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Activity of Esterases as Biomarkers of Metal Exposure in Spiders from the Metal Pollution Gradient

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Abstract

Spiders were studied as predators of the invertebrate epigeic fauna inhabiting forest and grassland ecosystems variously polluted with metals. The response of detoxifying enzymes in adult male and female spiders (a wolf spider *Pardosa lugubris* and a funnel weaver *Agelena labyrinthica*) was compared using material collected at five sites in forest and grassland transects along the metal pollution gradient. Carboxylesterases, acetylcholinesterase and metal concentrations were assayed in spiders during the season. In both species at the most polluted sites of forest and meadow transects CarE activity was higher. These animals effectively used quantitative compensatory strategy against pollutants, which demands extra energy. Comparisons between species showed a better adaptation to pollutants in the ground wolf spiders. In these animals from polluted meadows AChE activity was also higher than that in agelenids.

Keywords: Metal pollution gradient, *Pardosa lugubris, Agelena labyrinthica*, esterases, adaptation

Introduction

Spiders as predatory invertebrates are generally recognised as macroconcentrators of metals [1]. The levels of metals are species-specific and depend on many factors. Intensity of hunting, diet composition and form of feeding of their prey are of great importance [2]. In spiders physiological ability at effective rejection of metals seems to be less dependent on their trophic level than in insects [3]. Lycosids such as *Pardosa* species accumulate cadmium almost linearly during an accumulation period, whereas decontamination takes a very long time [4]. Metals might influence directly or indirectly metabolic processes in many soil-dwellers in areas contaminated with metals by depletion of cellular glutathione, disturbances in structure of lipids, and enhancement of lipid peroxidation. Metal-dependent cellular effects are manifested by inhibition or induction of various enzymatic pathways, including those of detoxifying character [5, 6]. We presumed that selection pressure favoured populations of spiders from the areas of the long metal-mining history, which should have allowed them to survive on a diet contaminated with metals with a concomitant better compensatory capacity to cope with other stresses of a chemical character [2]. In order to check whether biological features might influence detoxifying possibilities of spiders we selected the representatives of two different groups of spiders, wandering and net-constructing. The wolf spider *Pardosa lugubris* represented the first group and a funnel-web spider *Agelena labyrinthica* the second one. All animals were collected along a gradient of heavy metal pollution in meadow and forest ecosystems of an old industrial area in southern Poland. Both species differ also in their feeding habits, behaviour, physiology and life span [2, 7, 8]. The assays of activity of glutathione-dependent enzymes in these species demonstrated that *A. labyrinthica* is more tolerant to oxidative stressors while conjugation reactions with GST were more effective in *P. lugubris* from less polluted areas. Moreover, the wandering spiders are able to bioaccumulate metals, maintaining lower levels of detoxifying enzymes and glutathione [9]. This study is focused on the assays of

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enzymes used as specific biomarkers of effects caused by organophosphates and carbamates - acetylcholinesterases [10], and non-specific enzyme biomarkers sensitive to various chemicals, including metals, represented here by non-specific carboxylesterases [11]. This group of B esterases reacts with exogenous and endogenous esters in phase I of biotransformation of various toxic chemicals [10,12]. Trends in carboxylesterase activity have been studied in parallel with other groups of invertebrates, inhabiting five forest or meadow ecosystems situated in a gradient of metal pollution, mainly spiders [9], insects [13-15] and earthworms [16-17]. Data obtained in this study were related to the body burden of lead, cadmium, copper and zinc in spiders, and the level of metals in the humus layer of their habitats.

Material and Methods

Spiders were collected at five meadow (M1-M5) and five forest (F1-F5) sites along a gradient of heavy metal pollution in the vicinity of the town of Olkusz, in southern Poland (approx. 50°17'N/19°31'E to 50°32'N/19°39'E). Main pollutants are heavy metals: Pb, Zn, Cd, Cu; N-oxides; S-oxides; H_2S; PAHs and POB from natural sources, mining or the metallurgic plant 'Boleslaw', mining plants 'Pomorzany', 'Olkusz' and 'Boleslaw', all situated in close vicinity. In the late nineties the emission of dust to the atmosphere from these smelters reached about 45 tons per year. Annual soil deposits were calculated at more than 1000 kg Zn, nearly 200 kg Pb, 10 kg Cd and 31 Cu per km^2 [18].

