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5 This is a post-print of a paper published in Environmental Pollution (Elsevier, Oxford, England). The
6 contents are identical to those in the published version.

7
8 Full bibliographic citation (please cite as follows):

9 Vandegehuchte, M.B., De Coninck, D., Vandenbrouck, T., De Coen, W.M., Janssen,
10 C.R., 2010. Gene transcription profiles, global DNA methylation and potential
11 transgenerational epigenetic effects related to Zn exposure history in *Daphnia*
12 *magna*. Environmental Pollution 158, 3323-3329.

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

14 <http://dx.doi.org/10.1016/j.envpol.2010.07.023>
15

**Gene transcription profiles, global DNA methylation and potential
transgenerational epigenetic effects related to Zn exposure history in
*Daphnia magna***

Michiel B. Vandegehuchte^{1*}, Dieter De Coninck¹, Tine Vandenbrouck², Wim M. De Coen²,
Colin R. Janssen¹

¹Laboratory of Environmental Toxicology and Aquatic Ecology, Ghent University, J.
Plateaustraat 22, B-9000 Ghent, Belgium

²Laboratory for Ecophysiology, Biochemistry and Toxicology, University of Antwerp,
Groenenborgerlaan 171, B-2020 Antwerp, Belgium

*Corresponding author:

Michiel Vandegehuchte
Laboratory of Environmental Toxicology and Aquatic Ecology
J. Plateaustraat 22
B-9000 Ghent
Belgium

Michiel.Vandegehuchte@UGent.be

Tel +32 9 264 37 07

Fax +32 9 264 37 66

Abstract:

A reduced level of DNA methylation has recently been described in both Zn-exposed and non-exposed offspring of *Daphnia magna* exposed to Zn. The hypothesis examined in this study is that DNA hypomethylation has an effect on gene transcription. A second hypothesis is that accumulative epigenetic effects can affect gene transcription in non-exposed offspring from parents with an exposure history of more than one generation. Transcriptional gene regulation was studied with a cDNA microarray. In the exposed and non-exposed hypomethylated daphnids, a large proportion of common genes were similarly up- or downregulated, indicating a possible effect of the DNA hypomethylation. Two of these genes can be mechanistically involved in DNA methylation reduction. The similar transcriptional regulation of two and three genes in the F₀ and F₁ exposed daphnids on one hand and their non-exposed offspring on the other hand, could be the result of a one-generation temporary transgenerational epigenetic effect, which was not accumulative.

Capsule:

Zn induced DNA hypomethylation is related to gene transcription in *Daphnia magna* and Zn exposure potentially induced limited temporary transgenerational effects on gene transcription.

Keywords

Ecotoxicology, hypomethylation, microarray, multigeneration, zinc

Introduction

Environmental genomics is an emerging toxicological research field, in which the genomic signatures of environmental toxicants are studied and in which gene expression profiles are used to investigate gene-environment interactions and the exposure-effect relationship (Jayapal et al., 2010). The integration of this aspect of human toxicogenomics into the ecotoxicological research field termed ecotoxicogenomics has been defined by Snape et al. (2004) as ‘the study of gene and protein expression in non-target organisms that is important in responses to environmental toxicant exposures’. With genomic techniques, the mode of action of different chemicals in ecotoxicologically relevant organisms can be elucidated. Omics technologies can also contribute to the risk assessment process in aquatic toxicology, as part of a weight-of-evidence approach (Van Aggelen et al., 2010). One genomic tool that has been widely used by ecotoxicologists is the DNA microarray. This allows to evaluate the transcriptional response to toxicants of a large number of genes with a single RNA sample of exposed and control organisms (Poynton and Vulpe, 2009). In the past few years, microarrays have been developed for *Daphnia magna*, a key species in aquatic toxicology and ecology. These tools have subsequently been used to evaluate the effects of exposure to different types of chemicals on gene transcription in *D. magna* (Soetaert et al., 2007; Garcia-Reyero et al., 2009; Vandenbrouck et al., 2009). Transcriptional induction or repression of genes as a result of toxicant exposure can be mediated through several molecular pathways. Modulation of transcription can also occur through epigenetic mechanisms (Altucci and Stunnenberg, 2009). These are chromatin alterations in which the base sequence is not affected and include DNA methylation, histone modifications and interference of non coding RNA molecules with the transcriptional system (Feil, 2008). It has been shown that epigenetic modifications can be induced by

