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The Impact of Element–Element Interactions on Antioxidant Enzymatic Activity in the Blood of White Stork (*Ciconia ciconia*) Chicks

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Abstract The aim of this work was to determine interrelationships among macroelements Na, K, Ca, Mg, and Fe, microelements Zn, Cu, Mn, and Co, and toxic heavy metals Pb and Cd in the blood of white stork *Ciconia ciconia*, during postnatal development, in different Polish environments, and their impact on the activity of antioxidant enzymes. We considered the content of thiobarbituric acid-reactive substances (TBARSs), i.e., malondialdehyde (MDA), and activity of superoxide dismutase (SOD), catalase (CAT), ceruloplasmine (CP), glutathione peroxidase (GPx), and glutathione reductase (GR). Blood samples were collected from storks developing at Odra meadows (Kłopot; southwestern Poland). They were compared with blood of chicks from several suburban sites located 20 km away from Zielona Góra (0.1 million inhabitants; southwestern Poland) and near Głogów, where a copper smelter is situated. We also conducted research in the Pomeranian region (Cecenowo; northern Poland). We collected blood samples via venipuncture of the brachial vein of chicks in 2005–2007. They were retrieved from the nest and placed in individual ventilated cotton sacks. The blood was collected using a 5-ml syringe washed with ethylenediaminetetraacetic acid (EDTA). We found significant interactions between macro- and microelements and enzymatic activity and TBARS products. We noticed the predominance of Cd and Pb participation in element–enzyme interactions. Simultaneously, we found interrelationships between cadmium and Na, K, Ca, Mg, and Fe and the activity of antioxidant enzymes SOD, CAT, CP,

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GR, and TBARS products in the blood of white stork chicks. In the case of lead these relationships were not numerous and they were significant for Ca, Mg, Cu, Mn, and Co. Correlations with enzymes were significant for Pb-CAT and Pb-TBARS. We noted that activities of most enzymes (SOD, CAT, CP, GR) and TBARS products are determined by their interactions with physiological elements Na, Ca, Mg, Fe, and Zn and toxic heavy metals. White stork chicks ranged in age from 17 to 59 days. Concentrations of elements in the blood were age related. Among enzymes, only SOD, CAT, and GPx were age related. Young storks differed in the case of element concentration (except for Ca, Zn, and Cd) and enzymatic activity. We found that significant element–element interaction/enzyme activity predominated in the case of physiological elements and toxic metals, which we explain by the intensive and prevailing access of toxic metals in redox reactions. This causes changes in the priority of these metals, reflected by their influence on the enzymatic activity of antioxidant enzymes. The content of Cd and Pb in blood of young storks from different regions tends to affect the lipid peroxidation process negatively. However, in many cases we observed an increase in enzymatic activity with an increase in heavy metals. This indicates the changes in oxidative stress intensity in chicks in response to environmental differentiation. The increase in lipoperoxidation modifies antioxidant enzyme activity and causes changes in SOD, CAT, CP, GPx, and GR activity in chicks from various regions, principally increases in enzyme activity in chicks from polluted environments and suburbs. We suggest that the source of heavy metals in chicks' blood might be used as a biological test system of adaptation to oxidative stress. We also report that a high level of heavy metals is accompanied by increased lipid peroxidation. Thus young storks are probably significantly susceptible to environmental conditions. They demonstrated initiation of lipoperoxidation and oxidative modification of proteins that coincide with chemical elements, as a possible antioxidant defense system.

Introduction

Element–element interactions and their ecophysiological impact can be explained in a number of different ways. Most obvious is that interactions can be a chemical phenomenon which occurs in the lumen of the intestine along with other food ligands during the gastrointestinal phase of digestion. However, interactions might also be occurring at the site of chemical element binding to the brush border of the intestinal cell during its absorption. Moreover, as macroelements are incorporated into intestinal cell, there is also potential for cellular interactions of Ca, Mg, Fe, and trace elements. Finally, there might be competition in the plasma or basolateral phase where macroelements are transferred from the serosal side of the mucosal enterocyte to the accepting plasma protein transferrin (Smith 1988). It has been reported by Nielsen (1988) that environmental and nutritional circumstances can moderate element–element interactions. However, Giesy (1980) emphasized that toxic heavy metal interactions are moderated by their relations with naturally occurring organic ligands.

Chemical elements analyzed in this paper (Ca and heavy metals) were divided (according to Simkiss 1975; Underwood 1977; Fergusson 1990) into three categories. (1) The most important and indispensable elements for living organisms include, as the most necessary, Na, K, Ca, Mg, and Fe. (2) Trace elements, i.e., microelements, include mostly heavy metals, Zn, Cu, Mn, and Co. For any organism their concentrations are required to remain within relatively narrow limits for its proper (normal) functioning, on a limited scale (i.e., the organism has relatively narrow ranges of tolerance of these elements, whose concentrations in "excess" or "deficiency" are toxic). It is possible to include selenium here as a microelement, since it is known to interact and may be toxic itself (Hoffman 2002). However, selenium was not included in our studies, because of various technical difficulties with its proper identification. (3) Chemical elements are mainly toxic heavy metals. Even trace amounts in the living organism are very harmful and simply dangerous to it. The main such metals are Pb and Cd, though mercury can be included here also. Mercury was not included in our investigation, because of the fundamental difficulties in singling it out (the main reason is the necessity for a relatively low temperature (i.e., about 40°C) of sample mineralization [Hall et al. 1997]).

Metals are defined as being elements which have a characteristic lustrous appearance, are good conductors of electricity, and generally enter chemical reactions as positive ions or cations. However, sometimes the distinction between metals and nonmetals is not sharp; e.g., Sb, As, and Te have physical properties of metals and chemical properties of nonmetals. In biology, the distinction between metals and nonmetals is even less clear and depends very much on the personal prejudice of the author. Similarly, the distinction between metals which are heavy and those which are not is blurred. Many authors define metals as heavy if they have a relative density > 4.5 but there are many papers in which this term is used to describe elements which neither are heavy (e.g., Al) nor are metals in the strict sense (e.g., Se). The term "trace metals" is also difficult to define. In studies on vertebrates, metals are described as being present in trace amounts if their concentrations in the tissue are lower than that of Fe (Underwood 1977). This definition is unsatisfactory for

invertebrates because the normal levels of Fe in many species are much lower than those of Cu and Zn, considered to be trace metals under the vertebrate definition (e.g., spiders). These differences may be even greater in tissues of invertebrates from metal-polluted areas (Hopkin 1989).

It must be emphasized that the requirement for a particular element is demonstrated by comparing the growth, survival, and reproductive success of a control group with those of a group fed on a diet which is identical, except for the absence of the element under test. A number of criteria have been established, which must be satisfied before an element can be considered essential: (1) removal of the element from the diet should result in disturbed growth and reproduction; (2) these deficiency effects should be accompanied by changes in pathology and status of immunoactivity; (3) alleviation of deficiency effects should be dose dependent; and (4) the element should form an essential component of an enzyme, a hormone, or another biologically active substance (Hopkin 1989). Many elements considered to be essential for vertebrates satisfy only the first criterion. However, it may be impossible to disprove an essential requirement for some elements, as the theoretical lowest possible limit for essentiality is one atom for one specific gene in the chromosomes of a cell. The distinction between essential and nonessential elements is therefore equivocal (Hopkin 1989).

