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This is a post-print of a paper published in Chemosphere (Elsevier, Amsterdam, The Netherlands). The contents are identical to those in the published version.

Full bibliographic citation (please cite as follows):

Vandegehuchte, M.B., Vandenbrouck, T., De Coninck, D., De Coen, W.M., Janssen, C.R., 2010. Gene transcription and higher-level effects of multigenerational Zn exposure in *Daphnia magna*. Chemosphere 80, 1014-1020.

Link to published journal version (via digital object identifier):

http://dx.doi.org/10.1016/j.chemosphere.2010.05.032

Gene transcription and higher-level effects of multigenerational Zn exposure in *Daphnia magna*

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Abstract

Zn exposure of *Daphnia magna* during one generation has been shown to modulate gene transcription differently in Zn exposed organisms compared to their non-exposed offspring. Here we studied the transcriptional gene regulation with a cDNA microarray in *D. magna* exposed to Zn for three generations (F_0-F_2). For the first time molecular effects of multigeneration toxicant exposure in *D. magna* are described. Out of 73 differentially transcribed genes in the F_1 Zn exposed generation (compared to the F_1 control), only 7 genes were also differentially transcribed in the same direction in the F_0 Zn exposed daphnids (up or down, compared to the F_0 control). The majority of the differentially transcribed unigenes in F_1 Zn exposed daphnids (78 %) were not differentially transcribed in the F_0 Zn exposed organisms. This indicates that Zn exposure affected other molecular pathways in the second exposed generation, although a reduced reproduction and a reduction in juvenile growth were observed in both Zn exposed generations, compared to the respective controls. In the third Zn exposed generation (F_2), no reduction in growth or reproduction compared to the control was observed. This acclimation was reflected in a significantly lower number of differentially transcribed genes, compared to the Zn exposed F_0 and F_1 generations.

Keywords

Acclimation, microarray, ecotoxicology, stress, ecotoxicogenomics

1. Introduction

In the young and rapidly growing research field of ecotoxicogenomics, genomic tools are used to detect the molecular responses an organism experiences when exposed to pollutants, providing clues to the toxic effects in the organism and the compensatory mechanisms that are induced (Poynton and Vulpe, 2009). With DNA microarrays, ecotoxicological effects of exposure can be linked with transcription profiles of large numbers of genes. The transcriptional patterns obtained provide a means to identify complex pathways and strategies that are altered or induced in an organism when it is exposed to environmental stressors (Steinberg et al., 2008). In recent years, a number of studies has investigated the transcriptional responses of *Daphnia* sp. exposed to different types of environmental stress, using *Daphnia* microarrays. This way, molecular effects induced by exposure of daphnids to e.g. Cd, dietary Zn, fenarimol, Ni and even binary metal mixtures or munitions constituents have been discovered and elucidated (Soetaert et al., 2007; Connon et al., 2008; De Schamphelaere et al., 2008; Garcia-Reyero et al., 2009; Vandenbrouck et al., 2009).

Under continuous, multigenerational exposure to certain metals, *Daphnia magna* is known to develop tolerance to this stress. This was demonstrated in experiments with Cd, Cu and Zn (Bossuyt and Janssen, 2004; Muyssen and Janssen, 2004; 2005). Molecular analyses can reveal insights into the underlying mechanisms of tolerance development during the acclimation period. This knowledge may be useful for screening or monitoring potential tolerance development in response to chemical exposure, or for investigating other environmental factors that could affect this tolerance. Except for an investigation of metallothionein induction, related to multigenerational Cd acclimation (Guan and Wang,

2006), no molecular studies related to tolerance development in metal acclimated *D. magna* are available in the literature.

In a recent study, transcriptional patterns of *D. magna* exposed to Zn for one generation and cultured under non-exposed standard conditions for two subsequent generations were analyzed using a custom cDNA microarray (Vandegehuchte *et al.*, 2010b). This revealed transcriptional regulation of several genes, both in the exposed daphnids and in the two subsequent non-exposed generations. An interesting observation was that the differentially transcribed genes of the F_0 Zn exposed daphnids (compared to the F_0 control organisms) were different from those in their non-exposed F_1 and F_2 offspring (compared to F_1 and F_2 control daphnids).

