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15 Can metal stress induce transferable changes in gene transcription in *Daphnia magna*?

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Abstract

DNA methylation has recently been reported in *Daphnia magna*, which indicates the possible presence of epigenetic mechanisms regulating gene expression in this species. As such, effects of transient chemical exposure could be transferred through epigenetic inheritance to non-exposed generations. In this study, in the Zn-exposed daphnids, a large number of genes were found to be differentially transcribed, amongst which transcription and translation related genes (downregulated), genes associated with oxidative stress (upregulated) and different types of metabolism related genes (mostly upregulated). In the two subsequent generations of non-exposed daphnids, a considerable number of differentially regulated genes was observed, indicating an effect of Zn-exposure in the non-exposed progeny. However, none of the differentially transcribed genes observed in the Zn exposed generation were regulated in the same direction in both non-exposed subsequent generations. The exposure of *Daphnia magna* to a sublethal Zn concentration for one generation did not result in a stable transgenerational epigenetic effect with consequences for reproductive output nor was a stably epigenetically inheritable effect observed on the transcription of any of the studied genes. An important observation was the large number of genes that were differentially transcribed between different control generations with no pre-exposure history. These genes were not considered in the analysis of the effect of Zn exposure on gene transcription. This differential regulation between subsequent control generations was attributed to possible differences in synchronization of the molting and reproductive cycle of the daphnids in the different generations. This finding is of major importance for the interpretation and design of future microarray experiments with adult *Daphnia*.

Keywords: Transgenerational effect, epigenetics, mRNA, microarray, zinc, *Daphnia magna*

1. Introduction

Epigenetic changes involve stable changes in gene expression that occur without changes in the code of DNA-base pairs (Goldberg *et al.*, 2007). These changes are the result of e.g. changes in DNA methylation, histone modifications or RNA interference and can be induced by exposure to environmental contaminants (Reamon-Buettner *et al.*, 2008). Moreover, some epigenetic changes can be inherited by subsequent generations, even if the triggering environmental factor is removed (as reviewed by Youngson and Whitelaw (2008)).

A striking example of inheritable chemical-induced epigenetic effects was observed in pregnant rats exposed to the fungicide vinclozolin. This resulted in reduced reproduction, which correlated with altered DNA methylation patterns, of the male progeny up to four generations after the toxic exposure (Anway *et al.*, 2005). These results could not be reproduced by Schneider et al. (2008). However, the latter authors administered the vinclozolin orally, while Anway et al. (2005) used intraperitoneal injection.

If environmental exposure to chemicals induces inheritable epigenetic effects to non-exposed future generations and if this is wide-spread among species, this phenomenon may have major consequences for the way ecological risks assessments of chemicals are performed. In this case the effects of temporary exposures to contaminants on the future status of ecosystem structure and functioning should be considered.

Most of the research related to epigenetics has been performed with model animal species such as *Drosophila*, mice and rats and with plants like *Arabidopsis* (Youngson and Whitelaw, 2008). Although possibly of major importance to environmental toxicology and risk assessment, studies on transgenerational epigenetic effects in more environmentally relevant species are scarce, e.g. Brown et al.(2009). In *Daphnia magna*, one of the standard species in aquatic toxicology, DNA methylation has recently been detected (Vandegehuchte *et al.*, 2009a). Transgenerational effects of different maternal Zn exposures on the reproductive output of the next generation of *D. magna* have been reported, but effects on the subsequent generations have not been studied (Muyssen and Janssen,

2005). Maternal Zn exposure has also been shown to induce transgenerational changes in the overall methylated cytosine content of *D. magna* DNA (Vandegehuchte *et al.*, 2009b). These authors report a decrease in DNA methylation in the first non-exposed generation of offspring, which was not detected in the next generation. It should be stressed however, that the overall methylation status gives no information about the methylation of specific genes or about other epigenetic mechanisms possibly influencing specific genes.

Epigenetic changes can be measured directly at a molecular level, e.g. by detecting changes in DNA-methylation, by assessing the presence of elevated concentrations of specific interfering RNA molecules or by immunoprecipitation of modified histones (Barton *et al.*, 2005; Rassoulzadegan *et al.*, 2006; Waterland *et al.*, 2006). The genome sequence of *D. magna* is still largely unknown, which makes it difficult to investigate epigenetic changes at the level of specific genes. The field of transcriptomics on the other hand studies the presence and relative abundance of RNA transcripts in order to generate gene-specific mRNA expression profiles (Jamers *et al.*, 2009). The integration of this field into ecotoxicology has resulted in a large number of ecotoxicogenomic studies dealing with the transcriptional response of environmental toxic exposure (reviewed by Steinberg et al.(2008)). Through measuring the transcriptional pattern with microarrays, we studied possible epigenetically inheritable effects on gene transcription in an indirect way. Stable changes in the transcriptional pattern which can be transferred to subsequent non-exposed generations would indicate epigenetic inheritance (Bossdorf *et al.*, 2008). Recently, this method has been used to demonstrate a transgenerational effect of the endocrine disruptor vinclozolin on the transcriptome of embryonic rat testis (Anway *et al.*, 2008). The recent development of a custom cDNA microarray platform for *D. magna* enables the investigation of epigenetic changes in the gene transcription pattern of *D. magna* after a transient chronic exposure to a sublethal Zn concentration.