Concentrations of Cd, Pb, Zn, and other toxic metals in southwest Poland, an area that has been subjected to significant contamination, range from background levels to levels well in excess of the maximum tolerable limits in soils. Thus, Cd, Pb, and Zn ranged from 0.5 to 105, 14 to 7100, and 20 to 10,000 mg $\times$ kg^{-1} soil, respectively. Simultaneously, soil type, texture, organic matter, and cation exchange capacity did not influence the relative proportions of heavy metal forms in any consistent manner. However, the influence of soil pH was clear; soil of pH < 5.6 contained relatively more of all metals in the exchangeable form than soil of pH > 5.6. The most consistent distribution patterns were found when the soil samples were grouped according to their total metal contents. The inferred relative mobility of metals and their redistribution among different fractions are generally consistent with the findings of previous studies of contaminated soils and support the view that metals from anthropogenic sources are more mobile than those from soil parent materials (Chłopecka et al. 1996).

The white stork is a bioindicative species model for large-scale animal research (Kaługa 2006). The main threats to the breeding population of white stork include natural factors, e.g., unfavorable weather conditions, but are in great part due to anthropopression, e.g., lowering of groundwater level, drainage, transformation of grassland into arable fields, extensive networks of electric wires changes in roofing types, and the use of plastic strings in agriculture (Kaługa 2006; Profus 2006).

Although the white stork has been associated with human activities, significant changes in agricultural landscape and industrial management caused drastic changes in the numbers of this species and even its permanent retreat from considerable areas of Europe. On the other hand, this species is firmly settled in many national regions. Thus the decrease in numbers of this bird was able to be identified early. In many countries in western Europe in the last 20 years a decrease in white stork population size has been observed (Daniluk et al. 2006; Peterson and Jakubiec 2006). Also, central Europe is now under intense pressure of development and the white stork is under economical pressure in the changing landscape (Rubacha and Jerzak 2006). On the contrary, in Poland it is rather stable (Daniluk et al. 2006).

In Poland the white stork is generally moderately abundant, although in some regions it is infrequent (Tomiałojć and Stawarczyk 2003). Simultaneously, the white stork has become an umbrella species. In some parts of Poland, action is taken to protect nests from destruction and to rescue injured storks (Kaługa 2006). Due to its large body size, nesting in the vicinity of humans, and low timidity, this bird is easy to investigate. Moreover, it is well known and popular, and its characteristic appearance makes it difficult to mistake for any other bird species. Therefore it can be successfully watched during the breeding season, getting to know its ecology. The white stork needs vast and open terrains, abundant in a variety of small animals. For feeding grounds it prefers areas of high biodiversity. Because of the ease of finding storks' nests and determining hatching results, this species is ideal for population studies combined with hatching success and local conditions. Storks locate their nests in highly visible places, thus they are quite easy to watch during the whole season (Szulc-Guziak 2006). Simultaneously, as a migratory species, they encourage an acquaintance with the interesting and complex subject of bird migration. But, first of all, the white stork is a good indicator of the quality of the natural environment. In the wild, storks live only in places where the environment is not severely transformed and the birds are able to find rich feeding grounds assuring their survival. If storks leave an area, it indicates a decrease in its natural value. Therefore, analyzing the causes of changes in these birds' distribution and in the size of the population, on a regional scale, provides much valuable information on the threats to this species. So the white stork is an ideal object for birds population research because it helps in the acquisition of concrete knowledge about the environment and dependences, while shaping an active attitude toward the surrounding world. By actively participating in its protection, humans become convinced

of the need and effectiveness of their own activities (Szulc-Guziak 2006).

Investigations of the white stork population were carried out by Lack (1973), who attempted to analyze mechanisms regulating the number of the white stork population. According to Lack (1973), many years the white stork population is subjected to cyclic fluctuations in number, for insufficiently explained reasons. It effects distinct changes in its breeding territories in Europe, expressed by its decreased numbers and recession from the areas. On the basis of Jakubiec (1985) it may be concluded that since the 16th century environmental transformations favorable for the white stork in Europe have coincided with anthropopression and synanthropization of this species. The changes in population dynamics, number, fecundity, and mortality were the results of various processes, among which the impacts of environmental pollutants and changes in chemical element transfer were the most significant. However, particularly significant data given by Jakubiec (1985) demonstrate that despite a degradation in the environment, a local increase in the number of white storks can be observed. This shows distinctly that the number of white storks can remain below the environmental capacity in some parts of ecosystems.

Research on the effects of element concentration in birds under natural conditions has examined biogeochemical interactions affecting hepatic trace element levels in birds (Möller 1995). Benito et al. (1999) established the parameters which affect accumulation of trace elements in blood and suggested some regularities about Zn and Cu interactions with Pb and Cd. Kamiński and Warot (2005) studied relationships between elements in the organs of hole-nesting passerines during their nest development.

The aim of this work was to determine interrelationships among the macroelements Na, K, Ca, Mg, and Fe, microelements Zn, Cu, Mn, and Co, and toxic metals Pb and Cd in the blood of the white stork *Ciconia ciconia*, an altricial bird, during postnatal development, in different environments in Poland, and their impact on the activity of important antioxidant enzymes. We have taken under consideration the content of thiobarbituric acid-reactive substances (TBARSs), i.e., malondialdehyde (MDA), and activity of superoxide dismutase (SOD), catalase (CAT), ceruloplasmine (CP), glutathione peroxidase (GPx), and glutathione reductase (GR).

Study Area

Blood samples were collected from young storks developing immediately near Odra meadows (Kłopot village; 52°07′56.3″N, 14°42′10.4″E), southwestern Poland (Tryjanowski et al. 2005), henceforth called "Odra meadows" or "control," treated as the control environment. They were compared with blood samples from young storks at several suburban sites: four villages located about 20 km away from Zielona Góra (51°56′26.1″N, 15°30′38.9″E; about 0.1 million inhabitants, southwestern Poland), mainly Czarna (51°54′43.9″N, 15°42′01.1″E) and Czarnowo (52°02′03.''N, 14°57′24.7″E). In villages near Zielona Góra samples were collected at a distance of several kilometers from the city boundary. Thus hereafter we call these areas "suburbs." We also took our investigations near Głogów (51°39′32.6″N, 16°04′49.9″E), where a copper smelter is situated (polluted area). Głogów Copper Manufacture produces copper and lead from lead fields, therefore we call these areas "polluted." We also conducted research in Cecenowo, a small Pomeranian village near Słupsk (northern Poland; 54°38′34.5″N, 17°32′31″S), hereafter called "Pomeranian."

