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Lack of Evidence for Metallothionein Role in Tolerance to Copper by Natural Populations of *Daphnia longispina*

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Metallothioneins are a class of soluble, low molecular weight, heat-stable, cystein rich proteins, capable of selectively binding metal ions, such as zinc, cadmium and copper (Dabrio et al. 2002). They are believed to be involved in the regulation of essential metals (zinc, copper) and detoxification of nonessential ones (mainly cadmium, but also mercury, silver and gold). Their presence is almost ubiquitous in the animal kingdom, with homologous in plants and prokaryotes (Roesijadi 1992). Induction of metallothioneins or metallothionein-like proteins (MT) in mammals and other eukaryotes is usually related to heavy metal exposure (Hamer 1986, Roesijadi 1992). However, other physical or biological factors, such as starvation and molting cycle, can also influence MT production and concentration (Pedersen et al. 1997). Although a pool of MT is normally present in tissues of uncontaminated organisms, usually bound to zinc, copper or both (Roesijadi 1992), evidence for the existence of metal-induced specific MT isoforms has been found (Brouwer et al. 1992). It has also been found, in some species, that whereas some MT genes are activated by specific metals, others are activated by all metals (Laulier et al. 1999). The absence of MT production usually results in extreme sensitivity to metals (Hamer 1986), while duplication of MT genes is sometimes referred to as one of the mechanisms contributing to increased metal tolerance in natural populations (Maroni et al. 1995).

This study addressed two main objectives: (1) to adapt the differential pulse polarography (DPP) technique for MT quantification in daphnids, and (2) to ascertain if MT basal levels and/or MT induction were, at least partially, responsible for previously observed differences in the genetically-determined tolerance to lethal and sublethal levels of copper among cloned lineages of *Daphnia longispina*. The first objective was pursued using *D. magna* as test organism, since two previous studies (Bodar et al. 1990, Stuhlbacher et al. 1992) reported the presence of MT in this species and MT induction by cadmium, though different quantification techniques were used. In spite of *Daphnia* being a widely used standard test organism in ecotoxicology, only these two studies quantified MT levels in cladocerans. An association of MT levels (induced or basally expressed) to the genetically-determined tolerance to copper in *D.*

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longispina would contribute to explain the evolutionary process that occurred in a historically stressed field population of this species. The underlying hypothesis tackled in this study was that genotypes with differential MT expression were present in the original population, but due to the high directional selective pressure of the still ongoing long-term acid mine drainage contamination (Lopes et al. 2004), individuals were selected according to MT production capabilities, in a process that led to the "genetic erosion" of the original population (Van Straalen and Timmermans 2002).

MATERIALS AND METHODS

Daphnia magna Straus and *D. longispina* O.F. Müller were the two daphnid species used in this study. *D. magna* has been maintained in ASTM hardwater medium (ASTM 2002), 20 ± 1°C under a 14:10-hr light:dark cycle, for several years. Daphnids were fed daily with *Pseudokirchneriella subcapitata* (Korshikov) Hindak (formerly known as *Selenastrum capricornutum* Printz) ($3x10^5$ cells/mL/d), and culture medium, enriched with vitamins and the standard organic extract Marinure 25 (Glenside, Stirling, UK), was changed every other day. *D. longispina* individuals belonged to cloned lineages derived from field-collected females of two natural populations, one (I) inhabiting a site historically impacted with acid mine drainage, and the other (R) inhabiting a nearby reference (unpolluted) pond (Lopes et al. 2004). From each population, near 130 lineages were ranked according to their tolerance to lethal levels of copper, after acclimation for at least 15 generations to 25 ± 1°C and to laboratory conditions (Lopes et al. 2004). The three extremes of sensitivity (R1 to R3 and I1 to I3) and tolerance (R4 to R6 and I4 to I6) were selected for this study. Their $LC_{50,48hr}$ and $IC_{20,48hr}$ (inhibition of feeding on *P. subcapitata* during exposure) values of copper were not correlated (Lopes 2002) (Table 1).

For MT extraction, groups of 25 individuals were placed in previously weighed eppendorf tubes, with the aid of a plastic pipette. All water was carefully removed and samples were immediately frozen at -20°C. Homogenization was performed in 500 µL of 0.02M Tris-HCL (pH 8.6) on an ice bath, using an Ultra-Turrax T-25 homogenizer (IKA, Staüfen, Germany). Afterwards, 350 to 400 µL of homogenate (MT fraction) were placed in weighed eppendorf tubes, and weighed (homogenate weight). The remainder was weighed (wet weight) and placed for 24 hr at 80°C, for dry weight (d.w.) determination. The MT fraction was centrifuged at 30,000*g*, for 45 min at 4°C. The supernatant (first cytosolic fraction) was transferred to weighed tubes, weighed, and placed in a 80°C bath for 10 min, before being re-centrifuged under the previous conditions. The resulting supernatant, consisting of the purified MT fraction, was transferred to new tubes and frozen at -20°C for MT quantification.