In parallel with these two generations of non-exposed offspring, two generations of offspring were cultured under continuous Zn exposure. In the present study, gene transcription as well as higher-level effects in three generations of Zn exposed daphnids were studied to evaluate transcriptional effects of continuous multigeneration Zn exposure and to elucidate the acclimation process at a transcriptional level.

2. Materials and methods

2.1 Daphnia cultures and experimental design

D. magna Straus (clone K6) used in our experiments was originally collected from a pond in Kiel (Antwerp, Belgium) and has been successfully cultured under controlled laboratory conditions for more than 10 years in aerated carbon filtered tap-water, enriched with selenium (1 µg/L) and vitamins (7.5 mg/L thiamin, 100 µg/L cyanocobalamin and 75 µg/L biotin).

Daphnids were cultured in 10 mL medium per surviving daphnid during the first week and in 20 mL medium per surviving daphnid from the second week onwards, maintaining a constant density of organisms and food, as described by Vandegehuchte et al. (2010b). Culture media were renewed three times per week and juveniles were removed at these occasions. The experimental design used in the current study is as follows. A set of neonates (0-24h) taken from the laboratory culture was divided into two batches. One batch was transferred to modified standard M4 medium (Elendt and Bias, 1990) and cultured in this control medium for three generations (F_0C–F_2C). A second batch of neonates was transferred into the same medium, but with the Zn concentration adjusted to 388 µg/L and cultured in this Zn contaminated medium for three generations (F_0Zn–F_1Zn). Based on previous studies, the higher Zn concentration was estimated to be sublethal, with a significant effect on reproduction (Heijerick *et al.*, 2005; Muyssen and Janssen, 2005). Each combination of generation and exposure (control or Zn contaminated medium) is termed a 'treatment' throughout this paper (Fig. 1). The standard M4 medium was modified by replacing EDTA and Fe by 4 mg/L of natural Dissolved Organic Carbon (DOC) to avoid the use of excessively high metal concentrations due to EDTA complexation and to increase the environmental relevance of the medium. The dissolved organic matter was collected from a small unpolluted creek (Ruisseau de St. Martin, Bihain, Belgium) using a portable reverse osmosis system (PROS/2) (Sun *et al.*, 1995). It was stored in the dark at 4 °C in a 50 L barrel, at a concentration of approximately 400 mg/L DOC. This DOC stock was thoroughly mixed each time before the preparation of new medium. The same batch of DOC was used for all treatments and media renewals. The Zn concentration in the control medium was adjusted to 19 µg/L Zn, i.e. within the optimal concentration range of this essential element for daphnids (Muyssen and Janssen, 2004).

Reproduction as total number of living juveniles per surviving adult after 21 days was measured by counting the number of juveniles per organism three times per week for each individual daphnid. Ten individual daphnids were kept in plastic cages (fitted with 200 µm mesh size gauze) which were suspended in the same aquaria as the treatment cultures. The length from the top of the head until the base of the spine was measured for ten different individual organisms per treatment by analyzing a microscopic image with UTHSCSA Image Tool 3.0 (San Antonio, TX, USA). This was done on day 6, day 13 and one to three days after the fifth brood was observed in the aquarium, when sufficient 0-24h offspring were available to start the next generation treatment. Internal Zn concentrations were determined as described in Vandegehuchte et al. (2010b). All Zn concentrations were measured by atomic absorption spectrometry (SpectrAA-100, Varian, Mulgrave, Australia).

2.2 Statistical analysis

All statistics were performed with Statistica (Statistica, Tulsa, USA). Differences between the Zn exposed and the control daphnids in reproduction (total number of juveniles per surviving female), length or internal Zn concentration were assessed using t-tests. For the comparison of the internal Zn concentrations in daphnids from the three Zn exposed generations, a one-way ANOVA was used. Assumptions of normality and homoscedasticity were tested with Shapiro-Wilk's test and Bartlett's test, respectively. When one of these assumptions was not met, non-parametric Mann-Whitney U tests were performed to assess differences between exposed and control treatments (USEPA, 2000). In all tests, the limit of significance was set at $p = 0.05$.

