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Antioxidative defence enzymes in beetles from a metal pollution gradient

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Oxidative stress in insects may result from an imbalance of oxidants and antioxidants under a significant impact of metals. We studied variation in antioxidant enzyme activity in four species of beetles representing carnivores, carrion eaters, omnivores and phytophages in response to heavy metal pollution. Insects were collected at five forest sites along a gradient of heavy metal pollution in southern Poland. Assayed activity of superoxide dismutase (SOD), glutathione reductase (GR), glutathione S-transferase (GST), Se-dependent (GPOX) and Se-independent (GSTP) glutathione peroxidases and catalase (CAT) correlated with metal pollution levels and body concentrations of metals (Pb, Zn, Cu, Cd). Antioxidative enzyme activity patterns were species-dependent and were correlated with the levels of metal pollution or the body loads of metals. Correlations were predominantly positive in the case of Pb and Cd, and both positive and negative in the case of Zn. The largest difference between species was in the GST activity of the carnivorous *Pterostichus oblongopunctatus* and the phytophagous *Phyllobius betulae*. Activity of GSH-dependent peroxidases and GR was low in most of the species studied. Beetles from metal-contaminated sites showed higher within-species variance of enzyme activity, probably due to their higher polymorphism of antioxidative enzymes.

Key words: heavy metals, beetles, detoxification, antioxidative enzymes.

Introduction

Organisms may respond to environmental stressing factors either behaviourally through various avoidance mechanisms or by means of biochemical and physiological mechanisms used to compensate the negative effects. Natural selection is an important driving force of population differentiation, enhancing the fitness of individuals and leading to local adaptation (Posthuma & Van Straalen, 1993; Woods & Hoffmann, 2000). Adaptation to heavy metals has been reported in natural populations of terrestrial invertebrates (for review see Posthuma & Van Straalen, 1993). In laboratory conditions, metal-adapted individuals may appear in metal-stressed populations within some generations; in an environment naturally contaminated with metals, the stressing effects extend for many generations, and only organisms capable of overcoming the toxic effects can survive (Woods

& HOFFMANN, 2000). In industrially polluted, metal-contaminated soils, strong and directional selection by toxicants is expected to be more common (HOFFMANN & PARSONS, 1997; VAN STRAALEN & HOFFMANN, 2000). Insects from such areas might achieve higher metal tolerance through changes in avoidance mechanisms, which occur more often than genetic changes. Those insects acquire heavy metals mostly in ingested food, such as leaf litter, plant material and captured prey, or rarely through dermal absorption (HELIÖVAARA & VÄISÄNEN, 1993). Among the factors determining the metal bioaccumulation and bioelimination rates, food habits seem very important (VAN STRAALEN & VAN WENSEM, 1986; AUGUSTYNIAK & MIGULA, 2000). Recent studies, however, have demonstrated that physiologically effective rejection of metals is less dependent on the trophic level (LINDQVIST et al., 1995; KRAMARZ, 1999). The performance of insects depends on their tolerance of toxic substances. An excess of metal ions would impose additional energy costs for repair of metal-induced disturbances at the cellular level. Metals might increase the production of reactive oxygen species, and directly or indirectly cause oxidative damage by inhibiting antioxidant activity. An excess of copper acts directly, causing an increase in reactive oxygen species, while cadmium either indirectly leads to an increase in cellular iron levels or directly inhibits the antioxidant activity of glutathione-related enzymes and depletes cellular glutathione (KANG, 1997). Among insects, the beetles have been recognized as weak bioaccumulators of metal (HOPKIN, 1989; VAN STRAALEN & VAN WENSEM, 1986). Some species, such as the chrysomelid beetle *Chrysolina pardalina*, F., 1781, a monophagous feeder of the nickel-hyperaccumulating plant *Berkheya coddii* (Roessler), are able to complete the entire life cycle utilizing only the leaves of this species and effectively eliminating excessive amounts of metals with faeces (MESJASZ-PRZYBYŁOWICZ et al., 2002).

We propose that beetles inhabiting metal-contaminated areas may have adapted to metals by developing more effective bioelimination of metals and/or defence mechanisms against prooxidants than in beetles from an undisturbed environment. Our task was to check whether local populations of selected species of beetles with different feeding habits living along a metal pollution gradient differ in their bioconcentration of metals and antioxidative efficiency. The heavily industrialized vicinity of the town of Olkusz, Poland, an area rich in non-ferrous ores, where a metal pollution gradient can be demarcated easily, was selected for the study. Four species of beetles representing phytophages, detritivores, omnivores and predators from five forest sites along the gradient were used to assess the activity patterns of antioxidative enzymes and their interrelations with the metal loads in beetles and their habitat.