The methodology for MT determination was by DPP as described by Bebianno and Langston (1989). DPP is one of the standard methods for MT quantification and it is based on the electrochemical properties of the thiol groups present in the cysteines (Dabrio et al. 2002). Its applicability in cladocerans had never been

Table 1. Lethal ($LC_{50,48hr}$) and sub-lethal ($IC_{20,48hr}$, inhibition of feeding) concentrations of copper (μg/L), with the respective 95% CI (inside brackets), for the 12 cloned lineages (Linea) of *Daphnia longispina* (three sensitive and three tolerant), selected from the reference (R) and stressed (I) populations (adapted from Lopes 2002).

Linea	$LC_{50,48hr}$ of copper		$IC_{20,48hr}$ (feeding) of copper	
	R	I	R	I
1	59.9 (53-68)	183.2 (163-209)	28.9 (27-30)	61.7 (52-74)
2	75.5 (67-84)	136.8 (123-152)	25.2 (?-?)	84.0 (73-99)
3	95.3 (80-116)	74.2 (72-76)	23.0 (18-26)	47.5 (26-80)
4	292.3 (236-412)	368.6 (284-629)	35.9 (14-51)	90.2 (79-104)
5	232.3 (118-282)	254.9 (195-396)	21.2 (7-33)	85.7 (77-97)
6	236.1 (202-283)	217.5 (186-261)	29.3 (1-54)	82.7 (76-90)

tested. The DPP technique was performed using a Metrohm 747 VA stand (Metrohm, Herisau, Switzerland), equipped with a dropping mercury electrode as working electrode, an Ag/AgCl reference electrode and a platinum auxiliary electrode. This equipment was connected to a μAUTOLAB II with the GPES software (Ecochemie, Utrecht, Netherlands) for electrode control and signal analysis. Polarography was conducted under anaerobic conditions, in 20 mL of supporting electrolyte, to which 100 μL of sample and 250 μL of 0.02% (v/v) Triton X-100 were added. The supporting electrolyte was the Brdicka working electrolyte, consisting of 1M NH_4Cl, 1M NH_4OH and 2M $[Co(NH_3)_6]Cl_3$. Working conditions were: pulse (modulation) amplitude of 50 mV, potential scan from -1.4 to -1.74 mV, scan speed of 2 mV/sec, modulation time of 0.007 sec, normal pulse time of 0.05 sec, and 2 min of purge. Purified rabbit liver MT (Sigma, M5267, Munich, Germany) was used as standard solution (10 mg/L), due to the inexistence of MT standard for daphnids. MT concentrations were determined by adding twice 250 μL of MT standard solution and measuring the signal after each addition and expressed on a dry weight basis, following the calculation steps of Bebianno and Langston (1989).

To adapt the DPP technique for analysis of MT in *Daphnia*, MT levels in *D. magna* were investigated before and after cadmium exposure, in two separate experiments. In a first experiment, MT induction by exposure to increasing cadmium concentrations was evaluated. Five-days old individuals (3rd brood) were exposed for 24 hr to 0.0, 66.7, 100.0, and 150.0 μg/L of cadmium, under the same conditions as those described for culture maintenance. A stock solution of 100 mg/L of $CdCl_2$ (Merck, Darmstadt, Germany) was prepared in nanopure water and test solutions were prepared using the ASTM medium as dilution water, without food, organic extract or vitamins. Four replicates were used for each concentration, and each replicate consisted of 25 individuals, divided in groups of five in 120-mL glass vials filled with 80 mL of test water. For this first experiment, 100 μL of homogenate were used for d.w. determinations. In a second experiment, induction of MT by cadmium was repeated to improve the reproducibility of MT quantification. Five-days old individuals (3rd brood) were

Table 2. Mean (± SD) MT concentrations (mg/g d.w.) in *Daphnia magna* exposed to cadmium for 24 hr, in a gradient of increasing concentrations (first experiment) and in a single exposure of 100 μg/L (second experiment).