The bedrock of the sites M1–M3, situated 2.5 to 4.5 km from the major zinc smelter, is Jurassic or Triassic limestone, rich in zinc and lead blende. Metal concentrations (per kg of dry humus) along the pollution gradient range from 10,454 ± 2,618 mg Zn, 81.9 ± 17.2 mg Cd, 46.9 ± 4.6 mg Cu and 2,635 ± 120.4 mg Pb (site 1), to 151 ± 34.5 mg Zn, 0.8 ± 0.4 mg Cd, 10.7 ± 0.9 mg Cu and 136 ± 8.8 mg Pb in the least polluted site (site 5). More details are given in earlier studies [19]. The meadow sites are labelled here as M1 (nearest to the smelters) to M5 along a gradient of heavy metal pollution placed in a decreasing order. They are mostly xerothermic, acidic sandy soils covered by a reduced number of herbal and ruderal species of plants. The forest sites (F1-F5) are covered by dominating Scots pine (*Pinus silvestris)* with an admixture of oaks (*Quercus robur, Q. sessilis*), birches (*Betula* sp.), Norway maple (*Acer platanoides*) and alders (*Alnus glutinosa* and *A. incana*).

The laboratory assays were carried out on the adult males and females two of the most common species at all sites along the gradient of pollution: a wolf spider, *Pardosa lugubris,* Lycosidae (*P. l.*) and a funnel-web, *Agelena labyrinthica,* Agelenidae (*A.l)*. They differ in their hunting behaviour, catching intensity (web building – *A.l.* or non-web building - *P.l.*) and habitats [7]. The average fresh body weight in the web spider, *A.l.* was 0.155 ± 0.042 g and 0.111 ± 0.022 g for the females and males, respectively. They were almost twice the size of wolf spiders, *P.l.* (female – 0.024 ± 0.007 g and male– 0.016 ± 0.005 g). Metabolism rates in *A.l.* are based on oxidative processes to a lesser degree than that in *P.l.* [7]. Weighed spiders were anaesthetized on ice and homogenised at 4°C in Sorensen buffer pH 7.4 at 1:50 vv. Homogenates were centrifuged at 15,000 *g* for 10 minutes and supernatants were used for biochemical assays. Activity of carboxylesterases (EC 3.1.1.1) was measured spectrophotometrically at 400 nm (Helios Epsilon, UNICAM), in the submitochondrial fraction, according to Ljundquist and Augustinsson [20] with paranitrophenyl acetate (p-NPA) as a substrate. The results were expressed in nmol p-NPA min^{-1} mg $protein^{-1}$. Activity of acetylcholinesterase (EC 3.1.1.7) was measured at 410 nm in the same fraction, according to Ellman et al. [21], with acetylthiocholine iodide (AChI) as a substrate and expressed in nmol AChI min^{-1} mg $protein^{-1}$. A protein concentration in samples was determined by the dye-binding method of Bradford [22], using fraction V of bovine serum as a standard. Samples of spiders used for the determination of the metal concentration were dried, weighed and digested in a mixture of suprapure grade nitric acid and perchloric acid mixed in 4:1 proportions. Samples of spiders were analyzed for the metal content in their bodies using the atomic absorption spectrophotometer Solaar Unicam 939 in air-acetylene flame for zinc and copper and on the graphite furnace PU-93 090X for Cd and Pb, as described elsewhere [23]. Merck standards were used for construction of appropriate curves from the initial concentration of 1 g Me 1^{-1} water. The accuracy was controlled with reference materials: SRM-1577b bovine liver (US Dept Commerce, Ntl Inst. of Standards and Technology, Gaithensburg, MD) and BRC-185 bovine liver (IRMM, Geel, Belgium). The percentage recovery of spiked samples was high and equalled 93-96% for the measured concentration of Pb, Cd, Cu and Zn.