2. Materials and methods

2.1 Daphnia cultures and experimental setup

D. magna Straus (clone K6) used in all 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).

Organisms were cultured in a semi-static manner in plastic aquaria, in a volume of 10 mL per daphnid for the first week and 20 mL per daphnid from the second week onwards (Muyssen *et al.*, 2006). Media were renewed three times per week. One set of neonates (0 – 24 h) was cultured under these laboratory conditions for 21 days and 6, 13 and 21 day old organisms from this batch were used to create a reference pool of cDNA (see below). Another 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 for three generations (F_0C–F_2C). A second batch of organisms was transferred into the same medium, with the Zn concentration adjusted to 388 µg/L by adding a concentrated solution of $ZnCl_2$ (Merck, Germany) in deionized water. Based on previous studies, this Zn concentration was estimated to have a significant effect on reproduction (Heijerick *et al.*, 2005; Muyssen and Janssen, 2005). F_1 neonates born from this F_0Zn^+ generation were transferred back into the control medium (F_1Zn^-). In this way, F_1Zn^- daphnids were only briefly exposed to Zn during the first hours of their life-cycle. Offspring of the F_1Zn^- organisms were also cultured in the control medium (F_2Zn^-) (Fig. 1). Each combination of generation and exposure history will be named a 'treatment' throughout this manuscript. 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 and to increase the environmental relevance of the medium. The Zn concentration in the 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). Organisms were fed daily with

136 an algae mix consisting of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3/1
137 cell number ratio. Algal concentrations were measured each time before daphnid feeding with a
138 Coulter Counter (Beckman, USA) and were adjusted daily to obtain a concentration of 17.8 mg/L in
139 the first week and 26.7 mg/L from the second week onwards.

140 Reproduction as total number of living juveniles per surviving adult after 21 days was measured by
141 counting the number of juveniles per organism three times per week in individual daphnids. Ten
142 individual organisms were kept in plastic cages (fitted with 200 µm mesh size gauze) which were
143 suspended in the same aquaria as the treatment cultures.

144 For internal Zn concentration measurements, ten daphnids per treatment were collected and placed
145 for five hours into a low Zn reference medium consisting of consecutively carbon- and bio-filtered tap
146 water, with a low Zn concentration of 4.3 µg/L. The same algae concentration as used in the test
147 aquaria was provided to allow depuration of Zn-contaminated algae from the daphnids' gut (Gillis *et*
148 *al.*, 2005). Subsequently, they were placed into an EDTA solution (Na-EDTA, 5 x 10^{-3} M, Fluka) to
149 remove the Zn adsorbed to the carapax thus allowing quantification of internal Zn concentrations.
150 Three replicates of three or four daphnids were dried at 40 °C for 2 days. After weighing these three
151 replicates, the organisms were destructed per replicate in 14 mol/L HNO_3 (BDH Prolabo, Belgium, Zn
152 ≤ 0.005 mg/kg) by heating in a microwave at increasing power (90, 160 and 350 W) for four minutes
153 each time. Samples were diluted with deionized water to 10% HNO_3 (v/v) and the Zn concentration
154 was measured by atomic absorption spectrometry (SpectrAA-100, Varian, Mulgrave, Australia).

155 ***2.2 Statistical analysis***

156 All statistics were performed with Statistica (Statistica, Tulsa, USA). Differences between two
157 treatments in reproduction (total number of juveniles per surviving female) and internal Zn
158 concentration were assessed using t-tests. Assumptions of normality and homoscedasticity were
159 tested with Shapiro-Wilk's test and Bartlett's test, respectively. For the reproduction in the F_1

generation, the assumptions were not met and a Mann-Whitney U test was used. In all tests, the limit of significance was set at $p = 0.05$.