Materials and Methods

A total of 78, 86, and 146 white stork chicks from 36, 35, and 48 nests were surveyed in three breeding seasons, 2005, 2006, and 2007, respectively. The age of birds varied from 14 to 62 days. We studied 84 individuals from Odra meadows, 48 from Pomeranian, 85 from suburbs, and 53 individuals from polluted areas in the period 2005–2007 (Table 1). To eliminate diurnal rhythm changes, all examinations started at 10 and ended at 12 am. Samples of wing venous blood were taken for analyses of chemical element concentration. Contents of elements (ppm, dry weight) were then determined using atomic absorption spectrophotometry (Weltz 1985) with a Perkin-Elmer apparatus (type AAnalyst 800–RW0683/3PYC). Standard curves were prepared using standardized Merck samples.

We collected blood samples via venipuncture of the brachial vein of stork chicks. They were retrieved from the nest and placed in individual ventilated cotton sacks. Blood (5 mL) was collected using a 5-ml syringe washed with EDTA. Samples were kept in a chilled cooler before transport to the laboratory. After centrifugation, plasma samples were frozen at −20°C and stored until analysis. Our behavioral observations of storks as well as physical examinations of the birds suggested that all were physically healthy.

As a measure of oxidative stress, MDA, the last product of lipid breakdown (caused by oxidative stress), was measured in venous blood. The lipid peroxidation process was measured by the TBARS method (Timirbulatov and Selezniev 1981). The blood samples (as above) were used for SOD activity, which was estimated from the extent of inhibition of superoxide (O_2^-)-dependent quercetine transformation, according to the method of Kostyuk et al. (1990). CAT activity was determined in plasma blood by the amount of H_2O_2 consumed $\times$ min^{-1} $\times$ L^{-1} with

Table 1 Age range with mean age for each location and differences between concentration of elements and antioxidant enzymes activity in the blood of white stork *Ciconia ciconia* chicks from different environments (mean ± SD)

	N Age range	Odra meadows 14–59	*N*	Pomeranian 20–50	*N*	Suburbs 17–59	*N*	Polluted area 18–62	*P*
Mean age ± SD	84	34.09524 ± 9.280916	48	38.37500 ± 7.147757	85	36.28571 ± 9.612456	53	37.33962 ± 10.07079	
Element									
Na [mg/kg]	86	144.246 ± 13.5506	45	139.568 ± 22.164	80	139.413 ± 14.056	53	131.140 ± 24.544	0.000745
K [mg/kg]	86	5.062 ± 2.7527	45	7.013 ± 3.671	79	6.360 ± 3.245	53	14.156 ± 25.297	0.000109
Ca [mg/kg]	82	181.783 ± 54.5258	45	183.533 ± 58.794	77	185.282 ± 56.893	52	176.817 ± 59.721	0.869407
Mg [mg/kg]	83	2431.289 ± 946.9148	45	4326.133 ± 2269.722	78	3032.506 ± 1241.870	51	3931.078 ± 1573.469	0.000000
Fe [mg/kg]	83	26.033 ± 20.1952	45	37.200 ± 7.437	79	32.417 ± 19.133	53	29.543 ± 16.372	0.005082
Zn [mg/kg]	82	10.037 ± 5.0364	45	9.736 ± 0.561	78	10.393 ± 5.421	53	11.218 ± 6.016	0.449045
Cu [mg/kg]	48	7.424 ± 2.7175	45	10.695 ± 1.202	56	7.171 ± 3.201	37	9.866 ± 3.402	0.000000
Mn [mg/kg]	48	42.834 ± 8.5124	45	47.590 ± 6.074	56	44.424 ± 7.161	37	49.273 ± 3.736	0.000042
Co [mg/kg]	48	2.260 ± 1.0563	45	2.251 ± 0.711	56	2.749 ± 0.727	37	4.037 ± 1.996	0.000000
Cd [mg/kg]	81	2.351 ± 1.0555	45	2.364 ± 1.086	78	2.861 ± 1.384	51	2.977 ± 1.466	0.065950
Pb [mg/kg]	48	1.336 ± 0.6910	45	2.005 ± 1.205	56	2.276 ± 1.131	37	4.554 ± 3.156	0.000000
SOD [U/min mL]	**65**	**450.622 ± 275.0620**	**68**	**492.151 ± 299.899**	**78**	**385.321 ± 210.661**	**53**	**355.425 ± 190.509**	**0.009471**
CAT [μM/min L]	**61**	**1.046 ± 0.7467**	**54**	**2.091 ± 0.902**	**77**	**5.330 ± 7.728**	**53**	**5.216 ± 4.251**	**0.000000**
CP [mg/L]	61	**21.507 ± 14.1062**	**54**	**13.843 ± 9.351**	**79**	**24.018 ± 19.143**	**51**	**17.509 ± 9.238**	**0.000498**
GPx [nmol $NADPH_2$/min mL]	**65**	**714.349 ± 709.7167**	**55**	**551.617 ± 564.223**	**56**	**1183.933 ± 1291.315**	**37**	**3114.891 ± 3564.837**	**0.000000**
GR [nmol GSH/min mL]	**65**	**101.367 ± 108.8912**	**55**	**49.195 ± 48.525**	**56**	**27.322 ± 26.870**	**37**	**44.540 ± 42.971**	**0.000000**
TBARSs [μM/L]	**65**	**6.994 ± 4.1565**	**55**	**20.693 ± 23.558**	**77**	**4.989 ± 3.625**	**53**	**7.094 ± 7.738**	**0.000000**

Note: Significance level; *p*-value. One-way ANOVA followed by multiple-range test and using Tukey test (test of reasonably important difference for bumpy numerical force of attempt)

Bold values denote distinct demarcation of groups of chemical radicals and antioxidant enzymes

molibdate ammonium (Korolyuk et al. 1988). CP activity was estimated using *p*-phenilenodiamine (Ravin 1961).

Measurement of MDA continues to be a useful method for determination of the extent of lipid peroxidation, as it is the most abundant aldehyde formed as a by-product during this process. The level in the blood was estimated following the 2-thiobarbituric acid (TBA) method of Timirbulatov and Selezniev (1981) for determination of TBARS concentration. This method involves reaction of the degradation product of lipid peroxidation, MDA, with TBA under conditions of high temperature and acidity to generate a colored adduct that is measured spectrophotometrically. Briefly, 0.1 mL of blood was added to 2 mL of distilled water, which followed by 1 mL of trichloracetic acid and 1 mL of TBA reagent, and the mixture heated in a boiling water bath for 10 min before the addition of butanol. After cooling, the mixture was centrifuged for 10 min. Absorbance in the organic phase was measured at 532 nm and samples were compared to a blank. Micromoles of MDA per liter of blood was calculated using 1.56×10^5 $mol/L^{-1} \cdot cm^{-1}$ as the extinction coefficient.

SOD activity in blood was measured using quercetine as the substrate after suitable dilution following the method of Kostyuk et al. (1990). The assay mixture in a total volume of 1 mL consisted of 0.08 mmol/L EDTA and 0.1 mol/L sodium phosphate buffer (pH 7.8) at a 1:1 proportion. Briefly, 0.1 mL of blood (1:1000) after dilution was added to 2.3 mL of distilled water, after which 1 mL of assay mixture with EDTA and sodium phosphate buffer. The increase in absorbance due to oxidation of quercetine at 0 and 20 min was recorded at 406 nm using a spectrophotometer. In the blank, blood was substituted by equal quantities of distilled water. One unit of SOD activity is defined as the quantity of enzyme that inhibited quercetine oxidation by 50% under given experimental conditions.