Basic statistics and assumptions underlying the analysis (significance of regression slopes, analysis of variance for significance between sites and species for metals and enzyme activity, distribution testing) were conducted using the STATISTICA 6.0 package for PC. The data were analyzed for homogeneity of variance using Levene's test of equality of error variances or using LSD test if heterogeneity was identified with a confidence range of $p<0.05$.

Results and Discussion

Statistical analysis did not prove any significant site-dependent differences in the concentration of metals between both examined species (Table 1). Generally, high coefficients of variation characterized the metal bioaccumulation levels. High levels of site-independent variance about the means are important indicators of stress caused by chronic exposure to heavy metals demonstrating the diversity of response at the individual level [9, 23]. A decrease in the amounts of accumulated metals in tissues with the increased distance from the main pollution area was not always indicated in both species of spiders. In *P.l.* such relationships were noted in spiders of both sexes inhabiting forest sites in the case of Pb and Zn, whereas in the case of Cd only in the females from forest and meadow sites. A gradient of the body burden of metals in *A.l.* in relation to the concentration of metals at different sites was less clear than in *P.l.* In the case of cadmium it was well expressed only in the females from forest and meadow ecosystems, and in case of lead in the males from forest sites (Table 1).

Positive correlations between metal concentrations in the humus layer and the body burden of metals in net-constructing spiders (*A.l.*) from meadows were confirmed for Cd ($R^2 = 0.787$; $p < 0.028$ in the males and $R^2 = 0.984$; $p < 0.001$ in the females) and similarly for Pb ($R^2 = 0.860$; $p<0.015$ and $R^2 = 0.741$; $p < 0.039$ for the males and females, respectively). In forest inhabiting spiders of the same species such relationships were confirmed in the males for two metals – Pb ($R^2 = 0.954$; $p <0.015$) and Cu ($R^2 = 0.891$; $p < 0.037$).

Metal concentrations in wolf spiders (*P.l.*) correlated well with the levels of the same metals in the humus layer only in the case of the meadow-inhabiting animals. The square of correlation coefficient (R^2) of Pb in the males was 0.778 ($p < 0.03$) and of Zn 0.78 ($p < 0.03$). In the females it was 0.758 ($p < 0.035$) and 0.78 ($p < 0.03$) for Cd and Zn, respectively. Parallel studies of other soil dwelling and epigeic insect species occupying the same sites (beetles: predatory *Pterostichus oblongopunctatus, Poecilus versicolor*, Carabidae, detritivorous, *Geotrupes stercoros;* carcass eater, *Necrophorus humato;* omnivorous, *Staphylinus caesareus;* phytophagic, *Phyllobius betulae* [13, 15]*;* and grasshoppers (*Chorthippus brunneus* and *Euthystira brachyptera*) [14] made possible the comparison of similar relations between insects and spiders. Spiders had 2- to 5-fold higher concentrations of zinc, cadmium and copper, and in this group of soil-dwelling invertebrates the correlations between their body burden of metals and the level of the metals in soils are better. Concentrations of lead in the examined spider species were maintained at similar levels as in insects [14, 15]. Differences in the pattern of metal accumulation between both species of spiders resulted from their biological features [2, 24]. The higher body burden of metals in *P. l.* was probably fostered by more intensive hunting activity, lower bioelimination rates, and the type of prey or differences between habitats [7, 24-27].