environmental factors, such as exposure to toxicants and that epigenetic changes can be transgenerationally inherited, even when the triggering factor is removed (Suter and Aagaard-Tillery, 2009). When the inducing factor persists for several generations, the effects on epigenetic marks may accumulate and lead to a more extreme phenotype which can be transferred to non-induced subsequent generations (Jablonka and Raz, 2009). As such these observations can be of importance in the context of environmental toxicology and risk assessment as the epigenetic effects of temporary exposures could affect the future status of ecosystem structure and functioning.

A custom cDNA microarray was recently used to study transcriptional patterns of *D. magna* exposed to Zn for one generation and cultured under non-exposed conditions for two subsequent generations (Vandeghechuchte et al., 2010b). The transcription of several genes was modulated, both in the exposed daphnids and in the two subsequent non-exposed generations. However, none of the genes was regulated in the same direction (up or down) in the three generations, indicating the absence of a stably inheritable epigenetic effect on any of the 1207 unique identified DNA fragments in the array. Interestingly, in the F₀ Zn exposed daphnids, the differentially transcribed genes (compared to the F₀ control organisms) were different from those in their non-exposed F₁ and F₂ offspring (compared to F₁ and F₂ control daphnids). This suggested that direct Zn exposure and parental Zn exposure affect different molecular mechanisms. In parallel with the two generations (F₁ and F₂) of non-exposed offspring, two generations of offspring from the F₀ Zn exposed daphnids were cultured under continuous Zn exposure (Vandeghechuchte et al., 2010c). Additionally, global DNA-methylation was quantified in the F₀ Zn exposed daphnids and in both the Zn exposed and the non-exposed F₁ and F₂ offspring generations. A significant reduction in DNA

109 methylation was only observed in the F₁ generation and this for the Zn exposed as well as for
110 the non-exposed daphnids (Vandeghechuchte et al., 2009).

111 In the present study, gene transcription in the daphnids from the above described
112 treatments, supplemented with non-exposed offspring of daphnids with two generations of
113 Zn exposure history, was studied with the following aims. First, the relation between global
114 DNA methylation reduction and gene transcription in the first generation of offspring was
115 examined. Second, the hypothesis was tested that accumulative epigenetic effects can affect
116 gene transcription in non-exposed offspring from parents with two-generation exposure
117 history.

118

Materials and methods

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).

The culture of the daphnids is described by Vandegehuchte et al. (2010b). 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 for three generations (F_0C – F_2C). A second batch of neonates was transferred into the same medium, with the Zn concentration adjusted to 388 µg/L. A group of F_1 neonates born from this F_0Zn^+ generation was then cultured in this Zn contaminated medium (F_1Zn^+). Another group of these F_1 neonates was transferred back to 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 ($F_2Zn^{-/-}$, which were named F_2Zn^- in Vandegehuchte et al. (2009)). Offspring of the F_1Zn^+ daphnids were in turn divided in a similar manner into two batches, one of which was cultured further in Zn contaminated medium ($F_2Zn^{+/+}$) while the other one was transferred to the control medium ($F_2Zn^{+/-}$) (Fig. 1). Each combination of generation and exposure history is termed a ‘treatment’ throughout this paper. 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 Zn

concentration in the control test 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).

Internal Zn concentrations were determined as described before (Vandeghechuchte et al., 2010b). Body length from the top of the head until the base of the spine, was measured for ten daphnids 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. All reported Zn concentrations were measured by atomic absorption spectrometry (SpectrAA-100, Varian, Mulgrave, Australia).

