 25, 50, and 100 mg/kg in 24 and 48 h intervals. Immunological assessments included total hemocyte count, plasmatocytes, granulocytes, and phenol oxidase enzyme activity. The results demonstrated significant changes in immunological parameters compared to the control group. Notably, iron and zinc at 100 mg/kg exhibited the significant increase in enzyme activity at both 24 and 48 h. Conversely, iron caused a significant decrease in the activity of this enzyme after 24 h at 50 mg/kg when compared to other treatments. In terms of nutritional indicators, copper metal salt at 25 mg/kg enhanced the relative growth rate compared to the control. This preliminary study highlights the significant impact of zinc, copper, and iron salts on the biological characteristics and immune response of *E. kuehniella*. These findings suggest that the incorporation of small amounts of these metals into the insect's diet can induce biological and physiological disorders.

Keywords: Abundance of blood cells, nutritional index, biology, heavy metals, *Ephestia kuehniella*

Introduction

Currently, the abundance and diversity of insects are decreasing worldwide, and various factors contribute to this decrease. Besides biological factors, habitat destruction, and climate change, 30 pollutants are crucial in reducing insect populations. In addition to biological factors, habitat destruction, and climate change, pollutants are essential to insect population reduction. Multiple contaminants, such as pesticides, heavy metals, or airborne particulates from agricultural or industrial sources, may have lethal or sublethal toxicity on insects (Sanchez-Bayo and Wyckhuys, 2019; Wagner, 2020). A major part of environmental pollution is related to the impact of heavy metals, which have endangered the health of human societies (Duruibe *et al*., 2007) as well as terrestrial and aquatic organisms (Sanchez, 2008). The long-

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term accumulation and persistence of these metals in the environment have caused serious problems in different ecosystems (Malakar *et al*., 2009; Hejazizadeh *et al*., 2016). The heavy metals are divided into two groups: essential and non-essential. Heavy metals are classified into two essential and non-essential groups. A small amount of iron, copper, zinc, manganese, and nickel is necessary for physiological and biochemical activities. However, the elements, including cadmium, lead, arsenic, mercury, and chromium, are not necessary for the vital activities of living organisms. Therefore, it has been proven that the bodies of living organisms need essential metals for various important processes such as growth and development, metabolism, reproduction, and the immune system. Still, even a slight increase in these metals causes a change in the protein structure of enzymes and their accumulation between cells and toxicity (Witeska *et al*., 2014; Tuncsoy *et al*., 2016).

There are reports on the effects of heavy metals on the survival, biology, and immunity of various insects. These effects depend on metal concentration, application method, insect species, developmental stage, and sensitivity (Suganya *et al*., 2016). Borowska *et al.* (2004) reported that when *Musca domestica* (Diptera: Muscidae) feeds on artificial food containing copper, zinc, lead, and cadmium, these compounds accumulate in the body, reduce larval development and larval and pupal survival rates. The mortality rate of *Culex pipiense* L. (Diptera: Culicidae) larvae increases at high concentrations of copper, lead cadmium, and mercury (El Sheikh *et al*., 2010). Copper and zinc accumulate in the midgut and fat body of *Galleria mellonella* (Lepidoptera: Pyralidae) and change the number of antioxidants and activities of detoxifying enzymes (Tuncsoy and Mese, 2021). Also, Suganya *et al*. (2016) proved that the action of antioxidant enzymes of *Spodoptera litura* F. (Lepidoptera: Noctuidae) larvae changes against cadmium and lead metals.

There is also a report about the effect of heavy metals on the hemocyte profile of insects. (Borowska and Pyza, 2011, Kurt and Kayis, 2015). By decreasing the size of hemocytes, the granulocytes shrink in front of metal treatments (Borowska and Pyza, 2011). Granulocytes and plasmatocytes fight foreign agents through phagocytosis and nodulation (Lavin and Strand, 2002). Granulocytes are considered the most critical cells against foreign agents, which participate in phagocytosis, nodulation, and plasmatocytes. The reduced size of granulocytes will negatively affect the insect's immune response (Pourali and Ajamhassani, 2018). In another report, in larvae of *Mamestra brassicae* (Lepidoptera: Noctuidae), feeding copper-treated food increased plasmatocytes and decreased the level of phagocytosis (Kazimirova and Slovak, 1996).

Ephestia kuehniella is one of the important pests that cause economic damage to stored agricultural products yearly (Mostaghimi *et al*., 2013). The larvae of this pest primarily prefer floured grains, so flour and bran are their primary food. However, due to the polyphagous nature, the larvae of this species can feed on cereal grains such as wheat, corn, rice and dried fruits such as raisins, figs, apricots, and even fresh and dried mushrooms (Mehrkhou and Tarlak, 2016). In this study, we tried to investigate the effect of three heavy metals, zinc, copper, and iron, on some biological and immune systems of *E. kuehniella*. This study can be a prelude to additional studies on using these metals as nanocapsules against storage pests as one of the alternatives to chemical compounds.

Materials and Methods

Rearing of *E. kuehniella*

For research, sterilized plastic containers with dimensions of 30 × 30 × 20 cm were prepared containing 100 g of wheat flour and 3% yeast.
White organza lace fabric was covered for ventilation in the upper part of the containers. These containers were placed in the germinator at 25 ± 1 °C, relative humidity of 40 ± 5% and a photoperiod. 14:10 h (darkness: light) for the mass breeding of *E. kuehniella*. (Tavakoli and Ajamhassani, 2017). The female insects' eggs were isolated, and after hatching, larvae feed on

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wheat flour. The body length and head capsule width of the one-day-old fourth instar were used to determine larval instars using millimeter paper (Dyar, 1980).