The females of *A.l.* from both meadow and forest sites had, on average, two-fold lower concentrations of Cd, Pb, Zn and Cu in their tissues than the males (Table 1). The males, in comparison with the females, seem to be relatively less exposed to heavy metals. Their longer period of starvation suggest that the bioaccumulation rates of metals should be lower than of the females, due to their higher bioelimination rates. Our results indicated, however, quite opposite effects (Table 1). Soon after reaching maturity the males search for a sexual partner and die shortly after mating. The male characteristic of ceasing hunting activity suggests that lower amounts of metals may enter their bodies during a prolonged period of starvation. In this period of life the rate of production of metals containing granules could increase in a similar way as Alikhan [28] demonstrated in isopods or Djangmah, [29] in prawns, thus fostering bioaccumulation of metals. The females, despite higher hunting intensity and longer life span [7], were able to decontaminate metals more efficiently than the males, eliminating them with faeces [8]. This feature may be of selective character for spiders living in metal polluted areas, which may be regarded as an adaptative strategy protecting the genetic material against excessive amounts of heavy metals.

Both spider species from the most polluted meadow or forest site characterized the highest CarE activity, suggesting that excessive amounts of metals might induce activity of this enzyme (Fig. 1). This way the spiders might respond to higher levels of metals or, as well, to other organic contaminants, which could enter their body. This also means that metals, even in high concentrations, did not influence detoxifying abilities of the examined species negatively, confirming statements in our earlier studies on spiders [24]. Such effects of metals might be rather of an indirect character, because induced CarE detoxification activity is probably caused by various exogenous esters from captured prey. Nevertheless, we could not eliminate the direct effects of metals, as it was proved in the laboratory for *Galleria mellionella* [30] or *Triatoma infestans* [31], at least in the case of wandering spiders. In this species a similar pattern of CarE activity as in mentioned above study was also observed by Wilczek and Migula [24] in spiders from heavily polluted environments. Enhancement of CarE activity with increased metal levels was also demonstrated against another substrate (α-naphtyl acetate) than used in that study. The same enzyme in web-building Linyphiidae, Metidae and Araneidae spiders from areas under strong impact of industrial pollutants showed a significant decrease in its activity [2]. Lack of confirmed significant differences between the patterns of CarE activity measured in the web- and non-web building spiders against p-NPA suggests that within a range of metal pollution gradient in the studied area both species might respond in a similar way. Still unclear is whether animals maintain the same or different esterase isoforms in response to increased levels of metals in local populations. These aspects are the objectives of our recent studies.

The analysis of relations between the body burden of metals and enzyme activity in the examined species demonstrated that both genders of spiders did not differ generally along the pollution gradient when zinc or copper were used as independent variables ($p < 0.05$). In *P. lugubris* positive correlations were found between the body burden of cadmium and both CarE activity ($R^2 = 0.424$; $p <0.05$) and AChE activity ($R^2 = 0.737$, $p < 0.03$). The body burden of Pb in the lycosid spiders was also correlated positively with CarE activity in *A. labyrinthica* collected at both meadow and forest sites ($R^2 = 0.49$,

Table 1. Mean concentrations of four metals [µg Me g dry weght^{-1} ± SD] in the female and male spiders of *P. lugubris* and *A. labyrinthica* collected along the heavy metal gradient at meadow (M) or forest (F) sites. N = 6-8 per each site, species and gender. Different letters indicate site-dependent significant differences between metal levels within genders in both species at $p < 0.05$ [ANOVA, Least Signifcance Differences].