Study area, material and methods

Study sites

Beetles were collected at five forest sites along a gradient of heavy metal pollution in S Poland (approx. 50°17′ N, 19°31′ E to 50°32′ N, 19°39′ E), on a transect running 30 km northeast from the most polluted site (I) near Olkusz to the least polluted site (V) about 5 km north of the small town of Pilica. The main pollutants are heavy metals (Pb, Zn, Cd, Cu) from natural sources (zinc- and lead-containing ores) and from industry. The main sources of pollution are the Pomorzany and Olkusz mines and the Boleslaw mine and smelter. In the late 1980s, annual deposition per km^2 was calculated at more than 1000 kg Zn, nearly 200 kg Pb, 10 kg Cd and 31 Cu (KAPEJA et al., 1990). Since that time, technological improvements and reduction of mining activity have significantly decreased emissions of dust containing heavy metals. In the late 1990s, emission of dust to the atmosphere from these smelters reached about 45 tons per year. Other aerial pollutants of industrial origin are N-oxides, S-oxides, H_2S, PAHs and POBs (CIMANDER et al., 1998).

Sites I–V are situated 2.5, 3, 4.5, 8.5 and 30 km, respectively, from the major zinc smelter, Bolesław. Phytographically, sites I, IV and V belong to the association *Leucobryo-Pinetum* (W. MAT. 1962), and sites II and III to *Querco roboris-Pinetum* (W. MAT. 1981). The bedrock is Jurassic or Triassic limestone, rich in zinc and lead blend. Metal concentrations per kg dry humus at sites I–V along the pollution gradient ranged from 10.4 ± 26 g Zn, 82 ± 17 mg Cd, 47 ± 5 mg Cu and 2.6 ± 0.12 g Pb (site I) to 151 ± 34.5 mg Zn, 0.8 ± 0.4 mg Cd, 10.7 ± 0.9 mg Cu and 136 ± 8.8 mg Pb at the least polluted site (V). More details are given elsewhere (STONE et al., 2001; WILCZEK et al., 2003).

Insects

The following species of beetles were used for laboratory investigations: the predatory *Pterostichus oblongopunctatus* F., 1787 (Carabidae), a litter-soil dweller abundant at all sites; the detritivorous dung beetle *Geotrupes stercorosus* Scriba, 1791 (Scarabeidae); the omnivorous short-winged beetle *Staphylinus caesareus* Cederhjelm, 1798 (Staphylinidae); and the phytophagous curculionid beetle *Phyllobius betulae* F., 1801 (Curculionidae), occupying small birch trees mostly at the edges of forest stands. The insects were captured with pitfall traps checked and emptied once or twice a week throughout the 2000–2002 vegetation seasons. The *P. betulae* weevils were collected with a sweep-net on birch trees. All laboratory assays were carried out on adult individuals.

Heavy metal determinations

Samples of insects and plants were dried, weighed and digested in a mixture of suprapure grade nitric acid and perchloric acid mixed 4:1. Digests were analyzed for metal content using a Solaar Unicam 939 atomic absorption spectrophotometer in an air-acetylene flame for zinc and copper and in a PU-93 090X graphite furnace for cadmium and lead, as described elsewhere (Augustyniak & Migula, 2000). Merck standards were used for construction of the appropriate curves from the initial concentration of 1 g of metal dm^{-3} water. Accuracy of determinations was controlled with SRM-1577b bovine liver (U.S. Dept. of Commerce, Ntl. Inst. Stand. Technol., Gaithersburg, MD) and BRC-185 bovine liver (IRMM, Geel, Belgium) as reference materials. The percentage recovery of spiked samples was high: 93–96% for the measured concentrations of Pb, Cd, Cu and Zn.

Enzyme assays

Before sectioning, the insects were kept for a day for their gut content to empty, then anaesthetized on ice and weighed. Enzyme activity was determined in whole body samples of adult beetles with excised elytrae. Samples were prepared and homogenized at 0–4 °C in 2.5 ml 0.05 M Sorensen buffer, pH 7.4, using one (*G. stercorosus.*), two (*S. caesareus*), or five (*P. oblongopunctatus, P. betulae*) specimens per sample. The homogenates were filtered and centrifuged for 10 min at 15,000 g at 0 °C. Activity was measured in supernatants at 25 °C.

