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Metallothioneins and energy budget indices in cadmium and copper exposed spiders *Agelena labyrinthica* in relation to their developmental stage, gender and origin

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ABSTRACT

The aim of our studies was to explain the role of metallothioneins (MTs) in the neutralization of excessive amounts of metals (essential: copper (Cu) and toxic: cadmium (Cd)) and to describe the energy status in metal-exposed spiders *Agelena labyrinthica* in relation to its developmental stage, gender and origin. Juvenile, female and male spiders were collected from three variously polluted habitats, transferred to the laboratory and exposed to the metals in their diet. Cu and Cd accumulation in the body and exuviae, bioaccumulation factor, percentage of metallothionein positive cells, MT concentration, percentage of cells with depolarized mitochondria, ATP concentration and ADP/ATP ratio were measured and calculated. Cu appeared to be regulated and its excess is eliminated via, among others, the molting process, while Cd was rather accumulated by the spiders. The level of MTs increased significantly mainly in females exposed to both metals, irrespectively of the pollution degree of their site of origin, indicating a defensive role of the proteins. In general, even if both the MT level and the energy status indices were positively correlated with Cd and Cu concentrations in the spider body, the energy status of *A. labyrinthica* did not seem disturbed.

1. Introduction

In organisms exposed to stress two groups of responses can be described. The first group includes defensive reactions, leading to the neutralization, elimination or minimization of the negative effects of stressors (Wilczek et al., 2003a, b; Łaszczyca et al., 2004; Augustyniak et al., 2005; Babczyńska et al., 2006). The second group consists of all changes in physiological processes, described as stress reactions, resulting from the detrimental action of xenobiotics (Wilczek, 2005; Wilczek et al., 2008). The responses require energy expenditures (Calow, 1991; Migula et al., 1997; Stone et al., 2001) and depend on the environmental pressure, phenotypic plasticity, possible (pre) adaptations, gender, developmental stage as well as nutritional and physiological status (Kafel et al., 2003; Wilczek et al., 2003b; Augustyniak et al., 2008, 2009a, b). Metals of unknown biological role, such as Cd, and excessive concentrations of biogenic elements, such as Cu, belong to the most harmful factors due to their persistence in the environment, the tendency to accumulate in tissues and organs, their transfer along food chains, high reactivity and the ability to induce reactive oxygen species (Ahmad, 1995; Pardini, 1995; Kang, 1997; Lagadic, 1999). Spiders are known to survive in environments extremely polluted with metals (Łuczak, 1984; Majkus, 1988; 2003). The main way of metal neutralization is deposition in an inactive form of so-called "mineral granules" (Brown, 1982; Hopkin, 1989) or "temporal spherites" (Ludwig and Alberti, 1988)—lisosomal structures in digestive cells of the midgut glands in spiders' abdomen. According to the Dallinger's classification, spiders in general are the macroconcentrators of metals (Dallinger, 1993). In fact, various groups of spiders apply different strategies depending, to some extent, on their hunting behavior, concerning the construction, or not, of trap webs. From our previous works we know that actively-hunting spiders generally store metals while web-building species accumulate less amounts of the elements (Wilczek and Babczyńska, 2000; Wilczek et al., 2005). Although *A. labyrinthica* is a web-building species, its ecological niche is similar to wandering spiders (Foelix, 1996). It accumulates relatively high amounts of metals, similar to active hunters and, at the same time, significantly higher than other web-building species (Wilczek and Babczyńska, 2000; Wilczek et al., 2005). However, the possibility of elimination of metals by *A. labyrinthica*, cannot be excluded. Since spiders grow and molt all their life (Foelix, 1996) the elimination of excess metals with exuviae seems worth investigating in this group of animals. This mechanism is also observed in other invertebrates (Lindqvist, 1992; Bergey and Weis, 2007).

Both elimination and accumulation strategies require the synthesis of metal binding molecules such as metallothioneins (MTs). MTs are a group of low molecular (6–7 kDa), conservative, thermostable, cytosolic proteins, rich in cysteine –SH groups (Dabrio et al., 2002; Amiard et al., 2006). The chemical structure of these proteins determines their role in the regulation of homeostasis of biogenic elements and the elimination

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of their excess. They also take part in detoxification of xenobiotic metals and contribute to an antioxidative defense and the regulation of apoptosis and cell growth (Coyle et al., 2002; Amiard et al., 2006; Moulis, 2010). The induction of MTs is a biological response to high concentration of metals in the environment (Dallinger et al., 2004; Wu and Chen, 2005; Marie et al., 2006; Mićović et al., 2009). Very little is known about MTs in spiders. It has been found so far that the percentage of MT positive cells increases in response to dimethoate and heat shock in *Pardosa lugubris* and *A. labyrinthica* (Wilczek, 2005). Moreover, the percentage of MT positive cells in midgut glands of spiders depends strongly on the metal concentration in their body resulting from the pollution of their habitats (Wilczek et al., 2008).

An initialization of defensive mechanisms is often accompanied by an increase in metabolic activity and energy consumption. This ultimately leads to the changes in the adenine nucleotide (ATP, ADP, AMP) concentrations in cells (Giesy et al., 1983; Haya and Waiwood, 1983; Viarengo, 1989). Adenine nucleotides belong to the most universal enzymatic effectors. Among them ATP is a positive effector for the enzymes regulating anabolic pathways while AMP activates the enzymes regulating catabolic pathways. The evaluation of ATP concentration changes in relation to the remaining adenine nucleotides, mainly ADP, can be applied as a biomarker of metabolic status of cells. Basing on this comparison it is possible to predict further development of the cells, towards either proliferation or cell death processes (Richter et al., 1996). Energy status of the cells can be assessed by the analysis of their mitochondrial potential ($\Delta\Psi m$). It was stated that if at least 90% of mitochondria in the cell have high transmembrane potential, the cell has a strong energetic efficiency (Lecoeur et al., 2004). Sudden breakdown of the value of transmembrane potential in the majority of mitochondria in the cell, accompanied by the extinction of ATP resources, leads to necrosis. Therefore the measurements of $\Delta\Psi m$ are regarded as good indices of energy status and physiological fitness of either single cell, tissue or an organ as a whole (Tiano et al., 2001; Saleh et al., 2003) and can be applied as an effective biomarker of early subcellular effects of harmful chemicals (Videira et al., 2001).