Species	Site	Zn	Cu	Cd	Pb
P.l	M1	1209.0 ± 376.9^{a}	110.4± 34.3^{ab}	74.1 ± 5.2^{a}	2.4 ± 1.0^{a}
female	M2	1153.0 ± 226.7^{ab}	121.0 ± 16.0^{b}	59.7± 11.9^{b}	3.1 ± 0.3^{a}
	M3	907.4 ± 64.5^{c}	101.6± 23.1^{ab}	47.4 ± 4.4^{b}	3.6 ± 2.1^{a}
	M4	700.0 ± 76.4^{d}	85.8 ± 11.6^{c}	51.2 ± 4.0^{b}	0.8 ± 0.4^{b}
	M5	611.8 ± 59.4^{d}	78.4 ± 16.6^{ac}	29.6± 11.7^{c}	0.5 ± 0.1^{b}
	F1	1270.2± 189.3^{a}	198.8 ± 54.1^{a}	74.1± 18.2^{a}	5.0 ± 1.1^{a}
	F2	900.8 ± 76.1^{bc}	182.2± 53.4^{ab}	58.0 ± 4.3^{b}	3.6 ± 1.2^{bc}
	F4	923.6 ± 39.9^{b}	92.0 ± 22.5^{cd}	51.0± 17.3^{b}	3.1 ± 1.2^{c}
	F5	718.6 ± 99.3^{c}	118.6 ± 11.3^{d}	33.6 ± 5.9^{c}	0.7 ± 0.6^{d}
P.l.	M1	1540.7 ± 58.4^{a}	235.75 ± 8.2^{a}	59.6 ± 4.0^{a}	6.8 ± 2.5^{a}
male	M2	1236.8± 202.1^{b}	125.4 ± 3.0^{a}	59.5± 10.5^{a}	5.4 ± 2.2^{a}
	M3	1019.2 ± 43.1^{a}	112.0 ± 20.2^{b}	50.5 ± 7.4^{b}	4.6 ± 1.5^{b}
	M4	958.0 ± 136.5^{a}	119.0 ± 25.6^{b}	66.1± 13.2^{b}	2.2 ± 1.1^{b}
	M5	821.2 ± 102.8^{c}	93.6 ± 14.2^{b}	31.6 ± 5.9^{b}	2.5 ± 1.3^{b}
	F1	1714.0± 189.3^{a}	208.0 ± 35.0^{a}	82.0 ± 8.0^{a}	5.2 ± 2.7^{a}
	F2	1133.6± 214.0^{a}	140.0 ± 19.9^{b}	74.9± 11.3^{b}	3.6 ± 1.4^{b}
	F4	1272.2 ± 49.8^{a}	129.4 ± 26.2^{a}	44.8 ± 6.8^{b}	3.3 ± 0.61^{c}
	F5	1029.2 ± 99.3^{b}	170.8 ± 15.3^{a}	51.8 ± 7.9^{b}	0.7 ± 0.2^{c}
A.l.	M1	597.6 ± 268.1^{a}	129.2 ± 84.5^{a}	43.6 ± 9.0^{ab}	2.7 ± 0.3^{a}
female	M2	1021.2± 137.8^{b}	99.8 ± 28.7^{b}	33.4 ± 8.8^{ab}	1.2 ± 0.1^{a}
	M3	461.2 ± 120.8^{c}	64.2 ± 30.9^{b}	19.4± 11.7^{a}	0.2 ± 0.1^{b}
	M4	601.0 ± 86.7^{d}	83.6 ± 9.2^{b}	15.6± 11.6^{b}	0.6 ± 0.1^{c}
	M5	816.8 ± 121.2^{e}	73.6 ± 33.9^{b}	17.0± 10.5^{c}	0.4 ± 0.1^{c}
	F1	757.2 ± 220.9^{a}	94.0 ± 29.3^{a}	51.2 ± 5.8^{a}	3.3 ± 0.5^{a}
	F2	699.0 ± 90.7^{bd}	58.4 ± 55.8^{b}	30.8 ± 9.4^{a}	6.0 ± 1.4^{bc}
	F4	769.4 ± 71.1^{bc}	95.0 ± 27.6^{a}	26.4±15.7^{ab}	0.3 ± 0.1^{c}
	F5	471.6 ± 323.6^{d}	59.6 ± 40.4^{b}	21.8± 11.6^{b}	0.4 ± 0.1^{d}
A.l.	M1	796.8 ± 58.4^{a}	154.6 ± 18.9^{a}	50.4 ± 4.8^{a}	6.4 ± 1.5^{a}
male	M2	1159.2± 202.1^{b}	107.2 ± 16.9^{b}	31.8 ± 6.8^{b}	3.1 ± 0.2^{b}
	M3	493.2 ± 43.2^{c}	69.4 ± 8.3^{c}	12.4 ± 3.8^{c}	0.6 ± 0.1^{c}
	M4	686.6 ± 136.5^{a}	62.6 ± 6.9^{c}	11.7 ± 1.9^{c}	0.8 ± 0.2^{c}
	M5	749.0 ± 102.9^{a}	93.4 ± 8.3^{c}	22.0 ± 1.6^{bc}	0.7 ± 0.1^{c}
	F1	872.0 ± 227.2^{a}	142.6 ± 12.8^{a}	67.6 ± 7.9^{a}	2.9 ± 0.1^{a}
	F2	905.8 ± 17.0^{a}	98.0 ± 19.7^{b}	43.2± 31.7^{b}	2.7 ± 0.4^{b}
	F4	643.6 ± 74.6^{b}	67.2 ± 7.3^{bc}	20.6 ± 6.4^{c}	1.0 ± 0.1^{c}
	F5	589.6 ± 139.1^{b}	60.8 ± 18.2^{c}	40.4 ± 4.0^{b}	0.7 ± 0.1^{c}

