



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

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

ARTICLE INFO

Article history:

Received 8 February 2010

Received in revised form

13 July 2010

Accepted 18 July 2010

Keywords:

Ecotoxicology

Hypomethylation

Microarray

Multigeneration

Zinc

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 down-regulated, 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.

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1. 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; Vandembrouck 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 Agaard-Tillery, 2009). When the inducing factor persists for several

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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 (Vandegehuchte 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 F_0 Zn-exposed daphnids, the differentially transcribed genes (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). This suggested that direct Zn exposure and parental Zn exposure affect different molecular mechanisms. In parallel with the two generations (F_1 and F_2) of non-exposed offspring, two generations of offspring from the F_0 Zn-exposed daphnids were cultured under continuous Zn exposure (Vandegehuchte et al., in 2010c). Additionally, global DNA methylation was quantified in the F_0 Zn-exposed daphnids and in both the Zn-exposed and the non-exposed F_1 and F_2 offspring generations. A significant reduction in DNA methylation was only observed in the F_1 generation and this for the Zn-exposed as well as for the non-exposed daphnids (Vandegehuchte et al., 2009).

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

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).

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 (0–24 h) 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_0 C– F_2 C). 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_0 Zn⁺ generation was then cultured in this Zn contaminated medium (F_1 Zn⁺). Another group of these F_1 neonates was transferred back to the control medium (F_1 Zn⁻). In this way, F_1 Zn⁻ daphnids were only briefly exposed to Zn during the first hours of their life-cycle. Offspring of the F_1 Zn⁻ organisms were also cultured in the control medium (F_2 Zn^{-/-}), which were named F_2 Zn^{-/-} in Vandegehuchte et al., (2009). Offspring of the F_1 Zn⁺ daphnids were in turn divided in a similar manner into two batches, one of which was cultured further in Zn contaminated medium (F_2 Zn^{+/+}) while the other one was transferred to the control medium (F_2 Zn^{+/-}) (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

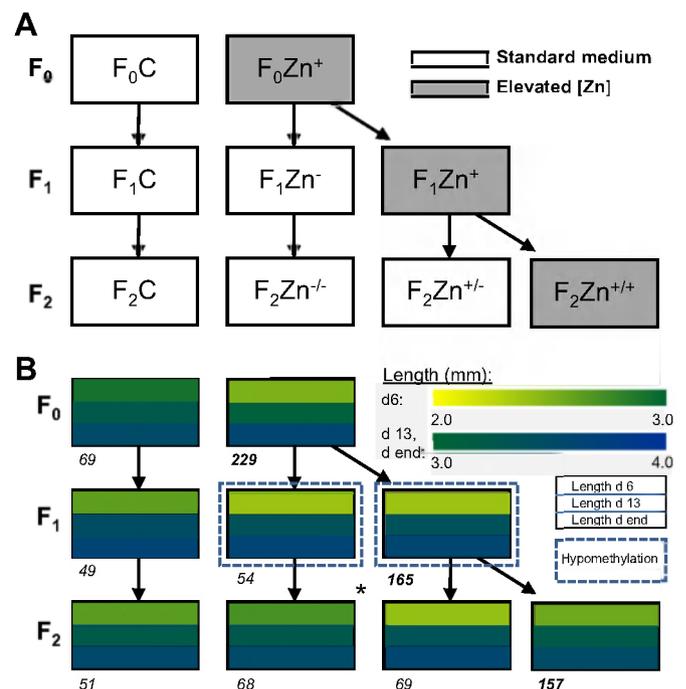


Fig. 1. (A) Overview of the experimental design. F_0 , F_1 , F_2 : generations. White = control medium. Grey = medium with average Zn concentration of 388 µg/L. Codes represent daphnids in specific 'treatments' (combinations of generation + exposure history). (B) Overview of the length (mm) of daphnids 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 (Vandegehuchte et al., 2009). The asterisk (*) denotes that no methylation data are available for F_2 Zn^{-/-}. Internal Zn body concentrations (µg 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.

optimal concentration range of this essential element for daphnids (Muyssen and Janssen, 2004).

Internal Zn concentrations were determined as described before (Vandegehuchte 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 1–3 days after the fifth brood was observed in the aquarium, when sufficient 0–24 h offspring were available to start the next generation. All reported Zn concentrations were measured by atomic absorption spectrometry (SpectraAA-100, Varian, Mulgrave, Australia).

2.2. Statistical analysis

All statistics were performed with Statistica (Statistica, Tulsa, USA). For the F_0 generation, differences between the Zn-exposed and the control daphnids in length or internal Zn concentration were assessed using *t*-tests. For the F_1 and F_2 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 $p = 0.05$.