CAT activity was estimated by measuring the breakdown of hydrogen peroxide in the reaction mixture using a spectrophotometer at the wavelength of 410 nm by the method of

Table 2 Element–element–enzyme interactions and age-related changes of elements level and antioxidant enzymes activity in the blood of white stork *Ciconia ciconia* chicks during nesting in different regions

	Age (days)	Na [mg/kg]	K [mg/kg]	Ca [mg/kg]	Mg [mg/kg]	Fe [mg/kg]	Zn [mg/kg]	Cu [mg/kg]	Mn [mg/kg]	Co [mg/kg]	Cd [mg/kg]	Pb [mg/kg]
Age (days)		**−0.1302**	0.0392	**0.1354**	0.0709	**0.3283**	**−0.1550**	−0.0071	**0.1826**	−0.1038	**−0.2831**	**−0.1179**
(*N*)		**(257)**	(256)	**(250)**	(251)	**(256)**	**(252)**	(185)	**(185)**	(185)	**(250)**	**(185)**
		*	ns	*	ns	***	*	ns	*	ns	***	*
Na [mg/kg]	**−0.1302**		−0.0785	**−0.2484**	0.1220	**−0.4272**	**0.1850**	−0.0115	**−0.3043**	0.0367	**0.2161**	−0.0465
(*N*)	**(257)**		(262)	**(251)**	(252)	**(252)**	**(253)**	(181)	**(181)**	(181)	**(250)**	(181)
	*		ns	***	ns	***	**	ns	***	ns	**	ns
K [mg/kg]	0.0392	−0.0785		**0.2794**	−0.1054	0.0102	**0.2467**	0.1216	**0.4009**	**−0.1679**	**0.1816**	−0.0668
(*N*)	(256)	(262)		**(251)**	(252)	(252)	**(253)**	(181)	**(181)**	**(181)**	**(250)**	(181)
	ns	ns		***	ns	ns	***	ns	***	*	**	ns
Ca [mg/kg]	**0.1354**	**−0.2484**	**0.2794**		**−0.5367**	**0.1978**	**0.3952**	0.1001	**0.4632**	**−0.2117**	**0.1422**	**−0.1001**
(*N*)	**(250)**	**(251)**	**(251)**		**(254)**	**(252)**	**(255)**	(186)	**(186)**	**(186)**	**(252)**	**(186)**
	*	***	***		***	**	***	ns	***	**	*	*
G [mg/kg]	0.0709	**0.1220**	−0.1054	**−0.5367**		**0.1379**	−0.0836	0.0594	**−0.3205**	**0.2249**	**−0.1826**	**0.2113**
(*N*)	(251)	**(252)**	(252)	**(254)**		**(253)**	(256)	(186)	**(186)**	**(186)**	**(253)**	**(186)**
	ns	*	ns	***		*	ns	ns	***	**	**	**
Fe [mg/kg]	**0.3283**	**−0.4272**	0.0102	**0.1978**	**0.1379**		**−0.4217**	0.0629	**0.3985**	−0.0615	**−0.6263**	0.0471
(*N*)	**(256)**	**(252)**	(252)	**(252)**	**(253)**		**(254)**	(186)	**(186)**	(186)	**(251)**	(186)
	***	***	ns	**	*		***	ns	***	ns	***	Ns
Zn [mg/kg]	**−0.1550**	**0.1850**	**0.2467**	**0.3952**	−0.0836	**−0.4217**		**−0.2000**	**−0.2931**	0.1295	**0.4968**	0.0590
(*N*)	**(252)**	**(253)**	**(253)**	**(255)**	(256)	(254)		**(186)**	**(186)**	(186)	**(254)**	(186)
	*	**	***	***	ns	***		**	***	ns	***	ns
Cu [mg/kg]	−0.0071	−0.0115	**0.1216**	0.1001	0.0594	0.0629	**−0.2000**		**0.3253**	0.0969	0.0528	**0.3063**
(*N*)	(185)	(181)	**(181)**	(186)	(186)	(186)	**(186)**		**(186)**	(186)	(186)	**(186)**
	ns	ns	*	ns	ns	ns	**		***	ns	Ns	***
Mn [mg/kg]	**0.1826**	**−0.3043**	**0.4009**	**0.4632**	**−0.3205**	**0.3985**	**−0.2931**	**0.3253**		0.0180	0.0250	**0.2438**
(*N*)	**(185)**	**(181)**	**(181)**	**(186)**	**(186)**	**(186)**	**(186)**	**(186)**		(186)	(186)	**(186)**
	*	***	***	***	***	***	***	***		ns	ns	**
Co [mg/kg]	−0.1038	0.0367	**−0.1679**	**−0.2117**	**0.2249**	−0.0615	0.1295	0.0969	0.0180		−0.0145	**0.6459**
(*N*)	(185)	(181)	**(181)**	**(186)**	**(186)**	(186)	(186)	(186)	(186)		(186)	**(186)**
	ns	ns	*	**	**	ns	ns	ns	ns		ns	***
Cd [mg/kg]	**−0.2831**	**0.2161**	**0.1816**	**0.1422**	**−0.1826**	**−0.6263**	**0.4968**	0.0528	0.0250	−0.0145		0.0633
(*N*)	**(250)**	**(250)**	**(250)**	**(252)**	**(253)**	**(251)**	**(254)**	(186)	(186)	(186)		(186)
	***	**	**	*	**	***	***	ns	ns	ns		ns
Pb [mg/kg]	**−0.1179**	−0.0465	−0.0668	−0.1001	**0.2113**	0.0471	0.0590	**0.3063**	**0.2438**	**0.6459**	0.0633	
(*N*)	**(185)**	(181)	(181)	(186)	**(186)**	(186)	(186)	**(186)**	**(186)**	**(186)**	(186)	
	*	ns	Ns	ns	**	ns	ns	***	**	***	ns	
SOD [U/min/mL]	**0.1122**	−0.0972	−0.0144	**0.3630**	**−0.3522**	**0.4247**	**−0.1162**	0.0979	**0.2762**	−0.0480	**−0.2190**	0.0742
(*N*)	**(230)**	(222)	(221)	**(216)**	**(216)**	**(217)**	**(218)**	(180)	**(180)**	(180)	**(216)**	(180)
	*	ns	ns	***	***	***	*	ns	***	ns	**	ns