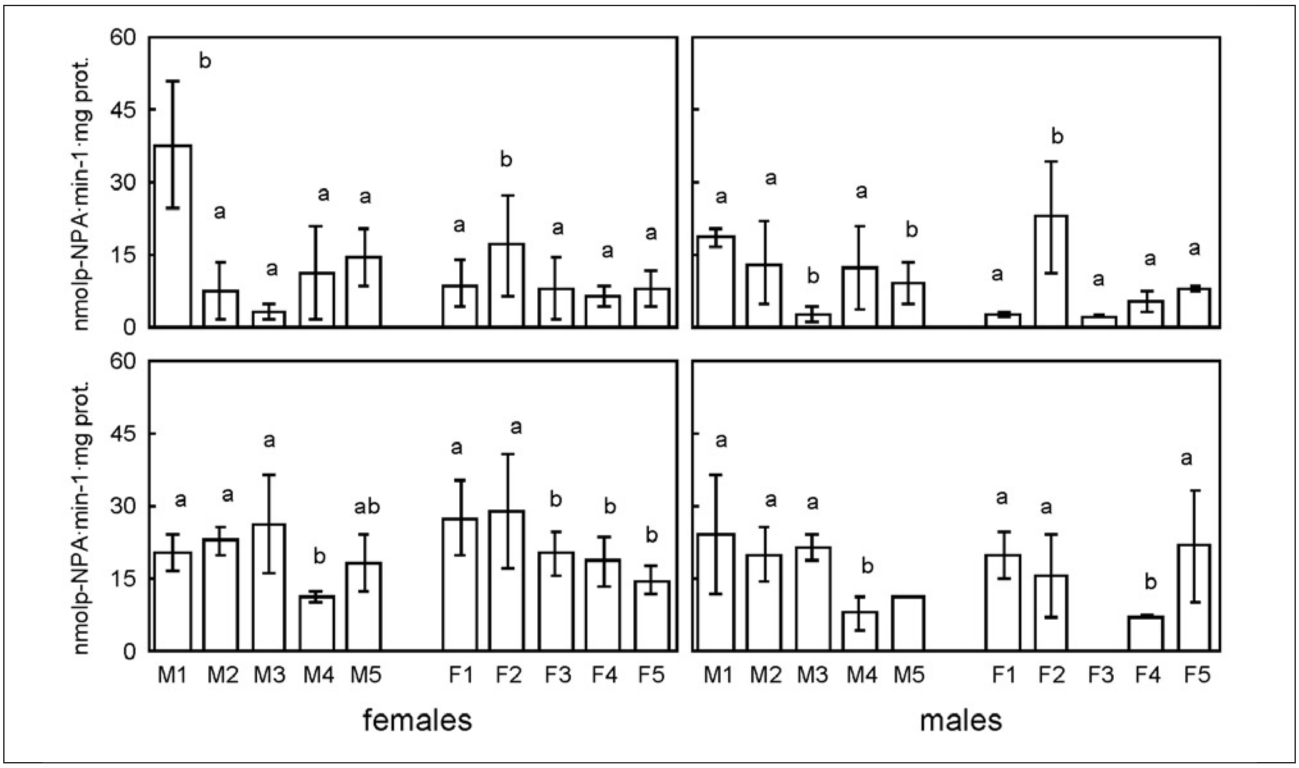

Fig. 1. Carboxylesterase activity (means ± SD) in the male and female *P. lugubris* (*P.l.*) and *A. labyrinthica* (*A.l.*) from five meadow (M) and forest (F) ecosystems along a gradient of heavy metal pollution. Different letters indicate site-dependent significance of differences within the same gender and species, separately for forests and meadows at $p < 0.05$.

