

**Effects of heavy metals and fluorine on phagocytosis and phenoloxidase activity
in *Mamestra brassicae* (Lepidoptera: Noctuidae)**

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Abstract. Phagocytic response to injected iron saccharate and yeast cells (heat-killed *Saccharomyces cerevisiae* or viable *Candida tropicalis*) and haemolymph phenoloxidase (PO) activity were studied in *Mamestra brassicae* larvae fed on diets contaminated by copper, lead, cadmium or fluorine ions. In copper-stressed larvae, increased and reduced percentage of plasmatocytes and granular cells, respectively, and a reduced phagocytosis of iron saccharate were found. Cadmium- and fluorine-treatment reduced phagocytosis of *S. cerevisiae*. Cadmium showed a stimulatory effect, copper an inhibitory effect on phagocytosis of *C. tropicalis*. PO activity was reduced by all toxicants, in copper-stressed larvae it reached only 22% of the activity in the control.

INTRODUCTION

Rapid industrialization causes an increase of the amount of trace elements in the environment. Heavy metals accumulated in insects can exert toxic effects at all levels of biological organization (Hare, 1992). Also fluorine, as a part of industrial immissions, affects negatively the development and survival of insects (Weismann & Reháková, 1993a).

In general, the toxicity of trace elements is primarily attributed to perturbation of biochemical functions by metal ions, probably due to their alteration of enzyme-ligand interaction and changes in the macromolecular structure of cellular components. For example, in crabs, raised levels of copper and cadmium were found to interfere with respiratory activity, osmoregulation and energy metabolism by influencing the activity of enzymes involved in these processes (Hansen et al., 1992a,b). Heavy metal ions also inhibit the activity of enzymes playing a key role in insect metabolism (Weismann & Reháková, 1993b). In aquatic molluscs, copper ions were found to cause lysosomal membrane destabilization and affect the activity and synthesis of acid phosphatase (Suresh & Mohandas, 1990). In these organisms, an altered immunocompetence has been observed after exposure to copper (Cheng, 1988b) or cadmium (Coles et al., 1995). However, immunotoxic and immunomodulating effects of heavy metals have been reported mainly in vertebrates (Benková et al., 1992, 1993; Morozzi et al., 1992; Daum et al., 1993; Rougier et al., 1994). In insects, cadmium and lead ions were found to cause reduction of total protein, lipid and glycogen content in ovaries (Jamil & Hussain, 1993) and a delay in development (Akins et al., 1992). Chronic exposure to toxins may affect genetic variation of populations and lead to genetic adaptation to heavy metals (Posthuma & Van Straalen, 1993).

As little information is available about insect immunity under stress imposed by environmental pollutants, it was thought worthwhile to investigate some aspects of immune response, namely phagocytic response of haemocytes and the activity of haemolymph phenoloxidase involved in insect defence mechanisms, in cabbage moth, *Mamestra brassicae* (L.) (Lepidoptera: Noctuidae) larvae exposed via food to sublethal doses of copper, cadmium, lead or fluorine ions.

MATERIAL AND METHODS

Insects

Eggs of *Mamestra brassicae* were obtained from a laboratory stock reared at $23 \pm 1^\circ\text{C}$, $65 \pm 5\%$ RH and 16:8 (L:D) h photoperiod. The first three larval instars were fed on leaves of Brussels sprouts, and subsequent instars on a semi-synthetic diet (Podmanická & Weismann, 1975).

From the 4th instar to the sampling of haemolymph, larvae were fed continually on diets contaminated with 5% $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 5% $\text{Pb}(\text{NO}_3)_2$, 5% $\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$, or 5% NaF, which were mixed with the liquid diet during its preparation.

Following concentrations were applied: 7.6 or 76 μg of Cu, 8.9 μg of Pb, 8 μg of Cd or 7.2 μg of F per g diet dry matter.

Haemocyte count and phagocytosis assay

Differential haemocyte count (DHC) and phagocytic activity were investigated in 4-day-old 6th instar larvae. Prior to injection, larvae were anaesthetized by immersion into cold water. Haemolymph was obtained by cutting off the first abdominal proleg.

A. DHC and phagocytosis of iron saccharate: larvae were injected with 5 μl of Ferrum-Lek (Ljubljana, Yugoslavia) in insect saline (1:9). After 1 h incubation period at $23 \pm 1^\circ\text{C}$ blood films were prepared, air-dried, fixed in formaldehyde: methanol (1:9), immersed into $\text{K}_4[\text{Fe}(\text{CN})_6] : \text{HCl}$ (1:1) and stained with basic fuchsin : neutral red (for details see Slovák et al., 1991). Under light microscope, 200 haemocytes were differentiated from each larva and examined for their phagocytic activity. The percentage of haemocytes which had phagocytized non-self material was recorded as the phagocytic index. The degree of phagocytosis was evaluated as follows: + cells with few small particles to small aggregates of engulfed material, ++ cells filled approximately to 75% with engulfed material, nucleus visible, +++ cells full of engulfed material.