Preparation of salt solutions

Based on the literature, 25, 50, and 100 mg of heavy metal salt/kg wheat flour with yeast were used (Parizanganeh *et al*., 2010; Nazir *et al.*, 2011). The mentioned amounts of heavy metal salts (Feso$_4$, Cuso$_4$, and Znso$_4$) were dissolved in distilled water, and a base concentration of 2000 ppm was prepared from each salt and added to flour containing yeast. Fourth-instar larvae did not die under the influence of these concentrations. Flour containing distilled water was used as a control.

Hemocyte counts

Separate Petri dishes containing larvae and one gram of wheat flour with three percent yeast were supplemented with 1 mL of a solution of 2000 ppm. The solution was prepared by dissolving heavy metal salts of iron (FeSO4), copper (CuSO4), and zinc (ZnSO4) at concentrations of 25, 50, and 100 mg, respectively. Each treatment consisted of four replicates and each replicate consisted of four larvae. Changes in the number of larvae hemocytes were investigated after 24 h and 48 h. In the control group, distilled water was substituted for the salts, with a volume of one mL. Two microliters of hemolymph from each larva were collected using a capillary tube, and the total hemolymph of four larvae (8 μL) was diluted with 100 μL of physiological buffer (Tyson's anticoagulant solution) (Mahmood and Yousuf, 1985). Blood cells were counted using a Neubauer hemacytometer and Olympus BH2 optical microscope with a magnification of 40X. The number of hemocytes in five wells of a Neubauer slide (one square millimeter each), was measured using the Jones equation (Jones, 1962; Jones, 1967).

$$\frac{\text{Depth of Neubauer slide cells} * \text{dilution rate} * \text{1mm2} * \text{number of hemocyte}}{\textit{number of counted the slide holders}} \quad (1)$$

The hemocyte lysate method was used to determine the effect of iron, copper, and zinc salts on the activity of the phenol oxidase enzyme of the tested larvae (Leonard *et al*., 1985). For this method, each treatment, consisting of four replicates, involved 40 fourth instar larvae treated with iron, zinc, and copper salts. The hemolymph of each of these larvae (a total of 80 μL of hemolymph) was collected and centrifuged at 10,000 rpm for five minutes. The supernatant was removed, and 100 μL of phosphate buffer (pH = 7) was added to the sediments and homogenized. The obtained solution was again centrifuged for 15 min at 12000 rpm. Then, 25 μL of the samples were added to 50 μL of 10 mM L-dihydroxyphenylalanine (L-DOPA) solution (as substrate) and 50 μL of phosphate buffer. This mixture was incubated for five min at 30 °C, and the absorbance was read at 490 nm using a microplate reader.

The nutritional indices

Each treatment consisted of four replicates. For each replication, four fourth-instar larvae of *E. kuehniella* were kept in a Petri dish with an opening diameter of 8 cm containing 1 g of wheat flour with 3% yeast and different concentrations of heavy metal salts. Like other treatments, the control treatment consisted of 4 replicates, and each replicate contained four fourth-instar larvae placed in a Petri dish containing 1 g of wheat flour with three percent yeast and no metal. The Petri dish was placed in the germinator set at 25 ± 1 °C, relative humidity of 40 ± 5%, and a photoperiod of 14:10 h (darkness: light). Feeding data, including the total weight of live insects, the total initial weight of insects, the weight of food eaten, the weight of food consumed in the treatment, and the weight of the food consumed in control, were measured. Subsequently, the effects of heavy metals in the obtained concentrations on the nutritional indices were analyzed using the equation (2-5) method (Huang and Ho, 1998):

Relative Growth Rate: $$RGR = \frac{A - B}{B \times Day} \quad (2)$$

Where A is the total weight of live insects (mg) after three days, and B is the initial weight of insects (mg).

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Relative Consumption Rate: $RCR = \frac{D}{B \times Day}$ (3)

D is the amount of food consumed (mg), and B is the total initial weight of insects (mg).

Efficacy of Conversion of Ingested food:

$$ECI\% = \frac{RGR}{RCR} \times 100 \quad (4)$$

Feeding Deterrent Index: $FDI\% = \frac{C - T}{C} \times 100$ (5)

C is the weight of food consumed in control (mg), and T is the weight consumed in treatment (mg).

Biological properties

Each treatment consisted of four replicates. Each replicate consisted of 10 larvae, 1 g of wheat flour with three percent yeast, and concentrations of 25, 50, and 100 mg/kg of iron, copper, and zinc salts, which were placed in separate Petri dishes. After treatment, the effect of different concentrations of iron, copper, and zinc heavy metal salts on the larval period, the pupal period, the weight of pupae, the percentage emergence of adults, and longevity were investigated.

Statistical analysis

The data analysis was performed in a completely random design using the SAS 9.4 software, and the means were compared using Tukey᾽s test at the one percent1% probability level.

Results

Total hemocyte counts

The variance of the data related to the test of the effect of iron, copper, and zinc salt on the average total hemocyte count (THC) showed that there is a significant difference between the treatments and the control at the probability level of 1% ($F = 80$; $df_{t,e} = 19, 60$; $P \leq 0.0001$) (Figure 1).

After 24 and 48 h, the total number of hemocytes decreased in the treatment with iron salt at 25 and 50 mg/kg concentrations. The 100 mg/kg concentration increased the total number of hemocytes compared to the control. In the treatment with copper and zinc salts at all concentrations, the average number of the total hemocyte count showed a significant increase compared to the control (Fig. 1). Analysis of variance of the data related to the test of the effect of iron, copper and zinc salts on the average number of plasmatocytes showed that there is a significant difference between the treatments and the control at the probability level of 1% ($F = 10.72$; $df_{t,e} = 19, 60$; $P \leq 0.0001$). In the treatment with iron salt at all concentrations after 24 and 48 h, a decrease in the average number of plasmatocytes was observed compared to the control. In the treatment with copper salt with 25 mg/kg after 24 h, plasmatocytes decreased significantly compared to the control and reached 148 ± 14.5 cell /mm ^{3} hemolymph. With zinc salt treatment, 50 mg/kg of plasmatocytes decreased after 24 and 48 h and reached 137 ± 5 and 145 ± 6.5 cell/mm ^{3}, respectively (Fig. 2).