2.3 Microarrays

Three *D. magna* cDNA libraries enriched with genes related to energy metabolism, molting and life stage specific processes have been developed by Soetaert et al. (2006; 2007a)using the suppression subtractive hybridization technique. Next to these cDNA libraries, two extra cDNA fragments, corresponding to expressed sequence tags (ESTs) from genes that are reported to be sensitive to Zn were spotted on the array: ESTs with homology to (1) ferritin (AJ292556) and (2) retinol dehydratase (DV437801) gene fragments (Poynton *et al.*, 2007). Finally, also two ESTs with homology to putative MTs (metallothioneins) (DV437799 and DV437826) were spotted because MTs have been shown to be induced by Zn (Fan *et al.*, 2009). These sequences were PCR amplified from a cDNA sample taken from a random treatment, checked by electrophoresis, purified by Montage PCR Plate (Millipore, USA) and loaded into 384-well plates (Genetix, UK) in 50% dimethylsulfoxide at a final concentration of 50–75 ng/µL. The isolated cDNA clones from the cDNA libraries were PCR amplified from the pGEM-T easy vector (Promega, USA) after which the same control, purification and loading steps were performed. The cDNA fragments (average length of 415 bp) were spotted in triplicate on aminosilane coated glass slides (Generoma microarray slides, Asper Biotech, Estonia). A set of artificial control genes (Lucidea Universal Scorecard, Amersham Biosciences, UK) were also spotted. After rehydration and drying, the cDNA fragments were cross-linked to the slides using UV-radiation at 300 mJ (UV Stratalinker 2400, Stratagene, USA). The cDNA microarrays were used as a dual color system where two samples are labeled with different dyes and hybridized together on one array.

2.4 Microarray preparation

Three replicates of ten adult daphnids per treatment ('treatment' = combination of generation and exposure type, see Fig. 1) were sampled for mRNA analysis. This was done one to three days after the fifth brood was observed in the aquarium, when sufficient 0-24h offspring were available to start

185 the next generation. After submerging the daphnids in RNALater (Qiagen, the Netherlands) they
186 were flash frozen in liquid N_2 and total RNA was isolated using the Trizol extraction method following
187 the manufacturers' protocol (Invitrogen, Belgium). Following DNase treatment (Fermentas,
188 Germany), RNA integrity was evaluated by denaturing formaldehyde-agarose gel–electrophoresis.
189 Lucidea test or reference mRNA spikes were added to the RNA samples. Probes were prepared by
190 converting 5 µg total RNA from each replicate into aminoallyl-dUTP (Sigma, Belgium) labeled cDNA
191 using the Superscript II Reverse transcriptase kit (Invitrogen, Belgium). Remaining RNA was
192 hydrolyzed and un-incorporated nucleotides were removed using the Qiaquick PCR purification
193 columns (Qiagen, the Netherlands) following a modified protocol (van der Ven *et al.*, 2005).
194 Treatment and reference pool aminoallyl cDNA were then covalently coupled with Cy5- or Cy3-
195 esters, respectively (or vice versa in dye-swap experiments), purified once more, and the labeling
196 efficiency was determined by spectrophotometry (Nanodrop ND-1000, Nanodrop Technologies,
197 USA). Each of the three replicates of the treatment daphnids was hybridized on a separate array
198 against a labeled reference pool sample, following a universal reference design.

199 ***2.5 Bioinformatic analysis of microarray data***

200 The microarrays were scanned using the Genepix personal 4100 Scanner (Axon instruments, USA).
201 Scanned images were analyzed using the Genepix Pro Software 4.0 (Axon Instruments) for spot
202 identification and for quantification of the fluorescent signal intensities. Subsequently, data were
203 further evaluated using the Bioarray Software Environment database (BASE 1.2.17, http://www.islab.

204 ua.ac.be/base/), i.e. a MIAME platform based microarray analysis method developed by the
205 Intelligent Systems Laboratory (University of Antwerp, Belgium). Spots were background corrected
206 by local background subtraction. Spots with saturated intensities were filtered out by visual
207 inspection. The Cy5/Cy3 ratio was calculated for each spot, $\log_2$ transformed, and normalized
208 between arrays using variance stabilization normalization (Huber *et al.*, 2002). Analysis of significant
209 differences in transcription between treatments was performed by using Limma (linear models for

microarray data) (Smyth, 2004; Smyth *et al.*, 2005). Fragments for which the p-value, adjusted for false discovery rate, was lower than 0.05, were retained as significantly up- or downregulated (Benjamini and Hochberg, 1995). Only those fragments for which the log2 ratio was outside the interval [-0.75, 0.75] were retained for further analysis. Sequence descriptions and annotations were obtained through Blast2GO (Conesa *et al.*, 2005)(www.blast2go.de), according to which genes were classified into functional groups (Table 2).

3. Results and discussion

Exposure to 388 µg/L Zn significantly reduced the *D. magna* reproduction in the F_0 generation (F_0Zn^+, Table 1). The F_1Zn^- and F_2Zn^- treatments, however, did not exhibit significant changes in reproduction compared to the control treatment of the same generation. From this it is concluded that the adverse effect on reproduction observed in the F_0Zn^+ treatment was not transferred to the non-exposed progeny.