Table 2 continued

	Age (days)	Na [mg/kg]	K [mg/kg]	Ca [mg/kg]	Mg [mg/kg]	Fe [mg/kg]	Zn [mg/kg]	Cu [mg/kg]	Mn [mg/kg]	Co [mg/kg]	Cd [mg/kg]	Pb [mg/kg]
CAT [μM/min L]	**−0.2115**	**0.1980**	0.0743	**0.1130**	**−0.2289**	**−0.6950**	**0.5553**	**0.2231**	0.0368	**0.2027**	**0.7093**	**0.2193**
(*N*)	**(229)**	**(221)**	(220)	**(215)**	**(215)**	**(216)**	**(217)**	**(179)**	(179)	**(179)**	**(215)**	**(179)**
	**	**	ns	*	**	***	***	**	ns	**	***	**
CP [mg/L]	0.0636	**−0.2649**	0.0068	0.0325	0.0589	**0.4813**	**−0.3540**	0.0741	−0.0119	−0.0632	**−0.3373**	−0.0422
(*N*)	(229)	**(220)**	(219)	(215)	(216)	**(216)**	**(217)**	(181)	(181)	(181)	**(215)**	(181)
	ns	***	ns	ns	ns	***	***	ns	ns	ns	***	ns
GPx [nmol $NADPH_2$/min mL]	**0.1535**	**−0.5124**	**0.4923**	**0.4009**	**−0.4017**	**0.3851**	**−0.1546**	0.0401	**0.3083**	−0.0695	0.0151	−0.0553
(*N*)	**(192)**	**(184)**	**(183)**	**(180)**	**(180)**	**(180)**	**(180)**	(180)	**(180)**	(180)	(180)	(180)
	*	***	***	***	***	***	*	ns	***	ns	ns	ns
GR [nmol GSH/min mL]	0.0258	**0.2256**	**−0.3435**	**−0.1203**	**0.1049**	**−0.1898**	**0.1516**	**−0.1970**	−0.1426	0.0348	**−0.1707**	−0.0643
(*N*)	(192)	**(184)**	**(183)**	**(180)**	**(180)**	**(180)**	**(180)**	**(180)**	(180)	(180)	**(180)**	(180)
	ns	**	***	*	*	*	*	**	ns	ns	*	ns
TBARSs [μM/L]	0.0307	0.0079	−0.0577	−0.0185	**0.1059**	**0.1255**	−0.0503	**0.1709**	0.1097	0.1212	**−0.1804**	**−0.1841**
(*N*)	(229)	(221)	(220)	(215)	**(215)**	**(216)**	(217)	**(179)**	(179)	(179)	**(215)**	**(179)**
	ns	ns	ns	ns	*	*	ns	*	ns	ns	**	*

Bold values denote significant element-element-enzyme interactions and age-related changes of elements level and antioxidant enzymes activity

Korolyuk et al. (1988). The reaction was started by the addition of 0.1 mL of serum to 2 mL of 0.03% H_2O_2 solution and 1 mL of 4% ammonium molybdate. One unit of catalase activity is defined as the amount of enzyme required to clear 1 μmol of H_2O_2 per minute per liter of blood.

The activity of GPx in the hemolysate and tissue homogenates was measured spectrophotometrically as described by Moin (1986). The assay mixture contained 0.8 mL of 0.1 mol/L Tris–HCl with 6 mmol/L EDTA and 12 mmol/L sodium aside, pH 8.9, 0.1 mL of 4.8 mmol/L GSH, 0.2 mL of 1:50 hemolysate or tissue homogenates, 1 mL of 20 mmol/L *t*-butylhydroperoxide, and 0.1 mL of 0.01 mol/L 5,5-dithiobis-2-nitrobenzoic acid. The rate of GSH reduction was followed spectrophotometrically at 412 nm. GPx activity is expressed as micromoles per minute per liter of blood or micromoles per minute per gram of tissue.

GR activity in the 1:20 hemolysate and tissue homogenates was measured according to the method described by Glatzle et al. (1974). The enzyme assay mixture contained 0.2 mL of 7.5 mmol/L oxidized glutathione, 0.1 mL of 1:4 hemolysate or tissue homogenate, 2.4 mL of 67 mmol/L sodium phosphate buffer, pH 6.6, and 0.2 mL of 6 mmol/L NADPH. The rate of NADPH oxidation was followed spectrophotometrically at 340 nm. A control without NADPH was used and the specific activity is expressed as micromoles per minute per milliliter of blood or micromoles per minute per milligram of tissue.

CP level was measured spectrophotometrically at the wavelength of 540 nm as described by Ravin (1961). The assay mixture contained 0.1 mL of serum, 8 mL of 0.4 mol/L sodium acetate buffer, pH 5.5, and 1 mL of 0.5% *p*-phenilendiamine. The mixture was incubated at 37°C for 60 min. Before cooling at 4°C for 30 min the mixture was added to 2 mL of 3% sodium fluoride for reaction inhibition. Ceruloplasmine is expressed as milligrams per liter of blood.

Statistical Analysis

Results are expressed as mean ± SD. Significant differences among means were measured using a multiple-range test at minimum $p < 0.05$. Data not having a normal distribution were log transformed. Significance of differences in element level and enzyme activity in the blood of white stork chicks (significance level, $p < 0.05$) was examined using one-way ANOVA followed by multiple-range test (significance level, $p < 0.05$) and RID Tukey test (test of reasonably important difference for bumpy numerical force of attempt). Arithmetic mean concentrations of elements and enzyme activity were compared using one-way ANOVA followed by multiple-range test and using RID Tukey test. Correlations between elements and enzyme activity in the blood of white stork chicks from different regions at the set significance level were determined by the

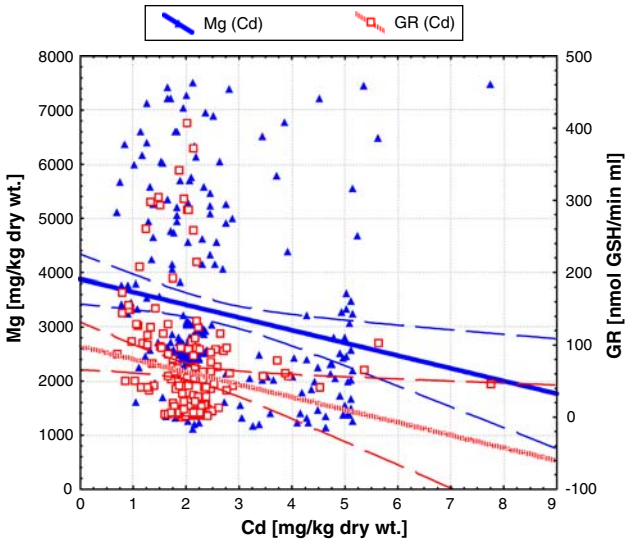

Fig. 1 Interrelationships between Mg−Cd interaction and GR activity in the blood of white stork *Ciconia ciconia* chicks from different regions of Poland

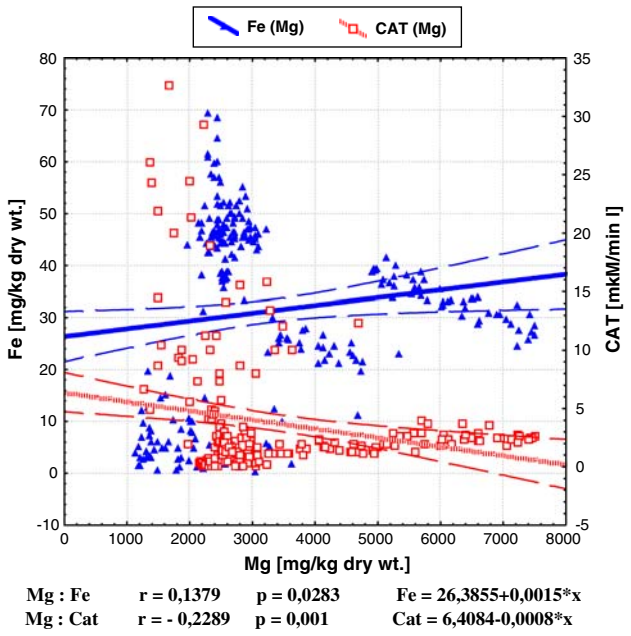

Fig. 2 Interrelationships between Fe−Mg interaction and CAT activity in the blood of white stork *Ciconia ciconia* chicks from different regions of Poland

regression method. Interactions were established by the Pearson test for linear correlation (Zar 1998).