2.3 Microarrays

147 Three *D. magna* cDNA libraries enriched with genes related to energy metabolism, molting
148 and life stage specific processes have been developed by Soetaert et al. (2006; 2007) using
149 the suppression subtractive hybridization technique. Next to these cDNA libraries, two extra
150 cDNA fragments, corresponding to expressed sequence tags (ESTs) from genes that are
151 reported to be sensitive to Zn were spotted on the array: ESTs with homology to (1) ferritin
152 (AJ292556) and (2) retinol dehydratase (DV437801) gene fragments (Poynton et al., 2007).
153 Finally, also two ESTs with homology to putative MTs (metallothioneins) (DV437799 and
154 DV437826) were spotted because MTs have been shown to be induced by Zn (Fan et al.,
155 2009). The preparation and spotting of the sequences are reported by Vandegehuchte et al.
156 (2010b).

157

158 ***2.4 Microarray preparation***

159 Three replicates of ten adult daphnids per treatment ('treatment' = combination of
160 generation and exposure type, see Fig. 1) were sampled for mRNA analysis on the day the
161 next generation was started (see above). The methods for RNA-extraction, conversion into
162 cDNA, labeling and hybridization following a universal reference design can be found in
163 Vandegehuchte et al. (2010b).

164 ***2.5 Bioinformatic analysis of microarray data***

165 The microarrays were scanned using a Genepix personal 4100 Scanner (Axon instruments,
166 USA). Scanned images were analyzed using Genepix Pro Software 4.0 (Axon Instruments) for
167 spot identification and for quantification of the fluorescent signal intensities. Subsequently,
168 data were further evaluated using the Bioarray Software Environment database (BASE

169 1.2.17, http://www.islab.ua.ac.be/base/), i.e. a MIAME based microarray analysis package
170 developed by the Intelligent Systems Laboratory (University of Antwerp, Belgium). Spots
171 were background corrected by local background subtraction. Spots with saturated intensities
172 were filtered out by visual inspection. The Cy5/Cy3 ratio was calculated for each spot, $\log_2$
173 transformed, and normalized between arrays using variance stabilization normalization
174 (Huber et al., 2002). Analysis of significant differences in transcription between treatments
175 was performed by using Limma (linear models for microarray data) (Smyth, 2004; Smyth et
176 al., 2005). Fragments for which the p-value, adjusted for false discovery rate, was lower than
177 0.05, were retained as significantly up- or downregulated (Benjamini and Hochberg, 1995).
178 Only those fragments for which the $\log_2$ ratio was outside the interval [-0.75, 0.75] were
179 retained for further analysis. Sequence descriptions and annotations were obtained through
180 Blast2GO (Conesa et al., 2005)(www.blast2go.de), which allowed genes to be classified into
181 functional groups (Fig. 2). A heat plot was created with MultiExperiment Viewer (MeV) 4.5.1
182 (Saeed *et al.*, 2006).

Results and discussion

Differences between exposed and control treatments will only be mentioned when they are statistically significant ($p < 0.05$).