The average number of granulocytes increased in the iron and copper salt treatment at all concentrations. ($F = 15.19$; $df_{t,e} = 19, 60$; $P = 0.0001$). Additionally, at concentrations of 25 and 50 mg/kg of zinc salt, there was an increase in the average number of granulocytes at 24 and 48 hours compared to the control. Also, compared to the control, the average number of granulocytes in the treatment with zinc salt was significantly reduced at a 100 mg/kg concentration in both periods (Fig. 3).

Phenoloxidase activity

The variance analysis of the experimental data of the effect of iron, copper, and zinc salts on the average activity of phenol oxidase enzyme showed that there is a significant difference between the treatments and the control at the probability level of 1% ($F = 108.44$; $df_{t,e} = 19, 60$; $P \leq 0.0001$) (Figure 4). The results showed that, compared to the control, the average phenol oxidase enzyme activity increased in the treatment with iron salts in concentrations of 25 and 100 mg/kg in both periods and decreased at the concentration of 50 mg/kg after 24 h.

The copper and zinc salts treatment showed increased enzyme activity in all concentrations and periods compared to the control (Fig. 4).

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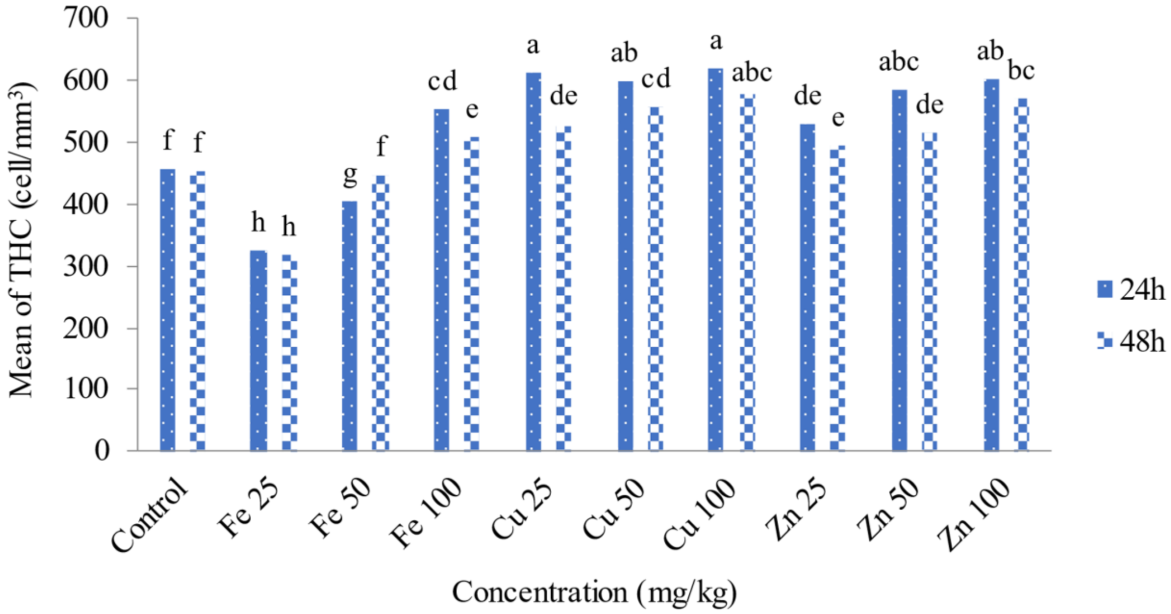

Figure 1 Mean ± SE total hemocyte count in fourth instars larvae of the Mediterranean flour moth under the influence of feeding with flour containing different concentrations of iron, copper and zinc after 24 and 48 h (Different letters show significance using Tukey's test at $p < 0.05$).