Statistical analysis

All statistics were performed with Statistica (Statistica, Tulsa, USA). For the F₀ generation, differences between the Zn exposed and the control daphnids in length or internal Zn concentration were assessed using t-tests. For the F₁ and F₂ generations, differences between treatments and control were assessed by a one-way ANOVA, followed by Dunnett's test. 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 treatments and the control (USEPA, 2000). In all tests, the limit of significance was set at p = 0.05.

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; 2007) using the suppression subtractive hybridization technique. Next to these cDNA libraries, four extra

cDNA fragments, corresponding to expressed sequence tags (ESTs) from genes that were expected to be responsive to Zn were spotted on the array. Isolation of these sequences and spotting of the array are reported by Vandegehuchte et al. (2010b).

Microarray preparation

Three replicates of ten adult daphnids each per treatment ('treatment' = combination of generation and exposure type, see Fig. 1) were sampled for mRNA analysis on the day the next generation was started (see above). The methods for RNA-extraction, conversion into cDNA, labeling and hybridization can be found in Vandegehuchte et al. (2010b). Briefly, RNA was isolated using the Trizol extraction method following the manufacturer's protocol (Invitrogen, Belgium). Probes were prepared by converting 5 µg total RNA from each replicate into aminoallyl-dUTP (Sigma, Belgium) labeled cDNA using the Superscript II Reverse transcriptase kit (Invitrogen, Belgium). Treatment and reference pool aminoallyl cDNA were covalently coupled with Cy5- or Cy3-esters. Each of the three replicates of the treatment daphnids was hybridized on a separate array against a labeled reference pool sample, following a universal reference design.

Bioinformatic analysis of microarray data

Microarray data analysis was performed as described in Vandegehuchte et al. (2010b). Briefly, the microarrays were scanned using a Genepix personal 4100 Scanner (Axon instruments, USA), upon which scanned images were analyzed using Genepix Pro Software 4.0 (Axon Instruments). Spots with saturated intensities were filtered out by visual inspection. Subsequently, data were evaluated with the MIAME platform based Bioarray Software Environment (BASE 1.2.17, <http://www.islab.ua.ac.be/base/>), using local background subtraction, variance stabilization normalization (Huber et al., 2002) and Limma

189 (linear models for microarray data) (Smyth, 2004; Smyth et al., 2005). Data can be accessed
190 at GEO (Gene Expression Omnibus) with access number GSE 22805 (temporary number, to
191 be changed to final number in proofs). Fragments with an adjusted p-value lower than 0.05
192 and a $\log_2$ ratio was outside the interval $[-0.75, 0.75]$, were retained as significantly up- or
193 downregulated for further analysis. Sequence descriptions and annotations were obtained
194 through Blast2GO (Conesa et al., 2005)(www.blast2go.de), which allowed genes to be
195 classified into functional groups (Fig. 2).

Results

Body lengths of 6-day old daphnids of the F_1Zn^- and $F_2Zn^{+/-}$ treatments were significantly lower than that of 6-day old daphnids in the respective F_1C and F_2C controls (Fig. 3). This indicates a significant effect on growth in the juvenile life stages of daphnids in these two treatments.

The average internal Zn concentration of $68 \mu g Zn/g$ dry weight in the non-exposed $F_2Zn^{+/-}$ daphnids did not differ significantly from that of the F_2C organisms, which was $51 \mu g Zn/g$ dry weight.

Redundant fragments on the array were grouped into contigs, resulting in 1207 unique identified fragments on the array, which are termed unigenes. When the gene transcription patterns of control treatments were compared (i.e. F_0C vs F_1C , F_1C vs F_2C or F_0C vs F_2C), a large number of genes were found to be differentially transcribed, as reported by Vandegehuchte et al. (2010b) (Table S1). This concerned more than 15% of the unigenes on the array. The differential transcription of these genes is likely due to differences in the molting phases and reproductive cycles of the daphnids in the different generations and is as such not specific to the Zn exposure. Therefore, those genes that significantly varied in transcription between different control generations, were removed from the microarray analysis result list of differentially transcribed genes between Zn treated organisms and controls. Thus, 38 to 46 % of the differentially transcribed unigenes between treatments and controls were retained for further analysis. In the following section of the manuscript, differential transcription will always be related to the control of the same generation. Differentially transcribed genes with a sequence description are listed in Fig. 2. Genes for which no homology was found are summarized in Table S2 in the online supplementary material.