B. Phagocytosis of yeast cells: *Saccharomyces cerevisiae* (Endomycetales: Saccharomycetaceae) (commercial brand) suspension (1 g/50 ml of insect saline) was boiled for 10 min. 2.5×10^5 cells in 10 μl of saline were injected per larva. Blood films were prepared after 5 h incubation at $23 \pm 1^\circ\text{C}$. Air-dried films were fixed in methanol for 10 min and stained with May-Grünwald solution (diluted 1:1 in phosphate buffer, pH 7.2) for 5 min.

Candida tropicalis (Deuteromycetales: Cryptococcaceae) (isolate CCY 29-7-6, State Yeast Collection, Chemical Institute, Slovak Academy of Sciences) culture was suspended in insect saline. Each larva was injected with 3.6×10^6 cells in 10 μl saline. After 3 h incubation (at $23 \pm 1^\circ\text{C}$) blood films were prepared, air-dried, fixed in methanol for 10 min and stained with 0.1% methylene blue for 3 min. Phagocytosis of yeast cells was evaluated as follows: phagocytic index was determined as the percentage of haemocytes that had phagocytosed yeast cells; endocytotic indices were evaluated as the percentages of phagocytizing haemocytes containing 1 to more than 5 endocytosed yeast cells.

Assay of phenoloxidase (PO) activity

10 μl of haemolymph were obtained from 4-day-old 6th instar larvae. Haemolymph samples from each individual were diluted 1:19 in chilled phosphate buffer by Sørensen (pH 6.8) and kept at 4°C for 1 h. The assay was performed in microtiter plates. To each well, 100 μl of haemolymph sample, 100 μl of DL-DOPA (Sigma) (0.02 M in phosphate buffer, pH 6.8) were added. The mixture was incubated at 30°C for 30 min. The reaction was stopped by 5 μl of 1% thiourea. The absorbance of the resulting dopachrome was measured at 490 nm using a microreader. PO units were expressed as absorbance units (at the specified wavelength multiplied by 1,000).

Data were analysed by one-way ANOVA and the Duncan multiple range test. Percentages were transformed to arcsine \sqrt{p} .

RESULTS

Differential haemocyte count

Five haemocyte types could be distinguished in *M. brassicae* larvae (Table 1). The bulk of the total haemocyte population 1 h post-injection with iron saccharate was made up of plasmatocytes (PL) and granular cells (GR) comprising 24–34% and 52–61% of the total, respectively.

Significantly higher percentage of PL was found in copper-stressed larvae. Chronic exposure to copper decreased the percentage of GR (though not significantly at $P < 0.01$).

TABLE 1. Differential haemocyte count in iron saccharate-injected *Mamestra brassicae* larvae fed on heavy metal- and fluorine-contaminated diets.

	n	Haemocyte types (mean percentage \pm S.E.M.)				
		Prohaemocytes	Plasmatocytes	Granular cells	Spherulocytes	Oenocytoids
Control	20	6.76 \pm 0.91 a	24.05 \pm 1.35 a	58.70 \pm 1.20 ab	8.74 \pm 0.84 a	1.75 \pm 0.26 ab
Copper	25	3.65 \pm 1.24 bc	32.73 \pm 1.41 cd	52.42 \pm 2.19 a	10.17 \pm 1.40 a	1.04 \pm 0.23 c
10 Copper	19	2.94 \pm 0.98 bcd	34.36 \pm 1.80 d	51.67 \pm 1.75 a	10.50 \pm 1.05 a	0.54 \pm 0.12 c
Lead	22	4.64 \pm 0.81 ab	26.80 \pm 1.02 ab	60.29 \pm 1.71 bc	6.40 \pm 0.57 a	1.87 \pm 0.23 a
Cadmium	20	3.68 \pm 0.85 bc	26.32 \pm 1.56 ab	59.00 \pm 1.22 bc	9.11 \pm 0.69 a	1.89 \pm 0.27 ab
Fluorine	20	2.54 \pm 0.82 bcd	25.29 \pm 1.57 a	61.28 \pm 1.78 bc	8.78 \pm 0.99 a	2.11 \pm 0.28 a

n – number of individuals in a treatment; means in a column followed by the same letter are not significantly different at $P < 0.01$ (ANOVA and Duncan multiple range test after arcsine transformation of data, actual percentages are presented). 10 Copper – 76 μ g Cu/g diet dry matter.