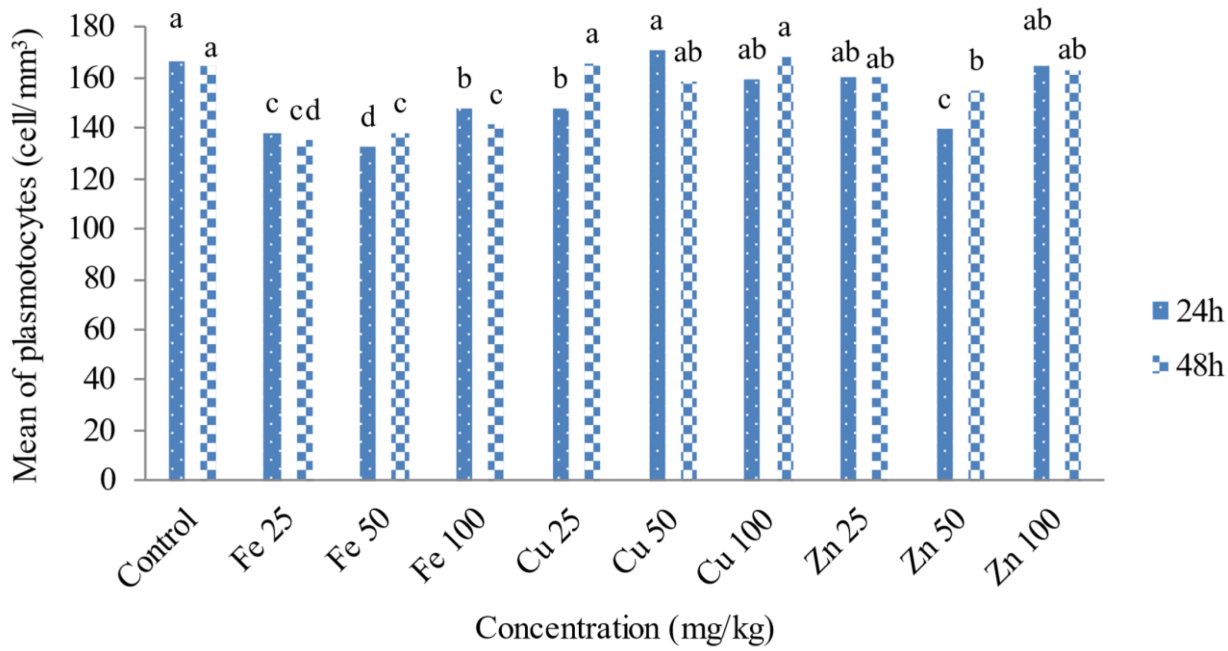

Figure 2 Mean ± SE plasmatocytes in fourth instars larvae of the Mediterranean flour moth under the influence of feeding with flour containing different concentrations of iron, copper and zinc after 24 and 48 h. (Different letters show significance using Tukey's test at $p < 0.05$).

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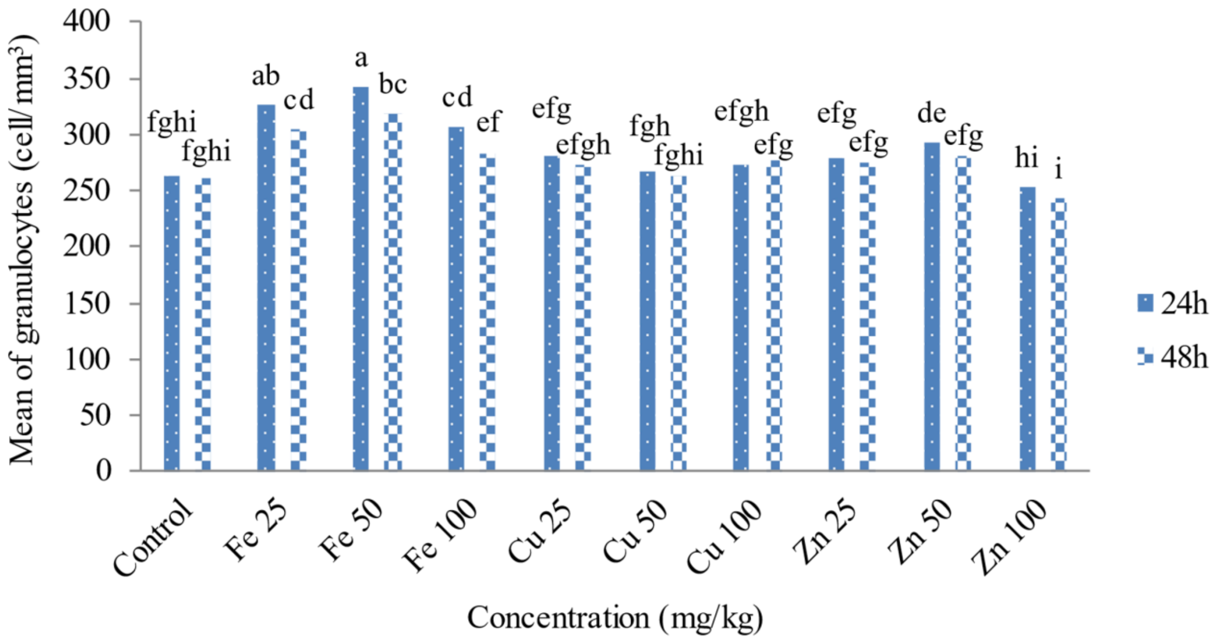

Figure 3 Mean ± SE granulocytes in fourth instars larvae of the Mediterranean flour moth under the influence of feeding with flour containing different concentrations of iron, copper and zinc after 24 and 48 h. (Different letters show significance using Tukey's test at $p < 0.05$).