Discussion

Gene transcription results and internal Zn concentrations of the F_0Zn^+ , F_1Zn^- and $F_2Zn^{-/-}$ treatments were reported in Vandegehuchte et al. (2010b). Results of F_1Zn^+ and F_2Zn^+ are presented in a paper discussing the effects of multigenerational Zn exposure and Zn acclimation (Vandegehuchte et al., 2010c). Some of these results are recapitulated in this paper to answer our research questions (Fig 2, Table 1). Considering the length measurements, it can be noted that a significant effect on growth (versus their respective controls) was present only in 6-day old daphnids of the F_0Zn^+ , F_1Zn^- and $F_2Zn^{+/-}$ treatments (Fig. 3, Table 1).

Numbers of differentially transcribed (up- and downregulated) unigenes in all treatments can be found in Table 1.

Like in the F_0 generation, a Zn-induced reduction in juvenile growth (compared to the respective control) was also observed in both the exposed and the non-exposed F_1 offspring (Figs. 1B, 3). However, no growth reduction was noted in the F_2 generation of the continuously Zn exposed lineage (compared to the F_2 control). The non-exposed $F_2Zn^{+/-}$ offspring of the Zn exposed F_1Zn^+ organisms, however, did exhibit a reduction in juvenile growth, while the non-exposed $F_2Zn^{-/-}$ descendants of the F_1Zn^- daphnids did not. The growth reduction observed in the F_1Zn^- organisms can be due to a maternal or possibly an epigenetic effect, which was not transferred to the $F_2Zn^{-/-}$ offspring. A similar transfer of effect may have occurred from F_1Zn^+ to $F_2Zn^{+/-}$. In both F_1 treatments, a reduction in overall DNA methylation has been reported by Vandegehuchte et al. (2009). This global hypomethylation was not observed in $F_2Zn^{+/+}$ or $F_2Zn^{-/-}$ daphnids. In a recent study on the effects of exposure to five chemicals on DNA methylation in *D. magna*, Vandegehuchte et al (2010a) observed a decreased overall DNA methylation which also coincided with a reduced size at day 7 in 5-

244 azacytidine exposed daphnids. Unfortunately, due to technical failure, no DNA methylation
245 data of the $F_2Zn^{+/-}$ daphnids are available to further evaluate the possible relationship
246 between DNA hypomethylation and growth reduction in *D. magna*.

247 Since the internal Zn concentration in the non-exposed $F_2Zn^{+/-}$ daphnids did not differ
248 significantly from that of the F_2C organisms, any possible Zn induced effect on gene
249 transcription in $F_2Zn^{+/-}$ cannot be attributed to a direct exposure to maternally transferred
250 excess internal Zn. The same observation was made for the F_1Zn^- and $F_2Zn^{-/-}$ treatments. The
251 internal concentration of 68 μg Zn/g dry weight in the $F_2Zn^{+/-}$ organisms is in line with the
252 internal Zn concentrations of the other non-exposed daphnids observed by Vandeghechuchte
253 et al. (2010b) (Fig. 1B).

254 The number of 73 unigenes which were differentially transcribed in the F_1Zn^+ daphnids, is
255 comparable to the 71 transcriptionally regulated unigenes in the F_0Zn^+ treatment, and higher
256 than the 42 differentially transcribed unigenes in the F_1Zn^- daphnids. However, only seven
257 genes were regulated in the same direction in F_0Zn^+ and F_1Zn^+ . On the other hand, in F_1Zn^+
258 and F_1Zn^- , which both exhibited reduced DNA methylation, twenty genes were regulated in
259 the same direction, of which twelve were upregulated (Fig. 2, Table S2). This amounts to
260 approximately 47% of the differentially transcribed genes in F_1Zn^- . Except for the gene with
261 homology to a phage related lysozyme, none of these genes were differentially transcribed
262 in the same direction in the next generation, in which no DNA hypomethylation was
263 observed. This suggests that the differential transcription of the other 19 genes may be
264 related to the reduction in DNA methylation in both F_1 treatments. Interestingly, the gene
265 with homology to sel-1 suppressor of lin-12-like was upregulated. This protein is known as a
266 repressor of the Notch signaling pathway (Rooman et al., 2006). Activation of the Notch