Superoxide dismutase (SOD; 1.15.1.1) was measured indirectly by monitoring the degree of inhibition of adrenalin auto-oxidation to adrenochrome in alkaline medium (Misra & Fridovich, 1972). The absorbance rate without SOD for 0.33 mmol adrenalin solution at 480 nm equals 0.025 absorbance unit/min. The activity unit was defined as the amount of enzyme that inhibits 50% of the control reaction/min/mg protein. Glutathione reductase (GR; EC 1.6.4.2) was measured according to Racker (1955) by spectrophotometric registration of NADPH consumption necessary for the reduction of a unit of oxidized glutathione (GSSG), at 340 nm. Activity was expressed in nmol NADPH/min/mg protein. Glutathione peroxidases (EC 1.11.1.9) – selenium-dependent (GPOX) and selenium independent (GSTP) – were determined spectrophotometrically at 340 nm, as in Simmons et al. (1989), by measuring the rates of hydrogen peroxide or cumine hydroperoxide reduction in the presence of NADPH and glutathione reductase. Sodium azide (NaN_3) was used as the catalase inhibitor. Activity was expressed in nmol NADPH/min/mg protein. Catalase (CAT; EC 1.11.1.6) was determined by monitoring the absorbance of UV radiation of hydrogen peroxide at 230 nm (Aebi, 1974). The specific ac-

Table 1. Mean concentrations of metals (μg g^{-1} dry body weight ± S.D.) in four species of beetles from five sites (I–V) along a decreasing heavy metal pollution gradient.

Species	Site	N	Zn	Pb	Cu	Cd
P. o.	I	6	220.1 ± 112.5^{a}	10.3 ± 0.1^{a}	14.2 ± 7.8^{c}	16.8 ± 12.2^{a}
	II	7	200.8 ± 112.5^{ab}	3.2 ± 2.5^{b}	34.4 ± 13.7^{b}	6.9 ± 6.2^{bc}
	III	7	179.3 ± 32.2^{ab}	1.8 ± 0.9^{b}	24.7 ± 9.5^{a}	11.3 ± 9.8^{ab}
	IV	6	176.2 ± 31.4^{b}	1.4 ± 1.5^{b}	25.0 ± 10.1^{b}	9.6 ± 9.2^{ab}
	V	8	230.8 ± 96.0^{a}	2.9 ± 1.9^{b}	42.4 ± 21.0^{b}	3.2 ± 0.8^{c}
S. c.	I	5	725.3 ± 121.2^{a}	9.7 ± 2.1^{a}	28.3 ± 4.1^{a}	26.1 ± 4.9^{a}
	II	6	597.7 ± 84.0^{a}	6.2 ± 4.2^{a}	21.7 ± 4.9^{ab}	22.2 ± 7.9^{abc}
	III	6	679.1 ± 14.7^{ab}	2.1 ± 0.4^{b}	7.9 ± 1.0^{c}	8.7 ± 2.5^{d}
	IV	6	629.8 ± 163.0^{ab}	2.9 ± 2.2^{b}	17.7 ± 3.9^{b}	11.5 ± 6.9^{bc}
	V	6	540.5 ± 65.0^{b}	1.8 ± 0.8^{b}	9.6 ± 2.1^{c}	4.2 ± 1.7^{d}
G. s.	I	7	451.2 ± 63.5^{a}	7.3 ± 1.3^{a}	21.5 ± 2.4^{a}	7.1 ± 1.3^{a}
	II	7	335.6 ± 52.6^{a}	5.5 ± 1.0^{a}	23.4 ± 3.5^{a}	6.6 ± 1.5^{a}
	III	7	221.0 ± 9.81^{b}	3.1 ± 0.3^{ab}	18.1 ± 3.6^{a}	4.5 ± 1.4^{ab}
	IV	7	214.0 ± 14.9^{b}	3.7 ± 1.0^{ab}	21.9 ± 1.3^{a}	5.6 ± 2.0^{a}
	V	7	329.0 ± 59.8^{ab}	1.9 ± 0.9^{ab}	16.1 ± 6.8^{a}	2.9 ± 2.0^{b}
P. b.	I	6	508.1 ± 68.5^{a}	9.9 ± 3.7^{ab}	40.0 ± 23.3^{b}	11.9 ± 4.9^{a}
	II	8	694.7 ± 49.1^{b}	17.3 ± 4.6^{a}	62.5 ± 7.1^{ab}	12.4 ± 6.5^{a}
	III	8	659.4 ± 80.3^{ab}	9.5 ± 2.9^{b}	27.1 ± 7.1^{b}	10.8 ± 3.4^{a}
	IV	5	660.4 ± 115.2^{ab}	28.9 ± 4.3^{c}	30.9 ± 6.2^{b}	28.9 ± 8.6^{b}
	V	7	353.0 ± 51.0^{c}	1.0 ± 0.2^{d}	31.3 ± 6.7^{b}	6.8 ± 2.2^{c}