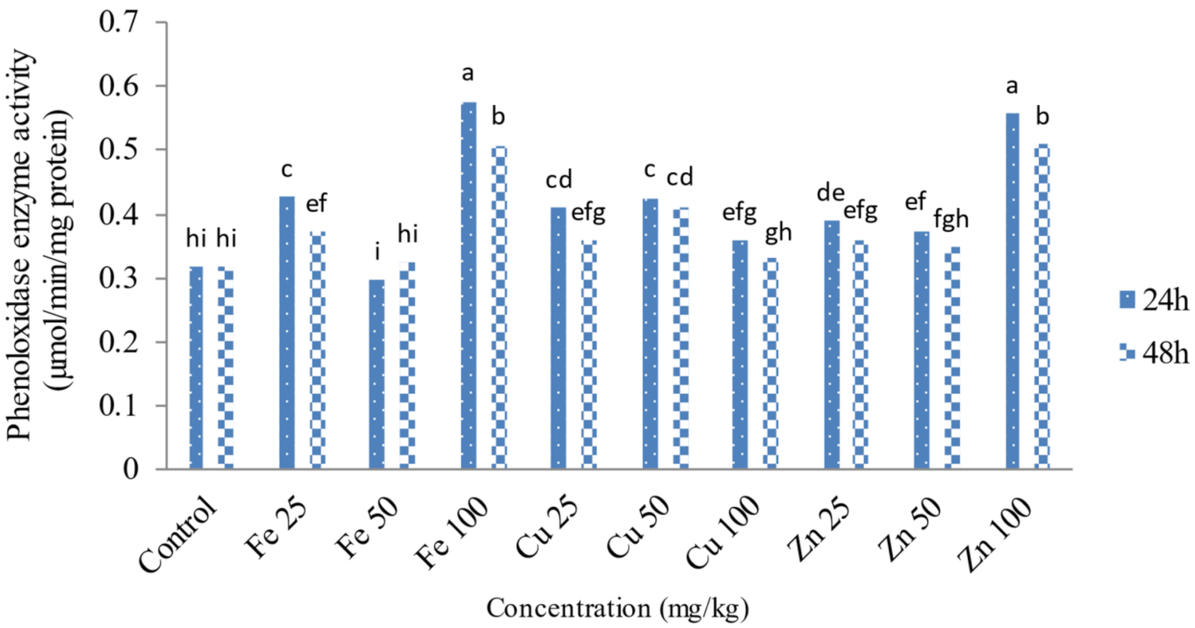

Figure 4 Mean ± SE phenoloxidase enzyme activity in fourth instars larvae of the Mediterranean flour moth under the influence of feeding with flour containing different concentrations of iron, copper and zinc after 24 and 48 h. (Different letters show significance using Tukey's test at $p < 0.05$).

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Nutritional indices

The effect of iron, zinc, and copper salts on the relative growth rate (RGR) ($F = 1.48$; $df_{t,e} = 9, 90$; $P = 0.0053$) and the feeding deterrence index (FDI) ($F = 223.41$; $df_{t,e} = 8, 80$; $P = 0.0001$) showed a significant difference. The highest amount of RGR was related to the larvae fed with copper salt with 25 mg/kg (0.145 ± 0.059 mg/mg/day). The highest amount of FDI was related to the larvae fed with copper (44.379 ± 1.27 mg/mg/day) and zinc salt (43.426 ± 0.8 mg/mg/day) with 100 mg/kg. However, there is no significant difference in relative consumption rate (RCR) and food conversion efficiency (ECI) in different treatments. (Table 1).

Biological characteristics

Variance analysis of the experimental data of the effect of iron, copper and zinc heavy metal salts on the larval period ($F = 19.52$; $df_{t,e} = 9, 30$; $P = 0.0001$), the pupal period ($F = 2.86$; $df_{t,e} = 9, 30$; $P = 0.01$), pupal weight ($F = 12.31$; $df_{t,e} = 9, 30$; $P = 0.0001$) and percentage of adult exit ($F = 34.81$; $df_{t,e} = 9, 30$; $P = 0.0001$) showed that there is a significant difference among all treatments at the probability level of 1% (Table 2).

At high concentrations of copper and zinc (treatments of 100 mg/kg of copper and zinc salts), the larval period was the highest and reached 17.75 ± 0.47 and 17 ± 0.40 days, respectively. The pupal duration was the longest (10.25 ± 0.85 day) in 100 mg/kg of copper treatments. At 25 mg/kg concentration of iron, the pupal weight was 0.018 ± 0.0004 mg placed in a statistical group with control.

The percentage exit of adults in some treatments was significantly lower than the control. The lowest value was related to the concentration of 100 copper (41.25 ± 0.85 %) and zinc (45.75 ± 1.37%).

Discussion

The results of recent research on all the concentrations of copper and zinc compounds and the concentration of 100 iron showed an increase in the total number of hemocytes. Lower concentrations of iron, 25 and 50 mg/kg, caused a significant decrease in blood cells compared to the control. The number of plasmatocytes also decreased significantly in all iron-treated concentrations compared to the control. In the case of granulocytes, concentrations of 25 and 50 mg/kg of iron caused a significant increase in this type of cell. More than other compounds could affect the number of immune cells in *E. kuehniella* larvae. Insect hemolymph does not contain hemoglobin, and iron forms the central core of this protein. Iron seems to affect the structure and activity of other blood factors, such as hemocytes. While zinc and copper ions have much fewer changes in the number of cells than iron.

Table 1 Mean ± SE relative growth rate (RGR), relative consumption rate (RCR), efficacy of conversion of ingested food (ECI) & feeding detterent index (FDI) in fourth instars larvae of the Mediterranean flour moth with flour containing different concentrations of iron, copper and zinc.

Concentrations (mg/kg)	Treatment	RGR (mg/mg/day)	RCR (mg/mg/day)	ECI (%)	FDI (%)
0	Control	0.061 ± 0.002 b	1.667 ± 0.07 a	3.797 ± 0.27 a	-
25	$FeSO_4$	0.075 ± 0.001 ab	1.699 ± 0.09 a	3.737 ± 0.24 a	12.777 ± 0.79 e
	$CuSO_4$	0.145 ± 0.059 a	1.267 ± 0.08 a	4.519 ± 0.22 a	13.691 ± 0.58 d
	$ZnSO_4$	0.069 ± 0.022 b	1.419 ± 0.01 a	4.411 ± 0.56 a	8.338 ± 0.51 f
50	$FeSO_4$	0.062 ± 0.002 b	1.656 ± 0.06 a	4.095 ± 0.18 a	17.616 ± 0.67 d
	$CuSO_4$	0.092 ± 0.001 ab	1.245 ± 0.11 a	4.123 ± 0.3 a	24.626 ± 1.08 c
	$ZnSO_4$	0.088 ± 0.023 ab	1.592 ± 0.17 a	4.022 ± 0.6 a	11.67 ± 0.27 ef
100	$FeSO_4$	0.045 ± 0.011 b	2.024 ± 0.31 a	3.815 ± 0.21 a	33.42 ± 0.81 b
	$CuSO_4$	0.057 ± 0.003 b	1.699 ± 0.16 a	3.956 ± 0.67 a	44.379 ± 1.27 a
	$ZnSO_4$	0.071 ± 0.006 b	1.601 ± 0.07 a	3.99 ± 0.25 a	43.426 ± 0.8 a

Means followed by the same letters in each column are not significantly different (Tukey's test, $P < 0.05$).