267 pathway was shown to be crucial for hypermethylation of a gene in *Drosophila* (Ferres-
268 Marco et al., 2006). A repression of the Notch pathway could be involved in the
269 hypomethylation in the F_1Zn^+ and F_1Zn^- daphnids in this study. Another interesting
270 observation is the transcriptional downregulation of a gene with homology to a Ser/Thr
271 protein phosphatase in the F_1Zn^+ as well as in the F_1Zn^- daphnids. The Ser/Thr protein
272 phosphatase PP1 is shown to form a complex with Nuclear Inhibitor of Protein Phosphatase
273 – 1, in combination with the Polycomb Repressive Complex (Roy et al., 2007). This complex is
274 involved in gene silencing, at least in part through trimethylating histone 3 Lys 27. It also
275 interacts with DNA methyltransferases and it is necessary for the methylation of target DNA
276 (Vire et al., 2006; Nuytten et al., 2008). Through these interactions, the downregulation of
277 the gene coding for Ser/Thr protein phosphatase in the F_1Zn^+ and F_1Zn^- daphnids could be a
278 reason for their reduced global DNA methylation. These data suggest an effect of parental Zn
279 exposure on the gene transcription in exposed and non-exposed offspring, possibly
280 mediated through changes in DNA-methylation. In F_1Zn^+ , a larger number of genes were
281 affected because of the combination of parental and direct Zn exposure.

282 An interesting differentially regulated gene in F_1Zn^+ is S-adenosylhomocysteine hydrolase
283 (SAHH). The transcriptional upregulation of this gene can be a mechanism to increase the
284 level of free homocysteine, which may be reduced due to conjugation with Zn-induced
285 metallothioneins (Barbato et al., 2007; Fan et al., 2009). Free homocysteine is a substrate for
286 the formation of methionine, which can be converted to S-adenosylmethionine (SAM), an
287 important methyl donor for DNA methyltransferases (Mason, 2003; Muskiet, 2005). Low
288 homocysteine levels were suggested to be an indirect cause for the observed DNA
289 hypomethylation in the F_1Zn^+ daphnids by Vandegehuchte et al. (2009). If this was indeed
290 the case, this was not restored by this transcriptional upregulation of SAHH. This is

291 consistent with results of Hermes et al (2008) in which overexpression of SAHH in human
292 embryonic kidney cells did not cause differences in global DNA methylation. The
293 upregulation of a gene coding for the heat shock protein Hsp90 in the F_1Zn^+ daphnids can be
294 a stress response leading to elevated levels of Hsp90, as observed in earthworms exposed to
295 Zn and Pb contaminated soils (Marino et al., 1999). An interesting aspect of Hsp90 is that it
296 has recently been proposed to be involved in chromatin remodeling and transgenerational
297 epigenetic inheritance (Ruden and Lu, 2008). Through this pathway, Zn exposure could affect
298 the gene transcription of non-exposed offspring in the $F_2Zn^{+/-}$ treatment.