Key: Different letters denote site-dependent differences within species (one-way ANOVA, LSD test; $P < 0.05$). From 3 (*G. stercorosus*) to 12 (*P. betulae*) specimens were used per digested sample from each site. *P. o.* – *P. oblongopunctatus*; *G. s.* – *G. stercorosus*; *S. c.* – *S. caesareus*; *P. b.* – *P. betulae.*

Table 2. Correlation (r) and regression coefficients for paired mean tissue concentrations of Pb, Zn, Cu or Cd in the examined species of beetles from the metal pollution gradient.

Species	Variables			Regression coefficients			
	Independent	Dependent	r	a	b	F	P
P. o.	Pb	Cd	0.675	5.830	0.953	7.2	0.030
	Cu	Cd	−0.855	22.520	−0.456	16.5	0.001
G. s.	Zn	Pb	0.684	0.394	0.011	7.4	0.025
	Pb	Cu	0.752	15.670	1.050	10.0	0.010
	Pb	Cd	0.929	2.111	0.751	57.7	0.001
	Cu	Cd	−0.821	−5.112	0.518	31.2	0.005
P. b.	Zn	Pb	0.720	17.580	0.053	8.4	0.021
	Pb	Cd	0.856	3.911	0.726	20.8	0.020
S. c.	Pb	Cu	0.892	6.337	2.350	28.8	0.001
	Pb	Cd	0.923	2.560	2.330	47.1	0.001
	Cu	Cd	−0.870	−2.804	1.023	21.7	0.01

Key: Regressions follow the model $Y = a \pm bX$ (μg g^{-1} dry body weight). Only statistically confirmed correlation coefficients are presented ($P < 0.05$). *: P. o. – P. oblongopunctatus; G. s. – G. stercorosus*; *S. c. – S. caesareus*; *P. b. – P. betulae.*

tivity unit was defined as the enzyme equivalent reducing 1 mmol H_2O_2/min/mg protein. Glutathione-S-transferase (GST; EC 2.5.1.18) was assayed by measuring the rate of conjugation of CDNB (1-chloro-2,4-dinitrobenzene) as substrate (Yu, 1982) with glutathione. Changes in absorbance were recorded at 340 nm. Activity was expressed in μmol CDNB/min/mg protein. Protein concentration was determined by the method of Bradford (1976) using bovine albumin as the standard.

Statistics

The data are averages of 5–9 replicates for each site and species. Site-dependent statistical differences between populations were examined using one-way analysis of variance. If significant differences were detected, means were separated by the least squares difference (LSD) test. Linear relationships between enzyme activity and metal concentrations were checked using regression analysis and compared with species as dependent factor (Statistica Package 5.0).

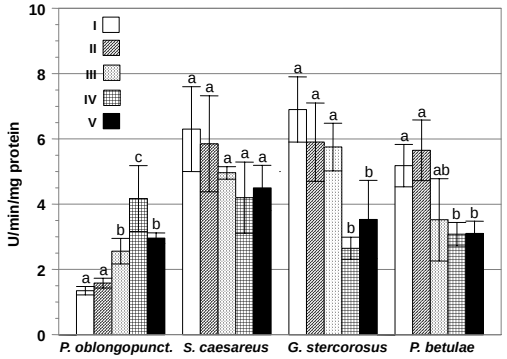

Fig. 1. Activity of superoxide dismutase in four species of beetles from five sites (I–V) in a heavy metal pollution gradient. N = 6–9 per site and species. Values represent means ± S.D., U (Misra units)/min/mg protein. Means within one species indicated by the same letters above columns do not differ significantly (one-way ANOVA followed by LSD test, $P \leq 0.05$). Results of ANOVA: *P. oblongopunctatus* ($F = 13.26$); *S. caesareus* ($F = 1.62$); *G. stercorosus* ($F = 3.44$); *P. betulae* ($F = 4.76$).