Results

We found significant interactions among macro- and microelements and activity of enzymes and TBARS products

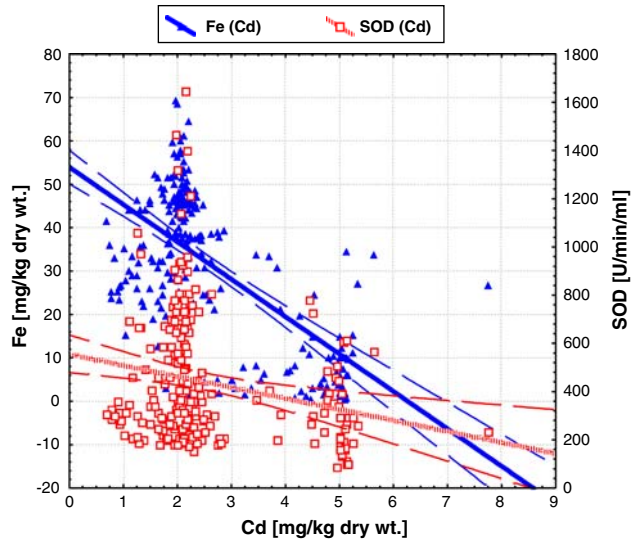

Fig. 3 Interrelationships between Fe−Cd interaction and SOD activity in the blood of white stork *Ciconia ciconia* chicks from different regions of Poland

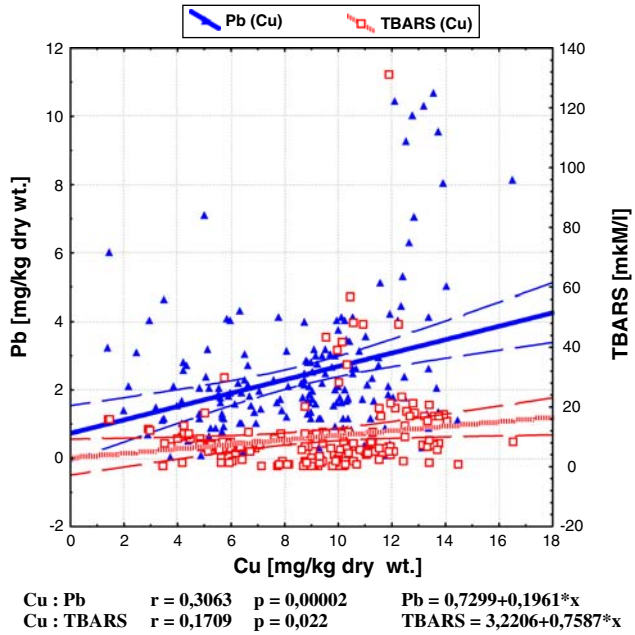

Fig. 4 Interrelationships between Pb−Cu interaction and TBARS products content in the blood of white stork *Ciconia ciconia* chicks from different regions of Poland

(Table 2, Figs. 1–5) simultaneously; that is, there were important interactions with enzymatic activity. It is important to note the predominance of cadmium and lead participation in element–enzyme interactions (e.g., Figs. 1, 3, 4, and 5, Table 2); Cd and Pb have a significant impact on the biochemical activity of most enzymes. Simultaneously, we found interrelationships between cadmium and

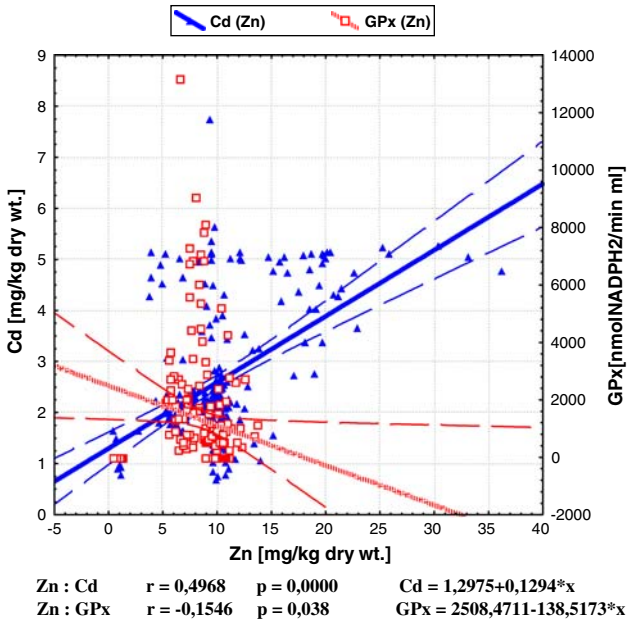

Fig. 5 Interrelationships between Cd−Zn interaction and GPx activity in the blood of white stork *Ciconia ciconia* chicks from different regions of Poland

macroelements Na, K, Ca, Mg, and Fe and the activity of the important antioxidant enzymes SOD, CAT, CP, and GR and TBARS products in the blood of white stork chicks. In the case of lead these relationships were not numerous and they were significant for Ca, Mg, Cu, Mn, and Co. Correlations with enzyme activity were significant only for Pb-CAT and Pb-TBARS products (i.e., MDA) as Table 2 reports. Thus cadmium has an important impact on increases in antioxidant enzymatic activity of most investigated enzymes (Table 2, Figs. 1 and 3).

We noted that the activity of most enzymes investigated (SOD, CAT, CP, GR) and TBARS products are determined by their interactions with physiological elements (Na, Ca, Mg, Fe, Zn) and toxic heavy metals, mainly Cd (Table 2). Microelements, however, influenced enzymatic activity through both excess and deficient concentrations in the blood of young storks from different environments, but they also modified oxidative stress by their impact on the level of free radicals, e.g., in the case of Cd-Fe-SOD (Fig. 3), Pb-Cu-TBARS (Fig. 4), or Cd-Zn-GPx (Fig. 5) dependences.