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, 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).

2.4. 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.

2.5. 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 (linear models for microarray data) (Smyth, 2004; Smyth et al., 2005). Data can be accessed at GEO (Gene Expression Omnibus) with access number GSE 22805. Fragments with an adjusted *p*-value lower than 0.05 and a log₂ ratio was outside the interval [−0.75, 0.75], were retained as significantly up- or down-regulated for further analysis. Sequence descriptions and annotations were obtained through Blast2GO (Conesa et al., 2005) (www.blast2go.de), which allowed genes to be classified into functional groups (Fig. 2).

3. Results

Body lengths of 6-day old daphnids of the F₁Zn[−] and F₂Zn^{+/-} treatments were significantly lower than that of 6-day old daphnids in the respective F₁C and F₂C 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 µg Zn/g dry weight in the non-exposed F₂Zn^{+/-} daphnids did not differ significantly from that of the F₂C organisms, which was 51 µg 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. F₀C vs F₁C, F₁C vs F₂C or F₀C vs F₂C), 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, 38–46% 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.

4. Discussion

Gene transcription results and internal Zn concentrations of the F₀Zn⁺, F₁Zn[−] and F₂Zn^{+/-} treatments were reported in Vandegehuchte et al. (2010b). Results of F₁Zn⁺ and F₂Zn⁺ 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 F₀Zn⁺, F₁Zn[−] and F₂Zn^{+/-} treatments (Fig. 3, Table 1).

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

Like in the F₀ generation, a Zn-induced reduction in juvenile growth (compared to the respective control) was also observed in both the exposed and the non-exposed F₁ offspring (Figs. 1B, 3). However, no growth reduction was noted in the F₂ generation of the continuously Zn-exposed lineage (compared to the F₂ control). The non-exposed F₂Zn^{+/-} offspring of the Zn-exposed F₁Zn⁺ organisms, however, did exhibit a reduction in juvenile growth, while the non-exposed F₂Zn^{+/-} descendants of the F₁Zn[−] daphnids did not. The growth reduction observed in the F₁Zn[−] organisms can be due to a maternal or possibly an epigenetic effect, which was not transferred to the F₂Zn^{+/-} offspring. A similar transfer of effect may have occurred from F₁Zn⁺ to F₂Zn^{+/-}. In both F₁ treatments, a reduction in overall DNA methylation has been reported by Vandegehuchte et al. (2009). This global hypomethylation was not observed in F₂Zn^{+/+} or F₂Zn^{+/-} 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-azacytidine exposed daphnids. Unfortunately, due to technical failure, no DNA methylation data of the F₂Zn^{+/-} daphnids are available to further evaluate the possible relationship between DNA hypomethylation and growth reduction in *D. magna*.

Since the internal Zn concentration in the non-exposed F₂Zn^{+/-} daphnids did not differ significantly from that of the F₂C organisms, any possible Zn-induced effect on gene transcription in F₂Zn^{+/-} cannot be attributed to a direct exposure to maternally transferred excess internal Zn. The same observation was made for the F₁Zn[−] and F₂Zn^{+/-} treatments. The internal concentration of 68 µg Zn/g dry weight in the F₂Zn^{+/-} organisms is in line with the internal Zn concentrations of the other non-exposed daphnids observed by Vandegehuchte et al. (2010b) (Fig. 1B).

The number of 73 unigenes which were differentially transcribed in the F₁Zn⁺ daphnids, is comparable to the 71 transcriptionally regulated unigenes in the F₀Zn⁺ treatment, and higher than the 42 differentially transcribed unigenes in the F₁Zn[−] daphnids. However, only seven genes were regulated in the same direction in F₀Zn⁺ and F₁Zn⁺. On the other hand, in F₁Zn⁺ and F₁Zn[−], which both exhibited reduced DNA methylation, twenty genes were regulated in the same direction, of which twelve were upregulated (Fig. 2, Table S2). This amounts to approximately 47% of the differentially transcribed genes in F₁Zn[−]. Except for the gene with homology to a phage related lysozyme, none of these genes were differentially transcribed in the same direction in the next generation, in which no DNA hypomethylation was observed. This suggests that the differential transcription of the other 19 genes may be related to the reduction in DNA methylation in both F₁ treatments. Interestingly, the gene with homology to sel-1 suppressor of lin-12-like was upregulated. This protein is known as a repressor of the Notch signaling pathway (Rooman et al., 2006). Activation of the Notch pathway was shown to be crucial for hypermethylation of a gene in *Drosophila* (Ferres-Marco et al., 2006). A repression of the Notch pathway could be involved in the hypomethylation in the F₁Zn⁺ and F₁Zn[−] daphnids in this study. Another interesting observation is the transcriptional down-regulation of a gene with homology to a Ser/Thr protein phosphatase in the F₁Zn⁺ as well as in the F₁Zn[−] daphnids. The Ser/Thr protein phosphatase PP1 is shown to form a complex with Nuclear Inhibitor of Protein Phosphatase – 1, in combination with the Polycomb Repressive Complex (Roy et al., 2007). This complex is