	Cadmium (μg/L)			
Experiment	0.0	66.7	100.0	150.0
First	5.45 (± 1.14)	4.51 (± 1.13)	4.64 (± 0.76)	4.26 (± 0.81)
Second	10.74 (± 2.48)		9.27 (± 1.72)	

exposed to 0 and 100 μg/L of cadmium, in ASTM medium for 24 hr. In this experiment, 150 μL of homogenate, rather than 100 μL, were used for d.w. determination, in order to increase precision and accuracy. Basal MT concentrations were determined in the 12 *D. longispina* cloned lineages. Five-day old individuals (3rd brood) from each lineage were placed for 24 hr in ASTM medium, under the culture conditions previously described for this species, without food, vitamins or organic extract. Four replicates were used in MT determination for each lineage. Each replicate consisted of 25 individuals, divided in groups of five in 60-mL beakers filled with 40 mL of ASTM medium. Afterwards, MT concentrations were determined for the most sensitive (R1) and for the most tolerant (I4) lineages, after the exposure to increasing copper concentrations. Five-days old individuals (3rd brood) were exposed to 0.0, 15.6, 25.0, and 40.0 μg/L of copper for 24 hr. A 140 mg/L copper stock solution was prepared with $CuSO_4$ (Merck) in nanopure water and test solutions were prepared using ASTM medium as dilution water. Replicates consisted of 25 individuals, divided in groups of five in 60-mL beakers filled with 40 mL of test medium.

Before and after each experiment, including those with *D. magna*, pH (WTW, 537, Weilheim, Germany) and dissolved oxygen (DO) (WTW OXI92) were measured. Values of both variables remained constant during the tests; pH ranged from 8.0 to 8.2 and DO was always above 8.1 mg/L. Mortality was only observed in lineage R1 when exposed to 25.0 and 40.0 μg/L of copper, where 50 and 100% individuals died, respectively. Differences between mean MT concentrations of individuals exposed to each treatment or metal concentration were tested using *t*-tests and ANOVAs. Homogeneity of variances was checked using Bartlett and Levene's test. Multiple range Tukey HSD tests were performed to identify homogeneous groups among treatments. Coefficients of variation were compared using the Miller-Feltz equation. Associations between mean basal MT levels of *D. longispina* cloned lineages and their sensitivities to lethal and sublethal levels of copper were calculated using simple correlation.

RESULTS AND DISCUSSION

To ascertain that the polarographic procedure was correctly quantifying MT in the samples, several steps were evaluated. Reliability of the measurements and stability of the samples were confirmed by repeating the polarographic procedure with random samples, with different sample amounts (50, 100 and 150 μL), and after some days of storage at -20°C. Measured signal within the referred range

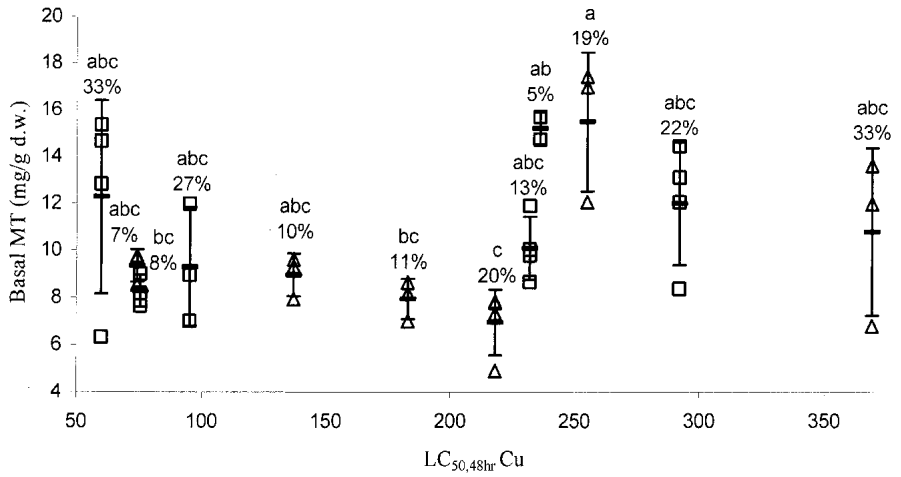

Figure 1. Mean (± SD) basal MT concentrations (mg/g d.w.) in 12 *Daphnia longispina* cloned lineages, belonging to a reference (squares) and an acid mine drainage historically-stressed (triangles) populations, plotted against their median lethal copper concentrations (μg/L). Numbers and letters above data values indicate coefficients of variation (%) and homogeneous groups obtained with the Tukey HSD test, respectively.

increased linearly with sample amount, signal being weak with 50 μL. Afterwards, 100 μL of sample were used for MT determination. Results were consistent among replicated measurements, with differences never exceeding 5%. The two 250 μL standard additions had always correlation values above 0.98. One extra 250 μL addition (up to 750 μL total standard) altered significantly neither the slope of the regression nor the correlation coefficient ($P > 0.05$).