The internal Zn concentration in the exposed F_0Zn^+ daphnids was 229 µg Zn/g dry weight. This was significantly higher than the 69 µg Zn/g dry weight observed in the F_0 control daphnids. The average internal Zn concentration in the non-exposed F_1 and F_2 Zn^- treatments was not significantly different from the average in the F_1 and F_2 controls: resp. 54 vs. 49 and 69 vs. 51 µg Zn/g dry weight. These concentrations are in the same range as the 96 µg/g and 173 µg/g observed by Muyssen et al. (2006) for *D. magna* in a control and a 170 µg/L Zn treatment, respectively. As such, any possible Zn induced effect on gene transcription in the F_1 or F_2 Zn^- treatments cannot be attributed to the direct exposure to maternally transferred excess internal Zn.

Comparison of Zn exposed and non-exposed daphnids of the F_0 generation resulted in 287 differentially regulated fragments. Omitting bad sequences and contamination sequences on the array, 263 differential fragments were retained from the 1975 valid reporter fragments on the array. However, redundant fragments were present on the custom microarray and therefore fragments

were grouped into contigs. This resulted in 291 contigs and 916 gene fragments not belonging to a contig on the array. These estimated 1207 unique identified fragments will subsequently be called unigenes. In this way, 178 differential unigenes representing 15% of the unigenes on the array were detected in the Zn exposed versus non-exposed F_0 daphnids, 119 of which were upregulated.

When the transcription profiles of F_0C and F_1C were compared with Limma, we observed that 105 unigenes were differentially transcribed between two generations of control organisms. Between F_0C and F_2C and between F_1C and F_2C there were 78 and 144 differentially transcribed unigenes, respectively. A total of 191 or more than 15% of the unigenes on the array were thus differentially transcribed between control treatments of different generations, despite the fact that exposure conditions in the control treatments were kept constant throughout the experiment. These transcription changes between the different control generations may possibly be due to the different phases in the molting and associated reproductive cycles of the daphnids sampled from different generations. Daphnids were sampled for RNA extraction at a non-standardized time point in the cycle around the release of the sixth brood. Many physiological processes in *D. magna* have a cyclic nature associated with molting which may be reflected in differences in gene transcription. Ecdysteroid levels for instance can increase more than fourfold in a period of 12 hours (Martin-Creuzburg *et al.*, 2007). In *Bombyx mori*, the transcription of epidermal genes changes considerably at different time points between two molts (Okamoto *et al.*, 2008). Significant differences in gene transcription between two control groups of adult organisms were also observed in the earthworm *Lumbricus rubellus* (Owen *et al.*, 2008). Worms sampled in November exhibited a different transcription profile from worms exposed to the same conditions but sampled in late December.

The comparison of control treatments has not been reported by other authors working with this custom *D. magna* microarray (Soetaert *et al.*, 2006; Soetaert *et al.*, 2007a; Soetaert *et al.*, 2007b). Some of the differentially transcribed genes that are described by these authors may be due to the above-mentioned differences in the molting phase and not to the chemical exposure. However, in most of these experiments, juvenile daphnids were exposed for maximum 96 h. As daphnids of this

262 age do not reproduce yet, fewer confounding influences can be expected on reproduction related
263 genes. The same remark holds for a study with *Daphnia magna* and another microarray with 24 h
264 exposed neonates (Watanabe *et al.*, 2008). Others used exposed adult *Daphnia* sp. of different ages
265 with replicates at different dates, or even a mixture of adults and their offspring, thereby averaging
266 out differences in molting cycles (Poynton *et al.*, 2007; Shaw *et al.*, 2007).

267 As the observed differential transcription between the non-exposed control generations was not due
268 to Zn, these genes were not considered in the comparison of Zn treatment effects. Applying this
269 filter, 71 differentially transcribed unigenes of which 44 were upregulated, were thus identified as
270 being affected by the Zn treatment in the F_0 generation. This is 39% of the originally detected total
271 number of differentially transcribed unigenes. The genes with a sequence description are
272 summarized in Table 2. Genes for which no homology was found are listed in the online
273 supplementary material. None of the genes that were added to the original custom array were
274 differently transcribed. Except for the retinol dehydratase gene, this corroborates the results of
275 Poynton et al. (2007), who did not observe significant transcription differences on the microarray for
276 the putative MTs or for ferritin. All ten differentially transcribed translation and transcription related
277 genes were downregulated in the Zn-exposed daphnids. This is in contrast with what Connon et al.
278 (2008) found in *D. magna* exposed to Cd for 24 hours. These authors observed both up and
279 downregulation of ribosomal proteins. Brown-Peterson et al. (2005) suggested that a decrease in
280 ribosomal protein synthesis is a stress induced energy saving mechanism. The oxidative stress
281 response genes peroxiredoxin and gluthathione S-transferase were upregulated, which was also
282 observed by Poynton et al. (2007) in adult daphnids exposed to Cd. These authors, however, did not
283 observe this in *D. magna* assays with Zn which may possibly be explained by their short exposure
284 time: i.e. 24 h versus 23 days in our study. Soetaert et al. (2007b) indeed demonstrated that
285 exposure duration can have an impact on the gene transcription profile. We also observed that eight
286 genes related to metabolism were upregulated in the Zn exposed F_0 daphnids, amongst which a
287 chitinase associated gene. Poynton et al. (2007), however, noted a downregulation of these genes in