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Table 2 Mean ± SE larval duration, pupal duration, pupal weight, percent of adult emergence & adult longevity of Mediterranean flour moth fourth instar larvae were fed with flour containing different concentrations of iron, copper and zinc salts. (Different letters in each column show significance using Tukey's test at $p < 0.05$).

Concentrations of heavy metal' salts (mg/kg)	Larval duration (day)	Pupal duration (day)	Pupal weight (g)	Percent of adult emergence (%)	Adult longevity (day)
Control	12.5 ± 0.28 d	7.5 ± 0.64 c	0.018 ± 0.0004 a	76.75 ± 3.85 a	5 ± 0.00 a
FeSO4 25	13.25 ± 0.25 dc	7.75 ± 0.47 bc	0.018 ± 0.0004 a	72.75 ± 3.35 a	5.25 ± 0.25 a
FeSO4 50	13.5 ± 0.28 dc	9 ± 0.70 abc	0.016 ± 0.0004 ab	71.5 ± 1.55 a	5.25 ± 0.25 a
FeSO4 100	15.5 ± 0.64 b	9.5 ± 0.28 ab	0.0137 ± 0.0006 bc	61.25 ± 1.25 b	6 ± 0.57 a
CuSO4 25	13 ± 0.40 dc	8 ± 0.40 bc	0.016 ± 0.0004 ab	71.75 ± 1.43 a	5.5 ± 0.28 a
CuSO4 50	14.25 ± 0.25 c	8.75 ± 0.62 abc	0.014 ± 0.0009 bc	62.75 ± 1.25 b	5.5 ± 0.28 a
CuSO4 100	17.75 ± 0.47 a	10.25 ± 0.85 a	0.008 ± 0.0006 d	41.25 ± 0.85 c	6.3 ± 0.25 a
ZnSO4 25	13 ± 0.40 dc	7.5 ± 0.5 c	0.016 ± 0.0004 ab	71.75 ± 2.86 a	5.75 ± 0.47 a
ZnSO4 50	13.75 ± 0.47 dc	8 ± 0.40 bc	0.014 ± 0.0001 bc	62 ± 1.87 b	5.75 ± 0.47 a
ZnSO4 100	17 ± 0.40 a	9.25 ± 0.47 ab	0.0132 ± 0.0001 c	45.75 ± 1.37 c	5.1 ± 0.9 a

Means followed by the same letters in each column are not significantly different (Tukey's test, $P < 0.05$).

The number and types of hemocytes play a significant role in the success of the insect defense system (Strand, 2008; Borges *et al.*, 2008; Ajamhassani, 2019; Duarte *et al.*, 2020). Accordingly, the number of hemocytes may indicate the stress level of many invertebrates (Correia, 2008). Invertebrate hemocytes (bivalves) have the potential to accumulate heavy metals in lysosomes (Matozzo *et al*., 2001). Accordingly, when these animals are exposed to heavy metal pollution, their defense response increases the number of blood cells (Coles *et al*., 1995; Mayrand *et al*., 2005). Recent studies have shown that some concentrations are consistent with this theory. Of course, it has been proven that the number of hemocytes does not always increase in the face of stress, and it may even decrease for different reasons, such as the transfer of cells to the fat body and basal membrane and the apoptosis or death of cells (Pipe and Coles, 1995). For instance, the reason for the decrease in the number of hemocytes of the fourth instar larvae of *E. kuehniella* at concentrations of 25 and 50 mg/kg of iron salt compared to the control can be explained by the fact that the cells have probably migrated to the tissues and the toxicity of these concentrations was not so high to create a robust immune response. The research results of Roesijadi *et al*. (1997) showed that heavy metals cause the production of metallothionein by hemocytes. Metallothioneins are responsible for the homeostasis and detoxification of heavy metals. Binding to heavy metals, these proteins reduce the amount of available free ions and return them to the system when required (Marino *et al*., 1998). Another reason for the change in the number of cells is that sometimes the cells are damaged or destroyed due to contamination with heavy metals; therefore, the cell ratio changes (Johansson *et al*., 2000; Jiravanichpaisal *et al*., 2006). In other studies, the number of hemocytes has shown noticeable changes under the influence of heavy metals introduced into the body. As such, the total number of hemocytes of *Achronia grisella* (Lepidoptera: Pyralidae) decreased against the concentrations of cadmium and lead (Gunduz *et al.*, 2020). In the last instar larvae of *S. litura,* the number of hemocytes decreased 48 h after digestive treatment with need gold (Sharma *et al.*, 2003).