Results

All examined species of beetles generally showed great species-related variability in metal concentrations, related to their taxonomic position and feeding habits (Tab. 1). Zn concentrations were species-specific, and correlations with Zn levels in the humus layer were statistically insignificant. The highest concentration of lead was in the predatory *P. oblongopunctatus* from site I. Site-related differences in Pb levels in this species were not significant. Significant correlations were found between the body load of Pb in *G. stercorosus* and *S. caesareus* and the Pb levels in soil ($P < 0.05$). Pb in *P. betulae* correlated positively with Pb contents in birch leaves ($r = 0.72$; $P < 0.05$). The mean Cu body levels varied between the examined species of beetles up to about six-fold (Tab. 1). In *P. oblongopunctatus* the body load of Cu correlated positively with distance from the major polluter ($r = 0.711$; $P < 0.05$). The lowest concen-

Table 3. Correlation (r) and regression coefficients of enzyme activity (units as in Figs 1–6) against body concentrations of metals (μg g^{-1} dry weight) calculated for each species separately.

Species	Metal	Enzyme			Regression coefficients	
			r	P	a	b
P. o.	Zn	GST	−0.772	0.010	−17.860	0.280
	Pb	SOD	−0.686	0.030	3.270	−0.215
		GSTP	0.678	0.050	1.230	0.134
		GR	0.806	0.005	1.320	0.172
	Cu	GPOX	0.726	0.015	0.344	0.103
	Cd	GSTP	0.718	0.020	0.728	0.019
		GPOX	−0.674	0.030	5.580	−0.220
		GR	0.702	0.030	0.978	0.110
G. s.	Zn	GPOX	0.771	0.010	0.463	0.021
		GR	0.758	0.006	−1.116	0.010
	Pb	SOD	0.720	0.015	2.354	0.603
		GSTP	0.853	0.001	1.887	0.911
		GPOX	0.646	0.006	1.772	1.052
		GR	0.706	0.008	0.018	0.462
	Cu	GSTP	0.731	0.002	−5.876	6.573
		GST	0.682	0.020	85.730	−2.820
	Cd	GSTP	0.890	0.001	−0.377	1.160
		GST	−0.682	0.005	58.820	−6.120
		GR	0.736	0.020	−0.605	0.502
S. c.	Zn	GSTP	0.720	0.030	−2.110	0.010
	Pb	SOD	0.910	0.001	4.060	−0.240
		GPOX	0.880	0.001	3.120	1.023
		GST	−0.821	0.010	158.800	−4.640
		GR	−0.723	0.050	2.760	−0.140
	Cu	SOD	0.760	0.010	3.821	0.073
		GPOX	0.890	0.010	0.932	0.412
		GST	0.910	0.001	161.500	1.830
	Cd	SOD	0.890	0.005	3.911	0.086
		GSTP	0.723	0.050	2.800	0.617
		GPOX	0.952	0.001	1.880	0.422
		GST	−0.930	0.001	174.200	−1.820
		GR	−0.710	0.050	3.060	−0.060
P. b.	Zn	GSTP	−0.710	0.020	10.180	−0.060
	Pb	GSTP	−0.670	0.005	7.467	0.078
	Cu	GPOX	0.740	0.010	−1.226	0.034

Key: Regressions follow the model $Y = a \pm bX$; ($P < 0.05$). *P. o.* – *P. oblongopunctatus; G. s.* – *G. stercorosus*; *S. c.* – *S. caesareus*; *P. b.* – *P. betulae*; abbreviations for names of enzymes as in Material and Methods.

tration of Cu was in beetles of this species from site I. Cd levels in the body of beetles and in the humus layer correlated only in the case of *S. caesareus* ($r = -0.69$; $P < 0.05$) and *G. stercorosus* ($r = -0.68$; $P < 0.05$). Body load of Cd correlated negatively with those of Pb and Cu in the remaining species, except for *P. betulae* (Tab. 2).

Apparent species-dependent differences in enzyme activity patterns versus the metal pollution gradient were found in the examined species. The superoxide dismutase (SOD) activity of *P. oblongopunctatus* from highly contaminated site I was the lowest, and it increased with the distance from the main pollution source (Fig. 1). SOD activity in the remaining species was highest in insects from the most polluted site, and about 2–3 times higher than in *P. oblongopunctatus*. Positive correlations between SOD activity and the body loads of Pb, Cu and Cd were documented for *G. stercorosus* and *S. caesareus*. No such correlations were found in the case of *P. betulae*. Pb concentration correlated negatively with SOD activity in *P. oblongopunctatus* (Tab. 3).