The cause for the variability found in the tests and the main error source of the procedure was probably not related to the adaptation of the DPP technique but it might have been the dry weight determination step. The gravimetric measurement of the dry weight of a part of each sample, though appropriate for larger organisms such as mollusks (Bebianno and Langston 1989), is probably not accurate enough for small organisms as daphnids, because the dry weight (between 1 and 0.2 mg) was close to the detection limit of the used scales (0.01 mg). Moreover, small differences in calibration or sample preparation (small droplets of water in the tube walls, pipetting errors) could also influence the final result. A possible alternative is the determination of the total protein concentration by the Bradford method (Bradford 1976) or other spectrophotometric methods, as used in other MT assays (Boutet et al. 2002). Nevertheless, the DPP technique was found to be suited to determine with precision MT levels in small organisms, using small tissue amounts.

Concentrations of MT have not changed in in *D. magna* exposed to cadmium ($F_{3,11} = 1.14$, $P = 0.375$ and $t_4 = 0.84$, $P = 0.450$, respectively) (Table 2). On the

Table 3. Mean (± SD) MT concentrations (mg/g d.w.) in two *Daphnia longispina* cloned lineages exposed to copper for 24 hr.

	Copper (μg/L)			
Lineages	0.0	15.6	25.0	40.0
R 1	11.34 (± 1.93)	11.76 (± 4.87)	4.61 (± 1.11)	---
I 4	18.55 (± 2.05)	12.87 (± 3.51)	15.17 (± 1.73)	11.27 (± 4.83)

contrary, Bodar et al. (1990) found that an exposure to 100 μg/L of cadmium caused a 3.8-fold increase of MT in whole tissues of previously unexposed *D. magna*. The basal MT concentration found in the first experiment was three times higher than those reported by Bodar et al. (1990) for non-exposed daphnids, being similar to MT levels of individuals chronically exposed to 1 μg/L of cadmium. Concentrations of MT in the second experiment almost doubled those found in the previous one, when daphnids were exposed to similar conditions, the only difference being the homogenate quantity (150 versus 100 μL, respectively). Coefficients of variation ranged from 16 to 25%, being higher than the average 8% found, for this species, by Bodar et al. (1990). This variation can be explained by the factors discussed above.

Significant differences were found in basal MT concentrations among the 12 *D. longispina* cloned lineages ($F_{11,27}$ = 4.13, P = 0.001); coefficients of variation were similar (χ^2_{11} = 12.3, P > 0.25) (Figure 1). However, these differences between lineages were not correlated with increased tolerance to lethal levels of copper (r =0.25, P = 0.43), although cloned lineages with an $LC_{50,48hr}$ of copper greater than 200 μg/L showed higher basal MT concentrations than those with a lower $LC_{50,48h}$ ($F_{11,27}$ = 10.20, P = 0.004). This fact indicates that MT might have been involved in the tolerance acquisition process, but this interpretation is only partially supported by the data, given the absence of correlation and the high MT level in the most sensitive lineage. In addition, as MT are part of a sublethal response, one would expect a strong association with sub-lethal parameters, such as feeding depression. However, no correlation was found between basal MT levels and the respective concentrations of copper causing a 20% depression in the feeding rate, for a 48-hr exposure (r = -0.07, P = 0.82). As with the experiments with *D. magna* and cadmium, no induction of MT by copper was observed, either in the most or in the least tolerant lineages of *D. longispina* ($F_{3,7}$ = 2.52, P = 0.142 and $F_{2,5}$ = 3.23, P = 0.136, respectively) (Table 3). However, MT levels of the most tolerant lineage (I4: $LC_{50,48hr}$ = 368.6 μg/L of copper) were higher than those of the most sensitive one (R1: $LC_{50,48hr}$ = 59.9 μg/L of copper) ($F_{1,14}$ = 9.41, P = 0. 008). In the later, a small decrease of MT levels in the 25 μg/L concentration was found. At this concentration 50% mortality was recorded and at 40.0 μg/L no individual survived. Therefore, the MT decrease in the 25 μg/L may indicate that the stress resistance threshold was broken (Roesijadi, 1992) and that severe damages had already occurred.

Other tolerance and/or detoxification mechanisms, that do not involve increased MT production, may be involved in the acquisition of metal tolerance by these

lineages, similarly to what was found in other works (Stuhlbacher et al 1992). However, slightly higher basal MT levels in the more tolerant lineages indicate that at least part of the increased tolerance of these lineages can be explained by an increased basal production of metallothioneins.

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