White stork chicks ranged in age from 14 to 62 days. This is a considerable age variation. Since the young are altricial, considerable age-related physiological changes occur during posthatching development, which would be expected to include changes in activity of various antoxidant enzymes and TBARS products concentrations as well. Furthermore, concentrations of some elements in the blood were age related (Table 2). Among enzymes, only SOD, CAT, and GPx were age related (Table 2). We could

Table 3 Differences between concentration of elements and antioxidant enzymes activity in the blood of white stork *Ciconia ciconia* chicks from different environments

	Differences between chicks from areas	*p*-value
Element		
Na	Odra meadows/Polluted area	0.001032
K	Odra meadows/Polluted area	0.000361
	Suburbs/Polluted area	0.003320
	Polluted area/Pomeranian	0.019481
Ca	ns	
Mg	Odra meadows/Polluted area	0.000009
	Odra meadows/Pomeranian	0.000008
	Suburbs/Polluted area	0.010510
	Suburbs/Pomeranian	0.000169
Fe	Odra meadows/Pomeranian	0.013176
Zn	ns	
Cu	Odra meadows/Polluted area	0.000849
	Odra meadows/Pomeranian	0.000008
	Suburbs/Polluted area	0.000166
	Suburbs/Pomeranian	0.000008
Mn	Odra meadows/Polluted area	0.000258
	Odra meadows/Pomeranian	0.004819
	Suburbs/Polluted area	0.011189
Co	Odra meadows/Polluted area	0.000008
	Suburbs/Polluted area	0.000019
	Polluted area/Pomeranian	0.000008
Cd	ns	
Pb	Polluted area/Odra meadows	0.000008
	Polluted area/Suburbs	0.000008
	Polluted area/Pomeranian	0.000008
	Odra meadows/Suburbs	0.031602
Enzyme		
CAT	Odra meadows/Suburbs	0.000012
	Odra meadows/Polluted	0.000052
	Suberbs/Pomeranian	0.002584
	Pomeranian/Polluted area	0.004478
SOD	Pomeranian/Polluted area	0.024650
CP	Odra meadows/Pomeranian	0.027812
	Suberbs/Pomeranian	0.001290
GPx	Odra meadows/Polluted area	0.000008
	Suburbs/Polluted area	0.000013
	Polluted area/Pomeranian	0.000008
GR	Odra meadows/Suburbs	0.000008
	Odra meadows/Polluted area	0.002214
	Odra meadows/Pomeranian	0.000439
TBARSs	Pomeranian/Polluted area	0.000008
	Suburbs/Pomeranian	0.000008
	Pomeranian/Odra meadows	0.000008

Note: ns, not significant. ANOVA *p*-values. One-way ANOVA followed by multiple-range test and using Tukey test (test of reasonably important difference for bumpy numerical force of attempt

thus analyze lipoperoxidation processes and their age dependences.

White stork chicks from investigated areas differed in the case of element concentration (except for Ca, Zn, and Cd) and enzymatic activity (Table 3). We found that significant element–element interaction/enzyme activity predominated, especially in the case of physiological elements Mg, Fe, Zn, and Cu and toxic heavy metals Cd and Pb (Figs. 1–5). We can thus explain this tendency by the intensive and prevailing access of toxic metals in redox reactions. This causes changes in the priority of these metals, reflected by their influence on enzymatic activity of antioxidant enzymes.

Our research emphasized the impact of toxic heavy metals in blood (Cd and Pb in chicks from polluted regions and suburbs) on the activity of antioxidant enzymes and TBARS products (MDA) (Tables 1 and 2). The higher concentration of Cd and Pb in storks from polluted areas and suburbs coincides with higher enzymatic activity of most enzymes studied (CAT, CP, GPx, GR), as Table 1 reports. Cd and Pb in blood of young storks from different regions tends to affect the lipid peroxidation process negatively (Cd-MDA, Pb-MDA) (Table 2). However, in many cases we observed an increase in enzymatic activity in the white storks' blood with an increase in heavy metal concentration (Table 2). Thus our results indicate changes in oxidative stress intensity in chicks in response to environmental differentiation (Table 1).

We can therefore summarized that increased lipoperoxidation modifies antioxidant enzyme activity and causes changes in SOD, CAT, CP, GPx, and GR activity in chicks from various regions, principally causing increased enzyme activity in chicks from polluted and suburb regions. We thus suggest that the source of heavy metals in chicks' blood might be used as a biological test system of adaptation to oxidative stress. We also report that a high level of heavy metals in chicks' blood is accompanied by increased lipid peroxidation (Table 1). Thus young storks are probably significantly susceptible to environmental conditions. They demonstrated initiation of lipoperoxidation and oxidative modification of proteins that coincide with chemical elements, as a possible antioxidant defense system.

Discussion

We conclude from our results that the physiological activity of the important enzymes SOD, CAT, and CP, and TBARS products, is determined by the concentrations of macro- and microelements and toxic metals (Tables 1 and 2, Figs. 1–5). We have available detailed data on normal concentrations (from the physiological point of view) of radicals, and their excess and deficiency in individual organisms, and the components of trophic chains. We can find and we thus know these "normal" ranges of element levels and their "excess" and "deficiency" for defined tissues and/or organs, and/or organisms, and also for their environments (e.g., Underwood 1966, 1977; Kabata-Pendias and Pendias 1984; Fergusson 1990; Volesky 1990; Merian 1991). Thus we conclude that macroelements, but particularly microelements also, influence enzymatic activity through both excess and deficiency of their concentration in the organism and in the environment.

Previously we analyzed pro-antioxidant balance processes in the blood of young storks and their age dependences in different environments (Kurhalyuk et al. 2006). On the basis of our research we conclude that significant interactions between the toxic heavy metals Pb, and Cd and the macroelements Na, Mg, and Fe and the enzymatic activity of SOD, CAT, and CP and TBARS products occurs in the blood of white stork chicks. These influenced and modified lipoperoxidation balance in the organism. However, lipoperoxidation processes in the cell membrane and subcellular organelles have been proposed as the primary mechanism for cellular membrane dysfunction and tissue injury associated with free radical-initiated processes. Elevated concentrations of lipid peroxides may disturb relations between protective and aggressive factors at the tissue and molecular level, evidencing a change in redox status.

Although much is known about the chemistry of lipoperoxidation processes and cellular defense mechanisms, chronobiological studies are needed to quantify the various cellular components involved in these processes to better understand the physiological processes. Chromosomes of putative anti- and pro-oxidants have been mapped to explore their putative role as markers in redox states in tissues (Aschoff 1981; Tan et al. 2000).

The correlations between elements and age of birds as well as between antioxidant enzymes and TBARS products and age of birds (Table 2) indicate that age is a determining factor for birds with respect to development of their ecophysiological responses to environmental stress. Hoffman et al. (1985) reported different age-related changes in blood and plasma enzymes of nestling kestrels (*Falco sparverius*), and Lanzarot et al. (2005) reported many plasma enzyme and plasma chemistry age-related changes in black stork (*Ciconia nigra*) chicks between 25 and 53 days of age.

Periodically changing environmental factors such as food availability, temperature, and social stimulation can synchronize avian rhythms, but the most important synchronizer is the periodic alteration of light intensity with melatonin as the effector molecule (Aschoff 1981). Melatonin has been found to be an effective antioxidant and oxygen free radical scavenger. Oxygen species, antioxidants, and their induced

redox alterations have been found previously to influence a number of gene expression and signal transduction pathways (Hardeland et al. 1995; Reiter et al. 1995; Sewerynek et al. 1995; Tan et al. 2000).