Phagocytosis

GR were the predominant phagocytic cells in *M. brassicae*, comprising 92% of the total. The remaining 8% were recognized as PL. The extent of the phagocytic response varied according to the injected foreign material.

A. Phagocytosis of iron saccharate. In most treatment groups except for that exposed to lead, phagocytic indices were lower than in the control. Copper- and fluorine-stress reduced significantly phagocytosis of this non-self material (Fig. 1). Iron saccharate was readily phagocytized by *M. brassicae* haemocytes. On average, the proportion of haemocytes engulfing this material to a certain extent, defined as the degree of phagocytosis, was 14:36:50% (i.e., degree + : ++ : +++). This ratio was not significantly affected by toxicant bioaccumulation.

B. Phagocytosis of yeast cells. Phagocytic indices after injection of unviable *S. cerevisiae* cells were lower (9–16%) than those obtained after injection of viable *C. tropicalis* cells (16–26%) or iron saccharate (35–46%).

Phagocytic response to *S. cerevisiae* was reduced significantly in cadmium- and fluorine-stressed larvae (Fig. 1). However, phagocytic response to viable *C. tropicalis* increased in cadmium-stressed larvae (Fig. 1). Chronic copper-stress did not affect considerably the phagocytosis of yeast cells. Endocytotic indices remained unaffected by toxicant-stress. On average, the proportions of phagocytizing haemocytes having engulfed 1, 2, 3, 4, 5 and > 5 yeast cells were 57:23:10:4:3:3% (*S. cerevisiae*) or

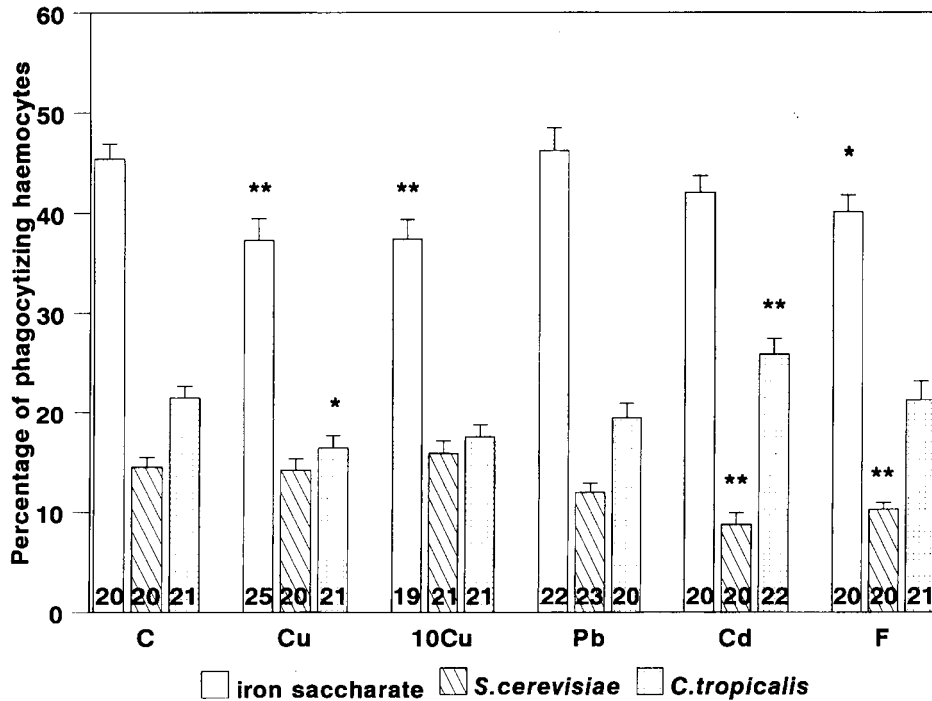


Fig. 1. Phagocytosis of iron saccharate, heat-killed *Saccharomyces cerevisiae* and viable *Candida tropicalis* cells by heavy metal- and fluorine-stressed *Mamestra brassicae* larvae. Data represent actual percentages of phagocytizing haemocytes (mean + SEM). Values at the base of each column represent numbers of individuals per treatment. *P < 0.05, **P < 0.01 indicate the significance of differences as compared with the control (one-way ANOVA and LSD of data transformed to arcsine \sqrt{p}); C-control; Cu (7.6 $\mu\text{g/g}$ diet dry matter), 10Cu (76 $\mu\text{g/g}$ diet dry matter).

34 : 26 : 16 : 9 : 5 : 10% (*C. tropicalis*) and did not differ significantly among the treatment groups.