their 24h experiments with Zn. Our results show that a gene coding for Cathepsin L, a proteolytic enzyme which is involved in the breakdown of MTs in lysosomes (Klaassen *et al.*, 1993), was downregulated. Its downregulation and thus the reduction of MT breakdown can be interpreted as a protective measure against Zn toxicity. Two transport related genes were induced, amongst which a vesicle associated membrane protein, which may be involved in the excretion of Zn (Pan and Wang, 2008). A gene with homology to a vitellogenin-like protein and a gene with homology to vitellogenin 2 (both of the copepod *Lepeophtheirus salmonis*; Blastx E-values of 6e-20 and 6e-6 respectively) were downregulated. Metal induced inhibition of vitellogenesis has been proposed as a toxicity mechanism (Hook and Fisher, 2002). However, in our study we noted that genes with homology to *D. magna* vitellogenin or vitellogenin fused with superoxide dismutase (Blastx E-values of 1e-148, 1e-98 and 1e-77) were upregulated. Vitellogenin was shown to have a protective effect against oxidative stress in honey bees (Seehuus *et al.*, 2006). As such, upregulation of these genes could be an additional oxidative stress response. Finally it is remarkable that none of the genes that were found to be differentially transcribed due to dietary Zn exposure by De Schamphelaere et al.(2008), were found to be affected in our study with waterborne Zn.

After filtering out the fragments that differed in transcription between controls of the three generations, 42 unigenes were found to be differentially transcribed between F_1Zn^- and F_1C. Of these 20 were upregulated in F_1Zn^-. Applying the same procedure to F_2, 56 differentially transcribed genes were found, 24 of which were upregulated in F_2Zn^- (Table 2).

Bossdorf et al. (2008) state that if environmental exposure causes a stable inheritable epigenetic effect, a stable change in gene transcription should be observed in the consecutive generations descending from the exposed organisms. In the present study, none of the putative 1207 unigenes on the array was consistently up- or downregulated in the three generations (Fig. 2, Table 3). Some, however, were regulated in the same direction in two consecutive generations. E.g. four genes were upregulated in both F_0Zn^+ and F_1Zn^-: three unidentified fragments and a mitochondrial rRNA methyltransferase 1 homolog. Ribosomal RNA methylation stabilizes the rRNA conformation

(Fromont-Racine *et al.*, 2003). As none of these genes was differentially transcribed in the F_2 Zn^- daphnids, it can be concluded that their upregulation in F_0Zn^+ and F_1Zn^- is not a stable inheritable epigenetic change. Two genes were differentially regulated in the same direction in F_1Zn^- and in F_2Zn^-: a gene with homology to a phage lysozyme was downregulated and a cuticular protein was upregulated. An epigenetic effect of the Zn exposure in F_0 is deemed to be an unlikely cause of these similarities in gene transcription for the following reasons. For the phage lysozyme it is possible that the spot on the array is derived from bacterial or phage cDNA that was retained during the creation of the library with the SSH method. This may occur if there is a difference between the associated bacterial/phage cultures present in the two populations used for the creation of the energy related library (Soetaert *et al.*, 2007a). A random difference between bacterial/phage cultures present in the F1 control and Zn^- treatments and which was passed onto the F_2 generation may be a possible explanation for the downregulation of this phage lysozyme gene in both generations. A large number of bacteria are indeed symbionts of *Daphnia* sp. (Qi *et al.*, 2009). The upregulation of the cuticle protein gene in F_1Zn^- and in F_2Zn^- is probably due to random differences in the molting phases, as mentioned in the discussion on the differences in gene transcription between the controls of different generations. Twenty-six of the 191 unigenes that showed differential transcription between the controls were homologous to cuticle proteins, indicating that this type of genes is subject to up- or downregulation independent of the Zn exposure.