Glutathione reductase (GR) activity in *P. oblongopunctatus* and *S. caesareus* increased with the increase in metal pollution. In both species GR activity correlated positively with the body loads

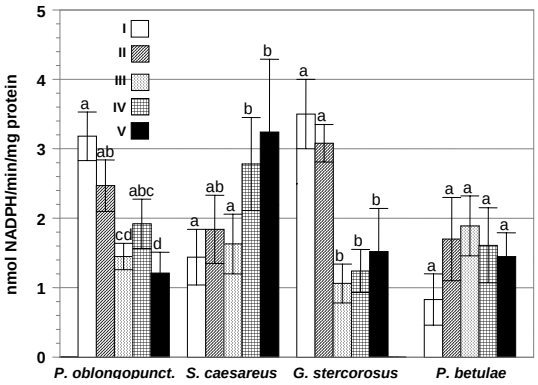

Fig. 2. Activity of glutathione reductase in four species of beetles from five sites (I–V) in a heavy metal pollution gradient. N = 5–9 per site and species. Values represent means ± S.D., nmol GSH/min/mg protein. Means within one species indicated by the same letters above columns do not differ significantly (one-way ANOVA followed by LSD test, $P \leq 0.05$). Results of ANOVA: *P. oblongopunctatus* ($F = 11.84$); *S. caesareus* ($F = 4.92$); *G. stercorosus* ($F = 8.92$); *P. betulae* ($F = 2.16$).

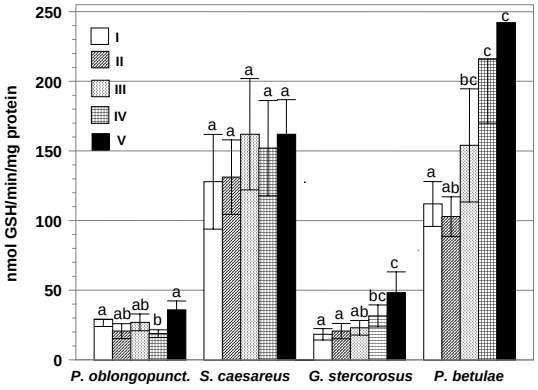

Fig. 3. Activity of glutathione S-transferase in four species of beetles from 5 sites (I–V) in a heavy metal pollution gradient. N = 7–9 per site and species. Values represent means ± S.D., nmol GSH/min/mg protein. Means within one species indicated by the same letters above columns do not differ significantly (one-way ANOVA followed by LSD test, $P \leq 0.05$). Results of ANOVA: *P. oblongopunctatus* ($F = 1.36$); *S. caesareus* ($F = 2.12$); *G. stercorosus* ($F = 3.58$); *P. betulae* ($F = 8.76$).

of Pb and Cd, but in *S. caesareus* the correlation was negative (Fig. 2, Tab. 3). There were no significant site- dependent differences in GR activity in *P. betulae*.

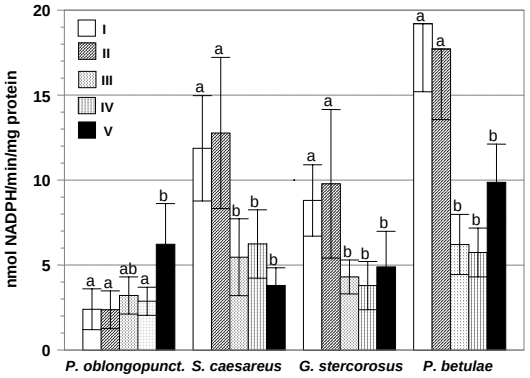

Fig. 4. Activity of selenium-dependent glutathione peroxidase with H_2O_2 as substrate in four species of beetles from five sites (I–V) in a heavy metal pollution gradient. N = 5–7 per site and species. Values represent means ± S.D., nmol NADPH/min/mg protein. Means within one species indicated by the same letters above columns do not differ significantly (one-way ANOVA followed by LSD test, $P \leq 0.05$). Results of ANOVA: *P. oblongopunctatus* ($F = 3.48$); *S. caesareus* ($F = 7.82$); *G. stercorosus* ($F = 6.32$); *P. betulae* ($F = 12.24$).

Glutathione S-transferase (GST) activity in the dung beetles and carabid beetles was 5–7 times lower than in *S. caesareus* and *P. betulae* (Fig. 3). Correlations between GST activity and body loads of metals were negative in the case of Zn (*P. oblongopunctatus)*, Pb and Cd (*S. caesareus*), and positive in the case of Cu (*G. stercorosus*, *S. caesareus*) (Tab. 3).