It has been shown that the increase in the activity of phenoloxidase enzyme resulting from heavy metal-containing foods is due to the increase in the immune response of insects (Van Ooik *et al*., 2008). Cellular and humoral immunity are closely related (Lavine and Strand, 2002; Jiravanichpaisal *et al.*, 2006). Possibly, in some concentrations, any increase in the number of hemocytes has led to a rise in the activity of the phenoloxidase enzyme because hemocytes such as oenocytoids are potential sources of phenoloxidase production in Lepidoptera (Lavine and Strand, 2002). Also, the decrease in phenoloxidase enzyme activity after exposure to heavy metals is the damage to the basal

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membrane of the midgut and, as a result, the release of some immune mediators into the hemocoel (Dobovskiy *et al*., 2011). The results of Baghban *et al.* (2018) showed that the level of phenoloxidase enzyme activity in *Helicoverpa armigera* Hübner in treatment with cadmium increased at 50 mg/kg and in therapy with zinc at 100 mg/kg and it decreased at 25 mg/kg of cadmium metal salt. In another study, the activity of the phenoloxidase enzyme decreased in the *Galleria mellonella* L. at concentrations of 5 and 10 μg/g of nickel metal and increased at higher concentrations (Dubovskiy *et al.*, 2011). Concentrations of 3 and 5 mM of copper and zinc increased the activity of this enzyme in *Hyphantria cunea* Drury (Lepidoptera: Arctiidae), probably due to some metal ions in the structure of the phenoloxidase enzyme (Ajamhassani *et al*., 2013). Copper, cadmium, and lead decreased phenoloxidase activity in *Mamestra brassicae* (Lepidoptera: Noctuidae) larvae (Kazimirova and Slovak, 1996).

In another part of the study, the effect of zinc, copper, and iron salt on feeding indicators of the pest was investigated. Studies have shown that the RGR index is a function of larval body weight (Srinivasan and Uthamasamy, 2005). In this study, only copper, at 25 mg/kg, caused an increase in larval weight and, as a result, increased relative growth rate index. The increase in larval body weight by feeding on the lowest concentration of copper ions has caused a two-fold increase in larval weight compared to the control. This confirms that food containing copper is somewhat more favorable for *E. kuehniella* larvae. The index of feeding inhibition in the larvae fed with the concentration of 100 zinc and copper was significantly higher than other treatments, indicating the low desirability of these treatments for feeding the larvae. The study indicates that heavy metals such as iron, copper, and zinc, essential for living things, could cause poisoning at high concentrations (Warrington, 1987). The zinc cofactor, having thousands of metalloenzymes and proteins, is involved in the active part of a wide range of enzymes (Dow, 2017). Copper catalyzes oxidation, reduction, and cell defense against oxygen radicals (Yazgan and Yazgan, 2014). Iron also forms the central core of blood hemoglobin and cytochrome P450 and is the most abundant metal in animals' bodies (Broadley *et al*., 2007). Zinc and copper play essential roles in metabolisms, such as enzyme agents and biological processes (Cass and Hill, 1980). Also, these two metals can bioaccumulate (Cheruiyot *et al*., 2013).

According to Hare (1992), copper and zinc can bond with particular metal-carrying proteins such as metallothioneins and release them when essential metal ions are lacking. Since copper is powerfully bound to metallothionein, the concentration of 25 mg/kg of this metal could increase the amount of fat, weight, and, as a result, RGR. However, zinc could increase RGR less than copper, even at a higher concentration (50 mg/kg), due to its lower binding power with metallothionein. On the other hand, since zinc is the second known metal after iron in terms of metabolism and abundance in living organisms (Broadley *et al*., 2007), insects seem to consume more zinc than copper. Since the nutritional inhibition index has also increased with heavy metal salt concentration, it can be assumed that low concentrations can also incur anti-nutritional effects. Research has shown that heavy metals cause damage to the epithelial membrane of the gut and destroy the midgut tissues of insects (Peric-Mataruga *et al*., 2018). The heart is the first organ exposed to metals and other environmental pollutants that enter the body with food. These foreign factors hurt intestinal enzymes' activity and disrupt food digestion and absorption (Wang *et al*., 2020). This could lead to a change in feeding indicators, as adding some nickel to the food of *Tribolium castaneum* caused a decrease in the feeding rate of the larvae and affected the feeding indicators (Goncalves, 2007). The increase in FDI in this study could be due to the insect's resistance to the toxicity of heavy metals and to reduce the damage to the digestive tube resulting from feeding on heavy metal salt-containing foods.

Compared to the control group, the growth stages of *E. kuehniella* also underwent significant changes under the influence of metal-

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contaminated food. According to the results, increased concentration of heavy metal salts has increased the larval period. Among them, copper and zinc at 100 mg/kg had the greatest effect in increasing the length of the larval period. Probably due to the high inhibitory effect of feeding (based on previous results about these treatments), the feeding of the mentioned treatments is greatly reduced. Therefore, it is obvious that the growth of the larvae is slowed down, and the larval period is completed in a longer period. It has been proven that the accumulation of metal compounds in the stomach and fat organs causes changes during the larval period and losses of larvae and pupae (Borowska *et al*, 2004). Research has shown that the larval period of *H. cunea* increases by increasing the amount of iron, copper, and cobalt in the diet (Tupkara and Yanar, 2020). Also, Kosalwat and Knight (1987) showed that the minimum concentration of copper in the *Chironomus decorus* Johannsen mosquito causes an increase in the length of the larval and pupal period and, as a result, a delay in the emergence of adults.