Unlike in the F_0Zn^+ treatment, oxidative stress response genes were not differentially transcribed in F_1Zn^- and in F_2Zn^-. This can be directly linked to the absence of Zn-induced oxidative stress in these non-exposed treatments (Lynes *et al.*, 2007). There was less uniformity in the direction of regulation of differentially transcribed genes in F_1Zn^-compared to the F_1C control within the functional gene groups. A ribosomal protein gene was upregulated, while an elongation factor gene was downregulated in the group of translation related genes. Out of five regulated metabolism related genes, four were downregulated. This is in contrast with the patterns in the F_0 generation, where all transcription and translation related genes were downregulated and all but one metabolism related

genes were upregulated in F_0Zn^+ compared to F_0C. The transcription pattern in the F_1 generation may for some genes be due to random differences in the molting phases, as was the case for the differential transcription observed between control treatments. A large number of the changes in gene transcription observed in the F_1 daphnids are probably attributable to the Zn exposure in the parent generation. It has been shown that previous Zn exposure of adult daphnids affects the fitness of their offspring (Muyssen and Janssen, 2005). Since the differentially transcribed genes between F_1Zn^- and F_1C were not the same as those between F_0Zn^+ and F_0C, the microarray data from this study suggest that the response to the F_0 Zn exposure in the F_1 offspring is the result of other mechanisms than those that are directly induced by the Zn exposure in the F_0 adults.

In the F_2Zn^- treatment, it is striking that all regulated vitellogenin related genes are downregulated compared to the F_2C control. The genes with homology to *D. magna* vitellogenin fused with superoxide dismutase were analyzed in detail with blastn. This resulted in good homologies (E-values < 5e-20) with one or both of two closely related *D. magna* vitellogenin genes, vtg1 and vtg2 (Tokishita *et al.*, 2006). The gene for vtg1 and fragments with homology to both vtg1 and vtg2 (vtg 1/2) were upregulated in F_0Zn^+ (Tables 1,2). Following the assumption that vitellogenin was induced in F_0 as an oxidative stress response, it could be speculated that an "over-compensation" mechanism occurred in F_2Zn^-. A possibly more likely explanation are random differences in the phases of the reproductive cycle and the associated vitellogenesis between the sampled F_2C and F_2Zn^- daphnids. Stibor (2002) has demonstrated large differences in yolk protein levels at different times between the deposition of two consecutive broods into the brood pouch. These random differences in reproductive phases can also be the explanation for the upregulation of these vitellogenin genes in F_0Zn^+ vs F_0C.

It should be noted that only a limited, albeit relevant for ecotoxicological studies, set of genes could be studied with this custom cDNA microarray which was not specifically developed to detect Zn stress. Therefore not all mechanisms of Zn toxicity and recovery could be elucidated. Also, a possible

365 epigenetic transgenerational effect on the transcription of a gene that was not present on the array
366 cannot be excluded.

367 It can be concluded that the exposure of *Daphnia magna* to a sublethal Zn concentration for one
368 generation did not result in a transgenerational effect on the reproduction. An important
369 observation was the presence of a large number of genes that were differentially transcribed in
370 different subsequent generations of non-exposed control daphnids cultured in the same
371 experimental conditions. This is likely due to differences in the molting phases and reproductive
372 cycles of the daphnids in the different generations. It is clear that when adult daphnids are used for
373 microarray experiments such unintended gene transcription patterns should be accounted for, e.g.
374 by comparing the gene transcription of different control treatments. After elimination of this gene
375 transcription pattern, our microarray results demonstrate that Zn induces differences in gene
376 transcription in adult daphnids exposed to Zn. In the non-exposed F_1 and F_2 offspring, a considerable
377 number of differentially transcribed genes was also observed. However, none of the differentially
378 transcribed genes observed in the non-exposed F_1 and F_2 offspring were regulated in the same
379 direction in as in the exposed F_0Zn^+ daphnids. As such, it is concluded that no Zn-induced stably
380 inheritable epigenetic change occurred on the transcription of any gene in the custom microarray
381 used.

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Tables

Table 1 – Reproduction (mean ± standard deviation) as total number of living juveniles per surviving adult at day 21 in the different treatments. The last column indicates the p-values of the t-test (F_0 and F_2) or Mann-Whitney U test (F_1) comparing the mean reproduction of the Zn^+ or Zn^- treatments with the mean reproduction in the control of the same generation.