It is important to note the predominance of Pb and Cd participation in element–enzyme interactions in the blood of young storks in our studies (Figs. 1, 3, 4, and 5). Moreover, our results emphasize the significant impact of toxic heavy metals (particularly Pb and Cd) on the biochemical activity of important enzymes, especially SOD and CAT. Uchida et al. (2004) also indicated that erythrocyte CAT and Cu/Zn-SOD activities are reduced as a result of long-term Cd exposure in Cd-polluted areas. They also suggested that erythrocyte CAT and Cu/Zn-SOD activities may be sensitive markers for predicting renal tubular damage due to chronic Cd exposure. Because Cd generates reactive oxygen substances (ROSs) and inhibits antioxidant enzyme activities in erythrocytes, these activities may be used as noninvasive biological markers for assessing and predicting renal tubular injury (Uchida et al. 2004). Similar results were obtained by Stajn et al. (1997). Those authors stated that Cd accumulation in kidneys of rats, due to chronic dietary intake of Cd, is associated with marked alterations of the antioxidant defense system (ADS). However, they do not indicate an obvious impairment of kidney ADS. Furthermore, Cd-induced injury is not prevented by simultaneous intake of Se, which induces a significant improvement in kidney ADS. Therefore, the role of altered ADS in the development of Cd-induced nephrotoxicity, although possible, is not completely clear (Stajn et al. 1997).

The great importance of Pb and Cd as modulators of antioxidant enzymatic activity in erythrocytes and plasma transaminases is emphasized by Zhikic et al. (2001). They concluded that Cd induces the appearance of anemia and alters the metabolism of proteins and carbohydrates. They stated that decreased activity of SOD in erythrocytes during Cd exposure indicates the presence of ROS-induced peroxidation, which leads to the destruction of RBC membranes. Moreover, Congiu et al. (2000) stated that chronic Cd administration in starlings yields a positively correlated increase in hepatic glutathione GSH levels (GSH is known to protect cells from oxidative damage through its oxidation as GSSG via Se-dependent GSH-Px; glutathione peroxidase [Congiu et al. 2000]). However, Nakahama et al. (2001) point out that Cd only slightly inhibits oxidant enzyme activities.

As stated above, significant element–element–enzyme interactions predominated, especially in the case of physiological elements (e.g., Fig. 2). We thus explain this tendency by the intensive and prevailing access of toxic metals in redox reactions. This causes degeneration of the priority of these metals to physiological elements, reflected by their influence on the enzymatic activity of antioxidant enzymes. These stages of reactions have been reported by Ercal et al. (2001). They found that transition metals act as catalysts in oxidative reactions of biological macromolecules, therefore toxicities associated with these metals might be due to oxidative tissue damage. Redox-active metals, such as Fe, Cu, and Zn, undergo redox cycling, whereas redox-inactive metals, such as Pb, Cd, and Hg, deplete cells' major antioxidants, particularly thiol-containing antioxidants and enzymes. In addition, either redox-active or redox-inactive metals may cause an increase in production of ROSs. Enhanced generation of ROSs can overwhelm cells' intrinsic antioxidant defenses and result in a condition known as "oxidative stress." Cells under oxidative stress display various dysfunctions due to ROS-induced lesions to lipids, proteins, and DNA. So it can be suggested that metal-induced oxidative stress in cells is partially responsible for the toxic effects of heavy metals (Shmueli et al. 2000; Ercal et al. 2001; Ermak and Davies 2002; Mateo et al. 2003). In accordance with this, we can consider that strict relationships exist among macroelements Mg, Fe, Na, and K and toxic heavy metals, regarding their participation in and modification of peroxidation processes (Möller 1995; Quig 1998; Shmueli et al. 2000; Patrick 2003). Cd, Pb, Hg, and Cr exposure also affects Mg, Fe, Na, and K status, which caused further decreases in antioxidation and detoxification processes. Thus early detection and treatment of metal burden are important for successful detoxification, and optimization of nutritional status is paramount to the prevention and treatment of metal toxicity (Quig 1998; Valko et al. 2005).

Some research has determined the effect of antioxidant supplementation following heavy metal exposure. It suggests that antioxidants may play an important role in abating the hazards of heavy metals in connection with interaction of physiological radicals. So multiple mechanisms may be responsible for ROS production in toxic metal exposure. Among them, alterations in thiol status, increased lipid peroxidation, production of ROSs, and damage to the cell's antioxidant defense systems are well known for all redox-active and inactive elements. Element chelators are given to increase excretion of metals, but unfortunately, their side effects are numerous (Congiu et al. 2000; Ercal et al. 2001; Patrick 2003). Heat-induced oxidative stress studies by Han et al. (2005) suggest a role for superoxide (O_2^-) and hydrogen peroxide (H_2O_2). However, heat shock increases cellular generation of O_2^- and H_2O_2 in proportion to the severity of oxidative injury. Under this condition the generation of O_2^- or H_2O_2 induced in excess of the ability of antioxidant enzymes to remove these toxic species causes cell injury and cytotoxicity. This damage is caused by lipid peroxidation leading to disruption of the cytoskeleton and Ca metabolism. Next, the flux of O_2^- and H_2O_2 generated by it induces synthesis of additional antioxidant enzymes.

On the basis of our research we summarize that significant interactions between the toxic metals Pb and Cd and the macroelements Na, K, Mg, and Fe and SOD, CAT, CP, GPx, and GR activity and TBARS product content modify oxidative processes in the blood of white stork chicks. We conclude that the physiological activity of enzymes and TBARS products is determined by the concentration of macroelements and toxic heavy metals. These chemicals influence enzymatic activity through both excess and deficiency of their concentration in the environment (Merian 1991).

As stated in our previous studies (Kamiński et al. 2007, unpublished) the concentrations of Na, K, Mg, and Fe and toxic heavy metals Pb and Cd were higher in the blood of white stork chicks from polluted and suburban environments than in those nesting near Odra meadows. It is important to notice the predominance of Cd and Pb in element–enzyme interactions. Most often research has emphasized the significant impact of toxic heavy metals on the biochemical activity of most enzymes, especially SOD and CAT. Significant element–enzyme interactions predominated in young storks, especially in the case of Mg, Fe, and Zn (Fig. 2). We explain this tendency by the intensive and prevailing access of toxic metals in redox reactions. This causes degeneration of the priority of these metals to physiological elements, reflected by their influence on the activity of antioxidant enzymes.

Our results indicate changes in oxidative stress intensity in chicks in response to environmental differentiation. We summarize that increased lipoperoxidation modifies antioxidant enzyme activity and causes changes in SOD, CAT, CP, GPx, and GR activity in chicks from various environments, principally causing increased enzyme activity in chicks from polluted regions. We thus suggest that the source of heavy metals in chicks' blood might be used as a biological test system of adaptation to oxidative stress. We also suggest that a high level of heavy metals in chicks' blood is accompanied by increased lipid peroxidation. Thus young storks are probably significantly susceptible to environmental conditions. They demonstrated initiation of lipoperoxidation and oxidative modification of proteins that coincide with chemical elements, as a possible antioxidant defense system.

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