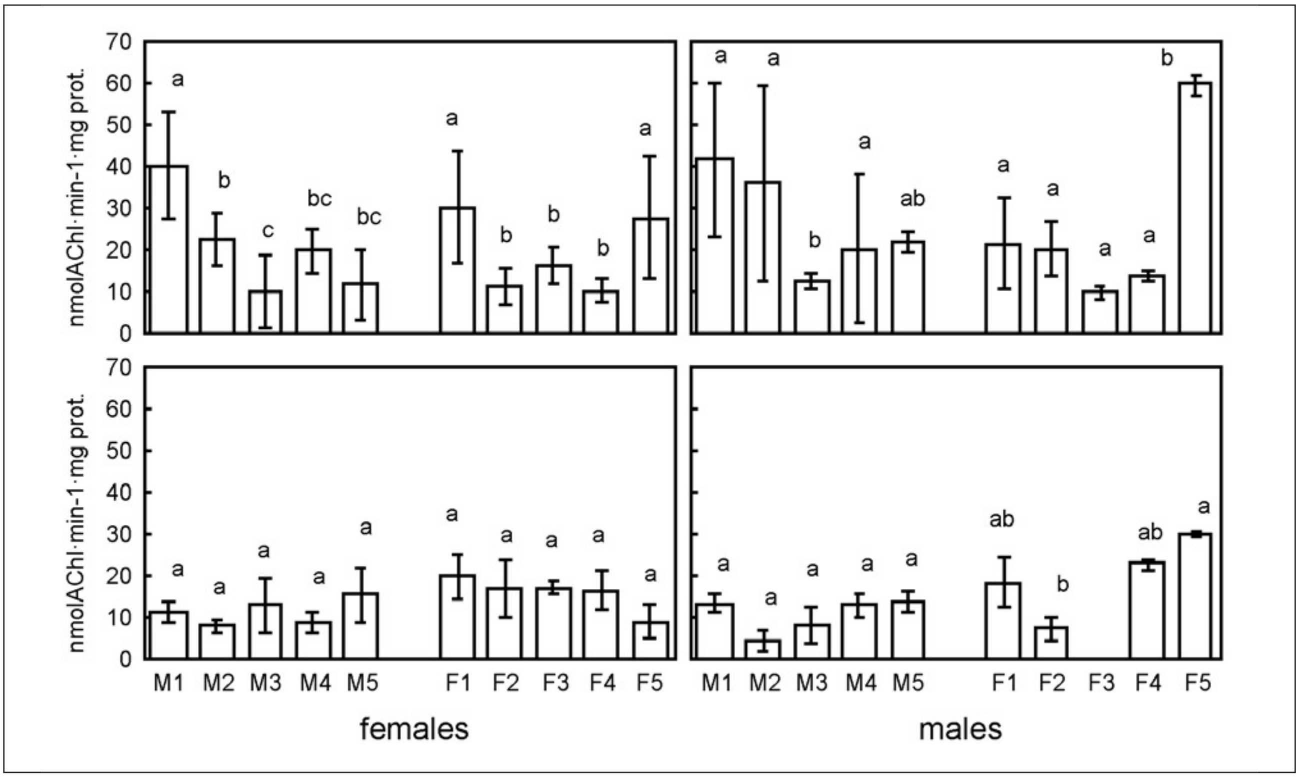

Fig. 2. Acetylcholinesterase activity (means ± SD) in the male and female *P. lugubris* (*P.l.*) and *A. labyrinthica* (*A.l.*) collected at five meadow (M) and forest (F) ecosystems along a heavy metal gradient. Different letters indicate site-dependent significance of differences within the same gender and species, separately for forests and meadows at $p < 0.05$.