	Exposure history		
Generation	Ctrl	Zn^+ (F_0) or Zn^- (F_1 and F_2)	p-value
F_0	86 ± 9	62 ± 8	0.000264
F_1	99 ± 5	100 ± 20	0.527701
F_2	64 ± 12	55 ± 8	0.097310

Table 2 – Significantly up- or downregulated genes (compared to the control treatment of the same generation) in Zn exposed adult F_0 daphnids and non-exposed adult F_1 and F_2 progeny. Only those genes for which a sequence description could be obtained through Blastx are represented. For some genes, Blastn descriptions are added in italics.

accession	sequence description	up(↑) or down (↓) regulation			biol. process/molecular function
		F_0	F_1	F_2	
transcription and translation					
FD466400	ribosomal protein l31	↓	-	-	
DW724534	60s ribosomal protein l12	↓	-	-	
FD466739	elongation factor 1 alpha isoform 2	↓	-	-	
FD466813	elongation factor-1 alpha	↓	-	-	
DW985494	ribosomal protein s8	↓	-	-	
DW724510	prohibitin 2	↓	-	-	
DW985443	ribosomal protein s20	↓	↑	-	
FD466735	ribosomal protein l10	↓	-	-	
FD466373	ribosomal protein s16	↓	-	-	
DV075844	lsm1u6 small nuclear RNA associated	↓	-	-	
FD482895	elongation factor-1 gamma	-	↓	-	
FD466655	ribosomal protein s12	-	-	↓	
DV075845	ribosomal protein l3	-	-	↓	
oxidative stress response					
DY037379	peroxiredoxin 6	↑	-	-	
DW724644	glutathione S-transferase theta 1	↑	-	-	
metabolism					
FD466516	lactate dehydrogenase d	↑	↓	-	
FD467047	26s proteasome-associated pad1 homolog	↑	-	-	
DW724638	cathepsin l	↓	-	↓	
FD466539	ser thr protein phosphatase	↑	↓	-	
DW985622	mitochondrial rRNA methyltransferase 1 homolog	↑	↑	-	
FD467186	brain chitinase and chia	↑	-	-	
EG565372	sphingomyelin phosphodiesterase 2, neutral membrane	-	↓	-	
FD466708	isoform b	-	↓	-	
FD466661	glucose-6-phosphate 1-dehydrogenase	-	-	↑	
DW724637	hiu hydrolase	-	-	↑	
DW985460	angiotensin converting enzyme	-	-	↑	
transport					
FD466420	vesicle-associated membrane protein 7	↑	-	-	
DW985497	organic anion transporter	↑	-	↑	
FD466488	carnitine o-acyltransferase	-	-	↑	
FD466588	solute carrier family 25 (carnitine acylcarnitine translocase) member 20	-	-	↑	
DW985584	lipophorin precursor	-	-	↓	
embryonic development					
DW724602	vitellogenin structural genes family member (vit-2)	↓	-	↓	
protein folding					
FD466402	chaperonin containingsubunit 6a (zeta 1)	↑	-	-	
various					
DW724563	long wavelength-sensitive opsin	↑	-	↑	*stimulus response*
FD466427	Na+ K+ atpase alpha subunit	↑	-		*stimulus response*
FD482885	subfamilymember 3	-	-	↑	*stimulus response*
FD466284	epithelial membrane protein	↑	-	↑	*defense response*
DW724673	aurora ipl1-related kinase 3	↓	↑	↓	*cytokinesis*
DW724676	2-domain hemoglobin protein subunit	↓	-	↓	*oxygen transport*
DV075792	cuticle protein 5a	↑	-	-	*cuticle constituent*
DY037411	endocuticle structural glycoprotein SgAbd-4	↑	-	-	*cuticle constituent*
DW724634	cuticular protein	-	↓	↓	*cuticle constituent*
DW985439	cuticle protein	-	-	↓	*cuticle constituent*
FD466847	histone 1	↓	-	-	*nucleosome assembly*
FD466943	dynactin 1	↓	-	-	*microtubule-based process*