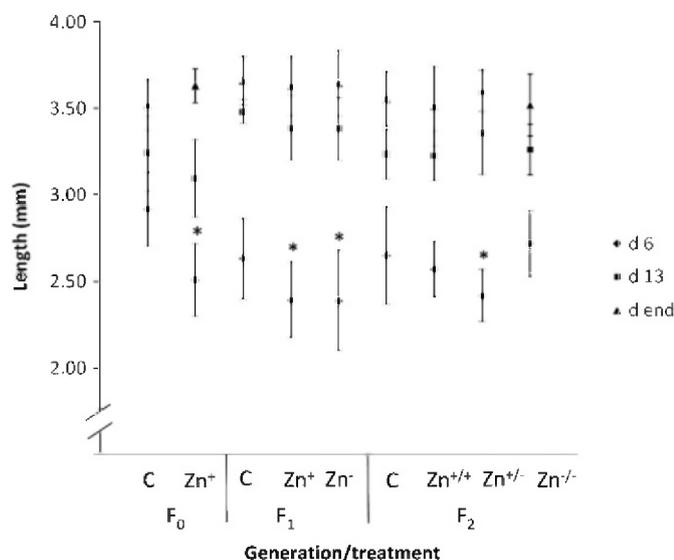


Fig. 3. Length of daphnids in different treatments. Error bars denote standard deviations. An asterisk denotes a significantly smaller value compared to the control of the same generation.

involved in gene silencing, at least in part through trimethylating histone 3 Lys 27. It also interacts with DNA methyltransferases and it is necessary for the methylation of target DNA (Vire et al., 2006; Nuytten et al., 2008). Through these interactions, the down-regulation of the gene coding for Ser/Thr protein phosphatase in the F_1Zn^+ and F_1Zn^- daphnids could be a reason for their reduced global DNA methylation. These data suggest an effect of parental Zn exposure on the gene transcription in exposed and non-exposed offspring, possibly mediated through changes in DNA methylation. In F_1Zn^+ , a larger number of genes were affected because of the combination of parental and direct Zn exposure.

An interesting differentially regulated gene in F_1Zn^+ is S-adenosylhomocysteine hydrolase (SAHH). The transcriptional upregulation of this gene can be a mechanism to increase the level of free homocysteine, which may be reduced due to conjugation with Zn-induced metallothioneins (Barbato et al., 2007; Fan et al., 2009). Free homocysteine is a substrate for the formation of methionine, which can be converted to S-adenosylmethionine (SAM), an important methyl donor for DNA methyltransferases (Mason, 2003; Muskiet, 2005). Low homocysteine levels were suggested to be an indirect cause for the observed DNA hypomethylation in the F_1Zn^+ daphnids by Vandegehuchte et al. (2009). If this was indeed the case, this was not restored by this transcriptional upregulation of SAHH. This is consistent with results of Hermes et al. (2008) in which overexpression of SAHH in human embryonic kidney cells did not cause differences in global DNA methylation. The

upregulation of a gene coding for the heat shock protein Hsp90 in the F_1Zn^+ daphnids can be a stress response leading to elevated levels of Hsp90, as observed in earthworms exposed to Zn and Pb contaminated soils (Marino et al., 1999). An interesting aspect of Hsp90 is that it has recently been proposed to be involved in chromatin remodeling and transgenerational epigenetic inheritance (Ruden and Lu, 2008). Through this pathway, Zn exposure could affect the gene transcription of non-exposed offspring in the $F_2Zn^{+/-}$ treatment.

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

The genes with similar up- or downregulation in F_1Zn^+ and $F_2Zn^{+/-}$ were not the same as 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

Table 1

Overview of the results in the different treatments. Differences in body length, numbers of up- and down-regulated 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, c). ↓: 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	Down-regulated 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

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 (Vandegehuchte et al., 2010b, c). 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.

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

Appendix. Supplementary information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envpol.2010.07.023.

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