$p < 0.05$ and $R^2 = 0.831$, $p< 0.05$, respectively). On the contrary, lead influenced AChE activity negatively in wolf spiders ($R^2 = 0.694$, $p < 0.05$).

CarE activity was generally higher in females than in males (Fig. 1). Lower esterase activity in the males may be explained by their specific behaviour at the adult stage. Cessation of feeding by the mature males may negatively influence the enzyme, considering that esterases should be involved in lipid metabolism [32]. It was proved for insects that the females have a better ability to metabolise xenobiotics [33]. Hydrolytic activity in this gender is generally higher, due to the reproduction processes, when effective detoxifying mechanisms are crucial for protecting genetic material against toxic substances. Perhaps similar mechanisms function effectively in spiders.

Inhibition of AChE is a valuable biomarker assay for organophosphate and carbamate compounds. Phosphorylation of the serine residue at its active site by organophosphates causes loss of AChE activity or its reactivation is slower [10]. In the examined spiders the AChE activity was species-specific, 2-3 fold higher in wandering *P. l.* than in web-building *A. l.*, irrespective of the spider's gender or inhabited areas (Fig. 2). This might be due to higher mobility of the wolf spiders [7]. Metals generally did not influence activity of this enzyme significantly as it was confirmed for *A. l.* in both sexes from meadows and forests and for the male spiders of *P.l.* (Fig. 2). All these groups were characterized by high coefficients of variability, indicating that enzymatic response of an individual is heterogenic. This also suggests a low sensitivity to metals, at least in the case of *A. l.* Lack of site-dependent differences in AChE activity may also come from effective compensatory mechanisms. Such evolutionary adaptation might be expected in terrestrial animals inhabiting areas rich in metal ores, with mining activity, where they have been exposed to excessive amounts of metals extending over many generations [11]. Such species-specific AChE properties have been described earlier by Scaps et al. [34] in *Eisenia fetida*. AChE was not inhibited when individuals had been exposed for 8 weeks to either 8 or 80 ppm of Cd or 1000 or 2 000 ppm of Pb. Also, in the case of grasshoppers (*Aiolopus thalassinus*) exposed to cadmium in their diet, the cholinesterase activity was not inhibited [35].

In many cases, the pattern of AChE activity had a U-shaped form, with the lowest AChE activity in the individuals collected at medium-polluted sites (both sexes of *P. l.* from forests, the females of *P. l* from meadows, the males of *A. l.* from forests), while the highest activity was in spiders collected at highly or weakly polluted areas (Fig. 2). We assume that in spiders from the moderately contaminated areas there might be much stronger substrate inhibition by its competition for binding with highly reactive serine groups. Metal bonds may block active centres of the enzyme. Animals from highly polluted environments probably developed better quantitative compensatory mechanisms, and higher AChE activity is possible due to increased synthesis of the enzyme molecules. Quantitative compensatory mechanisms towards AChE inhibition was proposed by Olima et al. [36] in a freshwater shrimp (*Parataya australiensis*) collected at variously polluted aquatic ecosystems. On this basis we assume that in spiders from highly polluted areas additional energy should be allocated to detoxification processes and subtracted from the energy needed for the general maintenance or production [37]. Another strategy in energetic trade-off history might be used by spiders from medium-polluted environments. In this case a pattern of maintained lower levels of AChE activity enables them to function/operate properly. Based on the stated patterns of AChE activity in the examined species of spiders we may conclude that compensatory mechanisms in web-building *A. l.* are more effective compared with wandering *P.l.* Taking into account that other groups of important detoxifying enzymes are more sensitive to metals than non-specific and specific esterases, it is possible that spiders might have developed other adaptative strategies in response to the metal pollution than these described in this paper. They require, however, further studies.

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