EH669336	protein disulfide isomerase	↓	-	-	*isomerase activity*
DW724675	fc fragment of binding protein	↓	-	↓	*cell adhesion*
DW724515	signal sequence beta	↓	-	-	*receptor activity*
EH669287	rab6 interacting protein 1	-	↓	-	*kinase activity*
DW985478	novel protein (zgc: 92136)	-	↑	-	*kinase activity*
EG565397	endou protein	-	↑	-	*kinase activity*
DW724470	glucosamine-phosphate n-acetyltransferase 1	↑	↓	-	*transferase activity*
EH669340	LDLa domain containing chiting binding protein 1, isoform A	-	↓	-	*chitin deacetylase activity*
DW724465	myosin light chain 2	-	↓	-	*ATPase activity*
DW724478	rhodopsin 4	-	↓	-	*phototransduction*
FD466492	phage related lysozyme	-	↑	↑	*catalytic activity*
FD467184	6-phosphofructo-2-kinase fructose--bisphosphatase short form	-	-	↓	*catalytic activity*
DV075812	accessory gland protein	-	-	↓	*mitotic spindle organization and biogenesis*
EH669353	sec63	-	-	↓	*protein binding*
DW724698	rna terminal phosphate cyclase domain 1	↓	-	↓	*RNA processing*
DY037250	vitellogenin fused with superoxide dismutase *(vtg1/2)*	↑	-	↓	*lipid transport*
DY037295	vitellogenin fused with superoxide dismutase *(vtg1/2)*	↑	-	↓	*lipid transport*
DY037287	vitellogenin fused with superoxide dismutase *(vtg 2)*	-	-	↓	*lipid transport*
DY037244	vitellogenin fused with superoxide dismutase *(vtg 1/2)*	-	-	↓	*lipid transport*
DY037239	vitellogenin fused with superoxide dismutase *(vtg 1)*	-	-	↓	*lipid transport*
DY037265	vitellogenin fused with superoxide dismutase *(vtg 1)*	-	-	↓	*lipid transport*
DW724641	vitellogenin *(vtg 2)*	-	-	↓	*lipid transport*
DW724656	vitellogenin *(vtg 1)*	↑	-	↓	*lipid transport*
FD466517	cg11160-isoform a	↑	-	-	
FD466968	elongation factor 2	↑	-	-	
DV075804	stromal cell derived factor 2-like protein	↑	-	-	
FD467111	f-box and leucine-rich repeat protein 2	↑	-	-	
DY037413	cleavage and polyadenylation specificity factor	↑	-	-	
FD466422	bromodomain and wd repeat domain containing 2	-	↑	-	
EH669340	chitin deacetylase 1	-	↓	-	
DW724564	male sterility domain-containing protein, putative	-	-	↑	
FD466998	UPF0183 protein CG7083	-	-	↑	
DW724527	vitellogenin 2	↓	-	↓	
FD467147	apolipoprotein d	-	-	↓	
DW985509	isoform b	-	-	↑	
EH669235	obstractor d	-	-	↑	
FD482904	cg15828-isoform b	-	-	↓	
DV075799	briggsae cbr-grsp-2 protein	-	-	↓	

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Table 3 – Genes with significant differences in transcription in more than one generation between the Zn^+ or Zn^- treatment and the control of the same generation. Log FC = logarithm of the fold change in transcription in the Zn^+ or Zn^- treatment, compared to the control of the same generation. "-" = no significant difference in transcription

accession nr.	sequence description	log FC F_0	log FC F_1	log FC F_2
FD466798	---no homology---	1.02	0.79	-
FD466892	---no homology---	0.94	0.84	-
FD466895	---no homology---	1.00	1.18	-
DW985622	mitochondrial rRNA methyltransferase 1 homolog	1.21	0.97	-
DV075828	---no homology---	0.82	-1.82	-
FD466516	lactate dehydrogenase d	1.08	-0.81	-
DW724470	glucosamine-phosphate n-acetyltransferase 1	0.76	-0.77	-
DW724619	---no homology---	-1.06	0.87	-1.46
DW724673	aurora ipl1-related kinase 3	-1.07	0.83	-1.14
DW985443	ribosomal protein s20	-0.94	0.79	-
DW985520	---no homology---	1.11	-1.27	1.21
FD466539	ser thr protein phosphatase	1.17	-1.06	-
FD466492	phage related lysozyme	-	0.81	1.13
DW724634	cuticular protein	-	-1.33	-0.90
FD467057	---no homology---	1.34	-	0.84
DW724454	peptidoglycan-binding domain 1 protein	0.83	-	0.94
DW724675	fc fragment ofbinding protein	-1.40	-	-1.44
DW724638	cathepsin l	-1.51	-	-1.70
DW724676	2-domain hemoglobin protein subunit	-1.19	-	-0.83
DW724563	long wavelength-sensitive opsin	0.94	-	1.04
FD466284	epithelial membrane protein	0.90	-	0.86
DW724602	vitellogenin structural genes family member (vit-2)	-1.41	-	-1.79
DW724527	vitellogenin 2	-0.99	-	-1.26
DW724698	rna terminal phosphate cyclase domain 1	-1.41	-	-1.39
DW985497	organic anion transporting polypeptide 26f	0.86	-	0.79
EG565381	---no homology---	1.15	-	0.92
DY037250	vitellogenin fused with superoxide dismutase *(vtg 1/2)*	0.78	-	-1.20
DY037295	vitellogenin fused with superoxide dismutase *(vtg 1/2)*	0.75	-	-1.23
DW724656	vitellogenin *(vtg 1)*	0.84	-	-1.29
DW724573	---no homology---	-0.76	-	1.33

Figure captions

Fig. 1 - Overview of the experimental design. F_0, F_1, F_2: generations. C = control medium. Zn = organisms cultured in medium with average Zn concentration of 388 µg/L. Codes represent daphnids in specific 'treatments' (combinations of generation + exposure history).

Fig. 2 – Number of differentially transcribed genes in the different generations, compared to the control of the same generation.