Toxic effects of xenobiotics depend on biological features of the organism, such as sex, age and developmental stage. Behavioral and physiological aspects connected with variable hormone levels during the development, growth and sexual maturation modify the energy allocation and differentiate the intensity of detoxification. They also determine the energetic budget of an organism. Different patterns of biochemical responses in various physiological forms of animals were found and described, among others, by Kotze and Rose (1987), Augustyniak and Migula (2000), Stone et al. (2002) and Wilczek et al. (2003b, 2004, 2008).

In this study metal-induced stress effects and its neutralization were investigated considering the influence of developmental stage, gender, and previous exposure to chemicals in the environment. In order to achieve this goal, juvenile, female and male *A. labyrinthica* spiders were transferred from three habitats polluted to various degrees to the laboratory and exposed to Cu (biogenic) and Cd (potentially toxic) in their food. To assess the metal neutralization strategy of *A. labyrinthica*, Cu and Cd concentrations were measured and their bioaccumulation coefficient was calculated. A possible role of MTs in these processes and their inducibility was estimated using two complementary, qualitative and quantitative methods of MT immunodetection. Moreover, indices of metal induced changes in the energy budget of spiders were analyzed in response to metals accumulated in their body.

2. Materials and methods

2.1. Sites

Spiders were collected from three sites with different degrees of metal pollution. The first site, Olkusz, O (50°16'05" N; 19°27'47" E) is a town in southern Poland. The main contaminants are metals (Pb, Zn, Cd, Cu), N-oxides, H2S, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). The pollutants are of both natural and industrial origin. The main emitters are the Pomorzany and Olkusz mines and the Boleslaw mine and smelter. Metal concentrations in soil have been reported in Łaszczyca et al. (2004).

Welnowiec, W (49°04'N, 18°55'E) is a large, post-industrial waste heap, localized on a flotation tailings sedimentation tank, containing wastes from ore processing. It consists mainly of clay, sludge and sinter and contains zinc, cadmium and lead complexes. Metal concentrations in soil are presented in Franiel and Więski (2005).

Pilica, P (50°51'N, 19°65'E) 30 km away from the contamination sources, was the reference site. The metal soil burdens are presented in Stone et al. (2001; 2002).

2.2. Spiders

A. labyrinthica (Agelenidae) are funnel-web spiders that builds their webs either just above the ground or among low bushes, between grass blades, plant stems or thin branches in sunny but humid places. Their diet consists mainly of Hymenoptera (29%), Nematocera, Brachycera (16%), Coleoptera (8%), Homoptera (7%) and Lepidoptera, Orthoptera, Trichoptera (12%) (Nentwig, 1987). The individuals were hand-collected in June (juvenile) and July (adult females and males) 2010. The developmental stage and the maturity of specimens were determined according to Foelix (1996). To avoid cannibalism, the spiders were transported to the laboratory separately in plastic tubes clogged with cotton wool. In the laboratory the spiders were placed separately in plastic, 150 mL containers with ventilation-assuring covers. At the bottom there was a layer of moist sand to maintain proper humidity. The containers were stored at suitable temperature (D: 25 °C; N: 15 °C), photoperiod (L:14 h; D:10 h) and relative humidity: 70 ± 10%. The juveniles, males and females from each study site were randomly divided into three experimental groups: control (C), Cd- and Cu-intoxicated groups (Cd-int and Cu-int, respectively). The control spiders were fed fruit flies *D. melanogaster* raised on a standard diet (Graf et al., 1992). Metal exposed spiders were fed fruit flies reared on a diet supplemented with Cd (0.29 mg $CdCl_2 \cdot g$ dry mass of the $medium^{-1}$) and Cu (0.24 mg $CuSO_4 \cdot g$ dry mass of the $medium^{-1}$). The concentration of metals in fruit-flies eaten by spiders are shown in Table 1. The treatment with metals extended over three weeks. Whenever, during that time, spiders molted, the exuviae were carefully collected for metal contents analyses. At the beginning of 4th week of the experiment (22nd–23rd day), the samples were prepared for further analyses. To avoid a loss in adenylate pool during preparation, ATP concentration and ADP/ATP ratio were measured in whole spider bodies. Metal concentrations were assayed in abdomens (6 replicates for each site, sex or age and experimental group; Wilczek and Babczyńska, 2000). For cytometric and immunoenzymatic tests midgut glands were dissected. 5 replicates of samples for each site, stage/gender and experimental group were prepared for each procedure.

2.3. Metal concentration

For metal concentration analyses the spiders were anesthetized at −20 °C for 10 min. Then they were immersed and gently shaken

Table 1
Cd and Cu concentration (mean ± SD, $\mu g \cdot g^{-1}$dry mass) in the body of *D. melanogaster* from control (C) group and reared on cadmium and copper containing media (Cd- and Cu-treated groups).

Group	Cd	Cu
C	4.0 ± 0.5	19.6 ± 1.4
Cd-treated	30.0 ± 6.2	
Cu-treated		152.6 ± 5.1