299 The number of 40 differentially transcribed unigenes in $F_2Zn^{+/-}$ is comparable to the 43
300 differentially transcribed unigenes in F_1Zn^- and higher than the 23 transcriptionally regulated
301 genes in $F_2Zn^{+/+}$ daphnids, which seemed to be acclimated to the Zn exposure
302 (Vandeghechuchte et al., 2010c). An accumulative epigenetic effect in $F_2Zn^{+/-}$ caused by the
303 continuous Zn exposure in F_0Zn^+ and F_1Zn^+ , as proposed by Jablonka & Raz (2009), was not
304 observed in this study. Similarly as in F_1Zn^- , only a minor fraction of the up- or
305 downregulated unigenes in the $F_2Zn^{+/-}$ daphnids was differentially transcribed in the same
306 direction in Zn exposed daphnids from the previous generation. Two genes with no
307 homology and one gene with homology to a hemoglobin chain were up- and downregulated,
308 respectively, in both F_1Zn^+ and $F_2Zn^{+/-}$ (Fig. 2, Table S2). As such, these results are in
309 agreement with Vandeghechuchte et al.(2010b), who observed that in non-exposed progeny of
310 Zn exposed daphnids the affected unigenes were to a large extent different from or
311 differently regulated than the affected unigenes in the exposed organisms.

312 The genes with similar up- or downregulation in F_1Zn^+ and $F_2Zn^{+/-}$ were not the same as
313 those regulated in the same direction in F_0Zn^+ and F_1Zn^- (Fig. 2, Table S2). Two of the latter

genes, a gene with no homology and a gene with homology to a mitochondrial rRNA methyltransferase, were also regulated in the same direction in F_1Zn^+ . This suggests that the differential transcription of these genes in both F_1 treatments could be due to a one-generation temporary transgenerational epigenetic effect, induced by the Zn exposure in F_0Zn^+ . The same holds for a gene coding for a 2-domain hemoglobin protein subunit and two other unigenes with no homology, which are differentially transcribed in the same direction in the F_1Zn^+ daphnids and in their $F_2Zn^{+/-}$ and $F_2Zn^{+/+}$ offspring.

In the $F_2Zn^{+/-}$ daphnids, only two genes, or 5% of all differentially transcribed genes, were regulated in a different direction than in the previous F_1Zn^+ generation. This is different from the F_1Zn^- daphnids, in which 20% of all differentially transcribed genes were regulated in the opposite direction compared to their parental F_0Zn^+ generation. The continuous Zn exposure elicited other transcriptional effects in F_1Zn^+ compared to F_0Zn^+ , which may have resulted in different transcriptional reactions in the non-exposed offspring. It is remarkable that approximately one third of all regulated genes in $F_2Zn^{+/-}$ are regulated in the same direction as in F_0Zn^+ (Fig. 2, Table S2). An epigenetic effect of Zn exposure on the transcription of these genes could be transferred from F_0 to F_2 , without being exerted in F_1 . This would be in accordance with Bygren et al. (2001), who reported an effect of food availability in grandparents (F_0) on longevity in their grandsons (F_2), but not in their children (F_1). This transgenerational effect was suggested to be related with genomic imprinting and thus with DNA-methylation. Also in $F_2Zn^{+/+}$ and in $F_2Zn^{-/-}$, a considerable proportion of unigenes were regulated in the same direction as in F_0Zn^+ : more than one fifth and one fourth of all differentially transcribed genes, respectively (Vandeghechuchte et al., 2010b; 2010c). However, an epigenetic mechanism by which this transfer of changed gene transcription could occur from F_0 to F_2 , skipping F_1 , has not been described.