Similarly, the results of the study on the growth of the predator *Podisus maculiventris* Say (Hemiptera: Pentatomidae) indicated that the minimum concentration of zinc and copper leads to a decrease in the adult weight and an increase in the time of conversion of immature to adult individuals (Cheruiyot *et al*., 2013). A lengthened period of larvae development can increase the probability of their parasitism (Kaitaniemi and Ruohomaki, 1999). In addition, an increased concentration of heavy metal salts could also increase the pupal period. Thus, the pupal period corresponding to 100 mg/kg of iron, zinc and copper concentrations was longer than that of other treatments and the control. In other words, inhibiting the feeding of larvae from higher concentrations has caused an increase in the pupation period and a delay in the birth of adults, indicating the effect of ion poisoning on insects' growth and molting processes. Other researchers also reported that high amounts of copper cause physiological disorders of *S. litura*. Huang *et al*. (2012) showed that high amounts of copper cause disturbance in physiological function in *S. litura*. Therefore, the increase in the duration of the pupa can be due to the disturbances created in the physiological processes of the pest. Also, Huang *et al*. (2012) showed that the pupal period of *S. litura* increases in treatment with copper at 200 mg/kg. Other effects of zinc, copper, and iron treatments included reduced pupal weight and a reduced exit percentage of *E. kuehniella* adults. The weight of the pupa and the percentage of healthy adults is a function of how the larvae are fed. Since the larvae could not have proper nutrition from the flour contaminated with high concentrations of compounds, the low weight of the pupae and the decrease in the birth of adults is justified. The results of this research are consistent with others. The destruction of the membrane of the epithelial cells of the gut leads to a decrease in the secretion of digestive enzymes and the lack of proper feeding of the larvae. Then, the weight of the resulting pupae and the emergence rate of healthy butterflies will decrease (Emre *et al*., 2013). In addition, it seems necessary to determine the amount of protein in the insect's body to ascertain whether the ingredients in the food consumed affect the insect's growth rate (Büyükgüzel, 2002; Büyükgüzel and İçen, 2004). The study by Tupkara and Yanar (2020) found that the amount of *H. cunea* pupal protein decreases with increased iron and copper in the diet, while with an increase in zinc and cobalt, the number of pupal protein increases. They concluded that the amount of pupal protein is negatively affected by iron and copper metals, resulting in decreased pupal weight.

Although iron, copper, and zinc did not significantly affect the longevity of *E. kuehniella* adults, some researchers showed that the impact of heavy metals on the longevity, survival, and reproduction of adults is positive. Longevity and fecundity of adults and survival of the *Chromatomyia milii* Kaltenbach (Diptera: Agromyzidae) have decreased with increased exposure to cadmium (Scheirs *et al*., 2006). High concentrations of selenium cause

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the death of crickets and grasshoppers by consuming the leaves of *Stanleya pinnata* Britton and *Brassica juncea* L. (Freeman *et al*., 2007). According to Görür (2010), cabbage and radish contaminated with zinc or cadmium had destructive effects on the growth of *Brevicoryne brassiccae*. In addition, it has been determined that the survival of *Heliothis virescens* Fabricius (Lepidoptera: Noctuidae) decreases with increased amounts of zinc and cadmium (Kazemi-Dinan *et al*., 2014).

Conclusion

In this research, 25, 50 and 100 mg/kg of iron, copper and zinc concentrations were investigated on the developmental, nutritional and immune characteristics of the fourth instar larvae of *E. kuehniella*. All the treatments related to copper and zinc and the concentration of 100 mg/kg of iron caused a significant increase in the total number of hemocytes, which indicates the effect of small amounts of contamination with food on the immune characteristics of insects. In addition, high concentrations of all compounds caused an increase in the larval and pupal period length and a decrease in pupal weight and the exit percentage of moth from the pupal cocoon. According to the present results and conducting additional research, similar compounds can be used in control methods against this important storage pest.

Conflicts of interest

The authors declare that they have no conflict of interest.

Author contributions

Bijan Ahangi Rashti performed experiments. Maryam Ajamhassani analyzed the data, and both authors wrote the paper and approved the final manuscript.

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تأثیر نمک‌های مس، روی و آهن بر فراوانی هموسیت‌ها، شاخص‌های تغذیه و ویژگی‌های بیولوژیکی شب پره مدیترانه ای آرد *Ephestia kuehniella* (Lepidoptera: Pyralidae)

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چکیده: تجمع فلزات سنگین در خاک منجر به سمّیت گیاهان، حیوانات و جوامع انسانی می‌شود. این مطالعه به بررسی تأثیر مقادیر کم نمک‌های آهن، روی و مس بر فراوانی هموسیت‌ها، شاخص‌های تغذیه‌ای و ویژگی‌های بیولوژیکی شب پره مدیترانه ای آرد *Ephestia kuehniella* Zeller در شرایط آزمایشگاهی می‌پردازد. لاروهای سن چهارم با نمک‌های آهن، روی و مس با دزهای ۲۵، ۵۰ و ۱۰۰ میلی‌گرم بر کیلوگرم در دوره‌های ۲۴ و ۴۸ ساعت تیمار شدند. مطالعات ایمونولوژیک شامل بررسی تعداد کل هموسیت‌ها، پلاسماتوسیت‌ها، گرانولوسیت‌ها و فعالیت آنزیم فنل‌اکسیداز بود. تیمارها نسبت به شاهد تغییر معنی‌داری نشان دادند. آهن و روی در غلظت ۱۰۰ میلی‌گرم بر کیلوگرم بیشترین تأثیر را در افزایش فعالیت آنزیم در ۲۴ و ۴۸ ساعت داشتند. همچنین آهن در غلظت ۵۰ میلی‌گرم بر کیلوگرم باعث کاهش معنی‌داری در فعالیت این آنزیم پس از ۲۴ ساعت نسبت به سایر تیمارها شد. از نظر شاخص‌های تغذیه‌ای، نمک فلز مس در غلظت ۲۵ میلی‌گرم بر کیلوگرم، سرعت رشد نسبی را نسبت به شاهد افزایش داد. این مطالعه مقدماتی تأثیر معنی‌دار نمک‌های روی، مس و آهن را بر ویژگی‌های بیولوژیکی و سیستم ایمنی *E. kuehniella* نشان می‌دهد. به‌نظر می‌رسد با افزودن مقادیر کمی از این فلزات به رژیم غذایی حشره می‌توان اختلالات بیولوژیکی و فیزیولوژیکی را در آن ایجاد کرد.

واژگان کلیدی: فراوانی هموسیت‌ها، شاخص تغذیه، زیست‌شناسی، فلزات سنگین، شب‌پره مدیترانه‌ای آرد

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