An effect of Zn exposure on growth (vs. the respective controls) was noted in 6-day old daphnids of the F_0Zn and F_1Zn treatments (Fig. 3A, Table 1). Growth reduction in juvenile daphnids is not uncommon and has been observed in toxicity tests with cetyltrimethylammonium bromide and 5-azacytidine (Knops *et al.*, 2001; Vandegehuchte *et al.*, 2010a). Like in the F_0 generation, a Zn induced reduction in juvenile growth (compared to the respective control) was also observed in their F_1Zn offspring. However, no growth reduction was noted in the F_2 generation (compared to the F_2 control). The absence of growth reduction in the F_2Zn daphnids can be interpreted as acclimation to Zn in the third exposed generation. This acclimation in the F_2Zn organisms is also suggested by the fact that their reproduction is not affected (compared to the F_2 control daphnids), although reproduction results in F_2 should be interpreted with care, considering the decreased control reproduction in F_2C. In the first and second generation of Zn exposed daphnids a reduction in reproduction was observed (compared to the control of the same generation, Fig. 3B, Table 1). Muyssen et al (2005) showed that exposure to Zn for six generations can increase or decrease the reproductive output, depending on the acclimation concentration and the test concentration to which the sixth-generation daphnids were exposed. These authors reported a significantly higher reproduction in daphnids of the sixth versus the first generation acclimated to 45 µg/L Zn^{2+} (which is higher than the optimal concentration range), when exposed to an optimal test concentration of 22 µg/L Zn^{2+}. In that study, reproduction in the actual acclimation treatments was not reported. Tolerance development/acclimation to a metal can occur even after two generations of exposure, as

207 demonstrated for net reproduction in *D. magna* exposed to 5 to 35 µg/L of Cu (Bossuyt and
208 Janssen, 2003). This is in accordance with our results on reproduction.

209 The average Zn body burdens of the exposed F_1Zn and F_2Zn treatments (resp. 165 and 157
210 µg Zn/g dry weight) were higher than those of the F_1C and F_2C controls (resp. 49 and 51 µg
211 Zn/g dry weight). This is in accordance with the previously reported internal Zn
212 concentrations of F_0Zn and F_0C (resp. 229 and 69 µg Zn/g dry weight, Vandegehuchte *et al.*,
213 2010b). There was no significant difference between the internal Zn concentrations of the
214 three Zn exposed treatments.

215 When the gene transcription patterns of control treatments were compared (i.e. F_0C vs F_1C, F_1C vs
216 F_2C or F_0C vs F_2C), a large number of genes were found to be differentially transcribed, as reported by
217 Vandegehuchte et al. (2010b). This concerned more than 15% of the unigenes on the array. The
218 differential transcription of these genes is likely due to differences in the molting phases and
219 reproductive cycles of the daphnids in the different generations and is as such not specific to the Zn
220 exposure. Therefore, those genes that significantly varied in transcription between different control
221 generations, were removed from the list of differentially transcribed genes between Zn treated
222 organisms and controls obtained with the microarray analysis. Thus, 38 to 46 % of the differentially
223 transcribed unigenes between treatments and controls were retained for further analysis. In the
224 following section of the manuscript, differential transcription will always be related to the
225 control of the same generation. Differentially transcribed genes for which a sequence
226 description could be obtained are listed in Fig. 2. Genes for which no homology was found
227 are summarized in the supplementary online material. Redundant fragments on the array
228 were grouped into contigs. The resulting 1207 unique identified fragments on the array are
229 termed unigenes (Vandegehuchte *et al.*, 2010b).

In the F_1Zn daphnids, 73 differentially transcribed unigenes were found (Table 1). This number is comparable to the 71 regulated unigenes in the F_0Zn treatment, where also a reduction in reproduction and in body length at day 6 were observed. Seven genes were regulated in the same direction in F_0Zn and in F_1Zn. However, another set of seven common genes were differentially transcribed in opposite directions in F_0Zn and F_1Zn (Fig. 2 and supplementary online table). Although some of the remaining 59 differentially transcribed unigenes in F_1Zn may belong to the same gene as fragments that were differentially transcribed in F_0Zn (such as genes with homology to *D. magna* vitellogenin or to a hemoglobin subunit), for most of these fragments this is not the case. Zn exposure in the second generation daphnids clearly elicited different effects at the transcriptional level compared to the first generation. Some differentially transcribed genes in F_1Zn for which a sequence description could be obtained through Blast will be discussed in the next paragraphs.