The activity of GPOX and GSTP in carnivorous (*P. oblongopunctatus*) and GSTP in omnivorous (*S. caesareus*) beetles was lower than in the other two species (Figs 4, 5). *G. stercorosus* from highly polluted sites showed high activity levels of these enzymes. In *P. betulae*, the highest GPOX values were recorded in beetles from the highly polluted sites (Fig. 4), while the values for GSTP were highest in beetles from the least polluted site (Fig. 5). Close relationships between GPOX activity and the body loads of metals were found in all examined species. For GSTP activity the correlations with body loads of Pb were negative in two cases; the correlations were all positive for the three other metals (Tab. 3). Catalase had the highest activity of the assayed antioxidant enzymes. CAT activity levels were high in the carnivorous beetles, moderate in the carrion eaters and dung beetles, and lowest in the herbivore *P. betulae* (Fig. 6). CAT activity correlated negatively with the environmental load of metal pollutants

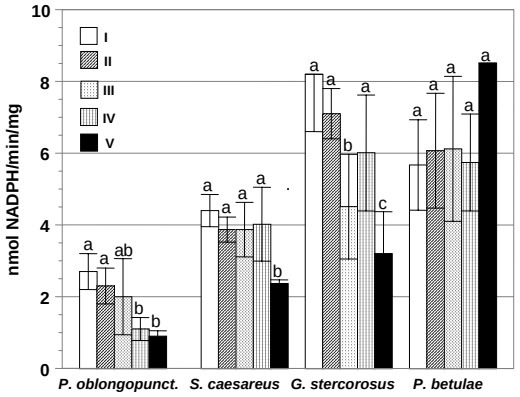

Fig. 5. Activity of selenium-independent glutathione peroxidase with cumine hydroperoxide as substrate in four species of beetles from five sites (I–V) in a heavy metal pollution gradient. N = 5–7 per site and species. Values represent means ± S.D., nmol NADPH/min/mg protein. Means within one species indicated by the same letters above columns do not differ significantly (one-way ANOVA followed by LSD test, $P \leq 0.05$). Results of ANOVA: *P. oblongopunctatus* ($F = 6.32$); *S. caesareus* ($F = 4.94$); *G. stercorosus* ($F = 7.86$); *P. betulae* ($F = 1.44$).

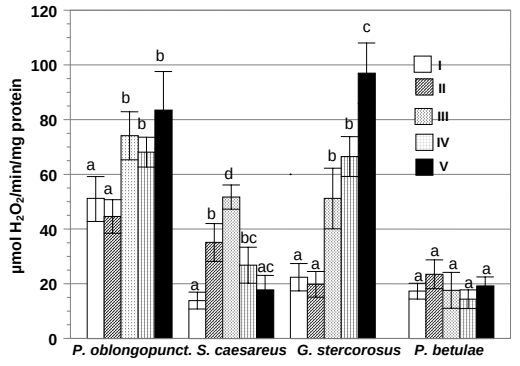

Fig. 6. Activity of catalase in four species of beetles from five sites (I–V) in a heavy metal pollution gradient. N = 7–9 per site and species. Values represent means ± S.D., μmol H_2O_2/min/mg protein. Means within one species indicated by the same letters above columns do not differ significantly (one-way ANOVA followed by LSD test, $P \leq 0.05$). Results of ANOVA: *P. oblongopunctatus* ($F = 9.12$); *S. caesareus* ($F = 13.22$); *G. stercorosus* ($F = 11.56$); *P. betulae* ($F = 1.68$).

in most of the examined species. Correlations between CAT activity and body loads of metals were not statistically significant ($P > 0.05$).

Discussion

Variation in the body content of metals in insects is attributable to a number of factors such as diet, age, sex or physiological state. In our study, variation was usually more pronounced in insects collected at more polluted sites. The body concentrations of metals in all examined species were within the levels previously documented in herbivorous and predatory beetles from the vicinity of the largest iron smelter in Poland (Migula et al., 1990) and within the levels reported for beetles from areas near the Kosogorsky smelter in Russia (Butovsky et al., 1999). In some coleopterans living near a lead and zinc smelter (Arnoldstein, Austria), Pb levels exceeded the usually reported maximum 20 μg g^{-1} by a factor of 30 (Rabitsch, 1995). Kramarz (1999) demonstrated that carabid beetles (*Poecilus cupreus* L., 1758) are poor accumulators of heavy metals. Heikens et al. (2001) drew similar conclusions, confirming that coleopterans are capable of regulating essential metals within the range of normal soil concentrations. Our study found positive correlations between cadmium and lead for each of the examined species, but not between zinc and cadmium. Although zinc can be replaced by cadmium (Migula, 2000), the examined species maintained high body concentrations of this biogenic metal, irrespective of feeding habits and their place in the food chain (Van Straalen & Van Vensem, 1986).