Phenoloxidase (PO) activity

Significant reduction of PO activity in *M. brassicae* haemolymph was detected following chronic exposure to all toxicants (Fig. 2). Copper (both concentrations) and cadmium reduced PO activity to 22% and 45% of the activity measured in control larvae. Both lead and fluorine reduced PO activity to 78%.

DISCUSSION

Immune response depends not only on the characteristics of the non-self object, but also on the physiological state of the organism. Wounding, injection of different foreign particles, application of several poisons can cause changes in insect haemocyte populations (Shapiro, 1979). Reduced haemopoiesis was observed in copper-stressed molluscs, while

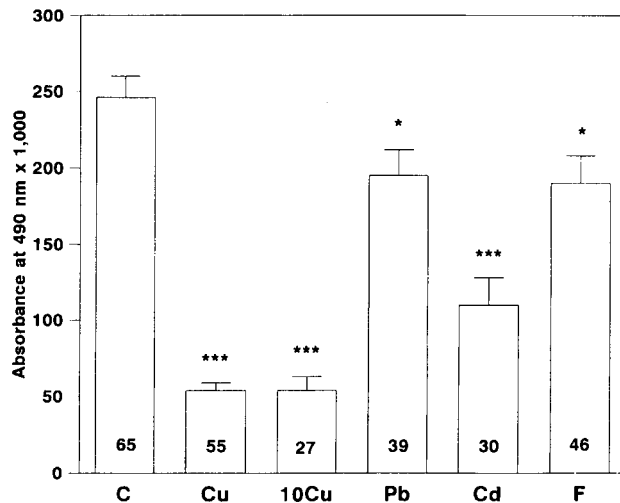


Fig. 2. Effect of heavy metal- and fluorine-stress on haemolymph phenoloxidase activity (mean + SEM) in *Mamestra brassicae* larvae. *** P < 0.001, for others see Fig. 1.

tion occurs after iron saccharate injection, the reduced phagocytic response in copper-stressed larvae might have been, in part, due to reduction of the number of GR (the main phagocytic cells in this insect species). Similarly, an inhibition of phagocytosis of bacteria was observed in copper-stressed bivalves (Cheng, 1988b). Copper has been reported to decrease the number of kidney-leucocytes and reduce the phagocytic response in fish (Rougier et al., 1994). Heavy metals immission inhibited up to 70% the phagocytic activity of the alveolar macrophages of mice (Morozzi et al., 1992), reduced phagocytic ability of peritoneal macrophages of guinea pigs (Benková et al., 1992) and that of leucocytes of sheep (Benková et al., 1993). However, copper-stressed cabbage moth larvae displayed only a slightly reduced phagocytic response to *C. tropicalis* and their response to *S. cerevisiae* did not differ from the control. Cadmium and fluorine appeared to inhibit phagocytosis of *S. cerevisiae*, while cadmium stimulated phagocytic response in *C. tropicalis*. Exposure to cadmium increased phagocytosis of bacteria (Cheng, 1988b) and neutral red (Coles et al., 1995) in aquatic molluscs. However, cadmium was found to inhibit the RNA, DNA and antibody synthesis in murine B-lymphocytes (Daum et al., 1993). Differences in the phagocytic response between the two yeast species might be due to the different surface characteristics of these objects, to the extent of nodule formation after their injection and to possible differences in the modulation of cellular immune response by various toxicants.

In this study, copper-stressed larvae appeared to bleed more readily than control larvae or those treated with other toxins, and their haemolymph PO activity was reduced to 22% of the control. Until now, inhibition or suppression of PO activity have been observed in insects parasitized by hymenopterous parasitoids (Stoltz & Cook, 1983) and

cadmium appeared to stimulate this process (Cheng, 1988a).

No alteration was demonstrated in proportions of circulating haemocyte types after exposure of aquatic molluscs to cadmium (Coles et al., 1995). Chronic copper-stress appeared to increase the number of PL and reduce that of GR in *M. brassicae* larvae, which were previously injected with iron saccharate. However, we suppose that the injection itself could not have led to dramatic DHC changes within a 1 h incubation period. As no nodule formation

entomopathogenic nematodes (Yokoo et al., 1992), or after administration of phenylthiourea (Brewer & Vinson, 1971).

In conclusion, this study has shown that cellular immune response and PO activity in insects may be influenced by heavy metals or fluorine. However, studying the effects of various environmental contaminants on insect immunity is a complex problem. Other facets remain unknown, e.g., the mechanism by which melanin biosynthesis is inhibited by toxins. Also more knowledge is required of histopathological changes in blood cells and the modulation of different aspects of immune function in insects under heavy metal-stress. The question whether insects and their immune system could adapt to sublethal doses of toxins requires investigation.

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