338 In conclusion, a one-generation temporary transgenerational epigenetic effect could be the
339 reason for the similar differential transcription of two and three genes respectively in F_0Zn^+
340 and F_1Zn^+ on one hand and their non-exposed offspring on the other hand. An accumulative
341 epigenetic effect after two generations of Zn exposure was not observed. On the contrary,
342 unigenes with a similar differential transcription in F_0Zn^+ daphnids and their offspring were
343 different from the unigenes with a similar differential transcription in F_1Zn^+ and their
344 offspring. A large number of common genes, differentially transcribed in the same direction
345 in the F_1Zn^+ and F_1Zn^- daphnids suggests a possible effect of reduced DNA methylation on
346 gene transcription in these treatments. Interestingly, several genes which can play a role in
347 the reduction of DNA methylation were differentially transcribed in those treatments.
348 Currently, the *D. magna* genome is being sequenced by the *Daphnia* Genomics Consortium,
349 coordinated at Indiana University. When this genome sequence becomes available,
350 sequence specific methylation patterns will be detectable by means of bisulfite sequencing,
351 which was performed on genes of the recently sequenced pea aphid *Acyrtosiphon pisum*
352 (Walsh *et al.*, 2010). Future research can unveil the specific methylation pattern of
353 (promoters of) genes differentially transcribed in daphnids that showed hypomethylation
354 after one generation of Zn exposure. Determining the susceptibility of genes for methylation
355 changes will help in selecting candidate genes to study longer lasting altered methylation, as
356 detected on a genomic level after exposure to 5-azacytidine (Vandeghehuchte *et al.*, 2010a).
357 Such alterations in gene expression and DNA methylation in several generations of non-
358 exposed offspring of daphnids exposed to chemicals, if coinciding with ecologically relevant
359 effects, may have consequences for the environmental risk assessment of chemicals.

Acknowledgement

The authors thank Emmy Pequeur, Leen Van Imp and Gisèle Bockstael 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). The authors thank the anonymous reviewers for their constructive comments which improved the manuscript.

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Tables

Table 1 – Overview of the results in the different treatments. Differences in body length, numbers of up- and downregulated genes and total nr of differentially transcribed genes compared to the control of the same generation. Some data from F_0Zn^+ , F_1Zn^- , $F_2Zn^{-/-}$, F_1Zn^+ and $F_2Zn^{+/+}$ were previously reported by Vandegehuchte et al. (2010b; 2010c). ↓: body length lower than the control; ↑: Internal Zn concentration higher than the control; ns: no significant difference with the control

Treatment	Body length			Internal Zn concentration	Upregulated genes	Downregulated genes	Differentially transcribed genes
	d6	d13	d21	d21	d21	d21	d21
F_0Zn^+	↓	ns	ns	↑	44	27	71
F_1Zn^+	↓	ns	ns	↑	43	30	73
F_1Zn^-	↓	ns	ns	ns	20	22	42
$F_2Zn^{+/+}$	ns	ns	ns	↑	11	12	23
$F_2Zn^{+/-}$	↓	ns	ns	ns	26	14	40
$F_2Zn^{-/-}$	ns	ns	ns	ns	24	32	56

Figure captions

Figure 1 – (A) Overview of the experimental design. F_0 , F_1 , F_2 : generations. White = control medium. Grey = medium with average Zn concentration of 388 $\mu\text{g/L}$. Codes represent daphnids in specific ‘treatments’ (combinations of generation + exposure history). (B) Overview of the length (mm) of daphnids at different ages in different treatments, indicated by a color scale. The positions of the treatments are identical to those in (A). Treatments in which hypomethylation was observed are indicated with an extra dashed rectangle (Vandeghehuchte *et al.*, 2009). The asterisk (*) denotes that no methylation data are available for $F_2\text{Zn}^{-/-}$. Internal Zn body concentrations ($\mu\text{g Zn/g dry body weight}$) are indicated below each treatment symbol in italics. **Bold font** indicates a significant difference with the control of the same generation.

Figure 2 - Heat plot of significantly up- or down-regulated genes (compared to the control treatment of the same generation) in non-exposed and Zn exposed adult F_1 offspring from F_0 Zn exposed daphnids, as well as in non-exposed adult F_2 offspring from Zn exposed F_1 daphnids. Data for $F_1\text{Zn}^-$ and $F_1\text{Zn}^+$ are also reported in Vandeghehuchte *et al.* (2010b; 2010c) and are added for comparison. Only those genes for which a sequence description could be obtained through Blastx are represented. For some genes, Blastn descriptions are added in italics. (a): This unigene was differentially transcribed in the same direction in $F_0\text{Zn}^+$ (b): This unigene was differentially transcribed in the same direction in $F_2\text{Zn}^{+/+}$

Figure 3 – Length of daphnids in different treatments. Error bars denote standard deviations. An asterisk denotes a significantly smaller value compared to the control of the same generation.