Metals are highly persistent and may exert strong selection pressure on multiple generations of low-dispersant populations of organisms (Stone et al., 2001). These cited authors did not register significant changes in the body mass of *P. oblongopunctatus* inhabiting the two most polluted sites in the same area as in our studies. They found that the life span of beetles exposed to multiple stress (pesticide, starvation) was shorter, because they had to pay the additional costs of metabolic detoxification, enhanced production of metal-binding proteins, and other means of eliminating toxic substances. In another study, we confirmed the inducibility of microsomal cytochrome c reductases NAD(P)H in *Pterostichus versicolor* Sturm, 1824 and *P. oblongopunctatus* (Bednarska et al., 2001); in *P. oblongopunctatus* from the most polluted forest site, the activity of cytochrome c was 3–10 times higher in the presence of NADH, and 2–3 times higher in the presence of NADPH, compared with the activity measured in beetles living at a less polluted site.

Metals can enhance oxidative stress and lipid peroxidation in insects, especially when other pro-oxidant constituents are present in their diet (AHMAD, 1995; FELTON & SUMMERS, 1995; CHRAŚCINA et al., 1996). The mechanisms of initiation of the Fenton reaction are poorly recognized in insects, with some exceptions for iron, copper, cadmium or mercury (AHMAD, 1995; MIGULA & GŁOWACKA, 1996). Both enhancement and inhibition have been reported for the activity of antioxidant enzymes such as SOD, CAT or GST, depending on the metal levels, form and period of exposure, and species (ZAMAN et al., 1994; CHRAŚCINA et al., 1996; MIGULA & GŁOWACKA, 1996). In the beetles studied here, there was high species-dependent variability of enzyme responses (Figs 1–6). The largest responses, opposite to the level of the metal load, were documented for GST and CAT activity in *P. oblongopunctatus* and *P. betulae*. Site-related differences in enzyme activity within species were smaller, from slight variations to 6–7-fold increases.

There should be a clear quantitative relationship between catalase and the activity of superoxide dismutase, which dismutates the free superoxide radical (O_2^-) to H_2O_2 and in turn has to be eliminated by catalase (FELTON & SUMMERS, 1995). Catalase is a widespread enzyme in various organelles of insect cells, and in all examined species of beetles it had the highest activity among the assayed antioxidative enzymes. The activity of SOD and CAT in predatory *P. oblongopunctatus* from the most polluted sites was lower than in those from less polluted sites. Insects influenced by metabolic generators of superoxide anions may increase SOD activity and decrease CAT activity (PRITSOS et al., 1988; PERIĆ-MATARUGA et al., 1997). Such effects observed in our study in *P. betulae* suggest joint effects of pro-oxidative activity and more intensive microsomal detoxification.

Glutathione transferases play an essential role in the overall fitness of insects exposed to potentially toxic exo- or endogenic substances. High levels of GSH activity indicate increased resistance to pollutants and imply intensification of detoxification processes. Some GST isoforms might play a protective role against oxidative stress, showing peroxidase activity (MIGULA et al., 1999). This may compensate low activity of GPOX, which is responsible for the reduction of H_2O_2 and ROOHs with GSH as the reductant (AHMAD, 1995). Such a detoxifying strategy might be important in *S. caesareus* from our study.

Among several factors of unknown undetermined importance, which might affect enzyme activity in the studied beetles, the effects of seasonal variation on the levels of glutathione and glutathione-dependent enzymes should also be taken into account. We found that the activity of antioxidant enzymes was generally enhanced within the growing season in the grasshopper *Chorthippus brunneus* Thunberg, 1815 (AUGUSTYNIAK et al., 2001). In the beetles in the present study such significant temporal differences were not found.

The high site-independent variation of enzymatic responses in the beetles from the metal pollution gradient seems to be an important indicator of heavy metal stress. CALLAGHAN et al. (1998) emphasized that many authors ignore this potential indicator due to difficulties in linking such effects with the expression of specific genes. Increased phenotypic variation may not always be coupled with increased genetic variation (CALLAGHAN et al., 1998). Thus, if the increase in genetic variation in insects from a heavily polluted site differs from that in insects from an unpolluted site, this should be a direct effect of environmental pollutants. The insects in our study were taken from unselected, natural, stationary populations inhabiting particular sites along a gradient of heavy metal pollution. High variability of response might result from high polymorphism of antioxidative enzymes in insects. Our laboratory is now studying whether genetic variation can increase after organisms are transferred to a novel environment (nontoxic or with higher multistress loads).

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