General trends per functional group of genes differ between F_0Zn and F_1Zn organisms. While in F_0Zn all affected transcription and translation related genes were downregulated, four out of five transcription and translation related genes are upregulated in F_1Zn. All five of these regulated unigenes are different from those in F_0Zn. The potential stress-induced energy-saving mechanism of decreasing ribosomal protein synthesis (Brown-Peterson et al., 2005), which was suggested based on the downregulation of ribosomal protein coding genes in F_0Zn, is not present in the second generation of Zn exposed daphnids anymore. Similarly, the oxidative stress response related genes peroxiredoxin 6 and glutathione S-transferase, which were upregulated in F_0Zn, were not differentially regulated in F_1Zn.

252 While most metabolism-related differentially transcribed genes were upregulated in F_0Zn,
253 this was the case for only four out of nine metabolism-related differentially transcribed
254 genes in F_1Zn. A gene coding for a serine threonine protein phosphatase, which was
255 upregulated in F_0Zn, was downregulated in F_1Zn. In the presence of Fe^{2+}, Zn^{2+} is known to
256 influence the activity of these phosphatases (Chu et al., 1996). It is hypothesized that in the
257 F_0Zn daphnids, the internally available Zn^{2+} concentration may have been high enough to
258 reduce the phosphatase activity compared to the control daphnids. A transcriptional
259 upregulation could compensate for this. Still following this hypothesis, the internally
260 available Zn^{2+} concentration may have changed in the F_1Zn daphnids, due to Zn induced
261 defense mechanisms, resulting in a phosphatase activity which is near the optimum and
262 higher than in the control, thus explaining the lower transcription. The upregulation of a
263 serine protease, as seen in the F_1Zn treatment, was also observed specifically after Zn
264 exposure in a study of transcriptional responses in *Daphnia magna* exposed to munitions
265 constituents, such as metals and nitroaromatic compounds (Garcia-Reyero et al., 2009).
266 Similarly, the observed downregulation of a chitinase is consistent with previous studies with
267 Zn exposed *D. magna*, where Zn toxicity was suggested to be associated with molting and
268 exoskeleton maintenance (Poynton et al., 2007; Garcia-Reyero et al., 2009).

269 The upregulation of a gene coding for the heat shock protein Hsp90 can be a stress response
270 leading to elevated levels of Hsp90 in Zn exposed daphnids, as observed in earthworms
271 exposed to Zn and Pb contaminated soils (Marino et al., 1999). Another likely stress
272 response, which was already noted in the F_0Zn treatment, is the upregulation of a gene
273 related to glutathione S-transferase, which is involved in oxidative stress abatement
274 (Newman and Clements, 2008). Also similar to F_0Zn, all differentially transcribed genes with
275 homology to *D. magna* vitellogenin, which is fused with a superoxide dismutase module

(Kato et al., 2004), were upregulated. These genes are involved with vitellogenesis, the production of yolk proteins in the oocytes. Their differential transcription is likely due to random differences in reproductive cycle phases and associated vitellogenesis between the F_1Zn and F_1C daphnids, as indicated by the differential transcription between two control treatments of a unigene with the same homology (Vandegehuchte *et al.*, 2010b). Stibor (2002) has demonstrated large differences in yolk protein levels at different times between the deposition of two consecutive broods into the brood pouch.

The transcriptional downregulation of genes coding for a hemoglobin protein subunit was already noted in F_0Zn. Martinez-Tabche et al. (2000) reported that Zn exposure decreased the hemoglobin level in the oligochaete worm *Limnodrilus hoffmeisteri*. These authors suggested that this was caused by a Zn induced inhibition of heme synthesis. If Zn inhibits heme synthesis, it can be speculated that transcription of hemoglobin related genes would not lead to the formation of hemoglobin protein and transcriptional downregulation could be an energy-saving mechanism. Zn exposure is indeed known to decrease the hemoglobin content in *D. magna* (Berglind, 1986). A last remarkable upregulated gene in the F_1Zn treatment showed homology to cytochrome p450. P450s are proteins involved with phase I detoxification, lipid metabolism and hormone synthesis/breakdown (Baldwin et al., 2009). Transcriptional upregulation of a P450 coding gene in *D. magna* was also observed after Cd exposure (Connon et al., 2008). Zn exposure, as well as Cu exposure, induced P450 activity in earthworms (Lukkari et al., 2004).

It is striking that in the third generation of Zn exposed daphnids (F_2Zn) a much lower number of genes than in the previous generations are differentially transcribed: only 23 of which 11 were upregulated (Table 1). Daphnids from this treatment seem to be acclimated to the Zn

299 exposure in the sense that no negative effects on reproduction or body length were
300 observed, although the internal Zn concentration of 157 µg Zn/g dry weight in body tissue
301 was still elevated and not significantly different from the previous Zn exposed generations.
302 Roelofs et al. (2009) also reported a smaller number of Cd-induced differentially transcribed
303 genes in a Cd tolerant versus a reference population of the springtail *Orchesella cincta*.
304 Additionally, these authors suggested that the absence of inhibitory effects on translation
305 and digestive enzyme related genes could explain the smaller growth reduction upon Cd
306 exposure in tolerant *Orchesella* populations (Posthuma et al., 1992). Our results for Zn are in
307 line with this suggestion. No growth reduction was observed in the ZnF_2 daphnids, for which
308 only one translation and two metabolism related genes were differentially regulated,
309 compared to six to seven and nine genes, respectively, in the previous generations with
310 juvenile growth reduction. Two notable differences between the present study and that of
311 Roelofs et al. (2009) can be remarked. First, springtails, unlike daphnids, are not
312 parthenogenetic and thus genetic variation was present in their populations. Second, the
313 springtails were selected from populations in different field sites, of which one had a long
314 history of metal pollution, whereas the daphnids in the present study originated from the
315 same parental generation and only differ in their three-generation exposure history. As such,
316 no genetic selection can have acted on the daphnids in this study.

317 The genes for hydroxyisourate hydrolase (HIU hydrolase, involved in purine metabolism) and
318 for obstractor d, involved in chitin metabolism, were downregulated in F_2Zn. Genes involved
319 in chitin metabolism have been observed to be both up- and downregulated in several
320 studies with metal exposed *D. magna* (Poynton et al., 2007; De Schamphelaere et al., 2008;
321 Vandenbrouck et al., 2009). As in the other Zn exposed treatments, a gene coding for
322 vitellogenin was upregulated and genes coding for hemoglobin subunits were

downregulated. Next to these, genes coding for a wd repeat protein and for a small nucleolar ribonucleoprotein involved in mRNA splicing or its regulation as well as genes with homology to chromosome 3 open reading frame 23 and to an inorganic pyrophosphatase were downregulated. Transcriptional upregulation was observed for genes coding for two proteins: one with homology to a hypothetical protein of the body louse *Pediculus humanus corporis* and another one with homology to a midline fasciclin, which mediates cell adhesion and signaling (Hu et al., 1998).

In conclusion, continuous Zn exposure resulted in acclimated *D. magna* in the third exposed generation, which exhibited no adverse effect on reproduction or growth. At the transcriptional level, few unigenes were regulated in the same direction in the three generations of Zn exposed daphnids: two genes with no homology, a vitellogenin coding gene and a hemoglobin chain coding gene. In the second Zn exposed generation (F_1Zn), a large number of the differentially transcribed genes were different from those in F_0Zn, although a reduction in reproduction and juvenile growth was observed in both treatments. Multigenerational exposure to Zn elicits different molecular effects in the different generations. The acclimation in the third exposed generation was reflected in a considerably smaller number of differentially transcribed genes. No direct molecular acclimation mechanisms could be deduced from the transcriptional results obtained with this custom cDNA microarray, on which a limited, although ecotoxicologically relevant, set of genes is represented. Currently, the *D. magna* genome is being sequenced by the *Daphnia* Genomics Consortium, coordinated at Indiana University. When this genome becomes available, wider transcriptome studies can be undertaken to elucidate the molecular mechanisms of metal acclimation in *D. magna*.

Acknowledgement

The authors thank Emmy Pequeur and Leen Van Imp for technical assistance. This study has been financially supported by the Ghent University Special Research Fund (GOA project No. 01G010D8) and by the Flemish Research Foundation (FWO-Vlaanderen, project No. 3G022909 09).

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